PHARMACOLOGICAL EVALUATION OF SOME POTENTIAL ACAT INHIBITORS AND AT_1 & α_1 RECEPTOR DUAL ANTAGONISTS

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By

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DECLARATION

In accordance with university ordinance number O.Ph.D.:8(iii), the undersigned states that, the work presented in this thesis entitled **"PHARMACOLOGICAL EVALUATION OF SOME POTENTIAL ACAT INHIBITORS AND AT_1 & α_1 RECEPTOR DUAL ANTAGONISTS"** comprises independent investigations carried out by me. Wherever references have been made to the work of others, it has been clearly indicated with the source of information under the title of references at end of each section.

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CERTIFICATE

This is to certify that the thesis entitled "PHARMACOLOGICAL EVALUATION OF SOME POTENTIAL ACAT INHIBITORS AND AT_1 & α_1 RECEPTOR DUAL ANTAGONISTS" submitted for the Ph. D. Degree in Pharmacy by Mr. Hardik Paragkumar Gandhi incorporates the original research work carried out by him under my supervision and no part of this work has been previously submitted for any degree.

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This is to certify that the following publications have arisen out of the research work carried out by my Ph. D. student Mr. Hardik Paragkumar Gandhi who wishes to submit thesis entitled **"PHARMACOLOGICAL EVALUATION OF SOME POTENTIAL ACAT INHIBITORS AND AT_1 & α_1 RECEPTOR DUAL ANTAGONISTS"** to The Maharaja Sayajirao University of Baroda, Vadodara for the award of Ph. D. in Pharmacy.

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Dedication

To

Family

(For their love, support & patience)

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IAEC Approval of Animal Studies

Animal husbandry, handling and treatments were performed in accordance with the CPCSEA guidelines. Animals were provided pelleted diet and water *ad libitum* unless otherwise indicated. They were housed in polypropylene cages with paddy husk as bedding under a temperature range and relative humidity of 22 ± 2°C and 50-75% RH respectively. A 12hr light/dark cycle was followed and animals were monitored daily.

All the studies mentioned in this document were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Dept., Faculty of Tech. & Engg., The M. S. University of Baroda *vide* the protocol numbers mentioned below:

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INTRODUCTION

INTRODUCTION

One of the quantitative traits in human physiology is blood pressure. The control of blood pressure is governed by multifactorial conduits. Well-coordinated regulatory systems mainly involving the brain, heart, conduction vessels and kidney govern the control of blood pressure. During the course of evolution and consequent natural selection, humans gradually became susceptible to genetically and environmentally induced hypertension. This in turn led to the evolution of regulatory systems to compensate the hypertensive phenotype. Blood vessel cross-sectional diameter, pressure natriuresis and resultant volume adjustments are frequently affected as a means for regulation of blood pressure and maintaining a normotensive phenotype. The available data suggests that faults in these regulatory systems can occasionally lead to the development of hypertension, a condition that stands as a root-cause for the development of several cardiovascular abnormalities in individuals (Kunes *et al*, 2012). Definitions of hypertension have metamorphosed over time owing to clinical studies in diverse populations and research elaborating the etiopathology of hypertension (Hajjar *et al*, 2006). It may be generalized on the basis of available guidelines (JNC, CHEP, ESH, ISH, ASH, etc.) that an individual is hypertensive if his/her blood pressure is above 140/90 mm Hg.

Recently, the problem of uncontrolled or resistant hypertension has gained enormous proportions (Daugherty *et al*, 2012). Further, diet modifications and exercise seldom cause any benefit in controlling this condition and the patient is usually recommended for pharmacological therapy. It is very well accepted that mono-drug therapy is no longer effective due to the multifactorial etiology and lack of proper lifestyle modifications by the patients. Clinical practice has adopted the use of two or more classes of antihypertensive agents (Elliott, 2002; Paulis and Unger, 2010) for effective control of blood pressure in hypertensive patients. The basis behind such a decision is that since the etiology of hypertension is complex, it is prudent to employ a parallel control of more than one systems affecting increase in blood pressure.

The homeostasis of blood pressure is maintained by two major systems: a) The Renin-Angiotensin-Aldosterone-System (RAAS) and b) The Sympathetic Nervous System (SNS) (Shepherd and Mancia, 1986; Hall *et al*, 1999; Lohmeier, 2001). Release of catecholamines upon SNS stimulation leads to vasoconstriction in the peripheral

beds, alongwith an increase in the chronotropic and inotropic action of the heart. On the other hand, RAAS stimulation modulates blood pressure directly and indirectly. Directly, through the vasoconstrictive action of the octapeptide angiotensin II (ang II) released upon ACE-mediated hydrolysis of angiotensin I and indirectly, through stimulating aldosterone release leading to sodium-water retention contributing to increase in total blood volume. A few clinical and experimental studies have demonstrated that the SNS and RAAS are in fact, intertwined in the homoestatic control of blood pressure (Shepherd and Mancia, 1986; Hall *et al*, 1999; Lohmeier, 2001). These demonstrations have generated the relevance of the concept of renin-angiontensin-sympathetic crosstalks in hypertension (Li *et al*, 1997; Grassi, 2001). The information regarding the mutual interaction of these systems may be derived from the literature which suggests that renin secretion and angII formation are amplified upon stimulation of the SNS (DiBona, 1989a; DiBona, 1989b). Norepinephrine modulates angII receptors via interaction with α_1 adrenergic receptors (Du *et al*, 1997; Li *et al*, 1997) while intracerebral injection of angII may trigger a sympathetically mediated rise in blood pressure(Wolff *et al*, 1984; Zimmerman *et al*, 1984; Hall *et al*, 1999). Stimulation of presynaptic angII receptors can stimulate norepinephrine release from nerve terminals (Starke, 1977; Zimmerman *et al*, 1984; Reid, 1992) and angII may amplify vasoconstrictor responses affected by the α_1-receptors. AT_1 and α_1 receptors are important targets in this regard and hence a simultaneous blockade of these targets might prove favorable towards the effective management of hypertension.

The past decade has witnessed the development of a concept termed as "Designed Multiple Ligands" (DMLs). DMLs are novel synthetic compounds possessing the pharmacological ability to bind to more than one target. This effect is supposed to be deliberate rather than by chance as observed in serendipitous discoveries. A superior therapeutic efficiency can be achieved through evenhanded modulation of multiple targets (Morphy *et al*, 2004). This may also be achieved through polypharmacy, administration of fixed dose combinations or an agent directed to all the required targets, i.e. a designed multiple ligand. When compared to the other alternatives, the administration of a DML may offer certain advantages like predictable pharmacokinetics, simple pharmacodynamic relationships, improved patient compliance and ease of therapeutic drug monitoring, if at all required (Morphy *et al*, 2004).

The present work describes the screening of a series of new chemical entities for potential dual-antagonist activity on the AT_1 and α_1 receptors. The compounds belong to a series of 6,7-dimethoxyquinazolines with different substitutions at 2nd position based on structural modifications involving prazosin and losartan. These compounds were assumed to show a balanced modulation of both the receptors in question. One of the compounds, **MCR-1329** (a 6,7-dimethoxyquinazoline derivative), has been evaluated pharmacologically for its potential as an antihypertensive agent.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

BLOOD PRESSURE AND SYSTEMIC HYPERTENSION: HEMODYNAMICS

By simple definition, pressure exerted by the flow of blood upon the walls of capillaries is termed as 'blood pressure'. The perception of pressure as the heart contracts against the resistance of blood vessels is in fact a simple application of the Ohm's law in physics which can be defined as "the product of the cardiac stroke volume, heart rate and systemic vascular resistance" where the product of cardiac stroke volume and heart rate is in fact, cardiac output (Guyton and Hall, 2006; Salvi, 2012). In this context, blood pressure is highest in the aorta and larger arteries and gradually falls down to zero as it reaches the larger veins and vena cava. The value of BP rises to as much as 120 mm Hg in a healthy adult individual during ventricular systole and falls to about 80 mm Hg during ventricular diastole. This pressure range is essential for maintaining an adequate perfusion rate and supply of nutrients to all the tissues of the body. Blood pressure is regulated within this range by several factors acting in concert with each other (Guyenet, 2006; Chopra *et al*, 2011). Traditionally, determinants of blood pressure have been known to be the renin-angiotensin-aldosterone-system, adrenergic stimuli to the blood vessels and the myocardium, baroreceptors, vagal responses, renal handling of salt & water and myogenic tone of vascular smooth muscles. All these systems are modulated in a fine-tuned manner by different modulators like renin, angiotensin II, ACE, AT_1R, noradrenaline, adrenoceptors, Ach, nitric oxide, L-type calcium channels, so on and so forth. It is these systems that regulate the blood pressure to ensure that none of the tissues in the body is deprived of the nutritional support achieved through blood. Based on our current understanding of blood pressure control, we still have a myopic view of the mechanisms involved in regulation of blood pressure. In spite of such a myriad assembly of factors maintaining normal blood pressure, individuals often tend to escape these control mechanisms and develop a condition of higher blood pressure termed as 'systemic hypertension'. Hypertension typically develops with an increase in systemic vascular resistance with or without increase in cardiac output (Kaplan, 2011). These two determinants are dependent upon kidney sodium excretion, baroreflexes and local autoregulation responses. Cardiac output may be modulated by extracellular fluid content which is actually given by total body sodium content (not plasma sodium concentration). This

parameter is entirely influenced by renal handling of sodium (Guyton and Hall, 2006; Vincent, 2008). Another factor modulating cardiac output is contractility & heart rate and these parameters may be in turn modulated by sympathetic tone and endogenous inotropic agents (Guyenet, 2006; Vincent, 2008). Modulation of systemic vascular resistance is more complex and involves an intricate balance between vasoconstrictor and vasodilatory mechanisms. These mechanisms may involve secretion of agents due to activation of the baroreceptor pathway, juxtaglomerular apparatus, local blood vessels and atria. Different factors like angiotensin II, noradrenaline, endothelin, thromboxane, prostaglandins, natriuretic peptides, bradykinin, excretion of Na^+ and nitric oxide among others are responsible for maintaining the intricate balance (Webb and Bohr, 1981; Umans and Levi, 1995; Carretero and Oparil, 2000; Oparil *et al*, 2003; Guyenet, 2006). Signals like blood pressure and blood viscosity lead to shear stress (Ku, 1997) thus influencing secretion of local effectors like endothelin or nitric oxide (Umans and Levi, 1995) for maintaining the appropriate cross-sectional diameter of the blood vessel.

PRIMARY AND SECONDARY HYPERTENSION

Hypertension is most commonly classified as primary or secondary. Primary hypertension (also known as *essential hypertension*) is a condition in which an individual develops higher blood pressure due to some unidentified etiology (Carretero and Oparil, 2000; Beilin, 1988; Johnson *et al*, 2008). More than 90% of individuals suffering from hypertension belong to this class with the age of onset being above 50 years and with a strong family history. Thus, this form of hypertension is also termed as hereditary hypertension. Primary hypertension shows stronger correlations between family members and races (Johnson *et al*, 2008). Secondary hypertension is the development of hypertension due to some identifiable etiopathologic factor. Most common causes are renal diseases, stenosis of renal arteries, pheochromocytoma and hyperaldosteronism. The underlying pathophysiologic factor is commonly diagnosed upon medical examinations and in most of the cases is potentially curable (Akpunonu *et al*, 1996; Faselis *et al*, 2011).

DEFINITIONS OF HYPERTENSION

Definitions of hypertension have metamorphosed over time owing to clinical studies in diverse populations and research elaborating the etiopathology of

hypertension. Different guidelines are elaborated by national agencies and these guidelines have minor differences based on the cohort studies in respective countries/ethnic groups. These differences are appreciated and taken into consideration while defining the intensity of therapy for any patient; however, due to its multifactorial etiopathology physicians are more likely to individualize diagnosis and therapeutic alternatives on a patient-to-patient basis. Guidelines published by the 'Joint National Committee (JNC)' set up by the American Medical Association (AMA) are the most commonly referred and cited set of guidelines (Chobanian *et al*, 2003) for defining hypertension. Other guidelines and their relative values are provided in the table below:

Table1: Classification of hypertension based on different national guidelines[1]

Sr. No.	Name of Guideline	Country of Publication (Year)	Definition or term	Values (mm Hg)	
				Systolic	Diastolic
1	Joint National Committee 8 (JNC 8) (James *et al*, 2014)	USA (2013)	Normal	< 120	< 80
			Prehypertension	120-139	80-89
			Stage 1	140-159	90-99
			Stage 2	≥ 160	≥ 100
2	World Health Organization/ International Society of Hypertension (WHO/ISH) (Weber *et al*, 2014)	UK (2013)	Grade1	140-159	90-99
			Grade2	160-179	100-109
			Grade3	≥ 180	≥ 110
3	European Society of Cardiology/ European Society of Hypertension (ESC/ESH) (Mancia *et al*, 2013)	UK (2013)	Normal	120-129	80-84
			High-Normal	130-139	85-89
			Grade1 (mild)	140-159	90-99
			Grade2 (moderate)	160-179	100-109
			Grade3 (severe)	≥ 180	≥ 110
			Isolated systolic hypertension	≥ 140	< 90
4	Canadian Hypertension Education Program (CHEP)	Canada (2013)	Grade A	< 130	< 85
			Grade B	130-179	85-109
			Grade C	180-199	110-119

	(Dasgupta *et al*, 2014)		Grade D	≥ 200	≥ 120
5	British Hypertension Society IV (BHS-IV) (Williams, 2004)	UK (2004)	Normal	< 130	< 85
			High-Normal	130-139	85-89
			Grade1 (mild)	140-159	90-99
			Grade2 (moderate)	160-179	100-109
			Grade3 (severe)	≥ 180	≥ 110
			Isolated Systolic Hypertension-Grade1	140-159	< 90
			Isolated Systolic Hypertension-Grade2	≥ 160	< 90
6	Indian Guidelines on Hypertension-III (IGH-III) (Shah *et al*, 2013)	India (2013)	Normal	< 130	< 85
			High-Normal	130-139	85-89
			Grade1 (mild)	140-159	90-99
			Grade2 (moderate)	160-179	100-109
			Grade3 (severe)	≥ 180	≥ 110
			Isolated Systolic Hypertension-Grade1	140-159	< 90
			Isolated Systolic Hypertension-Grade2	≥ 160	< 90

[1] While preparing this table, preference has been laid on guidelines most commonly referred in literature. Hence, chronological order is not followed

EPIDEMIOLOGY OF HYPERTENSION IN INDIA

There has been no national survey to identify the epidemiology of hypertension in the subcontinent. However, several authors have accumulated data from WHO publications and other regional studies in India to generate a consensus on the trends of hypertension in the country. Based on these publications, it could be concluded that hypertension is the major cardiovascular disorder responsible for the highest mortality caused by non-communicable diseases in India. While data compiled a decade ago showed that prevalence of hypertension is low among the rural population (Gupta, 2004), recent data analysis revealed that affluence does not have any bearing upon the occurrence of hypertension in the Indian population and hypertension occurs in both the urban and rural classes, almost equally (Moser *et al*, 2014). Risk factors for the occurrence of hypertension resemble the global trend and include increasing age, smoking, alcohol intake, concomitant diabetes and high salt intake in the diet (Devi *et al*, 2013). Important gender differences could be observed regarding the prevalence of hypertension in India. It was found that though prevalence rates did not differ much (26% men v/s. 23% women), diagnosis rates did. Women, more than 70% of them, had their blood pressures assessed with some health services. Age related prevalence indicates that 40% of individuals crossing 70 years of age are likely to suffer from hypertension. Again, alcohol consumption at any time of life increased the likelihood of an individual suffering from hypertension (Anand, 2010; Moser *et al*, 2014). The major issue among the Indian populace remains the ignorance and lack of awareness regarding diagnosis and treatment of hypertension. It has been observed that detection of hypertension remains the major concern while managing hypertension in India and hence if early detection of this condition can be handled, it can be managed in a far better manner (Devi *et al*, 2013).

ETIOPATHOGENESIS OF HYPERTENSION AND COMPENSATORY MECHANISMS

As mentioned above in *'blood pressure and systemic hypertension: hemodynamics'* section, blood pressure is the product of cardiac output and systemic vascular resistance. It is thus clear that the patient may either have increased systemic vascular resistance (which is often more common), increased cardiac output (which is less common) or both. The ultimate product of this situation is hypertension. It may be

observed that in most of the adult, middle aged and geriatric population systemic vascular resistance is increased. This population consists of more than 90% of the total patients suffering from hypertension in one or the other form (Kearney *et al*, 2004; Kumar *et al*, 2013). Increase in systemic vascular resistance is actually the increase in vascular tone and narrowing of lumen diameters in resistance vessels like arteries and arterioles. There are several etiologic factors responsible for increasing the vascular tone (Figure 1 below). This includes secretion of vasoconstrictive peptides like AngII and endothelin-I. Stimulation of α_1-adrenergic receptors by norepinephrine is another mechanism responsible for constriction of blood vessels (Calhoun *et al*, 2008). All these mechanisms converge to a final pathway where there is a GPCR mediated increase in cytosolic calcium, $[Ca^{++}]_i$, in vascular smooth muscle cells that causes vasoconstriction of the blood vessel (Koenigsberger *et al*, 2004) in a radial manner leading to narrowing of lumen diameter and consequent increase in vascular tone. If increase in vascular tone is accompanied by increasing vascular stiffness (or reduced elasticity of blood vessels), there is an enhanced load bearing onus on the left ventricle which, if persistent or not corrected, can lead to ventricular hypertrophy followed by ventricular dysfunction (Carretero and Oparil, 2000).

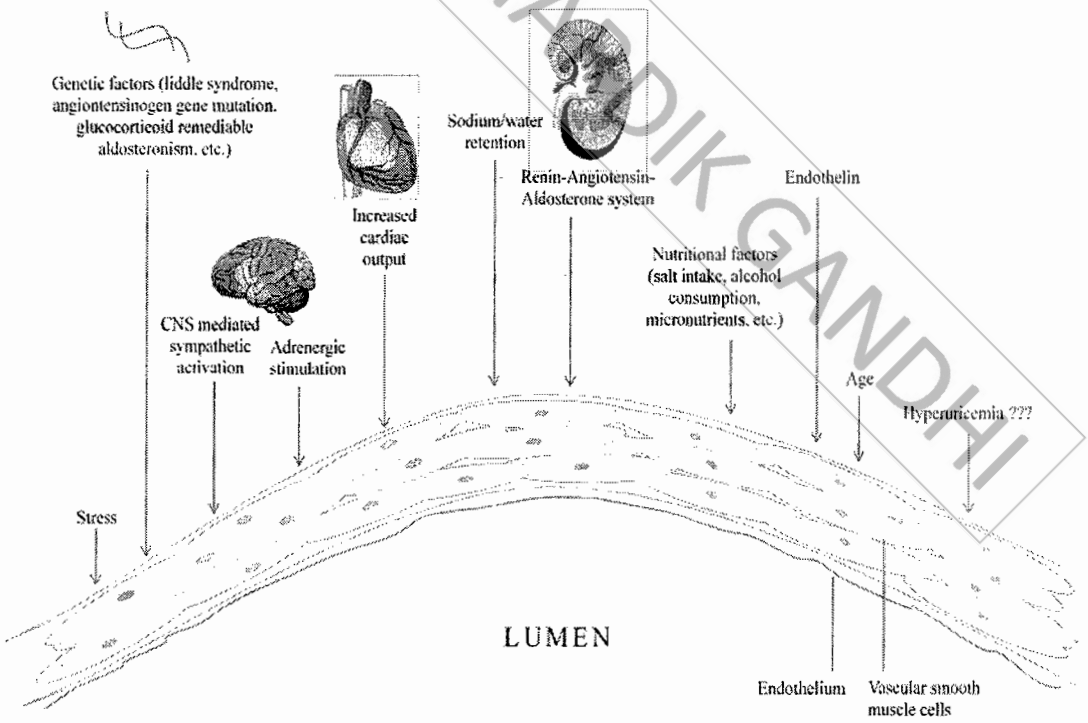


Figure 1: Etiopathological factors of hypertension [Redrawn from: Oparil *et al*, 2003]

The role of sympathetic nervous system in the pathogenesis of hypertension cannot be ignored. The major contribution provided by the sympathetic nervous system in the development of hypertension is in two forms: 1) increased release of norepinephrine which mediates direct constriction of blood vessels, and 2) enhanced sensitivity of norepinephrine in peripheral tissues (Mark, 1996; Brook and Julius, 2000) such that even traces of norepinephrine can shoot blood pressure values by 10-15 mm Hg. Environmental factors additionally play an important role in the pathogenesis of hypertension. Stress increases the peripheral sensitivity of circulating catecholamines thus predisposing the individual to increased vascular tone and heart rate (Esler, 2000). The compensatory mechanisms of the body responsible for control of blood pressure are dulled and thus elevations in blood pressure cannot be managed without pharmacotherapy. Abnormal handling of renal salt and water disturbs the natriuresis/volume/blood pressure balance (Chiolero *et al*, 2001), thus leading to hypertension. The renin-angiotensin-aldosterone-system is one of the key effectors of the etiopathogenesis of hypertension and is mostly involved in renovascular hypertension. Hypertension may occur independent of renin status, i.e. the patient may be classified as having low renin or high renin but the effector angII, is the main culprit responsible for all of the detrimental effects leading to hypertension and its complications (Gradman and Kad, 2008; Arnold *et al*, 2013;).

Different regulatory mechanisms exist in the human physiology as a means of controlling unanticipated elevations in blood pressure (Figure 2). These include the baroreceptor mediated regulation which reduces sympathetic outflow from the brain whenever increased pressure is sensed at the aortic and/or carotid baroreceptors (Guyenet, 2006; Xie *et al*, 1991; Guo and Abboud, 1984). Spatial changes induced in the blood vessels due to narrowing of the lumen diameter lead to secretion of endothelium derived relaxing factor, chemically known as nitric oxide (NO), which causes cGMP-mediated relaxation of vascular smooth muscle cells (Cai and Harrison, 2000). Renal capsule has a general role in maintaining the fluid-balance in the body. Renal diuresis mechanisms govern the overall blood volume and increased diuresis can reduce blood volume which improves cardiac output by reducing venous return (Legrand and Payen, 2011). The macula densa region of the kidney senses perfusion pressure of the tubules and thus regulates the release of the peptidase 'renin' which cleaves angiotensinogen to form ang I, the precursor for AngII (Persson *et al*, 2004;

Peti-Peterdi and Harris, 2010). One very important facet in the development of hypertension is that there is partial or complete blunting of the compensatory mechanisms that regulate blood pressure towards normotensive values.

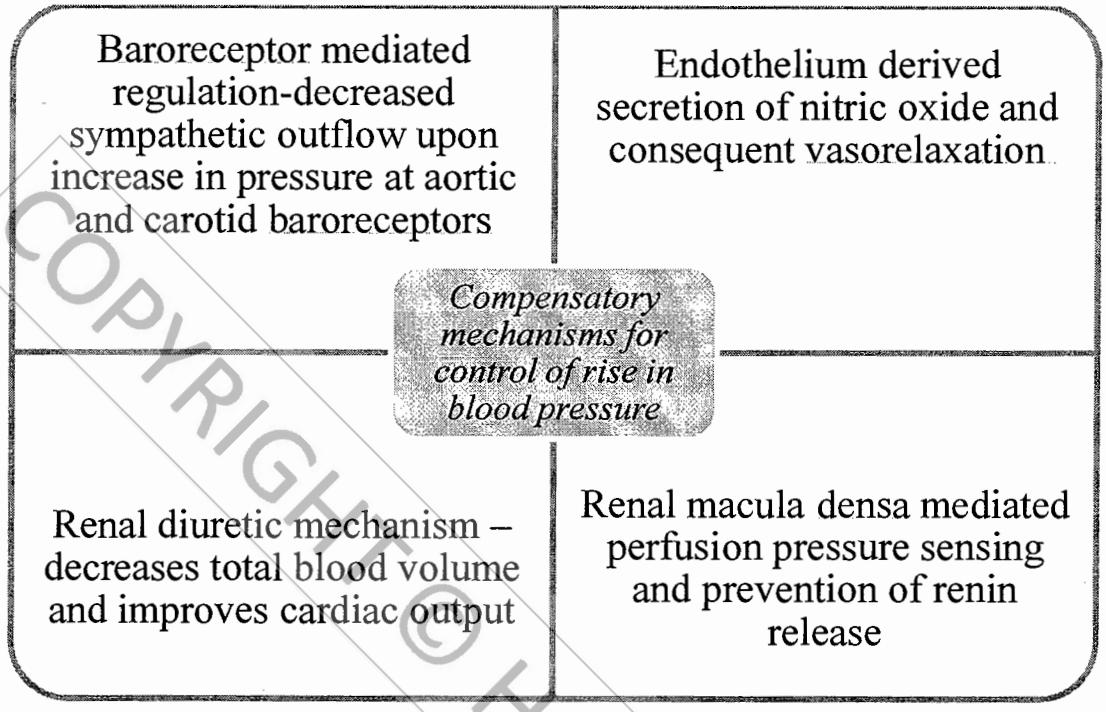


Figure 2: Different physiological mechanisms to prevent rise in abnormal blood pressure. These mechanisms operate through the brain, vasculature and kidneys.

Sympathetic nervous system

Several hemodynamic, rheologic and trophic changes in the cardiovascular system are maintained by a balance between the parasympathetic and sympathetic arms of the autonomic nervous system (Brook and Julius, 2000). Any imbalance in the autonomic system favouring the sympathetic arm, which is concomitant with reduced parasympathetic outflow, can lead to increased cardiovascular morbidity and mortality. Increased heart rate is usually normalized by increases in parasympathetic tone but in a situation of autonomic imbalance, reduced parasympathetic tone can lead to perpetual increases in heart rate and thus contributes to the pathological condition of hypertension (Brook and Julius, 2000; Oparil et al, 2003). Several population-based studies have concluded that increase in sympathetic tone is the ultimate cause of vascular remodeling which is preceded by vascular smooth muscle cell (VSMC) proliferation. Norepinephrine spillover studies, which afford the researchers with an index of norepinephrine release from sympathoeffector nerve terminals, are also in agreement

with the population-based studies and support the conclusion that one of the major contributors in the development of hypertension is elevated sympathetic tone (Esler, 2000).

The development of hypertension which is mediated through increased sympathetic tone is a complex network of different mechanisms. These involve alterations in the chemoreflex and baroreflex mechanisms, trophic changes in the heart and blood vessels and mainly renal sympathetic stimulation. There is central as well as peripheral resetting of baroreflexes under conditions of hypertension. In hypertensive patients, it could be observed that the arterial baroreceptors are reset to a higher pressure value, thus preventing the regulatory feedback. Also, there is a central resetting of the aortic baroreflexes (Xie *et al*, 1991; Guo and Abboud, 1984). Based on experimental data, it may be assumed that this resetting of the carotid and aortic baroreflexes is mediated, to a certain extent, by AngII (Xie *et al*, 1990; Li *et al*, 1996). Owing to its ability of 'presynaptic facilitatory modulation of norepinephrine release', AngII magnifies the peripheral responses to sympathetic stimuli (Abboud, 1974). Furthermore, reactive oxygen species and vascular factors like endothelin also cover out baroreceptor activity and contribute to the development of hypertension (Chapleau *et al*, 1992; Li *et al*, 1996). With respect to chemoreflex, stimuli such as hypoxia or apnea, lead to an overstated chemoreflex function leading to an obviously augmented sympathetic tone (Somers *et al*, 1995; Scultz *et al*, 2007). Sustained activation of the sympathetic arm of the autonomic nervous system leads to sustained release of norepinephrine and the cardiovascular system thus becomes a subject of the direct and indirect actions of norepinephrine (Louis *et al*, 1969; Goldstein, 1981). Norepinephrine also affects the release of various factors like FGF, TGF-β and IGF-1, all of which are trophic in nature (Grassi, 1998; Brook and Julius, 2000; Wakatsuki *et al*, 2004). Chronic sympathetic activation thus leads to development of left ventricular wall hypertrophy and consequent vascular remodeling ensues (Grassi *et al*, 1995; Oparil *et al*, 2003). As compared to normotensive individuals, hypertensive patients show an increased renal sympathetic tone. Direct stimulation of the renal nerves in animals has shown that there is an increased reabsorption of sodium-water and a fall in urinary output, both of which contribute to vascular volume expansion and thus cause increase in blood pressure *via* increase in cardiac output. Alternatively, renal denervation has been shown to reverse the raised blood pressure in experimental models of hypertension

(DiBona and Kopp, 1997; Kopp, 2011). This mode of therapy has also been found to be beneficial in the management of human hypertension and recently several regulatory authorities have added 'surgical renal denervation' as one of the major treatment alternatives for hypertensive patients (Schlaich *et al*, 2009; Krum *et al*, 2009). Precisely, these facts and results support the role of increased sympathetic activity in the etiopathogenesis of hypertension.

Vascular reactivity

Increased vascular reactivity is one of the facets of hypertension. It has been observed that hypertensive patients exhibit a higher vasoconstrictor tone in response to norepinephrine and other vasoconstrictive agents (Ziegler *et al*, 1991). Chronic high levels of circulating norepinephrine tend to cause down regulation of noradrenergic receptors but this does not occur in hypertensive states which cause an increase in peripheral resistance and rise in blood pressure (Oparil *et al*, 2003). Normotensive progeny born to hypertensive parents also show an increased sensitivity to the actions of norepinephrine. This suggests that vascular reactivity may be inheritable. Since offspring from parents without a history of hypertension do not show enhanced vascular reactivity, it may be concluded that hypersensitivity is of genetic origin and merely not a result of elevated blood pressure (Calhoun *et al*, 1993).

Endothelial dysfunction

NO is responsible for the maintenance of a continuous vasodilator tone in healthy individuals. NO, expressed by the normal vascular endothelium, is a potent vasodilator, inhibits platelet aggregation and adhesion and also prevents migration and proliferation of smooth muscles (Forstermann and Munzel, 2006; Pacher *et al*, 2007). NO may be released in response to stimuli like increased blood pressure, arterial stretch and/or increased shear stress. In case of human hypertension, macro- and micro-vessels of the periphery, heart and kidney undergo endothelial damage. Partial damage to the endothelium leads to dysfunction in the secretion of various factors that influence vascular tone and structure (Forstermann and Munzel, 2006). In case of hypertension, activation of an alternative cyclooxygenase pathway leads to generation of oxidative stress that reduces NO availability (Cai and Harrison, 2000; Puddu *et al*, 2000; Schulz *et al*, 2008). In the absence of NO, prostacyclin and an unidentified hyperpolarizing factor secreted from the endothelium maintain the relaxation. Other etiopathologic

factors like presence of concomitant hyperhomocysteinemia may further demoralize the compensatory actions of prostacyclin and the hyperpolarizing factor (Mitchell *et al*, 1992; Emsley *et al*, 1999; Matoba *et al*, 2000). Another factor secreted by the endothelium is endothelin-1 (ET-1) whose actions are opposite to that of NO, i.e. it is vasoconstrictive in nature. Under normal situations, ET-B receptor mediated NO elaboration keeps the ET-1 mediated vasoconstriction in check. However, under blunted NO release, ET-1 mediated vasoconstriction can overwhelm compensatory vasodilatory mechanisms and thus participates in increasing the blood pressure in patients with essential hypertension (Krum *et al*, 1998; Oparil *et al*, 2003).

Pro-angiogenic factors

Microvascular rarefaction is one of the discrete characteristics of hypertension (Goligorsky, 2010). It exemplifies a reduction in the parallel blood vessel circuits resulting in an alteration of microcirculation which ultimately contribute to end-organ damage. Impaired angiogenesis is one of the primary reasons for occurrence of rarefaction (Goligorsky, 2010; Feihl *et al*, 2006). Angiogenesis is a highly complex and regulated process which involves formation of vascular networks from microvessels. It is stimulated by hypoxic conditions (a compensatory mechanism to counteract ischemia) and is regulated by levels of NO. Lower than normal levels of NO mar the process of angiogenesis whereas normal or elevated levels support angiogenesis. Angiogenic factors like VEGF and FGF function only in presence of NO for promoting angiogenesis (Namikoshi et al, 2006; Goligorsky, 2010). Role of such growth factors is essential for angiogenesis to reduce target-organ damage. Such growth factors promote collateral vessel formation. Placenta inducible growth factor (PlGF) also acts by activating VEGF signaling (Carmeliet *et al*, 2001; Luttun *et al*, 2002). Lack of VEGF signaling can lead to limb ischemia or claudication as indicated by clinical trials (Baumgartner and Isner, 2000).

Vascular remodeling

Chronic elevated blood pressure leads to persistent systemic vascular resistance. This situation leads to an alteration in the structure of resistance vessels. These structural changes may be defined in terms of a ratio, media-to-lumen diameter (Rosei *et al*, 1995; Rizzoni *et al*, 2012). Thus, changes in media thickness and narrowing of the arterial lumen were initially perceived as means of vascular insufficiency but with the

observations made by Glagov *et al* (1987), it was acknowledged that in pathological conditions, arteries undergo several structural changes to preserve blood flow. This rearrangement of matrix and muscular material around the vessel's lumen, initially termed as vascular growth, was later dubbed as *vascular remodeling* by Mulvany (1993). Vascular remodeling may thus be defined as an active process that involves alterations in the cell growth, migration, survival and regulation of extracellular matrix, ultimately influencing vascular structure and function. Classification of different forms of vascular remodeling is given in figure 3 below:

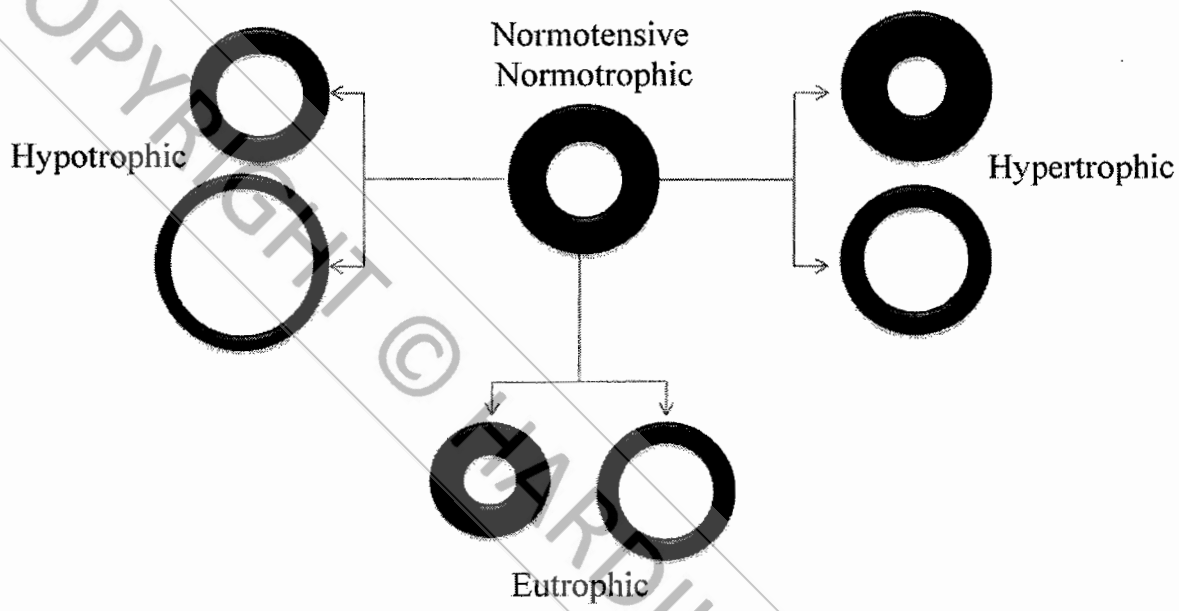


Figure 3: Remodeling may be hypertrophic (increased cross-sectional area as observed in severe hypertension), eutrophic (no change in cross-sectional area, mild-to-moderate hypertension) or hypotrophic (decreased cross-sectional area, following chronic antihypertensive therapy). As can be observed from the diagram, each form of remodeling may result either in increased lumen diameter (outward remodeling) or a decrease in lumen diameter (inward remodeling). Remodeling should not be confused with *rarefaction* where there is a decrease in the number of parallel blood vessel circuits as a result of increased peripheral vascular resistance.

Persistently elevated blood pressure stimulates arterial stretch leading to increased tensile forces in the arterial walls. This phenomenon further stimulates vessel wall thickening to normalize the changes in the tensile strength. Modifications in the structural content of the vessel walls result from several factors like growth, inflammation, apoptosis, fibrosis and cell-matrix interactions acting in concert with each other (Intengan and Schiffrin, 2001). While growth, inflammation and fibrosis result from secretion of several cytokines as a result of hypertensive pathophysiology,

apoptosis results from activation of programmed cell death cascades in blood vessels (Cho *et al*, 1995; Hamet *et al*, 1995). Inflammation triggers fibrosis to some extent and ultimately involves accumulation of remodeling components like collagen, fibronectin and other extra cellular matrix material. This kind of accumulation is further aggravated due to integrins (which are responsible for cell-matrix interactions) resulting in modulation of arterial structures. Other very important mechanism involved in remodeling is the imbalance between MMPs/TIMPs which may result in increased collagen turnover (Diez *et al*, 1995; Intengan and Schiffrin, 1999) and the balance may thus be tilted in favor of increased extracellular matrix remodeling (Galis *et al*, 1994). In case of chronic hypertension, the constricted vessel lumen is continuously enveloped in a gradually remodeling matrix. Thus the rearrangement of smooth muscles surrounding the lumen leads to inward remodeling. This type of process is actually compensatory in the beginning and behaves as an adaptive mechanism but later becomes maladaptive. It causes arterial stiffness and compromises end-organ function leading to complications of hypertension (Franklin, 2005; Payne *et al*, 2010).

Uric acid - a proposed pathological factor in hypertension

Hyperuricemia has been linked with human hypertension and CVD (Rich, 2000; Alderman *et al*, 1999) but there is no consensus regarding hyperuricemia whether it is a cause or effect of hypertension. Thus it remains to be classified either as a risk factor for hypertension or one of the markers of hypertension. Renal vasoconstriction and plasma renin activity, both relate positively to hyperuricemia, suggesting that uric acid may be an offshoot of the local RAAS in the kidney. Conclusions from major randomized trials like SHEP (Franse *et al*, 2000) did not find any correlation between uric acid levels of treated and untreated patients. Another major randomized trial, LIFE (Dahlof *et al*, 2002), suggested that higher baseline serum uric acid levels were associated with increased CVD risk. Mechanisms by which uric acid might be involved in the development of hypertension have been identified: 1) it stimulates renal arteriolopathy and tubulointerstitial inflammation, 2) it reduces NO excretion in the macula densa by reducing nNOS levels, 3) it is a marker for xanthine oxidase associated oxidative products which contribute to the development of hypertension and 4) it induced PDGF expression in VSMCs leading to smooth muscle proliferation (Johnson *et al*, 2003; Mazzali *et al*, 2010). One important aspect regarding experimental models used to study uric acid levels is that uric acid is metabolized to allantoin by an

enzyme uricase that is expressed in most experimental species except humans and other higher primates (Yeldandi *et al*, 1991; Szasz and Watts, 2010). This fact is important to note since canine and rat models are frequently used to study hyperuricemia where uric acid levels cannot be appreciated due to elaboration of uricase and thus interpretations should be done carefully if hyperuricemia is correlated to hypertension on the basis of such studies.

Salt-sensitive hypertension and renal microvascular disease

Evolutionary studies suggest that organisms evolved from salty environments of the ocean. This environment is analogous to extracellular fluid for mammalian cells which is rich in electrolytes. Further, the course of evolution offered remarkable challenges to the evolving organisms when they moved from salt-water to fresh-water (low in electrolytes) and ultimately to land. Regulatory systems developed in the human body to adjust with the low-salt environment on land so as to maintain homeostasis (Smith, 1959; Lifton *et al*, 2001; Fournier *et al*, 2012). Developing civilizations went through two major changes which contributed to increased salt intake by individuals: 1) They learnt to conserve food with the addition of salt and 2) daily nutritional intake in industrialized areas involved excessive salt in the diet. This led to sodium retention and ultimately water retention causing volume overload (Danziger, 2001; Lifton *et al*, 2001; Weder, 2007; Fournier *et al*, 2012). The renin-angiotensin-aldosterone system remains active *in continuum* to normalize this overload but has its detrimental effects on the kidney and cardiovascular system leading to hypertension. It has been argued that abnormal handling of salt/water by the kidney and concomitant renal vascular disease may be the primary cause of hypertension (Khalil, 2006; O'Shaughnessy and Karet, 2006). This argument has been the subject of exhaustive deliberations and has been supported by several researchers. It has been suggested that renal vasoconstriction for a prolonged period of time allows the development of renal arteriopathy, glomerulonephrosclerosis and tubulointerstitial disease which lead to sodium/water retention and thus contribute to the etiopathology of hypertension (Bidani and Griffin, 2002; Bidani and Griffin, 2004). Several stimuli like increased sympathetic tone and overactivity of the renin-angiotensin-aldosterone system lead to renal vasoconstriction. Renal handling of salt/water may be normal at this stage but chronic renal hypertension leads to initiation of renal injury which may involve renotubular ischemia, interstitial inflammation, local generation of AngII, decreased NO release, etc (Pohl, 1997;

Garovic and Textor, 2005). As mentioned earlier, renal arteries also undergo remodeling and lead to development of arterio- and arteriolo-pathy. This may be observed when kidneys of hypertensive animals show PAS-positive areas suggesting glomerulonephrosclerosis (Isaacson *et al*, 1991; Caetano *et al*, 2001; Marcantoni *et al*, 2002). All these factors result in increased renal vascular resistance wherein and single-nephron glomerular filtration rate is compromised. As an adaptive mechanism, the balance between vasoconstrictors and vasodilators shifts in favor of vasoconstrictors, and this kind of mechanism leads to sodium retention and increased systemic blood pressure (Bidani and Griffin, 2002). As a means for compensating rather than allowing maintenance of normal perfusion pressure, kidneys equilibrate at a higher blood pressure and thus sodium handling returns to normal but total systemic vascular resistance is increased as the pressure-natriuresis relations show a parallel rightward shift (Garovic and Textor, 2005).

The renin-angiotensin-aldosterone system

The physiology of the RAAS is a complex cascade of events which is the definitive blood volume and pressure regulation order of the human body and has important implications in the study of hypertension and related cardiovascular diseases (Carey and Siragy, 2003; Rosivall, 2009). The cascade starts with the release of renin, an aspartyl protease, from the juxtaglomerular glands of the kidney (**Figure 4**). This enzyme cleaves angiotensinogen, synthesized in the liver, which is the primary precursor of the RAAS to a decapeptide termed as angiotensin I (angI). Renin is the rate-limiting enzyme in this cascade and formation of ang I from angiotensinogen thus becomes the rate-limiting step. Stimuli for the release of renin include baroreflex response in the juxtaglomerular apparatus. The β-adrenergic sympathetic innervation increased chloride delivery at the macula densa division of the distal tubule. Renin also binds to a receptor termed the pro-renin receptor [(P)RR] which enhances the proteolytic activity of renin and also activates pro-renin, the precursor of renin (Nguyen et al, 2002; Nguyen, 2006; Nguyen, 2007). Activation of (P)RR has some effects which are independent of the RAAS pathway, yet have a bearing upon the pathophysiology of cardiovascular diseases. These include activation of the promyeloctic zinc finger (PLZF), activation of PI_3-Kinase and eventually MAPKs (Nguyen *et al*, 2004; Schefe *et al*, 2006). Activation of these factors result in increased proteosynthesis, proliferation and reduced apoptotic events (Nguyen *et al*, 2004).

AngI formed by the action of renin is majorly inactive and is cleaved to an octapeptide, AngII, by the action of angiotensin converting enzyme (ACE). ACE, a matrix metalloproteinase, may be available in circulation or may be expressed locally but majority of the AngII conversion takes place through ACE expressed by the pulmonary endothelial cells (Carey and Siragy, 2003; Rosivall, 2009). However, ACE is not the only enzyme with the ability to cleave angI to AngII. AngII formation may also occur due to the action of chymase, carboxypeptidase, cathepsin G or tonin (Clarke and Turner, 2012). ACE has the ability to hydrolyse bradykinin (Tom *et al*, 2003) and stimulate NO and prostacyclin, thus exhibiting vasodilatory properties. Increased angII levels, reduced baroreflex responses and sympathetic inhibition act as negative feedback for preventing/curtailing the release of renin (Harrison-Bernard, 2009; Rosivall, 2009). AngII formed through any of the aforementioned enzymes may eventually demonstrate different actions relevant to the pathophysiology of hypertension. AngII acts on the adrenal cortex, stimulating the release of aldosterone. Aldsoterone effects sodium reabsorption from the collecting ducts in the kidney thus contributing to reduced natriuresis (Mehta and Griendling, 2007). Aldosterone mediates its salt reabsorptive actions through the mineralocorticoid receptors. AngII itself has sodium reabsorption capacities and its activity in the brain leads to thirst stimulation and increased salt appetite (Fyhrquist *et al*, 1995). AngII also increases sympathetic outflow in an independent manner which is discussed below. Above all, the major mechanism by which the RAAS cascade is important in hypertension is the ability of angII to act directly on the vessel walls to mediate vasoconstriction and attendant rise in blood pressure. This vasoconstrictive action of angII is mediated through a *Gq* coupled GPCR, the angiotensin type 1 receptor (AT_1R). The AT_1R arbitrates vasoconstriction, inflammation, vascular hypertrophy and fibrosis (Touyz and Schiffrin, 2000; Kanaide *et al*, 2003).

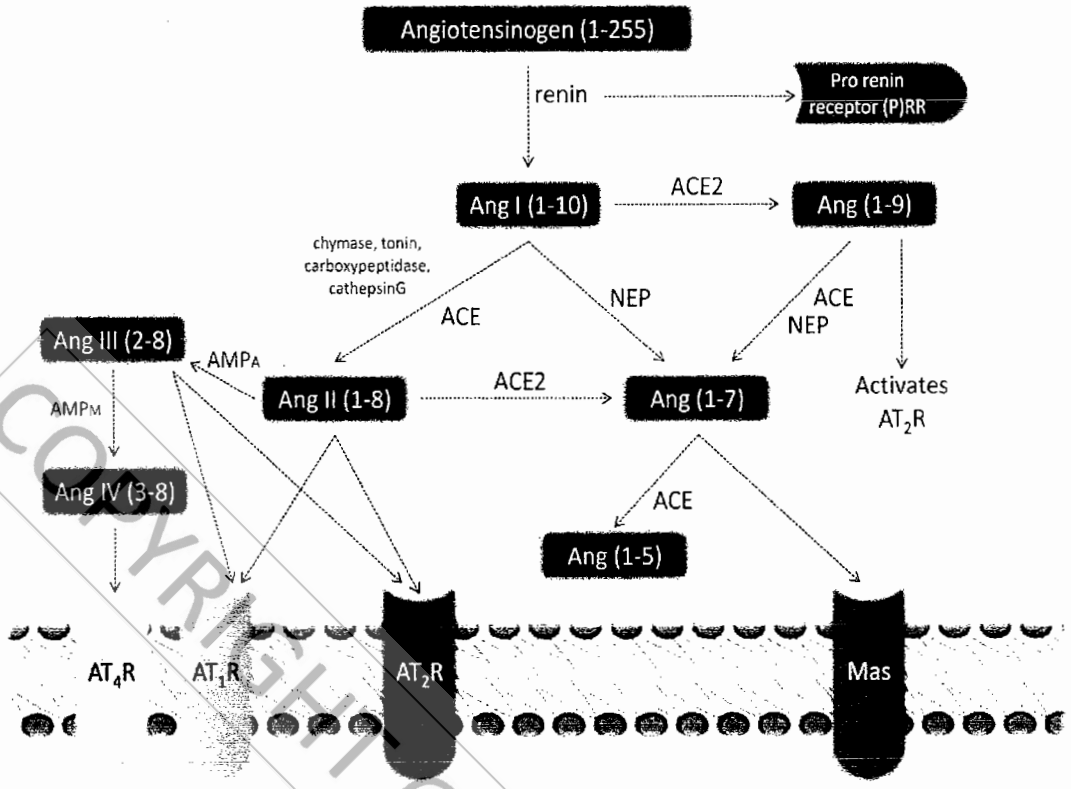


Figure 4: The complete renin-angiotensin cascade. AT_1R stimulation causes vasoconstriction, aldosterone release, hypertrophy, fibrosis and vascular remodeling. The pathophysiological effects of AT_1R stimulation are curtailed by AT_2R activation which activates NO°/cGMP pathway and inhibits MAPKs among other actions causing anti-proliferative, anti-inflammatory and vasodilatory effects. *Mas* receptor stimulation is analogous to AT_2R and promotes Akt phosphorylation, NO° release and vasodilation thus opposing the effects of AT_1R activation. The much less explored AT_4R stimulation by ang IV results in proinflammatory effects but its bearing upon the pathophysiology of human hypertension is yet to be known. ACE-Angiotensin Converting Enzyme; AMPA- Aminopeptidase M; AMPM-Aminopeptidase M; *Mas*- Meiosis activating sterol; NEP-Neutral Endopeptidase

Stimulation of the AT_1R results in activation of several tyrosine kinases which in turn phosphorylate the tyrosine residues in several proteins thus activating or inhibiting them. This results in vasoconstriction, cell growth and proliferation in blood vessels (Marrero *et al*, 1997; Dostal *et al*, 1997; Griendling *et al*, 1997). Activation of various signaling molecules like the small G-proteins (Ras, Rho, Rac, etc.), GPCR, second messengers, protein kinases, etc. are activated by AngII through the AT_1R (Robinson and Cobb, 1997) and contribute to vascular remodeling. Growth and migration of VSMCs in blood vessels is also mediated by AngII through *trans*-activation of EGFR (Greindling *et al*, 1994; Griendling *et al*, 2000). AngII mediated activation of the AT_1R stimulates PI3Kγ which ultimately activates the LTCCs in the smooth muscle cells leading to a contractile response. Akt or protein kinase B is a

signal molecule activated downstream to PI3Kγ (Saward and Zahradka, 1997; Wymann and Pirola, 1998; Takahashi *et al*, 1999a). It prevents the degradation of pore-forming subunit of the LTCC and thus maintains the Ca^{++}-influx and resulting contractile state (Ushio-Fukai *et al*, 1999). Pathological actions of angII in VSMCs implicate Rho and its effector Rho-kinases whereby the Rho-kinases phosphorylate the myosin subunit of myosin-light-chain-phosphatase (MLCP) leading to inhibition of the enzyme and maintenance of the smooth muscles in a contractile state (Loirand, 2006). Additionally, Rho-kinases negatively impact protein kinase A mediated phosphorylation of eNOS preventing the release of NO. AngII negatively regulates the NO signaling pathway and thus contributes to endothelial dysfunction (Ming, 2002; Lee, 2004). AngII is also linked to upregulation of p22phox mRNA in the vasculature. p22phox is a subunit of the NOX [NAD(P)H oxidases] group which are responsible for superoxide generation in the vasculature and contribute to oxidative vessel wall stress (Griendling *et al*, 1994; Pagano, 1998). AngII-dependent NOX activation leads to enhanced formation of $O2^{\cdot-}$, which readily reacts with NO forming peroxynitrite ($ONOO^-$) (Meier, 1996; Fukui, 1997). This reduces the available NO and enhances vasoconstrictor response of angII.

PHARMACOLOGICAL MANAGEMENT OF HYPERTENSION

Lifestyle modifications and dietary approaches to stop hypertension (DASH) (Sacks *et al*, 2001) are seldom effective in controlling blood pressure in hypertensive populations owing to non-compliance. It is almost always necessary to include drug-therapy as an appropriate measure to control blood pressure even in patients diagnosed with mild hypertension. Several agencies have drafted guidelines for the use of drugs in the management of hypertension and these guidelines are reinforced by data from landmark trials like the ALLHAT, UKPDS, INVEST and the HOPE trial among others (King *et al*, 1999; Sleight, 2000; Chrysant, 2003; Pepine *et al*, 2003). Although these reference guidelines may objectively provide information regarding the selection of appropriate class of agents from the vast therapeutic armamentarium, practical interpretations and patient-to- patient factors make pharmacologic therapy a subjective decision for the physicians and is ultimately individualized based on clinical judgment. An overview of the classes of medications typically utilized for the management of hypertension is given in Table 2. Diuretics, ACE-inhibitors and angiotensin receptor blockers form the first-line of therapy for any patient. Other agents may be added as required.

Several classes of novel antihypertensive agents have been approved in the recent past. Aliskiren, a rate-limiting inhibitor of renin in the RAAS, was approved in 2007 by the USFDA (Brown, 2008). It blocks the conversion of angiotensinogen to angI thus depleting the precursor for the formation of angII. However, the ALTITUDE trial (2011) concluded that aliskiren arm showed incidence of non-fatal stroke and renal impairment and thus appropriate contraindications were required to be added in the label of aliskiren (Parving *et al*, 2012). Other investigational renin inhibitors by Speedel Pharmaceuticals (Switzerland) and Vitae Pharmaceuticals (USA) are in different phases of clinical trials (Paulis and Unger, 2010). Clevidipine is a novel vasculo-selective calcium channel blocker indicated when oral therapy is not feasible (Ndefo *et al*, 2010). It was approved in 2008 and shows little effect on myocardial contractility. The ESCAPE clinical trials (ESCAPE-1 & 2) evaluated the efficacy of clevidipine in emergency settings where immediate reduction in blood pressure is necessary (Levy *et al*, 2007; Singla *et al*, 2008). The latest in class ARB, azilsartan medoxomil, was approved in 2011 for the treatment of essential hypertension (Jones *et al*, 2011). Levosimendan is an agent that has dual action, positive inotropy through calcium sensitization and vasodilatory action mediated by opening of ATP-sensitive potassium channels in vascular smooth muscles. Clinical studies have shown varying results with respect to its efficacy but it has been approved for use in patients with CVD accompanied by CHF (Antila *et al*, 2007; Packer *et al*, 2013). Another agent with a similar mechanism is nicorandil which has NO activation properties in addition to potassium channel opening action (Sakata *et al*, 2012; Wu *et al*, 2013). Other agents like cromakalim, rilmakalim and the likes were awaiting approvals from regulatory authorities (Gude, 2012). Rilmenidine, a preferential imidazoline receptor agonist, acts on the peripheral and medullary vasomotor structures thus mediating a sympatho-inhibitory effect and lowering blood pressure (Burke and Head, 2009).

Table 2: Oral antihypertensive drugs[2]

Sr. No.	Class	Prototypical Drug	Focal Mechanism	Major Adverse Effects	Clinical trials
1	Diuretics	Hydrochlorthiazide	Inhibition of ion transport proteins; ECF volume reduction	Hypokalemia	ALLHAT, PROGRESS
2	β-Blockers	Propranolol	Reduction of cardiac output; decreased renin release from JGA	Bradycardia, rebound hypertension	COPERNICUS, CAPRICORN, BHAT, UKPDS
3	Calcium channel blockers	Amlodipine	Reduce intracellular availability of Ca^{++}	Peripheral edema (DHP); Bradycardia (others)	INVEST, BEAT-HTN
4	ACE inhibitors	Captopril	Competitive inhibition of ACE and partial inhibition of AngII formation	Hyperkalemia, dysguesia, cough	SOLVD, HOPE, EUROPA, TRACE, UKPDS
5	Angiotensin receptor blockers	Losartan	Blockade of AT_1 receptor to prevent AngII mediated actions	Hyperkalemia	Val-HEFT, CHARM, LIFE, RENAAL
6	α_1-Blockers	Prazosin	Sympathetic blockade through α_1-blockade	Syncope, dizziness, palpitations	VHeFTI, ALLHAT
7	Aldosterone antagonists	Spironolactone	Prevent sodium/water retention	Hyperkalemia, gynecomastia	RALES, EPHESUS
8	Central α_2-agonists	Methyldopa	Stimulate α_2 receptors in the brain stem-decrease central sympathetic outflow	Transient sedation	POISE-2, ReHOT, BEAT-HTN
9	Direct vasodilators	Hydralazine	NO° mediated cGMP stimulation and/or K^+-channel activation	SLE	A-HeFT, BEAT-HTN

[2] Each class may have its own further sub-classifications which are not shown here. The clinical trials mentioned in the last column are suggestive of the class and not individual drugs *per se*. A-HeFT, African-American Heart Failure trial; ALLLHAT, Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial; BEAT-HTN, Black Education and Treatment of Hypertension study; BHAT, Beta-Blocker Heart Attack Trial; CAPRICORN, Carvedilol Post-Infarct Survival Control in Left Ventricular Dysfunction Trial; CHARM, Candesartan in Heart Failure Assessment of Reduction in Morbidity and Mortality Trial; COPERNICUS, Carvedilol Prospective Randomized Cumulative Survival Trial; EPHESUS, Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study; EUROPA, European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease Trial; HOPE, Heart Outcomes Prevention Evaluation Study; INVEST, International Verapamil-Trandolapril Study; LIFE, Losartan Intervention For Endpoint reduction in hypertension study; POISE-2, Perioperative Ischemic Evaluation study 2; PROGRESS, Perindopril Protection Against Recurrent Stroke Study; RALES, Randomized Aldactone Evaluation Study; ReHOT, Resistant Hypertension Optimal Treatment study; RENAAL, Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan study; SOLVD, Studies of Left Ventricular Dysfunction; TRACE, Trandolapril Cardiac Evaluation; UKPDS, UK Prospective Diabetes Study; Val-HeFT, Veterans Affairs Cooperative I study.

NOVEL THERAPEUTIC TARGETS FOR MANAGEMENT OF HYPERTENSION

In spite of a gigantic armamentarium of anti-hypertensive drugs and new drugs emerging every year, the personalized therapy for controlling hypertension has remained elusive. Several molecular modulators of hypertension have been identified in the past decade. These modulators offer several targets for a precise control of blood pressure. Myriad targets are currently being pursued experimentally as well as clinically to reinforce the management of hypertension. ACE cleaves the octapeptide angiotensin II to form ang(1-7) and angiotensin I to ang(1-9) (Donoghue *et al*, 2000; Tipnis *et al*, 2000). Several studies have shown cardioprotective role of Ang (1-7). It has been shown that systemic injection of Ang (1-7) leads to systolic BP reduction in SHRs. It has been shown that Ang (1-7) exclusively acts on the *Mas* receptor and a possibility of Ang (1-7) acting on either AT_1 or AT_2 receptor has been excluded. Downstream effects of *Mas* receptor involve phosphorylation of eNOS suggesting the role of Ang (1-7) in endothelium-dependent relaxation. Several formulations of Ang (1-7) including HPβCD-Ang (1-7) (Lula *et al*, 2007), liposomal Ang (1-7) (Silva-Barcellos *et al*, 2004) and cyclised Ang (1-7) (Kluskens *et al*, 2009) have been reported to improve the efficacy of Ang (1-7) for clinical application. AVE 0991 was the first synthetic non-peptide compound found to have agonistic activity on the *Mas* receptor. Two novel peptides, CGEN-856 and CGEN-857, were discovered via computational drug discovery platform. These peptides are unrelated in structure to Ang (1-7) and have high specificity for the *Mas* receptor (Shemesh *et al*, 2008). These peptides show a dose-dependent blood pressure reduction and their activity is inhibited by *Mas* antagonist, A-779. 1-[[2-(Dimethylamino)ethyl]amino]-4-(hydroxymethyl)-7-[[(4-methylphenyl)sulfonyl]oxy]-*9H*-xanthen-9-one (commonly referred to as *'xanthenone'*) and resorcinonaphthalein have been reported to enhance the levels of endogenous ACE2. While acute administration reduced blood pressure in both SHR and normotensive rats, chronic administration of these compounds reduced blood pressure only in SHRs (Savergnini *et al*, 2010; Savergnini *et al*, 2013). Since aldosterone antagonists are fraught with adverse effects (Maron and Leopold, 2010), an alternative strategy would be the prevention of aldosterone formation itself. This requires inhibition of the enzyme, aldosterone synthase, also known as CYP11B2 (Azizi *et al*, 2013). Thus, analogous to the ACE/AngII pathway, it will prevent formation of aldosterone and its

consequent actions. However, in slight contrast to the ACE/AngII formation, aldosterone is not formed by alternative enzymes thus reactive increase in aldosterone levels upon AngII stimulation would not be possible. FAD286, an enantiomer of fadrozol (Novartis, Switzerland) was first shown to lower blood pressure in rats over-expressing renin and angiotensinogen through inhibition of aldosterone synthase. Other investigational molecules, SPP2745 (Speedel Pharmaceuticals, Switzerland) and LCI699 (Novartis, Switzerland), were also found to have positive effects on kidney and heart owing to aldosterone inhibition in experimental studies (Fiebeler *et al*, 2005; Schumacher, 2013; Lea *et al*, 2009; Bertagna *et al*, 2014). The major reason to pursue aldosterone synthase inhibitors is to find alternatives for preventing the effect of aldosterone on cardio-renal physiology so that the novel agents/targets are non-inferior in efficacy and better tolerated as compared to currently used aldosterone antagonists. Cortisol, a physiological steroid, also activates mineralocorticoid receptors in altered redox states. This effect stands in the way of efficacy of aldosterone synthase inhibitors. Transient receptor potential (TRP) channels are part of a superfamily of cation channels that are formed by tetramers of six transmembrane domain subunits (Firth *et al*, 2007). The vanilloid type 1-TRP channels (TRPV1) are activated by several chemical and physical stimuli. The cation permeable pore of TRPV1 senses activation of GPCRs like adrenoceptors or AT_1R or stretch response (mechanotransduction) in the vascular smooth muscle cells, endothelial cells or pulmonary artery smooth muscle cells. This leads to increased influx of Ca^{++} within the cell. This increased $[Ca^{++}]_i$ has two actions: 1) it causes a Ca^{++} dependent increased activation of protein kinase A through phosphorylation and 2) it diminishes the inhibitory interaction between eNOS and caveolin-1. Displacement of caveolin-1 leads to binding of calmodulin with eNOS, which makes it a dock-site for Hsp90. Once Hsp90 binds to eNOS, the activated protein kinase phosphorylates the 1177 serine residue (1176 in mice) of human eNOS leading to its activation. This leads to a marked increase in formation of NO which causes guanylate cyclase mediated reduction of blood pressure (Sessa, 2010). TRPV1 channel is activated by capsaicin, a component of red-hot chilli peppers; rutaecarpine (a plant alkaloid) also activates TRPV1 (Deng and Li, 2005; Yang *et al*, 2010). Capsazepine, a TRPV1 channel blocker, opposes the action of TRPV1 activation (Yang *et al*, 2010).

RTX (resiniferatoxin) is an ultrapotent analog of capsaicin (structure shown below; Figure 5).

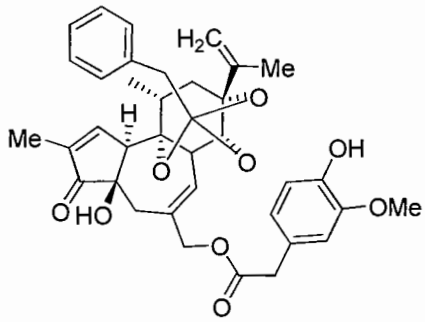


Figure 5: Resiniferatoxin (RTX) is a plant alkaloid obtained from the plant Euphorbia *resinifera*. It is an ultr-apotent analog of capsaicin and activator of TRPV1. It is an important pharmacological tool to study the effects of TRPV1 activation *in vivo*.

Iodinylated RTX, iRTX, is a potent blocker of TRPV1 channel (Sun *et al*, 2009). Based on previous studies it seems that activation of TRPV1 can prove to be useful in reducing the blood pressure. The angiotensin-independent effects of renin are mediated, in part, by binding to the pro-renin receptor, (P)RR (Nguyen *et al*, 2002). Activation of (P)RR mediates several effects that may not be favorable to the pathophysiology of hypertension like upregulation of PAI-1, TGFβ1, enhanced protein synthesis, cell proliferation and a reduction in apoptosis (Schefe, 2008; Huang *et al*, 2007). None of the currently available agents affecting RAAS is known to influence the (P)RR pathway. Investigational peptidic molecules that prevent binding of prorenin to (P)RR have indicated that blockade of (P)RR improves left ventricular function, reduces cardiac hypertrophy and fibrosis, and also prevents nephropathy (Ichihara *et al*, 2006; Susic *et al*, 2008). It is thus speculated that non-peptidic small molecule inhibitors of (P)RR may prevent the angiotensin-independent effects of renin and RAAS activation. Renalase, a flavoprotein, is known to be expressed strongly in the renal and cardiac tissue (Xu *et al*, 2005; Desir and Peixoto, 2014). It is an NAD(P)H oxidase, NOX, that effectively metabolizes catecholamines. Several studies in the recent past have demonstrated the involvement of NAD(P)H oxidase in different types of hypertension, renal and non-renal. The role played by renalase is depicted in Figure 6 below:

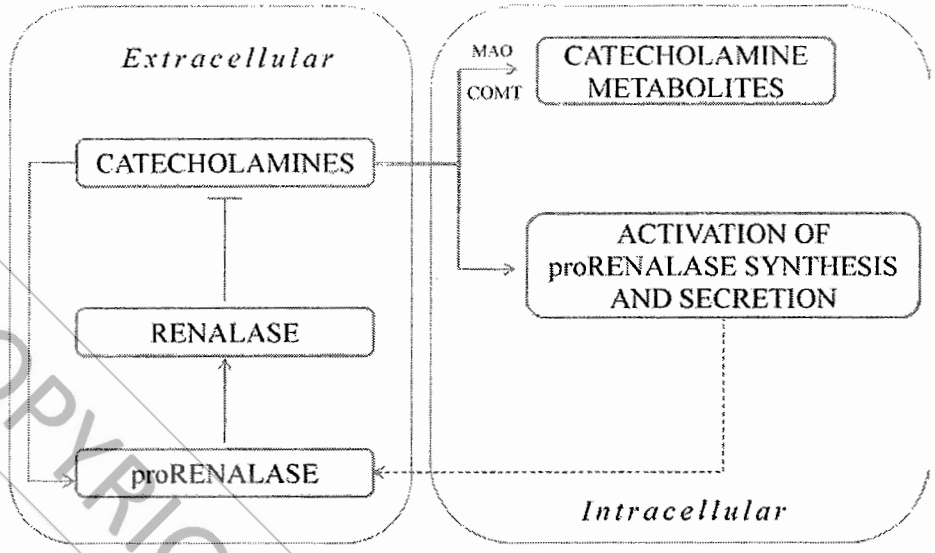


Figure 6: The physiology of renalase and its effect on catecholamine regulation. MAO, Monoamine Oxidase; COMT, Catechol-O-Methyl Transferase.

Renalase deficiency has been associated with intensified sympathetic tone and cardiovascular risk (Desir, 2009). It has been speculated that renalase-replacement therapy may improve cardiovascular outcome in patients suffering from hypertension. Vasopeptidase inhibitor is another very important target being pursued in the management of hypertension. Vasopeptidases, apart from ACE, involve other enzymes like the membrane-bound metalloproteinase, Neutral Endopeptidase (NEP or Neprilysin) and Endothelin-converting enzyme (ECE). Though NEP substrates might be vasodilatory or vasoconstrictive in nature, the prospect of successfully designing dual NEP/ACE- and triple NEP/ACE/ECE-inhibitors as a single molecule has motivated research in this direction (Gude, 2012). Ompatrilat, the prototypical NEP/ACE dual inhibitor, was shown to reduce blood pressure in experimental and clinical studies. Major trials like OCTAVE and OVERTURE showed that dual NEP/ACE inhibition might be effective in the management of hypertension (Packer *et al*, 2002; Kostis *et al*, 2004). Other agents like sampatrilat and fasidotril were also investigated. However, data from clinical studies raised concerns regarding angioedema. Safety profiles have remained an issue with these classes of agents. Instead of ACE inhibition, alternative strategy of combined NEP inhibition and AT_1R blockade is being chased to improve the safety profile of NEP inhibitors (Jandeleit-Dahm, 2006). Agents like ilepatril (Sanofi-Aventis, France) and daglutril (Solvay,

Belgium) are a few agents currently undergoing clinical trials to study the efficacy of NEP inhibition with or without AT_1R blockade (Paulis and Unger, 2010). NO donors and activators are being explored and newer alternatives keep emerging. These include soluble guanylate cyclase (sGC) activators like cinaciguat and riociguat, NO releasing agents like naproxcinod which is actually naproxen with NO releasing property (Merck, USA & Valbonne, France) and NCX-899 which is NO releasing enalapril (Valbonne, France) (Miller and Megson, 2007; Paulis and Unger, 2010). The tirade of biological therapeutics has penetrated in the management of hypertension as well, and as a result several biological products are being researched and tested upon for their potential as anti-hypertensive agents. CYT006-AngQb, a vaccine against AngII is technically made up of virus-like particles coupled to AngII (Tissot *et al*, 2008). Subcutaneous injection of this vaccine produces an immunological response equivalent to injection of foreign proteins in the systemic circulation and leads to production of antibodies against AngII. The preliminary idea behind this vaccine was to develop a safe and effective vaccine to reduce non-compliance in antihypertensive therapy (Brown, 2009; Maurer and Bachmann, 2010). However, another concern with this type of therapy is that the effects of the vaccine cannot be reversed or stopped easily. With these speculations, CYT006-AngQb underwent clinical trials in 2008 (Tissot *et al*, 2008) and showed mild reduction in blood pressure which was not comparable, infact, was inferior to ACE inhibitors and ARBs. It was also observed that blood pressure reduction was not reproducible across the dosing schemes that were detailed in the trial protocols and as a result progress of CYT006-AngQb to phase III was halted (Petrovsky, 2013). ATR12181 is another product which targets AT_{1A} receptor (Ming *et al*, 2006; Zhu *et al*, 2006). Similar vaccines with modified immunogenicity and different adjuvants are being investigated as potential future therapies. This discussion is by no means exhaustive and with several new therapies being investigated day-in and day-out, different therapeutic strategies would have surfaced at the time when this text is circulated.

PI_3K/Akt AS A POTENTIAL TARGET FOR ANTIHYPERTENSIVE THERAPY

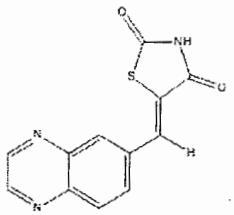
Constriction of vascular smooth muscle cells (VSMCs) as a result of increased myogenic tone, is one of the key elements that is responsible for maintenance of blood pressure (Guyton and Hall, 2006). This alteration of myogenic tone is not dependent on

neural mechanisms for blood pressure control and can be described due to the followings: 1) increased levels of intracellular calcium in the cell mediated through L-type calcium channels (LTCCs), 2) constriction in response to the increased levels of intracellular calcium and, 3) since the wall of the artery is unable to handle this tremendous pressure built within the VSMCs, there is forced dilation (Hill *et al*, 2006). Several pathways have been explored in the past to identify the agents or signals involved in the regulation of myogenic tone (Morello *et al*, 2009). Recently, phosphoinositide 3-kinase γ ($PI_3K\gamma$ for short) has been found to be a strategic regulator of vascular myogenic tone. PI_3Ks are implicated in several pathways related to varied responses in a cell. PI_3Ks, being kinases, phosphorylate the 3'-OH group of inositol ring in the PtdIns(4,5)P_2 (phosphatidyl inositol bis phosphate) molecule. This phosphorylation results in the formation of PtdIns(3,4,5)P_3 (inositol tris phosphate) which can be metabolized further (Morello *et al*, 2009; Vanhaesebroeck *et al*, 2010). The PI_3Ks have been classically divided into three classes: Class I, Class II and Class III (Katso *et al*, 2001, Carnevale *et al*, 2012a). The PI_3K of our interest, $PI_3K\gamma$ (EC 2.7.1.153) belongs to Class IB and has been believed to be restricted to leukocytes. However, some recent evidence suggests that $PI_3K\gamma$ has avenues outside the immune system as well and is also present in the cardiovascular system in general and in cardiomyocytes, VSMCs, vascular endothelial cells and platelets in particular (Oudit, 2004; Morello *et al*, 2009).

$PI_3K\gamma$ is the kinase that is typically activated upon activation of GPCRs (Macrez *et al*, 2001; Carnevale *et al*, 2012a). As stated previously, since $PI_3K\gamma$ is present in vascular smooth muscle cells and it was found to be a regulator of myogenic tone, it would not be an exaggeration to suspect its role in maintaining a delicate balance between vasoconstriction and vasorelaxation. Such vasoactive phenomenon may be induced by multitude of GPCR agonists/antagonists. Different researchers have already shown the role of $PI_3K\gamma$ in increased smooth muscle contractility mediated through angII (Carnevale *et al*, 2012b). Thus $PI_3K\gamma$ mediates the downstream signaling effects of the AT_1 receptors, specifically vasoconstriction as the end-result. $PI_3K\gamma$, upon stimuli received by activation of AT_1Rs (a GPCR coupled to G_q type of G-Protein) excites the L-type calcium channels in smooth muscle cells, thereby increasing intracellular calcium to such high levels that they cross the contractile threshold and result in a contractile response (Macrez *et al*, 2001;

Viard *et al*, 2004). This effect was confirmed using specific monoclonal antibodies against $PI_3K\gamma$ where $PI_3K\gamma$ blockade in rat portal vein myocytes inhibited the ang-II mediated downstream IP_3 production and ensuing calcium influx (Quignard, 2001; Viard *et al*, 2004). Another important aspect of L-type channel function is its degradation. The pore-forming subunit of the LTCC ($Ca_v\alpha 1c$) is susceptible to proteolytic degradation in an unphosphorylated state. But one of the agents, acting further downstream to that of $PI_3K\gamma$, Akt (the name of this molecule bears no functional importance since it was derived from the T-cells of an Ak strain of mice, hence Akt), phosphorylates this subunit and thus protects it from proteolytic degradation (Quignard, 2001). All these findings found pillars of support when mice lacking $PI_3K\gamma$ showed protection against chronic angII exposure and angII evoked L-type Ca^{++} influx (Carnevale *et al*, 2012b). Akt stands as a link between $PI_3K\gamma$ and LTCC in resistance arteries, regulating the increased myogenic tone upon stimulation (Shiojima and Walsh, 2002). Another very interesting effect mediated through $PI_3K\gamma$ signaling is that of "mechanotransduction". Patel *et al* have shown that even in the absence of angII, pressure exerted on the receptors may activate the AT_1R and could activate $PI_3K\gamma$ through $\beta\gamma$ signaling. Thus, modulation of the myogenic response mediated by $PI_3K\gamma$ may proceed in a ligand-dependent or independent approach (Patel *et al*, 2010). A point of concern that needs address at this juncture is that whether ARBs (Angiotensin Receptor Blockers, "*sartans*") fail to achieve adequate control of blood pressure due to this effect? In light of these findings, inhibition of $PI_3K\gamma$ and its downstream Akt signaling offers lucrative targets to relieve increased myogenic tonus and cause relaxation (Yang and Raizada, 1999; Kippenberger *et al*, 2005; Li and Malik, 2005a). Inhibition of kinase-dependent $PI_3K\gamma$ signaling and its downstream Akt signaling were found to markedly impair the myogenic tone in isolated vessels (Kippenberger *et al*, 2005; Carnevale *et al*, 2012b). Another finding of high-repute in this regard is of the fact that inhibition of $PI_3K\gamma$ signaling impairs accessory subunit ($Ca_v\beta 2a$) phosphorylation and thus decreases the probability of opening of LTCC (Viard *et al*, 2004). In the past decade, several small-molecule inhibitors of $PI_3K\gamma$ have been developed and explored in cardiovascular studies. Two small molecules are available commercially, though not for therapeutic use (Carnevale *et al*, 2012b):

- AS605240 (Cayman chemical, Sigma aldrich, Biovision)
- GE21 (under patent with IRCCS Neuromed, Italy)



(AS605240)

The antihypertensive effect of these inhibitors is manifested as: reduction of total peripheral resistance and counter action of development of myogenic tone, thus relaxing resistance arteries (Morello *et al*, 2009; Carnevale *et al*, 2012b). Based on the information available on the subject of $PI_3K\gamma$ specific inhibition, we can certainly build up on the premise that $PI_3K\gamma$-inhibition is a promising strategy to treat hypertension (Takahashi *et al*, 1999a; Dugourd *et al*, 2003). Recent genetic evidence in humans supports the association of $PI_3K\gamma$ to blood pressure regulation (Carnevale *et al*, 2012a) and thus inhibition of this pathway may be considered as a promising tool against hypertension.

THE ANGIOTENSIN RECEPTOR *TYPE 1*

The peptide angiotensin II (angII) mediates its effects through the angiotensin receptors in mammalian cells. Two types of receptor subtypes have been cloned and characterized: angiotensin receptor type 1, AT_1 and angiotensin receptor type 2, AT_2 (Murphy *et al*, 1991; Sasaki *et al*, 1991; Mukoyama *et al*, 1993). The AT_3 and AT_4 receptors are not fully characterized as of now (Wright and Harding, 1997; Krebs *et al*, 1996). All the subtypes, except AT_4, are known to bind angiotensin (Chaki and Inagami, 1992). Most of the perceptible actions of AngII are mediated through the type 1 receptor (IUPHAR Receptor Code ***2.1.Ang.01.000.00.00***). Experimental studies are yet dissecting the role of AT_2 receptors in different physiological functions (Dinh *et al*, 2001). The AT_1 receptor is coded by the human gene AGTR1 (a.k.a *at1*), located on chromosome 3q24. The gene sequence shares ~90 % sequence homology with rodent *agtr1* gene coding for the same protein [www.ensembl.org]. The human AT_1 receptor gene product consists of 359 amino acids with a molecular mass of about 41 kDa (Dinh *et al*, 2001). The AT_1 receptor belongs to the superfamily of GPCRs and consists of seven transmembrane units. Location of four cysteine residues in the extracellular domain results in formation of disulphide bridges which govern the tertiary structure of the receptor. The extracellular loops and the transmembrane domain (TMD) provide the site for binding of AngII. This receptor is

recognized by its ability to bind antagonistic ligands like losartan and the type, however, the antagonist binding site differs from the AngII-binding site (Dinh *et al*, 2001; Higuchi *et al*, 2007). Predominant expression of this receptor can be found in the liver, adrenal glands, kidney, vascular smooth muscles and lungs. Acutely increased levels of AngII are known to activate AT_1R, however, chronic exposure to AngII may lead to downregulation of the receptor (Lassegue *et al*, 1995; Griendling *et al*, 1996). While insulin and LDL are known to upregulate AT_1R (Nickenig *et al*, 1997a; Nickenig *et al*, 1997b); AngII, estrogen, EGF and PDGF are known to downregulate it (Gunther *et al*, 1980; Nickenig *et al*, 1996; Takeda *et al*, 2000). The effects of AngII upon target tissues are found to be momentary; the reason for this is the endocytosis of this receptor within 10 mins of its activation. One-fourth of the endocytosed receptors are then recycled back to the plasma membrane while the remaining are degraded by the lysosomes (Gunther *et al*, 1980). Phosphorylation of serine and threonine residues present on the C-terminal end of the cytoplasmic surface of AT_1R plays an important role in internalization of these receptors. This effect is mediated, in part, by caveola (Ishizaka *et al*, 1998). Intrinsic kinase activity is not present in any GPCR, but G-protein receptor kinases (GRKs) are responsible for phosphorylation of the serine and threonine residues (Oppermann *et al*, 1996). The AT_1R is also phosphorylated at various tyrosine residues and various tyrosine kinases like JAK, FAK and Src family kinases are known to arbitrate such actions (Oppermann *et al*, 1996; Kim *et al*, 2005).

Signaling pathways

The AT_1R being a GPCR, is coupled to the $G_{q/11}$ protein for effecting any downstream signal. This protein $G_{q/11}$ in turn mediates activation of second messenger systems like the IP_3/DAG pathway activated through phospholipase Cβ (Touyz and Schiffrin, 2000). Other non-G-protein related pathways are also activated upon AT_1R stimulation. Phospholipase Cβ cleaves PIP_2 leading to the formation of IP_3 and DAG (Alexander, 1985; Griendling *et al*, 1989; Touyz and Schiffrin, 2000). IP_3 binds to its receptor present on the sarcoplasmic reticulum leading to release of calcium from intracellular stores. This calcium forms a complex with calmodulin to activate MLCK which phosphrolyates the light chain of myosin to enhance its interaction with actin filaments (Touyz and Schiffrin, 1997; Touyz *et al*, 1999). This effect leads to smooth

muscle contraction and is predominantly observed in vascular smooth muscle cells. Normally, this effect is terminated by MLCP, which dephosphorylates myosin and thus its interaction with actin is abolished but Rho kinase inhibits MLCP leading to increasing contractions. On the other hand, DAG activates protein kinase C (PKC), which phosphorylates the Na^+/H^+ pump and increases the cellular *p*H during contraction phase which helps in sustaining the effect. Increased alkalinization of the cell retains $[Na^+]_i$ and thus increases $[Ca^{++}]_i$, leading to sensitization of the contractile apparatus to Ca^{++} and increased intracellular *p*H (Tepel *et al*, 1998; Touyz *et al*, 1999), potently stimulates DNA synthesis leading to cell growth and proliferation (Damron *et al*, 1998; Touyz *et al*, 1999). Thus by activating the Na^+/H^+ pump, AT_1R activation ultimately modulates VSMC contraction and growth (Touyz and Schiffrin, 1997; Touyz *et al*, 1999). DAG also contributes in the Ras/Raf/MEK/ERK pathway and exhibits activation/inactivation of different proteins through phosphorylation, the downstream molecules of which contribute in vasoconstriction (Kusuhara, 1998; Touyz *et al*, 1999). Agonist binding at AT_1R leads to activation of PLD which is responsible for hydrolysis of phosphatidylcholine to choline and phosphatidic acid. Phosphatidic acid is rapidly converted to DAG leading to activation of PKC and subsequent effects mediated through DAG (Alexander *et al*, 1985; Griendling *et al*, 1989). The AT_1R also leads to phosphorylation and activation of PLA_2, which results in the formation of arachidonic acid. Metabolites of arachdonic acid are known to regulate vascular tone and NAD(P)H oxidation of VSMCs. AT_1R activation also mediates stimulation of growth and migration related signaling, through a myriad of downstream proteins, which is above and beyond VSMC contraction (Griendling *et al*, 2000). The non-G-protein pathways include NAD(P)H and ROS signaling, MAP kinase activation, and stimulation of receptor & non-receptor tyrosine kinases (Griendling and Ushio-Fukai, 2000; Griendling *et al*, 2000).

AT_1R and PI_3K/Akt signaling

Phosphoinositide-3-kinases (PI_3Ks), a family of intracellular signal transducers, is also activated through AT_1 receptors. In vascular smooth muscle cells and cardiomyocytes, AngII mediates the activation, phosphorylation and migration of PI_3K through AT_1R activation. It also induces the translocation of the p85 subunit of PI_3K in various regions of

the cell from the perinuclear area to the foci, throughout the cytoplasm and to the cytoskeletal proteins (Saward and Zahradka, 1997). This action of AngII is reported to peak around 15 mins after binding at AT_1R and then subsides to baseline levels at 30 mins. It has been shown that small molecule inhibitors of PI_3K, wortmannin and LY294002 block AngII mediated hyperplasia and contraction in smooth muscle cells. A couple of years ago, Akt (protein kinase B), was identified to be an important downstream molecular target of PI_3K stimulation in VSMCs (Saward and Zahradka, 1997; Wyman and Pirola, 1998). It is not clear how exactly AT_1R activation leads to PI_3K-dependent Akt activation but it is suggested that it may involve redox-sensitive pathways and c-Src (Ushio-fukai *et al*, 1999). Akt is associated with VSMC proliferation and growth. Akt is known to regulate protein synthesis through activation of p70S6 kinase (Eguchi *et al*, 1999) and stimulates Ca^{++} currents in aortic smooth muscle cells, thus modulating AngII mediated contractions (Eguchi *et al*, 1999; Seki *et al*, 1999). AngII mediated activation of arachidonic acid catabolism generates metabolites like 5(S)- and 12(S)-hydroxyeicosatetraenoic acid through LOX, which activates Akt (Neeli *et al*, 2003; Moreno, 2009). PLD activation in CHO cells overexpressing endothelial differentiation receptor is known to activate Akt (Banno *et al*, 2001). However, it is not clear whether the same effect can be observed in VSMCs upon AngII mediated PLD activation. At present, it is speculated that AngII mediated Akt activation in VSMCs may be PLA_2- or PLD-dependent (Banno *et al*, 2001; Li and Malik, 2005). A peculiar effect observed with Akt is that it is known to inhibit caspases and influences Bcl-2 and c-myc expression, thus preventing apoptosis and promoting cell survival. Thus, phosphorylated and activated Akt is known to stimulate several downstream negotiators like Bad, GSK3, eNOS and mTOR among others which regulate events ranging from cell survival, cell cycle and protein synthesis to angiogenesis and vasomotor tone (Matsui *et al*, 2001; Shiojima and Walsh, 2002; Sata and Nagai, 2002; Downward, 2004).

THE ALPHA1 (α_1) ADRENERGIC RECEPTOR

The α_1-adrenergic receptors belong to the superfamily of 7TM-GPCRs and are responsible for most of the actions of noradrenaline *in vivo* and several physiological actions of adrenaline as well (Garcia-Sainz, 1993; Cotecchia, 2010). Molecular cloning has

revealed that the α_1-adrenergic receptor has three subtypes - α_{1A}, α_{1B} and α_{1D} in total (Schwinn *et al*, 1995; Graham *et al*, 1996) and all the three were found to have different amino acid sequences and consequently variable pharmacological actions (Table 3). All the receptors are known to bind to adrenaline and noradrenaline unequivocally, though they are also known to bind other synthetic agonists like phenylephrine and oxymetazoline selecti-

Table 3: IUPHAR data for the different alpha1-adrenergic receptor subtypes[3]

Receptor subtype	Gene symbol	Chromosomal loci	IUPHAR Code	Amino acid sequence length	Preferential human tissue distribution
Alpha1a (α_{1A})	ADRA1A	8p21-p11.2	2.1.ADR.A1A.000.00.00	466	Prostate, urethra, heart, liver, vasculature, cerebellum and cerebral cortex
Alpha1b (α_{1B})	ADRA1B	5q23-q32	2.1.ADR.A1B.000.00.00	519	Prostate, cervix, uterus, umbilical vein, spleen and coronary endothelium
Alpha1d (α_{1D})	ADRA1D	20p13	2.1.ADR.A1D.000.00.00	572	Aorta, bladder, iliac and femoral arteries

[3] Information retrieved from the family menu given at http://www.iuphar-db.org/DATABASE (Accessed on 27th Aug, 2014). This is the official website of the International Union of Basic & Clinical Pharmacology.

vely (Schwinn *et al*, 1991; 1988; Garcia-Sainz *et al*, 1992; Garcia-Sainz, 1993; Cotecchia *et al*, 2000). The hydrophobic transmembrane loops form the α-helices that are connected by alternating extracellular and intracellular hydrophilic loops. The hydrophobic regions form the ligand binding sites whereas the intracellular hydrophilic regions are responsible for interacting with G-proteins, signaling molecules and regulatory proteins (Wess, 1997; Bylund, 2007).

Signaling and regulation of α_1-adrenergic receptors

For the most part, actions of ligands interacting with the α_1-adrenergic receptors are elaborated through interaction with $G_{q/11}$ resulting in stimulation of phosphoplipase C activity and thus promote the hydrolysis of PIP_2 leading to the formation of IP_3 and DAG (Garcia-Sainz, 1993; Zhong and Minneman, 1999; Cotecchia, 2010). The details of this signaling are mentioned in *'The angiotensin receptor type 1'* section above. The three subtypes couple to phospholipase C with different levels of efficacy, with α_{1A} having the highest and α_{1D} the lowest (Theroux *et al*, 1996; Taguchi *et al*, 1998). The three subtypes also couple to other G-proteins at varied degrees and show myriad actions like induction of cardiac hypertrophy by interacting with $G_{12/13}$ (Hawrylyshyn *et al*, 2004). The resultant activation of proteins leads to mobilization of calcium from intracellular stores. In addition, these receptors also activate the influx of calcium through VDCCs and voltage-independent calcium channels (Muth *et al*, 1999; Petrasehvskaya *et al*, 2004). α_1-Adrenergic receptors also signal through the kinases of the MAPK family thus regulating the growth promoting effects of these kinases. MAPK signaling activated upon α_1-adrenergic receptor stimulation contributes to increased DNA synthesis and cellular proliferation in human VSMCs (Hu *et al*, 1999; Chang and Karin, 2001). In fibroblasts, cell proliferation and growth mediated through different JNKs (46 and 54 KDa), p38 MAPK is also mediated by α_1-adrenergic receptor stimulation (Alexandrov *et al*, 1999; Piascik and Perez, 2001; Clerk *et al*, 2001). It has also been shown that α_{1B} mediates cell-cycle progression while the other two subtypes mediate cell cycle arrest in fibroblasts. In hepatocytes, interleukin-6 signaling is inhibited through α_1-adrenergic receptor mediated MAPK activation (Auer *et al*, 1998; Nguyen and Gao, 1999). Stimulation of predominant subtypes (α_{1A} and α_{1B}) in cardiomyocytes *in vitro*, results in a hypertrophic response which in turn is accompanied by activation of

hypertrophic genes like *c-jun, c-fos* and *egr-1*(Iwaki *et al*, 1990; Knowlton *et al*, 1993). Also, there is an upregulation of contractile proteins like MLC-2 and reactivation of embryonic genes like ANF, myosin and actin (Iwaki *et al*, 1990; Knowlton *et al*, 1993; Cotecchia, 2010).

The regulation and desensitization of different α_1-adrenergic receptor subtypes follow contradictory patterns. The regulation of α_{1A} receptor subtypes is quite reticent and it undergoes, if at all, very modest agonist-induced endocytosis and recycling. In stark contrast, the α_{1B} receptor undergoes rapid phosphorylation by GRKs followed by desensitization and endocytosis when stimulated by an agonist (Diviani *et al*, 1996; Diviani *et al*, 1997; Stanasila *et al*, 2008). With the α_{1D} receptor, noradrenaline and protein kinase C mediated phosphorylation is connected to the desensitization of the receptor (Garcia-Sainz and Villalobos-Molina, 2004). β-Arrestin also plays a significant role in downregulating the effects of α_{1A} receptor, while α_{1B} receptor shows only weak interaction with β-arrestin (Stanasila *et al*, 2008).

Correlation of α_1 adrenoceptor subtypes with VSMC contraction

Previously, it was shown that α_1 receptor mRNA and receptor proteins were expressed on the peripheral arteries of the test species (rats or humans). After the subdivision of the α_1 adrenoceptor subtypes, question arose as to which vascular bed houses which kind of α_1 adrenoceptor subtype? Based on several studies, it was demonstrated that each vascular bed is host to one dominant α_1 adrenoceptor that mediates contraction (Yamada *et al*, 1980; Jones *et al*, 1985). Though there is a dearth of acutely specific receptor-subtype ligands, experiments utilizing different tissue preparations have been utilized to coherently demonstrate that the α_{1A} adrenoceptor mediates contraction of caudal and renal arteries whereas the α_{1D} adrenoceptor mediates aortic smooth muscles, mesenteric, femoral and iliac arteries (Piascik *et al*, 1995; Piascik *et al*, 1997; Hrometz *et al*, 1999). The α_{1B} adrenoceptor subtype is least involved in mediating contraction of any kind of vasculature. This is also evident from the study where phenylephrine (pan-specific α_1 adrenoceptor agonist) was shown not to have any pressor response in α_{1B} adrenoceptor knock out mice (Cavalli *et al*, 1997). Alternatively, overexpression of the α_{1B} adrenoceptor did not result in an increased systemic blood pressure thus confirming the paucity of this

subtype in regulating blood pressure (Zuscik *et al*, 2001). Similar studies were performed to evaluate the effects of such molecular and genetic manipulations on blood pressure. Rokosh and Simpson (2002) reported that α_{1A} adrenoceptor knock out mice show lower MAPs as compared to controls and their responses to phenylephrine are also understated. Similarly, α_{1D} adrenoceptor knock out mice did not show any changes in basal blood pressure values but the vasopressor response was impaired (Piascik and Perez, 2001). Overall, it may be concluded that in case of vascular contractility the dominance can be given as follows: $\alpha_{1A} > \alpha_{1D} >>> \alpha_{1B}$ (Piascik and Perez, 2001). The predominance of the α_{1D} subtype in major arteries suggests that this is the most significant receptor involved in maintenance of vascular tone. By using selective antagonists of this subtype, it was shown that pressor responses induced by neurogenic stimulation or by α_1 adrenergic agonists can be blocked, suggesting the functional role of α_{1D} receptors in development of vascular resistance (Tanoue *et al*, 2002). Catecholamines are known to induce proliferation, apart from contraction, in vascular smooth muscle cells. RASM cells show expression of different α_1 adrenoceptor subtypes involved in smooth muscle proliferation (Yu et al, 1996; Ulu *et al*, 2010). Prolonged stimulation of the α_1 adrenoceptors (by non-catecholamines as well), is known to increase the mRNA expression of α-actin, signifying the augmentation of the contractile phenotype of these cells. α_{1D} and α_{1B} receptors are supposedly the mediators of such a response. Chronic exposure to α_1 adrenergic agonists induces a hypertrophic response in these vascular smooth muscles with a concomitant rise in the volume and accumulation of contractile proteins (Bishopric *et al*, 1987; Long *et al*, 1989). α_{1A} Adrenoceptors are known to activate signaling pathways that are responsible for increasing the size of murine ventricular myocytes (Papay *et al*, 2013). Signaling is mediated through different kinases like PKC, PI3K, MAPK and the Ras proteins acting in concert with each other. Some signaling events of the different adrenoceptor subtypes may be contradictory to each other thus resulting in a cross talk; like activation of α_{1B} subtype inhibits signaling mediated through the α_{1A} subtype (Rossier *et al*, 1999). These phenomena are important in relation to hypertension since increase in blood pressure as well as upholding of increased blood pressure in SHRs involve hypersensitivity of vascular smooth muscles to α_1 adrenoceptors. Stimulation may either lead to increase in proportion of receptors that are able to respond to increased levels of catecholamines or there may be an

increase in the post-receptor events that mediate α_1 adrenoceptor responses in hypertension. It has been suggested that expression of α_{1D} receptor is increased in organs harvested from SHR or animals from other models of hypertension (Villalobos-Molina *et al*, 1999; Villalobos-Molina *et al*, 2008), however, radioligand binding studies have failed to detect these receptors (Hosoda *et al*, 2005). This paradoxical result raises doubts regarding the functional role of the α_{1D} subtype in development and maintenance of hypertension, yet the abundance of these receptors found from rat and human aortic smooth muscle cell membranes definitely point towards the prevalent role of the α_{1D} subtype in hypertension (Garcia-Sainz and Villalobos-Molina, 2004). α-Adrenergic responses in the aortic, carotid and mesenteric arteries of the normotensive as well as the hypertensive rats have been shown to be mediated through the α_{1D} adrenoceptors. In fact, it was also shown that the carotid and aortic rings from hypertensive rats were more reactive to noradrenaline as compared to those from normotensive animals (Tanoue *et al*, 2002). Another interesting observation is that α_{1D} adrenoceptors show an age-dependent rise in their expression (Ibarra *et al*, 1997). This can be correlated directly with hypertension as the population curve tends to show an increased occurrence of hypertension with increase in age. Thus it may be strongly suggested that α_{1D} adrenoceptors are involved in the pathogenesis and maintenance of hypertension.

α_1 Adrenoceptors and PI_3K/Akt signaling

PI_3K has been implicated in mediating the effects of several receptors including the pertussis-toxin insensitive GPCRs, of which α_1 adrenoceptors form the subset. Since these receptors are heterotrimeric in nature it is possible that any of the monomer units might be responsible for activation of PI_3K (Molkentin and Dorn, 2001). The p85 subunit of PI_3K is activated by activation of $G_{\beta\gamma}$ subunit of Gq receptors in platelets (Geltz and Augustine, 1998). It has been demonstrated that α_1 adrenoceptor mediated mitogenesis is a result of PI_3K activation in human VSMCs (Biesen *et al*, 2013). Specific information regarding the subtype involvement in PI_3K activation is scarce however it may be believed, based on the available evidence, that the three subtypes overlap in different tissues with regard to PI_3K activation. One study has reported that α_{1A} and α_{1B} but not the α_{1D} subtype are involved in PI_3K activation and downstream Akt signaling (Xiao *et al*, 2006). Norepinephrine mediated

the activation of PI_3K in stably transfected cell lines expressing the α_{1A} and α_{1B} subtypes but not in those expressing α_{1D} (Ballou *et al*, 2003). The authors concluded that α_{1A} receptors mediate PI_3K activation via the G_α subunits (Mohl *et al*, 2012) and α_{1B} receptors mediate PI_3K activation through $G_{\beta\gamma}$ subunits (Garcia-Sainz *et al*, 2011). Conversely, it was also shown that sequestration of the $\beta\gamma$ subunits from the GPCR did not result in any change in PI_3K activation in α_{1B} transfected cells (Hu *et al*, 1999a). Another study has reported that α_1 adrenoceptor mediated activation of PI_3K is associated with activation of Akt and Ras proteins. Initially there is PDK mediated phosphorylation of Akt (Thr 308) (Kuo *et al*, 2008) followed by rise in substrate specificity through subsequent phosphorylation (Ser 473) (Bayascas and Alessi, 2005). Full activation of this protein requires phosphorylation at both the residues. Yamboliev and colleagues (2005) have shown that noradrenaline induces PI_3K mediated membrane depolarization in canine mesenteric vein rings. They showed that norepinephrine leads to an increase in phosphorylated PI_3K and Akt upon adrenergic stimulation with noradrenaline. Budzyn *et al* (2005) also reported that wortmannin, a PI_3K inhibitor, attenuates contractile responses to phenylephrine in arterial preparations.

CROSS TALKS BETWEEN RAAS AND THE α-ADRENERGIC SYSTEM

Several clinical and preclinical studies have suggested that RAAS and the α-adrenergic system are involved in the homeostasis of blood pressure. Experimental evidence from several studies has implied that these two systems are not totally independent; in fact, they are intertwined through some common pathways and act in concert for the management of blood pressure. A liaison of sorts exists between the two for accomplishing the regulation of cardiovascular functions. The interactions between the adrenergic arm of the sympathetic nervous system and RAAS have physiological as well as pathophysiological consequences. The systems are known for mutually fortifying the responses to increases or decreases in blood pressure but may occasionally lose control over the response machinery leading to development of hypertension. In such situations, it becomes imperative that the entire compensatory system may be shut to prevent damage to vital organs caused by pressure overload. Several experimental studies have tried to delineate the occurrence of cross talks amongst the adrenergic system and RAAS.

Van Zwieten and de Jonge (1986) indicated that prolonged activation of the sympathetic nervous system and the RAAS are detrimental for the cardiovascular system. Renin release from the JG cells of the kidney is mediated through SNS stimulation while the anti-natriuretic and vasoconstrictor activity observed upon renal nerve stimulation is mediated by α_1 adrenoceptors. The root of the vasoconstrictor activity of AngII lies not only in the activation of post-synaptic AT_1 receptors but also in the activation of several processes related to the sympathetic nervous system like enhanced release and reduced uptake of noradrenaline, promotion of noradrenaline release through presynaptic AngII receptors and sensitization of peripheral α_1 adrenoceptors.

Seidelin *et al* (1987) initially interrogated whether the interaction between noradrenaline and angII in humans, if any, was presynaptic or postsynaptic? The authors studied the hemodynamic effects of noradrenaline released physiologically upon exogenous angII infusion and experimental SNS stimulation and they found that plasma noradrenaline responses were not enhanced by angII. Upon simultaneous infusion of angII and noradrenaline against angII only and control, it was observed that angII acts at some postsynaptic site to mediate the augmentation of noradrenaline mediated rise in systolic blood pressure.

Lang *et al* (1992) in a clinical study showed that AngII is able to induce sodium reabsorption in the proximal tubule and distal segment of the nephrons thereby exhibiting anti-natriuretic action without affecting the glomerular filtration rate. When non-depressor doses of prazosin were administered to animals the effects of AngII were blunted suggesting that there exists a renal interaction between the α_1 adrenoceptors and AngII.

Farivar *et al* (1995) evaluated the effects of losartan in phenylephrine mediated fibrosis. Phenylephrine is known to mediate fibroproliferative responses in cardiac fibroblasts. Since this effect is known to be mediated through the α_1 adrenoceptor, it may be assumed that prazosin might block these events. The authors studied the effects of losartan as well as prazosin in phenylephrine treated animals. Prazosin treated animals showed a normal histopathology. Interestingly cardiac histopathological sections from losartan-treated animals were also free from signs of fibrosis. The results of this study

suggest that AT_1 receptors are also involved in the fibroproliferative responses to phenylephrine.

Maeso *et al* (1996) utilized a functional antagonism assay on SHR aortic rings to demonstrate that AngII potentiates the contractile response of aortic rings to phenylephrine. Losartan was used as an antagonist to affect the contractile response produced by phenylephrine. It was observed that losartan did reduce the contractile response produced by phenylephrine without affecting the relaxation produced by acetylcholine or sodium nitroprusside, indicating a receptor mediated role. However, this attenuation was not observed in endothelium-denuded and *L*-NAME treated aortic rings suggesting a probable role of NO in causing this effect.

Li *et al* (1997) studied the effects of AngII stimulation on α_1 adrenoceptor subtype expression in ventricular myocytes. They found that AngII stimulation has no effect on α_{1B}/ α_{1D} receptor expression but α_{1A} receptor mRNA was found to be downregulated and this effect was mediated through the AT_1 type of angiotensin receptors. Transcription inhibitor studies found no changes in the transcription levels of α_{1A} receptor mRNA in AngII treated cells vs control cells and it was concluded that downregulation of the α_{1A} receptor mRNA was primarily a result of AngII mediated reduction in α_{1A} receptor mRNA stability.

Barki-Harrington *et al* (2003) showed that there is a physiological interaction between the β-adrenoceptors and the AT_1 receptors. This is the first report indicating a direct interaction between any 2 GPCRS. They showed that valsartan (an AT_1 antagonist) reduces isoproterenol-mediated elevation in heart rate in mice and selective blockade of β-adrenoceptors prevented angII-mediated contractility. They also demonstrated that a single antagonist, either valsartan or propranolol, may be able to inhibit the signaling by both the receptors in question. This mechanism was termed as 'transinhibition' since one molecule blocks its own receptors and downregulates the signaling pathways of the reciprocal receptor.

Abdullah *et al* (2011) studied the effects of carvedilol in intact rats and its response to AngII. The study involved injection of AngII in rats that may/may not be treated with carvedilol. This kind of adrenergic blockade did show an inhibitory effect on the vascular

responses of AngII suggesting the interactions between the adrenergic system and RAAS in normotensive animals.

Barrett-O'Keefe *et al* (2013) studied the effects of age related cross-talks between AngII and α_1 adrenoceptor mediated vasoconstriction. The study was based on the hypothesis that AngII mediated vasoconstriction would be more in the elderly as compared to young individuals owing to cross talks with α_1 adrenoceptors and this effect may subside in the presence of α_1 antagonism. The results of the study demonstrated that increased sensitivity to AngII mediated vasoconstriction may be attributed, in part, to potentiation of α_1 adrenoceptor mediated vasoconstriction caused by AngII. They also suggested that this consideration may be clinically applied to design optimally the therapy for patients of hypertension and heart failure.

Vittorio *et al* (2014) retrospectively reviewed the interactions observed between the adrenergic system and RAAS in major clinical trials like Val-HeFT and CHARM-added. The authors suggest that α_1 adrenoceptor and AT_1 receptor cross talk occurs at two levels: at the molecular receptor and the second messenger levels. Heterodimerization between the α_{1D} adrenoceptor and AT_1R has been observed in preeclamptic pregnant rats. Further, since both receptors are coupled to Gq subunit, the subsequent signaling mechanisms overlap significantly and result in second messenger level regulation.

Information on the renin-angiotensin-sympathetic interactions has also been extended to the possible sites of these interactions-stimulation of the sympathetic nervous system leads to renin secretion and AngII formation (DiBona, 1989b); released norepinephrine negatively regulates AngII receptors in cultured brain neurons (Mancia *et al*., 1995) and in vascular tissue through its interactions with α_1-ARs (Du *et al*., 1997). Evidence has also been provided that angII triggers a sympathetically mediated blood pressure rise associated with systemic vasoconstriction when dosed intracerebrally. It suggested a central facilitatory effect of AngII on sympathetic outflow (Wolff *et al*., 1984; Hall, 2004; Zimmerman, 1984). AngII plays a facilitatory role on the neuroadrenergic transmission across sympathetic ganglia (Zimmerman, 1984; Reid, 1992; Reit, 1972) and potentiates norepinephrine release from sympathetic nerve terminals via stimulation of presynaptic angiotensinergic receptors (Zimmerman, 1984; Reid, 1992; Starke, 1977).

AngII amplifies the α-receptor mediated vasoconstrictor responses to exogenously administered or endogenously produced norepinephrine. Furthermore, AngII has been shown to exert inhibitory effects on baroreceptor reflex control of heart rate and sympathetic nerve traffic (Zimmerman, 1984; Reid, 1992). In light of all these observations, blockade of a single receptor seems futile while simultaneous blockade of the α_1 adrenoceptor and AT_1 receptor seems a prudent strategy for the management of hypertension and related cardiovascular disorders.

COMBINATION THERAPY - THE CONCEPT OF DESIGNED MULTIPLE LIGANDS

Earlier research used to be centered on the one-target-one-disease paradigm. It was believed that desired therapeutic effect may be produced through selective manipulation of a single target and this would also prevent off-target effects. Hence, the concept of monodrug therapy was followed throughout the healthcare network but the professionals were not too late in realizing that intrinsic biological networks have a lot of cross-talks amongst them and hence modulation of a single target definitely produces a signal which is recognized by some other coherent network and results in a compensatory action (Wermuth, 2004; Morphy *et al*, 2004). This machinery was identified to be the cause of failure of different compounds in the clinic. Moreover, with increased understanding of the etiopathologies of different medical conditions, it was realized that many diseases are a result of multifactorial orchestra ultimately manifesting the symptoms and hence a single drug might not be sufficient to manage the entire situation. It was thus envisaged that a parallel modulation of different pathways by combining multiple therapeutic mechanisms would be a prudent strategy for the management of multifactorial disorders (Morphy *et al*, 2004; Morphy and Rankovic, 2005). This remains true for hypertension as well where several factors are acting in concert leading to rise in basal blood pressure. Hence combination therapy is usually preferred by clinicians over monodrug therapy for the management of hypertension (Gradman *et al*, 2011). Major clinical trials have also outlined the importance of modulating more than one target so as to achieve optimal blood pressure. The advantages of such a therapy would be increased efficacy in such a way that target BP is achieved more easily within a predicted time frame and a reduction in dose, as two- or

more drugs are being combined, leading to reduced side effects. The aspect of combination therapy initially involved polypharmacy where the patient was supposed to take a couple of pills, capsules or any other dosage form simultaneoulsy which frequently led to non-compliance. This problem was overcome with the advent of fixed-dose combinations (FDCs). FDCs involve formulation of two- or more pharmaceutically compatible drugs into a single tablet at required doses so that dosing regimen may be simplified and the patient is relieved from the burden of taking more than one pill (Gautam and Saha, 2008; Gupta *et al*, 2010; Huffman, 2014). This improves patient compliance especially in geriatric class of patients in whom swallowing pills is a common problem. Fixed dose combinations have ruled the roost since quite a few decades and newer combinations are coming up every year (Table 3). However, the concept of FDCs is also wrought with its own limitations. Though the drugs being combined may be pharmaceutically compatible but they do present with complex pharmacokinetic-pharmacodynamic relationships requiring special formulation techniques, less flexibility in dose adjustments and there is also a potential risk towards drug-drug interactions which could mar the basis of any FDC (Gautam and Saha, 2008; Hennekens, 2008).

The alternative to an FDC is development of a new chemical moiety that simultaneously modulates multiple pharmacological targets (Schyler and Horuk, 2006; Zimmerman *et al*, 2007). Any such drug available in the market today was not specifically designed to be multiply-targeted but was serendipitously discovered to be so. Labetalol and carvedilol are agents of this category with a dual antagonistic activity on adrenergic α_1 and β (pan) receptors (Rahn, 1992). Morphy *et al* (2004), first proposed the rational designing of molecules that may affect two- or more targets. They suggested that evenhanded modulation of multiple targets with a single chemical moiety can be deliberately designed to provide improved efficacy coupled to desirable xenobiotic behavior and minimal side effects.

Table 4: Novel fixed dose combinations being investigated for arterial hypertension[4]

Sr. No.	Compounds	Company	Mechanism
1.	Olmesartan medoxomil + Amlodipine + Hydrochlorthiazide	Daiichi-Sankyo, Japan	ARB + CCB + Diuretic
2.	Azilsartan kaemodoxomil + Chlorthalidone	Takeda Pharmaceuticals, Japan	ARB + Diuretic
3.	Aliskiren + Amlodipine	Novartis, Switzerland	Renin Inhibitor + CCB
4.	Aliskiren + Amlodipine + Hydrochlorthiazide	Novartis, Switzerland	Renin Inhibitor + CCB + Diuretic
5.	Lisinopril + Pyridoxal phosphate	Medicure, Canada	ACEI + cardioprotective vitamin

The major challenge in designing such compounds is not achieving target function, rather the challenge remains in accomplishing a balanced modulation of all the targets in question (Morphy and Rankovic, 2006; Costantino and Barlocco, 2012). The authors have coined the term '*designed multiple ligands (DMLs)*' and deliberated the two approaches that may be followed for designing such ligands (Morphy *et al*, 2004; Morphy and Rankovic, 2009). The first and more rational approach is the Pharmacophore-combination approach. In this approach, targets for desired actions are identified, pharmacophores are addressed from selective ligands acting on individual targets and then the pharmacophores are joined *in silico* through a cleavable or non-cleavable linker. This allows the medicinal

[4] This table is based on the clinically investigated compounds listed in PhRMA as on July, 2014. ACEI, angiotensin converting enzyme inhibitor; ARB, Angiotensin receptor blocker; CCB, Calcium channel blocker

chemist to understand the physicochemical and dynamic interactions following such pharmacophoric combinations (Morphy *et al*, 2004; Di Napoli and Papa, 2003; Buijsman *et al*, 1999; Murugesan *et al*, 2002). Alternatively, non-pharmacophoric structures, if at all present mutually in all prototypical structures being combined, may be overlapped and studied further. However, the onus ultimately remains on the lab chemist who synthesizes such compounds and the biologist who screens it to show final activity on the said targets (Morphy and Rankovic, 2007). Another approach is the Screening-approach. This involves screening of compound libraries for potential target activities. Such an approach has been attempted at various levels of success by different researchers (Walsh *et al*, 1995; Ryckmans *et al*, 2002). In this case absence of non-specific activities have to be ruled out and multiple screens are required to be conducted which raises logistical concerns since large compound libraries may not be located at the place where screening is carried out.

DUAL BLOCKERS INVOLVING AT_1R OR α_1-ADRENOCEPTOR BLOCKADE

α_1 and β receptor blockers

A simultaneous reduction in peripheral resistance and cardiac output can prove beneficial in the therapy of hypertension. Labetalol and carvedilol provide the best examples of this strategy and have been used in the management of hypertension since a very long time with good efficacy (Rahn, 1992). Other agents of this class include bucindolol, primidolol, etc. Clinical data regarding the efficacy of these compounds is quite strong and suggests that carvedilol and labetalol improve the hemodynamic profile of patients suffering from hypertension (Tomlinson *et al*, 1987; Cubeddu *et al*, 1987). Improvement in endothelial dysfunction is also observed and ejection fraction is significantly improved in geriatric patients (Katholi and Couri, 2011). Labetalol is also safe for use in pregnant patients.

α_1 and calcium channel blockers

Though none of the agents of this class is available clinically, experimental molecule S-2150 exerted vasorelaxation of rat aortic rings with an IC_{50} value of 190 nM. A clear hypotensive effect was observed in different models of hypertension including the

SHRs and two-kidney-one-clip rats (Iwaki *et al*, 1997). A similar effect was also observed in normotensive rats indicating the premise of calcium channel blockade.

Dual inhibition of AT_1R and Neprilysin

Neprilysin (*aka* neutral endopeptidase) inactivates several endogenous peptides including bradykinin. It is thus one of the regulators of the kinin-kallikrein system involved in control of blood pressure (Turner *et al*, 2001). Preventing the inactivation of vasodilatory bradykinin, when combined with AT_1R blockade can result in synergistic effect since control of blood pressure rise can be effected through two different systems. LCZ696, an investigational molecule from Novartis, Switzerland, is undergoing clinical trials and has previously shown blood pressure reduction to the tune of valsartan (Solomon *et al*, 2012). In one study, LCZ696 was found to be superior to placebo for treating patients suffering from mild-to-moderate hypertension (Solomon *et al*, 2012; Jhund *et al*, 2014). Other molecules having the same mechanism, daglutril and VNP489, are also in various phases of clinical development (Paulis and Unger, 2010).

Dual inhibition of AT_1R and ET receptor

The concept of dual AT_1R and ET receptor blocker surfaced when losartan and ET_A/ET_B receptor antagonist (SB 290670) produced additive reductions in blood pressure as compared to individual therapy (Kowala *et al*, 2004). This resulted in increased interest of pharmaceutical majors towards the development of dual AT_1R and ET receptor blockers. One investigational molecule, PS433540, showed good binding affinities for both receptors in radioligand binding assays (0.8 nM for AT_1 and 9.3 nM for ET_A). It has also been reported to be effective, safe and well tolerated and is currently in the final stages of clinical trials. Another molecule is BMS346567, which also shows good binding affinities for both the receptors (2 nM for AT_1 and 14 nM for ET_A) (Murugesan *et al*, 2005).

Dual AT_1R blockade and PPARγ agonism

Some AT_1R blockers are known to possess partial agonism of peroxisome proliferator-activated receptor gamma (PPARγ) receptor. It has been reported that telmisartan inhibits AT_1 receptor gene expression through PPARγ activation (Imayama *et*

al, 2006). The dual inhibition of angiotensin II function by telmisartan-AT_1 receptor blockade and downregulation would contribute to more complete inhibition of the RAAS. Telmisartan, an AT_1R blocker and a partial agonist of PPARγ, may be quite useful for the treatment of patients with hypertension with complications such as diabetes and atherosclerosis. A multitargeted ligand may be more useful for microalbuminuria reduction as compared to an AT_1R blocker with no PPARγ agonistic action. Telmisartan achieved more microalbuminuria reduction than other AT_1R blockers lacking PPARγ agonism, possibly through suppression of the inflammatory state in metabolic hypertensive patients (Miura *et al*, 2005; Yano ***et al***, 2007). Two more molecules azilsartan and PF-03838135 are reported to possess AT_1 receptor antagonism and a partial agonism of PPARγ (Paulis and Unger, 2010).

Dual AT_1R and calcium channel blockade

Hadizadeh *et al* (2010) reported the synthesis and evaluation of novel dihydropyridines prepared by connecting the imidazole nucleus of losartan to the dihydropyridine rings. Two compounds from their series were reported to have calcium channel blocking properties with parallel AngII antagonism observed on rat aortic rings. The authors have reported that the test compounds *Dimethyl 4-[2-butyl-1-(2'-carboxybiphenyl-4-yl)methylimidazol-4-yl]-1, 4-dihydro-2, 6-dimethylpyridine-3, 5-dicarboxylate* and *Diethyl 4-[2-butyl-1-(2'-carboxybiphenyl-4-yl) methylimidazol-4-yl]-1, 4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate* are 10^3 and 10^5 times more potent as compared to losartan respectively.

RESEARCH ENVISAGED

RESEARCH ENVISAGED

Recently, the problem of uncontrolled or resistant hypertension has gained enormous proportions (Daugherty *et al,* 2012). It may be very well accepted that mono-drug therapy is no longer effective due to the multifactorial etiology of hypertension and lack of proper lifestyle modifications by the patients. Several agents are employed in combination to manage the etiopathology and symptomatology of hypertension. Clinical practice has adopted the use of two or more classes of antihypertensive agents (Elliott, 2002; Paulis and Unger, 2010) for effective control of blood pressure in hypertensive patients. The basis behind such a decision is that since the etiology of hypertension is complex, it is prudent to employ a parallel control of more than one systems effecting increase in blood pressure. AT_1 and α_1 receptors are important targets in this regard and hence a simultaneous blockade of these targets might prove favorable. A superior therapeutic efficiency can be achieved through evenhanded modulation of multiple targets (Morphy *et al*, 2004). This may be achieved through polypharmacy, administration of fixed dose combinations or an agent directed to all the required targets, i.e. a multiple-targeted ligand. When compared to the other alternatives, the administration of a multiple-targeted ligand may offer certain advantages like more predictable pharmacokinetics, simple pharmacodynamic relationships, improved patient compliance and ease of therapeutic drug monitoring, if at all required (Morphy *et al*, 2004).

The compounds studied herewith belong to a series of 6,7-dimethoxyquinazolines with different substituents at 2^{nd} position based on structural modifications involving prazosin and losartan. These compounds were designed to show a balanced modulation of both the receptors in question i.e. AT_1 and α_1. This effect is supposed to be translated *in vivo* as the major mechanism involved in controlling the etiopathology of hypertension is targeted by these compounds.

Aim of this study involved screening of a series of 6,7-dimethoxyquinazolines for potential dual-antagonist activity on the AT_1 and α_1 receptors. Further to this, it was planned to evaluate the active compounds for toxicity and efficacy in the *in vivo* models of hypertension.

HYPOTHESIS

Structurally modified 6,7-dimethoxyquinazoline derivatives based on prazosin and losartan as respective parent compounds could be more effective in hypertension through simultaneous blockade of AT_1 and α_1 receptors

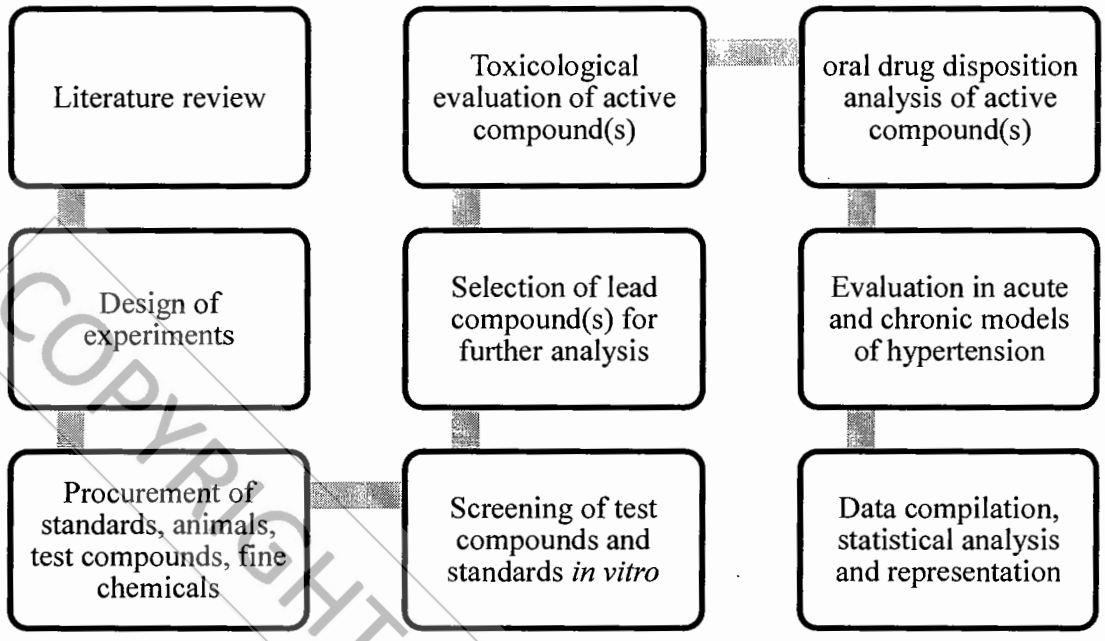
OBJECTIVES OF THE STUDY

1. To screen and identify the NCEs for potential dual-antagonist activity on the AT_1 and α_1 receptors using rat thoracic aorta preparation *in vitro*
2. To evaluate the active compounds for oral toxicity
3. To perform a pharmacokinetic evaluation of compounds showing positive activity on the rat thoracic aorta preparation
4. To evaluate the active compounds for their antihypertensive activity in acute and chronic models of hypertension in rodents

EXPERIMENTS PLANNED TO ACHIEVE THE OBJECTIVES

1. Functional antagonism assay on rat aortic strips using phenylephrine and angiotensin II
2. Single dose and repeat-dose toxicity evaluation of the selected NCE(s) by the procedures mentioned in OECD guidelines 423 and 407 respectively
3. Oral pharmacokinetics of the selected compound(s) in rats by HPLC-UV method
4. Inhibition of *in vivo* pressor response following intravenous injection of the selected compound(s) through invasive recording of arterial blood pressure
5. DOCA-salt induced hypertension in rats to determine the effect of selected NCE(s) upon chronic renal hypertension

FLOW OF WORK



RESULTS
&
DISCUSSION

RESULTS & DISCUSSION

FUNCTIONAL ANTAGONISM ASSAY: PRELIMINARY STUDIES

Establishing baseline values for agonists and studies with standard antagonists of α_1 and angII receptor

The primary aim of the studies was to identify a potential candidate that showed balanced modulation of the α_1 and the angII receptors. This was brought about by studying the antagonistic ability of different test compounds against phenylephrine and angII mediated contractions of the rat thoracic aorta. Similar studies were also performed with the standard compounds for the purpose of direct comparison. The studies were initiated with the evaluation of prazosin against phenylephrine mediated contractions. Receptor classification study was not performed since it has been very well reported that rat aorta expresses adrenoceptors of the α_{1D} type (Kenny *et al*, 1995; Deng *et al*, 1996; Stassen *et al*, 1997). Phenylephrine initiated contractions in the rat aortic strips at concentrations ranging from 15 nM or higher. It was observed that addition of 1μM prazosin caused a rightward parallel shift in the concentration-response-curve of phenylephrine (Figure 7). pA_2 calculations revealed a value of 8.08 ± 0.11 which was less than that reported in the available literature (Hussain & Marshall, 1997 [9.9]; Yamamoto & Koike, 2001 [9.65]). Higher (10μM) or lower (0.1μM) concentrations of prazosin did not induce significant changes in the pA_2 values. Hence, this value was considered as the standard value in all further studies and reported accordingly. It is known that prazosin is a specific α_1-receptor (subtype non-specific) antagonist. Prazosin mediates antagonism of specific and non-specific α_1-receptor agonists in different tissues like aorta (Stassen *et al*, 1997), vas deferens (Ohmura *et al*, 1992), anococcygeus muscle (Adenekan and Tayo, 1982), mesenteric arteries (Yamamoto and Koike, 2001), brain (Wee *et al*, 2008) and in different cell-types expressing the α_1-receptor subtypes. This study allowed establishment of baseline values for phenylephrine and simultaneously permitted calculation of pA_2 value of prazosin.

Similar studies were performed on separate set of aortic strips using angiotensin II as the agonist where losartan was used as the standard antagonist. Concentration Response Curve (CRC) was obtained with angII at concentrations ranging from 10 nM

and higher. Incubation of losartan (0.1μM) or higher concentrations (≤ 10μM) resulted in a rightward parallel shift of the CRC of angII (Figure 8) with pA_2 value being 8.43 ± 0.21.

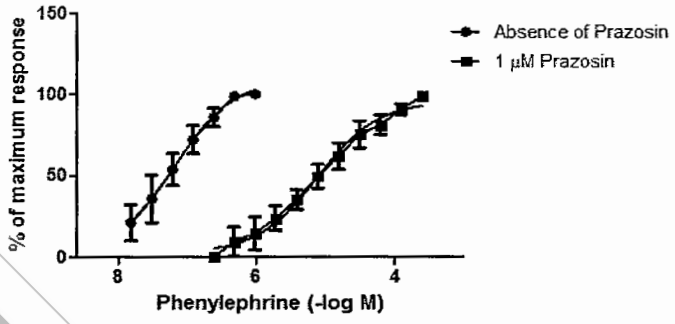


Figure 7: Concentratation response curves of phenylephrine in presence and absence of prazosin.

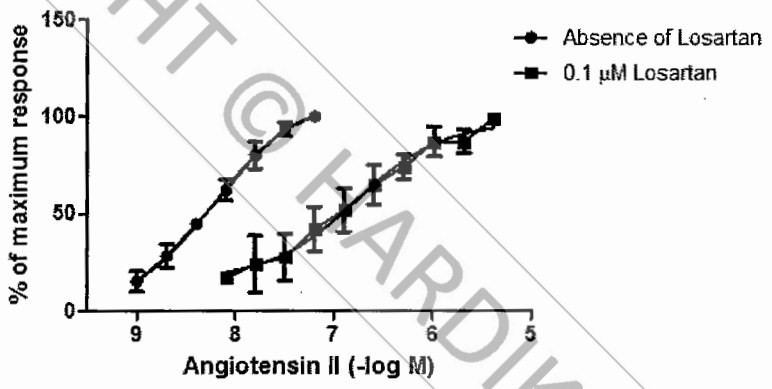


Figure 8: Concentration response curves to angiotensin II in presence and absence losartan.

Several studies have previously reported the pA_2 value of losartan and the results of the present study are in agreement with those studies (Rossi *et al*, 2006; Rossi *et al*, 2007; Laneri *et al*, 2011). Losartan, a non-peptide angII antagonist, is known to inhibit angII receptor activation by binding to it in a reversible manner (Johnston, 1995). Losartan mediated antagonism of angII receptor mediated effects has been shown in brain, hepatic, renal, pulmonary and arterial tissues (Guimaraes *et al*, 1998).

Since standard antagonists for both the receptors in question showed a rightward parallel shift in the CRC of their respective agonists and the antagonism mediated by them was found to be surmountable at higher concentrations of the agonist, it was

assumed that this method was suitable for studying the competitive antagonists of α_1 and angII receptors.

Cross screening of standard compounds on the α_1 and the angII receptors

A wide variety of chemical structures possess α_1-adrenoceptor blocking activity (Jain *et al*, 2008). Similar observations have been made for angII receptor antagonists (Naik *et al*, 2010). After studying the structure activity relationships of both the classes of drugs minutely it became evident that the drug binding sites of both the receptors could accommodate wide structural variations in the active molecules and if that presumption was correct, then designing of dual acting α_1 and angII antagonists should not be an unachievable task. Prazosin, the prototypical α_1-blocker was chosen as the lead molecule. Not many structural changes have been carried out in prazosin type of α_1-blockers except for some variations in the side chain at position-2 of the quinazoline ring system (Jain *et al*, 2008). On the other hand by considering losartan as the lead molecule of angII antagonists, it was noted that too many structural changes have been performed to obtain potent angII antagonists, like replacement of imidazole nucleus with other heterocyclic ring systems, replacement of the biphenylmethyl side chain with smaller and bigger groupings, and replacement of tetrazole moiety with other acidic groups (Naik *et al*, 2010). So, it was planned to choose hybrid structures that employed 6,7-dimethoxyquinazoline ring skeleton as the common structural motif for α_1- as well as angII antagonism and attach various types of side chains at its 2-and/or 3-positions. The main obstacle in the selection of the side chain was the nature of the side chain grouping - whether the attached side chains should have acidic functionality or a basic one because prazosin-type of α_1-blockers contained basic groupings in the side chain while all reported angII antagonists had an acidic functionality in the side chain. Ultimately it was decided to try all the three types of functional groups, acidic, basic as well as neutral in the attached side chain for designing of the compounds to justify the selection of the functionality. Assuming a high degree of structural tolerance for antagonistic activity by both the receptors, different compounds were selected from a series of compounds. Compounds with neutral groups like CH_3, OCH_3, Cl, Br, NO_2, CN etc. at different positions of the phenyl ring were studied initially as they offered synthetic convenience. They were screened for obtaining preliminary biological data. The results of this preliminary biological screening of all compounds in the preliminary selection showed dual antagonism to the phenylephrine and angII-mediated contractile

responses (Tables 7-12). These results forced us to have a relook at the mechanism of antihypertensive actions of both prazosin and losartan. When neutral molecules without any characteristic side chains could show dual α_1- and angII-receptor antagonism, was it possible for prazosin and losartan also to show dual antagonism at both the receptors? pA_2 value determinations of both of the standard lead molecules confirmed the correctness of our assumption of wide structural tolerance by both the receptors in their active spaces - prazosin exhibited potent dual antagonism at α_1- (pA_2 8.08±0.11) as well as angII receptors (pA_2 8.26±0.10) while losartan was found to be a potent antagonist at angII-receptor (pA_2 8.43±0.21) but a poor (pA_2 5.46±0.41) on eat α_1-receptor when evaluated on rat aortic strip using phenylephrine and angII as agonists. Other compounds were also screened and it was found that while doxazosin and terazosin also mediated angII receptor antagonism (pA_2 6.61 ± 0.4 and 6.39 ± 0.4 for doxazosin and terazosin, respectively) albeit only moderately, α_1-receptor antagonism potential was practically absent in valsartan and olmesartan (pA_2 values could not be calculated). In all these cases the slopes of the CRCs were not very different from unity (Figures 9 & 10).

This was a totally surprising finding unreported in the literature. It may be because prazosin possessed some stereochemical features required to bind to the AT_1 receptor or may possess some three dimensional features required to fill in AT_1 receptor pockets. Postural hypotension is one of the common side effects of α_1 receptor blockers frequently reported by patients. Prazosin, being the most prominent in this regard, causes orthostatic hypotension in majority of the patients (Take *et al*, 1998; Rieckert, 1996). Based on our findings, we take the liberty to suggest that prazosin mediates fall in blood pressure through blockade of not only α_1-receptors but AT_1 receptor as well. An absence of baroreceptor mediated reflex in patients receiving prazosin therapy is responsible for orthostatic hypotension (Gupta & Lipsitz, 2007). The baroreceptor reflex is regulated through the sympathetic as well as the parasympathetic nervous system. An activation of the SNS results in a consequential release of norepinephrine (Rowell, 1993). The primary action of norepinephrine released in this manner is to increase the resistance of blood vessels, ultimately maintaining the blood pressure. Secondly, the released norepinephrine also stimulates the secretion of rennin (Takagi *et al*, 1992) and ultimately, formation of angII. This *de novo* formation of angII can cause direct vasoconstriction mediated through AT_1 receptors. But as per our findings,

prazosin blocks this arm through competitive blockade of AT_1 receptors. As a novel finding, we can therefore assume, that potency of α_1-blockers is, additionally a function of AT_1 receptor antagonism also.

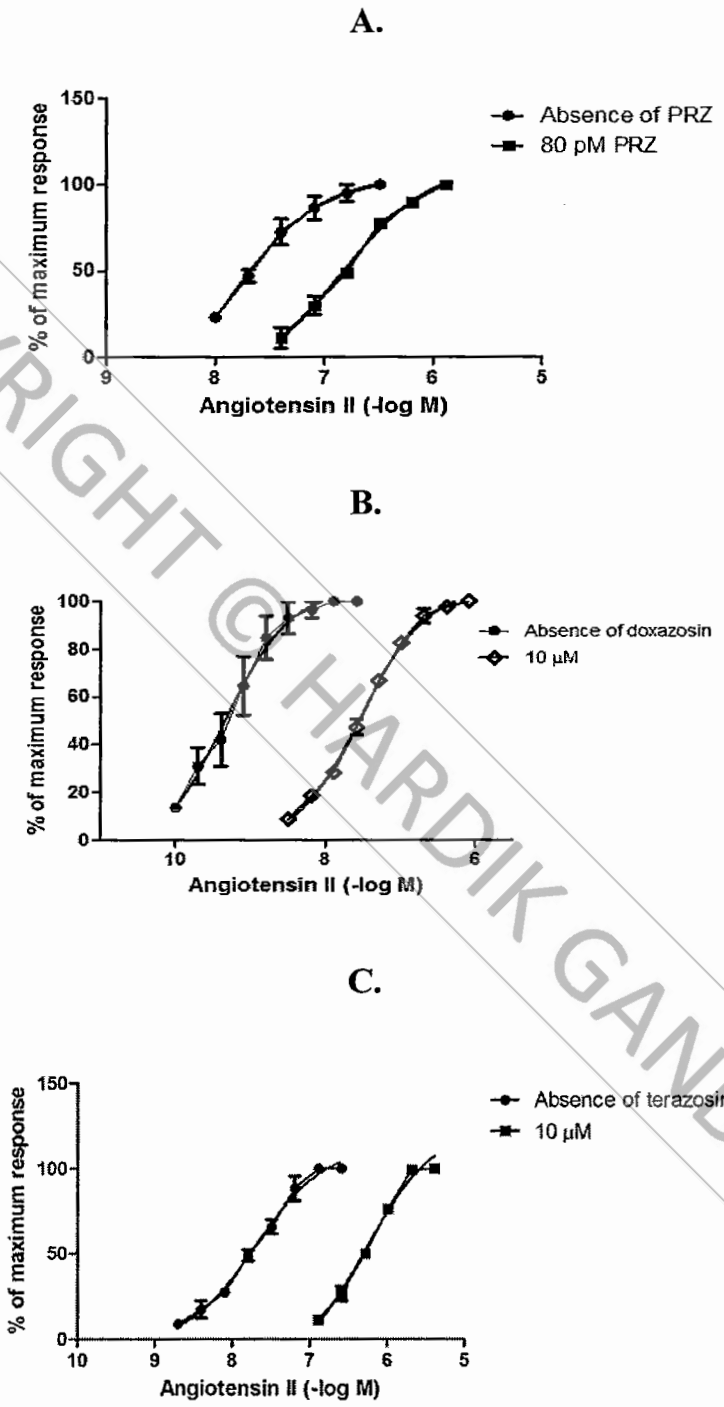
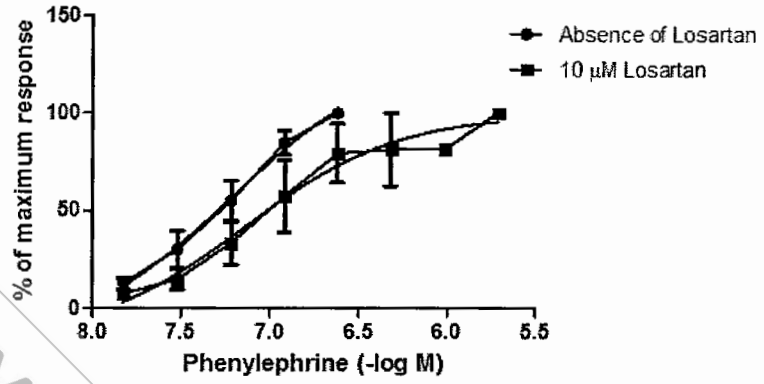
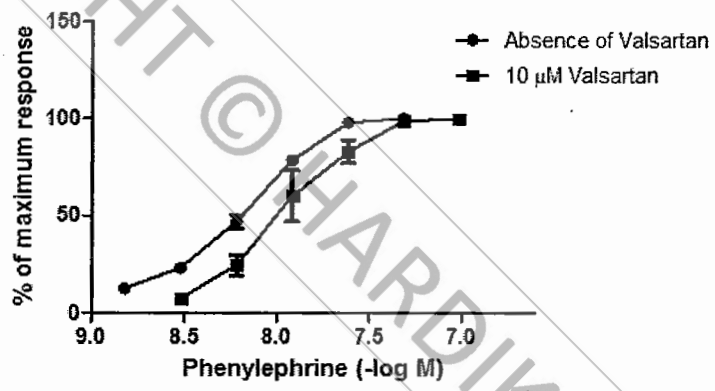


Figure 9: Concentration response curves of angiotensin II against, A) prazosin (80pM), B) doxazosin (10µM) and C) terazosin (10µM).

A.



B.



C.

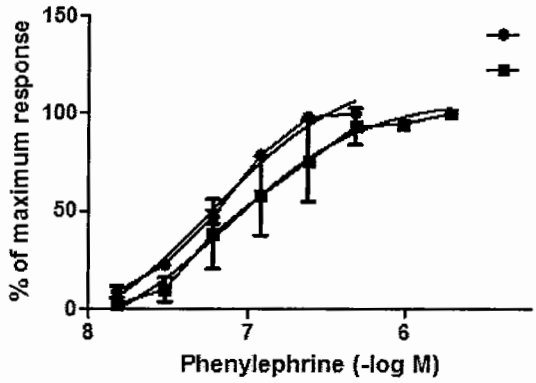


Figure 10: Concentration response curves of phenylephrine against, A) losartan (10µM), B) valsartan (10µM) and C) olmesartan (10µM).

Screening of potential compounds for dual antagonism of α_1- and angII- receptors antagonism

Based on the studies with standard compounds and the encouraging results obtained therewith, it was decided to screen six series (I-VI) of compounds for potential dual antagonistic activity on the α_1- and angII- receptors (Figure 11). Details about the series and the activity of different compounds therein are given in tables 5-10.

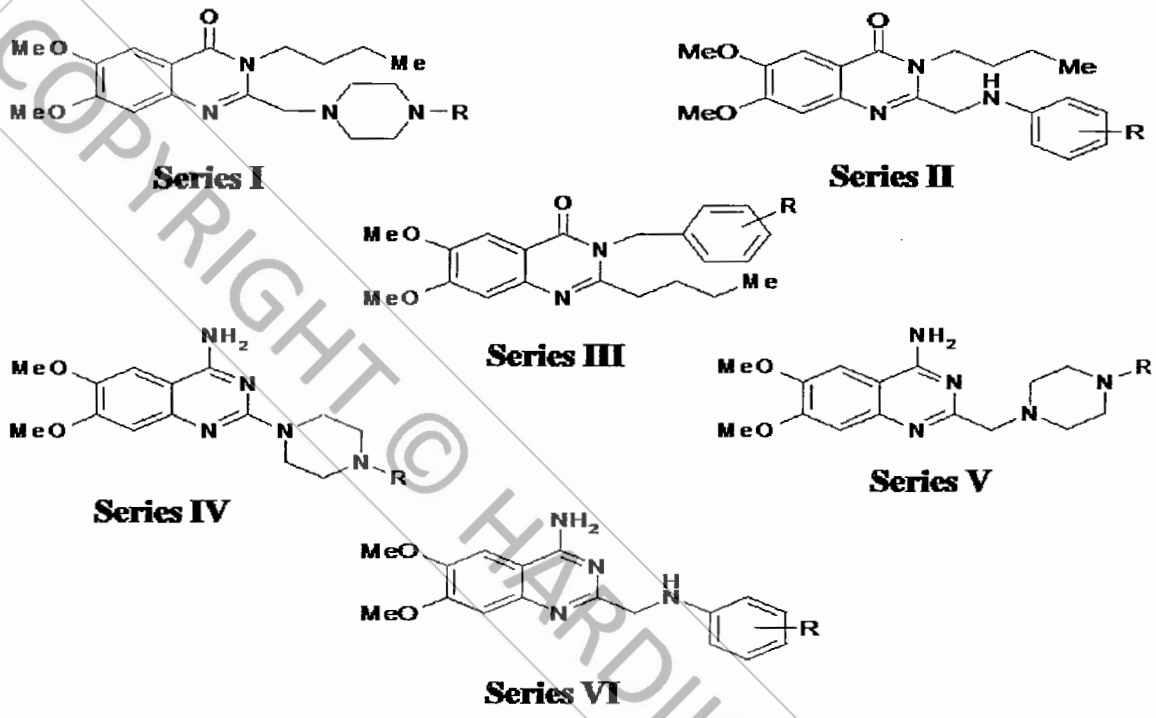
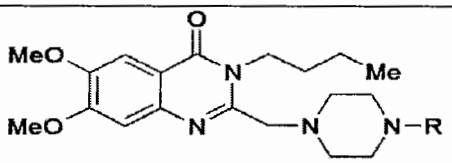


Figure 11: This figure represents the six series of compounds that were chosen as part of the preliminary screening protocol to identify a potential dual antagonist. 6, 7-Dimethoxyquinazoline ring system is native to all the series with series I-III being 4-keto derivatives and series IV-VI being 4-amino derivatives. Other differences are observed at the substitutions on 2nd and 3rd positions.

Table 5: Preliminary studies on compounds of series I

Compound	 *Where –R is*	*p*A_2 values	
		α_1	AT_1
I-1	$-CH_3$	4.18 ± 0.09	NC
I-2	$-CH_2CH_3$	5.28 ± 0.08	NC
I-3	$-C_6H_5$	5.45 ± 0.24	3.82 ± 0.09
I-4	$-C_6H_{11}$	6.22 ± 0.10	4.98 ± 0.09

I-5	-C_6H_4CN (*o*)	**7.71 ± 0.10**	**7.41 ± 0.10**
I-6	-$C_6H_4OCH_3$ (*o*)	7.21 ± 0.09	5.03 ± 0.11
I-7	-C_6H_4F (*o*)	6.33 ± 0.13	5.68 ± 0.05
I-8	- C_5H_4N	6.49 ± 0.11	5.91 ± 0.15
I-9	-C_6H_4OH (*p*)	NC	5.8 ± 0.09
I-10	-$CH(C_6H_5)_2$	6.07 ± 0.06	NC

Table 6: Preliminary studies on compounds of series II

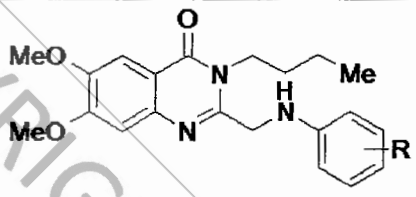
Compound	 *Where -R is*	**pA_2 values**	
		α_1	**AT_1**
II-1	-COOH (*m*)	7.45 ± 0.16	6.14 ± 0.14
II-2	-COOH (*p*)	5.27 ± 0.28	5.75 ± 0.28
II-3	-NH_2 (*p*)	5.48 ± 0.15	5.28 ± 0.27
II-4	-$NHSO_2CH_3$(*m*)	6.06 ± 0.10	5.13 ± 0.33
II-5	-$NHSO_2CH_3$(*p*)	5.69 ± 0.29	5.62 ± 0.09
II-6	-tetrazole *(m)*	5.63 ± 0.21	4.9 ± 0.14
II-7	-tetrazole *(p)*	4.53 ± 0.08	5.19 ± 0.13

Table 7: Preliminary studies on compounds of series III

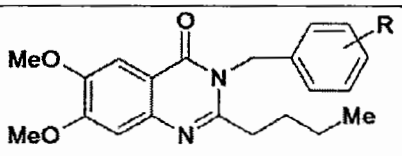
Compound	 *Where -R is*	**pA_2 values**	
		α_1	**AT_1**
III-1	-$COOCH_3$ *(m)*	6.42 ± 0.13	5.53 ± 0.11
III-2	-$COOCH_3$ *(p)*	**6.97 ± 0.12**	**7.00 ± 0.11**
III-3	-COOH *(p)*	5.50 ± 0.16	6.01 ± 0.21
III-4	-NO_2 *(m)*	7.13 ± 0.20	6.44 ± 0.15
III-5	-CN *(m)*	6.88 ± 0.13	6.65 ± 0.09
III-6	-CN *(p)*	6.40 ± 0.11	6.92 ± 0.12
III-7	-tetrazole *(m)*	**7.10 ± 0.12**	**7.03 ± 0.12**

Table 8: Preliminary studies on compounds of series IV

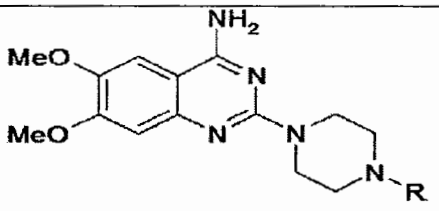
Compound	 *Where –R is*	pA_2 values	
		α_1	AT_1
IV-1	$-CH_3$	**9.23 ± 0.12**	5.75 ± 0.07
IV-2	$-CH_2CH_3$	**10.41 ± 0.12**	5.08 ± 0.08
IV-3	$-C_6H_5$	8.74 ± 0.08	3.31 ± 0.13
IV-4	$-C_6H_4CN$ (*o*)	**8.59 ± 0.51**	**9.04 ± 0.23**
IV-5	$-C_6H_4OCH_3$ (*o*)	7.26 ± 0.12	6.54 ± 0.51
IV-6	$-C_6H_4F$ (*o*)	10.52 ± 0.14	5.52 ± 0.09
IV-7	$-C_5H_4N$	6.95 ± 0.08	5.09 ± 0.12
IV-8	$-CH(C_6H_5)_2$	**7.09 ± 0.15**	**7.59 ± 0.58**

Table 9: Preliminary studies on compounds of series V

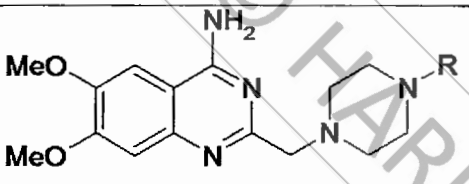
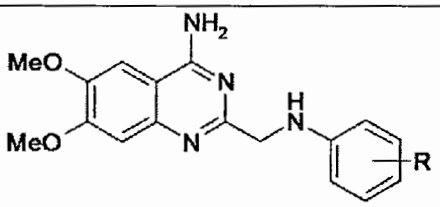
Compound	 *Where –R is*	pA_2 values	
		α_1	AT_1
V-1	$-CH_3$	6.79 ± 0.22	5.1 ± 0.38
V-2	$-CH_2CH_3$	4.19 ± 0.08	3.22 ± 0.10
V-3	$-C_6H_5$	6.49 ± 0.13	6.78 ± 0.10
V-4	$-C_6H_{11}$	4.90 ± 0.06	3.01 ± 0.25
V-5	$-C_6H_4CN$ (*o*)	**10.1 ± 0.20**	**8.83 ± 0.38**
V-6	$-C_6H_4OCH_3$ (*o*)	**7.45 ± 0.09**	6.34 ± 0.07
V-7	$-C_6H_4F$ (*o*)	6.76 ± 0.10	6.09 ± 0.13
V-8	$-C_5H_4N$	5.47 ± 0.48	3.65 ± 0.15
V-9	$-CH(C_6H_5)_2$	5.32 ± 0.07	6.36 ± 0.34
V-10	$-C_6H_4COONa$ (*o*)	3.64 ± 0.11	6.28 ± 0.28

Table 10: Preliminary studies on compounds of series VI

Compound	 *Where –R is*	pA_2 values	
		α_1	AT_1
VI-1	-H	**9.87 ± 0.15**	**8.37 ± 0.43**
VI-2	-CH_3(*m*)	6.16 ± 0.11	5.38 ± 0.14
VI-3	-CH_3(*p*)	5.28 ± 0.20	3.45 ± 0.34
VI-4	-OCH_3(*p*)	NC	6.29 ± 0.10
VI-5	-COOH(*m*)	5.52 ± 0.36	4.87 ± 0.17
VI-6	-COOH(*p*)	4.49 ± 0.20	6.43 ± 0.38
VI-7	-$COOCH_3$(*m*)	6.37 ± 0.13	6.27 ± 0.10
VI-8	-$COOCH_3$(*p*)	6.86 ± 0.12	**10.64 ± 0.10**
VI-9	-NO_2(*m*)	**9.38 ± 0.18**	**7.64 ± 0.46**
VI-10	-NO_2(*p*)	**8.09 ± 0.12**	**9.04 ± 0.15**
VI-11	-$NHSO_2CH_3$(*m*)	4.48 ± 0.29	3.41 ± 0.21
VI-12	-$NHSO_2CH_3$(*p*)	NC	4.38 ± 0.44
VI-14	-$NHCOCH_3$(*p*)	6.67 ± 0.12	NC
VI-15	-Cl(*m*)	7.02 ± 0.08	6.27 ± 0.12
VI-16	-Cl(*p*)	4.89 ± 0.27	4.65 ± 0.28
VI-17	-Br(*m*)	6.78 ± 0.14	7.23 ± 0.12
VI-18	-Br(*p*)	6.33 ± 0.10	6.75 ± 0.23
VI-19	-F(*p*)	6.60 ± 0.11	7.70 ± 0.20
VI-20	-Naphthyl	**8.37 ± 0.27**	**7.07 ± 0.16**
VI-21	-C_5H_4N(*o*)	6.91 ± 0.23	5.90 ± 0.14
VI-23	-C_5H_4N(*p*)	5.86 ± 0.13	4.68 ± 0.09
VI-24	-morpholinyl	5.26 ± 0.16	6.15 ± 0.25
VI-25	-$C_5H_{10}N$	3.01 ± 0.06	3.70 ± 0.17
VI-26	-triazolyl	5.93 ± 0.13	5.47 ± 0.13
VI-27	-C_4H_8N	3.49 ± 0.07	3.63 ± 0.08
VI-28	-$C_7H_5N_2$	4.05 ± 0.10	4.15 ± 0.12

Based on the results of these preliminary findings, a few compounds were shortlisted as potential antagonists exhibiting dual antagonism of α1-adrenoceptors and angII receptors. These compounds included I-5, III-2, III-7, IV-4, IV-8, V-5, V-6, VI-1, VI-9, VI-10 and VI-20 (marked **bold** in the tables). Out of these compounds, V-5 was selected for further studies as it was found to demonstrate most potent antagonism at both the receptors in question and the activity was even better than prazosin and losartan on the α_1-adrenoceptors and angII receptors respectively. This compound was further coded as ***MCR-1329*** as per coding norms of our laboratory.

Elaborated functional antagonism assay of potent compound

The antagonism afforded by **MCR-1329** on rat aortic strips was evaluated at 3 different concentrations (1, 5, 10 μM) only to find that each higher concentration resulted in a further rightward parallel shift in the CRC of phenylephrine as well as angII. It has been suggested that if a series of antagonist concentrations yield linear Schild regression with a slope of unity, then the pA_2 vlaue obtained may be considered as the actual affinity of the ligand to the receptor (Kenakin, 2009; Dale and Haylett, 2009), otherwise at a single concentration, $pA2$ value remains only an empirical measure of antagonist potency (Kenakin, 2009). The pA_2 value calculated at different concentrations of **MCR-1329** remained the same as that observed in preliminary studies. Accordingly, the pA_2 value of **MCR-1329** against phenylephrine mediated contractions was 10.10 ± 0.20 (Figure 12A) and for that against angII was found to be 8.83 ± 0.38 (Figure 12B).

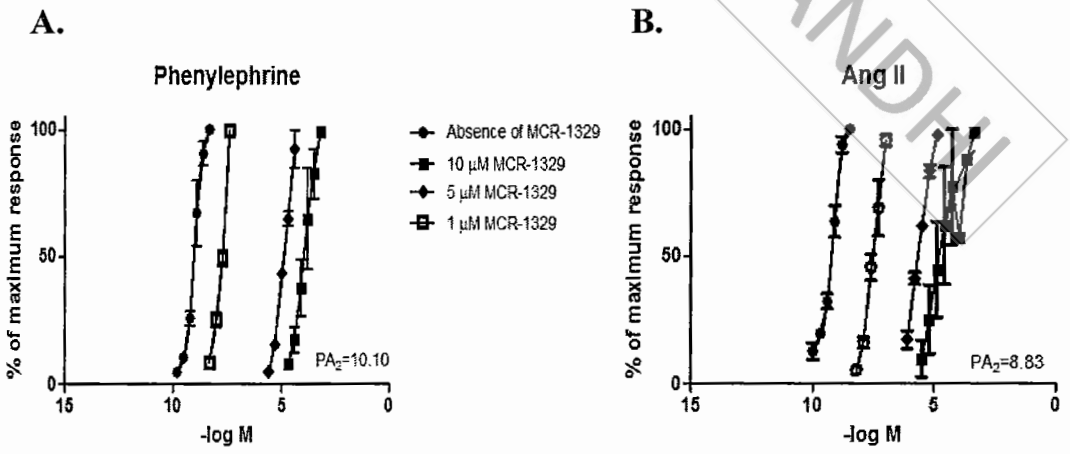


Figure 12: Concentration response curves to phenylephrinne (A) and angiotensin II (B) in presence of different concentrations of **MCR-1329** (0, 1, 5, 10 μM).

IN VIVO PRESSOR RESPONSE EVALUATION

Unmasked pressor response

The effects of **MCR-1329** *in vitro* were so profound that it was decided to challenge it against the *in vivo* effects of phenylephrine and angII on rat arterial blood pressure. Intact animals have been utilized by different research groups to evaluate the effects of angII (Liles *et al*, 2006; Padia *et al*, 2006) and phenylephrine (Smith *et al*, 2006; Cunha *et al*, 2005) on arterial blood pressure. This method involves measurement of arterial blood pressure after cannulation of the carotid artery and offers a direct measurement of blood pressure (Kurtz *et al*, 2005). This method may also be used to study arterial reactivity. Accordingly, two dose levels were chosen and the effects of **MCR-1329** were evaluated against intravenous injections of phenylephrine and angII (6µg/kg, i.v. each). The effects were compared with equimolar doses of prazosin and losartan as standards. It was found that prazosin-mediated effects were stronger as compared to **MCR-1329** in inhibiting the pressor response to phenylephrine. At a lower dose of 0.36 µmol/kg, prazosin exhibited near 50% inhibition of pressor response to phenylephrine whereas **MCR-1329** showed about 20% inhibition. At higher doses (0.72 µmol/kg), the effects were more prominent and prazosin almost completely blocked the pressor effects of phenylephrine while **MCR-1329** also significantly blocked the responses of phenylephrine to about 70% (Figure 13A). Separate dose levels were employed for the evaluation of antagonistic effects of losartan and **MCR-1329** against angII mediated pressor responses. Evaluation of mean arterial pressures revealed that losartan at a dose level of 0.72 µmol/kg produced around 50% inhibition of the pressor response to angII. At an equimolar dose the response of **MCR-1329** may be considered feeble and produces only about 10% inhibition of pressor response. Administration of 3.6 µmol/kg losartan prior to angII challenge brought about a complete inhibition of angII mediated rise in blood pressure. However, **MCR-1329** negotiated only about 40% inhibition of pressor response at the equimolar dose (Figure 13B). The results were intriguing since there was a vast difference between the *in vitro* data and preliminary *in vivo* investigations.

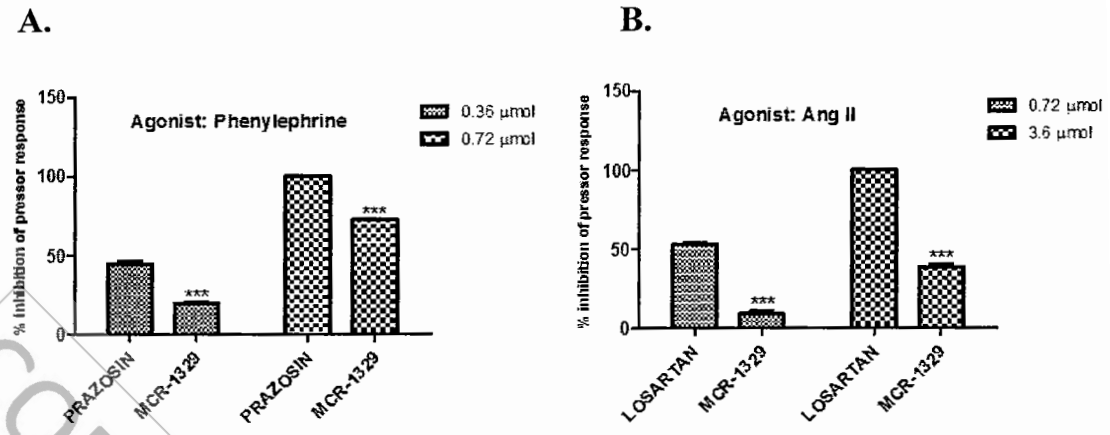


Figure 13: Mean arterial pressor response inhibition of phenylephrine (A) and angiotensin II (B) in animals previously dosed with **MCR-1329** or standards at equimolar levels (prazosin for phenylephrine and losartan for angiotensin II).

While *in vitro* data showed quite high potency of the test compound, **MCR-1329**, which was even higher than the standard compounds, results from the *in vivo* studies did not concur the same. This uncertainty was deliberated upon and the causes for such inconsistency were evaluated. It was presumed that probably **MCR-1329** undergoes rapid metabolism in the blood stream leading to a laid-back inhibition of pressor response. This was unlikely since the standard and test drugs were injected intravenously and the effects were observed immediately upon administration. Thus it could be a rare possibility that **MCR-1329**, a quinazoline derivative is metabolised immediately in contact with blood. Another speculation was that **MCR-1329** undergoes extensive plasma-protein binding, which prevents the compound from binding to the respective receptors. This was more likely to happen as it is known that derivatives of quinazoline are known to undergo extensive plasma protein binding. An *in vitro* study was planned to compare the plasma protein binding profiles of prazosin, losartan and **MCR-1329**. The results of this study revealed that this compound does not have major difference between itself and prazosin w.r.t plasma protein binding. The results of this study are presented in detail in the *PHARMACOKINETICS* section. On the basis of protein binding studies, lesser *in vivo* potency of **MCR-1329** could not be explained.

While mulling over these alternatives, one fact was clearly overlooked. Since **MCR-1329** shows dual action *in vitro*, it is possible that the actual concentration of the drug reaching at a particular receptor was much lesser while administering the drug on a molar basis. The reasons for such an abridged response may therefore be the

distribution of the drug upon both the receptors in question suggesting that only a fraction of **MCR-1329** is available at a given time to act upon a particular population of receptors since **MCR-1329** was designed as a multitargeted ligand. Multitargeted ligands are particularly suggested for conditions that have manifold etiopathologies so that a single agent can be utilized for direct inhibition of the causative factors. This fact may be correlated with several other investigational multitargeted ligands studied for the likely management of complex diseases (Wei *et al*, 2008; Bajda *et al*, 2011) which show differential inhibition of the targets involved (Bolognesi *et al*, 2010).

This speculation was based on *in vitro* studies performed earlier on isolated rat aortic strips. In those experiments, **MCR-1329** showed competitive antagonism against phenylephrine and angiotensin II, respective specific agonists of the α_1- and AII-receptors. This was evident from the rightward parallel shift observed in the dose-response plots for MCR-1329. This competitive inhibition of the contractile response in isolated vascular tissue showed that **MCR-1329** had the ability to bind to both the receptors in question in a dose-dependent manner. This condition of competitive binding to both the receptors was expected to be prevalent in the *in vivo* model as well, thereby causing reduction in the inhibition response of **MCR-1329** on both the receptors in comparison to the standard drugs. However, with this speculation it was not assumed that **MCR-1329** might show an equivalent distribution on both the receptors in question and hence the disparity among responses to phenylephrine and angII remained open to experimental conclusion. The reflection of this hypothesis is directly seen from the results which show that equimolar doses of **MCR-1329** show lesser amount of inhibition when compared to prazosin or losartan.

Masked pressor response

To provide credence to the hypothesis proposed above, it was planned to evaluate the pressor-inhibition potential of **MCR-1329** under masked conditions. *In vivo* inhibition of phenylephrine mediated arterial pressor response by **MCR-1329** was measured in those animals in which 12.5 mM losartan was pre-administered. The idea behind such a protocol was to mask the effects of **MCR-1329** on angII receptor. Similarly, the other set involved measurement of inhibition of angII mediated arterial pressor response in those animals in which 400 µM prazosin was pre-administered to mask the effects of **MCR-1329** upon α_1 receptor. The drugs and standard compounds

were administered at equimolar concentrations. Such a protocol for studying the effects of agonists under masked conditions has not been reported in the literature previously. The results obtained are shown in figure 14.

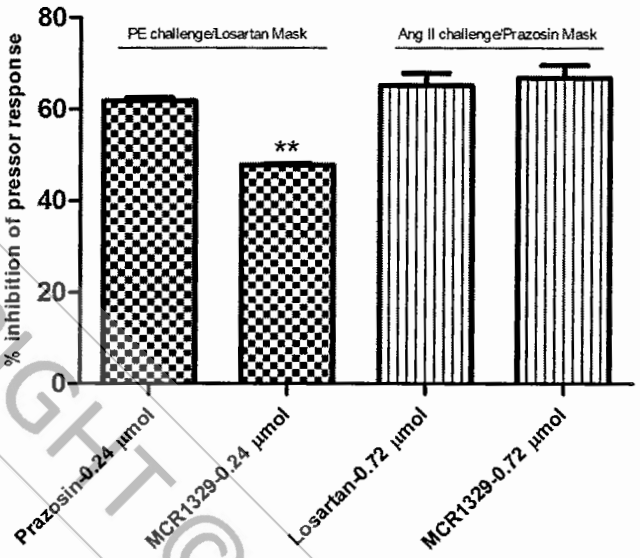


Figure 14: Mean arterial pressor responses to phenylephrine under losartan masking (checkerd columns) and to angII under prazosin masking (ruled columns). In case of phenylephrine challenge, the effect of **MCR-1329** was found to be moderate in comparison to prazosin.

It can be observed that the hypothesis stands true as **MCR-1329** was equipotent to losartan after masking the α_1-receptors with prazosin and slightly less active than prazosin after masking A-II receptors with losartan. Though compartmentalization of the drug was excluded with the possibility of high-degree of plasma protein binding (Roberts *et al*, 2013), the disparity of receptor distribution could not be ruled out. Disparities in receptor distribution owing to different receptor densities have been studied previously on neuropeptide (Beaudet *et al*, 1998) and cannabinoid receptors (McPartland *et al*, 2007).

As stated above, we have previously shown that prazosin shows potent antagonism of the angII receptors as well. Such type of action is responsible for an enhanced antihypertensive response afforded by prazosin. However, this effect can play a dual role in our masking study, again by acting on both α_1 and angII receptors. Hence we performed the same study using terazosin as a standard α_1 antagonist (Figure 15), which we have shown to have negligible action on angII mediated

vascular smooth muscle contraction. Activity of terazosin is exclusive for the α_1 receptor and hence we can safely assume that the effects of terazosin are mediated due to binding with α_1 receptor only. No significant differences could be observed between the effects of terazosin (as a masking agent and a standard α_1-antagonist) and that of **MCR-1329** as shown in figure 15.

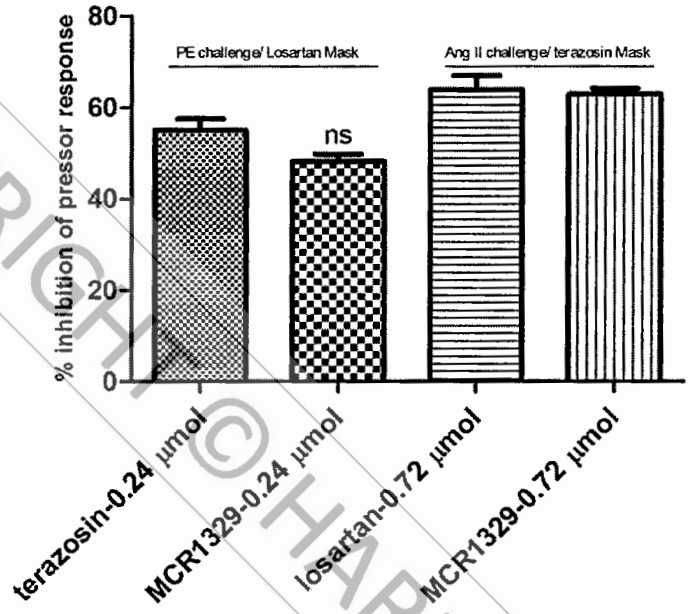


Figure 15: Mean arterial pressor responses to phenylephrine under losartan masking (checkered colums) and to angII under terazosin masking (ruled columns). In case of phenylephrine challenge, the effect of **MCR-1329** was found to be statistically comparable without any significant difference.

Such a masking study has not been reported in the literature. The results clearly shed light on the multiple effects shown by drugs used in clinical practice. The results suggest that the drugs used in clinical practice may have certain off-target effects not known at present. Simultaneously, it is also evident that it is possible to design ligands which have multiple targets for action. Such compounds may form the basis for favourable management of complex disorders like hypertension, atherosclerosis, alzheimer's and the like.

TOXICOLOGICAL EVALUATION OF MCR-1329

Single dose acute oral toxicity

Studies with different quinazoline derivatives have not shown any untoward toxicity signs belonging to this class of compounds (Zayed and Hassan, 2013; Alafeefy *et al*, 2010; Sinha *et al*, 2013). These studies have reported a safety of assorted quinazoline derivatives upto 2000 mg/kg and beyond. However, to ensure the safety of the test compound, **MCR-1329** was administered at the recommended dose. The post-treatment examination period was 14 days from the date of dosing. Body weights of the animals were recorded on days 0, 7 and 14. Slight fluctuations were observed in the body weight of animals but since they were within 20% of the mean body weight no additional measurements were taken and any other precaution was not followed. The animals were closely observed during the first 6 hours after dosing. The animals were starved during this period with access to water. No significant observations were recorded during this period. This part coincided with the light cycle and most of the time animals were asleep. When awake, the animals showed normal grooming behavior and water intake was also normal. During the entire post-treatment observation period special attention was paid to alteration of skin or fur, abnormal locomotion or breathing and changes in the eye. No untoward observations were made in this regard until the terminal day of the study. Mortality was recorded twice daily but no mortality was found in any dose group till day 14. At the end of the study period, the animals were euthanized and major organs (brain, heart, lung, liver, kidney, spleen) were harvested. Gross necropsy was performed by an individual blinded to the groups. No macroscopic lesions were recorded. Viscera, gastrointestinal tract and mucous linings appeared normal. Major blood vessels did not show any abnormalities. Detailed report on toxicity evaluation is presented as Appendix I.

Administration of 2000 mg/kg of **MCR-1329** showed no signs of toxicity or mortality during the test period. The LD_{50} of **MCR-1329** in rats was thus found to be >2000mg/kg.

Repeat dose oral toxicity

At the end of the study, no untoward observations were made regarding body weight, food intake or normal behavior. Gross necropsy did not reveal any suggestive lesions or abnormal anatomical feature. The most plausible side effect related to the mechanism of action of **MCR-1329** is hypotension. This effect was not evident from the tail-cuff recordings. Biochemical estimations did not suggest any major digression

from normal values. Urinary output and hematological data appeared normal. Studies with other quinazoline derivatives like fenazaquin have shown that this class of compounds is safe for daily administration upto a dose level of 30 mg/kg/day (Francis *et al*, 1992). Detailed report on repeat dose toxicity evaluation is presented as Appendix II. It was concluded that chronic administration of **MCR-1329** at a dose level of 10 mg/kg was safe.

PHARMACOKINETIC STUDIES

HPLC method

The HPLC analysis of prazosin and related quinazoline compounds has been successfully attempted by different researchers. Sultana *et al* (2013) has reported the analysis of prazosin by HPLC as API, in dosage forms and serum samples using a mobile phase composition of acetonitrile : water (75:25, *p*H=3.2). Ahmed *et al* (2010) showed the extraction and estimation of prazosin hydrochloride from pharmaceutical dosage forms using a mobile phase composition of 20 mM OPA: acetonitrile (70:30, *p*H=2.5). In both the analyses, the wavelength of detection was 254 nm. Shrivastava and Gupta (2012) have reported a method for simultaneous estimation of prazosin, doxazosin and terazosin. Rao *et al* (2006) also developed a method which could be applied to the quality assurance of 6,7-dimethoxyquinazolines. Before establishing the HPLC method for our test compound, several trials were performed to optimize the parameters for analysis of **MCR-1329** on HPLC. 10 mg of **MCR-1329** was dissolved in 10 ml of 0.1% OPA by vortexing to give a final concentration of 1 mg/ml. 0.1% of OPA was used as a blank solvent. Spectral scan of the stock solution on UV-1800 (Shimadzu, Japan; scan range 200-400 nm) gave the following data (Table 11):

Table 11: Absorbance of MCR-1329 at different wavelengths

Wavelength (nm)	OD
220	1.019
243	***0.833***
254	0.754
323	0.352

Different wavelengths have been utilized in the literature for estimation of quinazoline derivatives by HPLC. While Sultana *et al* (2013) utilized 254 nm for prazosin, Rao *et al* (2006) studied dimethoxyquinazolines at 240 nm. The Indian Pharmacopoeia and its European counterpart both indicate that 254 nm be used as the wavelength for estimation of prazosin. Based on these values and our observations 243 nm was selected as the wavelength for estimation since it showed the highest absorbance after 220 nm in the spectral scans. 220 nm was not chosen as the λmax for estimation since this wavelength falls below the UV cut-off of several organic solvents which are commonly used as mobile phases for HPLC.

Next, several mobile phases were tried based on the data available for related class of compounds. Different mobile phase compositions evaluated to obtain a good resolution for **MCR-1329** are shown in table 12.

Table 12: Mobile phase combinations evaluated for elution of MCR-1329

Mobile Phase ID	Solvent Mixture	Ratio
I	Methanol: Water (*p*H=6)	50:50
II	Acetonitrile: Methanol: Water (*p*H=6)	10:70:20
III	Acetonitrile: Methanol: Water (*p*H=6)	10:55:35
IV	Acetonitrile: Methanol: Water (*p*H=6)	22:22:56
V	Acetonitrile: Methanol: Water (*p*H=6)	20:40:40
VI	Acetonitrile: 0.2% OPA (pH=3)	40:60
VII	***Acetonitrile: 0.04M Na_2HPO_4 (pH=3)***	***40:60***
VIII	Acetonitrile: Methanol: 0.04M Na_2HPO_4 (*p*H=3)	20:70:10

Mobile phase I through V were chosen based on the solubility of **MCR-1329** in these mobile phases without the need for adjusting an acidic *p*H. Mobile phase II showed better solubility at normal *p*H of distilled water. However, when **MCR-1329**

was eluted using these mobile phases, very broad peaks were obtained. With mobile phases III & IV, tailing was also observed. It is known that *p*H of the mobile phase and its composition can be useful parameters in determining the elution of an analyte based on its *p*Ka value (Heinisch and Rocca, 2004). It was thus decided to use an acidic mobile phase for better and faster resolution of the test compound. **MCR-1329** was easily eluted in mobile phases VI-VIII, the reason being enhanced solubility due to an acidic *p*H. In some cases, it was found that lower concentrations of the test compound (below 10 ppm) were not resolved properly. The peak intensity and R_t of the analyte fluctuated on a case-to-case basis, i.e change in mobile phase and flow rate adjustments. No such problems were observed with mobile phases VII and VIII. Analyte peaks obtained with mobile phases VII and VIII were sharp, symmetric and free from tailing or shouldering effects. It was finally decided to utilize mobile phase VII for the purpose of analysis simply because of the relative ease of preparation.

Flow rate is known to affect not the resolution but only the retention time of the analyte in HPLC (Pous-Torres *et al*, 2009). Flow rate adjustments showed that a flow rate of 0.8 ml/min gave a peak between Rt of 3.5-4.5 mins, so that the time of actual analysis would be less than 5 mins, however, for the sake of brevity the time of analysis was kept 15 min. Most of the times, the analyte was eluted at an R_t of 4.2 ± 0.1 min. An R_t of less than 3 min at this flow rate would mean that the analyte is eluted in the dead volume of the 250 mm column. This combination of mobile phase and flow rate gave a

Table 13: Optimized HPLC parameters for quantification of MCR-1329

System	Shimadzu make
Column	Octadecyl silane (C18), Purospher
Mobile phase	ACN: 0.04M Na_2HPO_4 (*p*H=3); 40:60
Flow rate	0.8 ml/min
Pressure	108 ± 6 kgf
Injection Volume	20 µl
Temperature of analysis	25 ± 2°C
Detection wavelength	243 nm
Retention time (R_t)	4.2 ± 0.1 mins
Run time	15 mins

pressure of about 108 ± 6 *kgf* on the column which was of sufficient length for the analysis. Extraction efficiency of the sample preparation method was found to be greater than 90% at evaluated concentrations. The optimized HPLC conditions are summarized in the table 13.

Construction of calibration curves and linearity

Based on these parameters, calibration curves were constructed for **MCR-1329**. Two calibration curves were constructed, first after dissolving known amount of **MCR-1329** in mobile phase and preparing aliquots by serial dilutions (62.5-1000 ng/ml) and the second by spiking rat plasma samples from control animals with known amounts of **MCR-1329** (62.5-1000 ng/ml) and following the entire sample extraction and preparation procedure. The chromatograms, graphs, regression equations and linear correlation coefficient values for the same are presented in figures 16-19 below:

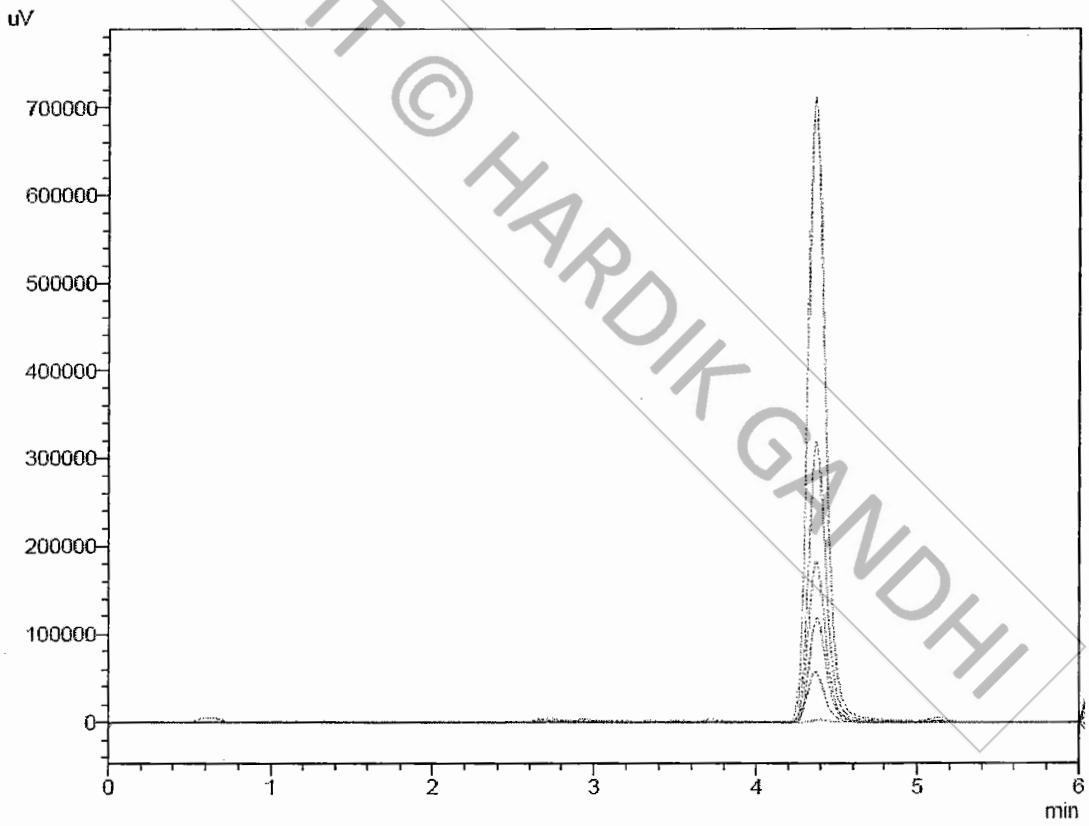


Figure 16: Chromatogram showing an overlay of peaks for calibration curve of analytical aliquots of the **MCR-1329**. The abscissa represents time in mins while the ordinate represents peak intensity in microvolts.

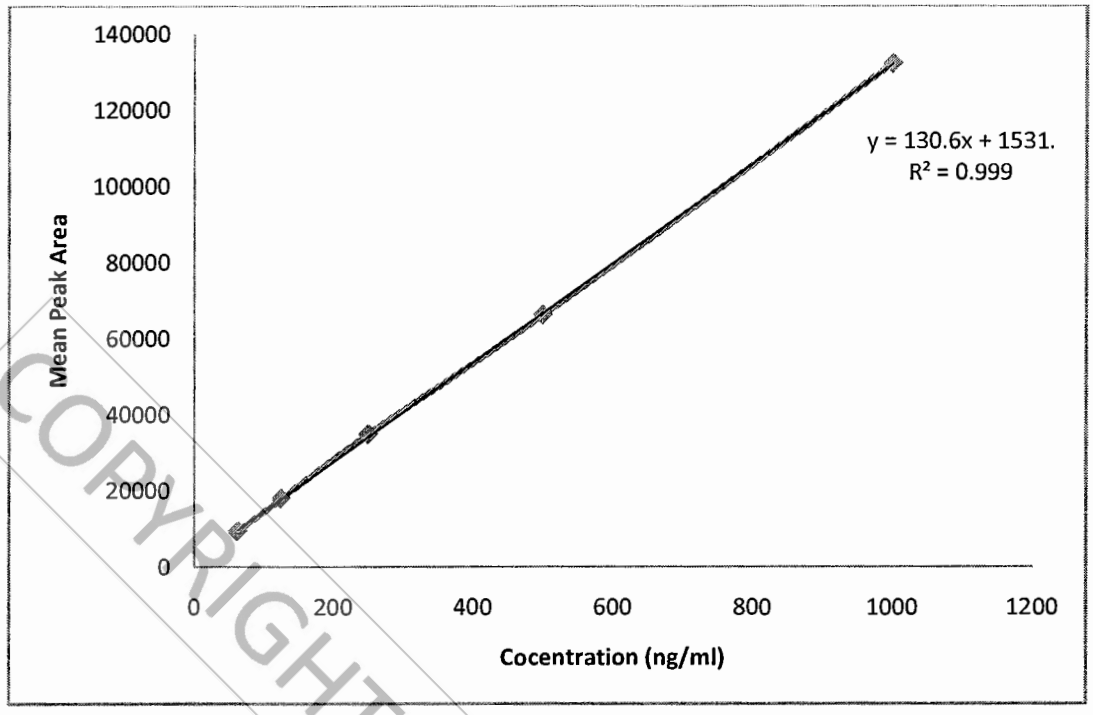


Figure 17: Calibration curve of **MCR-1329** from analytical samples dissolved in mobile phase. This graph was plotted using mean peak areas obtained for each peak at different concentrations.

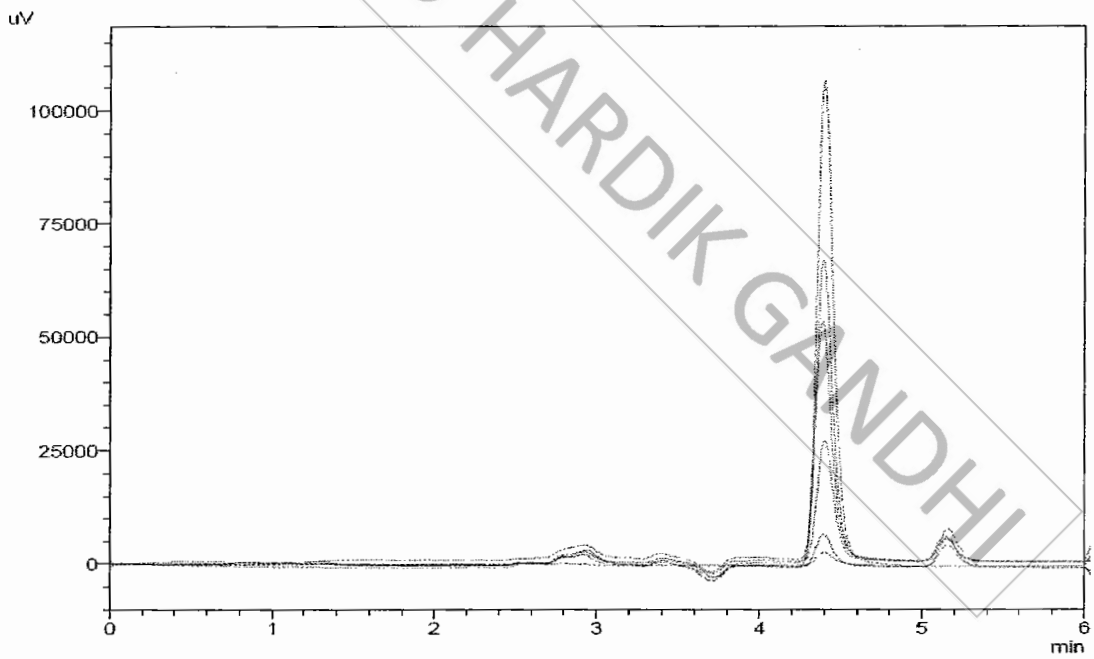


Figure 18: Chromatogram showing an overlay of peaks for calibration curve of spiked plasma samples. The abscissa represents time in mins while the ordinate represents peak intensity in microvolts.

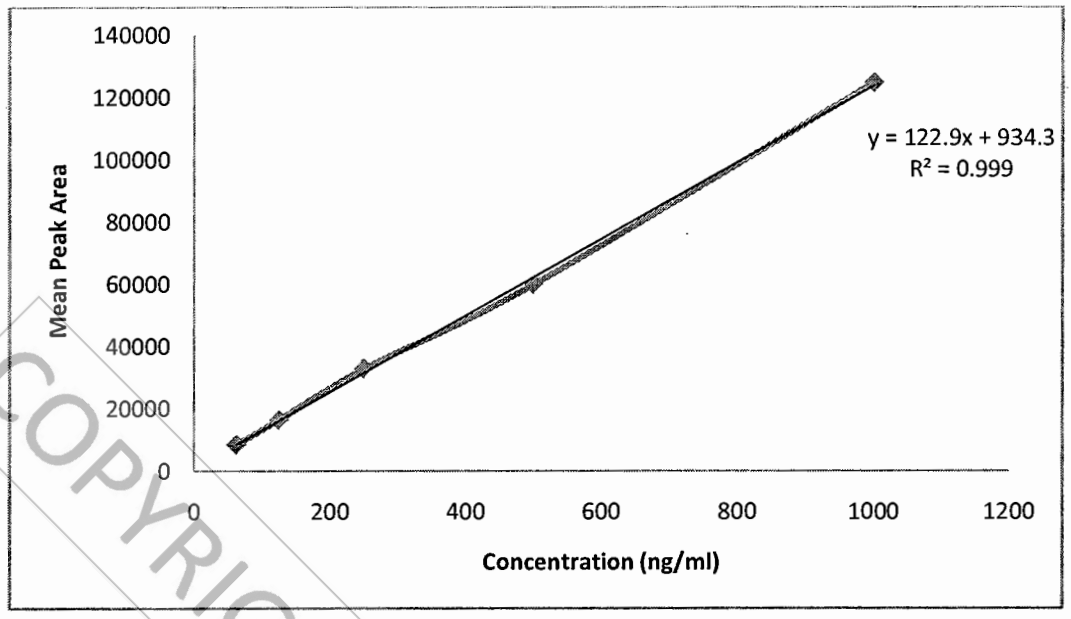


Figure 19: Calibration curve of **MCR-1329** from spiked plasma samples. This graph was plotted using mean peak areas obtained for each peak at different concentrations.

Determination of accuracy and precision

The repeatability of sample application and measurement of concentrations based on peak areas were expressed in terms of %RSD and are depicted in the table below. Table 16 shows the intra- and inter-day variations in concentrations of **MCR-1329** at three different levels (125, 250 and 500ng/ml). The proposed method afforded a recovery of between 94-106% which is well within the 85-110% range suggested in the

Table 14: Intra-day and inter-day precision of the method and recovery data

Concentration (ng/ml)	Intra-Day			Inter-Day		
	Conc. Found (ng/ml)	Accuracy (%recovery)	Precision (%RSD)	Conc. Found (ng/ml)	Accuracy (%recovery)	Precision (%RSD)
125	126.36 ± 3.68	101.08 ± 2.94	2.96	123.38 ± 5.87	98.94 ± 4.69	4.74
250	252.88 ± 10.16	101.15 ± 4.06	4.04	244.48 ± 5.89	96.86 ± 2.36	2.43
500	498.52 ± 10.24	99.70 ± 2.05	2.05	493.36± 10.16	96.58 ± 2.03	2.10

CDER guideline for bioanalytical methods (USFDA) indicating that the method is accurate and can be used for the quantification of **MCR-1329**. All RSD values for intra-day and inter-day precision fell within 5% which is again in agreement for the bioanalytical methods (Table 14). No major fluctuation was observed in the signal, and analyses between days were in close conformity with each other.

Oral dose disposition analysis

The mean concentration time course of **MCR-1329** after oral administration is shown in figure 20. The curve shows an asymmetric morphology, a deviation from the Gaussian distribution, suggesting that the elimination phase is prolonged and lasts longer as compared to the absorption phase. The fluctuations obtained at different concentrations may be a result of the dose adjustments made as per the body weight of animals used in replicate analysis. There is absence of any trough and a single peak is obtained, which indicates that the test compound is highly bound to plasma proteins and does not leave the vascular compartment. Binding of **MCR-1329** to plasma proteins was observed in a separate *in vitro* study. A shouldering concentration is found at 10 hrs after oral administration, which seems incidental since the remaining curve does not present any anomalies w.r.t disposition of **MCR-1329**. Measurable levels of **MCR-1329** were present in the serum upto 24 hr post dose but not beyond 28 hr. Overall, the results suggest that **MCR-1329** is rapidly absorbed from the stomach and upper part of the intestine after oral administration and is eliminated at a rate which is moderate as

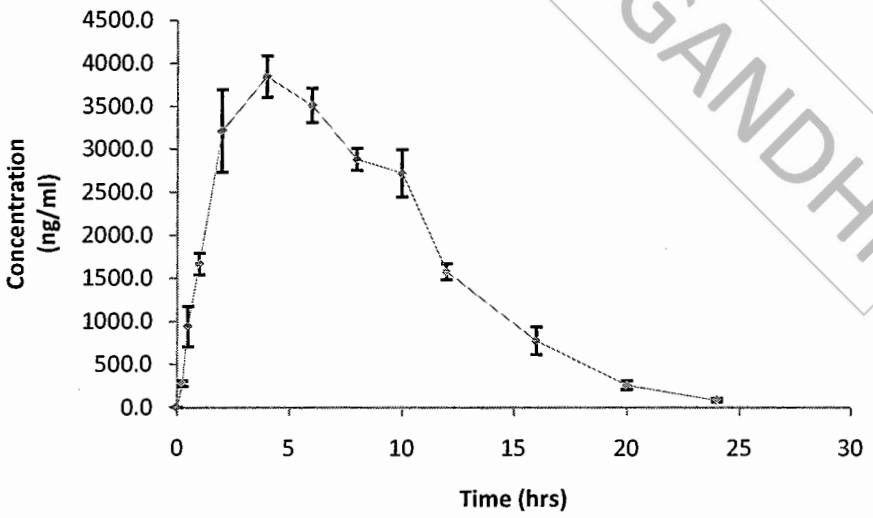


Figure 20: The figure shows the plasma concentration v/s time profile of **MCR-1329** after oral administration to rats (10 mg/kg, n=6).

compared to the absorption rate. The results obtained herewith can be interrelated with associated compounds like prazosin and other dimethoxyquinazolines (Chen *et al*, 1999; Sripalakit *et al*, 2005; Jaillon, 1980). Model independent pharmacokinetic parameters are listed in table 15.

Table 15: Summary of pharmacokinetic data based on non-compartmental model

Parameter	Dose (10 mg/kg)
C_{max} (ng/ml)	3840.8 ± 241.4
T_{max} (hr)	~ 4.0
K_{el} (h^{-1})	0.184
$t_{1/2}$ (hr)	3.77
MRT (hr)	7.68
AUC_{0-24} (ng.h/ml)	41495.9
$AUC_{0-\infty}$ (ng.h/ml)	41505.1

Human plasma protein binding study

MCR-1329 was allowed to bind to human plasma proteins present in pooled plasma and later on the release of this bound **MCR-1329** across a semi-permeable membrane was studied over a period of time. It was found that **MCR-1329** was gradually released into the stirred layer through the semi-permeable membrane as the bound form remained in equilibrium with the stirred layer which did not contain any amount of **MCR-1329**. The figure indicates the time-course of release of **MCR-1329** over a period of 24 hrs. It may be observed that even after 24 hrs of stirring (with sink conditions maintained by replacing fresh buffer), nearly 40% of **MCR-1329** still remained bound to the proteins in the plasma and was not released in the stirred layer (Figure 21). This study suggested that **MCR-1329** might remain in the vascular compartment upon absorption since it is highly bound to plasma proteins. As per the findings of the present study with **MCR-1329**, other dimethoxyquinazolines have been found to show a high degree (>90%) of plasma protein binding. Hence the results in this regards are in agreement with findings of other quinazoline derivatives (Jaillon, 1980; Uckun *et al*, 1999).

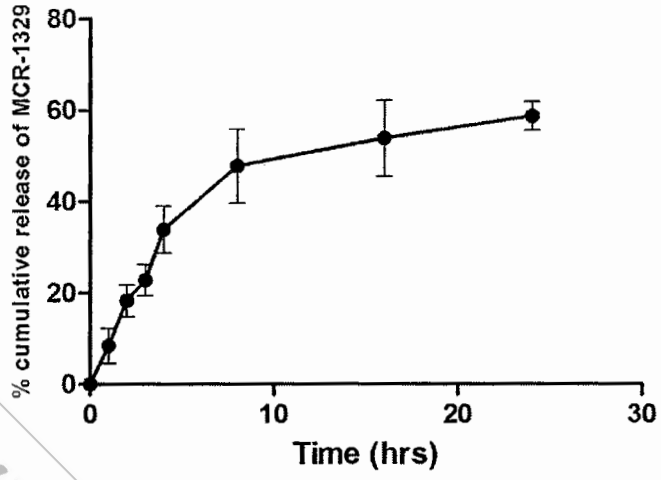


Figure 21: Time course of release of **MCR-1329** from human plasma protein binding. It is important to note that even after 24 hrs more than 40% **MCR-1329** remained bound to the plasma proteins and the slope of the curve is reduced after about 8 hrs.

DOCA SALT MODEL

MCR-1329 abates rise in blood pressure

Tail-cuff data

Pre-induction tail-cuff recordings showed that nearly all the animals utilized for the study were normotensive and no extreme levels of systolic blood pressure were recorded. DOCA administration alongwith salt intake is known to induce renal hypertension related to its sodium water retention effects (Seifi *et al*, 2010). DOCA being the precursor to aldosterone, is converted to aldsoterone *in vivo* through the action of the enzymes 11β-hydroxylase and subsequently aldosterone synthase. This *de novo* formation of aldosterone from exogenously aldministered DOCA leads to sodium retention in the distal tubules of the kidney (Iyer *et al*, 2010; Tomaschitz *et al*, 2010). However, the effects of such an exogenous administration may not be perceived until DOCA administration is also accompanied by salt intake (Badyal *et al*, 2003). Increase in blood pressure is mediated mainly through plasma volume expansion accompanied by sympathoexcitation and increased vasopressin levels (O'Donaughy and Brooks, 2006). Alteration of the central baroreflex (Schenk and McNeill, 1992), involvement of NOX pathways (Iwashima *et al*, 2008) and endothelin-I induced vasoconstriction (Callera *et al*, 2003) are also signatory to DOCA-salt mediated hypertension.

Severe hypertension was induced in the animals treated with DOCA-Salt (>160 mm Hg, $P<0.001$) towards the end of the study, whereas the UNX group showed non-significant rise in blood pressure (97.33 ± 2.22 v/s 108.3 ± 3.63 mm Hg). Control animals remained normotensive throughout the duration of the study. Animals treated with **MCR-1329** showed that **MCR-1329** was able to nullify the effects of DOCA-salt mediated renal hypertension and a 4-week regimen could conclude the antihypertensive effects of **MCR-1329** in animals. Likewise, the standard group also showed the beneficial effects of prazosin and losartan combination in animals treated with DOCA-salt. Figure 22 shows that there is scarcely any difference between the blood pressure values of the standard and the test group.

Invasive recording data

Intraarterial recordings were also comparable to those of the tail-cuff ones. Mean arterial pressure recordings showed that there was a difference of more than 60 mmHg units between the control and DOCA-salt group. Since pre-induction and post-induction levels could not be compared the terminal MAPs were directly evaluated by one-way ANOVA. The results indicated that DOCA-salt treated animals had significantly higher MAPs as compared to control and UNX animals whereas the treated animals (**MCR-1329** or standard combination) showed significant prevention in the elevation of MAP (Figure 23). Several studies have shown that unilateral nephrectomy induces negligible rise in blood pressure as compared to control animals (Giachini *et al*, 2011; Ndisang and Jadhav, 2010) which are in agreement with the results of the present study. No difference was observed between the pressure levels of **MCR-1329** and the standard group. As a matter of fact, the MAPs from the treatment groups were in close agreement with the UNX group suggesting that the treatments invalidated the hypertensive effects of DOCA salt. Since the molecular weights of **MCR-1329**, losartan and prazosin are in close agreement to each other, the doses reported in this study (*EXPERIMENTAL Section*) can be considered equivalent on a molar basis.

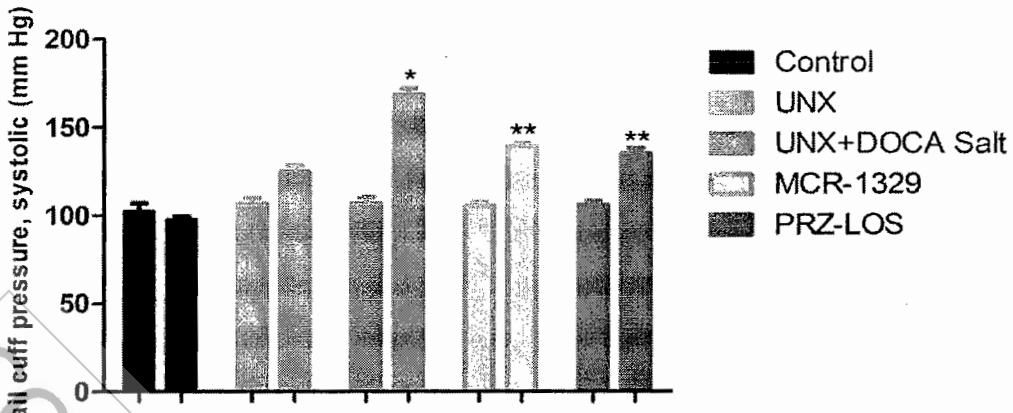


Figure 22: Figure shows the effects of mineralocorticoid induced hypertension in different groups of animals before and after induction/treatment. The figure clearly shows that all the groups are normotensive in the pre-induction phase whereas severe hypertension is evident in the UNX-DOCA Salt group. The animals treated with **MCR-1329** or a combination of prazosin and losartan were able to prevent DOCA-salt mediated hypertension in the animals. *indicates P<0.001 as compared to control group and **indicates P<0.001 as compared to the UNX-DOCA salt group.

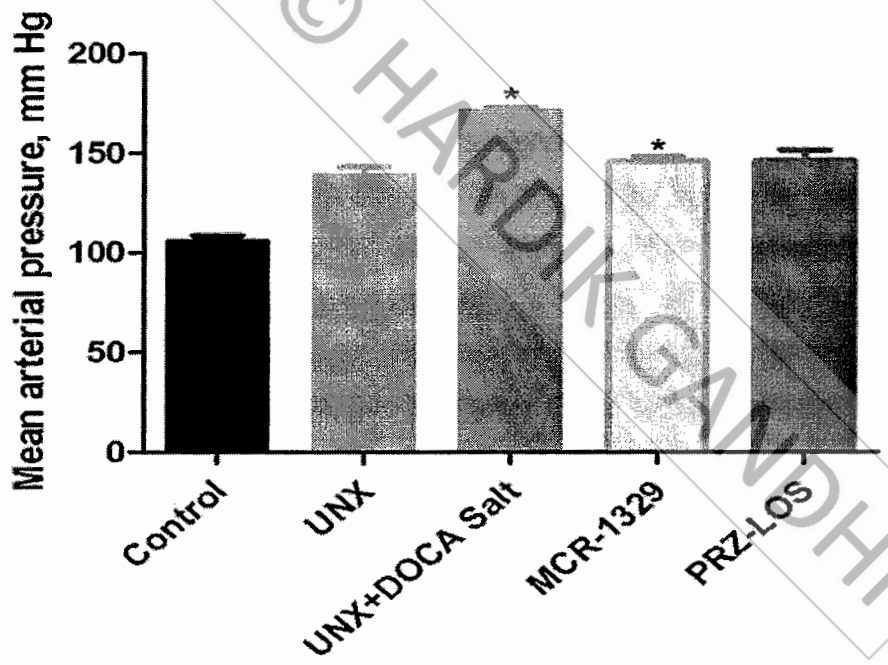


Figure 23: The columns indicate mean arterial pressures from different animals recorded at the carotid artery. It is evident from the graph that UNX-DOCA salt animals were severely hypertensive at the terminal stage of the study. Treatment with **MCR-1329** was able to ablate these destructive effects of DOCA salt and was comparable to those of standards. * indicates P<0.05 v/s control or UNX-DOCA Salt group.

MCR-1329 halts renal damage induced by nephrectomy and mineralocorticoid

Renal hypertrophy

Figure 24 indicates that UNX by itself does not lead to renal hypertrophy but mineralocorticoid induced pressure overload leads to enlargement of the renal capsule to a significant extent as suggested by Ndisang and Jadhav (2010). The unilateral renal capsule size increased nearly 2-fold suggesting the additional work done by the kidney. Moderate renal hypertrophy was also evident in the treated groups but was significantly lower as compared to the UNX-DOCA salt group. This suggested that **MCR-1329** by virtue of its effects on blood pressure and annulment of DOCA-salt mediated renal damage was able to prevent renal hypertrophy to a significant extent.

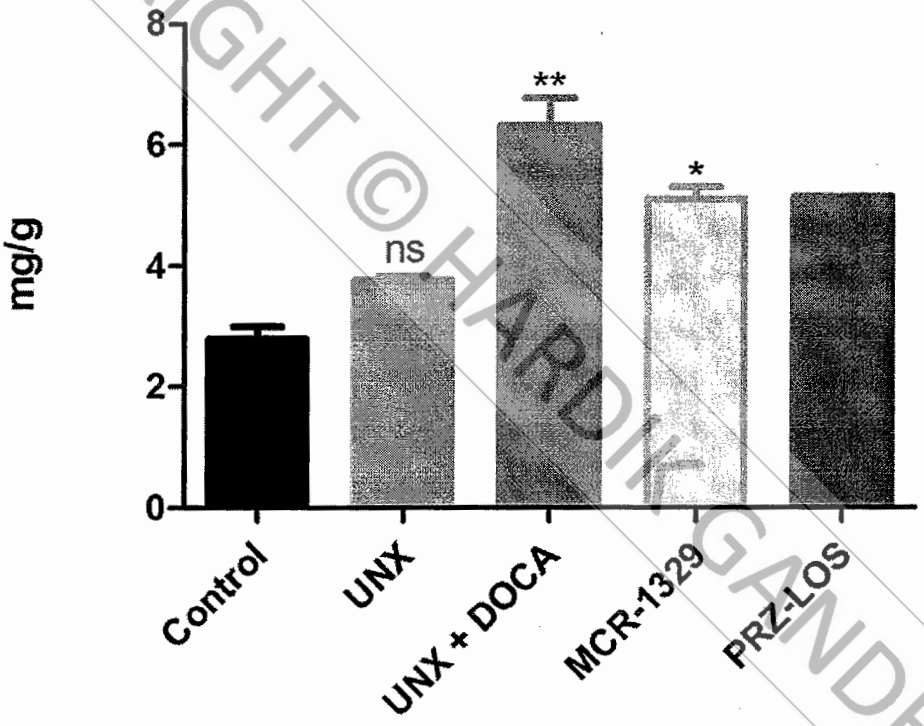


Figure 24: Figure demonstrates the ratio of kidney:body weight in different groups of animals. It is evident from the figure that UNX *per se* does not lead to kidney hypertrophy but additional overload produced by DOCA-salt causes the kidney to increase in size to compensate for GFR and other renal activities. 'ns' indicates non-significant change; *indicates P<0.05; **indicates P<0.01.

Urinary indices

Urinary parameters are important while studying mineralocorticoid induced hypertension since the major affected organ is the kidney. Hypertension mediated

overload leads to functional damage to the kidney which may be suggested by changes in excretion of electrolytes, creatinine, protein and glucose (Blasi *et al*, 2003; Ortmann *et al*, 2004; Artunc *et al*, 2006). Accordingly, it was decided to evaluate a battery of parameters which provide an index of renal function. It is known that mineralocorticoids like DOCA favour sodium/water retention and in turn supports excretion of potassium. In mild contrast to this finding, we found that urinary output was increased about 3-fold in DOCA-salt treated animals (82.7 ± 3.8 ml/day). This group was supplemented with 1% NaCl and 0.2% KCl in drinking water which increases the osmolarity of the drinking solution thus leading to increased volume intake and ultimately output as reported by Jia *et al* (2010) and Quigley *et al* (2009). Treated animals were also supplemented with 1% NaCl and 0.2% KCl in drinking water but the results indicated that **MCR-1329** and the standard drug therapy was able to prevent the effects of DOCA-salt on urinary output (Figure 25A). However, volume of intake was not measured as a part of this study. In concert with findings reported by others (Jia *et al*, 2010; Zhou *et al*, 1999), we also found that sodium excretion was significantly decreased (101.8 ± 6.5 mmol/lit) and potassium excretion increased (204.8 ± 8.2 mmol/lit) about 4-fold in the DOCA-salt treated group (Figure 25B, 25C). This was suggestive of increased sodium retention in the positive control group which might contribute to increased vascular volume factoring the rise in blood pressure. This data is in agreement with the findings reported by Jennings *et al* (2013), Zhou *et al* (1999) and Yemane *et al* (2010). It is important to note that **MCR-1329** was able to normalize urine (54.3 ± 4.5 ml/day) and sodium (123.1 ± 2.0 mmol/lit) output while reducing potassium excretion (158.4 ± 6.4 mmol/lit). The effects of standard therapy were found comparable to that of **MCR-1329** and were harmonious with the UNX group. The beneficial effects of **MCR-1329** in managing this condition may be attributed to its potential in controlling rise in blood pressure through antagonism of α_1 and AT_1 receptors and preventing the consequent effects of angII and other endogenous vasoconstrictor molecules.

Kidneys are known to effectively excrete creatinine in the urine and block the spillage of glucose and proteins in the urine. However, when renal structure is marred due to mineralocorticoid insult reinforced by overload it is possible that creatinine secretion is reduced (Sahan-Firat *et al*, 2010) and glucose/albumin (Rhaleb *et al*, 2011; Morrison *et al*, 2005; Quigley *et al*, 2009) or other proteins may spill in the urine.

Elevated levels of glucose (26.5 ± 2.7 mg/dl/day) and albumin (41.2 ± 2.7 µg/day) were observed in the DOCA-salt group (Figure 25D, 25E). Creatinine values in urine reduced about 4-fold (Figure 25F). Normally, glucose and proteins are absent in the urine (Ndisang and Jadhav, 2010; Dawson *et al*, 2000) but DOCA-salt groups showed elevated excretion of glucose and albumin which were reduced to a significant extent by **MCR-1329** (14.5 ± 1.1 mg/dl/day glucose and 17.7 ± 1.01 µg/day albumin). Creatininuria has been suggested to be a marker of renal function (Mombelli *et al*, 2013). Creatinine secretion improved in the **MCR-1329** treated group indicating normalization of renal function.

Based on these calculations, urine osmolality and creatinine clearance were derived using formulae and the results are summarized in figure 25 alongwith other urinary indices. Urine osmolality increased by 2-fold in the DOCA-salt group (Figure 25G) which may be attributed to the excretion of electrolytes, urea and glucose in the urine as a result of damage to the Bowman's capsule reducing its filtering capability. These results contradict the findings reported by Sahan-Firat *et al* (2010) and Bae *et al* (2009) who reported a decrease in urine osmolality with DOCA-salt groups. This discrepancy in the result may be a result of reduced fluid intake in some animals from the groups. Since this parameter is derived on the basis of several other parameters it is likely that actual urine osmolality is dependent on the values of the electrolytes, proteins and glucose. A decline in glomerular filtration rate was also evident from the creatinine clearance values which were reduced to about 3-fold in the DOCA-salt group as compared to the control group. Treatment with **MCR-1329** improved the individual urinary indcies but this was not reflected on creatinine clearance (Figure 25H). As can be seen from the figure there was only modest improvement in creatinine clearance, even with standard pharmacotherapy. Urine osmolality recuperated to a certain extent following and this effect was found to outshine that of the standard combination (Figure 25G). Upon closer examination, it was observed that potassium excretion was lesser in the **MCR-1329** group which was responsible for its improved urine osmolality values. This also suggested that risk of kaluria and consequent hypokalemia is lesser with **MCR-1329**, though not a known problem with quinazoline class of compounds. The effects of DOCA-salt and **MCR-1329** upon different indices of urinary function and creatinine clearance and urine osmolality values are summarized in figure 25.

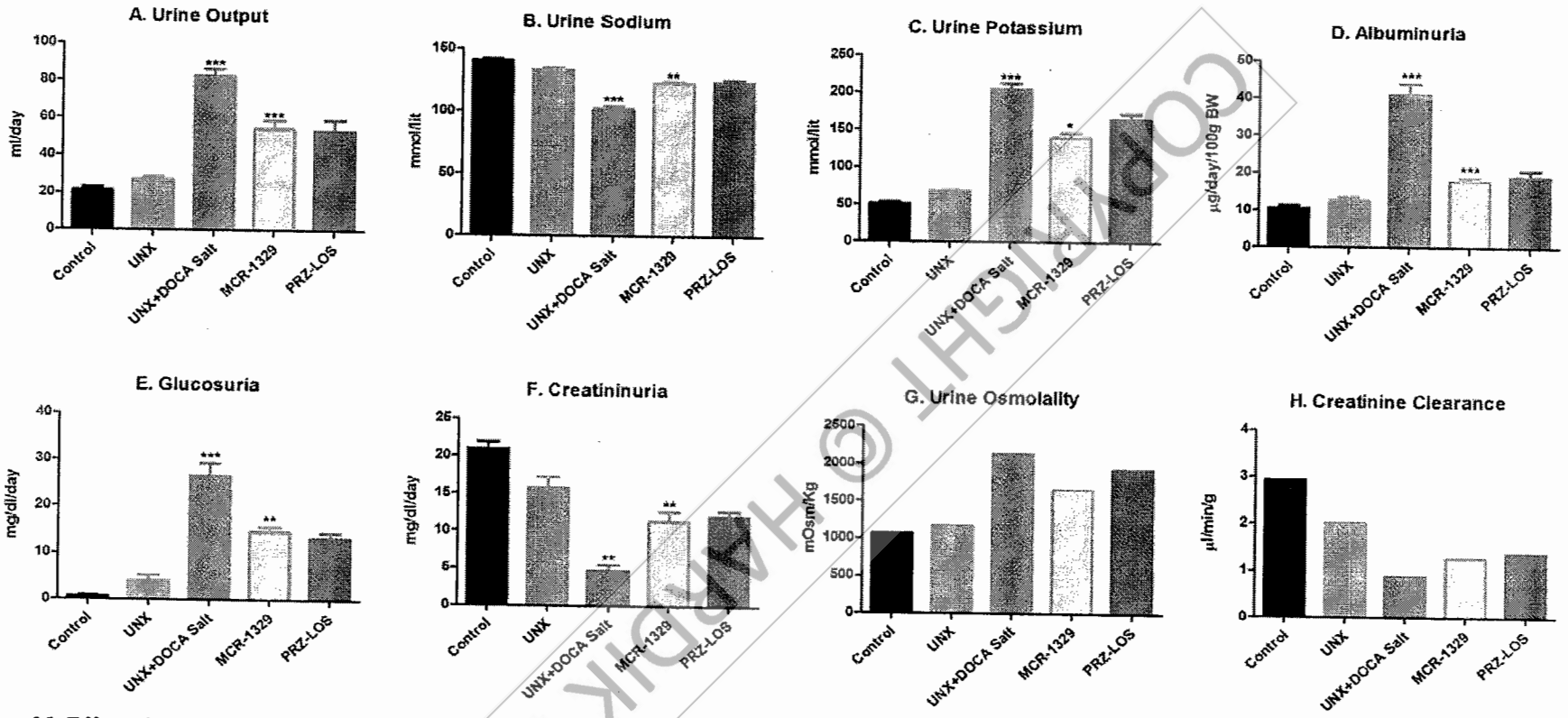


Figure 25: Effect of **MCR-1329** in UNX-DOCA salt hypertension. The figure summarizes the effect of **MCR-1329** on urinary indices like A) urine output is increased in the DOCA-salt group and normalized with **MCR-1329**, B) sodium retention is the major pharmacological effect of mineralocorticoids which is evident here. **MCR-1329** prevents retention of sodium, C) urinary potassium excretion is increased as a result of sodium retention in the DOCA-salt group. This effect was also improved with **MCR-1329** as a result of prevention of sodium retention, D, E) albuminuria and glucosuria may be considered as surrogates for glomerular function and are increased in the DOCA-salt mediated renal insult. Albuminuria reaches normalcy in the **MCR-1329** group, while glucosuria is reduced following **MCR-1329** treatment, F) DOCA-salt affected creatinine clearance is improved by **MCR-1329** therapy, G) Increased spillage of electrolytes and glucose increases urine osmolality and is prevented by **MCR-1329**, H) the unit of renal function, CrCl, is reduced in DOCA-salt group. **MCR-1329** mediated improvements are only modest. *P<0.05 as compared to UNX-DOCA salt group; **P<0.01 as compared to control or UNX-DOCA salt group; ***P<0.001 as compared to control or UNX-DOCA salt group.

Endothelial dysfunction: role of uric acid and effect of MCR-1329 on uric acid levels

It is known that hypertension leads to endothelial dysfunction. The major reason for this type of damage is the increase in pressure-mediated shear stress on the inner walls of arteries. This leads to gradual erosion of the endothelial layer and ultimately the effects of Ach/NO cannot be perceived (Sahan-Firat *et al*, 2010; Nunes *et al*, 2000; Jimenez *et al*, 2007). The present study dealt with evaluation of endothelial dysfunction in aortic strips from different groups of animals and comparison with sodium nitroprusside mediated relaxations. Figure 26 shows that relaxation was incomplete in strips obtained from DOCA-salt group, whereas Ach mediated complete relaxation of aortic strips from all other animals including those from the **MCR-1329** treated group. This suggested that since **MCR-1329** reins the rise in blood pressure, it might have a beneficial effect in preventing attrition of the endothelium thus maintaining the relaxant effect of endogenous vasodilators. Similar effects were observed in the standard treatment group as well.

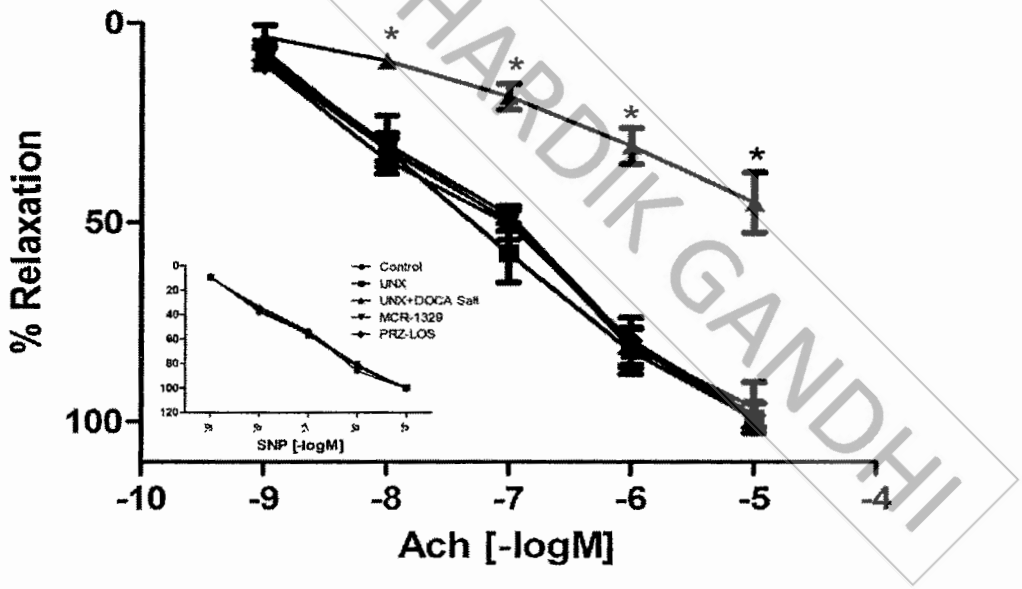


Figure 26: Cumulative concentration response curves to Ach ($10^{-9} – 10^{-5}$ M) in endothelium-intact strips precontracted with phenylephrine (1.5×10^{-6} M) from all the groups (n=3). Relaxation was significantly marred in strips from the DOCA-salt group. Inset shows sodium nitroprusside (SNP) mediated relaxant effects on the same strips. It is evident that SNP causes complete relaxation of the aortic strips irrespective of endothelial damage. * indicates $P<0.001$ as compared to control and **MCR-1329** group.

Uric acid has been traditionally considered as an inert metabolic product but off-late several studies have identified hyperuricemia to be a risk factor for occurrence of cardiovascular diseases (Johnson *et al*, 2003; Feig *et al*, 2008). It is not yet clear whether uric acid causes is a causative factor or a consequence of the effect of cardiovascular diseases including hypertension. Szasz and Watts (2010) evaluated the effects of uric acid on endothelial function in DOCA-salt hypertensive rats and reported that endothelial dysfunction is not predicted by uric acid levels. On the same lines we chose to evaluate the relation between endothelial dysfunction observed in the DOCA-salt group and serum uric acid levels. Contrary to the published reports it was found that uric acid levels in serum could not be correlated to any other parameter since no change was observed in the serum uric acid levels in animals from all the groups (Figure 27). One reason for such an observation would be the expression of the uric acid metabolising enzyme, uricase, in rodents which is not expressed in higher primates. This enzyme is responsible for metabolising any excess uric acid produced in the body. However, this study did not deal with estimation of allantoin which is the catabolic product of uricase activity on uric acid.

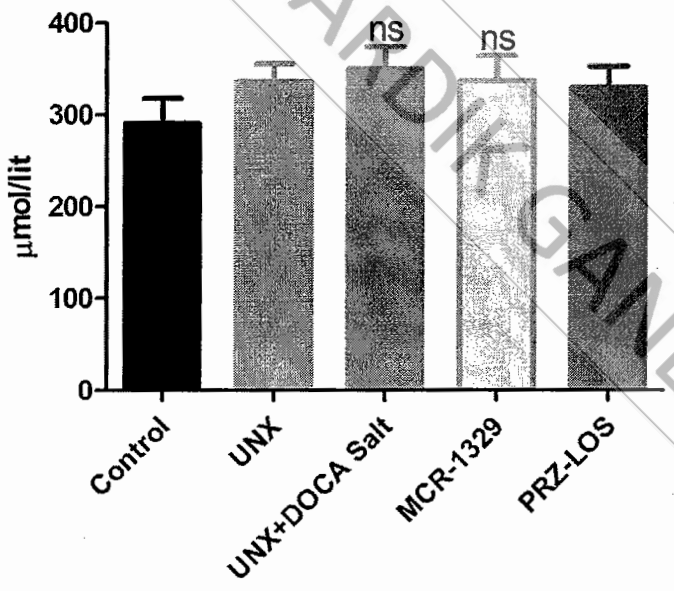
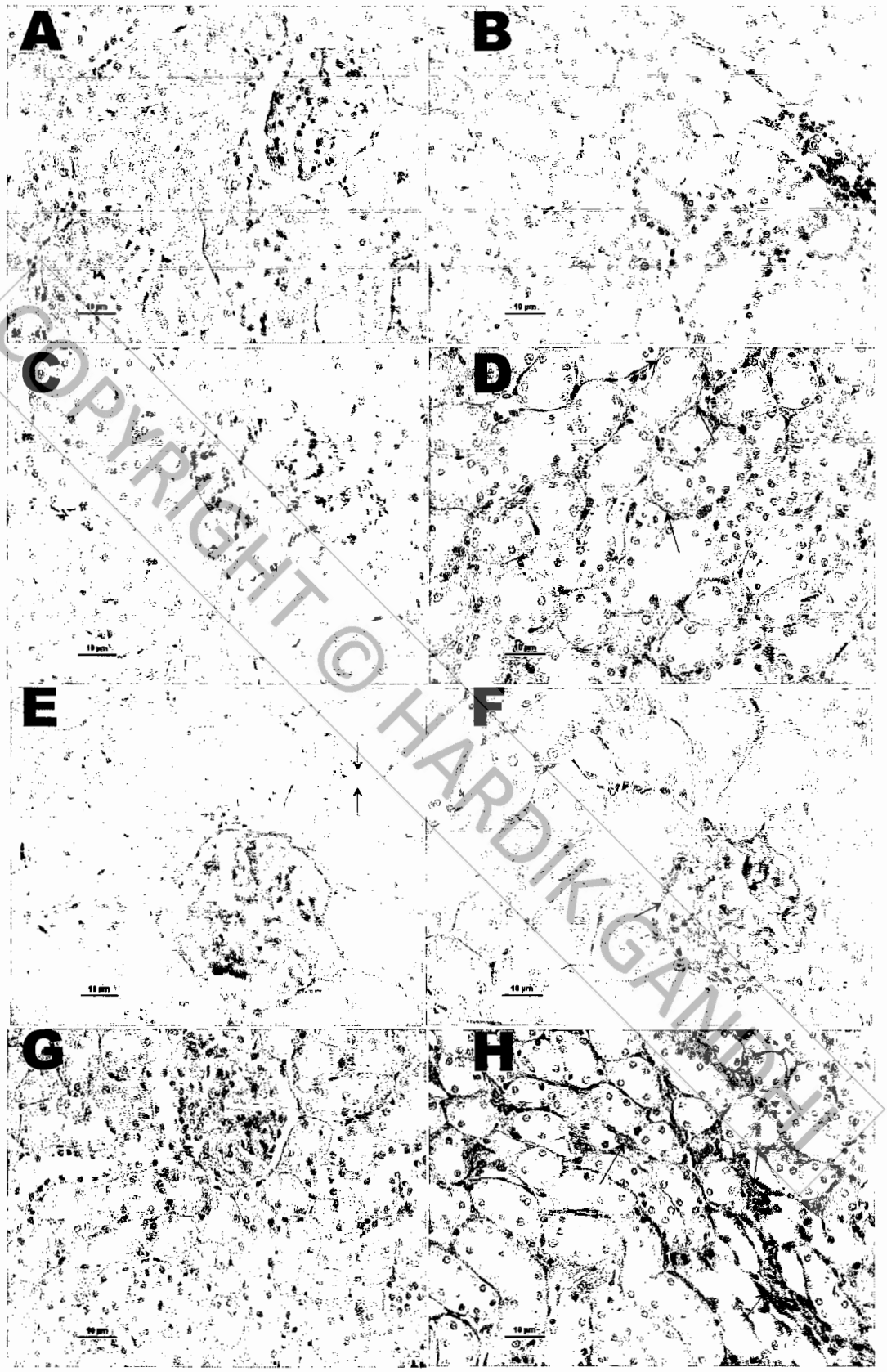


Figure 27: Uric acid levels in the serum of UNX, UNX-DOCA salt and **MCR-1329** treated animals were found to be comparable (n=5). Statistical evaluation did not show any significant difference between the groups regarding uric acid levels. ns=non significant.

Renal histopathology

Deposition of extracellular matrix components in the renal capsule and assessment of hypertensive damage was performed by Periodic-acid Schiff (PAS) staining (Figure 28) as described by Kiernan *et al* (1999). PAS staining is typically used to identify carbohydrate macromolecules like glycogen, proteoglycans and glycolipids deposited as matrix components in tissues. In a controlled reaction, the vicinal diols of the sugars present in these carbohydrates undergo oxidation with periodic acid and result in the formation of a pair of aldehydes. These aldehydes react with the Schiff reagent to give purple/magenta coloured complexes (Kiernan *et al*, 1999). Since nuclei are not stained with this stain, hematoxylin is used as a basic counter stain to visualise the nuclei. This technique is also utilised for the visualization of renal histoarchitecture. Several studies have reported the renal damage occurring in rats following DOCA-salt administration. It has been shown that mineralocorticoid and salt administration leads to cortical damage, glomerulosclerosis and tubulointerstitial damage (Blasi *et al*, 2003; Kim *et al*, 1994; Peng *et al*, 2001). There is damage to renal blood vessels resulting in luminal obliteration and blockade of blood flow in respective regions supplied by those blood vessels (Blasi *et al*, 2003; Lezin *et al*, 1999; Wang & Wang, 2009). Renal sections from the DOCA-salt group of the present study showed several PAS-positive sections indicating extracellular matrix deposition in the form of glycans. These were identified by dark purple regions in the sections. Glomerular damage was also evident and it was observed that Bowman's capsule had lost its normal morphology with the capillary tufts losing their normal formation and touching the glomerular walls (Figure 28E). Thickening of cortical cells (Figure 28F) due to matrix deposition and presence of hyaline casts were evident (Figure 28E). Glomerular basement membrane damage was identified by an absence of clear demarcation between the tufts and Bowman's capsule. Recuperation of these damages was observed in the treatment groups. A normal cortical area was observed in renal sections from **MCR-1329** and standard groups. PAS-positive areas were observed at the bowman's capsular walls in the **MCR-1329** group (Figure 28H) and near the cortical cells in the standard group (Figure 28J). The UNX group did not show any major pathological changes except for a few PAS-positve areas. The control group sections remained PAS-negative.



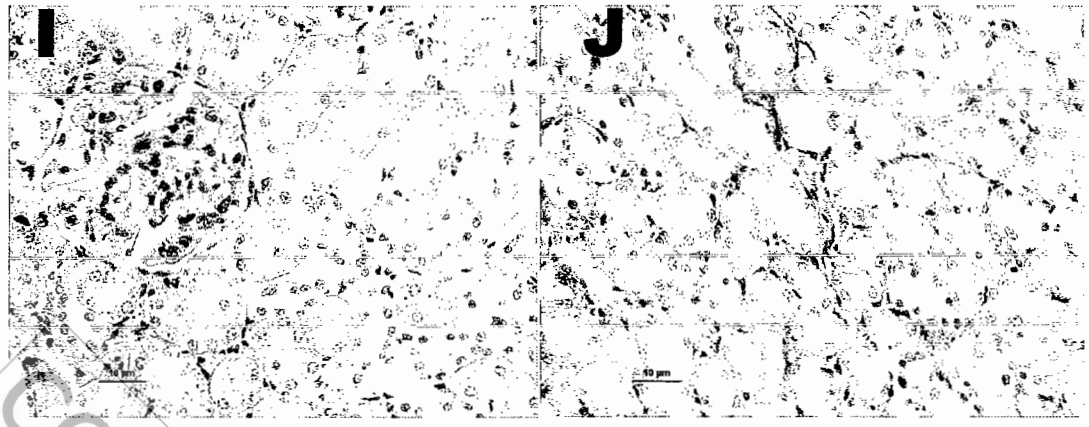


Figure 28: Light micrographs representing renal histology of different groups (Periodic acid schiff stain, 40X, bars represent 10µm). Control group (A, B), UNX (C, D), DOCA-salt (E, F), DOCA-salt + **MCR-1329** (G, H) and DOCA-salt + standard (I, J). While figures A-B virtually show no pathological changes, E-F are indicative of hypertensive renal damage in the cortical region as well as glomeruli. Derangements in the cortical cells and capillary tufts are evident. In contrast, figures G-J are relatively protected from damage and cortical cells appear normal, however, reduction in the space between capillary tufts and glomerular inner walls are still evident at the terminal stage.

CELL CULTURE STUDIES

Cytotoxicity assay

Before initiating the cell-signaling studies, it was important to determine the extent of cytotoxicity produced by **MCR-1329** on the cell line utilised. If the cells produce major cytotoxicity at concentrations used for the study, then cell signaling studies cannot be performed as treatment might lead to death of cells. Accordingly, cytotoxic potential of **MCR-1329** was evaluated on the rat aortic smooth muscle cell line by the MTT assay. The results of the study are shown in figure 29.

Cell signaling studies

The PI_3K pathway in VSMCs is important in relation to proliferation, mitogenesis, cell cycle progression and a range of cellular processes (Shigematsu *et al*, 2000; Katso *et al*, 2001). Its role has also been implicated in the pathogenesis of hypertension (Yang and Raizada, 1999; Quignard *et al*, 2001; Carnevale *et al*, 2012b). PI_3K is directly involved in vascular remodelling and proliferation of major and minor blood vessels (Saward and Zahradka, 1997). There are several downstream targets of

PI_3K like PDK-1 (Phosphoinositide-dependent Kinase 1), Akt and $p70^{S6K}$ (p70 ribosomal protein S6 kinase) among others.

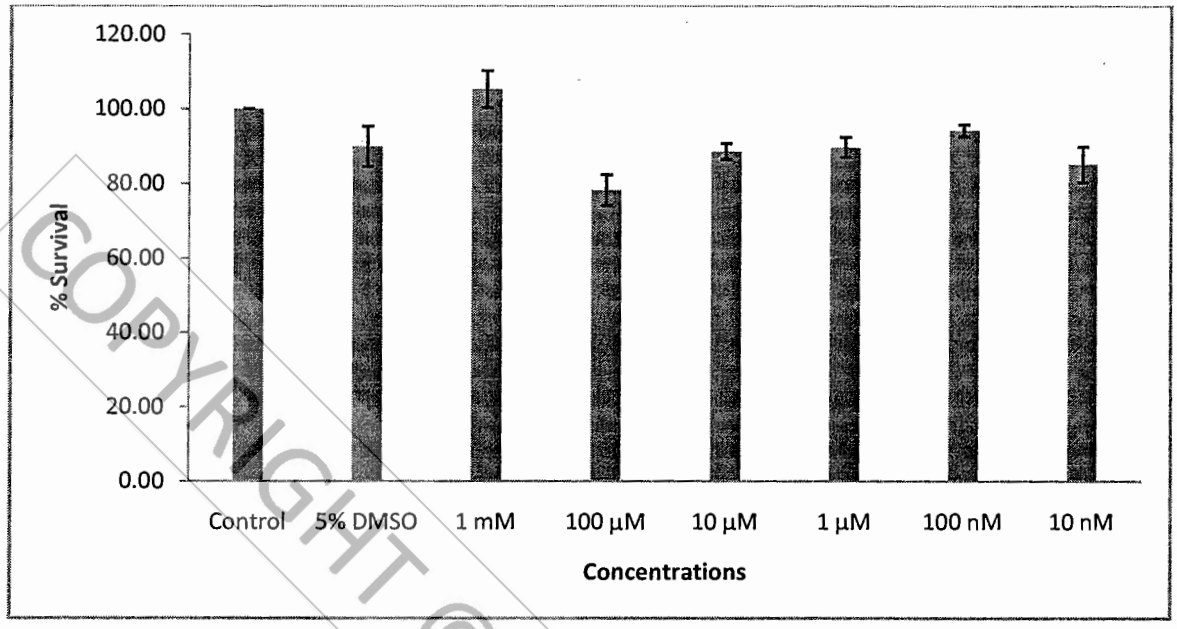


Figure 29: Survival of cells post-incubation with different concentrations of **MCR-1329**, vehicle and control cells are shown (n=6). Survival was found to be >80% at most of the concentrations suggesting that **MCR-1329** is not cytotoxic upto a concentration of 1mM. Statistical analysis did not find any significant difference between the groups.

Of these, Akt is one of the major downstream targets (Quignard *et al*, 2001). PI_3K mediated activation of Akt is induced through phosphorylation of Akt at Thr-308 (Alessi *et al*, 1997) however, some researchers have also indicated phosphorylation at Ser-473 (Toker and Newton, 2000; Atwell *et al*, 2000). Several lines of research indicate that PI_3K signaling activated through hypertensive afferents like angII and $\alpha 1$ agonists involve Akt as the major downstream molecule (Takahashi *et al*, 1999b; Dugourd *et al*, 2003). It has been shown that angII, norepinephrine and phenylephrine are able to increase the phosphorylation of Akt at Thr-308 and/or Ser-473 (Li and Malik, 2005b). In addition, antagonists of AT_1 and α_1 receptors are able to prevent or reduce agonist-mediated Akt-phosphorylation (Dugourd *et al*, 2003). All such studies have utilised either wortmannin or LY-294002, known inhibitors of PI_3K, to show that agonist-mediated Akt-phosphorylation is mediated through PI_3K signaling (Kippenberger et al, 2005; Dugourd *et al*, 2003). Based on these evidences, it was decided to evaluate **MCR-1329** for its potential to block agonist-mediated Akt-

phosphorylation. Since Ser-473 is debatable (Toker and Newton, 2000; Atwell *et al*, 2000), it was chosen to evaluate the Thr-308 phosphorylation of Akt using flow cytometry. Results indicated that both angII and phenylephrine were able to induce Akt-phosphorylation to a significant extent in rat aortic smooth muscle cells. Figure 30 shows the controls indicating that cell treatments and the 2° antibody by itself do not show fluorescence as such, but as shown in Figure 31A, phenylephrine causes significant increase in the fluorescence which is a result of Akt-phosphorylation at Thr-308 and consequent binding of the antibodies (indicated by the presence of the P3 population in the histogram).

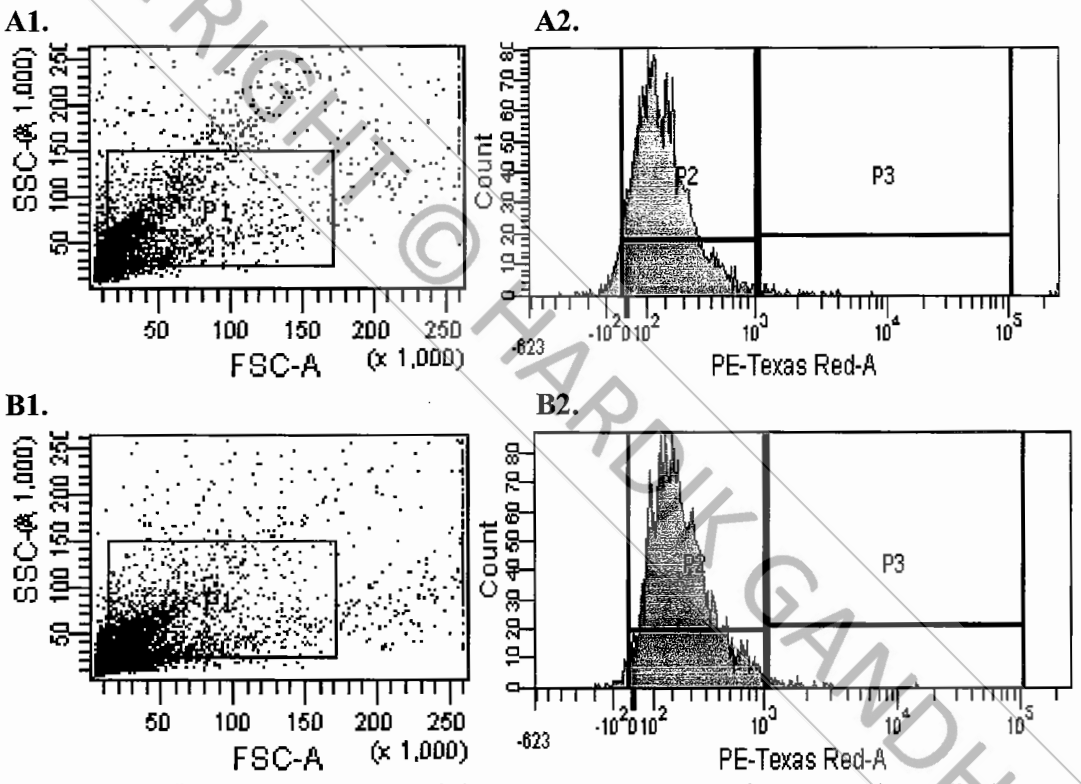


Figure 30: This figure indicates the controls in the flow cytometry experiment. Unstained cells shown no deviation from the parent population (A) and treatment with only 2° antibody did not show any major shift towards the P3 population (B) suggesting that washing efficiency and dilutions are optimal.

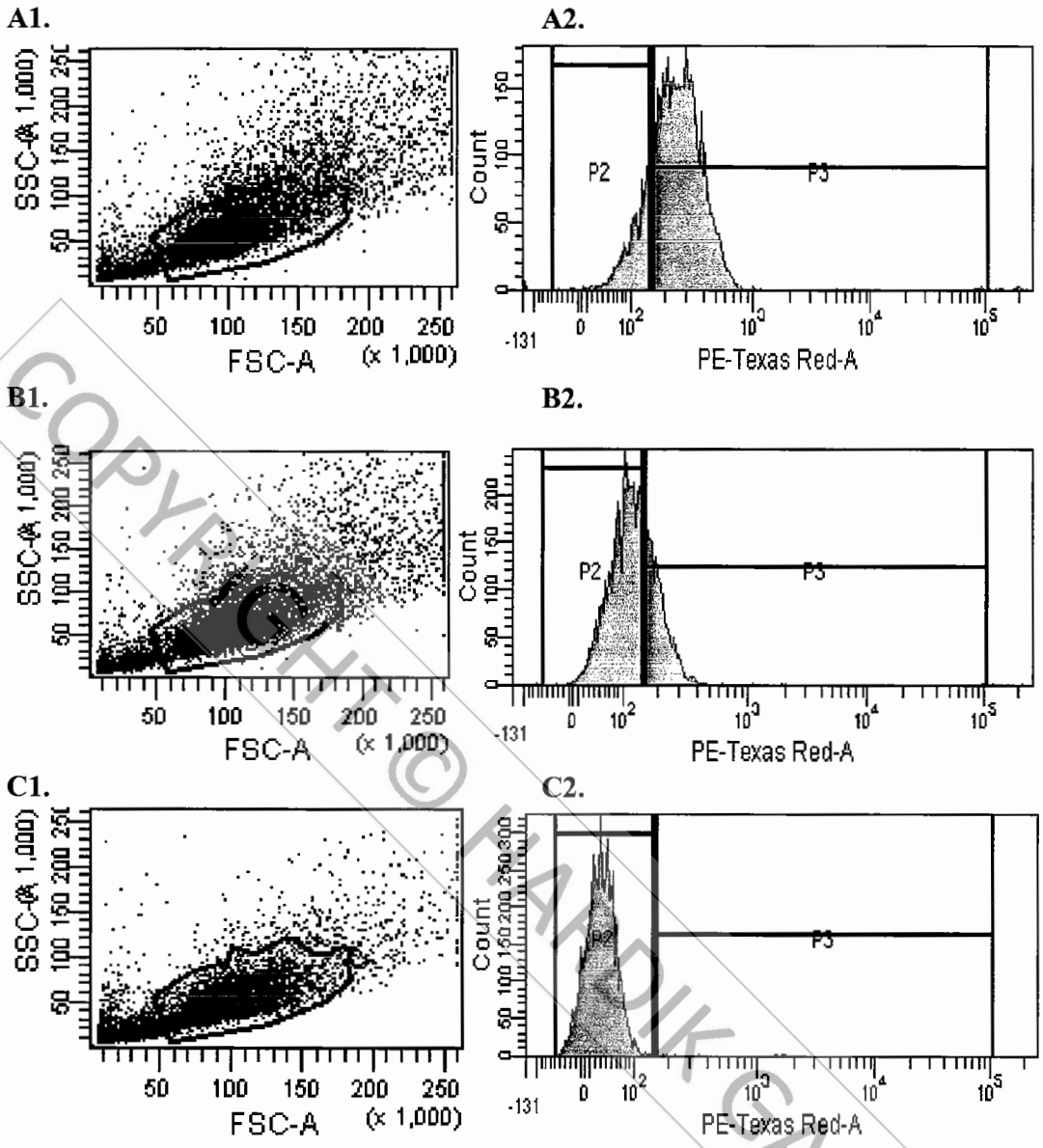


Figure 31: The scatter obtained from flow cytometry and corresponding histograms are shown here. P1 is the entire population gated from 10000 events. The figures represent different treatments: A, Stim1 (phenylephrine); B, Stim1 + **MCR-1329**; C, Stim1 + **MCR-1329** + LY294002

Similar results were obtained when angII was used to stimulate the cells (Figure 32A). Treatment with **MCR-1329** showed a 40-50% reduction in Akt-phosphorylation (Figures 31B and 32B). LY294002 is a selective inhibitor of PI_3K isoforms, comparable to wortmannin (Dugourd *et al,* 2003; Yang and Raizada, 1999). When LY294002 was used in conjunction with **MCR-1329**, it was observed that Akt-phosphorylation was completely blocked (Figures 31C and 32C) suggesting that **MCR-1329** prevents PI3K mediated Akt-phosphorylation. These results are in conjunction with previous studies

showing the role of AT1 or α1 antagonists in preventing PI3K-mediated activation of Akt. Dugourd *et al* (2003) and Yang and Raizada (1999) respectively showed the effect of irbesartan and losartan in preventing Akt-phosphorylation. Wang *et al* (2005), were able to show that prazosin prevents phenylephrine-mediated activation of Akt. These studies also showed that agonist-mediated effects were doctored by PI_3K as suggested through complete inhibition when LY294002 was employed.

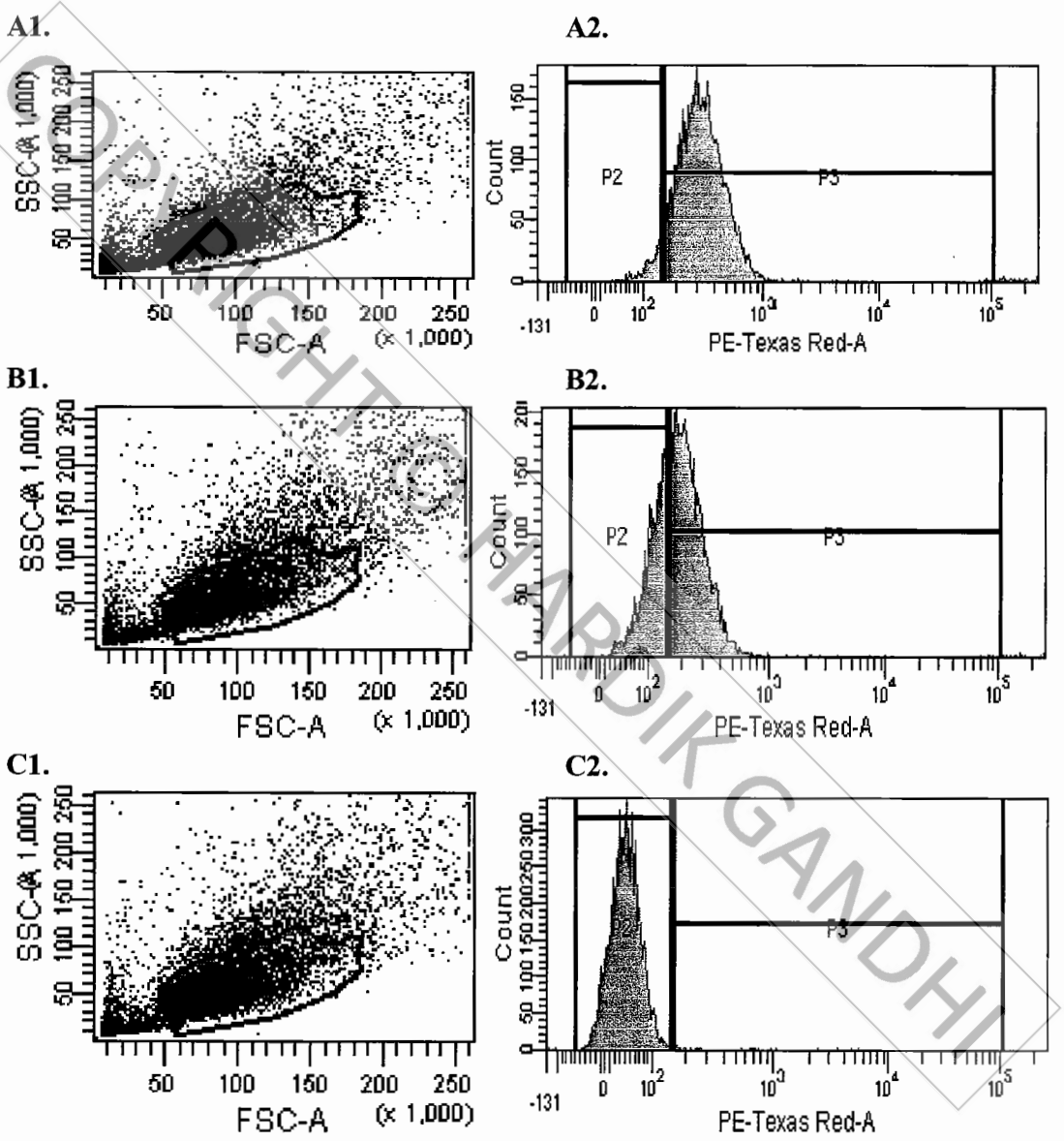


Figure 32: The scatter obtained from flow cytometry and corresponding histograms are shown here. P1 is the entire population gated from 10000 events. The figures represent different treatments: A, Stim2 (AngII); B, Stim2 + **MCR-1329**; C, Stim2 + **MCR-1329** + LY294002

Based on these results it was concluded that the beneficial effects of **MCR-1329** are, in part, mediated through inhibition of PI_3K-mediated Akt-phosphorylation. The quantitative results are summarized in Table 16.

Table16: Quantitative summary of flow cytometry data

Sr. No.	Treatment	P1 population (no. of cells)	P2 (% of P1)	P3 (% of P1)
CONTROLS				
1	Control (Unstained)	5134	96.9	1.2
2	Secondary Antibody	5456	94.0	1.4
PHENYLEPHRINE STIMULUS				
3	Stim1 (PE)	5338	16.8	77.6
4	Stim1 + **MCR-1329**	5667	66.8	25.3
5	Stim1 + **MCR-1329** + LY294002	4855	99.8	0.2
ANGIOTENSIN II STIMULUS				
6	Stim2(AngII)	4571	5.4	91.2
7	Stim2 + **MCR-1329**	5543	32.9	59.2
8	Stim2 + **MCR-1329** + LY294002	5614	99.3	0.5

On the basis of the present findings, it may be said that receptor-mediated activation of PI_3K and subsequent AKt-phosphorylation may be the key events related to the development of hypertension and that antagonism of α_1 and angII receptors can certainly abrogate hypertension mediated through these mechanisms.

EXPERIMENTAL

EXPERIMENTAL

MATERIALS

Test compounds were synthesized by the Pharmaceutical Chemistry lab of Pharmacy Dept., Faculty of Tech. & Engg., The M. S. University of Baroda. Rat aortic smooth muscle cell line, A10 clone, was procured from National Centre for Cell Sciences (NCCS), Pune, India. Phenylehprine hydrochloride, angiotensin II, prazosin, acetylcholine chloride, deoxycorticosterone acetate and bovine serum albumin were procured from Sigma Aldrich, St. Louis, MO, USA. Valsartan, olmesartan medoxomil and losartan potassium were kind gifts from Torrent Pharmaceuticals Ltd., Gujarat, India. Dulbecco's minimum essential medium (DMEM, High Glucose), Trypsin: EDTA solution, Fetal bovine serum (Australian donor herd origin), Antibiotic-antimycotic solution, tissue culture flasks (T-25 and T-75), MTT and lyophilized phosphate buffered saline (PBS, *p*H 7.2) were procured from HiMedia, Mumbai, India. 1° antibody directed against Thr-308 phosphorylated site of Akt (rabbit origin; Cat# sc-16646-R) was procured from Santacruz Biotechnology Inc., USA and 2° Alexa Fluor 594 conjugated goat anti-rabbit antibody was purchased from Life Technologies, USA. Heparin, ketamine, diazepam, tramadol, doxazosin and terazosin were procured locally. Other reagents were of analytical grade and procured from local companies.

ANIMALS

Animals were procured from licensed animal breeders. Animals were housed in an air-conditioned room (25 ± 2 °C, 50-65 % RH) in plastic cages (NMT 3 animals per cage) having paddy husk (Shree Dutt Agro Pvt. Ltd., Vadodara) as bedding with 12 hr light-12 hr dark cycles. They had free access to pelleted diet (Pranav Agro Foods Pvt. Ltd., Pune, India) and tap water. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Pharmacy Department, Faculty of Tech. & Engg., The M. S. University of Baroda, Vadodara. All experimental procedures were carried out as per CPCSEA guidelines.

FUNCTIONAL ANTAGONISM ASSAY USING ISOLATED RAT THORACIC AORTA PREPARATION

This assay was selected to determine the antagonistic effect of test compounds against angII and phenylephrine mediated contractions of rat aortic strips. Male wistar rats (14-16 weeks old; 200-250 g) were used for the study. They were sacrificed by cervical dislocation; descending thoracic aortas were removed immediately and placed in ice-cold Kreb's bicarbonate solution of the following composition (mM): NaCl 112, $NaHCO_3$ 12, glucose 11.1, KCl 5.0, $MgSO_4$ 1.2, KH_2PO_4 1.0 and $CaCl_2$ 2.5. The tissue was aerated with 95 % O_2 and 5 % CO_2. Peri-adventitious tissue was removed, taking care not to stretch the tissue. A spinal needle was inserted in the tissue and rotated gently to denude the endothelium. Following this, the tissue was cut spirally into a helical strip (20 mm × 3 mm) using a surgical blade. The strip was tied at both ends using a cotton thread and suspended in a 25 ml organ tube under an initial resting tension of 2 g. The *p*H of the Kreb's solution was 7.4 and maintained at 37°C using a thermostat. The Kreb's solution in the organ tube was changed every 10 mins during an equilibration period of about 90 mins. Denudation of the endothelium was confirmed by observing the "absence of relaxation" on strips precontracted with phenylephrine. Isometric contractions were recorded using a force transducer (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy). Contractions were induced in rat aortic strips with graded, cumulative concentrations of phenylephrine or angII. Losartan, valsartan, olmesartan or test compounds were added to organ tubes 30 mins prior to recording of the addition of phenylephrine. Similar procedure was followed to record graded cumulative response curves of angII against prazosin, doxazosin, terazosin and the test compounds. Control strips were incubated with solvent [DMSO (0.5%) or normal saline] for 30 mins before recording the concentration response curves. All the tissues were incubated with antagonists for 30 mins but incubation upto 45 mins did not affect the results. Reversal of inhibition induced by the antagonists was evaluated following repeated washings. pA_2 values were calculated by the method described by Arunlakshana and Schild (1959). Any effect mediated by the endothelium was not expected since the endothelial layer was removed while preparing the aortic strip. The primary basis for selecting this assay was cross-screening of angII blockers against phenylephrine mediated contractions and that of α1-adrenoceptor blockers

against angII mediated contractions. This assay was also utilized for screening of the test compounds for AT_1 and α_1 antagonism.

IN VIVO PRESSOR RESPONSE EVALUATION

After anesthetizing the animals (ketamine 100 mg/kg, i.p; diazepam 10 mg/kg, i.p.), righting reflex and pinching response were confirmed. A blunt dissection was performed on the ventral neck region and sternohyoid muscles were removed to expose the trachea. A partial transverse cut was put between the cartilagenous regions and a PE tube was inserted in the trachea which was connected to a ventilator (70 strokes/min; stroke volume 10 ml) for allowing unobstructed respiration. Following this, vagotomy was performed to exclude the effect of vagus. The carotid artery was isolated and cannulated with a PE catheter attached to a pressure transducer for measurement of intra-arterial blood pressure. The transducer and cannula were filled with heparinized saline (100 IU/ml) to prevent accidental clotting of blood inside. The transducer was connected to Powerlab-4/35 data acquisition system (AD Instruments, Australia) for recording the blood pressure. The left femoral vein was cannulated using a scalp cannula (27 gauge; 1 inch length) for administration of test compound (MCR-1329) dissolved in saline with 0.5% DMSO. Baseline blood pressure was recorded for 30 mins following which pressor responses to phenylephrine (6 µg/kg, i.v. bolus) and angII (6 µg/kg, i.v. bolus) were observed in absence and presence of MCR-1329. Inhibition of pressor response was observed at 2 dose levels; 0.36 µmol/kg and 0.72 µmol/kg, i.v. bolus.

Masked Conditions: It was planned to evaluate the pressor-inhibition potential of MCR-1329 under masked conditions. *In vivo* inhibition of phenylephrine mediated arterial pressor response by MCR-1329 was measured in those animals in which 3.6 µM losartan was pre-administered. The idea behind such a protocol was to mask the effects of MCR-1329 on AT_1 receptor. Similarly, the other set involved measurement of inhibition of angII mediated arterial pressor response in those animals in which prazosin (0.72 µM) or terazosin (0.72 µM) were pre-administered to mask the effects of MCR-1329 upon α_1-receptor. The standard drugs and MCR-1329 were administered at equimolar concentrations. Surgical protocol remained the same as described above.

TOXICOLOGICAL EVALUATION OF MCR-1329

Single dose acute oral toxicity study-OECD 423

This study was performed using male wistar rats (10-12 weeks old, 200-230g). Although the guideline suggests that female animals may be preferred, it was decided to evaluate the test drug in male animals to avoid the protective effects of estrogens upon the cardiovascular system which may become evident when female animals are used. This study does not allow calculation of precise LD_{50} values; rather it allows determination of exposure range where lethality might be expected. The test compound (MCR-1329) was suspended freshly in Na-CMC (0.5 %) just before dosing and administered by oral gavage on day 0 in overnight-fasted animals. Urinary and biochemical parameters were recorded at day 0 and day 14. There was no reason to believe that therapeutic doses may range beyond the highest selected dose (i.e. 2000 mg/kg). During the period of study, animals were observed closely, their weight and food intake was recorded twice-a-week and any abnormal behavior, if observed, was documented. Since no preliminary information regarding toxicity of the test substance was available, it was decided to omit the limit test and directly the main test was conducted. Annex 2c of the main guideline document (OECD 423) was followed unless otherwise indicated. Accordingly, 3 animals were dosed in each group and the subsequent dosing depended on the condition of the previously dosed animals. At the end of the observation period for each group, the animals were humanely sacrificed and gross necropsy was performed by closely observing all the major organs. Histopathological and microscopic observations were performed only in case of any untoward observation. Category 5 evaluation was precluded from the evaluation since a dose beyond 2000 mg/kg was unlikely to be used for any therapeutic studies.

Repeat dose oral toxicity study – OECD 407

The purpose of this study was to evaluate the effect of the test compound when administered for chronic regimens at the selected dose. Based on preliminary acute toxicology data and literature review of related class of compounds, 10 mg/kg was chosen to be the animal therapeutic dose for pharmacodynamic studies. Accordingly, this dose was selected for this toxicity evaluation, since this dose is twice that of maximum intended therapeutic dose. The guideline mentions the use of a range finding test or a limit test with a dose of 1000 mg/kg but since such a dose level is unlikely to

be used and corresponding human dose may never be applied in practice, we preferred using a dose of 10 mg/kg. This study was performed in healthy adult wistar rats (male and female, 10-12 weeks old, 200-230 g). At the end of the dosing period, tail-cuff pressures were recorded, blood samples were collected in 3.2 % citric acid for hemoatology, and serum & urine biochemistry was performed. At the end of the study, gross necropsy was performed and major organ weights were recorded (brain, heart, lung, liver, spleen, kidney). No adverse effect was observed during the period of dosing (0-28 days) or during the post-dose observation period (29-42 days). Neither kidney nor brain (organs most likely to be affected) showed any signs of hypotension-mediated damage which was expected with this category of compounds.

PHARMACOKINETICS OF MCR-1329

Pharmacokinetic studies can be performed by non-compartmental or compartmental methods. Non-compartmental methods estimate the exposure to a drug by estimating the area under curve of a concentration-time graph whereas the compartmental methods estimate the concentration-time graph using kinetic models. Non-compartmental methods are often more versatile in that they do not assume any specific compartmental model and produce accurate results acceptable for initial drug discovery programs.

Oral dose disposition

Healthy, male wistar rats (14-16 weeks old; 200-250 g) were used for the study (n = 6). All the rats were fasted overnight and one pre-dose blood sample was withdrawn from all animals under mild ether anesthesia. Then, all the animals were administered 10 mg/kg of MCR-1329 suspended in 0.5 % solution of sodium carboxymethylcellulose in water. Following administration of the compound, blood samples were withdrawn at 1, 2, 4, 8, 12, 16 and 24 hrs from all the animals into heparinised tubes. Plasma was obtained by centrifugation at 4 °C (1200×*g* for 10 mins). Compound (MCR-1329) was extracted from plasma by protein precipitation with 3 volumes of acetonitrile, followed by centrifugation (800×*g* for 10 mins). The extract was evaporated to dryness and reconstituted with mobile phase before injection into HPLC (Purospher, reverse phase [C_{18}], Merck). The analytical column was fitted with a guard column. Detection was performed at 243 nm. For concentrated samples, required dilution was performed using blank plasma. Based on the peak areas obtained,

calibration curve was prepared by linear regression and concentration in the plasma samples was determined. AUC_{0-t}, C_{max} and t_{max} were calculated from the plasma concentration vs. time curve. The area under the plasma concentration-time curves from time zero to time infinity (AUC) was calculated by the trapezoidal rule-extrapolation method. This method employs the logarithmic trapezoidal rule for the calculation of area during the declining plasma-level phase, and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to infinity was estimated by dividing the last concentration by the apparent terminal rate constant.

Reverse phase HPLC method for analysis of MCR-1329

The method was optimized on a binary pump Shimadzu LC-20AT instrument with Shimadzu Prominence UV detector (SPD-20A). The mobile phase chosen for analysis was 40 mM sodium phosphate buffer: acetonitrile (3:2; *p*H 3.0). Samples were injected through a 20 µl Rheodyne 7725i injection loop and eluted with the mobile phase at a flow rate of 0.8 ml/min (pressure 108-114 *kgf*). The run time was optimized to be 15 mins with an R_t of ~ 4.2 mins. Stock solution of the test compound was prepared by dissolving 10 mg of MCR-1329 in 10 ml of acetonitrile to give a final concentration of 1 mg/ml. Further aliquots from the stock were prepared by serial dilution using blank plasma as a diluent to give concentrations ranging from 0-1000 ng/ml. These aliquots were treated in the same manner as mentioned above for oral dose disposition samples and analysed on the RP-HPLC to construct a calibration curve based on the peak areas generated by the LC Solution software. All the samples were filtered through a 0.22 µm syringe filter before injection into the column. The method was validated for linearity, precision and accuracy before analysis of samples.

Human plasma protein binding/ In vitro plasma release

Binding of the test compound, MCR-1329 to plasma proteins was determined using a modified *in vitro* assay. Cellophane paper was activated by incubation in 90% ethanol for 24 hrs. It was then soaked in phosphate-buffered saline (PBS, *p*H 7.4) for another 24 hrs to remove any traces of ethanol. The cellophane paper was then folded into a cylindrical tube (available surface area 3 cm^2) and used for evaluation. To evaluate the degree of plasma protein binding, dilutions of the test compounds were prepared in PBS and spiked into blank human plasma at known concentrations and

vortexed for 10 mins and incubated at 37 ºC for 4 hrs to allow proper binding of the compounds to plasma proteins. This spiked plasma was pipetted in the cellophane cylinder and the cylinder was tied at both ends in such a way that no plasma could leak from the tied ends. These cylinders were then suspended into beakers filled with PBS maintained at 37°C with stirring. Samples were withdrawn at different time points and the amount of test compounds present in it were evaluated by HPLC as mentioned in the previous paragraph. Release of the test compounds through the semi-permeable cellophane was compared over a period of 24 hrs by calculating the % cumulative drug release from the known amount spiked in each sample.

DOCA-SALT INDUCED HYPERTENSION IN RATS

Adult Wistar rats (20-24 weeks; 250-350 g) were used for the study. Left unilateral nephrectomy (UNX) was performed in all the animals and accordingly left kidney was surgically removed under anesthesia (ketamine 100 mg/kg, i.p.; diazepam 5 mg/kg, i.m.). Briefly, after confirming anesthesia through loss of righting reflex and pinching response, peritoneal laparotomy was performed 2 cm dorsolateral to the diaphragmal line (the waist region) and the left kidney was exposed. The kidney was brought out by holding the surrounding fat. The adhering fat and periadventitious tissue was separated using a pointed forcep and the adrenal gland was detached from the kidney. The kidney was gently pulled towards the exterior side of the peritoneal cavity to expose the ureter, renal vein and renal artery. They were clipped together using a hemostatic forcep and tied towards the kidney with 2 silk sutures (no.4), one distal and the other proximal. The kidney was now severed away from the knot and the remaining portion was allowed to retract by removing the hemostatic forcep. The incision was closed by ethicon® braided-silk sutures. Since ketamine functions as a preemptive analgesic, immediate post-surgical anaesthesia was not required, however, a dose of tramadol (12.5 mg/kg, i.p.) was administered as soon as the animal showed signs of recovery from anesthesia. The animals were allowed to recover from surgery for two weeks. Two weeks after the surgery, animals were divided into the following groups:

1) Negative control - Normal animals (no treatment or surgery)
2) Positive Control – UNX + DOCA + Salt
3) UNX Control – UNX without DOCA/Salt administration
4) Treatment group - UNX + DOCA + Salt + MCR-1329

5) Standard group - UNX + DOCA + Salt + prazosin + losartan

Animals were switched to 1 % NaCl and 0.2 % KCl in drinking water and were given injections of deoxycorticosterone acetate in olive oil (25 mg/kg, s.c., twice a week) upto 4 weeks. Uninephrectomized (UNX) rats received vehicle without DOCA or salt. To study the effect of the test compound, treatment (10 mg/kg MCR-1329 in 0.5 % sodium CMC, p.o., daily) was initiated in all the groups simultaneously, except positive (UNX+DOCA+Salt), sham and negative controls. Standard group received a combination of prazosin and losartan (5 mg/kg/day each). Non invasive tail-cuff systolic pressures were measured every week. On the penultimate day of the study, animals were placed in metabolic cages for collection of urine over a 24-hour period. At the terminal day of the study, final tail-cuff pressures were recorded, blood samples were withdrawn before sacrificing the animals, after which kidneys were removed for evaluating extracellular matrix deposition and aortae were isolated for studying endothelial dysfunction. Parameter evaluation in the DOCA-Salt hypertension mode was performed as below:

Table 17: Parameter evaluation detail in the DOCA model

Parameter	**Method/Instrument**
Systolic blood pressure-non invasive	Tail cuff apparatus, PANLAB-letica instruments, Spain
Systolic blood pressure-invasive	Arterial pressure transducer, POWERLAB-AD Instruments, Australia
Endotheilial Dysfunction-Aortic strip relaxation	Two Channel Recorder with Force Transducer, GEMINI 7070-UGO BASILE, Italy
Tubuloglomerular matrix-renal capsule	Periodic Acid – Schiff (PAS) Staining for identification of extracellular matrix
Creatinine, Uric acid, BUN-serum	Commercial kit as per manufacturer's protocol
Urine Output	Metabolic cage
Proteinuria, albuminuria, Urea Nitrogen, Na^+, K^+, creatinine, glucose-24 hr urine	Commercial kit as per manufacturer's protocol
Urine Osmolality	[2×{[Na^+ (mg/dl)]+[K^+ (mg/dl)]}]+[Urea nitrogen (mg/dl)/2.8]+[Urine Glucose (mg/dl)/18]
Creatinine Clearance	[{(Urea creatinine×Urine output)/serum creatinine}×(1000/Body weight)/1440]

Non-invasive recording of blood pressure

One week after the UNX surgery, the animals were trained for non-invasive recording of tail-cuff blood pressures. All recordings were performed using the tail-cuff pressure storage meter (Letica Instruments, Barcelona, Spain). Entire protocol for training was same as that of actual recordings, except that the readings were not recorded during the training period. For training purpose, the animals were restrained using suitable size rodent restrainers such that the tail remained freely hanging and movable from one end of the restrainer. The restrainer assembly was placed inside an external heating chamber which was maintained at 39 ± 1°C. The tail-cuff was placed towards the proximal end of the tail while the pulse transducer was placed distal to the cuff. The animal was allowed to remain in this position, until the instrument showed a ready signal. Once ready the cuff was inflated by pressing a button, whereby it deflated automatically, and recordings were stored inside the instrument. This procedure was repeated in a set of 3×3 for a period of 7 days. During actual recordings, pressure was recorded repeatedly until two consecutive readings showed the same systolic blood pressure.

Invasive recording of blood pressure

The procedure for invasive recording of blood pressure in this model is same as that mentioned in the '*in vivo pressor response evaluation'* section.

Endothelial dysfunction

Endothelial dysfunction was measured by the relaxation of aortic strips from different groups of animals by acetylcholine. Briefly, after dissecting the aorta from the animal, periadventitious fat was removed and care was taken to preserve the endothelium in intact form while preparing the strips. The strips were mounted as mentioned in the *'functional antagonism assay'* section. Endothelial dysfunction was evaluated by means of graded, Ach-mediated relaxations on strips precontracted with phenylephrine as compared to sodium nitroprusside mediated relaxations on the same strips.

CELL CULTURE AND SIGNALING STUDIES

Culture and maintenance of cells

Preparation of complete medium for cell culture: The commercially available media are incomplete and do not support the growth of cells as they lack in growth factors. Fetal bovine serum was thawed and was added to the commercially procured DMEM in a final concentration of 10 %. To this solution, antibiotic-antimycotic solution was added in a final concentration of 1 %. Since all the ingredients are available in sterile form, preparation of the final solution was performed under a laminar air-flow hood to prevent accidental contamination. Sterile Pasteur pipettes were used for effecting the transfer of solutions. If required, the final complete medium was filtered through a 0.22 µm syringe filter before use. Serum deprived medium was prepared similarly, except that the final concentration of FBS in the medium was 1 %.

Upon receipt of the cell line, the cells were observed under a light microscope for morphology and confluency, and maintained in a CO_2-incubator (95 % Air + 5 % CO_2). Healthy cells remain adherent to walls of the culture flask and exhibit fibroblastic nature with spindle shape and may be clearly observed as a monolayer. Fresh medium was replaced every week. The cells were subcultured when they reached 80-90 % confluency which was judged by visual observation under a light microscope.

Subculturing of cells: It is essential to dislodge the adherent layer of cells for subculturing. Trypsin-EDTA solution can disrupt the collagen between the cells and hence aid in dislodging cells from the adherent sites. The tissue culture flask was removed from the incubator and the medium was discarded in a waste beaker containing sodium hypochlorite. The monolayer of cells was washed twice with sterile PBS to remove traces of media as FBS present in the media can inactivate trypsin. 1 ml of Trypsin-EDTA solution was added to each flask and allowed to stand for about 5 minutes. To check whether the cells have properly dislodged or not, the flasks were tilted and checked for turbidity of solution due to presence of dislodged cells. Once it was assumed that maximum cells have dislodged, trypsin was inactivated by addition of an equal volume of the medium and the contents were gently pipetted into a microcentrifuge tube. This procedure was termed as trypsinization of cells. The cells were spinned down at 125×*g* for 5 mins to obtain a cell pellet. The pellet was washed twice with sterile PBS and then the cells were resuspended in about 900 µl of fresh

medium. The cell clumps were broken down by trituration with the pipette. This cell suspension was divided to new T-25 flasks, each already containing about 5 ml of complete medium. Normally, a subculturing ratio of 1:3 was maintained for this cell line.

Counting of cells: The cells were counted by trypan-blue exclusion method using a cytometer slide. After trypsinization of cells, the cells were centrifuged and resuspended in 1 ml of sterile PBS. An aliquote of 10 µl was taken from these cells and admixed properly with 10 µl of trypan blue solution. A coverslip was placed on the cytometer slide and small drop was allowed to aspirate by capillary action onto the slide. The cells were counted in all the four corners and their corresponding squares. The total count was multiplied by $1.1\times10^4\times2$ to give the actual number of cells/ml. If required the stock suspension of cells may be diluted to adjust the number of cells/ml.

Cytotoxicity assay

This assay involved treatment of cells with the test compound at different concentrations and evaluation of cytotoxicity produced by means of reaction with MTT. For this purpose, cells were trypsinized and counted. The final concentration of cells was so adjusted that 200 µl suspension of cells prepared in the media contained nearly 10^4 cells. This final suspension was seeded onto different wells of a sterile 96-well tissue culture plate and then the plate was placed in the incubator to allow adherence of cells. Usually, 24 hrs were required for the cells to adhere. Meanwhile a stock solution of test compound, MCR-1329, was prepared in DMSO and from this stock solution further aliquots were prepared in serum deprived medium. The concentration of the aliquots was adjusted in such a way that 200 µl of the medium contained the required concentration of MCR-1329 and the overall concentration of DMSO did not exceed 5 %. A concentration range of 10 nM to 1 mM was utilized for the assay. After a 24-hr incubation period, the complete media was removed from the wells by inverting the plate forcibly on a paper towel. Serum deprived medium containing the test compound was added in different wells with appropriate controls for DMSO. All the concentrations were utilized in 6 replicates. The cells were allowed to remain in contact with the test compounds for 12-hrs after which the media was removed and 200 µl of complete media containing MTT (500 µg/ml) was added to each well. The plate was then put inside the incubator and MTT was allowed to react with the cells for nearly 4

hrs. Dehydrogenases from live cells metabolize MTT to formazan crystals. These crystals are insoluble in aqueous media and may be solubilized in DMSO. After 4 hrs of incubation the medium is removed and 200 µl DMSO (filtered through a 0.22 µm syringe filter) is added to each well to solubilize the formazan crystals. The plate is covered and shaken on a plate-shaker for 5 mins. The lid is then removed and the purple color developed is read at 570 nm with a correction applied at 620 nm to account for unmetabolized MTT that may be present in the wells. Higher absorbance is directly proportional to the number of live cells present in each well. % Survival in each well was calculated by the following formula:

% Survival = (OD of test compound well/OD of Control well) × 100

Akt signaling study

This study was undertaken to study the effect of MCR-1329 on AT_1- and α_1-receptor mediated Akt phosphorylation. The effect of phenylephrine and angII mediated stimulation of Akt phosphorylation may be studied using flow cytometry after incubation with appropriate primary and fluorochrome-conjugated secondary antibodies. The treatments and treatment protocols were followed as given below:

Table 18: Treatment indications for cell signaling studies[6]

Sr. No.	Title	Treatment
1	Control	Trypsinised cells (untreated)
2	2° Antibody	Trypsinised cells treated with vehicle and 2° antibody
3	Stim 1 (PE)	Cells treated with Phenylephrine for 30 mins
4	Stim 2 (AngII)	Cell treated with AngII for 30 mins
5	Stim1 + MCR-1329	Cell treated with phenylephrine and MCR-1329 for 30 mins
6	Stim2 + MCR-1329	Cell treated with AngII and MCR-1329 for 30 mins
7	Stim1 + MCR-1329 + LY294002	Cells treated with phenylephrine, MCR-1329 and LY294002
8	Stim2 + MCR-1329 + LY294002	Cells treated with AngII, MCR-1329 and LY294002

[6] While giving the different treatments, as and when indicated above, MCR-1329 was added first, followed by the PI_3K inhibitor LY294002 and then stimulated with respective agonists

Concentrations and dilutions utilized

Phenylephrine: 50 µM

Angiotensin II: 10 µM

MCR-1329: 100 µM

LY294002: 10 µM

1° Antibody: 1:100

2° Antibody: 1:500

Preparation of solutions for flow cytometry

Phosphate buffered saline (1X): 1.04 g of lyophilized powder of PBS was dissolved in 100 ml of double-distilled water, filtered through 0.22 µm nylon membrane and autoclaved before use. This solution may be stored at RT for 1 week.

Formaldehyde (methanol free): 2 gm paraformaldehyde was added to about 80 ml filtered 1X PBS and heated upto 60 °C. This heated mixture was intermittently sonicated to give a cloudy suspension. The cloudy suspension showed appearance of precipitate when sonication was stopped. While on the water bath, 2N NaOH was slowly added to the cloudy suspension until the solution clears completely. The solution was then allowed to cool to RT and *p*H was adjusted to 7.4 with 2N HCl. The final volume was made upto 100 ml and the solution was filtered through a 0.22 µm nylon membrane. This solution may be stored for 4-6 weeks at 2-8°C.

Incubation buffer: 500 mg of bovine serum albumin was dissolved in 100 ml of sterile 1X PBS and stored at 4°C. This solution may be filtration sterilized as BSA may precipitate upon autoclaving.

Flow cytometry protocol

Fixation of cells: All the required flasks were treated as mentioned in the above table. Treatments were given in serum-deprived media. It was necessary to fix the cells so as to freeze the protein signaling cascade. The cells were trypsinized and collected by centrifugation. The cell pellet was suspended in about 1 ml of sterile PBS and formaldehyde was added to a final concentration of 2 %. The fixation was allowed to proceed for 10 mins at RT after which the tubes were chilled on ice for 1 min.

Permeabilization of cells: To permeabilize the cells, initially the fixation was removed by centrifuging the cells and resuspending them in 90 % methanol. The cells were again

collected by centrifugation and resuspended in ice-cold methanol and permeabilization of the membranes was allowed to proceed for 30 mins on ice.

Immunostaining: The permeabilized cells were collected by centrifugation and suspended in incubation buffer. The cells were blocked in incubation buffer for about 10 mins at RT to prevent non-specific binding of 1° antibody. The unconjugated antibody against Thr-308 Akt-P was added at a dilution of 1:100 and incubated at RT for 1 hr. After the incubation period the cells were collected by centrifugation and the supernatant (which may contain unbound 1° antibodies) was saved for future studies. The cell pellet was rinsed in incubation buffer by centrifugation and then resuspended in incubation buffer containing the 2° Alexa Fluor 594 conjugated antibody (1:250). Incubation was allowed to proceed for 30 mins at RT after which the cells were collected by centrifugation and the supernatant (which may contain unbound 2° antibodies) was saved for future studies. The cell pellet was resuspended in incubation buffer and analysed on BD FACSAria III flow cytometer with an excitation laser at 590 nm and detection at 617 nm. Parameters like forward scatter, side scatter and histogram analysis were used to quantify the fluorescence emitted from cells treated with 2° antibody as compared to untreated cells.

STATISTICAL ANALYSIS

For all the studies, $3 \leq n \leq 6$. The results were compared by Student's *t*-test, one-way or two-way ANOVA as appropriate. Bonferroni's multiple comparisons were employed as the *post hoc* test wherever required. All the statistical analyses were performed using Graphpad Prism, San Diego, CA, USA (Ver. 05) or Excel Spreadsheet program, Microsoft Corp., Redmond, WA, USA (2007). Results were considered to be statistically significant when $P<0.05$.

Concentration versus area obtained from the LC Solution software after HPLC detection was treated by linear least square regression for linearity determination. Data from the validation study were presented as mean, standard deviation and % relative standard deviation [(SD*100)/mean].

SUMMARY
&
CONCLUSIONS

SUMMARY & CONCLUSIONS

In an effort to identify a novel therapeutic agent for hypertension, that acts as multi-targeted ligand towards the α_1 and AT_1 receptors, different series of 6,7-dimethoxyquinazolines were screened on rat aortic strip preparation using specific agonists like phenylephrine and angII for α_1 and AT_1 receptors respectively. This method allowed calculation of pA_2 values for different test compounds. One interesting speculation was raised during the screening studies that if different substituents on the quinazoline nucleus may antagonise the effects of phenylephrine and angII on rat aortic strips, it is likely that the known drugs of this category might also show these effects. To substantiate this speculation prazosin and other compounds were cross-screened for angII antagonism and the *sartans* were cross-screened for phenylephrine antagonism likewise. In a remarkable and motivating observation, it was found that only prazosin, no other agent screened, was found to antagonize the effects of angII mediated contractions on rat aortic strips. It was concluded that the potency of prazosin rests not only in its α_1 blocking activity but in the angII–antagonizing property as well.

Further, the test compounds from different series of 6,7-dimethoxyquinazolines were screened and the compound showing highest as well as balanced inhibition of agonist-mediated contractions of rat aortic strips, i.e. **MCR-1329**, was chosen for further analysis. Toxicological evaluations were performed by administration of the test compound, **MCR-1329**, through oral route as it was the intended route of administration. Acute toxicity study revealed that the compound showed no signs of toxicity at a single dose administration of 2000 mg/kg. Further to this, repeat dose oral toxicity was performed at the intended dose for *in vivo* studies. The intended dose presented deviation from the guideline, and the dose range study mentioned in the guideline suggested a pilot study at 1000 mg/kg which is 100 times the intended dose. Hence the pilot study was not performed and safety over a repeat-dosing period of 28-days was evaluated at 10 mg/kg. It was observed that animals exhibited normal behavioural patterns, eating and drinking habits were regular and no untoward observations were made in any regard. Hence, it was concluded that repeat dosing at the intended dose-level is safe in rats. Oral dose pharmacokinetics was performed assuming non-compartmental models of analysis. The dose disposition curve for **MCR-1329** was asymmetric and represented a linear time-dependent absorption pattern, reaching a peak

followed by a time-dependent elimination pattern. A minor anomaly was noticed as a shoulder in the elimination curve which was treated as an incidental observation since exclusion of that data did not affect the results of the study. *In vitro* plasma protein binding study was performed with human plasma and it was found that over a period of 24 hrs, only a small fraction of **MCR-1329** is secreted into the physiological buffer. On the basis of oral dose-disposition and the plasma-protein binding study, it was concluded that **MCR-1329** remains highly bound to plasma proteins, has a relatively faster absorption rate suggesting its absorption in the upper part of the GIT, compared with a moderate elimination rate. Peak plasma levels were obtained around 4 hrs post-dosing with an elimination half-life of 3.77 hrs.

Acute *in vivo* studies involving measurement of pressor responses to phenylephrine and angII revealed important particulars about **MCR-1329**. Initial studies showed that inhibition of pressor response by prazosin and losartan was more significant as compared to that of **MCR-1329.** This was intriguing since the *in vitro* potency of **MCR-1329** was significantly better than the respective standards. It was thus discerned that **MCR-1329** might distribute on both the receptors *in vivo* leading to a submissive response. This inconsistency was improved when the pressor response was recorded under masked conditions under which the pressor response to phenylephrine was recorded under losartan pre-administration to block the effects on angII receptors and responses to angII were recorded under prazosin administered in tandem. Results of this data assisted in concluding our previous findings showing that prazosin shows affinity towards angII receptors and blocks the angII mediated contractile responses. Since prazosin also antagonises both the agonists studied herewith, results produced by prazosin masking of angII pressor responses could not be justified for **MCR-1329**. It was thus decided to use terazosin as the masking agent for recording angII mediated contractions since terazosin was found not to have antagonistic actions on angII mediated responses. At the end of the study, it was concluded that **MCR-1329** showed a similar inhibition of pressor response as compared to prazosin and losartan both.

Efficacy of **MCR-1329** in chronic hypertension was evaluated using the DOCA-salt model of renal hypertension. DOCA-salt administration coupled with uninephrectomy of animals was utilised for the induction of hypertension in animals. Results of the study demonstrated that administration of **MCR-1329** could prevent

development of hypertension mediated through salt intake and aldosterone turnover. This conclusion was supplemented from tail-cuff data and mean arterial pressures of animals recorded at the terminal stage of the study. At the same time, it was also revealed that **MCR-1329** prevented hypertension mediated endothelial dysfunction and that this kind of dysfunction in rats is unrelated to serum uric acid levels in rats. Electrolyte imbalance and urinary markers of hypertension were also controlled by **MCR-1329** as indicated by urinary indices. Other parameters including urine output, albuminuria, glucosuria, creatininuria, natriuria and kaluria were also significantly improved in animals treated with **MCR-1329** as compared to the DOCA-salt group. Urine osmolality and creatinine clearance, surrogate indices based on preliminary electrolyte and solute levels, were also improved in **MCR-1329** treated in animals. All the findings were in close agreement with the animals from the standard group in which losartan & prazosin were co-administered so as to assess the worth of multi-targeting. It could be concluded that **MCR-1329** is safe and effective in the management of mineralocorticoid induced hypertension in rats.

Overall, the study sheds new light on the paradigm of multiply-targeted ligands for the effective management of multifactorial conditions. The era of serendipitous discoveries takes a backseat with designed multiple ligands entering the mainframe of the therapeutic armamentarium. The results of this study indicate that safe and efficacious multiply-targeted ligands can be designed to show an evenhanded modulation of the desired targets to achieve therapeutic goals. Further, the compounds presented in the present study may serve as potential examples or lead candidates which could be further explored for multiply targeting complex disorders.

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INTRODUCTION

INTRODUCTION

Development of chronic hypercholesterolemia predisposes an individual to the development of a metabolic condition termed as atherosclerosis. Atherosclerosis is a condition characterized by formation of lipid-rich plaques in the inner walls of arteries. Technically, the entire process is a remodeling event in the inner walls of the arteries that leads to subendothelial deposition of fatty substances (Vassiliadis *et al*, 2013). This event is initiated by acute or chronic injury to the arterial endothelium which leads to endothelial dysfunction. Dysfunction of a few endothelial cells leads to activation of the surrounding cells which begin attracting leukocytes and vascular smooth muscle cells (VSMCs) at the site of injury. VSMCs activated in presence of the leukocytes activate, proliferate further and secrete unwarranted amounts of connective tissue matrix that forms the mass of the plaque. The convoluted sequence of events occurring during atherosclerosis progression resemble to that of a chronic inflammatory process (Falk, 2006). The end result of such a process is formation of thick, mature and obstructive plaque which is fibrous in nature. Though this situation is detrimental, compensatory flow adjustments are sufficient to handle the narrowing of the luminal diameter in the arteries as long as the plaque remains stable. Complications arise when the plaque ruptures leading to hemorrhage of the ruptured plaque and formation of emboli or thrombosis at the lesion (Falk, 2006). A thorough understanding of the pathogenesis of atherosclerosis is essential for the development of strategies for the prevention of the disease, and for the development of new and effective treatments. Cholesteryl esters contribute significantly to the metamorphosis of an atherosclerotic plaque. It has been suggested that cholesteryl esters present in LDL can be considered not only as a risk factor but rather a diagnostic marker for atherosclerosis progression (Spector and Haynes, 2007).

It was routinely believed that esterification of cholesterol in the body is catalyzed solely by LCAT (Glomset, 1973). This perspective was changed with the discovery of ACAT (Chang *et al*, 1993) which catalyses the linkage between fatty acyl CoA and polar cholesterol, resulting in the formation of non-polar cholesteryl esters (Buhman *et al*, 2001). Two isoforms of ACAT are known: ACAT1 and ACAT2. Apart from the physiological aspect, production of cholesteryl esters by ACAT isoforms makes a noteworthy contribution to the pathogenesis of atherosclerosis. ACAT1 aids

the progression of atherosclerosis via accumulation of cholesteryl esters in macrophages and leading to conversion of smooth muscle cells to foam cells, ultimately leading to plaque initiation and subsequent events (Fazio *et al*, 2001; Linton and Fazio, 2003). In macrophages, which form an important part of the pathophysiology of atherosclerosis, ACAT1 regulates the allocation of intracellular cholesterol to esterified- and free-cholesterol pools (Akopian and Medh, 2006). This is a very important event for any cell, as esterification of cholesterol sequesters free cholesterol in the form of esterified lipid droplets by making it unavailable for ABCA1-mediated efflux (Akopian and Medh, 2006; Voloshyna and Reiss, 2011; Sorci-Thomas and Thomas, 2012). This massive accumulation of cholesteryl esters in the form of lipid droplets leads to the formation of lipid-laden foam cells. Cholesterol ester accumulation in vascular smooth muscle cells is also controlled by ACAT1 (Yagyu *et al*, 2000; Rong *et al*, 2005; Rong *et al*, 2013). Both of these events contribute significantly to the development of the lipid-rich core of the plaque and are the hallmarks of atherosclerosis (Chang *et al*, 2006a; Chang *et al*, 2006b). ACAT2 significantly contributes to the absorption of dietary cholesterol to deliver it to the lymph and plasma by esterifying it in the enterocytes. This absorbed cholesterol, alongwith cholesterol synthesized *de novo* by the HMG-CoA reductase pathway, contribute to the total pool of cholesterol in the body. In this manner, both the isoforms of ACAT are responsible for the development of atherosclerosis. Owing to the direct as well as indirect role played by ACAT in development of the atherosclerotic plaque, inhibition of ACAT has been a potential pharmacological target for researchers for preventing atherosclerosis progression (Chang *et al*, 2006a). Pan-specific or non-specific ACAT inhibition has been tried by different researchers with varying levels of success. Several studies identifying potential ACAT inhibitors for the management of hyperlipidemia and atherosclerosis have been reviewed (Pal *et al*, 2012). The past 2 decades have seen a generous number of publications on the subject of ACAT inhibitors. Filing of more than 150 patents (and still counting…) suggests a keen interest amongst researchers and in the commercial arena about ACAT inhibition as a potential therapeutic strategy for atherosclerosis. The development of several synthetic, herbal or microbial origin ACAT inhibitors has allowed the researchers to understand the role of ACAT in cholesterol turnover. The field of ACAT studies saw great impetus after the discovery of ACAT cDNA by Chang and colleagues (1993). Since then, abundance of research in this area has resulted in thorough understanding of the structure, function, localization and inhibition of ACAT

isoforms. In fact, a few studies have also found ACAT inhibition to be a detrimental strategy since it prevents esterification of toxic polar cholesterol leading to its accumulation in cells. Though variety exists in the observations made from studies that have evaluated different aspects of ACATs, their physiological role has been well-studied and understood.

The present work is aimed at identifying ACAT inhibitors as potential drug molecules to be used in atherosclerosis. For this purpose, an HPTLC-based analytical method was developed for the estimation of cholesteryl esters.

This method could be utilized for quantifying cholesteryl esters and screening of potential ACAT inhibitors by observing their ability to prevent formation of cholesteryl esters. Consequently, screening of a series of NCEs for potential ACAT inhibitory activity (present in liver microsomes) is reported. Chemically, these NCEs belong to a class of urea derivatives. Based on the preliminary screening results, a few potent compounds were selected for evaluation of their effect on triglyceride turnover and the potential compound(s) were further studied in an animal model of diet-induced atherosclerosis.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Cholesterol is known to be a vital chemical required by the human body for viability of all types of cells (Chang *et al*, 2006b). Excess of cholesterol is however toxic to the cells and leads to cell death (Tabas, 2002). For the same reason, excess cholesterol is accumulated by the cells as cholesterol esters (Chang *et al*, 2006b). Cholesterol esters form the neutral lipid that is imperative to the transport and storage of cholesterol in plasma and steroidogenic tissues like the adrenals respectively (Chang *et al*, 2009). It was customarily believed that the enzyme responsible for the esterification of cholesterol in blood was Lecithin: cholesterol acyltransferase (LCAT) (Glomset, 1973). This perspective, however, got changed with the discovery of Acyl CoA: cholesterol acyltransferase (ACAT, EC 2.3.1.26). Discovery of the enzyme ACAT (previously termed as *SOAT*; Sterol O-acyltransferase) dates back to the 1950s (Goodman *et al*, 1964). The credit of isolating ACAT cDNA is attributed to Chang and colleagues (1993), who made the task possible using the complementation of a mutant cell line devoid of ACAT activity. Consequently, a 4 kb cDNA encoding an ACAT was isolated from human macrophages. ACAT is an enzyme involved in the catalysis of the reaction which covalently links fatty acyl CoA to cholesterol, resulting in the formation of cholesteryl esters (Figure 1) (Buhman *et al*, 2001).

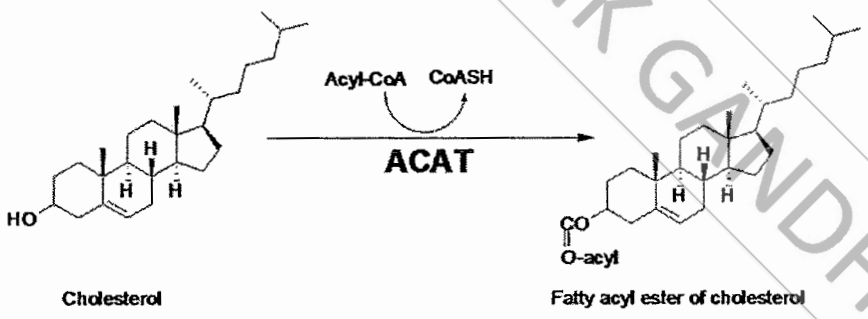


Figure 1: The classical reaction catalysed by ACAT. ACAT catalyses the formation of ester bond between fatty acyl-coA and 3-OH group of cholesterol present within cells. This results in formation of fatty acyl cholesteryl esters like cholesteryl oleate and cholesteryl palmitate.

Cholesterol is just one of the substrates as ACAT utilizes other oxysterols, plant sterols and various other sterols for enzymatic catalysis, but plant sterols are poor substrates (Liu *et al*, 2005). Apropos cholesterol homeostasis, ACAT plays an important role in lipoprotein assembly, dietary cholesterol absorption and intracellular cholesterol metabolism (Buhman *et al*, 2000a). Meiner *et al* (1996), disrupted the mouse ACAT gene and, contrary to popular belief, showed that ACAT activity still retained in the liver and intestine insinuating the likelihood of other isoforms of ACAT. The cloning of ACAT1 gene thus escorted the cloning of the other isoform and taking cue from the work of Meiner *et al* (1996), cDNA for human ACAT2 was cloned in 1998 by three independent research groups (Anderson *et al*, 1998; Cases *et al*, 1998; Oelkers *et al*, 1998). Two isoforms of ACAT have thus been discovered till present day in mammalian species, ACAT1 and ACAT2, both of which are encoded by different genes (Farese, 2006). ACAT1 is ubiquitously expressed whereas expression of ACAT2 is restricted to liver and enterocytes. Both these isoforms have unique functions and belong to the *Acact* gene family. The *Acact* gene family also encodes an enzyme termed the acyl CoA: diacylglycerol transferase-1 (DGAT1) and its orthologues (Burnett *et al*, 1999; Buhman *et al*, 2000a). These three enzymes namely ACAT1, ACAT2 and DGAT form the initial members of the family of enzymes termed as membrane bound O-acyltransferase (MBOAT) (Chang *et al*, 2011). The enzymes of this family form the multispan membrane-bound enzymes that employ fatty acyl-CoAs (long or medium chain) and another hypdrophobic substance like cholesterol as their substrate. This results in the formation of a neutral hydrophobic ester like cholesteryl oleate and coenzyme A. An important characteristic of the enzymes of the MBOAT family is the presence of an invariant histidine and a near-invariant asparagine within a long stretch of hydrophobic and moderately hydrophilic residue (Chang *et al*, 2011). Above and beyond cholesterol homeostasis, ACAT1 aids the progression of atherosclerosis via accumulation of cholesteryl esters in macrophages leading to conversion of smooth muscle cells to foam cells, ultimately leading to plaque initiation and subsequent events (Fazio *et al*, 2001; Linton and Fazio, 2003). ACAT2 functions in a more dedicated manner to facilitate cholesterol absorption and lipoprotein secretion (Shelness and Sellers,

2001; Nguyen *et al*, 2012). In lieu of these findings, ACAT inhibitors have remained attractive targets, though elusive, for pharmaceutical companies to prevent plaque development and cholesterol absorption through intestine. An attempt has been made here to recapitulate the cellular and tissue localization, biochemical regulation, active site and catalytic residues as well as attempts towards therapeutic inhibition of the ACAT isoforms. Significant events in the history of ACAT are listed in Table1.

Table 1: Significant events in the history of ACAT

SR. NO.	EVENT	YEAR	REFERENCE
i.	Cholesterol esters first identified as components of blood	1895	Hurthle, 1895
ii.	Cholesterol esters were shown to be present in arterial atheromatous plaques	1910	Windaus, 1910
iii.	Cholesterol esters are formed in blood	1935	Sperry, 1935
iv.	Rat liver homogenates can esterify cholesterol with palmitic acid	1958	Mukherjee *et al*, 1958
v.	A liver enzyme was found to be responsible for esterification using cholesterol and fatty acyl coA as substrates and identified as ACAT	1964	Goodman *et al*, 1964
vi.	LCAT catalyses the esterification of cholesterol in blood	1968	Glomset, 1968
vii.	ACAT activity was identified in macrophages apart from other tissues	1979	Brown *et al*, 1979
viii.	Unsuccessful attempt to purify ACAT	1982	Doolittle and Chang, 1982
ix.	Brown and Goldstein discovered that cholesteryl esters in OxLDL particles taken up by macrophages are first	1983	Brown and Goldstein,

	hydrolyzed in lysosomes and then re-esterifed prior to storage as lipid droplets		1983
x.	ACAT1 cDNA was cloned after a decade of efforts using the complementation of a mutant CHO cell line devoid of ACAT activity	1993	Chang *et al*, 1993
xi.	Disruption of mouse ACAT gene revealed that esterification activity is still retained in the liver and intestine suggesting the presence of another isoforms	1996	Meiner *et al*, 1996
xii.	cDNA for human ACAT2 was cloned	1998	Anderson *et al*, 1998; Cases *et al*, 1998; Oelkers *et al*, 1998
xiii.	Eflucimibe, Phase I trial concluded and molecule enhanced to Phase II	2002	Burnett, 2003
xiv.	Avasimibe & Progression of Lesions on Ultrasound (A-PLUS) trial halted as a result of lesion progression and suggested lack of activity	2003	Tardif *et al*, 2004
xv.	Pactimibe trial, ACAT Intravascular Atherosclerosis Treatment Evaluation (ACTIVATE) suspended	2006	Nissen *et al*, 2006
xvi.	Liver specific inhibition of ACAT2 using antisense oligonucleotides	2006	Bell *et al*, 2006
xvii.	CAPTIVATE randomized trial prematurely terminated	2009	Meuwese *et al*, 2009

ACAT GENE AND PROTEIN STRUCTURE

The human ACAT1 gene (*SOAT1*-Sterol O-Acyltransferase 1) is located on chromosome 1q25, the approximate molecular mass of the gene product being 65 KDa (Chang *et al*, 1994). However, it functions as a homotetramer (Yu *et al*, 1999) and the

molecular mass of the active molecule was actually found to be 263 KDa. Human ACAT2 gene (*SOAT2*-Sterol O-Acyltransferase 2) is located on chromosome 12 and the region of interest has a locus affecting the response of plasma LDL and VLDL to high-fat or high-cholesterol intake in the diet (Cases *et al*, 1998). The human ACAT2 gene encodes a single 46 KDa protein. Corresponding genes for ACAT1 and ACAT2 in mice are found on chromosomes 1 and 15 respectively (Uelmen *et al*, 1995; Cases *et al*, 1998). Predominance regarding the tissue distribution of any of the isoforms is controversial and is detailed in the succeeding section. Efforts by different groups of researchers have identified four mRNA transcripts for human ACAT1 (7, 4.3, 3.6 and 2.8 Kb) (Chang *et al*, 1993; Pape *et al*, 1995; Matsuda *et al*, 1996; Wang *et al*, 1996). All these transcripts have the same open reading frame but differ in the length of their untranslated regions (Li *et al*, 1999) ultimately responsible for formation of the homotetramer. The 4.3 Kb transcript originates from an atypical RNA recombination which involves trans-splicing of two precursor RNAs from chromosomes 1 and 7 (Li *et al*, 1999). A single mRNA transcript (2.2 Kb) is encoded by the human ACAT2 gene (Buhman *et al*, 2001). Both the isoforms share a lot of sequence identity near the -COOH terminus and are nearly 40 % identical in their gene products (Anderson *et al*, 1998; Cases *et al*, 1998; Oelkers *et al*, 1998).

Amino acids 403-409 of human ACAT1 form a highly conserved sequence (the FY*X*DWWN motif) which might be involved in binding to the acyl-CoA during the catalytic process (Buhman *et al*, 2001). This sequence is a communal occurrence in members of the *Acact* gene family. The MK*XX*SF motif (amino acids 265-270 of human ACAT1) is another conserved motif in the family members and the serine residue present in this motif is essential for ACAT activity (Cao *et al*, 1996; Joyce *et al*, 2000; Buhman *et al*, 2001). Other potential motifs common to the family members of *Acact* include a tyrosine phosphorylation motif and an N-linked glycosylation site. The N-terminal of both the isoforms contains a leucine-zipper motif but it is not known whether this motif has any role in formation of homotetramers (Chang *et al*, 1993; Buhman *et al*, 2000a). An interesting point in the structural biochemistry of ACAT proteins is that ACAT1 and ACAT2 do not form hetero-oligomeric complexes with each other (Chang *et al*, 2000).

ACAT1 contains 1 disulfide linkage and 7 free cysteines (Guo *et al*, 2005a) none of which are required for the activity of the enzyme but the disulfide linkage is essential for its structural stability.

Several investigations have been performed to study the membrane topology of human ACAT1 albeit without reaching a consensus. Lin and colleagues first studied the membrane topography of ACAT1 and suggested that ACAT1 contains at least 7-TMDs (Lin *et al*, 1999). This proposition was revised by the work of Guo and colleagues who proposed a 9-TMD model (Guo *et al*, 2005b). Again, Joyce *et al* (2000) proposed a 5-TMD model with 4 TMDs lying near the -NH_2 terminal and 1 TMD near the -COOH terminal. The method used by Lin *et al* (1999) as well as Joyce *et al* (2000), suffered from a common flaw that the actual membrane topography of the enzyme might be altered in the detection process (Guo *et al*, 2005b). Joyce and colleagues employed successive –COOH terminal truncations which could alter the structural stability of the protein in question (Joyce *et al*, 2000). Further, they have not shown whether ACAT activity was retained in each truncated subunit (Joyce *et al*, 2000). Lin and co-workers used the method of epitope insertion and double cytoimmunofluorescence to discern their topological model (Lin *et al*, 1999). Though the authors did report that each tagged protein was partially active, we do not have concrete evidence to believe that structural conformation remained unchanged in the entire protocol. Guo *et al* (2005b), were able to show that modest truncations near the -COOH terminal (the method used by Joyce *et al* (2000) for topographical studies) led to a near complete loss of enzymatic activity for ACAT1. Hence the model proposed by Joyce and colleagues seems unacceptable (Joyce *et al*, 2000). The topographical model suggested by Lin and colleagues indicated that ACAT2, being an integral membrane protein, contained two detectable TMDs (Lin *et al*, 2003). The authors asserted that other transmembrane domains may be found in the lipid bilayer but were not reported by them. On the contrary, Joyce and co-workers determined the topography of ACAT2 and reported that ACAT2 has five and not two TMDs (Joyce *et al*, 2000). However, data regarding ACAT2 TMDs is not concrete, as Chang and co-workers have reviewed (Chang *et al*, 2009). The authors argue that as was the case with ACAT1, the approaches used by Joyce *et al* (2000) and Lin *et al*

(1999), have an apparent possibility that the actual membrane topography of ACAT2 is altered during the process. The transfection procedure used by both the groups has a propensity to produce leaky cells and this might have definitely been stained by antibodies (Chang *et al*, 2009). This may not be acceptable because the results are rendered non-specific due to the permeable or dying cells.

CELLULAR LOCALIZATION AND TISSUE DISTRIBUTION

ACAT1 is normally found to be present in a variety of tissues including liver, sebaceous glands, adrenal glands, macrophages, fibroblasts and monocytes (Lee *et al*, 1998; Chang *et al*, 2000; Sakashita *et al*, 2000) while ACAT2 is found in intestinal mucosa and liver microsomal fraction (Joyce *et al*, 1999; Smith *et al*, 2004). Figure 2 indicates the location and physiological functions of ACAT isoforms of major importance. In humans, ACAT2 is the prime ACAT isoform found in the small intestine while ACAT1 protein expression is high in adrenals, macrophages and sebaceous glands. In the liver, ACAT activity is present not only in hepatocytes but also in *Kupffer* cells which are nothing but stellate macrophages (Buhman *et al*, 2001). In terms of cellular localization, expression of ACAT1 mRNA is ubiquitous in mammalian tissues (Chang *et al*, 1993; Uelmen *et al*, 1995; Meiner *et al*, 1997). ACAT1 is chiefly found to be present in the ER, spanning the ER membrane 5 or 7 times (Lin *et al*, 1999; Joyce *et al*, 2000), however such localization may change with different cell conditions like cholesterol loading. For example, cholesterol loaded human macrophages produce more numbers of ER-derived vesicles that are rich in ACAT1 (Temel *et al*, 2003; Liu *et al*, 2005; Sakashita *et al*, 2010). Some expression of the ACAT1 mRNA in mouse macrophages has been reported near trans-Golgi network (Khelef *et al*, 1998). As mentioned above, ACAT2 is expressed in the microsomes of liver and small intestine exclusively. More specifically, ACAT2 has been found to be present in the apical region of the intestinal villi (Chang *et al*, 2000; Lin *et al*, 2003) whereas ACAT1 is uniformly distributed over the villus-crypt axis (Chang *et al*, 2000), albeit in a lower amount. Immunological methods have helped in determining the predominance of the two isoforms in given tissues. Sakashita and colleagues demonstrated the relative prevalence of

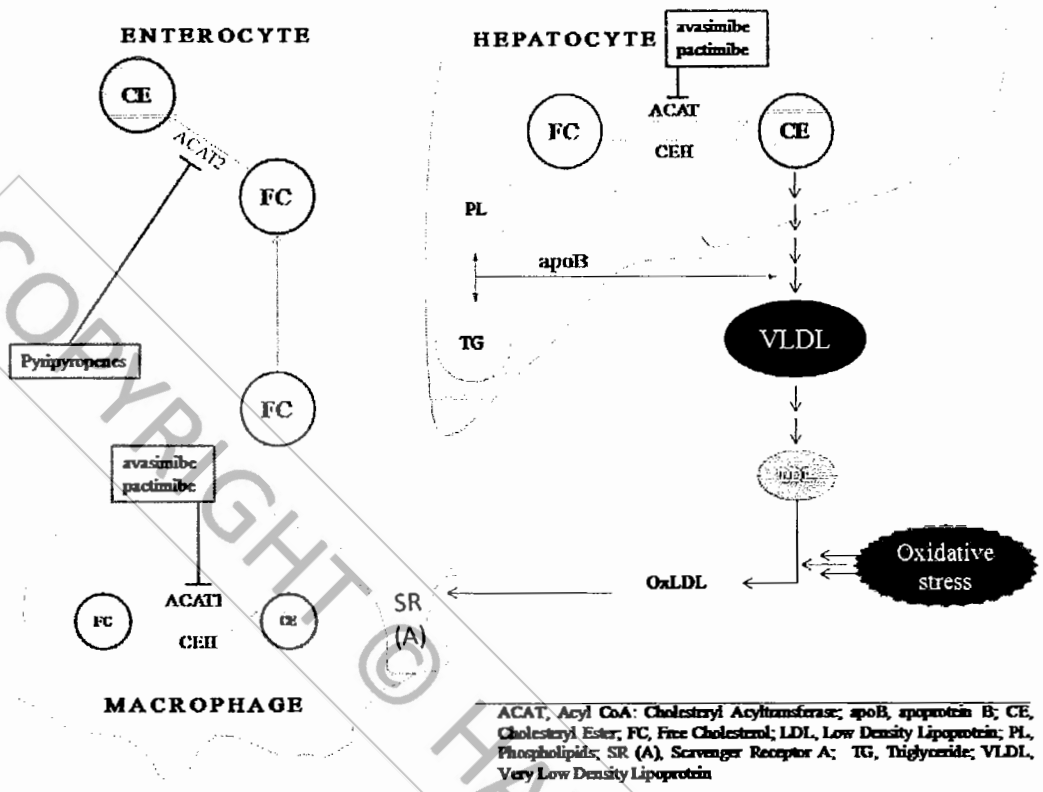


Figure 2: Physiological functions of ACAT. ACAT reduces the toxicity of free cholesterol by converting it from a polar derivative to a non-toxic ester form which can be stored in the cells in the form of lipid droplets. Apart from being stored lipid reservoirs, the cholesteryl esters formed as a result of this esterification process are utilized in the assembly of lipoproteins. In the macrophages, these cholesteryl esters combine with oxidised LDL leading to formation of the foam cell. The catalysing function of the ACAT isoforms can be inhibited (blunt arrows) by non-specific inhibitors like avasimibe and pactimibe or isoform-specific agents like pyripyropenes (boxes).

ACAT1 in normal human tissues using immunohistochemical detection methods (Sakashita *et al*, 2000). The authors report that prevalence of ACAT1 is highest in Kupffer cells, adrenal cortex & alveolar macrophages to be specific and macrophages of the liver, spleen and kidney in general (Sakashita *et al*, 2000). Occurrence of ACAT1 is also substantial in hepatocytes, oesophagal and fundic glands, mucosal epithelial cells of the large intestine, transitional epithelial cells of the urinary tract, epithelial cells of proximal and distal tubules in the kidney, myentric glia, neurons, bronchiolar and alveolar epithelia

(Sakashita *et al*, 2000). They have specifically utilized an anti-ACAT1 antibody, DM10, specifically immunoreactive against ACAT1 (Chang *et al*, 1995; Miyazaki *et al*, 1998) for immunodetection of ACAT1. The samples used for this study were all autopsy cases. The exact orientation of ACAT2 in the ER is unknown but data supporting the alignment of active site towards cytosol have been reported (Lin *et al*, 2003). Rudel and colleagues have inferred that active sites of ACAT1 and ACAT2 are, in fact, located on opposite sides of the ER membrane (Rudel *et al*, 2000). Thus, contradictory to previous reports, the active site of ACAT1 is located facing the cytosolic side of the ER whereas the active site of ACAT2 faces the lumen of ER (Joyce *et al*, 2000; Lada *et al*, 2004). It is hypothesized that since fatty acyl-CoA is not permeable to the ER membrane, the most plausible alignment of the active sites of ACAT is towards the cytosol (Coleman and Bell, 1983; Lin *et al*, 1999). Overall, it has been postulated that ACAT1 is present wherever cholesterol is required to be esterified for storage whereas ACAT2 is present in tissues involved in esterification of cholesterol for lipoprotein assembly and secretion (Anderson *et al*, 1998).

ACTIVE SITE RESIDUES AND CATALYTIC ACTIVITY

Both the isoforms of ACAT are homologous in their amino acid sequence near their C-termini but they are quite different in their intracellular functions due to their distinct nucleotide sequences (Das *et al*, 2008). A few studies have identified different residues required for ACAT activity. Kinnunen and colleagues showed that histidine residue(s) are required for ACAT activity (Kinnunen *et al*, 1988). This is quite plausible because histidine residues are frequently involved in enzymatic catalysis due to the reactive imidazolium nitrogen which may function as hydrogen bond acceptor/donor (An *et al*, 2006). Other groups of researchers have supported this finding and suggested that a histidine residue at C-terminal of ACAT is one of the active site residues (Lin *et al*, 2003; Guo *et al*, 2005b). Further, Sojin An and colleagues demonstrated that the histidine residues required for activity in ACAT1 are different from those required for activity of ACAT2 (An *et al*, 2006). They have shown that H386 and H460 are essential for ACAT1 catalysis whereas for ACAT2, the corresponding counterparts are H360 and H399 (An *et*

al, 2006). A vital step in the cholesterol esterification process is the cleavage of the thioester bond in the acyl-CoA molecule. The energy released during this process propels the esterification process. ACAT enzymes possess intrinsic thioesterase activity and the crystal structure of this thioesterase domain reveals a triplet residue, serine-histidine-aspartic acid, as the active site of the enzyme (Chakravarty *et al*, 2004; Das *et al*, 2008). Much of the work regarding identification of presumed active sites came from the work of Das and co-workers (2008). After several chemical modifications and site-directed mutagenesis studies, they reported several important residues required for ACAT activity (Das *et al*, 2008). Their results are consistent with previous reports indicating the role of the amino acid triad, serine-histidine-aspartic acid, for activity of ACAT (Guo *et al*, 2001). Das & colleagues have specifically identified that serine at position 456, histidine at 460 and aspartic acid at 400 is necessary for activity of ACAT1(Das *et al*, 2008). Additionally, aspartic acid residue at 400^{th} position is also essential for appropriate folding and structural stability of the protein (Das *et al*, 2008). Moreover, full enzymatic activity of ACAT2 is dependent upon a histidine residue at 438^{th} position (Das *et al*, 2008). Studies with tyrosine mutant enzymes revealed the importance of a tyrosine residue at 404^{th} position for ACAT1 and at 382^{nd} position for ACAT2. Mutation led to about 20 % decrease in cholesterol esterification activity (Das *et al*, 2008). Further to this, ACAT1 has a highly conserved sequence from amino acids 265 through 270 which contains a serine residue that is fundamental to the cholesterol esterification process. With regard to the highly conserved sequence, human ACAT1 has a conserved serine residue, Ser-269, which was found to be essential for activity because replacement of this serine residue with leucine resulted in loss of activity (Joyce *et al*, 2000). A corresponding serine residue is also present in the active site of ACAT2 (Joyce *et al*, 2000). Thus it was assumed that Ser-269 forms a part of the active site of ACAT1 (and for that matter ACAT2 as well). However, this may be considered only as a timid suggestion since there is a possibility that a serine-to-leucine mutation causes the mutated enzyme to be degraded at a faster rate as compared to the wild variety of the enzyme thus leading to a loss of activity not because of the structural modification but as a result of altered physiological metabolism (Chang *et al*, 2001). A

very interesting hypothesis proposed by Chang and colleagues states that the catalytic function of ACAT may be accomplished within the plane of the ER membrane (Chang *et al*, 2001). The authors argue that both the isoforms are able to form neutral lipid droplets that are found in the cytoplasm (earlier attributed exclusively to ACAT1) as well as contribute to the assembly of lipoproteins (attributed to ACAT2), a process which occurs in the ER lumen (Chang *et al*, 2001; Temel *et al*, 2003). The substrates for the reaction, fatty acyl-CoA and cholesterol are readily available from the ER membrane. Fatty acyl-CoA being impermeable, partitions on the cytoplasmic side of the ER membrane (Boylan and Hamilton, 1992), while cholesterol may equilibrate on either side of the ER membrane. Along these lines, it can be assumed that both fatty acyl-CoA and cholesterol diffuse laterally along the ER membrane, where at some point, they encounter ACAT, bind to it, leading to a conformational change in the enzyme so that the catalytically active sites are optimally exposed to the substrates for enzymatic catalysis to occur (Chang *et al*, 2001). Since these arguments have not been corroborated at biochemical or cell-biology levels, they are open to contemplative criticisms. A point worth noting in this observation is that if the catalytic function happens within the plane of the membrane, then CE generated in the lipid bilayer may leave the cytoplasmic layer to form lipid droplets or the CE may be recruited to form VLDL particles and thus the hypothesis proposed by the authors, that both isoforms are involved in neutral lipid and VLDL formation, will stand true.

BIOCHEMICAL REGULATION

The catalytic activities of ACAT1 and ACAT2 are similar in lot respects, but their biochemical activity may not be considered as identical to each other. Potential substrates subject to ACAT-mediated catalysis are listed in table 2. In addition to cholesterol, ACAT1 esterifies numerous other oxysterols (Cases *et al*, 1998; Buhman *et al*, 2001) suggesting a pivotal role in regulating bile acid and cellular cholesterol metabolism (Cases *et al*, 1998; Buhman *et al*, 2001). A variety of long chain fatty acyl-CoAs are also substrates to ACAT1 mediated catalysis (Table 2) (Yang *et al*, 1997; Cases *et al*, 1998). This broad substrate specificity is also present in murine ACAT1 (Cases *et al*, 1998).

Table 2: Potential ACAT substrates

Sterols	Fatty acyl-CoAs
Cholesterol 27-Hydroxycholesterol 25-Hydroxycholesterol 24(*S*)-Hydroxy cholesterol 7α-Hydroxy cholesterol 24(*S*),25-Epoxycholesterol Cholestanol	Arachidonyl CoA Palmitoyl CoA Linoleoyl CoA Oleoyl CoA

ACAT1 happens to be regulated at both, transcriptional and post-translational levels (Chang *et al*, 1997; Yang *et al*, 2001). *In vivo*, diet rich in fats, cholesterol (Pape *et al*, 1995; Uelmen *et al*, 1995) or dexamethasone treatment (Cheng *et al*, 1995b; Yang *et al*, 2004) led to increased ACAT1 activity. *In vitro*, cholesterol-loading, differentiation of monocytes to macrophages (Wang *et al*, 1996) and treatment with free fatty acids (Seo *et al*, 2001) are reported to increase ACAT1 activity. TGF-β (Hori *et al*, 2004) and TNF-α (Lei *et al*, 2009) are implicated in mediating the increased ACAT1 activity in differentiating monocytes thus leading to the formation of cells loaded with cholesteryl esters whereas adiponectin, an adipocytokine (Gandhi *et al*, 2010), shows a negative effect in this case (Furukawa *et al*, 2004). ACAT1 responds to cholesterol as its sterol substrate in a sigmoid habit (Chang *et al*, 1998), with a certain predilection over other oxysterols (Chang *et al*, 2009). Kinetic evidence has suggested that there is presence of a sterol-substrate site and a sterol-activator site in the structure of ACAT1 (Chang *et al*, 2009). Cholesterol increases the activity of ACAT1 several folds and it can be clearly seen that ACAT1 holds a typical bias towards cholesterol (Chang *et al*, 2009) as its substrate when compared with other variety of sterols having a 3β-OH configuration (Zhang *et al*, 2003; Liu *et al*, 2005). It has been discussed that cholesterol stimulates ACAT activity not only

by increasing substrate availability but also by acting as an allosteric activator of ACAT. The superiority of cholesterol to act as an allosteric activator of ACAT1 has been demonstrated against other sterols (Zhang *et al*, 2003; Liu *et al*, 2005) like 7-ketocholesterol and 7α-hydroxycholesterol. Liu and coworkers (2005) have reported that the stereochemistry of the 3-OH in the A ring is critical towards determining the ability of any sterol to behave as a substrate for ACAT1 (Liu *et al*, 2005). The authors further asserted that the axial orientation of the 3α-OH thus prevents the binding of epicholesterol, making it a poor substrate for ACAT1 (Liu *et al*, 2005). However, studies with *ent*-cholesterol, the mirror image of cholesterol with an equatorial orientation of the 3α-OH (same as that of 3β-OH in cholesterol), have revealed that it is the overall shape of the sterol molecule that is recognized by the substrate-binding site and not just the axial- or equatorial-orientation of the 3-OH group (Westover and Covey, 2004; Liu *et al*, 2005). As mentioned earlier, ACAT1 exists as a homotetramer. There is a dimer-forming motif (one which results in the formation of a dimer from a dimer, a tetramer) near the N-terminus, deletion of which converts the tetrameric enzyme to a functionally active dimeric form (Yu *et al*, 2002).

The *SOAT2* promoter contains *cis* elements [those regions within the same DNA strand that can regulate gene (*here SOAT2*) expression] for two transcription factors, Cdx2 and HNF1α, both of which are required for expression of the gene product ACAT2 (Song *et al*, 2006). While HNF1α is expressed in tissues like kidney, stomach, intestinal epithelium and hepatocytes, Cdx2 is constrained to the differentiated cells of the intestinal microvilli (Song *et al*, 2006; Chang *et al*, 2009).

ACAT2 also utilizes various oxysterols as substrates (Cheng *et al*, 1995a). It recognizes cholesterol with a higher specificity over ACAT1 and 25-OH cholesterol is also utilized more efficiently in comparison to ACAT1. With respect to fatty acyl-CoAs, ACAT2 has preference for linoleoyl-CoA or palmitoyl-CoA over oleoyl-CoA (Cases *et al*, 1998; Buhman *et al*, 2000a). Activity of ACAT2 is lowest with arachidonyl-CoA.

When the substrate specificities of ACAT1 and ACAT2 were put head-to-head, it was found that ACAT2 exhibits more specificity towards cholesterol in presence of plant sterols like sitosterol as compared to ACAT1 (Temel *et al*, 2003). Also, the rate of esterification of cholesterol and that of oleate incorporation into sterol esters is many times greater for ACAT2 as compared to ACAT1 (Temel *et al*, 2003). Reasons for such higher specificity and activity may be cited that on a mole-to-mole basis ACAT2 is more efficient than ACAT1 but with near 40% sequence homology none of the currently available methods can determine the specific activity of the two isoforms. Another likely explanation is the metabolic half-life of ACAT1 which is fairly shorter than that of ACAT2 (30 min v/s 6 hr), indicating that the rapid rate of ACAT1 turnover makes it less stable as a protein in comparison to ACAT2 leading to consequential decline in specific activity (Temel *et al*, 2003).

When ACAT activity was compared in overnight-fasted and -fed rats, it was found that the corresponding activity was two-fold higher in the fasted animals (Helgerud *et al*, 1982). Thus, nutritional status is yet another factor involved in the regulation of catalytic activity of ACAT isoforms.

A couple of studies in the past have deliberated upon the circadian regulation of ACAT activity. Since cholesterol biosynthesis follows a diurnal pattern in mammalian physiology, it was only prudent to check the effect of this circadian variation on activity of ACAT. It was found that the enzymatic activity of ACAT hits a higher point paralleling to that of the peaking cholesterol biosynthesis in mammalian systems. Thus, the circadian peak of cholesterol biosynthesis coincides with the physiological peak of ACAT catalysis (Erickson *et al*, 1980a). On the other hand, the circadian peak of adrenal corticosteroid was also found to be correlated to cholesterol ester levels and hence ACAT activity. Civen and co-workers showed that with circadian variation in adrenal corticosteroid levels, the ratio of esterified: free cholesterol was affected (Civen *et al*, 1982). Incidentally, they showed that the ratio of esterified: free cholesterol was lowest at the peak of corticosterone secretion. One of the major observations in this study was that as the levels of cholesteryl

esters begin to decline, ACAT activity was reduced to its minimum value. Another important observation put forward by Civen and colleagues was that the circadian change in ACAT activity is biphasic. ACAT activity was found to be at the maximum as the corticosterone levels begin to rise but reach a minimum as corticosterone levels peak (Civen *et al*, 1982). This suggested that as ACAT catalysis proceeded the amount of free cholesterol went down, leading to an increased requirement of the steroid precursor. Recent studies on diurnal variation of ACAT isoforms are lacking.

ACAT - A THERAPEUTIC TARGET?

ACAT in Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia characterized by abnormal deposits of β-amyloid (Aβ) in the brain and progressive neurodegeneration (Echavarri *et al*, 2011). It has been shown that cholesterol turnover in the brain can affect the development of AD (Shobab *et al*, 2005; Hirsch-Reinshagen *et al*, 2009) by influencing Aβ catabolism. Puglielli and colleagues (Puglielli *et al*, 2003) have associated the intracellular distribution of cholesterol with regulation of Aβ generation. Analysis of genetic data from AD patients has suggested an association of the *ACAT1* gene to that of AD risk (Bertram *et al*, 2007). Esterification of neuronal cholesterol has been implicated in the pathogenesis of AD (Chang *et al*, 2010). Further genetic evidence has also shown that lowered or nil activity of ACAT can confer protection against AD (Wollmer *et al*, 2003). Using specific ACAT1 inhibitors and $ACAT^{-/-}$ mice, it has been shown that ACAT plays an important role in processing of APP and generation of APP C-terminal fragments, Aβ40 & Aβ42 (Colell *et al*, 2009; Huttunen *et al*, 2009; Maulik *et al*, 2013). Thus, ACAT plays a modulatory role in promoting β- and γ-secretase activities for generation of Aβ fragments. Furthermore, ACAT increases the availability of APP from its early secretory pathway thus facilitating the activities of β- and γ-secretase (Puglielli *et al*, 2001; Hutter-Paier *et al*, 2004). However, these mechanisms are not well defined as of now and researchers are searching for other possibilities like the effect of ACAT in APP processing in the lipid rafts. Altered lipid droplet formation by modulating the activity of ACAT can also affect

APP trafficking and hence its processing. Also, patients undergoing cholesterol lowering therapies have been found to be among the lower risk groups for acquiring AD. This made ACAT a lucrative target among pharmaceutical companies pursuing novel strategies for treatment of AD.

ACAT & Atherosclerosis

Several reports have shown that the advent of foam cells rich in cholesteryl esters within the intima of arteries is an early event mediating atherosclerosis (Ross, 1999). Additionally, an alarming fact is that lipid-rich plaques are more prone to rupture as compared to lipid-poor plaques (Falk *et al*, 1995; Libby and Aikawa, 2002). A normal LDL particle is usually very slowly taken up by the monocytes or macrophages but oxidative stress can generate a special type of LDL particle, oxidized LDL (*OxLDL*), which can be very efficiently and rapidly taken up by the scavenger receptors of the macrophages (Itabe *et al*, 2011). After this oxidative modification, the uptake of OxLDL in a macrophage proceeds in an unregulated and unhindered manner. Upon uptake, the cholesteryl esters present in the lipoprotein are hydrolyzed and immediately re-esterified by ACAT1 and "the cholesteryl ester cycle" (Brown and Goldstein, 1983) begins. In macrophages, which form an important part of the pathophysiology of atherosclerosis, ACAT1 regulates the allocation of intracellular cholesterol to esterified- and free-cholesterol pools (Akopian and Medh, 2006). This is a very important event for any cell, as esterification of cholesterol sequesters free cholesterol in the form of esterified lipid droplets by making it unavailable for ABCA1-mediated efflux (Akopian and Medh, 2006; Voloshyna and Reiss, 2011; Sorci-Thomas and Thomas, 2012). This massive accumulation of cholesteryl esters in the form of lipid droplets leads to the formation of lipid-laden foam cells (Webb and Moore, 2007; Ouimet and Marcel, 2012). Cholesterol ester accumulation in vascular smooth muscle cells is also controlled by ACAT1 (Yagyu *et al*, 2000; Rong *et al*, 2005; Rong *et al*, 2013). Both of these events contribute significantly to the development of the lipid-rich core of the plaque.

Since ACAT plays a noteworthy role in the development of atherosclerotic plaque, it has been a potential pharmacological target for researchers. Pan-specific or non-specific ACAT inhibition has been tried by different researchers with varying levels of success. The following section describes the successes and failures of ACAT inhibitors.

ACAT inhibitors as potential therapeutic agents for atherosclerosis

Several studies identifying potential ACAT inhibitors for the management of hyperlipidemia and atherosclerosis have been reviewed (Pal *et al*, 2012). The past 2 decades have seen a generous number of publications on the subject of ACAT inhibitors (Figure 3). More than 150 patents have been filed suggesting a keen interest amongst researchers and the commercial arena regarding ACAT inhibition as a potential therapeutic strategy for atherosclerosis and AD. The development of several synthetic, herbal or microbial origin ACAT inhibitors has allowed the researchers to understand the role of ACAT in cholesterol turnover. However, at the same time the therapeutic potential of ACAT depletion has been questioned and marred by several unfavorable reports. A major hiccup in the future of ACAT inhibitors as potential drugs is the failure of almost all known compounds in clinical trials with the exception of melinamide. Melinamide, a non-competitive ACAT inhibitor, has been the only compound to be approved for treatment in Japan (Natori *et al*, 1986). Apart from this, all other compounds have failed miserably in advanced stages of clinical trials with avasimibe leading the brigade. A two-year long study [The A-PLUS trial (Tardif *et al*, 2004)] where patients were administered avasimibe on a daily basis failed to show any reduction in atherosclerotic plaques. Apart from that, avasimibe also raised the levels of LDL by about 9 percent generating chaos about the beneficial effects in atherosclerosis. The study with pactimibe [The ACTIVATE trial, (Nissen *et al*, 2006)] was more upsetting where patients with atherosclerosis were treated for 18 months with pactimibe not only failing to show any reduction in the atherosclerotic plaques but secondary endpoints of the study actually demonstrated a worsening in their condition. There were two major concerns: 1) the drug was non-specific with respect to

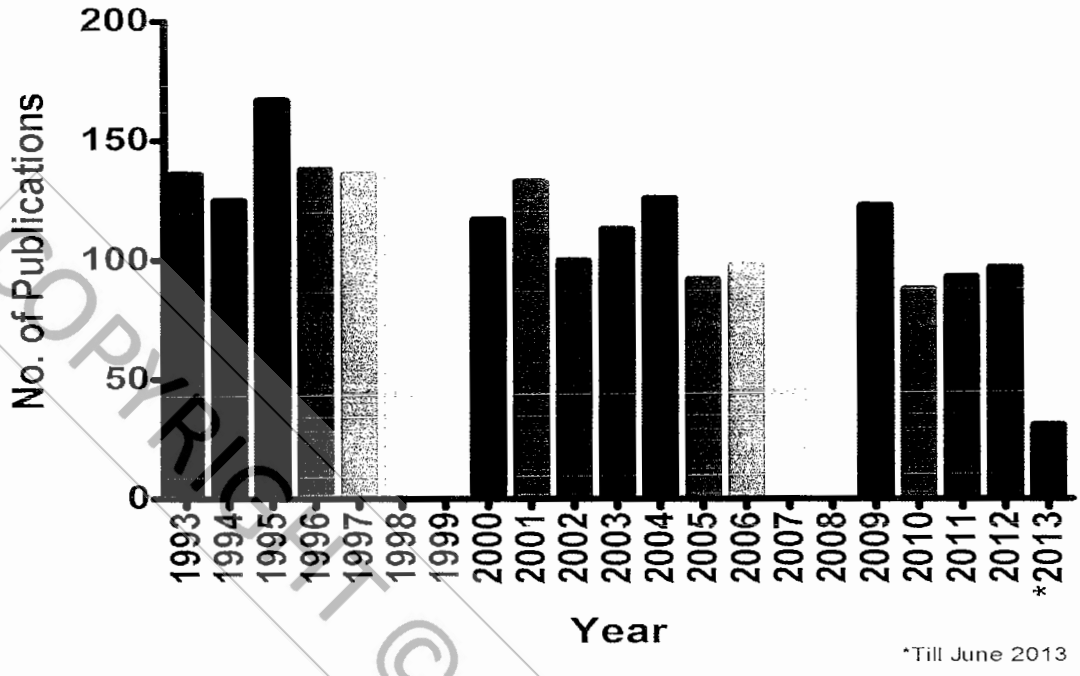


Figure 3: Trends of publication on ACAT. The graph indicates the no. of publications arisen after the discovery of ACAT1 cDNA by Chang & Colleagues (1993). A steady no. of publications shows that the interest of researchers has waxed and waned over a period of time. (Based on data retrieved from PUBMED using keywords like ACAT, acyltransferase and acyl coA: cholesterol acyltransferase. No. of publications for each keyword were noted for the indicated time period and the repetitions were eliminated to avoid duplication of data.)

ACAT inhibition, 2) plasma cholesterol levels remained unchanged, suggesting that inhibition of liver and intestinal ACAT2 is unsatisfactory (Nissen *et al*, 2006). The publication of the results of this trial was very unsettling and nearly put the concept of ACAT inhibition for atherosclerosis on a full stop. In lieu of the conclusions drawn from the ACTIVATE trial, the CAPTIVATE study was prematurely terminated (Meuwese *et al*, 2009). This seemed to be a prudent move from the sponsors as the results from the 15-month study in the CAPTIVATE trial had shown no major benefit with the administration of pactimibe as compared to placebo and in fact had resulted in an increase in the carotid intima media thickness (CIMT). This was accompanied by a rise in LDL cholesterol levels

and the pactimibe group was associated with a higher occurrence of major cardiovascular events like myocardial infarction and stroke in comparison to the placebo group (Meuwese *et al*, 2009). F12511 (generic name *eflucimibe*), chemically an anilide derivative, is a non-specific ACAT inhibitor developed by Eli Lilly in collaboration with French research group Pierre Fabre (Burnett, 2003). Eflucimibe has been previously reported to inhibit ACAT1 (IC_{50} 39 nM) over ACAT2 (IC_{50} 110 nM) with a certain predilection (Chang *et al*, 2000). Eflucimibe has been found to have favorable effects on serum cholesterol in cholesterol-fed animals (Junquero *et al*, 2001a; Junquero *et al*, 2001b; Rival *et al*, 2002). Eflucimibe cleared Phase I trials and was advanced to phase II of clinical trials in 2002 (Burnett, 2003). However, no further reports are available regarding the results of this phase II trial or progression to phase III or any randomized trials.

In experimental studies most of the ACAT inhibitors administered *in vivo* had shown two major manifestations: adrenotoxicity and cutaneous xanthomatosis [reviewed in (Buhman *et al*, 2000a)]. Both the effects are a direct result of inhibition of cholesterol esterification in the adrenal gland and skin respectively, leading to accumulation of toxic free cholesterol. Cholesterol crystal deposits have also been found in the brains of animals administered ACAT inhibitors [reviewed in (Chang *et al*, 2006a)]. Mutant mice lacking the ACAT1 gene ($ACAT1^{-/-}$) also showed these effects when fed with a high cholesterol diet (Fazio *et al*, 2001). An additional observation which was very useful in identifying the $ACAT1^{-/-}$ mice was the atrophy of meibomian glands. This led to narrowing of the eye openings by the age of 3-4 weeks in mice (Yagyu *et al*, 2000). With these effects becoming apparent in mice being treated with ACAT inhibitors, the question of safety also arose for the individuals participating in the clinical trials.

The identification of two isoforms of ACAT and their involvement in the pathogenesis of different conditions raised speculations about selective pharmacological inhibition of ACAT isoforms as a viable therapeutic option. Studies with $ACAT1^{-/-}$ and/or $ACAT2^{-/-}$ mice have provided useful insights regarding the depletion of specific isoforms and their role in conditions like atherosclerosis and AD. Some studies showed that ACAT

depletion in macrophages is actually pro-atherogenic due to the cytotoxicity of accumulated free cholesterol whereas aortic smooth muscle cells are relatively resistant to cytotoxic cholesterol accumulation and ACAT inhibition (Rong *et al*, 2005). In light of these findings, a very common observation was made suggesting that specific inhibition of ACAT1 can actually be detrimental due to accumulation of free polar cholesterol within the cells. Specific inhibition of macrophage ACAT1 can lead to increase in aortic lesion size and consequent detrimental effects. ACAT1$^{-/-}$ mice showed deposits of unesterified cholesterol on the skin, and brain histopathology showed similar deposits of cholesterol crystals in various regions of the brain. Alternatively, specific inhibition of intestinal ACAT2 was found to be beneficial in preventing diet-induced hypercholesterolemia and gall-stone formation (Kusunoki *et al*, 1995; Buhman *et al*, 2000b). Since ACAT2 is the predominant isoform present in the intestinal villi, inhibition of this isoform can lead to reduced esterification of dietary cholesterol marring its absorption. This shall ultimately reduce the production of apoB containing lipoprotein like cholesterol ester rich LDL. Another important observation made in this study was that ACAT2$^{-/-}$ mice were resistant to development of atherosclerosis when fed with a high-cholesterol diet. This again raised a speculation about the usefulness of ACAT2 specific inhibitors for the treatment of atherosclerosis and interestingly selective ACAT2 depletion showed induction of ABCA1 mediated free cholesterol efflux (Zhang *et al*, 2012). Selective ACAT2 inhibition increases the secretion of triglycerides into the newly forming VLDL particles leading to hypertriglyceridemia (Lee *et al*, 2004; Bell *et al*, 2006). Thus, selective inhibition of the ACAT isoforms is a deceitful path at present which should be trodden incisively. The research on ACAT2-specific inhibitors breathed a new life with pyripyropene-A, an ACAT2-specific inhibitor of fungal origin, exhibiting 2000-fold higher selectivity for ACAT2 over ACAT1 (Ohshiro *et al*, 2011; Ohtawa *et al*, 2013a; Ohtawa *et al*, 2013b). This molecule has shown very good results *in vitro* and in rodent models of hypercholesterolemia and atherosclerosis. Alternatively, pan-specific inhibitors of ACAT have shown very good results in models of diet-induced atherosclerosis with characteristic regression of aortic lesions. These molecules have also shown favorable effects on Aβ

turnover suggesting an advantageous role in AD-like condition. Liver-specific ACAT2 inhibition using antisense oligonucleotides has its own implications and is discussed in the subsequent sections.

Is it sensible to use anti-sense oligonucleotides for ACAT inhibition?

Among dwindling hopes and failing clinical trials, a study by Bell and colleagues smeared a ray of hope by utilizing antisense oligonucleotides (ASOs) for ACAT inhibition (Bell *et al*, 2006). The authors observed that ASOs against hepatic ACAT2 do reduce hepatic ACAT2 mRNA levels and activity without affecting the activity of ACAT1 in liver or affecting ACAT isoform activity in other tissues. Many endpoints, important to the pathophysiology of atherosclerosis were improved following biweekly ASO therapy to $LDLr^{-/-}$ mice for 16 weeks. Consequently, ASO treatment reduced hepatic cholesteryl ester content, plasma and LDL cholesterol and most importantly shifted plasma cholesteryl ester content from a saturated or monounsaturated to a principally polyunsaturated form. All these factors collectively resulted in a reduction in aortic cholesteryl ester content illustrating a reduction in the acuteness of atherosclerosis. In spite of these encouraging results, two important findings in this study featured against the use of liver-specific ASOs for treatment. One, liver specific ACAT2 inhibition led to increased triglycerides in nascent VLDL particles. This effect is a compensatory mechanism leading to increased plasma triglyceride levels (Lee *et al*, 2004; Bell *et al*, 2006). It is disturbing because hypertriglyceridemia can have its own implications in relation to hyperlipidemia or metabolic syndrome. Two, one of the ASOs used in the study led to elevation of transaminases, reflecting a possible hepatotoxic potential of these kind of agents. When ASO therapy has not yet received full clinical support such reports can certainly have a negative impact on the liability that ASOs might put forward when such agents are advanced towards clinical trials. Another point of concern in this study was whether ASO treatment was initiated after establishment of hypercholesterolemia or not. Because, even though the authors have used adequate controls for their experiments, amelioration of hypercholesterolemia after it has been ascertained for a few weeks can prove daunting.

Given these advantages and drawbacks of ASO therapy, it would be prudent to compare and contrast pharmacological ACAT inhibition *vis-à-vis* ASO-mediated ACAT inhibition. Most of the pharmacological ACAT inhibitors used in clinical trials have been non-selective to the ACAT isoforms ultimately leading to adrenal toxicity as one of the major concerns. Additionally, agents like avasimibe and pactimibe have failed to show efficacy in randomized clinical trials. However, non-selective inhibition has one clear advantage that since pharmacological inhibition is incomplete by nature, it leads to retention of ABCA1 activity. Thus free cholesterol efflux might counter the accumulation of free cholesterol alleviating the possibility of xanthomatosis or adrenotoxicity as observed in ACAT1$^{-/-}$ mice. Furthermore, non-selective inhibition can alter the composition of chylomicrons and VLDL decreasing the atherogenicity of these lipoproteins (Akopian and Medh, 2006). But clearly, as these agents have failed clinical trials, it is very difficult to say at this point that these advantages actually matter or not. On the other hand, ASO therapy is relatively free of side effects and animal studies show promising endpoints in relation of atherosclerosis progression. However, the risk of hepatic damage and hypertriglyceridemia may prove to be hurdles in the progress of ASOs as therapeutic alternatives. The relative lack of experience related to ASOs in human can also affect the relative sentiments regarding acceptance of this novel form of therapy. At present, it is unclear whether tissue-specific antisense inhibition or ACAT-inhibitory pharmacotherapy would offer better prognosis in experimental models and clinical cases of atherosclerosis.

ACAT2-selective inhibition

With encouraging results from the studies of Bell and coolleagues it became reasonably important for researchers to identify ACAT2-specific inhibitors that could be studied *in vivo* (Bell *et al*, 2006). The atheroprotection strategy with ACAT2-specific inhibition would have wider acceptability if it could be demonstrated; using an ACAT2-specific inhibitor that apart from ASO mediated ACAT2 inhibition, pharmacological inhibition of ACAT2 is also possible. Pyripyropenes are known ACAT inhibitors of fungal origin. Pyripyropene-A is a highly selective and potent inhibitor of ACAT2 (IC_{50} 70 nM)

(Ohshiro *et al*, 2011). Ohshiro and colleagues evaluated this orally active ACAT2-specific inhibitor in a mouse model of atherosclerosis and found that ACAT2-selective inhibition can be beneficial for atherosclerosis (Ohshiro *et al*, 2011). Atherogenic animals treated with pyripyropene-A showed reduced intestinal cholesterol absorption and ultimately reduced accumulation of cholesterol and cholesteryl esters in LDL and VLDL suggesting a protective effect against development of atherosclerotic lesions. Thus orally active inhibitors of ACAT2 hold latent anti-atherogenic ability. When more research groups show interest in this matter and when more ACAT2-selective agents are discovered, the picture might become clearer. Further studies in robust animal models can direct the progression of ACAT2-selective compounds towards clinical trials.

CURRENTLY USED METHODS FOR SCREENING OF ACAT INHIBITORS

Specific and non-specific inhibition of ACAT isoforms has been attempted by several researchers for the control of atherosclerosis with varying levels of success. All of these studies involve determination of the effect of inhibitors on the quantum of cholesteryl oleate formed by ACAT catalysis. Different methods have been employed for the estimation of cholesteryl esters in general and cholesteryl oleate in particular. By far the most commonly employed method is the one involving the use of radioactive substrates like [^{3}H] cholesterol or [^{14}C]oleoyl CoA, for the ACAT assay. This leads to production of radioactive cholesteryl oleate which is quantified by liquid scintigraphy (Erickson *et al*, 1980b; Chang *et al*, 1998; Temel *et al*, 2003). Hashimoto *et al* (1973) and Largis *et al* (1989) used a similar method for the determination of cholesteryl palmitate. In both the assays either cell homogenates or microsomes were used as a source of ACAT enzyme. Lada and co-workers developed a rapid and high-throughput cell-based assay for determination of cholesterol esters. This assay exploits the mimicry of fluorescent NBD-cholesterol (22-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3-ol) to that of native cholesterol. NBD-cholesterol shows relatively higher fluorescence in a non-polar milieu. Thus, esters of NBD-cholesterol are strongly fluorescent and this fluorescence can be measured with the help of fluorescence microscopy, HPTLC (High

Performance Thin Layer Chromatography) or fluorescent ACAT assay (Lada *et al*, 2004). Mizoguchi and associates have proposed a direct measurement method for the enzymatic determination of cholesterol esters by either colorimetry or fluorimetry. Cholesteryl esters are converted to free cholesterol with the help of cholesterol esterase and the liberated cholesterol is decomposed by H_2O_2, which on further treatment with 4-aminoantipyrine or amplex red produces a product which can be estimated by colorimetry or fluorimetry respectively. The inherent drawback of this method involves the need to isolate cholesteryl ester products from the reaction mixture using an efficient method like HPTLC (Mizoguchi *et al*, 2004). All these methods are laborious, time-consuming and/or expensive. Moreover, these methods possess a limitation of not being able to directly estimate cholesteryl ester levels in plasma.

RESEARCH ENVISAGED

RESEARCH ENVISAGED

Two isoforms of ACAT are known: ACAT1 and ACAT2. Apart from the physiological aspect, production of cholesteryl esters by ACAT isoforms makes a noteworthy contribution to the pathogenesis of atherosclerosis. ACAT1 aids the progression of atherosclerosis via accumulation of cholesteryl esters in macrophages leading to conversion of smooth muscle cells to foam cells, ultimately leading to plaque initiation and subsequent events (Fazio et al. 2001; Linton and Fazio 2003). ACAT2 has been found to be responsible for dietary cholesterol absorption through intestinal microvilli by converting the polar cholesterol to non polar esters. Pan-specific or non-specific ACAT inhibition has been tried by different researchers with varying levels of success. Several studies identifying potential ACAT inhibitors for the management of hyperlipidemia and atherosclerosis have been reviewed (Pal *et al*, 2012). The past 2 decades have seen a generous number of publications on the subject of ACAT inhibitors. More than 150 patents have been filed suggesting a keen interest amongst researchers and in the commercial arena regarding ACAT inhibition as a potential therapeutic strategy for atherosclerosis. The development of several synthetic, herbal or microbial origin ACAT inhibitors has allowed the researchers to understand the role of ACAT in cholesterol turnover. Between the day when the first ACAT inhibitor was discovered and to this day, lot of progress has been made in the understanding of structure, function, localization and inhibition of ACAT isoforms. However, there is yet a lacuna in the therapeutic class of ACAT inhibitors. Despite several discouraging attempts, the search for a safe and effective ACAT inhibitor for the management of atherosclerosis persists.

The present study deals with development of a screening method for ACAT inhibitors and then screening a series of potential compounds for ACAT-inhibitory activity. These compounds belong to a class of urea derivatives and are assumed to show inhibition of ACAT catalytic activity. This inhibitory activity is supposed to be effective in controlling hyperlipidemia and the hallmark features of atherosclerosis.

Primary aim of this study involved development and validation of a simple assay method which can be used to assess the ACAT-inhibition potential of the small molecules in question without the use of radiometric facilities which are more commonly utilised for the same purpose. Succeeding this, it was aimed to screen a

series of test compounds using this novel method and thus identify potential active compound(s) which could be studied for their efficacy in an animal model of atherogenesis.

HYPOTHESES

1. Cholesteryl esters formed as catalytic products of ACAT reaction can be quantified by planar chromatography
2. Urea-based derivatives can be effective in the prevention of atherosclerosis through ACAT-inhibitory mechanisms

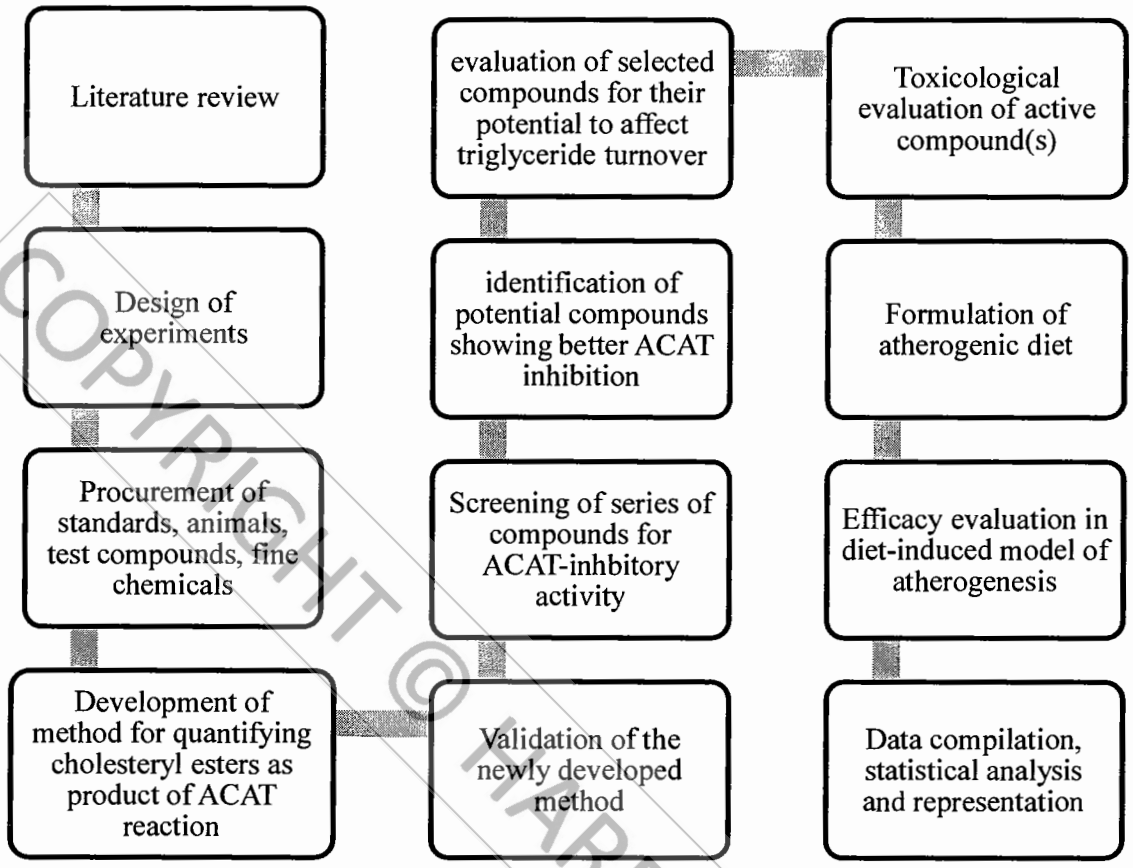
OBJECTIVES OF THE STUDY

1. To develop a method for the screening of ACAT inhibitors
2. To screen new chemical entities for potential ACAT-inhibition activity
3. To evaluate the active compounds for oral toxicity
4. To evaluate the active compounds for anti-hyperlipidemic and anti-atherosclerotic activity

EXPERIMENTS PERFORMED TO ACHIEVE THE OBJECTIVES

1. Development and validation of novel HPTLC-based method for screening of cholesteryl esters
2. Screening and identification of NCEs for potential inhibition of microsomal ACAT activity using rat liver microsomes-*ACAT Assay*
3. Evaluation of selected NCEs for their potential to affect triglyceride turnover *in vivo* using the Poloxamer-407 induced lipoprotein lipase inhibition model in rats
4. Single dose and repeat-dose toxicity evaluation of the selected NCE(s) by the procedures mentioned in OECD guidelines 423 and 407 respectively.
5. Determination of the efficacy of the selected NCE(s) in a model of diet-induced atherogenesis

FLOW OF WORK



RESULTS
&
DISCUSSION

RESULTS & DISCUSSION

SCREENING OF POTENTIAL ACAT INHIBITORS

The aim of this study was the screening of ACAT inhibitors. The screening process required that an *in vivo* or *in vitro* enzymatic reaction should take place so that the test compounds could be added/administered at appropriate time-points to evaluate their inhibition potential. It was chosen to utilize the *in vitro* cell-free ACAT enzyme based assay for evaluation of the test compounds since this assay was relatively simple, many test compounds could be studied simultaneously and the methods for determining completeness of the reaction were known, *i.e.* through quantification of cholesteryl esters formed in the reaction mixture. Based on a review of literature, it was known that radiometric scanning was the most commonly employed technique for the detection of cholesteryl esters. Researchers most commonly used radiolabeled substrates like C^{14}-oleoyl CoA, H^3-cholesterol or C^{14}-cholesterol. This resulted in formation of radiolabeled products having the respective labelled atoms in their structure. This radioactivity could be quantified simply using either a liquid scintillation counter or through radiometric scanning (Erickson *et al*, 1980b; Chang *et al*, 1998; Temel *et al*, 2003). One major caveat in these studies was that it required separation of the labelled products from the reactants and substrates otherwise the radioactivity measurements would give false negatives since the entire substrate might not be used up in the reaction. As an alternative, different research groups utilized thin layer chromatography as a means of separating the products formed as a result of the catalytic reaction, sprayed the plates with iodine vapours to visualize the cholesteryl ester bands and then scrapped the regions from the silica plates to process them for liquid scintillation based quantification. This method was tedious and employed radiometry as a technique for quantification (Largis *et al*, 1989; de Medina *et al*, 2004; Lada *et al*, 2004; Mizoguchi *et al*, 2004). Handling of radiometric reagents is a complex issue with lot of precautions and specialized areas and training being needed for handling such material. Apart from this, disposal of radiometric waste is another global concern. In lieu of this, it was decided to develop a non-radiometric, planar chromatography-based technique which could allow densitometric quantification of cholesteryl esters.

STANDARDIZATION OF ACAT ASSAY

This assay is a cell-free enzyme-based *in vitro* assay where substrates are added to the reaction mixture, enzyme and the test compounds are incubated previously and the reaction is allowed to proceed for a finite time at the end of which the products are extracted from the reaction mixture and quantified. Several reports have shown different modifications of the ACAT assay procedure (Tomoda *et al*, 1992; Cases *et al*, 1998; Lada *et al*, 2003). Accordingly, it was decided to standardize the reaction based on the available literature. As shown in the '*EXPERIMENTAL*' section, the reaction utilizes phosphate buffer, bovine serum albumin (BSA), a source of ACAT enzymes and cholesterol & oleoyl CoA as substrates. Different buffers have been indicated as a reaction medium for the ACAT assay like 10 mM Tris-buffer, 50 mM Tris-sucrose buffer, 0.154 M potassium phosphate buffer or 0.1 M potassium phosphate buffer (Chang *et al*, 1993; Temel *et al*, 2003; Liu *et al*, 2005). However, all the buffers are known to have good buffer-capacity and allow maintenance of normal tonicity and *p*H (~7.4) at 37 °C. Assay components are known to remain stable in these buffers; preparation of all the buffers is relatively simple and any buffer does not offer advantage over the other. Hence, 0.1 M potassium phosphate buffer was arbitrarily chosen for further protocols. BSA is added to prevent the small quantities of the enzyme being used in the assay from being lost by adhering to the reaction vessel walls. Alternatives to the use of BSA are egg albumin and fetal bovine serum (FBS). However, the purity of BSA is higher as compared to these reagents. Additionally, unnecessary enzyme and test compound binding may occur with FBS which would not be acceptable. Some researchers have also suggested removal of BSA altogether from the reaction mixture to reduce non-specific binding of test components (Llaverias *et al*, 2003) but in the present study no difference was observed in the data of standard inhibitor when BSA was present or absent in the reaction media. Microsomal ACAT protein was used as the source of the enzyme to catalyze the esterification process. Alternatively, S9 fraction of the liver homogenate (supernatant of a liver homogenate centrifuged at 9000×*g* for 20 mins) may also be utilized as a source of the enzyme. This fraction may be lyophilized and used as a crude enzyme source after protein quantification. However, presence of a lot of non-specific and uncharacterized substances in this fraction is liable to give false-positive and false-negative data. Another alternative is to use the purified enzyme obtained from recombinant sources or chemically-synthesized in commercial labs through peptide synthesis. However, such

sources are rarely available and if at all, are extremely costly and do not offer rationale for a screening process (Cho *et al*, 2003). Hence, microsomal ACAT was utilized for all assays in this study. Cholesterol is a hydrophobic molecule and is not soluble in aqueous solvents. Use of organic solvents in the assay may negatively affect enzyme activity and hence cholesterol was solubilized using 45% w/v hydroxypropyl β-cyclodextrin since this excipient has been reported not to affect the activity of biological enzymes (Karuppiah *et al*, 1993). The bulky and hydrophobic cholesterol is easily lodged inside the cyclodextrin cavity and thus becomes available for an aqueous medium. Cholesteryl oleate is by far, the most abundant cholesteryl ester in any mammalian cell and hence it was decided to use oleoyl CoA as the other substrate (Liu *et al*, 2005). Alternatives to oleoyl CoA include palmitoyl- and stearoyl CoA (Liu *et al*, 2005). After optimization of the assay reagents and their concentrations, it was important to extract the reaction products. Since cholesteryl esters are freely soluble in organic solvents like chloroform and methanol, a mixture of both in a ratio of 2:1 was utilized for the extraction purpose. However, the experimental trials indicated that volume of the extraction solvents are critical for achieving a reliable extraction of the product formed. When equal volumes of extraction solvent were used (*i.e.* equal to the volume of reaction mixture), no spot of the ester was obtained on TLC. Similar issue remained for upto 2-3 volumes of the extraction solvent even when multiple extractions were performed. It was realized that extraction of the cholesteryl esters might not be complete with such lower volumes and when 6-8 volumes of extraction solvent was employed for extraction, appropriate spot were obtained. When 6-8 volumes were utilized for extraction, the extract was concentrated and dried under N_2 vapors. The dried extract of cholesteryl esters were reconstituted in 500µl mixture of chloroform: methanol (2:1) before application to TLC plates.

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Optimization studies were carried out using standard solution of cholesteryl oleate, which was the analyte of interest in the present study. Based on the literature available for separation of cholesteryl esters like cholesteryl oleate and cholesteryl palmitate, several mobile phases were tried to obtain a good resolution of cholesteryl oleate (Largis *et al*, 1989; de Medina *et al*, 2004; Lada *et al*, 2004; Mizoguchi *et al*, 2004). The basic idea was to find a mobile phase which showed good resolution of cholesteryl oleate from other reagents like cholesterol and oleoyl CoA, had an R_f value

between 0.2-0.8, showed no tailing or fronting abnormalities and did not produce unnecessary charring or pigmentation of the samples before sample derivatization and quantification is performed. Different mobile phases, involving combinations of hexane, ether, acetic acid and ethyl acetate (Kupke and Zeugner, 1978; Touchstone, 1995, de Medina *et al*, 2004), evaluated for resolving cholesteryl oleate, are enlisted in table 3 below:

Table 3: Different mobile phase systems for resolution of cholesteryl oleate

Sr. No.	Mobile Phase Composition (ratio; v/v/v)	Comments
1.	*n*-Hexane: diethyl ether: acetic acid (70: 30: 1)	R_f is greater than 0.9; resolution of assay products is inadequate; tailing is another issue
2.	*n*-Hexane: diethyl ether: acetic acid (50: 50: 1)	R_f value too low; not resolved from cholesterol
3.	*n*-Hexane: diethyl ether (70: 30)	R_f is greater than 0.9
4.	*n*-Hexane: diethyl ether (80: 20)	R_f is greater than 0.9
5.	*n*-Hexane: diethyl ether (90: 10)	R_f is $\geq$ 0.9
6.	*n*-Hexane: diethyl ether (94: 6)	Optimal R_f (0.6-0.7)
7.	*n*-Hexane: diethyl ether (95: 5)	Optimal R_f (~ 0.6)
8.	Petroleum ether: diethyl ether: acetic acid (70: 30: 0.5)	Tailing is observed in samples
9.	*n*-Hexane: ethyl acetate (90: 10)	R_f is $\geq$ 0.8
10.	*n*-Hexane: ethyl acetate (95: 5)	R_f is greater than 0.9
11.	*n*-Hexane: ethyl acetate (93: 7)	Tailing and bizarre run, R_f is greater than 0.9
12.	Diethyl ether: petroleum ether (95: 5)	Tailing is observed in samples
13.	***n*-Hexane: diethyl ether: acetic acid (90: 10: 1)**	**Optimal R_f of analyte (~ 0.6) and very low R_f for substrate cholesterol**

The mobile phase optimized to *n*-hexane: diethyl ether: glacial acetic acid (90:10:1, v/v/v) gave a well-resolved peak with Gaussian symmetry (R_f = 0.59 ± 0.02,

Figure 4) and the same was used for further analysis. No immiscibility issues were observed with the chosen composition of mobile phase. Another advantage of this composition of mobile phase is that the R_f value of the substrate cholesterol is very low (0.05) hence it does not interfere with the detection of the cholesteryl oleate even if it is added in excess amount. Chamber saturation time was found to strongly affect the R_f value of cholesteryl oleate and was therefore optimized to be 30 minutes. Apart from the chamber saturation time, it was also observed that volume of mobile phase in the twin trough chamber was critical to the optimal R_f of the analyte. While higher volumes led to washing of the applied samples, very low volumes led to increased run time that affected R_f values. After trial and errors, the volume of mobile phase was decided to be 36-40 ml so that it got distributed equally to both the troughs and each trough therefore contained 18-20 ml of the mobile phase. Since the mobile phase contained highly volatile organic solvents, it was freshly prepared for each run. After several experiments it was concluded that the chosen mobile phase required nearly 18 minutes of run time. The effect of temperature and humidity was also considered for the experiment and at all the times the experiments were conducted at 25± 2 °C at an ambient relative humidity of 40-60%. Ambient temperature was not considered for the experiments since temperature and humidity variations at ambient conditions could affect the mobile phase composition and hence quantification of the analyte. The analyte of interest, cholesteryl oleate, could be analysed using an ultraviolet wavelength of 205 nm but at such low wavelengths background signal and noise might result in errors in the analysis and it is routinely necessary to derivatize the analyte spots before they can be detected. In the preliminary stages of the study, iodine vapors were used for detection and scanning of the spots of cholesteryl oleate (de Medina *et al*, 2004). Use of iodine is convenient and allows detection of the samples in visible light but iodine being volatile in nature, errors occurred in the detection of samples due to a gradual decrease in intensity of the bands. Another alternative for detection purpose was a combination of 50 mM $MnCl_2$ and 6% H_2SO_4 (Musanti *et al*, 1996) which resulted in production of $MnSO_4$ *in situ*. However, when this detection reagent was utilized, the intensity of the bands was very less and heating of the TLC plates at 120 °C for 15-20 mins after derivatization did not result in an increase in intensity. Henceforth, anisaldehyde-sulphuric acid reagent was used for spot identification and detection. This reagent was found to give an intense purple color with cholesteryl oleate after heating (120 °C for 8 mins) and the intensity also remained stable over the period of analysis.

METHOD VALIDATION

Linearity and Range

Least square regression method was used for calculation of correlation coefficient, slope and intercepts. The calibration curve was found to be linear in the range of 100-500 ng/band. The linearity of the calibration curves was validated by a value of correlation coefficient closer to unity. Each reading was an average of 3 determinations (Figures 5, Table 4).

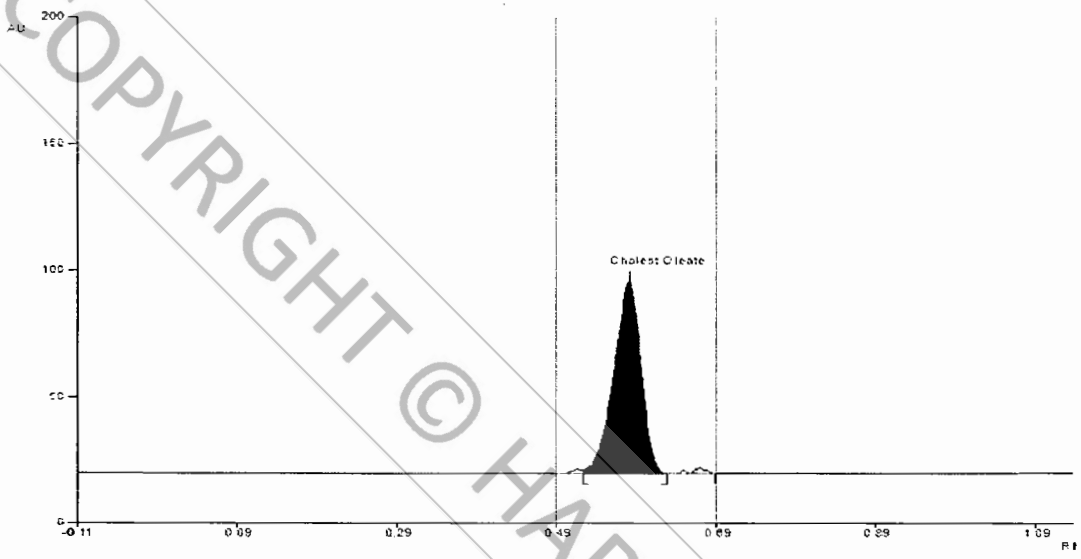


Figure 4: A typical chromatogram of cholesteryl oleate. This representative chromatogram depicts the R_f value of standard cholesteryl oleate (~ 0.59) obtained upon using the current method. A concentration of 400 ng/band was utilized to obtain this chromatogram.

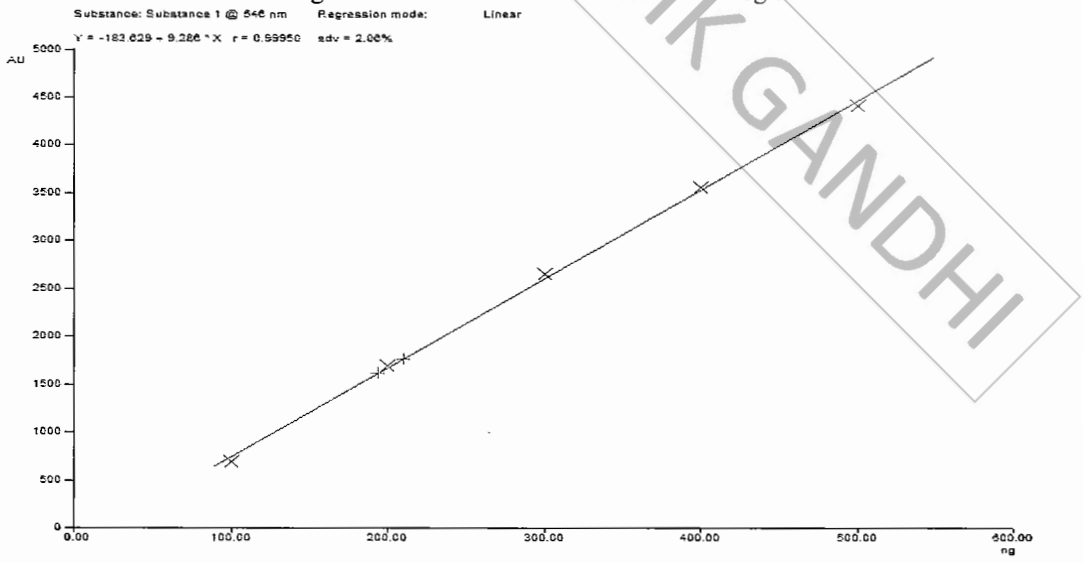


Figure 5: A typical regression curve obtained for standard and test samples of cholesteryl oleate. Coefficient of correlation closely approaches unity and quantity of cholesteryl oleate in the test samples also falls on the linearity curve.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated as per the formulae mentioned in the materials & methods section. Based on the formulae, LOD and LOQ for cholesteryl oleate were found to be 6.45 ng and 19.54 ng respectively (Table 4). However, by experimentation LOD and LOQ were found to be 10 ng and 25 ng respectively. Such low values of LOD and LOQ suggest that the method is adequately sensitive for analytical purposes.

Precision and Accuracy

The repeatability of sample application and measurement of concentrations based on peak areas were expressed in terms of % RSD and are depicted in table 5. Table 5 shows the intra- and inter-day variations of cholesteryl oleate at three different levels (200, 300 and 400 ng/band). The proposed method afforded a recovery within the range of 96.88-103.01% suggesting that the method is accurate and can be used for the quantification of cholesteryl oleate.

Specificity

The method was found to be robust as no interfering substances were found near the R_f value of cholesteryl oleate when the standard and sample lanes were compared. Further, there was no difference between the peak of standard cholesteryl oleate and the one obtained from the sample as determined by peak start, peak apex and peak end positions. An overlay of chromatograms from standard and samples of cholesteryl oleate is shown in figure 6. Further to this, it was observed that the biological matrix does not lead to any interference *per se* (Figure 7).

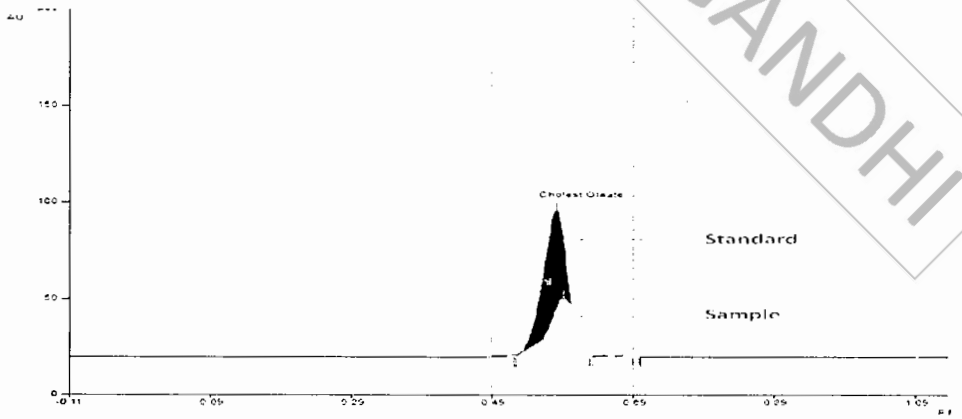


Figure 6: An overlay of the peaks obtained from standard and samples of cholesteryl oleate. This figure shows that the relative retention of standard cholesteryl oleate and from analyte samples remains the same. It may also be observed that no interference could be found in the analyte detection range.

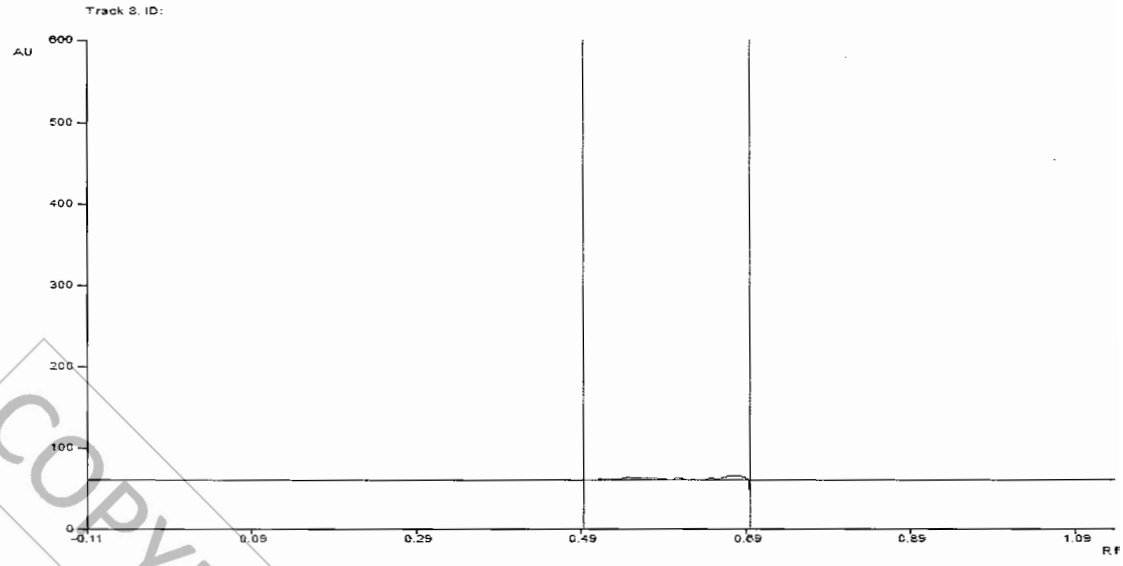


Figure 7: No peaks were found at the sample Rf when blank matrix was utilized for spotting

Robustness

The SD of peak areas was calculated for each determined parameter and %RSD was calculated. The values of %RSD (as shown in table 6) indicate that the method is robust and minor changes in experimental conditions do not affect analysis of cholesteryl oleate.

Validation of the method in plasma samples

The results of the validation of the method are shown in table 13. The % recovery of this method was found to be between 101.05-105.87 % suggesting the accuracy of the method. The low RSD values (Table 7) indicate that the method may be applied to the estimation of cholesteryl esters in pathological samples.

Table 4: Linear regression data of calibration curves[†1]

Range (ng/band)	r ± SD	Slope ± SD	Intercept ± SD	LOD (ng/band)	LOQ (ng/band)
100-500	0.9996 ± 0.0002	10.295 ± 0.95	205.703 ± 0.63	6.45	19.54

r, correlation coefficient; † n = 6

Table 5: Precision and Recovery[2]

Nominal Concentration (ng/band)	Intra-Day			Inter-Day		
	Conc. Found (ng/band)	Accuracy (%recovery)	Precision (%RSD)	Conc. Found (ng/band)	Accuracy (%recovery)	Precision (%RSD)
200 ng	193.76 ± 1.19	96.88 ± 0.59	0.61%	198.28 ± 2.61	99.14 ± 1.31	1.32%
300 ng	309.04 ± 3.73	103.01 ± 1.24	1.21%	302.02 ± 2.07	100.67 ± 0.69	0.68%
400 ng	401.94 ± 4.77	100.49 ± 1.19	1.19%	394.99 ± 3.19	98.75 ± 0.80	0.80%

[1] Linear regression data for cholesteryl oleate in the range of 100-500 ng/band
[2] Intra- and inter-day precision of the HPTLC method and recovery studies

Table 6: Robustness of the method[3]

Parameter	Mean peak area	SD of peak area	%RSD
Mobile phase composition	4765.20	51.10	1.07
Mobile phase volume	4251.00	46.43	1.02
Temperature	4555.90	55.47	1.30

Table 7: Validation using plasma samples[4]

Nominal Concentration (ng/band)	Intra-Day			Inter-Day		
	Conc. Found (ng/band)	Accuracy (%recovery)	Precision (%RSD)	Conc. Found (ng/band)	Accuracy (%recovery)	Precision (%RSD)
200 ng	211.35 ± 3.43	105.67 ± 1.71	1.62%	207.66 ± 3.24	103.83 ± 1.62	1.56%
300 ng	317.61 ± 3.02	105.87 ± 1.01	0.95%	316.82 ± 5.43	105.61 ± 1.81	1.72%
400 ng	406.84 ± 4.28	101.71 ± 1.09	1.05%	404.20 ± 7.90	101.05 ± 1.97	1.96%

[3] Robustness was evaluated by utilizing 3 different compositions of mobile phase. Total volume of the mobile phase and temperature of analysis were varied by ± 5%.

[4] Validation of the estimation method in pooled plasma samples. Results indicate the accuracy and precision of the method for estimation of cholesteryl esters in plasma samples. See 'experimental' section for details.

VALIDATION OF THE NEWLY DEVELOPED METHOD USING THE STANDARD INHIBITOR (AVASIMIBE)

The ACAT assay mentioned in section 2.4 was applied for the evaluation of avasimibe (known inhibitor of ACAT isoforms (Llaverias *et al*, 2003). The basic aim was to determine the IC_{50} value of avasimibe for ACAT inhibition and compare it with IC_{50} values determined by other methods and reported in the literature. The IC_{50} value of avasimibe was found to be 4.019 ± 0.064 µM which was found to be in agreement with the value (IC_{50} 4.0 µM (Burnett et al., 1999)) reported in literature. This finding indicates that the reported method can be effectively used for screening of novel ACAT inhibitors which otherwise becomes prohibitive for many researchers due to non-availability of facilities for radiometric method.

QUANTIFICATION OF CHOLESTERYL ESTERS IN PLASMA SAMPLES

It has been reviewed that cholesteryl esters present in plasma as a part of LDL-cholesterol are responsible for the development of atherosclerosis (Spector and Haynes, 2007, Ghosh *et al*, 2010). In lieu of these reports, we chose to evaluate pathological samples having known high total plasma cholesterol levels for their cholesteryl ester content. Interestingly, a close correlation between total cholesterol levels and cholesteryl ester levels (Figure 8) was found in the present study. Previous reports have underlined a positive correlation between total cholesterol and cholesteryl esters (Zhang *et al*, 2005, Matsumura *et al*, 1999). Most of the methods employed for estimation of total cholesterol levels use cholesterol esterase to hydrolyze the cholesteryl esters (Mizoguchi *et al*, 2004). Thus cholesterol esters are indirectly estimated as a difference of total cholesterol and free cholesterol (Levy *et al*, 2007). This suggests that a significant portion of total cholesterol is derived from the hydrolysis of cholesteryl esters. It is known that the concentration of cholesteryl esters is very high in the plasma and it was customarily necessary to dilute the samples with phosphate-buffered saline before analytical measurements could be performed. Since, the curve of cholesteryl oleate is not linear at very high concentrations (>100mg/dl), results presented herewith were obtained after dilution of the samples and then multiplying the results with an appropriate dilution factor. However, care was taken to ensure that dilution patterns remained similar across all the samples of analysis so as to eliminate errors of dilution and calculation. The figure below indicates the correlation between cholesterol ester

levels and total cholesterol levels from normocholesterolemic and hypercholesterolemic individuals. The trend of the curve shows that the level cholesteryl esters are directly

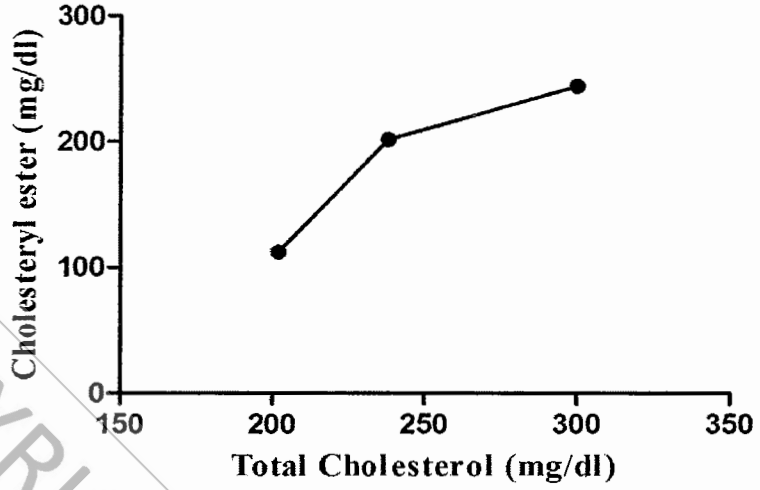


Figure 8: A plot showing the relation between total cholesterol and cholesteryl esters from plasma samples of 3 patients having total cholesterol levels higher than 200 mg/dl. Cholesteryl esters are expressed as a function of cholesteryl oleate. Results are expressed as a mean of triplicate analysis

proportional to that of total cholesterol. This finding may be in concert to that of other researchers (Spector and Haynes, 2007, Ghosh *et al*, 2010) who have suggested that cholesteryl esters may be a diagnostic factor and/or risk-marker for atherosclerosis.

ADVANTAGES OF THE METHOD

The method was found to have a total-run time (sample application through) of about 50-60 minutes after sample preparation hence, it can be claimed that the time of analysis is relatively short when compared to the HPLC-FLD and LC-ESI-MS/MS methods (Cao *et al*, 2013, Miyoshi *et al*, 2013). Currently, this method has been developed on a 10×10 cm^2 plate where 7-8 samples can be simultaneously estimated. It is also possible to use a 20×10 cm^2 plate where 17-18 samples can be estimated. Increasing the number of samples for analysis does not increase the time of analysis significantly as the spots are run simultaneously. The concentration range employed in the present study is 100-500 ng/band but the limit of quantification was found to be 19.54 ng/band (Table 4). Hence, this method may be applied in lower concentration ranges (i.e. 20-100 ng/band) indicating the sensitivity of this method. This sensitivity is comparable to that achieved with LC-ESI-MS/MS (30ng - 1µg; (Miyoshi *et al*, 2013). Samples and standards are estimated simultaneously on the same plate. Chances of

poisoning the sample are negligible since there is no carry over. The present method works as an open system and visual detection of the sample is possible post derivatization. Further, derivatization can be performed on the plate *in situ*. Derivatization with anisaldehyde-sulphuric acid reagent leads to the development of a purple band, so separate hydrolysis step is avoided. Using the HPTLC method, we have estimated cholesteryl esters *at par* with radiometric estimation. We were able to reproduce the data from literature regarding estimation of ACAT inhibitor avasimibe (Llaverias *et al*, 2003, Burnett *et al*, 1999). Cholesteryl ester content in plasma samples from hypercholesterolemic individuals was also estimated by this method.

PRELIMINARY SCREENING OF TEST COMPOUNDS FOR THEIR ACAT-INHIBITION POTENTIAL

After validation of the ACAT assay and quantification of cholesteryl oleate as a product of the ACAT catalytic reaction, this method was employed for the screening of five series of urea-based novel synthetic compounds to evaluate them for their potential ACAT inhibitory activity. Since, the compounds were screened using microsomal ACAT, the activity of the compounds is represented herewith as pan-ACAT and they were not evaluated for inhibition of individual isoforms. The results of preliminary screening are depicted in the tables (8-12) below:

Table 8: Preliminary screening of compounds from series I

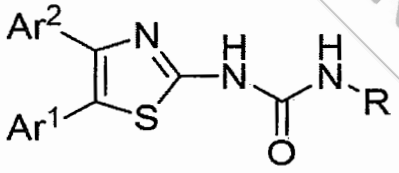
Comps	 (I)			% Inhibition (at 10 µM conc)
	Ar^1	Ar^2	R	
Ia	Ph	Ph	Ph	10.56
Ib	*4*-ClPh	4-OCH_3Ph	Ph	**56.86**
Ic	4-ClPh	4-OCH_3Ph	*n*-Butyl	27.58
Id	4-ClPh	4-CH_3Ph	*n*-Butyl	**36.37**
Ie	4-ClPh	4-FPh	2,4-F_2Ph	**42.39**

Table 9: Preliminary screening of compounds from series II

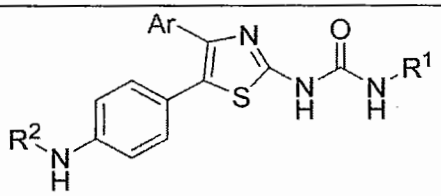
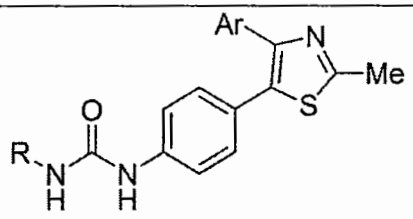
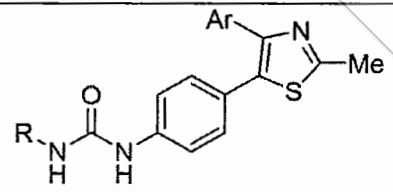
Comps	 (II)			% Inhibition (at 10 µM conc)
	Ar	**R^1**	**R^2**	
IIa	4-ClPh	2,4-F_2Ph	$COCH_3$	2.03
IIb	4-ClPh	2,4-F_2Ph	SO_2CH_3	6.39
IIc	4-ClPh	*n*-Butyl	$COCH_3$	3.75
IId	4-ClPh	*n*-Butyl	SO_2CH_3	5.63
IIe	4-FPh	2,4-F_2Ph	$COCH_3$	23.32
IIf	4-FPh	2,4-F_2Ph	SO_2CH_3	18.9
IIg	4-FPh	*n*-Butyl	$COCH_3$	15.4
IIh	4-FPh	*n*-Butyl	SO_2CH_3	0.43
IIi	4-CH_3Ph	2,4-F_2Ph	$COCH_3$	3.06
IIj	4-CH_3Ph	2,4-F_2Ph	SO_2CH_3	2.17
IIk	4-CH_3Ph	*n*-Butyl	$COCH_3$	5.23
IIl	4-CH_3Ph	*n*-Butyl	SO_2CH_3	32.67
IIm	4-OCH_3Ph	2,4-F_2Ph	Isopropyl	**51.73**
IIn	4-OCH_3Ph	2,4-F_2Ph	*n*-Dodecyl	1.77
IIo	4-OCH_3Ph	2,4-F_2Ph	$COCH_3$	**38.98**
IIp	4-OCH_3Ph	*n*-Butyl	$COCH_3$	**39.45**
IIq	4-OCH_3Ph	*n*-Butyl	SO_2CH_3	5.45

Table 10: Preliminary screening of compounds from series III

Comps	 (III)		% Inhibition (at 10 µM conc)
	Ar	**R**	
IIIa	4-ClPh	Ph	6.88

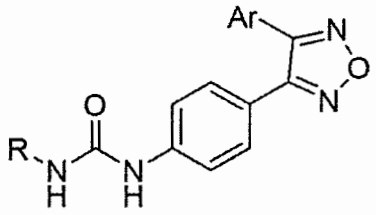
IIIb	4-ClPh	2,4-F_2Ph	1.3
IIIc	4-ClPh	2,6-$(C_2H_5)_2$Ph	**63.32**
IIId	4-ClPh	*n*-Butyl	**60.71**
IIIe	4-ClPh	*n*-Heptyl	**52.18**
IIIf	4-ClPh	*n*-Dodecyl	5.75
IIIg	4-FPh	Ph	16.81
IIIh	4-FPh	2,4-F_2Ph	4.97
IIIi	4-FPh	2,6-$(C_2H_5)_2$Ph	**46.68**
IIIj	4-FPh	*n*-Butyl	**59.91**
IIIk	4-FPh	*n*-Heptyl	**62.96**
IIIl	4-FPh	*n*-Dodecyl	34.47
IIIm	4-CH_3Ph	2,6-$(C_2H_5)_2$Ph	30.94
IIIn	4-CH_3Ph	*n*-Butyl	**64.91**
IIIo	4-CH_3Ph	*n*-Heptyl	33.41
IIIp	4-CH_3Ph	*n*-Dodecyl	**46.19**
IIIq	4-OCH_3Ph	Ph	11.37
IIIr	4-OCH_3Ph	2,4-F_2Ph	22.27
IIIs	4-OCH_3Ph	2,6-$(C_2H_5)_2$Ph	31.62
IIIt	4-OCH_3Ph	*n*-Butyl	**40.02**
IIIu	4-OCH_3Ph	*n*-Heptyl	22.21

Table 11: Preliminary screening of compounds from series IV

Comps	 **(IV)**		**% Inhibition (at 10 µM conc)**
	Ar	**R**	
IVa	4-ClPh	3,4,5-$(OCH_3)_3$Ph	24.44
IVb	4-ClPh	2,6-$[CH(CH_3)_2]_2$Ph	12.64
IVc	4-ClPh	Morpholinoethyl	12.31
IVd	4-FPh	3,4,5-$(OCH_3)_3$Ph	**41.98**
IVe	4-FPh	2,6-(*iso*-propyl)$_2$Ph	**42.12**
IVf	4-CH_3Ph	3,4,5-$(OCH_3)_3$Ph	21.88

IVg	4-CH_3Ph	2,6-(*iso*-propyl)$_2$Ph	11.19
IVh	4-OCH_3Ph	3,4,5-$(OCH_3)_3$Ph	25.44
IVi	4-OCH_3Ph	2,6-(*iso*-propyl)$_2$Ph	30.13
IVj	4-OCH_3Ph	Morpholinoethyl	21.46

Table 12: Preliminary screening of compounds from series V

Comps	 (V)		% Inhibition (at 10 µM conc)
	Ar	R	
Va	4-ClPh	2,4-F_2Ph	21.23
Vb	4-ClPh	2,6-$(C_2H_5)_2$Ph	8.25
Vc	4-ClPh	*n*-Butyl	23.03
Vd	4-ClPh	*n*-Heptyl	4.3
Ve	4-ClPh	*n*-Dodecyl	18.51
Vf	4-FPh	2,4-F_2Ph	**47.06**
Vg	4-FPh	2,6-$(C_2H_5)_2$Ph	**44.85**
Vh	4-FPh	*n*-Butyl	**54.5**
Vi	4-FPh	*n*-Heptyl	30.93
Vj	4-FPh	*n*-Dodecyl	**50.7**
Vk	4-CH_3Ph	2,4-F_2Ph	13.52
Vl	4-CH_3Ph	2,6-$(C_2H_5)_2$Ph	0.34
Vm	4-CH_3Ph	*n*-Butyl	2.83
Vn	4-CH_3Ph	*n*-Heptyl	18.65
Vo	4-CH_3Ph	*n*-Dodecyl	9.15

Based on the preliminary screening data, it was observed that several compounds showed good inhibition (some of them ≥ 60%) which was comparable to that of known pan-ACAT inhibitor avasimibe. This data was used further to select compounds for determination of IC_{50}. A threshold value of 35% inhibition was arbitrarily selected and all those compounds showing ≥ 35% inhibition of microsomal

ACAT activity were selected for IC_{50} determination at five different concentrations. The execution of IC_{50} determination was identical to that of the preliminary screening except that five non-zero concentrations were used to determine ACAT-inhibition potential for each compound. The results are shown below (Table 13):

Table 13: Determination of IC_{50} on ACAT for selected compounds

Comps	IC_{50} (μM)	Comps	IC_{50} (μM)
Ib	10.66 ± 1.02	IIIj	9.58 ± 0.81
Id	93.95 ± 1.97	IIIk	**5.69 ± 0.75**
Ie	18.37 ± 1.26	IIIl	46.83 ± 1.67
IIm	12.11 ± 1.08	IIIn	**2.43 ± 0.38**
IIo	31.86 ± 0.71	IIIp	12.18 ± 1.08
IIp	41.29 ± 0.58	IVd	12.62 ± 0.82
IIIc	**4.04 ± 0.60**	IVe	11.84 ± 0.84
IIId	**6.27 ± 0.80**	Vf	20.09 ± 1.30
IIIe	**7.85 ± 0.89**	Vg	9.73 ± 0.98
IIIi	**8.40 ± 0.92**	Vj	12.44 ± 1.09

Table 13 shows that the IC_{50} values of several compounds (IIIc, IIId, IIIe, IIIi, IIIk, and IIIn) are well below 10 μM. Several urea based compounds available in the literature are known to show good inhibition of microsomal ACAT activity. These compounds are usually tri- or di-substituted derivatives of urea with myriad of substituents on either side. All of the series listed above involve di-substituted derivatives of urea. Several researchers have reported individual SAR studies with their own di-substituted urea derivatives which have shown promise for ACAT-inhibition *in vitro* and good anti-hypercholesterolemic activity. These compounds were found to exhibit microsomal ACAT-inhibition in the IC_{50} range of 0.007-5.300 μM (Kimura *et al*, 1993; Kumazawa *et al*, 1995; Trivedi *et al*, 1995; Gelain *et al*, 2006; Asano *et al*, 2009). These activities may seem promising and comparable to the standard inhibitors of ACAT like avasimibe and pactimibe but some of these compounds had their inherent problems like low water solubility, low absorption through the GIT, adrenal toxicity and complexity of synthetic process which led to retraction in their pursual (Kimura *et al*, 1993; Dominick *et al*, 1993a; 1993b; Tawada *et al*, 1994; Trivedi *et al*, 1995).

Change in substituents, inclusion of heteroaryl moieties and hydrohphilic groups were tried to improve the overall pharmacological acceptability of these compounds. Similar approaches were carried out in the synthetic series used for the present study to generate a range of novel compounds which could show potent inhibition of ACAT. As is evident from the results, compounds of series III have shown good inhibition of microsomal ACAT. So far, compound **IIIn** was found to be the most potent inhibitor of microsomal ACAT *in vitro*. The *in vitro* data for **IIIn** is comparable to that reported for avasimibe (Llaverias et al, 2003). These active compounds were further evaluated in a model of triglyceride turnover.

POLOXAMER-407 INDUCED LIPOPROTEIN LIPASE INHIBITION MODEL

Triglycerides are water insoluble molecules that are considered to be rich sources of energy for extrahepatic tissues. They are secreted from the liver as subsets of VLDL particles. It has been observed that overproduction of VLDL leads to a direct disposition of an individual towards CHD. Increase in triglyceride production rates have been observed after inhibition of plasma lipoprotein lipase (responsible for TG hydrolysis) and thereafter observing the temporal changes in plasma TG levels (Johnston and Palmer, 1993; Chang *et al*, 2006a; Temel *et al*, 2007). Several studies have utilized Triton WR-1339 (*tyloxapol*®) for inhibition of lipoprotein lipase where TG production rate was calculated over time after Triton WR-1339 injection (Erickson *et al*, 1980a; Aragane *et al*, 1998). However, it has been observed that there are several possible variables which may induce errors in the results (Millar *et al*, 2005). These variables include fasting/fed conditions, fat-free diet, plasma sampling period, plasma holding time and methods of TG analysis over linearity curves. However, these variables can be controlled at the experimenter's discretion but there are few physiological effects related to Triton WR-1339 which might suggest that an alternative agent may be used. These effects include dissociation of ApoA-I and ApoC-II from HDL particles, accumulation into lysosomes and causing formation of autophagic vacuoles, secretion in bile and excretion *via* the liver which might point towards a reduction in biliary cholesterol output and most importantly a gradual decline in TG production rate after the first hour making it difficult to interpret data after 3-4 hours. Alternatively, Poloxamer-407 or P-407 (*Pluronic® F-125*) a non-ionic detergent also inhibits lipoprotein lipase. Initially it was used as a co-polymer for controlled drug

delivery but was later found to be an inhibitor of lipoprotein lipase. The advantages of P-407 over Triton WR-1339 are (Millar *et al*, 2005):

1. Flexibility in sampling with same production rates
2. Study can be extended upto 48-72 hrs
3. Dissolution of HDL particles is not an issue
4. Preferential renal excretion owing to the HLB difference
5. No accumulation in lysosomes so TG trafficking remains unaffected
6. Hepatic lipid production is not affected

Figure 9 shows the effects of different compounds on triglyceride (TG) turnover following P-407 administration. It indicates that, commensurate with the *in vitro* data, compound **IIIn** is also active *in vivo*. The effect of **IIIn** is evident from the fact that the **IIIn** administered group shows only moderate changes in TG turnover (~20 fold) over a period of 24 hrs. Similar effect was also observed in the avasimibe group. While control animals showed a mean 80-fold change in baseline TG levels, other

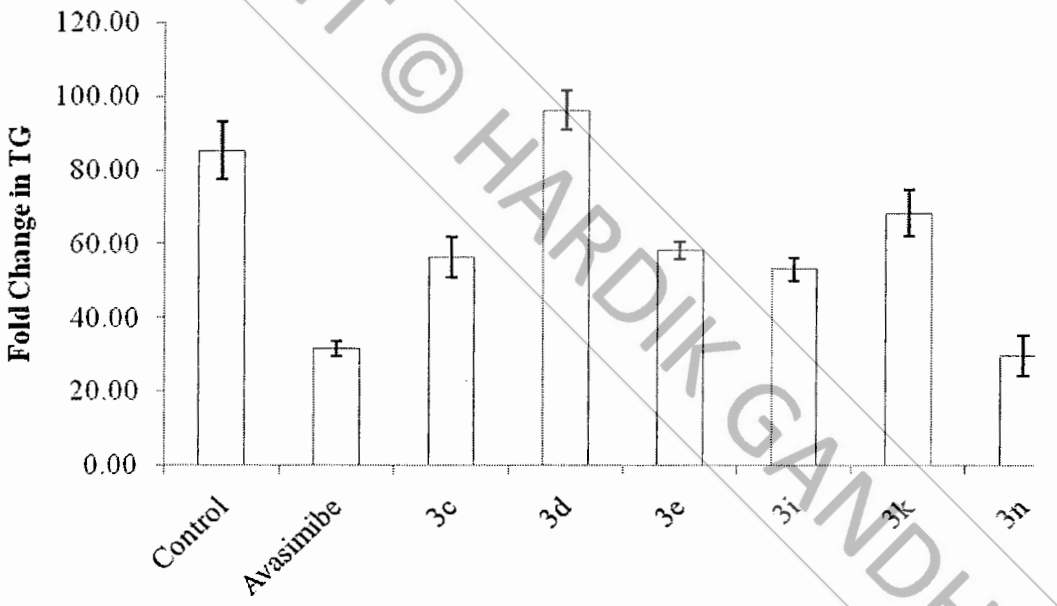


Figure 9: effects of P-407 on 24 hr triglyceride turnover measured as fold-change in TG at 0 hr and 24 hr after administration of P-407.

groups showed 50-90 fold changes in TG turnover which was exceedingly high in comparison to the standard and **IIIn** group. Since this study has already established that **IIIn** is a pan-ACAT inhibitor, it was interesting to identify the effects of this compound on TG turnover. However, these findings stand in stark contrast to those reported by Erickson *et al*, (1980b) who showed that administration of triton WR 1339 showed modest reduction in ACAT activity by itself and ACAT-inhibition, *per se*, has no effect

on lipid turnover. On the other hand Aragane *et al*, (1998) and Uchida *et al*, (1998) reported that known inhibitors of ACAT prevent the cholesteryl-ester input in plasma and reduce hepatic TG secretion rate. The present experiment revealed that there might be correlation between ACAT inhibition and TG turnover since avasimibe was also active against TG turnover mediated through LPL inhibition. It is known that chylomicrons (which form the significant chunk of TG), after losing triacylglycerols acquire cholesteryl esters from other lipoproteins. Since administration of ACAT-inhibitors can prevent *de novo* formation of cholesteryl esters, the pool of cholesteryl esters that remains available for acquisition by chylomicrons is depleted. This biochemical inhibition thus correlates with the observations of the present study where administration of ACAT inhibitors like avasimibe and the test compound **IIIn** resulted in a reduced TG turnover *in vivo*. Based on the results, it was decided to evaluate compound **IIIn** in a model of athersoclerosis since compound **IIIn**, apart from microsomal ACAT inhibition, also showed favourable effect on serum lipids.

Henceforth, compound **IIIn will be encoded as MCR-788** as per laboratory norms.

TOXICOLOGICAL EVALUATION OF MCR-788

Single dose acute oral toxicity

Studies with different urea-based derivatives belonging to this class of compounds have not shown any fatal toxicity signs. These studies have reported a safety of assorted urea-derivatives upto 2000 mg/kg and beyond (Lee *et al*, 2013; Robertson *et al*, 2000). Some compounds were reported to exhibit adrenal toxicity at high doses which may be an indication of non-specific cytotoxicity rather than their ACAT-inhibition potential (Dominick *et al*, 1993a; Roth, 1998). To ensure the safety of the test compound, **MCR-788** was administered at the recommended dose. The post-treatment examination period was 14 days from the date of dosing. Body weights of the animals were recorded on days 0, 7 and 14. Slight fluctuations were observed in the body weight of animals but since they were within 20% of the mean body weight no additional measurements were taken and any other precaution was not followed. The animals were closely observed during the first 6 hours after dosing. The animals were starved during this period with access to water. No significant observations were recorded during this period. This part coincided with the light cycle and most of the

time animals were asleep. When awake, the animals showed normal grooming behavior and water intake was also normal. During the entire post-treatment observation period special attention was paid to alteration of skin or fur, abnormal locomotion or breathing and changes in the eye. No untoward observations were made in this regard until the terminal day of the study. Mortality was recorded twice daily but no mortality was found in any dose group until day 14. At the end of the study period, the animals were euthanized and major organs (brain, heart, lung, liver, kidney, spleen and adrenals) were harvested. Gross necropsy was performed by an individual blinded to the groups. No macroscopic lesions were recorded. Viscera, gastrointestinal tract and mucous linings appeared normal. Adrenal glands exhibited normal size & shape and any signs of shrinkage were absent. Detailed report on toxicity evaluation is presented as Appendix III.

Administration of 2000 mg/kg of **MCR-788** showed no signs of toxicity or mortality during the test period. The LD_{50} of **MCR-788** in rats was thus found to be >2000mg/kg.

Repeat dose oral toxicity

At the end of the study, no untoward observations were made regarding body weight, food intake or normal behavior. Gross necropsy did not reveal any suggestive lesions or abnormal anatomical feature. Adrenal glands appeared normal. The most plausible side effect related to the mechanism of action of **MCR-788**, upon repeat-dose administration, could be cutaneous xanthomatosis (Yagyu *et al*, 2000; Farese, 2006; Ohshiro *et al*, 2011). This effect was not evident in any subject of the study even at the extended 14-day period. Biochemical estimations did not suggest any major digression from normal values. Urinary output and hematological data appeared normal. Urea based compounds promoted as ACAT inhibitors have been known to be safe at doses upto 60 mg/kg or less. Detailed report on repeat dose toxicity evaluation is presented as Appendix IV.

It was concluded that chronic administration of **MCR-788** at a dose level of 60mg/kg was safe.

EVALUATION OF MCR-788 IN A MODEL OF DIET-INDUCED ATHEROGENESIS

A model of diet-induced atherogenesis has been reported to be a valid model for study of compounds expected to be effective against atherosclerosis.

Significance of ingredients in the atherogenic diet

Cholesterol and coconut oil provide the caloric intake in the form of fat calories and thus carbohydrate calories are reduced. It has been studied that casein promotes casomorphin- or peroxidase-dependent oxidation of LDL through generation of tyrosyl free radicals (Kritchevsky, 1995; Tailford *et al*, 2003). Additionally, it may also promote atherogenesis by causing endothelial dysfunction mediated through invasion of the endothelia by monocyte/macrophages (Tailford *et al*, 2003; Matsuzawa *et al*, 2007). Cholic acid helps in the absorption of cholesterol through micellar solubilisation (Nishina *et al*, 1990; Vergnes *et al*, 2003). This property prevents the excretion of excess cholesterol taken in the form of diet and helps in the development of hypercholesterolemia. However, wistar rats are very robust and have high HDL-C and low LDL-C concentrations in plasma. It is very difficult to induce atherosclerosis in such wild-type animals without the inclusion of 2-thiouracil. 2-Thiouracil is a thyroid hormone inhibitor which prevents thyroid hormones from reducing elevated LDL-C, triglycerides, cholesterol and lipoprotein a [Lp(a)] (Joris *et al*, 1983). All these changes prompt the development of atherogenic lesions.

Body weight

The curve of body weight remained relatively unaffected for all the groups. No significant change in body weight was observed between the final and initial stages of the study. At the end of the study there was no significant difference between the progression control group (290.3 ± 8.1 g), **MCR-788** (10 mg/kg) treated group (294 ± 14.3g) and **MCR-788** (30 mg/kg) treated group (299 ± 6.6 g). Previous studies on similar lines have also reported that body weight remains comparable in all the groups (Maechler *et al*, 1992; Kusunoki *et al*, 2001; Ohshiro *et al*, 2011). Figure 10 shows the difference in body weights among the groups.

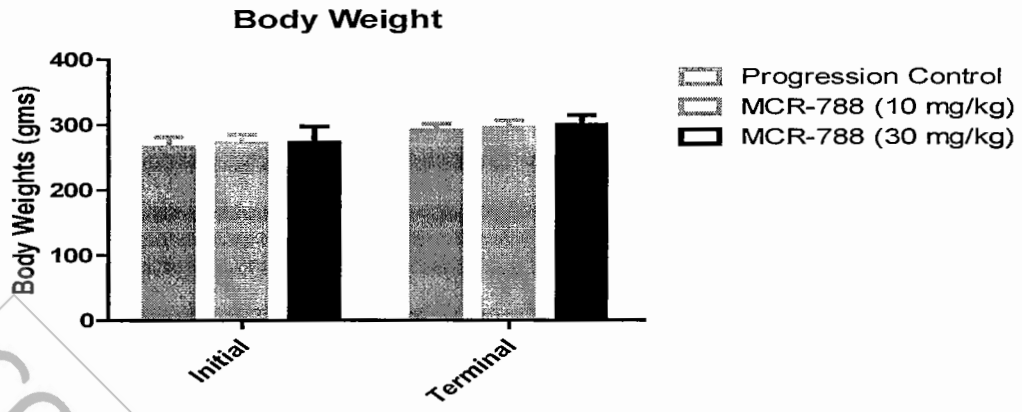


Figure 10: Figure shows mean body weight of all the groups before and after treatment. It may be noted that no significant change was observed between any groups towards the end of the study.

Analysis of serum lipids and lipoproteins

Effects of atherogenic diet and treatment with **MCR-788** on lipid profile are depicted in figure 11. At the initiation of the study the mean cholesterol values for all the animals averaged around 91.16 ± 4.54 g. It was observed that cholesterol levels start rising dramatically after about 2-weeks of HCD initiation and keep rising towards the terminal stage of the study in the progression control group. These findings are similar to those reported by other groups utilizing similar models for development of atherosclerosis (Maechler *et al*, 1992; Kusunoki *et al*, 2001). A dose-dependent prevention of serum total cholesterol elevation was observed in the groups (Figure 11A) that were co-administered **MCR-788**. The effect of 10mg/kg in preventing cholesterol elevation was not found to be significant but the 30 mg/kg dose-group showed drastic prevention of cholesterol elevation with serum total cholesterol levels being 138 ± 9.50 g at the end of the study. On the contrary, as expected HDL-C values showed a downward trend as the study progressed and a 2-fold reduction in serum HDL-C levels was observed in the control group. Such changes are similar to the reduced HDL-C levels reported by other groups (Maechler *et al*, 1992) Treatment with **MCR-788** was able to show a dose-dependent prevention of HDL-C reduction (Figure 11B). Minor elevations in triglyceride levels between the initial and final stages of the study were observed but were not found to be statistically significant (*P* value 0.251; Figure 11C). Analysis of lipoprotein cholesterol levels, LDL-C and VLDL-C, were based on the Friedewald's formula and showed that VLDL-C elevations were not significant among the groups (Figure 11D). However, a sharp rise in LDL-C (more than 12-fold) was observed in the progression control group ($P<0.001$). The lower dose group showed

only modest prevention in LDL-C elevation but the **MCR-788** 30 mg/kg dose group indicated a significant prevention ($P<0.001$; Figure 11E) of LDL-C elevation as compared to the progression control group. Based on these data, the atherogenic index was calculated, which indicates the prevalence of non-HDL cholesterol over HDL-C. This data indicated that after 8 weeks of HCD administration the atherogenic index peaks to 19.59 ± 5.8. Such a high value of the atherogenic index indicates that the animals were prone to the development of atherosclerotic lesions. Co-administration of **MCR-788** in the diet reduced the atherogenic index to 7.55 ± 0.9 and 1.82 ± 0.34 respectively in the 10 mg/kg and 30 mg/kg groups (Figure 11F).

Different disubstituted urea-based ACAT inhibitors reported in the literature have shown good cholesterol lowering properties *in vivo* (Kimura *et al*, 1993; Reindel *et al*, 1994; Gelain *et al*, 2006). It has been shown that these derivatives exhibit excellent efficacies for development as anti-atherosclerotic agents. These compounds not only improve total cholesterol levels but also increase the HDL-C titre which might be related to their protective effects. White *et al*, (1996) and Ohnuma *et al*, (2004) have reported substituted ureas as potent agents which improve triglyceride and cholesterol levels in plasma and have shown efficacy comparable to that of atorvastatin, the drug of choice in atherosclerosis. The animal models utilized in these studies were rats, mice, rabbits or genetically modified mice but the results shown by them indicated that these urea-derivatives showed potent inhibition of ACAT, *in vivo,* culminating in atheroprotection. Hence, the beneficial effect of **MCR-788** in similar conditions may be anticipated in light of the data presented above.

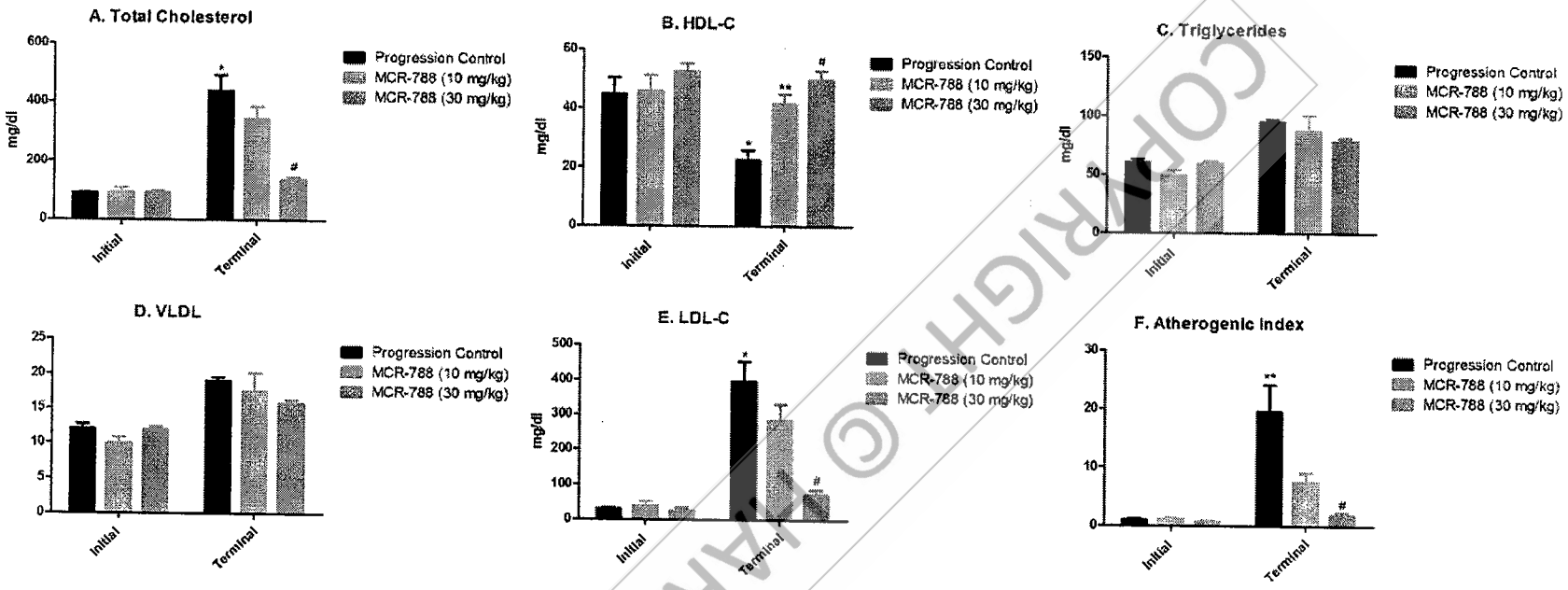


Figure 11: Figures represent the lipid profile of all the groups at the initial and terminal phases of study. A, Total cholesterol levels are increased in the progression control group at the end of 8 weeks. Significant prevention of cholesterol elevation is observed in the **MCR-788** high-dose treated group. B, Pathological development of hypercholesterolemia has led to significant reduction in the levels of HDL-C. Such abnormalities are not observed with the treated animals. C, No major fluctuation in the triglyceride levels was observed between groups. D, Since VLDL-C values were derived from triglyceride levels, the results were expected to be similar. E, Friedewald's equation revealed that LDL-C was significantly elevated in the progression control group. Treatment with low dose **MCR-788** showed only modest effect but high-dose **MCR-788** led to a significant prevention in elevation of LDL-C levels and were found to be similar to those at the beginning of the study. F. Atherogenic index was very high in the progression control group as compared to the treatment group indicating the protective effect of **MCR-788**. For all the observations, '*' indicates $P<0.05$ as compared to initial data at Day 0; '**' indicates $P<0.01$ as compared to initial data at Day 0 or as compared to the progression control group at 8 weeks; '#' indicates $P<0.01$ as compared to the progression control group at 8 weeks.

Lipid accumulation lesion area in the aortae

Lesion area in the aortic strips were characterized by *en face* lipid staining with Sudan red IV. Photographs of pinned aortae (Figure 12) upon area analysis by ImageJ revealed that 22.35±7.9 % area from the progression control group was stained as compared to the 16.21±1.07% ($P>0.05$) in the 10 mg/kg groups and 8.42±2.7% ($P<0.05$) in the 30 mg/kg group. Several studies have identified the potential of ACAT inhibition in reducing the lesion area. Ohshiro *et al*, (2011) represented a dose-dependent effect of ACAT-inhibition on lesion development while the results of Kusunoki *et al*, (2001) showed that F-1394 (a pan-ACAT inhibitor) prevented the development of atherosclerotic lesion to more than 40%. Chiwata *et al*, (2001) also showed dose-dependent protective effects of F-1394 on atherosclerotic lesion development. Results of the present study indicated that treatment with 30 mg/kg **MCR-788** shows nearly 3-fold reduction in the lesion area as compared to the progression control (Figure 13).

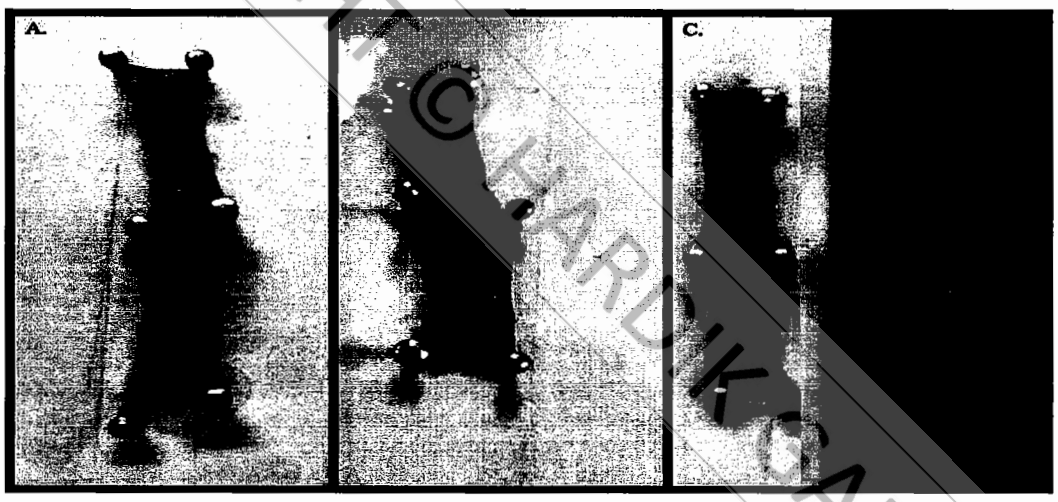


Figure 12: This figure shows representative aortae from each group. A, Accumulation of lipids in the intimal area result in the formation of fatty streaks which are stained maroon in color in the progression control group. This type of streaks are evident along the length of the aorta. B, The 10 mg/kg group also shows such intermittent streaks but as it is evident from the photograph, deposition has been minimised to a reasonable extent and C, Absence of any fatty streaks can be observed in this image. This is indicative of the atheroprotective role of **MCR-788** (30 mg/kg).

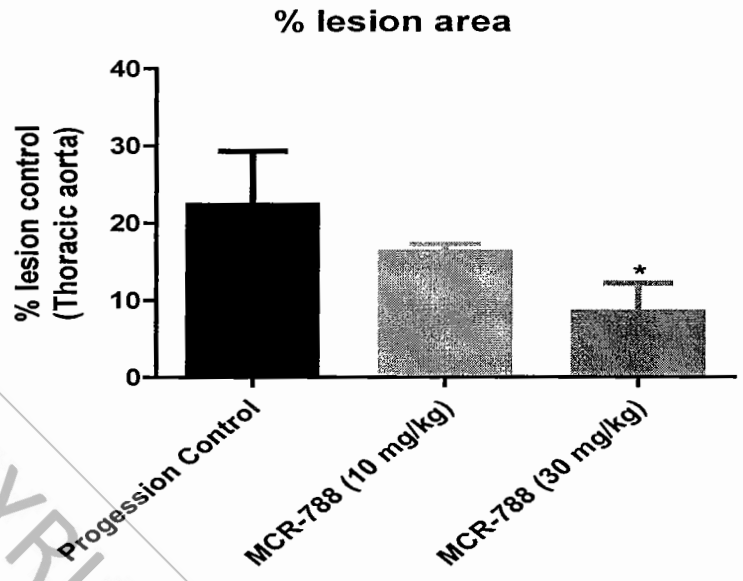


Figure 13: Fatty streaks observed by *en face* lipid staining were analysed by the ImageJ image analysis software and the lesion areas (stained maroon) were calculated respective to the total aortic strip area. Data are presented in the figure as mean ± SEM of lesion area. It can be observed that 30 mg/kg of **MCR-788** resulted in a significant prevention of lesion development. '*' indicates P<0.05 as compared to the progression control group at the end of 8 weeks.

Lumenal lipid plaque identification

Lumenal plaque formation was studied by taking multiple sections of the aortae and then staining them with Sudan Red IV to stain the lipid core of the plaque. Cryosections were used for the present study since conventional paraffin-block sectioning and deparaffinization methods utilize organic solvents which can crucially affect lipid content in the plaque core and give false-negative results. Since cryosectioning methods are solvent-free, they are recommended for such studies where solvents might affect the results. The sections were stained with Sudan Red IV to visualize the lumenal lipids.

Representative images from different groups are shown in the figure 14. The figures indicate that long-term consumption of an atherogenic diet has led to development of a plaque core which has obscured the lumen of the aortic root (*a.k.a.* aortic sinus). Aortic root is the most putative site for formation of lipid-rich plaques, although a plaque may be formed in any anatomical region of the artery. Such observations have been reported by Ohshiro *et al*, (2011) who have shown thickened intima in progression control groups. Other researchers like Kusunoki and colleagues (2001) suggest typical lesions in the aortic root cross sections and prevention of such lesions following ACAT-inhibitor therapy. Namatame *et al*, (2004)

also showed development of proximal root lesions following a high-cholesterol diet and its prevention with fungal ACAT-inhbitors (beauverolides). No evidence for the development of lipid-rich cores was found in animals treated with **MCR-788** in the present study.

Figure 14: This figure represents the cross-sectional areas from aortic roots after sacrificing animals of the different groups. Streaks in the intimal area are not observed in any group but development of plaque is evident in the HCD progression control (A). Minor damage to the arterial wall is evident at the region where plaque development is initiated. Intimal linings appear normal and there is no evidence of diameter obscuration in the treated groups (B and C). The scale bar in figure C represents 500 μm.

It was notable that protective effects of **MCR-788** reported in the study were obtained without any evidence of cutaneous or adrenal toxicities. In contrast, the study revealed that **MCR-788** was not only safe but also effective in reducing the biochemical profile and lesions in an atherogenic model. Based on this study, the potential of this compound as a lead candidate against atherosclerotic disease cannot be denied.

EXPERIMENTAL

EXPERIMENTAL

DEVELOPMENT AND VALIDATION OF HPTLC BASED METHOD FOR QUANTIFICATION OF CHOLESTERYL ESTERS AND SCREENING OF ACAT INHIBITORS

Materials and solvents

Rat liver microsomes (500 µg protein/ml) were procured from Krishgen Biosystems, Mumbai, India. Cholesteryl oleate standard was synthesized in the Pharmaceutical chemistry laboratory of Pharmacy Department, The M. S. University of Baroda. Cholesterol and oleoyl CoA lithium salt were obtained from Sigma, St. Louis, MO. Aluminium TLC plates pre-coated with silica gel $60F_{254}$ (10×10 cm^2, 250 µm thickness) were procured from Merck, Germany. Anisaldehyde-sulphuric acid reagent was prepared in the following manner: A) 0.5 ml of anisaldehyde was dissolved in 10 ml of glacial acetic acid. B) 5 ml of conc. sulphuric acid was dissolved in 85 ml ice-cold methanol. Final working reagent was prepared by mixing solution A & B. The working reagent appears colorless and should be discarded when a pink tinge appears. Other reagents and chemicals used in the present study were of analytical grade.

Standards and working solutions

A stock solution of cholesterol oleate was prepared by dissolving 100 mg of accurately weighed cholesteryl oleate in a mixture of chloroform-methanol (2:1, v/v) to make the volume upto 100 ml. Working standards were prepared by appropriate dilutions of the stock solution with chloroform-methanol (2:1, v/v). The stock and working solutions were stored at -20 °C.

High-Performance Thin Layer Chromatography

Before sample application, chromatography plates were pre-washed using methanol as a mobile phase and dried for 10 minutes at 120 °C to activate the plates. Samples were applied to the plate as 6 mm wide bands, 10 mm apart by means of Linomat V sample applicator (Camag, Switzerland) fitted with a 100 µl Hamilton syringe. A constant rate of application of 150 nl/s was used. After sample application, the plates were dried in a current of dry air and developed in a linear ascending manner using *n*-hexane-diethyl ether-glacial acetic acid (90:10:1, v/v/v) as mobile phase. 18 ml of the mobile phase was used for development of each plate. Development was performed in a 10×10 twin-trough chamber (Camag, Switzerland) which was previously saturated with mobile phase for 30 minutes. All the steps were performed at

25 ± 2 °C and ambient relative humidity. The solvent front position was fixed at 80 mm from the point of application. After running the mobile phase, plates were dried and dipped in a solution of anisaldehyde-sulphuric acid reagent. The plates were dried and heated at 120 °C for 8 minutes. This led to the development of purple colored bands at solute fronts. Densitometric scanning was performed with Scanner III (Camag, Switzerland) in absorbance mode at 546 nm. The slit dimension was set at 6mm × 0.45mm and scanning speed was 20 mm/s. Calculations were performed with the help of WinCats software (version 1.4.4, Camag, Switzerland).

Calibration curve of cholesteryl oleate

Calibration curve was prepared over a concentration range of 100-500 ng/band. Five dilutions (100, 200, 300, 400, 500 µg/ml) were prepared from the stock solution and further aliquots of these solutions were applied to the TLC plate. This procedure was repeated 6 times. The plates were developed, scanned and the data of peak areas of the developed spots versus concentrations were treated by linear least square regression to obtain the calibration curve.

VALIDATION

Method validation parameters like linearity, range, precision, accuracy, LOD (limit of detection), LOQ (limit of quantification), specificity and robustness were checked as per ICH guidelines [Q2R1]. The methods specified in the text of the guideline were followed unless indicated otherwise.

Precision and accuracy

Repeatability of sample application was carried out by taking six replicates of the same spot (200 ng/band of cholesteryl oleate) and measurement of the peak areas. The intra- and inter-day variation for the determination of cholesteryl oleate was carried out at three different concentrations (200, 300 & 400 ng/band). Accuracy was performed by the method of standard addition. A known amount of cholesteryl oleate was added to a previously analyzed sample at three different levels and % recovery was determined. Three determinations were performed on three different occasions for each level and the results obtained were compared with the expected results.

Limit of detection and limit of quantification

LOD and LOQ were determined by the formulae 3.3σ/S and 10σ/S respectively, where σ corresponds to the average standard deviation of the y-intercepts of regression lines and S corresponds to the average slope of the regression lines.

Specificity

Specificity of the method was tested by analyzing sample and standard on the same plate. The spot for cholesteryl oleate in sample lane was confirmed by comparing the retardation factor (R_f) values. Peak purity was adjudged by comparing peak start, peak apex and peak end values of samples with that of standard. In addition, we also performed an extraction procedure on the microsomal reaction mixture in which oleoyl CoA was not added. The entire procedure was followed as stated above and the extract was then spotted on the plate and scanned in a similar manner to that of other samples. Peaks in the region surrounding the R_f of cholesteryl oleate were observed for any interference due to the biological matrix involved.

Robustness

Robustness was determined by making deliberate changes in the mobile phase composition. Mobile phases having varying compositions of *n*-hexane-diethyl ether-glacial acetic acid (90:10:0.5, 92:8:1 and 88:12:1; v/v/v) were utilized and chromatograms were obtained. Volume of mobile phase and temperature of analysis were also varied in a range of ± 5 %. The effect of these changes on peak areas and R_f value of cholesteryl oleate were observed and data presented as % RSD.

Validation of the method in plasma samples

The aforementioned method was validated for accuracy and precision using pooled human plasma. Plasma samples from 5 different voluntary male donors were provided by a local blood bank (Suraktam Blood Bank, Vadodara, Gujarat, India). Equal volumes of these samples were pooled and used for validation. Briefly, 200 µl aliquot of the collected plasma sample was subjected to extraction with 800 µl mixture of chloroform-methanol (2:1, v/v) in a centrifuge tube. Extraction was effected by vortexing the mixture for 3 mins. The lower organic phase was separated out using a pipette and a known volume of standard cholesteryl oleate was added to the organic phase and further processed as mentioned in section 2.4. The volume of standard cholesteryl oleate was such that it resulted in final concentrations of 8, 12 and 16 µg/ml

after reconstitution. This afforded concentrations of 200, 300 and 400 ng/band when the samples were spotted on the TLC plate. The inherent cholesterol esters present in the pooled plasma samples were nullified from the final results of the spiked ones. Based on these experiments, intra-day and inter-day precision, accuracy and % recovery were calculated.

Quantification of cholesteryl esters in clinical plasma samples

Plasma samples of patients were collected from local pathology laboratories. These samples were originally collected for the estimation of total cholesterol levels by different labs upon referral by physicians. This study included plasma samples for 3 patients whose total plasma cholesterol levels were reported to be above 200 mg/dl by the respective pathology laboratories. 200 µl aliquot of the collected plasma sample was subjected to extraction with 800 µl mixture of chloroform-methanol (2:1, v/v) in a centrifuge tube. Extraction was done by vortexing the mixture for 3 mins. The lower phase was separated out using a pipette and subjected to drying under a stream of N_2 gas. The remaining procedure was same as that described in the *ACAT assay* section.

ACAT assay for screening of potential ACAT inhibitors

The ACAT assay mixture consisted of potassium phosphate buffer (0.1 M), BSA (5 mg/ml), microsomal protein (200 µg) and cholesterol solubilized in 45% w/v hydroxypropyl β-cyclodextrin (2 mM). Final reaction volume was made upto 850 µl with the help of 0.1M potassium phosphate buffer. Vehicle, test compounds or the standard ACAT inhibitor (avasimibe, sigma) were added at this point (final volume NMT 10 µl) and incubated for 15 mins to allow proper binding of the inhibitor with the ACAT enzymes. The reaction was initiated by the addition of 200 µM oleoyl CoA and allowed to proceed for 10 minutes at 37°C. The reaction was terminated by the addition of 6 ml of a mixture of chloroform-methanol (2:1, v/v) to the reaction mixture. The biphasic mixture was shaken in a separating funnel and both the phases were allowed to separate. The lower organic phase was collected and evaporated to dryness under a stream of N_2 gas. The residue was then redissolved in 500 µl of chloroform-methanol (2:1, v/v) and a volume of 25 µl from this solution was spotted on the TLC plates for quantification. Each sample was applied to the TLC plates at least in triplicate. Further analysis was performed as per the method given above.

POLOXAMER-407 INDUCED LIPOPROTEIN LIPASE INHIBITION MODEL

Poloxamer-407 induced lipoprotein lipase inhibition model [Johnston and Palmer, (1993); Millar *et al*, (2005)] was used to determine change in glyceride level by the test compounds.

Materials

Poloxamer-407 was procured as a generous gift sample by Wockhardt Ltd., Aurangabad, India. Avasimibe (cat. # PZ0190) was procured from Sigma Aldrich, St. Louis, MO, USA. The test compounds synthesized in Pharmaceutical Chemistry Laboratory of Pharmacy Dept., The M. S. University of Baroda, Gujarat, India were selected on the basis of preliminary *in vitro* screening results. Triglyceride estimation was performed by using commercial kits as per manufacturer's protocol (GPO-PAP method, Coral Clinical Systems, Vapi, India).

Experimental Protocol

Experiments were performed on healthy adult wistar rats (20-24 weeks; 250-350 g). Baseline triglyceride levels were estimated in serum samples from all animals using commercial kit on Day 0. Vehicle, test compounds (20 mg/kg) and avasimibe (20 mg/kg) were suspended in 0.5 % sodium CMC and administered on Day 1 by oral gavage in fasted animals. 4 hours after the administration of agents, lipoprotein lipase inhibitor, Poloxamer-407 (1000 mg/kg), (prepared as a 10 % solution in 0.9 % NaCl) was administered intraperitoneally. On Day 2, blood samples were withdrawn to obtain serum, and triglyceride levels were estimated (method described below in the succeeding sections) in the same. Results were analyzed to determine fold-change in triglyceride levels over the 24 hour test period.

TOXICOLOGICAL EVALUATION OF TEST COMPOUND (MCR-788) *(Jonsson et al, 2013)*

Single dose acute oral toxicity study - OECD 423

This study was performed using male wistar rats (10-12 weeks old, 200-230 g). Although the guideline suggests that female animals may be preferred, it was decided to evaluate the test drug in male animals to avoid the protective effects of estrogen upon the cardiovascular system which may become evident when female animals are used.

This study does not allow calculation of precise LD_{50} values, rather it allows determination of exposure range where lethality might be expected. The test compound (**MCR-788**) was suspended freshly in 0.5% Na-CMC just before dosing and administered by oral gavage on day 0 in overnight-fasted animals. Urinary and biochemical parameters were recorded at day 0 and day 14. There was no reason to believe that therapeutic doses may range beyond the highest selected dose. During the period of study, animals were observed closely, their weight and food intake were recorded twice-a-week and any abnormal behavior, if observed, was documented. Since no preliminary information regarding toxicity of the test substance was available, it was decided to omit the limit test and directly the main test was conducted. Annex 2c of the main guideline document (OECD 423) was followed unless otherwise indicated. Accordingly, 3 animals were dosed in each group and the subsequent dosing depended on the condition of the previously dosed animals. At the end of the observation period for each group, urinary and biochemical parameters were recorded (submitted herein as appendix III to the thesis), the animals were humanely sacrificed and gross necropsy was performed by closely observing all the major organs. Histopathological and microscopic observations were performed only in case of any untoward observation. Category 5 evaluation was precluded from the evaluation since a dose beyond 2000 mg/kg was unlikely to be used for any therapeutic studies.

Repeat dose oral toxicity study – OECD 407

The purpose of this study was to evaluate the effect of the test compound when administered for chronic regimens. Based on preliminary acute toxicology data and literature review of related class of compounds, 10 mg/kg & 30 mg/kg were chosen to be the animal therapeutic doses for pharmacodynamic studies. Accordingly, a dose of 60 mg/kg was selected for this toxicity evaluation, since this dose is twice that of maximum intended therapeutic dose. The guideline mentions the use of a range finding test or a limit test with a dose of 1000 mg/kg but since such a dose level is unlikely and corresponding human dose may never be applied in practice, we preferred using a dose of 60 mg/kg. Other studies have reported appearance of cutaneous toxicity like xanthomatosis in animals dosed chronically with ACAT inhibitors due to accumulation of free cholesterol in the skin. This study was performed in healthy adult Wistar rats (male and female, 10-12 weeks old, 200-230 g). No adverse effect was observed during the period of dosing (0-28 days) or during the post-dose observation period (29-42

days). Neither liver nor skin (organs most likely to be affected) showed any signs of free cholesterol deposition-mediated damage which is expected with this category of compounds.

EFFICACY EVALUATION OF MCR-788 IN A MODEL OF DIET-INDUCED ATHEROGENESIS

Materials

The list of material used in the study and their sources are given in table 14.

Table 14: List of materials and their sources for the atherogenic-diet model

Reagent/Chemical/Ingredient	***Source***
Pelleted chow for rats	Amrut Feed, Pranav Agro, India
Cholesterol	Spectrochem, India
Coconut oil	HiMedia, Mumbai, India
Cholic acid	HiMedia, Mumbai, India
2-Thiouracil	HiMedia, Mumbai, India
Casein	SD Fine Chemicals, India
Sudan Red IV	HiMedia, Mumbai, India
Hydroxypropylcellulose (HPC)	Gift sample from Wockhardt Ltd., Aurangabad India
Test compound (**MCR-788**)	Synthesized in Pharmaceutical Chemistry Laboratory of Pharmacy Dept., The M. S. University of Baroda, Gujarat, India
Kits for Total cholesterol, triglyceride and HDL-C estimation	Coral Clinical Systems, Mumbai, India
Solvents (diethyl ether, methanol, isopropyl alcohol)	SD Fine Chemicals, India

Atherogenic Diet

The composition of the diet was as follows: Cholesterol (2%), Coconut oil (1%), Cholic acid (1%), Propylthiouracil (0.5%), Casein (16.5%), powdered chow and hydroxypropyl cellulose (HPC) binder (q.s. to 100%). After accurately weighing all the ingredients casein, cholic acid and propylthiouracil were ground in a mortar pestle until

a homogenous solid mixture was formed. This dry mixture was added to a large vessel and mixed with required amount of powdered chow. Next, cholesterol was dissolved in appropriate volume of diethyl ether and this ethereal solution was properly mixed with the powder mass. The ether was allowed to evaporate for 2-3 hours. Once the ether evaporated, required amount of coconut oil was mixed properly. For every kilogram of diet to be prepared 800 ml of 1% HPC solution was prepared by stirring for about half an hour. This solution was added at once to the dry mass and vigorously kneaded to evenly mix all the HPC. The consistency achieved was such that the lumps formed neither crumbled nor were too sticky. The lumps were hand cured to form suitably shaped lumps which looked like pelleted chow. The final pellets were dried overnight in a hot-air oven at 45-50°C. This step improves long term storage and prevents microbial growth. The pellets were stored in a perforated bag at 2-8°C. The formed pellets were not stored for more than 7 days.

Experimental protocol

The animals (adult male Wistar rats, 230-300 g) were randomized into 3 groups of 6 animals each. The groups were as follows:

1. Progression Control-received atherogenic diet only
2. Treatment Group I-received atherogenic diet containing **MCR-788** such that final daily dose was 10 mg/kg
3. Treatment Group II-received atherogenic diet containing **MCR-788** such that final daily dose was 30 mg/kg.

The protocol of the study was in the following manner:

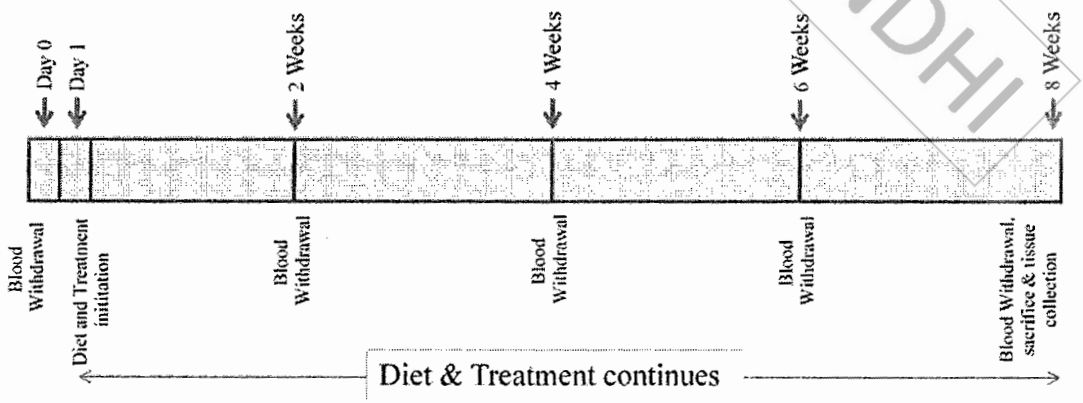


Figure 15: Protocol for diet administration, sampling and tissue collection in the atherogenic diet model.

All the blood samples were allowed to clot at room temperature for 15 mins and centrifuged at 800×*g* for 15 mins at 25°C. The supernatant serum was collected and subjected to biochemical estimation of total cholesterol, triglycerides and HDL-C using commercially available diagnostic test kits. Other parameters like VLDL, LDL-C and atherogenesis index were derived by formulae on the basis of these values. The principles and methodology of these biochemical estimations are given below. Towards the terminal stage of the study, animals were humanely sacrificed after blood sampling and thoracic aortae and aortic roots were carefully dissected.

Biochemical estimations of serum lipids and lipoproteins

Estimation of total cholesterol: (CHOD/POD-Phosphotungstate method)

Determination of the quantity of cholesterol in serum was done using enzymatic kit (Reckon Diagnostics Pvt. Ltd., Vadodara, India).

Principle:

Cholesterol esterase (CHE) hydrolyses cholesterol esters. Free cholesterol is oxidized by the cholesterol oxidase (CHO) to choloest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce pink colored quinoneimine dye. The intensity of color produced is proportional to cholesterol concentration.

Protocol:

Test tubes labeled Blank (B), Standard (S) and Test sample (T) were managed as shown in table 5 below:

Table 15: Protocol for total cholesterol estimation

Addition	**Blank**	**Standard**	**Test Sample**
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard	-	20 µl	-
Specimen	-	-	20 µl
Incubation at 37°C for 10 minutes.			
Distilled water	2.0 ml	2.0 ml	2.0 ml

The absorbance was read against Blank at 505 nm.

Calculations:

Total Cholesterol (mg/dl) = (OD of T / OD of S) X 200

Estimation of triglycerides: (GPO Method)

In vitro quantitative measurement of triglyceride (neutral fat) concentration in serum was done by using kit (Reckon diagnostics (India) Pvt. Ltd.).

Principle:

Triglycerides in the sample are hydrolyzed by microbial lipase to glycerol and free fatty acid (FFA). Glycerol is phosphorylated by adenosine 5-triphosphate (ATP) to glycerol 3-phosphate (G-3-P) in a reaction catalyzed by glycerol kinase (GK). G-3-P is oxidized to dihydroxy acetone phosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts with 4-aminoantipyrine and ADPS in the reaction catalyzed by peroxidases (POD). The result of this oxidative coupling is a purple colored dye quinoneimine. The absorbance of this dye in solution is proportional to the concentration of triglycerides in sample.

Table 16: Protocol for triglyceride estimation

Addition	**Blank**	**Standard**	**Test**
Working reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	20 µl	-
Specimen	-	-	20 µl
Incubation at 37 ºC for 15 minutes.			
Distilled water	1.5 ml	1.5 ml	1.5 ml

The absorbance of test and standard were read against the blank at 546 nm.

Calculations:

Triglycerides (mg/dl) = (Abs. of Test/ Abs. of Std.) X 50

Estimation of HDL-cholesterol: (CHOD/POD-Phosphotungstate method)

In vitro quantitative determination of the activity of cholesterol in serum was done using enzymatic kit (Reckon Diagnostics Pvt. Ltd., Vadodara, India).

Principle:

The VLDL and LDL fractions of serum sample are precipitated using PTA and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the ester into cholesterol. Then cholesterol is oxidized by cholesterol oxidase (CHO) to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) reacts with 4-aminoantipyrine and phenol to produce a red colored complex, whose absorbance is proportional to HDL-cholesterol concentration.

The following samples and reagents were pipetted into microcentrifuge tubes:

Table 17: Sample preparation for HDL-cholesterol estimation

Serum	0.2 ml
Precipitating Reagent	0.3 ml

Samples were vortexed and incubated at 25°C for 10 mins. The samples were centrifuged at 300×*g* rpm for 10 minutes to get a clear supernatant. If the supernatant was not clear (high TGL level) the samples were diluted in 1:1 ratio with normal saline. Pipetted into 3 test tubes labeled Blank (B), Standard (S) and Test sample (T) as shown in table 7 below:

Table 18: Protocol for HDL-cholesterol estimation

Addition	**Blank**	**Standard**	**Test sample**
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard	-	20 µl	-
Supernatant	-	-	20 µl
Incubation at 37 °C for 5 minutes			
Distilled water	2.0 ml	2.0 ml	2.0 ml

The absorbance is read against Blank at 505 nm.

Calculations:

HDL Cholesterol (mg/dl) = (Abs. of T / Abs. of S) X 50

Estimation of VLDL-cholesterol:

Estimation of VLDL-cholesterol was done using the Friedewald formula.

VLDL cholesterol (mg/dl) = triglycerides / 5.

Estimation of LDL-cholesterol:

Estimation of LDL-cholesterol was done using the Friedewald formula.

LDL cholesterol (mg/dl) = total cholesterol – (HDL cholesterol + VLDL cholesterol)

Estimation of Atherogenic index:

Atherogenic index = log [TG / HDL-C]

En face lipid staining

It is known that the site for atherogenesis is unpredictable along the length of the thoracic aorta, hence the entire thoracic aortae were subjected to *en face* staining with Sudan red IV solution (5 mg/ml in 70 % isopropyl alcohol, vortexed and filtered). The thoracic aortae were isolated and cleaned of periadventitious tissue as it stains heavily with Sudan red IV. Care was taken to prevent damage to intima as lipid deposits may be dislodged upon mishandling. It was split from the lumen to give a strip. The strips were pinned to a white plate and immersed in Sudan red IV solution at 37°C for 45 minutes. After this maneuver, the samples were destained for 10 mins in 40 % isopropyl alcohol. The stained tissue was photographed using a digital camera and images were analyzed using NIH (USA) image analysis software, ImageJ (ver. 1.43u, written by Wayne Rasband). Gross extent of atherosclerosis within the thoracic aorta was calculated by estimating the percent lesion area as compared to total area. Data obtained were subjected to statistical analysis.

Cryosectioning and Sudan Red IV staining

The aortic roots were immediately washed with ice-cold PBS, cleared of surrounding adventitious fat, carotid connections were severed and immersed in 10 % buffered formalin for cryosectioning. At the time of sacrifice, liver lobes were also removed to serve as support while sectioning the aortic roots. A small cavity was formed into the pith of the liver and one end of the aortic root was inserted inside the cavity. This whole lobe was now mounted on a chuck and put inside the sectioning instrument. The tissue was allowed to freeze for about 15-20 mins and then the chuck was placed near the blade-rotor assembly. The blade was moved by the rotor to give a slicing motion and the sections were collected on a slide. The sections easily stick on the slide as soon as the slides are brought to room temperature due to the proteins in the section. The sections were further stained with Sudan red IV solution (5 mg/ml in 70 % isopropyl alcohol, vortexed and filtered). The slides were put inside a coplin jar and the jar was filled with Sudan red IV solution. The jar was incubated in a water bath at 37°C for 45 mins. The slides were then destained for 10 mins in 40 % isopropyl alcohol. Slides were cleaned after removing, observed at 10X magnification and photographed.

STATISTICAL ANALYSIS

For all the studies, $3 \leq n \leq 6$. Data from the validation study were presented as mean, standard deviation and % relative standard deviation [(SD*100)/mean]. Concentration versus area obtained from the WINCATS software after HPTLC detection was treated by linear least square regression for linearity determination. Data from the ACAT screening assay and the atherogenesis study were presented in the form of mean ± SEM. Data from the lipoprotein lipase inhibition model were calculated as fold-change over a period of 24 hours and then presented as mean ± SEM.

The results were compared by Student's *t*-test, one-way or two-way ANOVA as appropriate. Bonferroni's multiple comparisons were employed as the *post hoc* test wherever required.

All the statistical analyses were performed using Graphpad Prism, San Diego, CA, USA (Ver. 05) or Excel Spreadsheet program, Microsoft Corp., Redmond, WA, USA (2007). Results were considered to be statistically significant when $P<0.05$.

SUMMARY
&
CONCLUSIONS

SUMMARY & CONCLUSIONS

Based on the review of literature, the urea-based test compounds were required to be screened for their ACAT-inhibition potential. Since, the methods available in the literature involved mostly the use of radioactive compounds it was decided to develop a planar-chromatography based method for the quantitative determination of cholesteryl esters, which could be used for the screening of compounds having their effect on ACAT catalytic activity. An HPTLC-based ACAT assay was developed using alumina-backed silica gel 60F254 as the stationary phase, *n*-Hexane: diethyl ether: acetic acid (90: 10: 1; v/v/v) as the mobile phase and anisaldehyde-sulphuric acid as the detection reagent. This method was validated for quantification of cholesteryl esters as products of the ACAT assay and it was also found to be suitable for quantification of cholesteryl esters in plasma samples. It was validated for linearity over the selected range, accuracy, precision, sensitivity and specificity. Robustness was also studied by making minor alterations in the parameters of analysis. This method was then utilized for the screening of five series of urea-based test compounds for their potential to block ACAT-mediated catalysis. Based on the screening results, one compound **MCR-788** (or **IIIn**) was chosen for further evaluation. Toxicity studies with this compound revealed that the compound did not show any signs of distress/morbidity. Single dose and repeat-dose studies confirmed that the expected adverse effect of this category of compounds, *i.e.* cutaneous xanthomatosis, was not observed with **MCR-788** at the doses intended for the studies. The animals, dosed with **MCR-788**, neither showed deviation from their normal behaviour, food and water intake nor in locomotor and grooming functions. The test compound was assumed to be safe for evaluation in further animal models.

Phamacodynamic studies performed with the chosen test compound **MCR-788**, presented interesting observations. An acute model of triglyceride turnover, poloxamer-407 mediated LPL inhibition, was utilized to evaluate the effects of **MCR-788** on 24-hr triglyceride turnover in rats. This model has been summarily utilized to study the compound showing positive effects on lipid turnover. It was found that prevention of TG elevation showed by **MCR-788** was comparable to the standard ACAT inhibitor, avasimibe. It could be concluded that this compound may reduce the availability of cholesteryl esters to be incorporated in the chylomicrons. This effect can certainly be of

therapeutic advantage to increase the excretion of chylomicrons and reduce their recycling. This acute model presented the efficacy of **MCR-788** *in vivo* and hence it was decided to evaluate this compound in a chronic model of atherosclerosis as a proof of efficacy. A model of diet-induced atherogenesis in rats over a period of 8-weeks was utilized for this purpose. A long protocol was selected to allow development of atherogenic lesions in the test animals. An atherogenic diet was custom-prepared to meet the needs of the study and to allow reproduction of the pathogenic condition of atherosclerosis in rats. Different parameters relevant to the study like body weight, serum lipid profile, plaque formation and development of atherogenic lesions along the aortae were studied as endpoints for the study. **MCR-788** was administered at two doses, 10 and 30 mg/kg. It was observed that both the dose groups resulted in beneficial effect considering the levels of lipoproteins in serum. This beneficial effect was also dose-dependent. It was found that administration of **MCR-788** prevented elevation of total cholesterol and simultaneously also prevented a decrease in HDL-C levels. Modest prevention in the elevation of triglyceride and VLDL-C levels were also evident in both the treatment groups. Parameters derived on the basis of these lipid levels, i.e. LDL-C and atherogenic index, were consequently found to be improved in the treated groups. These surrogate parameters pointed towards the protective effects of **MCR-788** on the lipid profile however, arterial deposition of lipids was evaluated to estimate a *bonafide* protective action of **MCR-788**. This study indicated reduced deposition of intra-arterial lipids. Further to this, plaque formation in the aortic root was also evaluated since this site is known to be a critical region for the formation of atherosclerotic plaques in animal models. It was concluded that **MCR-788** not only prevents the disparaging effects of an atherogenic diet on the lipid profile but also prevents lipid deposition in the aortae and prevents plaque development.

Finally, it may be summarized that the planar chromatography-based technique is suitable for the quantification of cholesteryl esters in different biological samples. The method is sensitive, accurate, precise, cost-effective and robust for use with different biological matrices. It can be applied to screening of multiple compounds due to the relative ease with which samples may be handled on an HPTLC. Further to this, urea based inhibitors were screened and one potent compound, **MCR-788**, was identified. This compound was subjected to toxicity studies and was found to be safe for repeat-dose oral administration. This compound showed protective action against

triglyceride turnover and was found to be efficacious in an animal model of atherogenesis. The synthetic series utilized in preparing such compounds may serve as lead for the synthesis and evaluation of better ACAT-inhibitors.

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TOXICITY REPORT

ACUTE ORAL TOXICITY STUDY – MCR 1329

SUMMARY

Title Acute oral toxicity study of MCR-1329 in rats

Test Compound MCR-1329

Species Strain Rat istar

ender Male

No of Test Animals 1

Duration an

Route of Administration Oral avage

Maximum Dose level 2 mg kg

olume of administration 1ml kg

water

Post treatment examination period 1 days

Type of examinations ody weight

Clinical symptoms

Mortality

ross necropsy

Results of the Study Administration of 2 mg kg MCR-1329

showed no signs of toxicity or mortality during the test period

THE LD50 OF MCR-1329 IN RATS IS >2000MG/KG

GENERAL INFORMATION

Type of Study:

The study was performed in accordance with the OECD gudielines No 23 2 1 Annex 2C of the guideline document was followed unless indicated otherwise Accordingly the initiation dose was 3 mg kg and extended upto 2 mg kg Category evaluation was precluded as a dose beyond 2 mg kg was unlikely to be ever used in practice

Place of Study:

Shri Patel Pharmacy uilding Donors Plaza atehgunj adodara-39 2 A constituent of Pharmacy Dept aculty of Tech Engg The M S niversity of aroda

Study Sponsored by:

PhD Contingency of Mr ardik andhi PhD Student at Pharmacy Dept aculty of Tech Engg The M S niversity of aroda

TEST SUBSTANCE INFORMATION

Name Code MCR-1329

Source Synthesized and purified in the Pharmaceutical Chemistry lab of Pharmacy Dept aculty of Tech Engg The M S niversity of aroda

Reference M R adav P P Naik P andhi S Chouhan and R iridhar Design and synthesis of - dimeth

α1- and AII-receptors antagonism ioorg Med Chem Lett 23 39 9-39

Appearance Pale yellow powder

Storage Room temperature away from light

-1329 is weighed and

administration

ANIMALS USED FOR THE TEST

Age at the commencement of test 1 -
ody weight range 2 -23 g
Sex Male
Total no of Animals used 1

preferred for studies on compounds acting on the cardiovascular system They present an appropriate mammalian system for replication of effects that might be observed upon administration of the test compounds to humans Although the guideline suggests that female animals may be preferred it was decided to evaluate the test drug in male animals to avoid the protective effects of estrogen upon the cardiovascular system which may become evident when female animals are used

usbandry The animals were housed in polypropylene cages 19× 2×28 cm^3 with paddy husk as bedding Pelleted chow diet and drinking water were provided *ad libitum* The room for the animals was maintained at 22°C ± 3°C with an R of - % Temperature and humidity were recorded using a thermohygrometer

DOSING

roups

Sr. NO.	Substance	Dose (mg/kg)	Volume (ml/kg)	No. of Animals
1	Carboxymethyl cellulose ehicle		1	3
2	MCR-1329 suspended in ehicle	3	1	*
3	MCR-1329 suspended in ehicle	2	1	*

*3 animals dosed twice

Dose Selection Since no previous information about the in vivo data regarding the test compound was available Annex 2C of the guideline was followed for dose selection No pilot study or dose-ranging study was performed

Mode of administration orally via gastric tube
Justification for route of administration ROA This ROA is the intended ROA for further preclinical and clinical studies
Dosing re uency Single dose
Applied maximum dose and volume 2 mg g 1 ml g
Dosing protocol The animals were fasted overnight before dosing On the day of dosing they were manually restrained to facilitate insertion of the gastric tube through which the test compound was administered in the said dose and volume The date of administration was 28th September 2 11

POST-TREATMENT EXAMINATION

The post-treatment examination period was 1 days from the date of dosing

ody. weight examination ody weights of the animals were recorded on days and 1 Slight fluctuations were observed in the body weight of animals but since they were within 2 of the mean body weight no additional measurements were ta en and any other precaution was not followed Table1

eneral behavior The animals were closely observed during the first hours after dosing The animals were starved during this period with access to water No significant observations were recorded during this period This part coincided with the light cycle and most of the time animals were asleep hen awa e the animals showed normal grooming behavior and food Table2 water inta e was also normal During the entire post-treatment observation period special attention was paid to alteration of s in or fur abnormal locomotion or breathing and changes in the eye No untoward observations were made in this regard until the terminal day of the study

Mortality Mortality was recorded twice daily but no mortality was found in any dose group until day 1

Pathological Necropsy At the end of the study period the animals were euthanized and major organs brain heart lung liver idney spleen were harvested ross necropsy was performed by an individual blinded to the groups No macroscopic lesions were recorded iscera gastrointestinal tract and mucous linings appeared normal Major blood vessels did not show any abnormalities

Table1 ody eight

Day 0	ody weight in gms *				
Animal No	Control	3 mg g set1	3 mg g set2	2 mg g set1	2 mg g set2
1	21	211	21	22	22
2	213	2	211	2 2	21
3	2	2 9	213	212	2 8
Day 7	ody weight in gms *				
Animal No	Control	3 mg g set1	3 mg g set2	2 mg g set1	2 mg g set2
1	218	21	223	231	23
2	22	213	22	21	222
3	21	22	221	223	22
Day 14	ody weight in gms *				
Animal No	Control	3 mg g set1	3 mg g set2	2 mg g set1	2 mg g set2
1	229	228	2 1	2 3	2
2	23	23	238	229	223
3	229	232	239	2 1	2 2

*results are rounded off to nearest whole number

Table2 Daily food inta e

Day No	Total food inta e cage 3 animals in gms *				
	Control	3 mg g set1	3 mg g set2	2 mg g set1	2 mg g set2
1	31	33	29	33	3
2	33	3	33	29	33
3	31	33	3	3	3
	29	32	32	3	32
	3	31	33	3	31
	33	3	31	3	3
	3	38	32	3	33
8	3	3	3	3	33
9	32	33	29	33	3
1	31	28	29	3	33
11	29	31	3	3	32
12	3	33	33	3	3
13	3	3	3	32	32
1	32	3	3	31	29

*results are rounded off to nearest whole number

DEVIATIONS FROM THE GUIDELINE

Although the guideline suggests that female animals may be preferred it was decided to evaluate the test drug in male animals to avoid the protective effects of estrogen upon the cardiovascular system which may become evident when female animals are used No other deviations were attempted perceived from the guideline for the toxicity study

CONCLUSION

At the end of the study no untoward observations were made regarding body weight food inta e or normal behavior ross necropsy did not reveal any suggestive lesions or abnormal anatomical feature ence it was concluded that the LD_{50} of MCR-1329 upon oral administration is >2 mg g

- - - - - - - - -

End of Report

TOXICITY REPORT

REPEAT DOSE ORAL TOXICITY STUDY – MCR 1329

SUMMARY

Repeat Dose oral toxicity study of MCR-

-

-1329

Maximum Dose level: 10 mg/kg

Volume of administration: 1ml/kg

Vehicle: 0.5% sodium carboxymethyl cellulose in distilled water

Post treatment examination period: 14 days

Type of examinations: Body weight
Clinical symptoms
Hematology

Serum biochemistry
Urine biochemistry
Mortality
Gross necropsy

Results of the Study: Administration of 10mg/kg MCR-1329 for 28-days showed no signs of toxicity or mortality during the test period

The dose level of 10 mg/kg is safe for chronic administration in rats.

GENERAL INFORMATION

Type of Study:

g 199 Text
was follow
prelim
related class of com

ev

Place of Study:

Shri Patel Pharm
atehg -39 2 A constituent of Pharmacy
Dept aculty of Tech Engg The M S University of
aroda

Study Sponsored by:

PhD Contingency of Mr ardik andhi PhD Student at
Pharmacy Dept aculty of Tech Engg The M S
University of aroda

TEST SUBSTANCE INFORMATION

Name Code MCR-1329
Source Synthesized and purified in the Pharmaceutical
Chemistry lab of Pharmacy Dept aculty of Tech Engg
The M S University of aroda
Reference M R Yadav P P Naik P andhi S
Chouhan and R iridhar Design and synthesis of -
dimeth
α1- and AII-receptors antagonism ioorg Med Chem
Lett 23 39 9-39

Storage

Expected Pharmacologica

-

suspended in 1 ml of %

administration

ANIMALS USED FOR THE TEST

Age at the commencement of test 1 -
-23 g
Sex Male emale nulliparous
Total no of Animals used 3

initiation of test compound
administration

preferred for studies on compounds acting on the

humans

ylene cages

3

ad libitum The room

-

thermohygrometer

DOSING

roups

Sr. NO.	Substance	Dose (mg/kg)	Volume (ml/kg)	No. of Animals
1			1	1 males females
2	MCR-1329 suspended in	1	1	1 males
3	MCR-1329 suspended in	1	1	1 females

*3 animals dosed twice

Dose Selection ased on preliminary acute toxicology data

selected

No pilot

study or dose-

he

intended ROA for further preclinical and clinical studies

Repeat dose

1

Dosing protocol

conditions manually

administered

s to facilitate urine collection

POST-TREATMENT EXAMINATION

The post-
last date of dosing

recorded on days 1 Slight fluctuations

during

food
also normal During the entire post-

until the terminal day of the study ematology serum and
th day

calculated at the terminal stage of the study Results are

lucose Total cholesterol AST ALT

Tail-cuff pressure At the end of the study period tail

MCR-

igure1

d in any dose group until the end of the test period

Pathological Necropsy At the end of the study period

days

in the report

Day No				
	Control male #	Control female #	male $	female $
	221	213	22	2 9
	221	21	232	21
	23			22
21		23		233
			2 2	

#
$

Day No				
	Control male #	Control female #	male $	female $
	11	12 3	11	12
3	11 3	11	11 9	11
	12 1	11	11 3	1
1	11 3	12	11	11 1
	1	11 2	11	12
1	11	1 9	12 1	12 1
21	11 3			11
	12			1 9
	11 1	11	1 9	

first decimal

#
$

	6 3	3 3		
#				
#				
$	8.4	10.9	12.1	36.3
10 mg/kg (female)$	7.7	11.7	12.0	36.0

#Mean of 5 observations, $Mean of 10 observations, *hematocrit was derived by triplicating Total Hb values.

Table4: Serum Biochemistry

Groups	Turbidity	Appearance	Total Cholesterol	Glucose (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (mg/dl)
Control (male)#	NIL	Pale Yellow	132.					
#					3	2	9	1
1 ale $	NI	Pale ello	1 3 2	111 2	1	2 3 2	9 9	
1 fe ale $	NI	Pale ello	13 9	1 3 3	3 9	2 2 2	9 1	2 9

#Mea of o ser atio s $Mea of 1 o ser atio s

APPENDIX II- Repeat Dose Oral Toxicity Study of MCR-1329

Ta le ri e A alysis

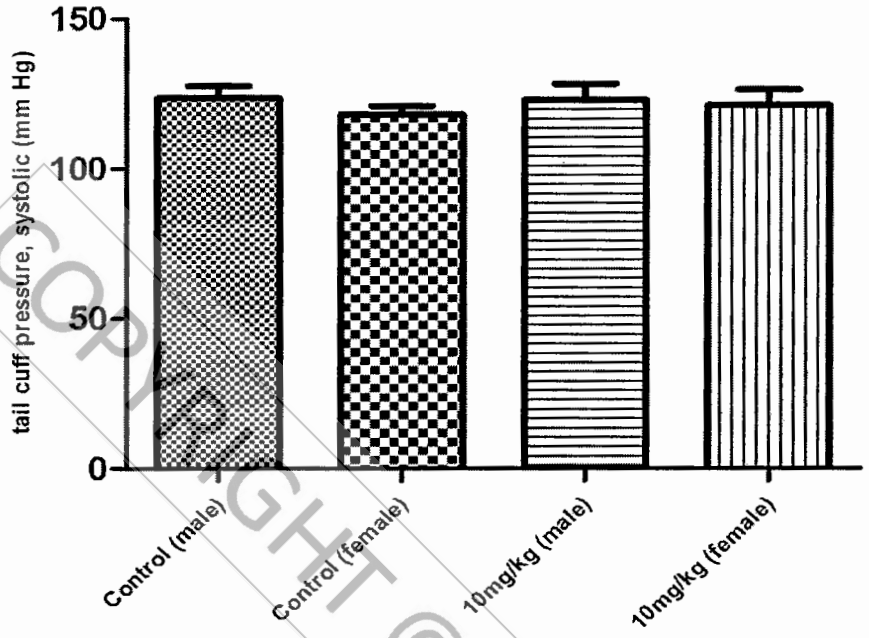
roups	Mea olu e	Appeara ce	Tur idity	Mea p	lucose	Protei
Co trol ale #		Pale ello	NI		1 dl	NI
Co trol fe ale #	9	Pale ello	NI		1 dl	NI
1 ale $		Pale ello	NI		1 dl	NI
1 fe ale $		Pale ello	NI		1 dl	NI

#Mea of o ser atio s $Mea of 1 o ser atio s

[#]					0.07	1.80 ± 0.05
Control (female)[#]	1.86 ± 0.04	0.66 ± 0.08	0.90 ± 0.11	5.98 ± 0.67	0.39 ± 0.04	1.35 ± 0.05
10mg/kg (male)[$]	2.13 ± 0.08	0.98 ± 0.04	1.25 ± 0.06	6.89 ± 0.91	0.51 ± 0.04	1.75 ± 0.07
10 mg/kg (female)[$]	1.93 ± 0.03	0.84 ± 0.06	1.06 ± 0.07	6.01 ± 0.42	0.42 ± 0.06	1.57 ± 0.05

*Both the capsules were weighed, [#]Mean of 5 observations, [$]Mean of 10 observations

Figure1: Tail cuff pressure comparison between groups*



*For control groups, n=5; for test groups, n=10.

DEVIATIONS FROM THE GUIDELINE

The guideline mentions the use of a range finding test or a limit test with a dose of 1000 mg/kg but since such a dose level is unlikely and corresponding human dose may never be applied in practice, we preferred using a dose of 10 mg/kg. No other deviations were attempted/perceived from the guideline for the toxicity study.

CONCLUSION

At the end of the study, no untoward observations were made regarding body weight, food intake or normal behavior. Gross necropsy did not reveal any suggestive lesions or abnormal anatomical feature. The most plausible side effect related to the mechanism of action of MCR-1329 is hypotension. This

effect was not evident from the tail-cuff recordings. Biochemical estimations did not suggest any major digression from normal values. Urinary output and hematological data appeared normal. Hence it was concluded that chronic administration of MCR-1329 at a dose level of 10mg/kg was safe.

- - - - - - - -

End of Report

TOXICITY REPORT

ACUTE ORAL TOXICITY STUDY – MCR 788

SUMMARY

-788

-788

-

-NA-

Mortality

-

788

THE LD50 OF MCR-788 IN RATS IS >2000MG/KG

GENERAL INFORMATION

Type of Study:

Place of Study:

-

Study Sponsored by:

CSIR-

TEST SUBSTANCE INFORMATION

-788

- -788

-788

ANIMALS USED FOR THE TEST

3

ad libitum

-

DOSING

Sr. NO.	Substance	Dose# (mg/kg)	Volume (ml/kg)	No. of Animals
	MCR-788			
	MCR-788			

#admixed in the diet; *3 animals dosed twice

-

NMT -

preparation

th

POST-TREATMENT EXAMINATION

recorded 7

food

No

Day 0					
Day 7					
	7				8
Day 14					

Day					
7					
8					

DEVIATIONS FROM THE GUIDELINE

-

suitable vehicle

CONCLUSION

50 of MCR-788

- - - - - - - -

End of Report

TOXICITY REPORT

REPEAT DOSE ORAL TOXICITY STUDY – MCR 788

SUMMARY

Repeat Dose oral toxicity study of MCR-788

-

-788

Repeat dose; daily

for 28 days

Route of Administration: Oral (diet-admixture)

Maximum Dose level: 60 mg/kg

Volume of administration: -NA-

Vehicle: Pelleted chow

Post treatment examination period: 14 days

Type of examinations: Body weight

Clinical symptoms

Hematology

Serum biochemistry

Urine biochemistry

Mortality

Gross necropsy

Results of the Study: Administration of 60mg/kg MCR-788 for 28-days showed no signs of toxicity or mortality during the test period

The dose level of 60 mg/kg is safe for chronic administration in rats.

GENERAL INFORMATION

Type of Study:

The study as perfor ed in accordance ith the OECD udielines (No. 407, . Text of the uideline docu ent as follo ed unless indicated other ise. Based on preli inary acute toxicolo y data and literature revie of related class of co pounds, 60 as chosen to be the ani al therapeutic dose for phar acodyna ic studies. Accordin ly, this dose as selected for this toxicity evaluation, since this dose is t ice that of axi u intended therapeutic dose.

Place of Study:

Shri . H. Patel Phar acy Buildin , Donors Plaza, Fateh unj, Vadodara- 0002 (A constituent of Phar acy Dept., Faculty of Tech. & En ., The M. S. niversity of Baroda

Study Sponsored by:

. PhD Contin ency of Mr. Hardi andhi, PhD Student at Phar acy Dept., Faculty of Tech. & En ., The M. S. niversity of Baroda
2. CSIR-SRF Contin ency of Mr. Hardi andhi, PhD Student at Phar acy Dept., Faculty of Tech. & En ., The M. S. niversity of Baroda

TEST SUBSTANCE INFORMATION

Na e Code: MCR-788

Source: Synthesized and purified in the Phar aceutical Che istry lab of Phar acy Dept., Faculty of Tech. & En ., The M. S. niversity of Baroda.

Reference: Co pound Data-Sheet of MCR-788 by Palash Pal, Phar aceutical Che istry lab of Phar acy Dept., Faculty of Tech. & En ., The M. S. niversity of Baroda.

Appearance: hite po der

Stora e: Roo te perature, a ay fro li ht

Safety re uire ent: Not no n

Expected Phar acolo ical Effect: Prevention of cholesterol accu ulation and i prove ent of lipid profile

Expiry Date: Not no n

Preparation: Re uired a ount of MCR-788 is ei hed and ad ixed in the diet based on daily re uire ent and uantity of food consu ed by each individual ani al e. . if an ani al (B. . 200 consu es of food pellets on day , then to ad inister a dose of 000 the co pound-feed ad ixture is prepared so as to contain 200 test co pound per or .4 test co pound per 00 of feed . The odified food pellets ere for ed everyday and stored at 2-8°C until provided to the ani als.

ANIMALS USED FOR THE TEST

Species Strain: Rat istar Albino

A e at the co ence ent of test: 0- 2 ee s

Body ei ht ran e: 200-2 0

Sex: Male & Fe ale (nulliparous

Total no. of Ani als used: 0

Source of Ani als: Zydus Research Centre, Ah edabad, India

Accli itization: ee

Rando ization: day prior to initiation of test co pound ad inistration

Justification for species and sex: istar albino rats are preferred for studies on co pounds actin on the cardiovascular syste . They present an appropriate a alian syste for replication of effects that i ht be

observed upon ad inistration of the test co pounds to hu ans.

Husbandry: The ani als ere housed in polypropylene ca es (42 28 c ^{3} ith paddy hus as beddin . Pelleted cho diet and drin in ater ere provided *ad libitum* for the ani als as aintain

-

ther oh

DOSING

Sr. NO.	Substance	Dose# (mg/kg)	Volume (ml/kg)	No. of Animals
	Co trol roup - Pelleted c o	—		ales fe ales
	MCR-788 suspe ded i Ve icle			ales
	MCR-788 suspe ded i Ve icle			fe ales

#admixed in the diet; *3 animals dosed twice

Dose Selectio : Based o preli i ary acute toxicolo y data a d literature revie of related class of co pou ds, or less as c ose to be t e a i al t erapeutic doses for p ar acody a ic studies. Accordi ly, t is dose as selected for t is toxicity evaluatio , si ce t is dose is t ice t at of axi u i te ded t erapeutic dose. No pilot study or dose-ra i study as perfor ed.

Mode of ad i istratio : orally via feed ad ixture

Justificatio for route of ad i istratio ROA : T is ROA is t e i te ded ROA for furt er precli ical studies.

Dosi Fre ue cy: Repeat dose, daily for 8 days

Applied axi u dose a d volu e: ; NMT diet-ad ix

Dosi protocol: T is study as co ducted u der fed co ditio s. O t e day of dosi , t e or al diet as replaced by t e odified diet a d co ti ued for 8 days. T e a ou t of odified diet replaced eac day as depe de t upo t e diet co su ed by eac a i al o t e previous day. Differe t roups ere i ducted o subse ue t days to facilitate uri e collectio .

POST-TREATMENT EXAMINATION

T e post-treat e t exa i atio period as 4 days fro t e last date of dosi .

Body ei t exa i atio : Body ei ts of t e a i als ere recorded o days , 7, 4, a d 8. Sli t fluctuatio s ere observed i t e body ei t of a i als but si ce t ey ere it i % of t e ea body ei t o additio al easure e ts ere ta e a d a y ot er precautio as ot follo ed Table .

e eral be avior: T e a i als ere closely observed duri t e first ours after dosi . No si ifica t observatio s ere recorded duri t is period. T is part coi cided it t e li t cycle a d ost of t e ti e a i als ere asleep. e a a e, t e a i als s o ed or al roo i be avior a d food Table & ater i ta e as also or al. Duri t e e tire post-treat e t observatio period special atte tio as paid to alteratio of s i or fur, ab or al loco otio or breat i a d c a es i t e eye. No u to ard observatio s ere ade i t is re ard u til t e ter i al day of t e study. He atolo y, seru a d uri e bioc e istry ere perfor ed o 8th day.

He atolo y: RBC a d BC cou t, total Hb a d e otcrit ere calculated at t e ter i al sta e of t e study. Results are prese ted i Table .

Seru bioc e istry: lucose, Total c olesterol, AST, ALT, ALP a d creati i e ere esti ated a d results are prese ted i Table4.

ri e bioc e istry: ri e volu e, pH, lucose a d protei ere esti ated at t e e d of t e dosi period a d results are prese ted i Table .

Mortality: Mortality as recorded t ice daily but o ortality as fou d i a y dose roup u til t e e d of t e test period.

Pat olo ical Necropsy: At t e e d of t e study period 4 days , t e a i als ere eut a ized a d ajor or a s brai , eart, lu , liver, id ey, splee ere arvested. ross ecropsy as perfor ed by a i dividual bli ded to t e roups. No acroscopic lesio s ere recorded. Viscera, astroi testi al tract a d ucous li i s appeared or al. Major blood vessels did ot s o a y ab or alities.

Or a ei ts: After dissecti t e a i als, ajor or a s ere ei ed a d t eir ei ts ere recorded. A y ajor differe ce fro co trol roup as oted do a d prese ted i t e report Table .

Table : Body ei t

Day No.	Mea Body ei t per roup i s *			
	Co trol ale #	Co trol fe ale #	ale $	fe ale $
		7		4
7				
4	4			
	4		4	8
8	48			44

*results are rou ded off to earest ole u ber
#Mea of observatio s
$Mea of observatio s

Table : Daily food i ta e

Day No.	Mea food i ta e per a i al i s *			
	Co trol ale #	Co trol fe ale #	ale $	fe ale $
	.	.	.7	.
	.	.4	.	.4
7	.8	.4	.	.
	.	.	.	.
4	.	.8	.	.7
7	.7	.	.	.
	.	.	.8	.
4	.	.	.	.
8	.7	.7	.	.

*results are rou ded off to first deci al
#Mea of observatio s
$Mea of observatio s

	6 3	3 3		
#				
#	7			
$	7.4	10.2	12.0	34.8
60 mg/kg (female)$	7.9	11.1	12.1	40.2

#Mean of 5 observations, $Mean of 10 observations, *hematocrit was derived by triplicating Total Hb values.

Table4: Serum Biochemistry

Groups	Turbidity	Appearance	Total Cholesterol	Glucose (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (mg/dl)
Control (male)#	NIL	Pale Yellow	135.2 ± 10.2	90.3 ± 5.9	65.3 ± 10.2	24.9 ± 5.3	88.4 ± 13.1	0.50 ± 0.09
Control (female)#	NIL	Pale Yellow	128.3 ± 14.3	111.8 ± 10.1	68.5 ± 9.4	27.3 ± 3.9	93.2 ± 14.7	0.53 ± 0.11
10mg/kg (male)$	NIL	Pale Yellow	122.8 ± 9.6	109.5 ± 7.9	67.4 ± 11.3	28.4 ± 6.2	91.6 ± 10.2	0.45 ± 0.06
60 mg/kg (female)$	NIL	Pale Yellow	125.6 ± 11.2	100.2 ± 12.4	69.8 ± 10.3	29.2 ± 4.2	95.3 ± 16.2	0.54 ± 0.10

#Mean of 5 observations, $Mean of 10 observations

APPENDIX IV- Repeat Dose Oral Toxicity Study of MCR-788

Table5: Urine Analysis

Groups	Mean volume	Appearance	Turbidity	Mean pH	Glucose	Protein
Control (male)[#]	6.1	Pale Yellow	NIL	6.5	<10mg/dl	NIL
Control (female)[#]	5.7	Pale Yellow	NIL	6.9	<10mg/dl	NIL
10mg/kg (male)[$]	5.5	Pale Yellow	NIL	6.6	<10mg/dl	NIL
60 mg/kg (female)[$]	5.9	Pale Yellow	NIL	6.8	<10mg/dl	NIL

[#]Mean of 5 observations, [$]Mean of 10 observations

#			1.27 ± 0.14	7.54 ± 1.10	0.45 ± 0.05	1.78 ± 0.29
Control (female)#	2.11 ± 0.18	0.078 ± 0.05	1.13 ± 0.10	6.79 ± 0.96	0.51 ± 0.03	1.65 ± 0.33
10mg/kg (male)$	2.21 ± 0.22	1.04 ± 0.09	0.97± 0.15	7.92 ± 1.03	0.53 ± 0.01	1.53 ± 0.27
60 mg/kg (female)$	1.99 ± 0.08	0.83 ± 0.1	1.19 ± 0.09	8.02 ± 1.23	0.55 ± 0.08	1.80 ± 0.31

*Both the capsules were weighed, #Mean of 5 observations, $Mean of 10 observations

DEVIATIONS FROM THE GUIDELINE

The guideline mentions the use of a range finding test or a limit test with a dose of 1000 mg/kg but since such a dose level is unlikely and corresponding human dose may never be applied in practice, we preferred using a dose of 60 mg/kg.

Doses were administered in the form of diet-admixture as this was the intended route for further preclinical studies.

No other deviations were attempted/perceived from the guideline for the toxicity study.

CONCLUSION

At the end of the study, no untoward observations were made regarding body weight, food intake or normal behavior. Gross necropsy did not reveal any suggestive lesions or abnormal anatomical feature. The most plausible side effect related to the mechanism of action of MCR-788 is free cholesterol deposition and associated xanthomatosis. This effect was not evident from visual observations nor did the cholesterol levels suggest any such effects. Biochemical estimations did not suggest any major digression from normal values. Urinary output and hematological data appeared normal. Hence it was concluded that chronic administration of MCR-788 at a dose level of 10mg/kg was safe.

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End of Report

SPi	Journal Code				Article ID				Dispatch: 21.10.13	CE:
	B	M	C		3	0	7	5	No. of Pages: 6	ME:

Research article

Biomedical Chromatography

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(wileyonlinelibrary.com) DOI 10.1002/bmc.3075

An HPTLC method for quantification of cholesteryl esters from human plasma and rat liver microsomes

Hardik Gandhi, Palash Pal, Rajani Giridhar and Mange Ram Yadav*

ABSTRACT: Cholesteryl oleate present as a neutral lipid in low-density lipoprotein has been speculated to be a biomarker for atherosclerosis. Methods which are at hand for the quantification of cholesteryl oleate are either costly or entail the use of radioactive compounds. Charring of TLC plates has been used to identify cholesteryl esters for a long time but has never been applied to quantification of cholesteryl esters in biological matrices. Here, we report a novel method based on planar chromatography for the analysis of the products of the acyl CoA–cholesterol acyltransferase (ACAT) assay, viz. cholesteryl esters. Using silica gel 60 F_{254} as stationary phase, compounds were spotted on the plate and run using a solvent system comprising *n*-hexane–diethyl ether–glacial acetic acid (90:10:1, v/v/v). The plates were developed by dipping in anisaldehyde–sulfuric acid reagent and were scanned at 546 nm for quantification. The developed method shows good linear relationship in the concentration range of 100–500 ng/band with a correlation coefficient (*r*) value of 0.9996. The method was validated for accuracy, precision and robustness. Percentage recovery of the method was found to be in the range 96.88–103.01% with intra- and inter-day precision analysis yielding <2% relative standard deviation at nominal concentrations for analysis. The limits of detection and quantification were found to be 6.45 and 19.54 ng, respectively. The method was validated for robustness by making deliberate changes in mobile phase composition, volume and temperature of analysis, and the standard deviations of peak areas for these intentional changes were found to be 1.07, 1.02 and 1.30 respectively. The method was applied to the estimation of cholesteryl esters in plasma samples from patients diagnosed with hypercholesterolemia. No interferences were found from the biological matrices used in the assay. The proposed method could be of immense potential for estimation of cholesteryl oleate as a marker of ACAT activity, for screening of ACAT inhibitors in drug discovery process and in the prognosis of atherosclerosis. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: ACAT; cholesteryl oleate; HPTLC; microsomes; validation

Introduction

Free cholesterol serves an important role in maintaining the structural integrity of plasma membrane of the cell. It also acts as a precursor for the biosynthesis of a number of biologically vital metabolites. However, excess of free cholesterol is toxic to the cells and leads to cell death. Therefore, human body has a simple yet unique way of shuttling excess of free cholesterol accumulating within the cells through the formation of cholesteryl esters. Cholesterol is comparatively a polar precursor while cholesteryl esters are neutral nonpolar lipids which are stored as droplets within the cell (Buhman *et al.*, 2000). Cholesterol taken up as a part of the daily calorific intake is esterified intracellularly in the body with the help of two enzymes belonging to the ACAT (acyl CoA–cholesterol acyltransferase) gene family, ACAT1 and ACAT2 (Anderson *et al.*, 1998; Cases *et al.*, 1998a, 1998b; Farese, 1998; Oelkers *et al.*, 1998). Cholesterol utilizes fatty acyl CoAs as substrates to form a covalent linkage at the 3-OH position with the help of catalyzing enzymes, resulting in the formation of cholesteryl esters (Chang *et al.*, 1997). The most commonly utilized substrates are oleoyl-CoA and palmitoyl-CoA, with the former having a higher precedence over the latter. Further, it has been shown that over-accumulation of cholesterol esters in arteries leads to the development of atherosclerotic lesions. Cholesteryl esters accumulate in the macrophages and vascular smooth muscle cells of major arteries, leading to the formation of foam cells, the events that hallmark atherosclerosis progression (Akopian and Medh, 2006; Peng *et al.*, 2000). Based on previous studies Spector and Haynes (2007) have raised a speculation that low-density-lipoprotein (LDL) cholesteryl oleate content may be an indicator of risk factor for the development of atherosclerosis and that LDL cholesteryl ester content may be used as a biomarker for atherosclerosis. Parallel to these studies, specific and nonspecific inhibition of ACAT isoforms has been attempted by several researchers for the control of atherosclerosis with varying levels of success. All of these studies involve determination of the effect of inhibitors on the quantum of cholesteryl oleate formed by ACAT catalysis.

Different methods have been employed for the estimation of cholesteryl esters in general and cholesteryl oleate in particular. By far the most commonly employed method is the one involving the use of radioactive substrates like [^{3}H] cholesterol or [^{14}C] oleoyl

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Abbreviations used: ACAT, acyl CoA–cholesterol acyltransferase.

CoA, for the ACAT assay. This leads to production of radioactive cholesteryl oleate which is quantified by liquid scintigraphy (Chang *et al.*, 1998; Erickson *et al.*, 1980; Temel *et al.*, 2003; Choi *et al.*, 2012; Levy *et al.*, 2007). Hashimoto *et al.* (1973) and Largis *et al.* (1989) have used a similar method for the determination of cholesteryl palmitate. In both the assays either cell homogenates or microsomes were used as a source of ACAT enzyme. Lada *et al.* developed a rapid and high-throughput cell-based assay for determination cholesterol esters. This assay exploits the mimicry of fluorescent NBD-cholesterol {22-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3-ol} of that of native cholesterol. NBD-cholesterol shows relatively higher fluorescence in a nonpolar milieu. Thus, esters of NBD-cholesterol are strongly fluorescent and this fluorescence can be measured with the help of fluorescence microscopy, HPLC with fluorescence detection (HPLC-FLD), HPTLC (high-performance thin layer chromatography) or fluorescent ACAT assay (Lada *et al.*, 2004; Cao *et al.*, 2013). Mizoguchi *et al.* have proposed a direct measurement method for the enzymatic determination of cholesterol esters by either colorimetry or fluorimetry. Cholesteryl esters are converted to free cholesterol with the help of cholesterol esterase and the liberated cholesterol is decomposed by H_2O_2, which on further treatment with 4-aminoantipyrine or amplex red produces a product which can be estimated by colorimetry or fluorimetry. The inherent drawback of this method involves the need to isolate cholesteryl ester products from the reaction mixture using an efficient method like HPTLC (Mizoguchi *et al.*, 2004). Alternatively, Montoudis and colleagues have reported methods involving HPLC and solid-phase extraction for the determination of cholesteryl esters (Montoudis *et al.*, 2004). Recently, Miyoshi and colleagues have reported an LC-ESI-MS/MS based method for the detection of cholesteryl ester ozonolysis products (Miyoshi *et al.*, 2013). This method involves ozone treatment of samples, isolation and characterization followed by further oxidation and derivatization with dansyl hydrazine for detection by LC-ESI-MS/MS. Given the reputation of LC-ESI-MS/MS, it can be agreed that the analytical method will be most sensitive and accurate; however, the cost of analysis and the elaborate process raise concerns about the use of this method for routine analysis involving a large number of samples. Some researchers utilize an indirect method for the estimation of cholesteryl esters in plasma levels (Levy *et al.*, 2007). This method essentially requires calculation of the difference between total and free cholesterol to determine the concentration of free cholesterol.

All the methods mentioned above are laborious, time-consuming and/or expensive. An HPLC method requires repeated injections which can lead to contamination of samples owing to 'carry over' effect. Additionally, standard and samples need to be injected separately and derivatization *in situ* is not possible for detection of cholesteryl esters. Fluorimetric analysis requires a closed system where any exposure to the external environment can lead to erroneous results. Moreover, some of these methods possess a limitation of not being able to directly estimate cholesteryl ester levels in plasma. Therefore, we describe in this paper the development and validation of an HPTLC-based simple, sensitive and cost-effective method for the rapid determination of cholesteryl oleate from biological matrices. The primary objective of this study was to develop a method for quantifying the products formed from the ACAT reaction. The secondary objective entailed estimation of cholesteryl ester levels from pathological plasma samples.

Experimental

Materials and solvents

Rat liver microsomes (500 µg protein/mL) were procured from Krishgen Biosystems, Mumbai, India. Cholesteryl oleate standard was synthesized in the Pharmaceutical Chemistry Laboratory of the Department. Cholesterol and oleoyl CoA lithium salt were obtained from Sigma (St Louis, MO, USA). Aluminum TLC plates pre-coated with silica gel 60 F_{254} ($10 \times 10\,cm^2$, 250 µm thickness) were procured from Merck, Germany. Anisaldehyde–sulfuric acid reagent was prepared in the following manner: (A) 0.5 mL of anisaldehyde was dissolved in 10 mL of glacial acetic acid; (B) 5 mL of concentrated sulfuric acid was dissolved in 85 mL ice-cold methanol. Final working reagent was prepared by mixing solutions A and B. The working reagent appears colorless and should be discarded when a pink tinge appears. Other reagents and chemicals used in the present study were of analytical grade.

Calibration standards and working solutions

A stock solution of cholesterol oleate was prepared by dissolving 100 mg of accurately weighed cholesteryl oleate in a mixture of chloroform–methanol (2:1, v/v) to make the volume upto 100 mL. Working standards were prepared by appropriate dilutions of the stock solution with chloroform–methanol (2:1, v/v). The stock and working solutions were stored at −20 °C.

High-performance thin-layer chromatography

Before sample application, chromatography plates were pre-washed using methanol as a mobile phase and dried for 10 min at 120 °C to activate the plates. Samples were applied to the plate as 6 mm-wide bands, 10 mm apart, by means of Linomat V sample applicator (Camag, Switzerland) fitted with a 100 µL Hamilton syringe. A constant rate of application of 150 nL/s was used. After sample application, the plates were dried in a current of dry air and developed in a linear ascending manner using *n*-hexane–diethyl ether–glacial acetic acid (90:10:1, v/v/v) as mobile phase. An 18 mL aliquot of the mobile phase was used for development of each plate. Development was performed in a 10×10 twin-trough chamber (Camag, Switzerland) which was previously saturated with mobile phase for 30 min. All the steps were performed at 25 ± 2 °C and ambient relative humidity. The solvent front position was fixed at 80 mm from the point of application. After running the mobile phase the plates were dried and dipped in a solution of anisaldehyde–sulfuric acid reagent. The plates were dried and heated at 120 °C for 8 min. This led to the development of purple-colored bands at solute fronts. Densitometric scanning was performed with Scanner III (Camag, Switzerland) in absorbance mode at 546 nm. The slit dimension was set at 6×0.45 mm and scanning speed was 20 mm/s. Calculations were performed with the help of WinCats software (version 1.4.4, Camag).

ACAT assay

The ACAT assay mixture consisted of potassium phosphate buffer (0.1 M), bovine serum albumin (5 mg/mL), microsomal protein (200 µg) and cholesterol solubilized in 45% w/v hydroxypropyl β-cyclodextrin (2 mM). Final reaction volume was made upto 850 µL with 0.1 M potassium phosphate buffer. Vehicle or the ACAT inhibitor (Avasimibe, Sigma) was added at this point (final volume NMT 10 µL) and incubated for 15 min to allow proper binding of the inhibitor with the ACAT enzymes. The reaction was initiated by the addition of 200 µM oleoyl CoA and allowed to proceed for 10 min at 37 °C. The reaction was terminated by the addition of 6 mL of a mixture of chloroform–methanol (2:1, v/v) to the reaction mixture. The biphasic mixture was shaken in a separating funnel and both the phases were allowed to separate. The lower organic phase was collected and evaporated to dryness under a stream of N_2 gas. The residue was then redissolved in 500 µL of chloroform–methanol (2:1, v/v) and a volume of 25 µL from this solution was spotted on the TLC plates for quantification. Each sample was applied to the TLC plates at least in triplicate.

Calibration curve of cholesteryl oleate

The calibration curve was prepared over a concentration range of 100–500 ng/band. Five dilutions (100, 200, 300, 400 and 500 μg/mL) were prepared from the stock solution and further aliquots of these solutions were applied to the TLC plate. This procedure was repeated six times. The plates were developed, scanned and the data of peak areas of the developed spots vs concentrations were treated by linear least square regression to obtain the calibration curve.

Method validation

Method validation parameters like linearity, range, precision, accuracy, LOD (limit of detection), LOQ (limit of quantification), specificity and robustness were checked as per International Conference on Harmonization guidelines (ICH, 2005). The methods specified in the text of the guideline were followed unless indicated otherwise.

Precision and accuracy. Repeatability of sample application was carried out by taking six replicates of the same spot (200 ng/band of cholesteryl oleate) and measurement of the peak areas. The intra- and inter-day variation for the determination of cholesteryl oleate was carried out at three different concentrations (200, 300 and 400 ng/band). Accuracy was performed by the method of standard addition. A known amount of cholesteryl oleate was added to a previously analyzed sample at three different levels and percentage recovery was determined. Three determinations were performed on three different occasions for each level and the results obtained were compared with the expected results.

Limit of detection and limit of quantification. The LOD and LOQ were determined by the formulae $3.3\sigma/S$ and $10\sigma/S$, where σ corresponds to the average standard deviation of the *y*-intercepts of regression lines and *S* corresponds to the average slope of the regression lines.

Specificity. Specificity of the method was tested by analyzing sample and standard on the same plate. The spot for cholesteryl oleate in sample lane was confirmed by comparing the retardation factor (R_f) values. Peak purity was adjudged by comparing peak start, peak apex and peak end values of samples with that of standard. In addition, we also performed an extraction procedure on the microsomal reaction mixture in which oleoyl CoA was not added. The entire procedure was followed as stated above and the extract was then spotted on the plate and scanned in a similar manner to that of other samples. Peaks in the region surrounding the R_f of cholesteryl oleate were observed for any interference owing to the biological matrix involved.

Robustness. Robustness was determined by making deliberate changes in the mobile phase composition. Mobile phases having varying compositions of *n*-hexane–diethyl ether–glacial acetic acid (90:10:0.5, 92:8:1 and 88:12:1; v/v/v) were utilized and chromatograms were obtained. Volume of mobile phase and temperature of analysis were also varied in a range of ±5%. The effect of these changes on peak areas and R_f value of cholesteryl oleate were observed and data presented as percentage relative standard deviation (RSD).

Validation of the method in plasma samples. The aforementioned method was validated for accuracy and precision using pooled human plasma. Plasma samples from five different voluntary male donors were provided by a local blood bank (Suraktam Blood Bank, Vadodara, Gujarat, India). Equal volumes of these samples were pooled and used for validation. Briefly, a 200 μL aliquot of the collected plasma sample was subjected to extraction with an 800 μL mixture of chloroform-methanol (2:1, v/v) in a centrifuge tube. Extraction was afforded by vortexing the mixture for 3 min. The lower organic phase was separated out using a pipette and a known volume of standard cholesteryl oleate was added to the organic phase and further processed as mentioned above. The volume of standard cholesteryl oleate was such that it resulted in final concentrations of 8, 12 and 16 μg/mL after reconstitution. This afforded concentrations of 200, 300 and 400 ng/band when the samples were spotted on the TLC plate. The inherent cholesterol esters present in the pooled plasma samples were nullified from the final results of the spiked ones. Based on these experiments, intra-day and inter-day precision, accuracy and percentage recovery were calculated.

Quantification of cholesteryl esters in clinical plasma samples

Plasma samples of patients were collected from local pathology laboratories. These samples were originally collected for the estimation of total cholesterol levels by the different laboratories upon referral by physicians. This study included plasma samples for three patients whose total plasma cholesterol levels were reported to be above 200 mg/dL by the respective pathology laboratories. A 200 μL aliquot of the collected plasma sample was subjected to extraction with 800 μL mixture of chloroform–methanol (2:1, v/v) in a centrifuge tube. Extraction was afforded by vortexing the mixture for 3 min. The lower phase was separated out using a pipette and subjected to drying under a stream of N_2 gas. The remaining procedure was same as that described in the 'ACAT assay' section.

Results and discussion

Optimization of chromatographic conditions

Optimization studies were carried out using standard solution of cholesteryl oleate. Several mobile phases were tried to obtain a good resolution of cholesteryl oleate. The mobile phase optimized to *n*-hexane–diethyl ether–glacial acetic acid (90:10:1, v/v/v) gave a well-resolved peak with Gaussian symmetry ($R_f = 0.59 \pm 0.02$, Fig. 1) and the same was used for further analysis. No immiscibility F1
issues were observed with the chosen composition of mobile phase. Another advantage of this composition of mobile phase is that the R_f value of the substrate cholesterol is very low (0.05), hence it does not interfere with the detection of the cholesteryl oleate even if it is added in excess amount. Chamber saturation time was found to strongly affect the R_f value of cholesteryl oleate and was therefore optimized to be 30 min. In the preliminary stages of the study, iodine vapors were used for detection and scanning of the spots of cholesteryl oleate. However, iodine being volatile in nature errors occurred in the detection of samples owing to a gradual decrease in intensity of the bands. Henceforth, anisaldehyde–sulfuric acid reagent was used for spot identification and detection. This reagent was found to give an intense purple color with cholesteryl oleate and the color intensity also remained stable over the period of analysis.

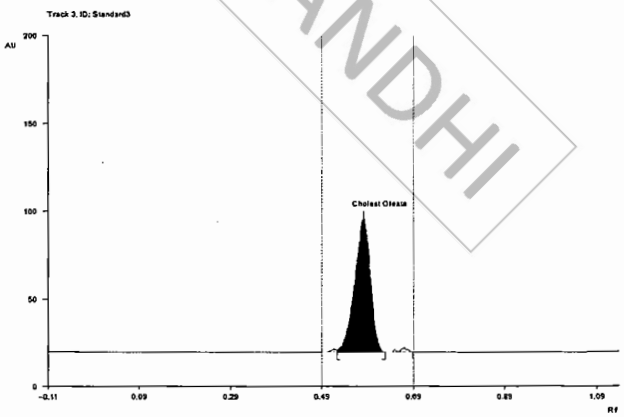

Figure 1. A typical chromatogram of cholesteryl oleate. This representative chromatogram depicts the R_f value of standard cholesteryl oleate (~0.59) obtained using the current method. A concentration of 400 ng/ Q3
band was utilized to obtain this chromatogram.

Table 1. Linear regression data of calibration curves[a,b]

Range (ng/band)	$r \pm$ SD	Slope ± SD	Intercept ± SD	LOD	LOQ
100-500	0.9996 ± 0.0002	10.295 ± 0.95	205.703 ± 0.63	6.45	19.54

[a]Linear regression data for cholesteryl oleate in the range of 100-500 ng/band.
[b]$n = 6$.
r, Correlation coefficient; SD, standard deviation; LOD, limit of detection; LOQ, limit of quantitation.

Table 2. Precision and recovery[a]

Nominal concentration (ng/band)	Intra-day			Inter-day		
	Concentration found (ng/band)	Accuracy (percentage recovery)	Precision (RSD)	Concentration found (ng/band)	Accuracy (percentage recovery)	Precision (RSD)
200 ng	193.76 ± 1.19	96.88 ± 0.59	0.61%	198.28 ± 2.61	99.14 ± 1.31	1.32%
300 ng	309.04 ± 3.73	103.01 ± 1.24	1.21%	302.02 ± 2.07	100.67 ± 0.69	0.68%
400 ng	401.94 ± 4.77	100.49 ± 1.19	1.19%	394.99 ± 3.19	98.75 ± 0.80	0.80%

[a]Intra- and inter-day precision of the HPTLC method and recovery studies.

Method validation

Linearity and range. The least square regression method was used for calculation of correlation coefficient, slope and intercepts. The calibration curve was found to be linear in the range of 100–500 ng/band. The linearity of the calibration curves was validated by a value of correlation coefficient closer to unity (Table 1). Each reading was an average of three determinations. T1

Limit of detection and limit of quantification. LOD and LOQ were calculated as per the formulae mentioned in the 'Materials and methods' section. Based on the formulae, LOD and LOQ for cholesteryl oleate were found to be 6.45 and 19.54 ng respectively (Table 1). However, by experimentation, the LOD and LOQ were found to be 10 and 25 ng, respectively. Such low values of LOD and LOQ suggest that the method is adequately sensitive for analytical purposes.

Precision and accuracy. The repeatability of sample application and measurement of concentrations based on peak areas were expressed in terms of RSD and are depicted in Table 2. Table 2 shows the intra- and inter-day variations of cholesteryl oleate at three different levels (200, 300 and 400 ng/band). The proposed method afforded a recovery within the range of 96.88–103.01%, suggesting that the method is accurate and can be used for the quantification of cholesteryl oleate. T2

Specificity. The method was found to be robust as no interfering substances were found near the R_f value of cholesteryl oleate when the standard and sample lanes were compared. Further, there was no difference between the peak of standard cholesteryl oleate and the one obtained from the sample as determined by peak start, peak apex and peak end positions. An overlay of chromatograms from standard and samples of cholesteryl oleate is shown in Fig. 2. Further to this, it was observed that the biological matrix does not lead to any interference *per se*. F2

Robustness. The SD of peak areas was calculated for each determined parameter and the RSD was calculated. The values of RSD (as shown in Table 3) indicate that the method is robust and minor changes in experimental conditions do not affect analysis of cholesteryl oleate. T3

Validation of the method in plasma samples. The results of the validation of the method are shown in Table 4. The percentage recovery of this method was found to be between 101.05 and 105.87%, suggesting the accuracy of the method. The low RSD values (Table 4) indicate that the method may be applied to the estimation of cholesteryl esters in pathological samples. T4

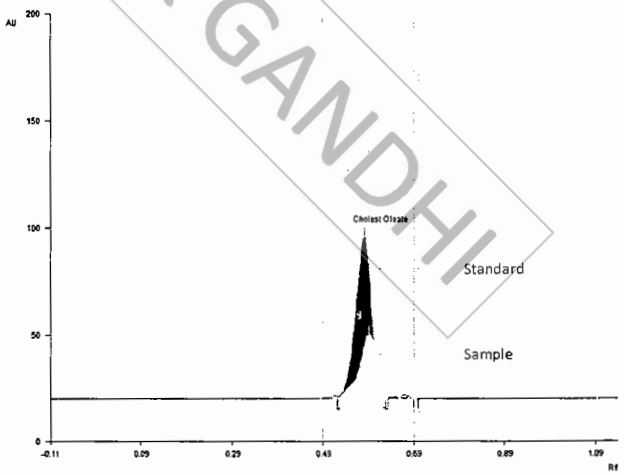

Figure 2. An overlay of the peaks obtained from standard and samples of cholesteryl oleate. This figure shows that the relative retention of standard cholesteryl oleate and from analyte samples remains the same. It may also be observed that no interference could be found in the analyte detection range.

Colour online, B&W in print

Table 3. Robustness of the method[a]

Parameter	Mean peak area	SD of peak area	RSD
Mobile phase composition	4765.20	51.10	1.07
Mobile phase volume	4251.00	46.43	1.02
Temperature	4555.90	55.47	1.30

[a]Robustness was evaluated by utilizing three different compositions of mobile phase. Total volume of the mobile phase and temperature of analysis were varied by ±5%.

ACAT assay with inhibitor

The ACAT assay mentioned above was applied for the evaluation of avasimibe (known inhibitor of ACAT isoforms; Llaverias *et al.*, 2003). The basic aim was to determine the IC_{50} value of avasimibe towards ACAT inhibition and compare it with IC_{50} values determined by other methods and reported in the literature. The IC_{50} value of avasimibe was found to be 4.019 ± 0.064 μM, which was found to be in agreement with the value (IC_{50} 4.0 μM; Burnett *et al.*, 1999) reported in the literature. This finding indicates that the reported method can be effectively used for screening of novel ACAT inhibitors which otherwise become prohibitive for many researchers owing to nonavailability of facilities for radiometric method.

Quantification of cholesteryl esters in plasma samples

It has been reported that cholesteryl esters present in plasma as a part of LDL-cholesterol are responsible for the development of atherosclerosis (Spector and Haynes, 2007; Ghosh *et al.*, 2010). In lieu of these reports, we chose to evaluate pathological samples having known high total plasma cholesterol levels for their cholesteryl ester content. Interestingly, we found a close correlation between total cholesterol levels and cholesteryl ester levels (Fig. 3). Previous reports have underlined a positive correlation between total cholesterol and cholesteryl esters (Zhang *et al.*, 2005; Matsumura *et al.*, 1999). Most of the methods employed for estimation of total cholesterol levels use cholesterol esterase to hydrolyze the cholesteryl esters (Mizoguchi *et al.*, 2004). Thus cholesterol esters are indirectly estimated as a difference between total cholesterol and free cholesterol (Levy *et al.*, 2007). This suggests that a significant portion of total cholesterol is derived from the hydrolysis of cholesteryl esters.

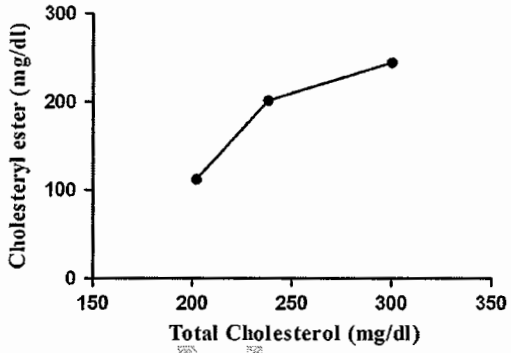

Figure 3. A plot showing the relation between total cholesterol and cholesteryl esters from plasma samples of three patients having total cholesterol levels higher than 200 mg/dL. Cholesteryl esters are expressed as a function of cholesteryl oleate. Results are expressed as a mean of triplicate analysis.

Advantages of the method

The method was found to have a total-run time (sample application through) of about 50–60 min after sample preparation and hence it can be claimed that the time of analysis is relatively short when compared with the HPLC-FLD and LC-ESI-MS/MS methods (Cao *et al.*, 2013; Miyoshi *et al.*, 2013). This method has been developed on a 10 × 10 cm^2 plate on which seven or eight samples can be simultaneously estimated. It is also possible to use a 20 × 10 cm^2 plate on which 17–18 samples can be estimated. Increasing the number of samples for analysis does not increase the time of analysis significantly as the steps of estimation are run simultaneously. The concentration range employed in the present study is 100–500 ng/band but the limit of quantification was found to be 19.54 ng/band (Table 1). Hence, this method may be applied in lower concentration ranges (i.e. 20–100 ng/band), indicating its sensitivity. This sensitivity is comparable to that achieved with LC-ESI-MS/MS (30 ng to 1 μg; Miyoshi *et al.*, 2013). Samples and standards are estimated simultaneously on the same plate. The chances of poisoning the sample are negligible since there is no carry over. The present method works as an open system and visual detection of the sample is possible post derivatization. Further, derivatization can be performed on the plate *in situ*. Derivatization with anisaldehyde–sulfuric acid reagent leads to the development of a purple band, so a separate hydrolysis step is avoided. Using the HPTLC method, we estimated cholesteryl esters *at par* with radiometric estimation. We were able to reproduce data from

Table 4. Validation using plasma samples[a]

Nominal concentration (ng/band)	Intra-day			Inter-Day		
	Concentration found (ng/band)	Accuracy (percentage recovery)	Precision (RSD)	Concentration found (ng/band)	Accuracy (percentage recovery)	Precision (RSD)
200 ng	211.35 ± 3.43	105.67 ± 1.71	1.62%	207.66 ± 3.24	103.83 ± 1.62	1.56%
300 ng	317.61 ± 3.02	105.87 ± 1.01	0.95%	316.82 ± 5.43	105.61 ± 1.81	1.72%
400 ng	406.84 ± 4.28	101.71 ± 1.09	1.05%	404.20 ± 7.90	101.05 ± 1.97	1.96%

[a]Validation of the estimation method in pooled plasma samples. Results indicate the accuracy and precision of the method for estimation of cholesteryl esters in plasma samples. See 'Experimental' section for details.

the literature regarding estimation of ACAT inhibitor avasimibe (Llaverias *et al.*, 2003; Burnett *et al.*, 1999). Cholesteryl ester content in plasma samples from hypercholesterolemic individuals was also estimated by this method.

Conclusion

The proposed method for estimation of cholesteryl oleate by HPTLC was found to be precise, accurate, specific and robust. The method is free from interference from the biological matrix used for the ACAT assay. Statistical data showed that the method can be applied to the analysis of cholesteryl oleate as a product of the ACAT assay. Inherent advantages of the application of HPTLC for analysis involve low cost and the applicability of the method to estimate several samples on a single plate simultaneously in a very short span of time. This method may have numerous applications in future but one application which could be most vital to the healthcare fraternity would be the extension of this method to the estimation of cholesteryl esters in plasma samples from patients predisposed to or suffering from hypercholesterolemia and atherosclerosis. The reported method can also be used for the screening of ACAT inhibitors in the absence of facilities for radiometric method. For the first time, we report in this paper a quantification method for products of the ACAT assay without the use of any radiolabeled compounds.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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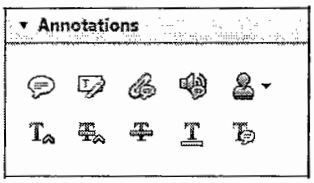

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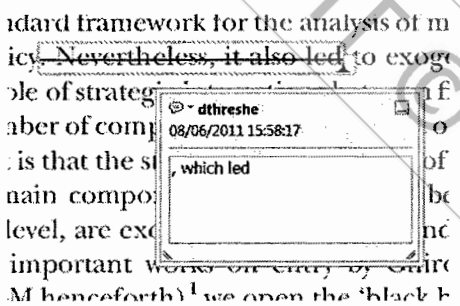

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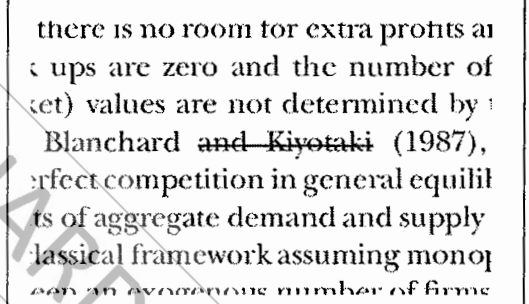

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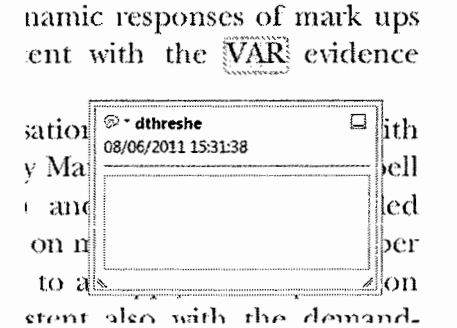

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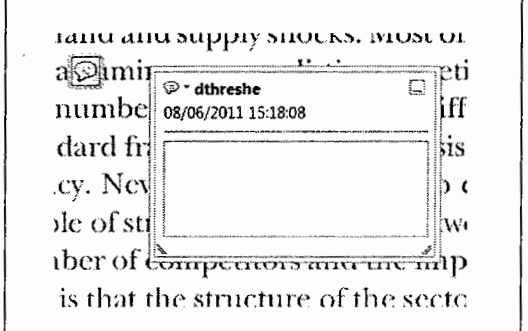

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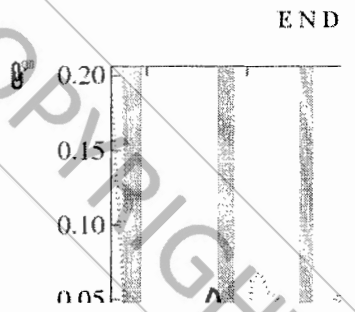

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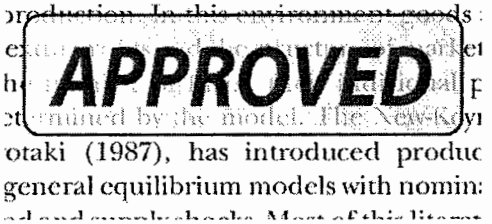

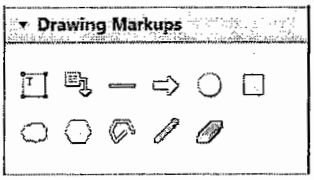

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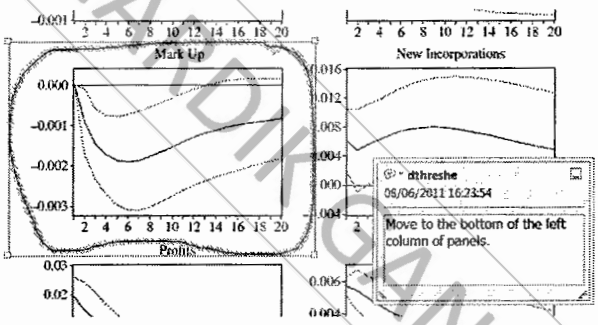

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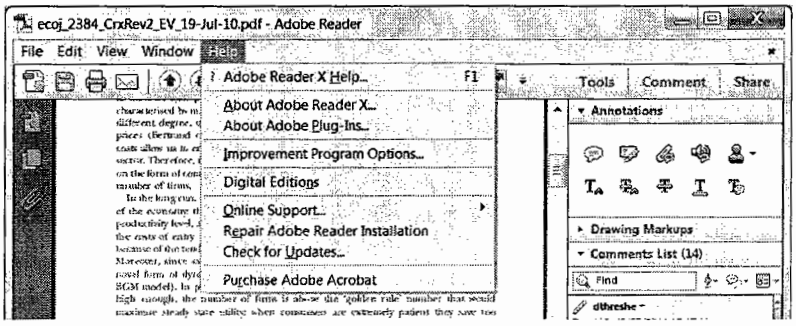

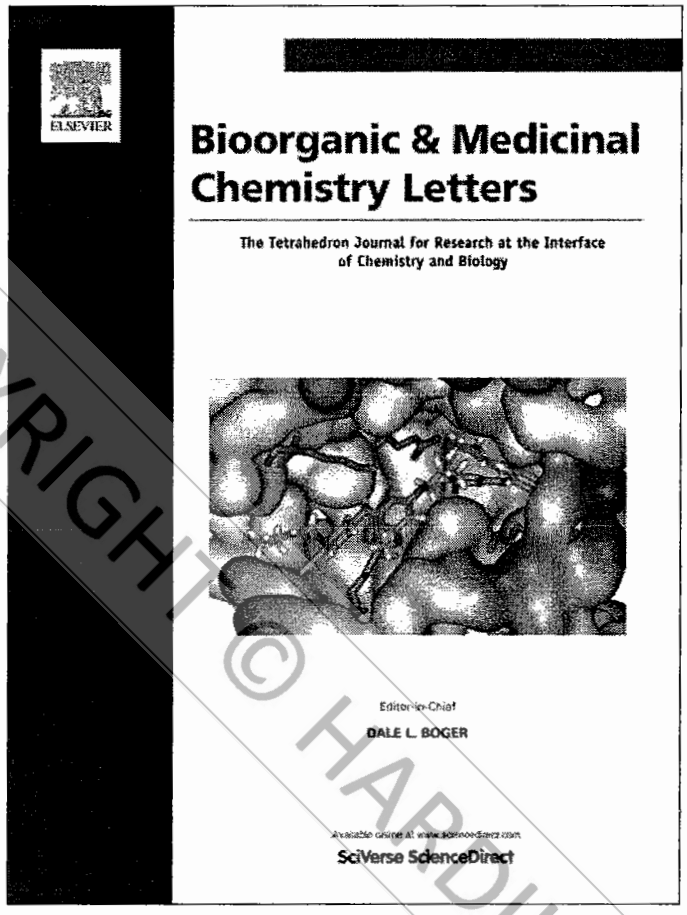

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Design and synthesis of 6,7-dimethoxyquinazoline analogs as multi-targeted ligands for α_1- and AII-receptors antagonism

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ABSTRACT

Multiple-targeted ligands can have certain advantages for the management of hypertension which has multiple controls. Molecules with dual bioactivities are available in literature for treating metabolic disorders like diabetes, hypertension and hypercholesterolemia. After scrutinizing the SAR of prazosin-type α_1-blockers and AII-antagonists it was planned to develop dual α_1- and AII-antagonists. Five series of quinazoline derivatives were synthesized and evaluated as dual α_1- and AII-antagonists on rat aortic strips for the blockade of known α_1- and AII-agonist mediated contractions. Many compounds showed balanced activity on both the receptors but compound (**22**) was found to be the most active derivative having higher antagonistic activity on both the receptors. In the in vivo experiments the chosen compound (**22**) was slightly less active than prazosin but was found to be equipotent to losartan. These findings shed a new light on the structural requirements for both α_1- as well as AII-receptor antagonists.

It is increasingly being recognized that a balanced modulation of multiple targets can provide a superior therapeutic efficiency in comparison to targeting a single enzyme/receptor in a diseased state.[1] There is no single drug treatment of hypertension due to differences in etiology, risk factors and body constitution of individuals.[2] It requires a combination therapy as a single drug of any class of antihypertensives proves ineffective.[3,4] The conventional treatment of hypertension involves combination of drugs from such categories of drugs as diuretics, α_1-adrenergic receptor antagonists, β-blockers, calcium channel blockers, ACE inhibitors, AII-receptor antagonists etc.

Compared to drug combinations, there are certain advantages associated with a multiple-targeted ligand as a therapeutic agent, such as more predictable pharmacokinetic and pharmacodynamic relationship and improved patient compliance as a consequence of administration of a single drug at a time. It is interesting to note that of the known multiple targeted (multi-action) drugs only a few have been designed to act on the intended targets. Rest others have been serendipitously discovered wherein the modes of action of the drugs were elucidated retrospectively to be multiple targeted. Increase in the number of publications on the designing and development of multiple targeted molecules in recent times is an indication of developing interest in the field.[5–7] Rational designing approaches, in which structural features of selected ligands are combined into one single entity, have produced new ligands that act on a variety of targets. A key challenge in the designing of such multiple-targeted ligands is attaining a balanced activity at each target of interest while achieving a higher selectivity and a suitable pharmacokinetic profile simultaneously.[8] The so called dual acting drugs (dimeric ligands) are designed by joining together the pharmacophores by a cleavable/noncleavable linker or more commonly by overlapping the pharmacophores of two drugs into a single chemical entity by taking advantage of common structural features of two or more classes of drugs. To integrate the pharmacophores of two drugs common structural features of both of the drugs are overlapped.

There are numerous examples of hybrid molecules wherein dual bioactivities have been packed into a single chemical entity. Glitazones (insulin sensitizers) and fibrates (lipid lowering agents) act through PPARγ and PPARα receptors respectively. The aryloxazole (**1**) is a combination of thiazolidinedione derivative (**2**) and fenofibric acid[9] (**3**). A dual acting hybrid sulphonamide (**4**) was obtained by linking propranolol, a β-blocker with mefruside, a diuretic.[10] In prizidilol (**5**), hydralazine a well known vasodilator was integrated with a pharmacophoric group responsible for β-blocking activity to afford a dual acting drug.[11]

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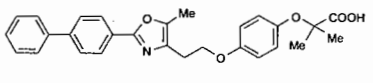

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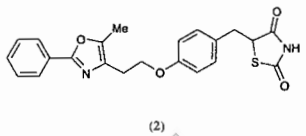

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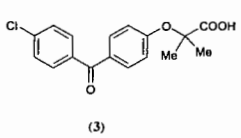

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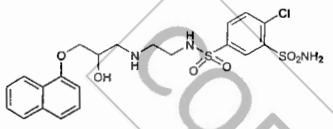

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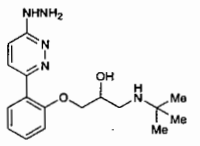

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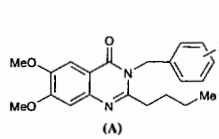
Several experimental and clinical studies have pointed towards the linked action of RAAS (renin angiotensin aldosterone system) and SNS (sympathetic nervous system) in the homeostatic control of cardiovascular functions.[12,13] Evidences show that these two systems interact mutually with each other to effect their cardiovascular regulatory roles.[14,15] Stimulation of SNS results into vasoconstriction and increased inotropic and chronotropic effects of heart while stimulation of RAAS results in increased production of active hormone angiotensin II (AII) which raises blood pressure. The RAAS–SNS interactions have physiological as well as pathophysiological relevance; a reciprocal reinforcement of the favorable as well as unfavorable cardiovascular, renal, metabolic and reflex effects of the two systems have been reported in a variety of cardiovascular conditions like hypertension.[15–17] SNS and RAAS become important targets in order to control the blood pressure as both of the systems work in coordination. Simultaneous blockade of both the systems should prove to be beneficial in the management of hypertension. Two important targets belonging to the RAAS–SNS systems are the α_1- and AII receptors.

α_1-adrenergic receptor blocking agents and AII receptor antagonists both are important classes of antihypertensive drugs. A wide variety of chemical structures[18] possess α_1-adrenoceptor blocking activity. Similar observations have been made for AII receptor antagonists.[19] After studying the structure activity relationships of both the classes of drugs minutely, it became evident that the drug binding sites of both the receptors could accommodate wide structural variations in the active molecules. And if that presumption was correct, then designing of dual acting α_1 and AII antagonists should not be a distant dream.

Prazosin (**6**), an important α_1-blocker was chosen as the lead molecule. Not many structural changes have been carried out in prazosin type of α_1-blockers except for some variations in the side chain at position-2 of the quinazoline ring system. On the other hand, by considering losartan (**7**) as the lead molecule of AII antagonists, it was noted that too many structural changes have been performed to obtain potent AII antagonists, like replacement of imidazole nucleus with other heterocyclicring systems, replacement of the biphenylmethyl side chain with smaller and bigger groupings, and replacement of tetrazole moiety with other acidic groups. So, it was planned to design hybrid structures by employing 6,7-dimethoxyquinazoline ring skeleton as the common structural motif for α_1 as well as AII antagonism and attaching various types of side chains at its 2-and/or 3-positions. The main obstacle in the selection of the side chain was the nature of the side chain grouping—whether the attached side chain should have acidic functionality or a basic one because prazosin-type of α_1-blockers contained basic groupings in the side chain while all reported AII antagonists had an acidic functionality in the side chain. It was envisaged to make a try with all the three types of functional groups, acidic, basic as well as neutral in the attached side chain for designing of the compounds. Assuming a high degree of structural tolerance for antagonistic activity by both the receptors, the given five types (**A–E**) of compounds were designed:



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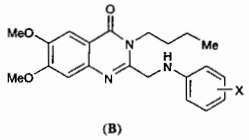

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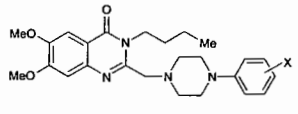

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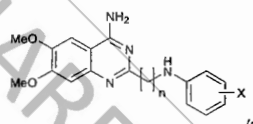

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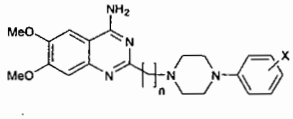
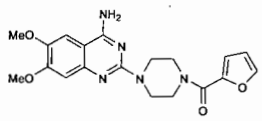


(n = 0,1)

(E)

Work was initiated for the preparation of series **A** compounds. For synthetic convenience, compounds with neutral groups like CH_3, OCH_3, Cl, Br, NO_2, CN etc. at different positions of the phenyl ring were prepared first. Compounds so synthesized (Scheme 1) were screened for obtaining preliminary biological data. The results of this preliminary biological screening were a bit shocking to us. Surprisingly, all of the screened compounds showed dual antagonism to the phenylephrine and AII responses in the in vivo normotensive rat model; although the level of antagonism was low to moderate in comparison to prazosin and losartan, the two prototype lead antagonists. In vitro experimentation on isolated rat aortic strip could not be performed for these neutral compounds due to their solubility problem in aqueous solutions. These results forced us to have a relook at the mechanism of antihypertensive actions of both prazosin and losartan. When neutral molecules without any characteristic side chains could show dual α_1- and AII-receptor antagonism, was it possible for prazosin and losartan also to show dual antagonism at both the receptors? pA_2 value determinations of both of the standard lead molecules confirmed the correctness of our assumption of wide structural tolerance by both the receptors in their active spaces—prazosin exhibited potent dual antagonism at α_1- (pA_2 8.91) as well as AII-receptors (pA_2 8.26) (Table 1) while losartan was found to be a potent antagonist at AII-receptor (pA_2 8.08) but a poor (pA_2 5.46) one at α_1-receptor when evaluated on rat aortic strip using phenylephrine and AII as agonists.[20]



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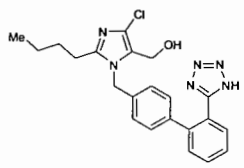

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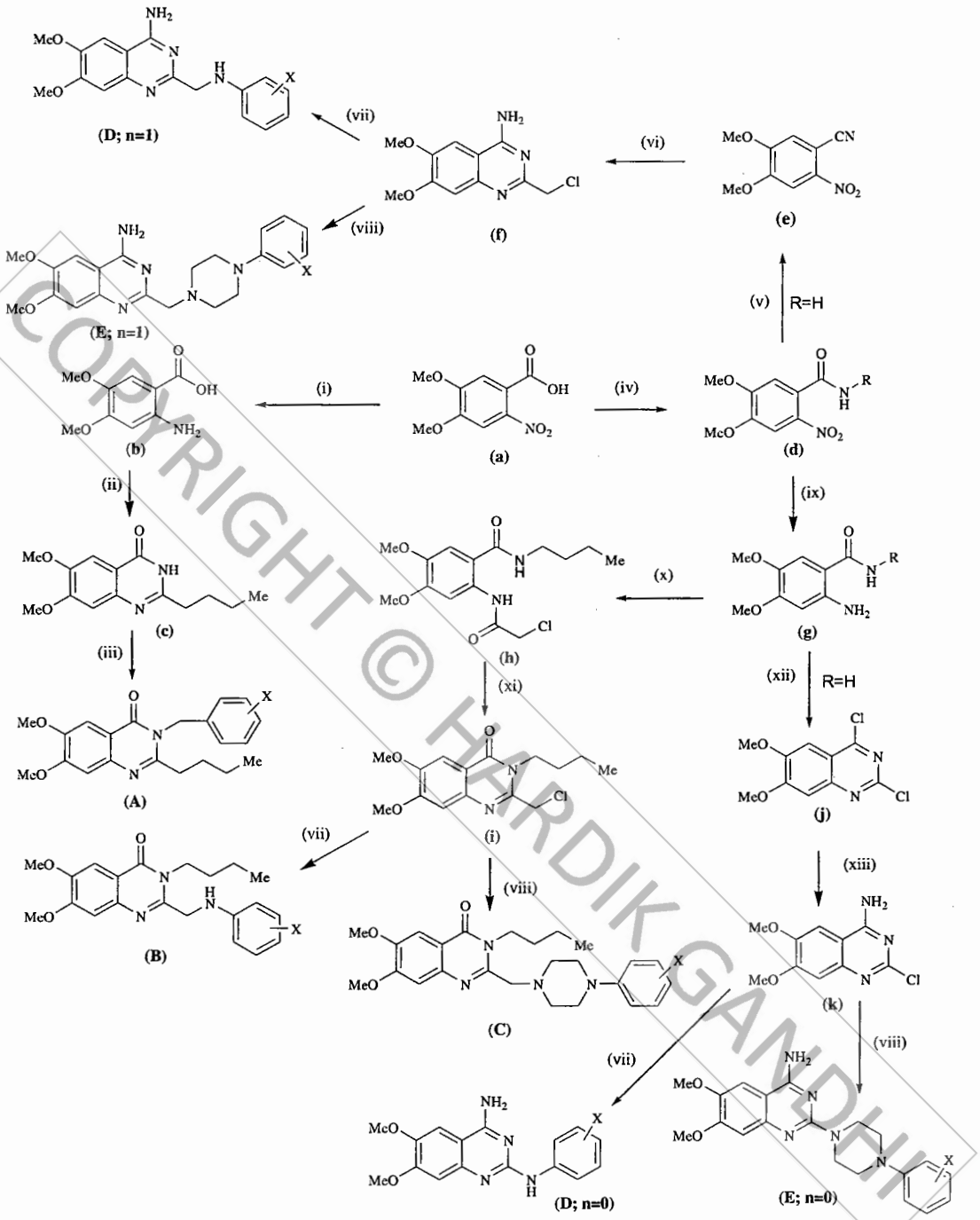


Scheme 1. Synthetic steps for various compounds (series **A–E**). Reagents: (i) H_2, Pd/C; (ii) $CH_3CH_2CH_2COCl$, NH_4OAc; (iii) Br-CH_2-C_6H_4X; (iv) $SOCl_2$, *n*-$BuNH_2/NH_3$ (v) $POCl_3$; (vi) (a)-Fe, NH_4Cl; (b)-$ClCH_2CN$, HCl; (vii) H_2N-C_6H_4X; (viii) 4-*Piperazine*-C_6H_4X; (ix) Fe, NH_4Cl; (x) $ClCH_2COCl$; (xi) KTB (xii) Urea, HCl; (xiii) NH_3.

Buoyed by these results a large number of compounds representing all of the above described five categories of chemical structures were synthesized (Scheme 1). The synthesized compounds were evaluated on rat aortic strip for their pA_2 value determinations using phenylephrine and AII as agonists for α_1- and AII receptors respectively. The results are described in Table 1. 4-Quinazolinonoes belonging to series (**A–C**) did not yield much promising dual inhibitors. 3-*n*.Butyl substitution yielded more potent compounds (**10** and **11**) than the 2-*n*.butyl substituted derivatives (**8** and **9**). Among the 2-*n*.butyl-3-benzyl substituted derivatives *m*-cyano (**8**) and *m*-tetrazoly1 (**9**) derivatives were found to be the most active compounds. Introduction of basic nitrogen in the side chain at position-2 of the 4-quinazolinones improved α_1-antagonistic activity. Compound (**11**) was found to be the best among them having a balanced activity at both the receptors but it was somewhat less potent than prazosin (**6**). Restoring 4-amino group (as existed in prazosin) from the 4-keto function improved the activity profile of the synthesized derivatives. In the **D**-series, compounds with one

Table 1
pA_2 values of the prominent synthesized compounds on rat aortic strips

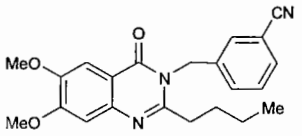
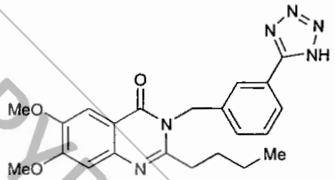
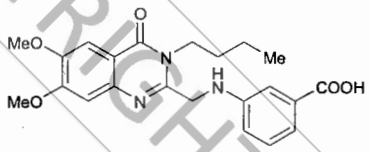
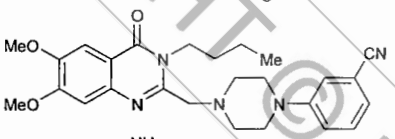
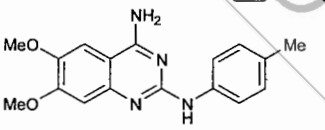
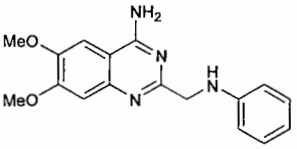
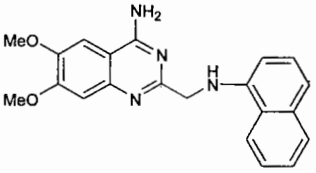
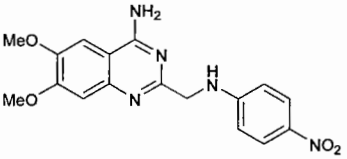
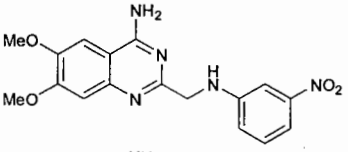
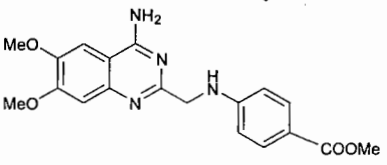
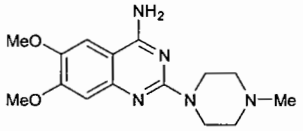
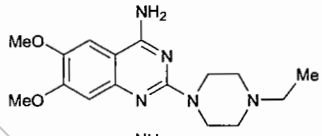
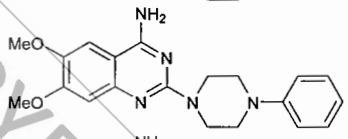
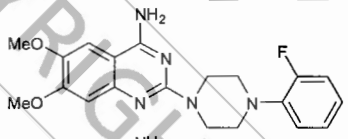
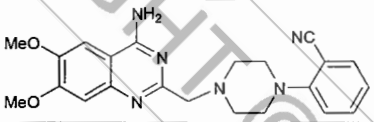
Compd	Structures	pA_2 values	
		α_1-Receptor	All-receptor
—	Terazosin	8.65 ± 0.37	6.39 ± 0.40
6	Prazosin	8.08 ± 0.11	8.26 ± 0.10
7	Losartan	5.46 ± 0.41	8.43 ± 0.21
8		6.88 ± 0.13	6.65 ± 0.09
9		7.10 ± 0.12	7.03 ± 0.12
10		7.45 ± 0.16	6.14 ± 0.14
11		7.71 ± 0.01	7.41 ± 0.10
12		8.53 ± 0.12	7.65 ± 0.09
13		9.87 ± 0.15	8.37 ± 0.43
14		8.37 ± 0.27	7.07 ± 0.16
15		9.38 ± 0.18	7.64 ± 0.07
16		8.09 ± 0.12	9.04 ± 0.15
17		6.86 ± 0.12	10.64 ± 0.10

Table 1 (*continued*)

Compd	Structures	pA_2 values	
		α_1-Receptor	AII-receptor
18		9.23 ± 0.12	5.75 ± 0.07
19		10.41 ± 0.12	5.08 ± 0.08
20		8.74 ± 0.08	3.31 ± 0.13
21		10.51 ± .014	5.52 ± 0.10
22		10.10 ± 0.20	8.83 ± 0.04

carbon linkage between the quinazoline ring and the basic nitrogen offered more potent compounds than those in which nitrogen was directly linked to the quinazoline nucleus. One such compound having the highest activity was the *p*-tolyl derivative (**12**) which was almost equipotent to prazosin. Among the anilinomethyl analogs large variations in the substituents were tolerated for potent activity against both the receptors. With the introduction of a methylene group in between the amino and the quinazoline nucleus, antagonistic potency increased against both the receptors. An unsubstituted phenyl ring (**13**) offered the most potent α_1-blocker while *p*-methylcarboxylate group yielded the most potent (**17**) AII antagonist in the series.

Direct attachment of piperazine ring as it existed in prazosin, in the **E**-series of compounds, increased the potency against the α_1-receptors. But these compounds (**18–21**) started showing imbalance in potency against both the receptors. Potency against the α_1-receptor increased but the potency declined against the AII receptor. Compound[21] (**21**) was about 100 times more potent than prazosin on α_1-receptor but was poorly active against the AII receptor. Introduction of methylene group in-between the piperazine and quinazoline rings gave the most useful compounds. *o*-Cyano substituted compound[22] (**22**) was found to be the most useful compound amongst the whole lot as it showed potent activity against both the receptors.

Biological data of the synthesized compounds offered some startling results as for as the existing knowledge about α_1-receptor blocking and AII antagonist drugs are concerned. The results showed that it was not at all necessary to have an acidic side chain to obtain a potent AII antagonist even a neutral group like CN in compound (**22**) offered a potent AII antagonism. 4-Aminoquinazolines in general yielded much more potent and balanced compounds than 4-quinazolinones. Placing a carbon linker in between the quinazoline ring and the basic nitrogen offered more potent and balanced dual acting compounds than directly linking the basic nitrogen to the quinazoline ring.

To corroborate the results obtained from experimentations on isolated rat aortic strips, it was planned to evaluate the most active compound from series **E** (**22**) in the in vivo studies. Compound (**22**) was evaluated for its ability to inhibit the pressor responses induced by phenylephrine (6 μg/kg, iv bolus) and AII (6 μg/kg, iv bolus) in anesthetized normotensive rats. The effects are summarized in the Figure 1. The results demonstrated that compound (**22**) has dual inhibitory action upon pressor responses induced by agonists, phenylephrine and AII. Thus, compound (**22**) has independently shown dual-inhibiting potential to antagonize the effects of endogenous pressor substances like noradrenaline and AII. But the results of this comparative study were somewhat not as per expectations, given the potent in vitro activity exhibited by compound (**22**) on isolated aortic strips. Administration of compound (**22**) on equimolar basis had only a modest inhibitory response at the most when compared to the pressor-response inhibition mediated by the standard drugs prazosin or losartan. Since compound (**22**) shows dual action, it was speculated that the actual concentration of the compound reaching at a particular receptor could be much lesser while administering it on a molar basis. The reason for such an abridged response could be the distribution of the drug upon both the receptors in question suggesting that only a fraction of the compound (**22**) is available at a given time to act upon a particular population of receptors. This speculation is based on in vitro studies performed earlier on isolated rat aortic strips. In these experiments, compound (**22**) showed competitive antagonism against phenylephrine and angiotensin II, respective specific agonists of the α_1- and AII-receptors. This is evident from the rightward parallel shift observed in the dose–response plots for compound (**22**). This competitive inhibition of the contractile response in isolated vascular tissue shows that compound (**22**) has the ability to bind to both the receptors in question in a dose-dependent manner (Fig. 2). This condition of competitive binding to both the receptors is expected to be prevalent in the in vivo model also, thereby causing reduction in the inhibition response

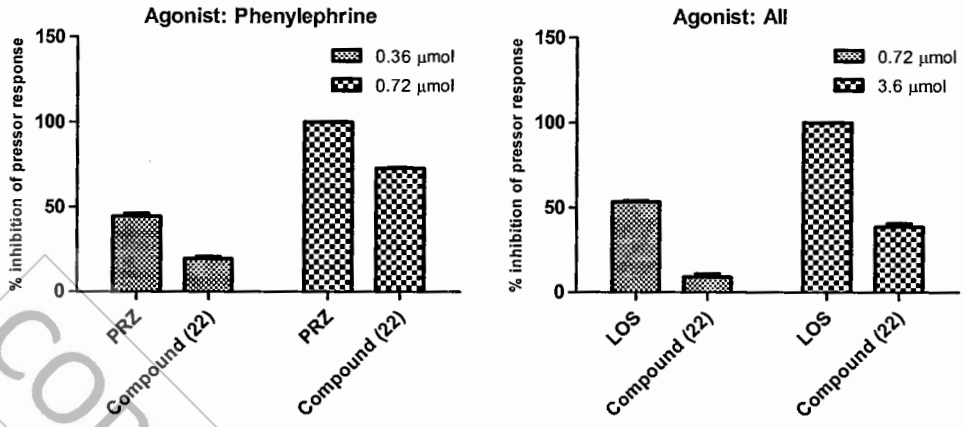


Figure 1. In vivo pressor inhibition effect of compound (**22**) on normotensive rats at two different doses in comparison to the standard drugs. Data is presented as mean ± SEM of six animals.

of compound (**22**) on both the receptors in comparison to the standard drugs.

To provide credence to the above proposed hypothesis, it was planned to evaluate the pressor-inhibition potential of compound (**22**) under masked conditions. In vivo inhibition of phenylephrine mediated arterial pressor response by compound (**22**) was measured in those animals in which 3.6 μM losartan was pre-administered. The idea behind such a protocol was to mask the effects of our compound (**22**) on AII receptor. Similarly, the other set involved measurement of inhibition of AII mediated arterial pressor response in those animals in which 0.72 μM prazosin was pre-administered to mask the effects of compound (**22**) upon α_1-receptor. The standard drugs and the compound (**22**) were administered at equimolar concentrations. The results obtained are shown in Figure 3.

It can be observed that the hypothesis stands a chance as the compound (**22**) was equipotent to losartan after masking the α_1-receptors with prazosin but slightly less active than prazosin after masking AII receptors with losartan. Slightly lesser activity of compound (**22**) on α_1-receptors could be due to differences in pharmacokinetic parameters from the standard drug prazosin. Predicted plasma protein binding values for compound (**22**) and prazosin are 97.5% and 96%, respectively, suggesting that plasma-protein binding efficiency of compound (**22**) is slightly higher than prazosin.[23] To evaluate the degree of plasma protein binding, we tried to compare the in vitro release profile of compound (**22**) and prazosin from human plasma. For the plasma protein binding study, both

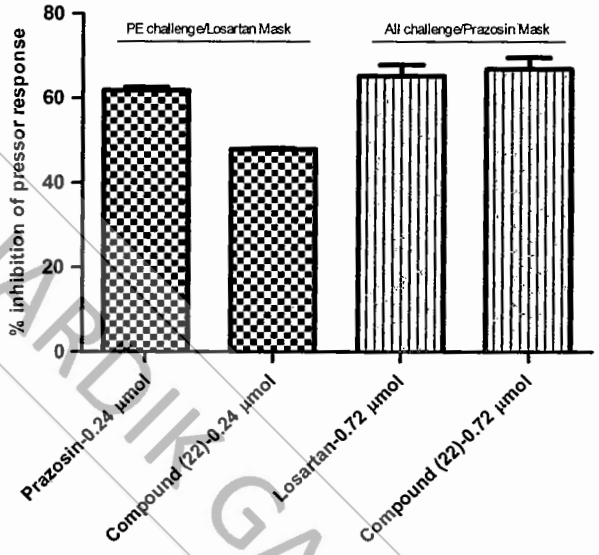


Figure 3. Evaluation of pressor response of compound (22) and prazosin for α_1-antagonism (losartan was used for masking AII-receptors), and of compound (**22**) and losartan for AII-antagonism (prazosin was used for masking of α_1-receptors) at equimolar concentrations (mean ± SEM of six animals).

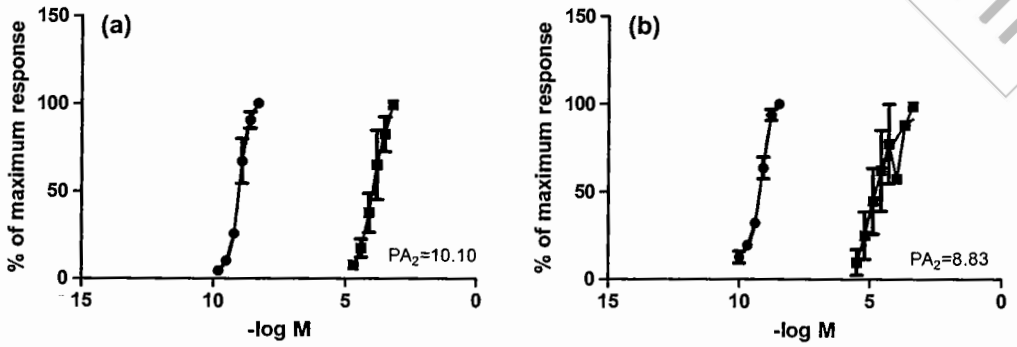


Figure 2. (a) Dose–response plot of compound (**22**) against phenylephrine on rat aortic strips. A rightward parallel shift of the curve can be observed in presence of compound (**22**), $n = 4$. (b) Dose–response plot of compound (**22**) against angiotensin II on rat aortic strips. A rightward parallel shift of the curve can be observed in presence of compound (**22**), $n = 4$.

the drugs were spiked into blank human plasma and their release through a semi-permeable membrane into physiological buffer (phosphate buffered saline, pH 7.4) were compared over time. It was observed that the release of prazosin is somewhat faster (~10%) than that of compound (**22**) (Fig. 4). Assuming that this would be true for the in vivo environment, then the amount of prazosin reaching the receptor would be higher than compound (**22**), which could account for the somewhat lesser response observed with compound (**22**) in vivo. This could be observed from the cumulative drug release profile shown below. Further, we have also compared the release profile of losartan and compound (**22**). Based on these studies, we excluded the possibility of differential tissue distribution of compound (**22**) and losartan which was corroborated by the inhibition of pressor responses of compound (**22**) and losartan in the in vivo model.

Though compartmentalization of the drug was excluded with the possibility of high-degree of plasma protein binding, the disparity of receptor distribution cannot be ruled out. As stated above, we have previously shown that prazosin shows potent antagonism of the AII receptors as well.[20] Such type of action is responsible for an enhanced antihypertensive response afforded by prazosin. However, this effect can play a dual role in our masking study, again by acting on both α_1 and AII receptors. Hence we performed the same study using terazosin as a standard α_1 antagonist (Fig. 5), which we have shown to have negligible action on AII mediated vascular smooth muscle contraction. Activity of terazosin is exclusive for the α_1 receptor and hence we can safely assume that the effects of terazosin are mediated due to binding with α_1 receptor only. No significant differences could be observed between this study involving terazosin (as a masking agent and a standard α_1-antagonist) and that of prazosin as shown in Figure 3.

To conclude, rational designing of hybrid molecules with balanced biological activities on multiple targets can be a daunting task. As mentioned earlier, there are few molecules that exhibit such dual activity. In this paper, we have reported for the first time, synthesis and biological evaluation of novel antagonists which have dual activity of antagonizing the α_1- and AII-receptors simultaneously. Further investigations on these compounds are underway. These investigations include in vivo evaluation of active compounds in rodent models of hypertension, identification of their pharmacokinetic profile in rats, and further optimization of the leads to obtain more potent dual active compounds with balanced activities on both of the targets.

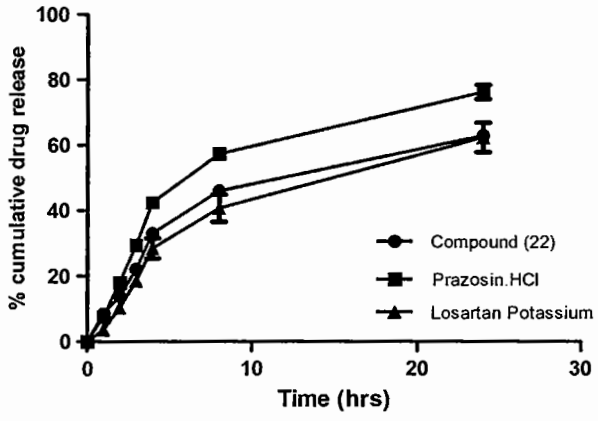


Figure 4. In vitro cumulative release profile of compound (**22**) in comparison to that of prazosin hydrochloride and losartan potassium. All the compounds were spiked in blank human plasma, filled in a semi-permeable dialysis bag which was suspended in a beaker filled with physiological buffer maintained at 37 ± 2 °C. Samples were withdrawn at different time intervals (replacing equal volume of buffer) and analyzed for drug contents by high performance liquid chromatography. All studies were performed in triplicate.

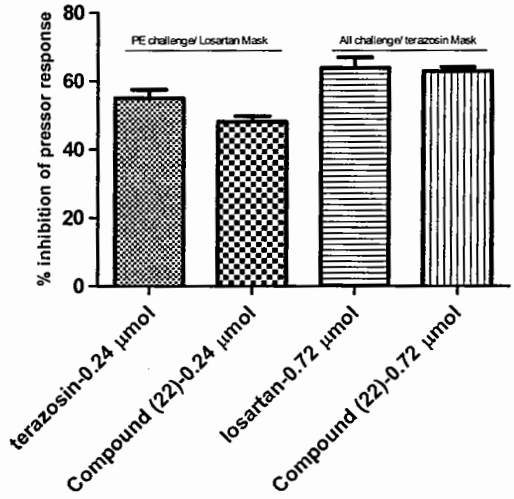


Figure 5. Evaluation of pressor response of compound (**22**) and terazosin for α_1-antagonism (losartan was used for masking AII-receptors to prevent binding of compound (**22**) to AII-receptors), and of compound (**22**) and losartan for AII-antagonism (terazosin was used for masking of α_1-receptors to prevent binding of compound (**22**) to α_1-receptors) at equimolar concentrations (mean ± SEM of six animals).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.04.054.

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RESEARCH ARTICLE

Revelation on the potency of α_1-blockers – parallel blockade of angiotensin II receptor: A new finding

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Abstract

Context: The problem of hypertension has gained enormous proportions in the past decade. Multifactorial etiology and complex pathophysiology of the disease has rendered the treatment of the disease a hard task. Sympathetic nervous system and the renin–angiotensin–aldosterone system are primary contributors of blood pressure homeostasis.

Objective: Structural similarities were identified among AT_1 and α_1-antagonists, initiating a speculation that α_1-antagonists could possibly block the AT_1 receptor and vice-versa.

Methods: To corroborate this speculation, we screened prototypical α_1-antagonists such as prazosin, doxazosin, and terazosin for antagonism of angiotensin II on rat aortic strips. We also examined the AT_1 antagonists losartan, valsartan, and olmesartan for their possible antagonistic effect, on contractions of rat aortic strips induced by phenylephrine.

Results: To our astonishment, we found that prazosin and its analogs which have been reported to have α_1-antagonistic activity only, were able to shift concentration response curves of angiotensin II.

Conclusion: Our findings suggest that the potent antihypertensive effect of prazosin-type α_1-antagonists is not purely due to α_1-receptor blocking activity of these compounds but also due to blockade of AT_1 receptors. This finding may lead to the development of more potent dual inhibitors which would prove to be of immense value in the control of the scourge of hypertension.

Keywords: α_1 Antagonist, AT_1 antagonist, prazosin, losartan

Introduction

Hypertension is recognized as one of the leading risk factors for human morbidity and mortality. On a worldwide basis, hypertension has been ranked first as a cause of disability adjusted life years (Global Health Risks, 2009). Hypertension is known as a silent killer because the symptoms do not appear until it is severely high. The blood pressure is derived from the hemodynamic properties of the systemic circulation.

Hypertension is influenced by both function and structure of blood vessels. The purpose of the "blood pressure system" is to maintain blood flow to all of the tissues of the body at rest or during movements. There are two important contributors to the control of blood pressure, sympathetic nervous system (SNS) and renin–angiotensin–aldosterone system (RAAS). Over the years, a number of experimental and clinical investigations have shed light on the key role exerted by RAAS and SNS in the homeostatic control of blood volume and blood pressure (Sealey & Laragh, 1995; Shepherd & Mancia, 1986). Straightforward evidence has also been provided that these two systems do not operate independently but interact mutually with each other in accomplishing their cardiovascular regulatory functions. (Grassi, 2001; Rang et al., 2007).

Stimulation of SNS results into vasoconstriction and increased inotropic and chronotropic effect of heart, while stimulation of RAAS results in increased production of active hormone angiotensin II (Ang II), which raises blood pressure in two ways. First, Ang II is a potent vasoconstrictor that raises systemic vascular resistance and second, it indirectly influences blood pressure through release of aldosterone and noradrenaline.

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Both SNS and RAAS also appear to modulate fluid volume through the kidney. The kidney is a vital organ involved in long-term control of blood pressure. The renal-body fluid feedback mechanism couples the long-term regulation of arterial pressure to extracellular volume (sodium and water) homeostasis via pressure natriuresis whereby the kidneys respond to changes in arterial pressure by altering urinary sodium and water excretion (Cowley, 1992). Both SNS and RAAS systems are primary modulators of renal effects on circulating blood volume. The α-adrenergic receptors are involved only when associated renal hemodynamic changes occur with decrease in renal blood flow (RBF), glomerular filtration rate (GFR) and urinary sodium excretion. Renal α_1-adrenergic receptors mediate renal (including preglomerular) vasoconstriction and tubular gluconeogenesis. These effects are coupled to tubular Na^+ reabsorption. The direct effects of renal nerve stimulation through renal tubular α_1-adrenergic receptors were observed *in vivo* in the dog (Summers, 1984) and rabbit (Wolff et al., 1985) and *in vitro* in isolated buffer-perfused kidney preparation of the rat (Drew & Whiting, 1979; Horn et al., 1982; Wolff et al., 1984). On the other hand, Ang II causes vasoconstriction and diminishes blood flow through the kidneys, thereby increasing the reabsorption of salt and water retention (Hall, 2004).

Information on the renin–angiotensin–sympathetic interactions has also been extended to the possible sites of these interactions:

- Stimulation of the sympathetic nervous system leads to renin secretion and Ang II formation (DiBona, 1989).
- It has been shown that released norepinephrine negatively regulates Ang II receptors in cultured brain neurons (Mancia et al., 1995) and in vascular tissue through its interactions with α_1-adrenoceptor (α_1-ARs) (Du et al., 1997). In neonatal rat, cardiac myocytes Ang II selectively down-regulates α_1-AR subtype mRNA and its corresponding receptors (Li et al., 1997).

Evidence has also been provided that Ang II:

- Triggers a sympathetically mediated blood pressure rise associated with systemic vasoconstriction when dosed intracerebrally. It suggested a central facilitatory effect of Ang II on sympathetic outflow (Wolff et al., 1984; Hall, 2004; Zimmerman, 1984).
- Plays a facilitatory role on the neuroadrenergic transmission across sympathetic ganglia (Zimmerman, 1984; Reid, 1992; Reit, 1972)
- Potentiates norepinephrine release from sympathetic nerve terminals via stimulation of presynaptic angiotensinergic receptors (Zimmerman, 1984; Reid, 1992; Starke, 1977).
- Amplifies the α-receptor mediated vasoconstrictor responses to exogenously administered or endogenously produced norepinephrine. Furthermore, Ang II has been shown to exert inhibitory effects on baroreceptor reflex control of heart rate and sympathetic nerve traffic (Zimmerman, 1984; Reid, 1992).

The renin–angiotensin–sympathetic interactions have physiological, as well as pathophysiological relevance; a reciprocal reinforcement of the favorable as well as unfavorable cardiovascular, renal, metabolic and reflex effects of the two systems have been reported in a variety of cardiovascular conditions like hypertension (Zimmerman, 1984; Reid, 1992; Reit, 1972). Multiple signaling pathways and redundant feedback mechanisms, both positive and negative, contribute to the hypertensive disease process.

Due to its multifactorial etiology, it has become difficult to control the hypertension with one particular class of therapy. Each class of antihypertensive agents has its own limitations. Chronic administration of particular class of antagonist causes one or the other adverse effects. Therefore, combinations of two or more types of antihypertensive agents are preferred in medical practice for effective control of blood pressure (Elliott, 2002; Paulis & Unger, 2010).

Parallel modulation of multiple biological targets can be beneficial for the treatment of disease with complex etiologies like hypertension. Both SNS and RAAS become important targets in order to control the blood pressure as these systems work in coordination. Simultaneous blockade of both systems would be beneficial. A number of clinically used drugs have been found to have activity at more than one target, which in some cases is associated with increased efficacy (Morphy & Rankovic, 2009).

This laboratory is actively engaged in developing newer antihypertensive agents. While studying the structure–activity relationship (SAR) of prazosin class of α_1-antagonists and AT_1 antagonist, it was realized that molecules with dual activities (α_1- and AT_1-receptors blocking) could be designed and synthesized. Working on the hypothesis of developing dual inhibitors, a large number of molecules were synthesized and evaluated biologically for α_1 and Ang II receptor blocking activities (unpublished results). Some of the synthesized compounds showed dual inhibition of both the receptors, comparable in potency to prazosin and losartan. Since these compounds were chemically related to the structure of prazosin and losartan and possessed dual activity, it struck to us whether prazosin and losartan could also possess dual activities and their potencies as antihypertensive agents could be due to dual inhibition of both the types of receptors and not due to singular effects. To support this assumption it was decided to cross-screen the clinically used drug molecules (α_1- and AT_1-receptor blockers) of one class on the other class of receptors. The α_1 receptor antagonist like prazosin, doxazosin, and terazosin were thus screened for AT_1 receptor antagonistic and AT_1 antagonist like losartan, valsartan, olmesartan were screened for α_1 receptor antagonistic activities.

Materials and methods

In vitro functional antagonism and potency assay

Male Wistar rats (200–250 g) were used for the study. They were sacrificed by cervical dislocation; descending thoracic aortas were removed immediately and placed in ice-cold Kreb's bicarbonate solution of the following composition (mM): NaCl 112, $NaHCO_3$ 12, glucose 11.1, KCl 5.0, $MgSO_4$ 1.2, KH_2PO_4 1.0, and $CaCl_2$ 2.5. The tissue was aerated with 95% O_2 and 5% CO_2. Periadventitial tissue was removed, taking care not to stretch the tissue. A spinal needle was inserted in the tissue and rotated gently to denude the endothelium. Following this, the tissue was cut spirally into a helical strip (20 mm × 3 mm) using a surgical blade. The strip was tied at both ends using a cotton thread and suspended in a 25 mL organ tube under an initial resting tension of 2 g. The pH of the Kreb's solution was 7.4 and maintained at 37°C using a thermostat. The Kreb's solution in the organ tube was changed every 10 min during an equilibration period of about 90 min. Denudation of the endothelium was confirmed by observing the "absence of relaxation" on strips precontracted with phenylephrine. Isometric contractions were recorded using a force transducer (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy). All the experimental protocols were approved by the Institutional Animal Ethics Committee of Pharmacy Department, the M. S. University of Baroda (Protocol No. PHR/GHPC/CPCSEA/183/06). The experiments were carried out in accordance to the guidelines of CPCSEA, India.

Contractions were induced in rat aortic strips with graded, cumulative concentrations of phenylephrine or Ang II. Losartan, valsartan, olmesartan, or solvent were added to organ tubes 30 min prior to the addition of phenylephrine. Similar procedure was followed to record graded cumulative response curves of Ang II against prazosin, doxazosin, and terazosin. Control strips were incubated with solvent [DMSO (0.5%) or normal saline] for 30 min before recording the concentration response curves. The pA_2 values were calculated by the method described by Arunlakshana and Schild (1959).

Results and discussion

Calculations based on Schild plots revealed that the α_1-antagonists prazosin, doxazosin, and terazosin cause a dose-dependent shift in the concentration response curves to Ang II. Any effect mediated by the endothelium was not expected since the endothelial layer was removed while preparing the aortic strip. The pA_2 values obtained after regression analysis were found to be 8.26 ± 0.7, 6.61 ± 0.4, and 6.39 ± 0.4 for prazosin, doxazosin, and terazosin, respectively. The slopes in these cases were not very different from unity. As opposed to these findings, the AT_1 blockers like losartan, valsartan, and olmesartan did not cause any major shifts in the concentration response curves to phenylephrine. All the tissues were incubated with antagonists for 30 min but incubation up to 45 min did not affect the results. Inhibition induced by the antagonists was reversible after the antagonists were removed following repeated washings. The pA_2 value is an index of antagonist potency and a higher pA_2 reflects strong competitive antagonism. As is evident from Figure 1A, prazosin causes major shift in the Ang II concentration response curves suggesting that this might be the subsidiary supporting mechanism by which prazosin acts, ensuing a higher potency as compared to its counterparts, doxazosin (Figure 1B).

The pA_2 value of prazosin (8.26 ± 0.7) is almost equal to losartan (8.08 ± 0.3) on Ang II receptor. This is a totally new and surprising finding unreported in the literature. It may be because of prazosin possessing some stereochemical features required to bind the AT_1 receptor or may possess some three-dimensional features required to fill in AT_1 receptor pockets. Further work on the structurally related analogs of prazosin is in progress.

Postural hypotension is one of the common side effects of α_1-receptor blockers frequently reported by patients. Prazosin, being the most prominent in this regard, causes orthostatic hypotension in majority of the patients. Based on our findings, we take the liberty to suggest that prazosin mediates fall in blood pressure through blockade of not only α_1-receptors but also AT_1 receptor as well. An absence of baroreceptor-mediated reflex in patients receiving prazosin therapy is responsible for orthostatic

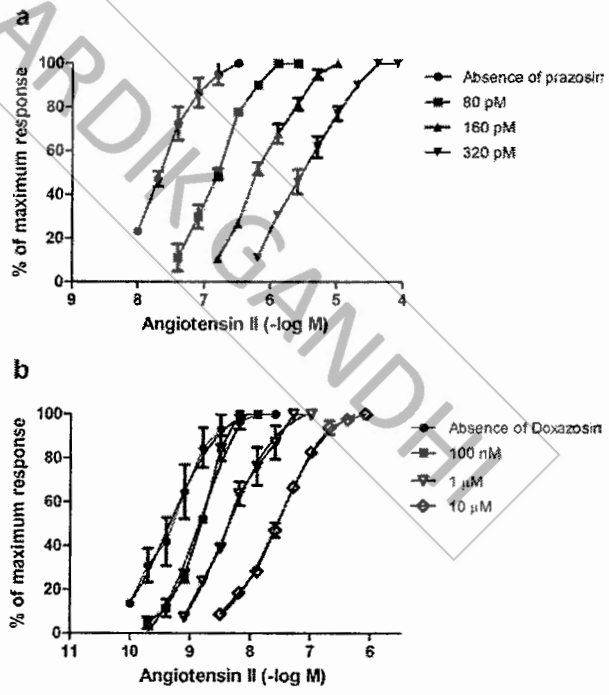

Figure 1. Inhibition observed with prazosin and doxazosin in Ang II-induced contractions of rat thoracic aorta. Results are expressed as percentage of maximum contractions obtained on aortic strips incubated with solvent. Values are represented as mean ± SEM of 4–5 experiments.

hypotension. The baroreceptor reflex is regulated through the sympathetic as well as the parasympathetic nervous system. An activation of the SNS results in a consequential release of norepinephrine. The primary action of norepinephrine released in this manner is to increase the resistance of blood vessels, ultimately maintaining the blood pressure. Second, the released norepinephrine also stimulates the secretion of renin and, ultimately, formation of Ang II. This *de novo* formation of Ang II can cause direct vasoconstriction mediated through AT_1 receptors. But as per our findings, prazosin blocks this arm through competitive blockade of AT_1 receptors. As a novel finding, we can therefore assume that potency of α_1-blockers is additionally a function of AT_1 receptor antagonism also. These results are amazing and throw new light on the antihypertensive effect of prazosin type of compounds. Such a novel finding will be able to delineate new facets of mechanism of α_1-antagonists. The high potency of this class of antihypertensive agents receives a good stand based on our findings. This finding will be of use in designing potent dual-acting antihypertensive agents.

Declaration of interest

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