Abstract

Of

The Thesis Entitled

“Design, Synthesis and Biological Evaluation of Novel PI3Kδ Inhibitors for the Treatment of Leukemia and Inflammatory Diseases”

To be submitted to The Maharaja Sayajirao University of Baroda

For the Degree

Of

DOCTOR OF PHILOSOPHY

In Chemistry

By

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Under guidance of

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To be submitted to The Maharaja Sayajirao University of Baroda,
For the degree of DOCTOR OF PHILOSOPHY

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Title of the Thesis: "*Design, Synthesis and Biological Evaluation of Novel PI3Kδ Inhibitors for the Treatment of Leukemia and Inflammatory Diseases*"

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CHAPTER I

1. Introduction

1.1. Inflammation

Inflammation is derived from the Latin word inflammare which means "to set on fire with passion”. Inflammation is a part of body's defence mechanism through which the immune system recognizes and removes harmful foreign particle. It also initiates the healing process. Key symptoms of inflammation are redness (rubor), swelling (tumor), heat (calor), pain (dolar) and loss of tissue function. Unresolved acute inflammation conditions generally lead to chronic inflammatory condition, which result into many inflammatory diseases.

1.1.1. Pathophysiology of inflammation

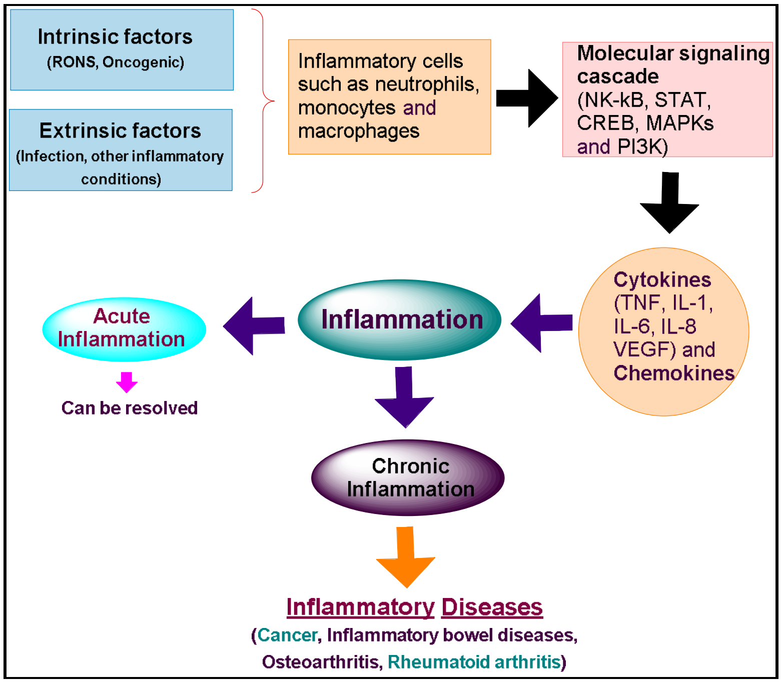


Figure 1: Pathophysiology of inflammation

Inflammation is a protective mechanism initiated in response to tissue damage caused by infection, trauma and chemical exposure **[Figure 1]**. As the process of inflammation proceeds, various other players of inflammation such as lymphocytes and macrophages are stimulated and recruited to the site of inflammation. When inflammatory responses become chronic, cell mutation and proliferation can result, often creating an environment that is conducive to the development of cancer or can trigger the development of various types of inflammatory diseases such as Cardio vascular diseases (CVD), Neurological Disease, Autoimmune Disease (AD), Rheumatoid arthritis (RA) and Cancer.

1.1.2. Current Pharmacologic Therapies for the treatment of RA

Medication for RA mainly divided into two categories; Traditional therapies include Non-steroidal anti-inflammatory drugs (NSAIDs), Corticosteroids (Steroids), Diseases modifying anti-rheumatic drugs (DMARDs). Other category is targeted therapies such as Biological agents (proteins) and Kinase inhibitors (small molecules). There are many kinase inhibitors approved for RA such as Tofacitinib, Baricitinib (JAK inhibitors), Fostamatinib (SYK inhibitors) and some are under late stage (Phase 3) of clinical development such as Leniolisib, Nemiralisib and Seletalisib (PI3Kδ inhibitors).

1.1.3. Current Pharmacologic Therapies for the treatment of cancer

There are many therapies available for the treatment of cancer. Cancer therapies are divided in to two main types traditional and targeted. Traditional therapy includes Surgery, Chemotherapy, Radiation and Bone marrow transplant, whereas target therapy includes Immunotherapy (biological therapy) and small molecule inhibitors.

All traditional therapeutics were developed using a practical approach without detailed understanding of their mode of action and therapeutic target. Similarly, biological agents have many drawbacks such as high cost, cold storage condition, stability, logistic and immunological adverse effects. Idelalisib (PI3Kδ inhibitor) is currently approved for chronic lymphatic leukemia (CLL), follicular lymphoma (FL). Due to adverse side effect associated with liver, lungs and immune system, Idelalisib is not approved for treatment of inflammatory diseases.

In this regards, as a part of our ongoing research, we found development of PI3Kδ inhibitor could be an attractive targeted based therapy for safe and effective treatment of RA and cancer. We decided to develop selective, potent and orally bioavailable PI3Kδ inhibitor.

1.2. Kinase

Kinases are enzyme which catalyzed the phosphorylation of substrate (Protein, Carbohydrate and Lipid) via transfer of phosphate from high energy ATP molecule to give phosphoanhydride and ADP, **Figure 2**. These phosphorylated substrate act as a messenger in signal transduction pathway to activate various cellular function such as cell growth, cell division, metabolism, protein regulation etc. which are important for normal cell physiology.

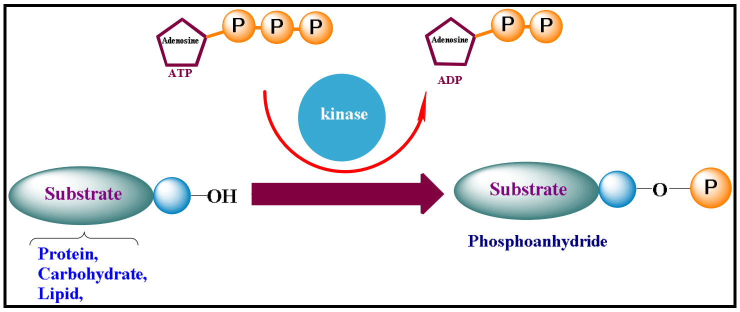


Figure 2: Kinases; catalyzed the phosphorylation of substrate

Depending upon substrate type, kinases are classified in the three main types: Protein kinase, Carbohydrate kinase and Lipid kinase. In protein kinase substrate is amino acid. In carbohydrate kinase substrate is sugar whereas in lipid kinase the substrate is lipid of plasma and cell membrane. **Phosphatidylinositol-3-kinase** (PI3K) belongs to lipid kinase family.

1.2.1. Classification of PI3K

PI3Ks has been classified into three classes (I, II and III) based on substrate specificity, sequencing homology and types of regulatory subunits, **Table 1**. The class I PI3Ks consists of four kinases (PI3K-α, β, δ and γ) and further grouped into two sub-classes: class IA and class IB. The PI3Kα and β are expressed in a wide variety of tissues and organs. PI3Kγ is found mainly in leukocytes, while expression pattern of PI3Kδ is restricted to spleen, thymus, hematopoietic cells and peripheral blood leukocytes. PI3Kγ and PI3Kδ are mainly expressed in rheumatoid arthritis (RA) synovium, which regulate innate and adaptive immune responses.

Table 1: PI3K enzyme classification

Class	**Structural features of catalytic subunits**	**Subunits**	
		Catalytic	**Regulatory (Adaptor)**
IA		**p110 α, β and δ**	**p85α, p85β and p55γ**
IB		**p110γ**	**p101**
II		**PI3K-C2α,β and δ**	**C2 domain at C-terminal**
III		**Vps34p analogues**	**p150**
● **(Regulatory binding)** ◆ **(Ras binding)** ⬬ **(C2 Domain)** ■ **(Phosphoinositide kinase)** ⬬ **(Kinase main domain)**			

Class I PI3Ks are heterodimers composed of catalytic subunit (p110) and a regulatory (adaptor) subunit (p85). It is further subdivided into class IA and IB on the basis of regulatory sub units. Class IA PI3K has three isoforms namely PI3Kα, PI3Kβ and PI3Kδ, with the respective p110 catalytic subunit bound to the p85 regulatory subunit. Class II PI3K has a single catalytic subunit (adaptor subunit is absent) with C2 domain at C-terminal and has three PI3K isoforms; PI3KC2α, PI3KC2β and PI3KC2γ. Class III PI3K consists of a single catalytic Vps34 subunit and regulatory subunit p150.

1.2.2. Biochemistry of Phosphatidylinositol-3-kinase

In the cell, the main biochemical role of PI3Ks is to phosphorylate 3'-OH position of the inositol ring of PIP_2 (Phosphatidylinositol 4,5-bisphosphate) to give PIP_3 (Phosphatidylinositol 3,4,5-trisphosphate) which work as secondary messenger to activate other pathways **[Figure 3]**. It is negatively regulated trough dephosphorylating by phosphatase and tensin homolog (PTEN) gene.

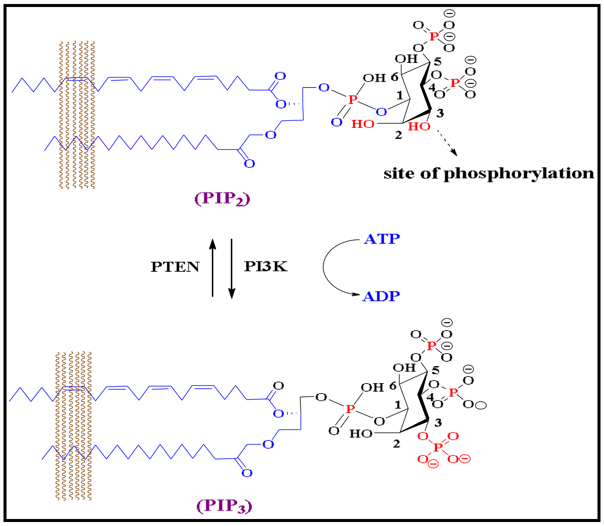


Figure 3: Phosphorylation with PI3K enzyme in cell

1.2.3. PI3K Signal transduction pathways

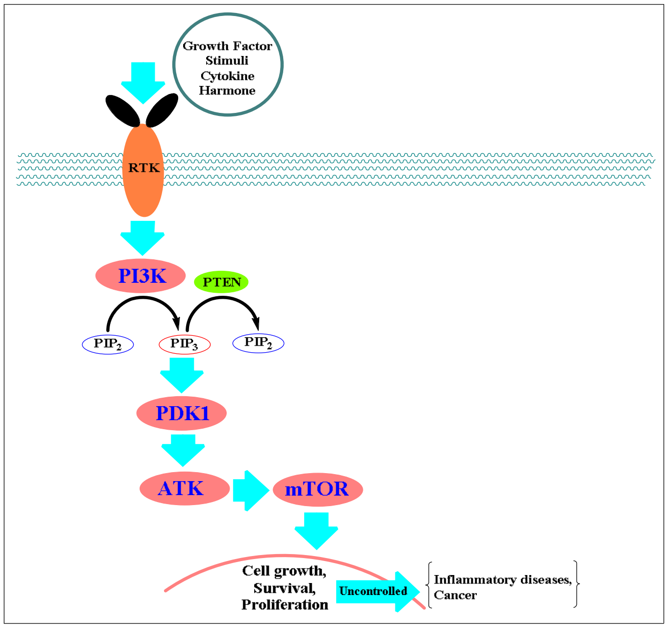


Figure 4: PI3K activation pathway

Initially RTK (Receptor tyrosine kinase) is normally activated by extracellular stimuli, growth factors, cytokines or hormones, leading to dimerization to release specific protein to activate PI3K **[Figure 4]**. Upon activation, PI3K catalyzes the phosphorylation of PIP_2 to produce PIP_3, which trigger many other signaling pathways such as PDK_1 (3-phosphoinositide-dependent protein kinase-1), ATK or PKB (Protein kinase B) and m-TOR (Mammalian target of rapamycin) pathway. Above signal transduction is important to control many cellular functions such as cell growth, cell survival, cell proliferation, apoptosis etc. Uncontrolled activation of any enzyme (PI3K, RTK, PDK_1, ATK and m-TOR) in this pathway leads to many inflammatory diseases ultimately ending with cancer. PTEN prevents activation of above mention downstream kinases.

1.2.4. Clinical Significance for targeting PI3Kδ isoform

PI3K is believed to be one of the key therapeutic targets for inflammatory diseases and cancer treatment because it is observed that hyperactivity of PI3K signaling is significantly correlated in autoimmune disorder and tumor progression. The p110δ isoforms of PI3K have gained more attraction as pharmacological targets in the treatment of RA and hematological malignancies because of its known effects on a variety of immune cells. Selectively inhibition of PI3Kδ isoform with suitable inhibitor is already documented in the literature. It is also supported through *in vivo* studies (PI3K gene knock out/deletion) where in PI3Kδ gene deleted mice showed depressed immunological response. Recently, more efforts are directed towards the development of isoform selective inhibitors, particularly PI3Kδ selective inhibitors, for the safe and effective treatment of hematological malignancy and inflammatory disorders. There are many clinically approved PI3Kδ inhibitors, some are under clinical development.

1.2.5. PI3K Inhibitors

PI3Kinhibitors are listed in below table 2, some of them are launched and some are in clinical trials.

Table 2: PI3K Inhibitors in clinical development

Sr. No.	Selectivity	Generic Name	Development Phase	Indication	Originator
1	PI3K (δ)	Idelalisib Zydelig™	**Approved (July 2014)**	Chronic lymphocytic leukemia, Small lymphocytic lymphoma	Gilead Science
2	PI3K (δ)	Leniolisib	Late III	Activated PI3Kδ syndrome COPD, Asthma	Novartis
3	PI3K (δ)/ CK1ε	Umbralisib Ukoniq	**Approved (February 2021)**	Marginal zone lymphoma, Follicular lymphoma	TG Therapeutic
4	PI3K (δ)	-	II	Rheumatoid arthritis	Amgen
5	PI3K (δ)	Parsaclisib	II	Hematological malignancies, Solid tumors	Incyte Corporation
6	PI3K (α/δ)	Copanlisib Aliqopa™	**Approved (September 2017)**	Hematological and solid malignancies	Bayer
7	PI3K(δ/γ)	Duvelisib Copiktra™	**Approved (September 2018)**	Chronic lymphocytic leukemia, inflammatory Diseases	Infinity/ Verastem
8	PI3K (δ/γ)	Tenalisib	II	Relapsed/Refractory Indolent Non-Hodgkin's Lymphoma	Rhizen
9	PI3K α	Alpelisib Piqray™	**Approved (May 2019)**	Advanced or Metastatic breast cancer	Novartis
10	PI3K α	Taselisib	Late III	Metastatic breast cancer	Roche
11	PI3K α	Serabelisib	II	Breast cancer, Endometrial cancer Renal cell carcinoma	Takeda
12	PI3K/mTOR	Gedatolisib	III	Solid Tumor, Brest Cancer	Pfizer

13	PI3K/mTOR	Dactolisib	III	Advance solid tumor	Novartis
14	PI3K/mTOR	Samotolisib	II	Neoplasm	Eli Lilly and Co.
15	PI3K/mTOR	Bimiralisib	II	Solid Tumor, Brest Cancer	Piqur Therapeutic
16	PI3K/mTOR	Omipalisib	II	Advance Solid Tumor	GSK
17	PI3K/mTOR	Apitolisib	II	Breast cancer, Endometrial cancer	Genentech
18	Pan PI3K	Pictilisib	Terminated	Solid Tumor, Brest Cancer	Roche
19	Pan PI3K	Buparlisib	III	Breast Neoplasm	Novartis
20	Pan PI3K	Pilaralisib	II	Endometrial Neoplasm	Sanofi

Idelalisib (GS-1101/CAL-101/ZydeligTM) is the only PI3Kδ selective inhibitor launched till now (July 2014) for Chronic lymphocytic leukemia, Small lymphocytic lymphoma. The most common side effects of Idelalisib are decreased in neutrophil count, hypertriglyceridemia, hyperglycemia, hepatotoxicity, ALT (alanine aminotransferase) increased and fever. Leniolisib (CDZ173) is an oral and selective inhibitor of the PI3Kδ isoform develop by Novartis. It is under clinical trial for activated PI3Kδ syndrome (APDS). Umbralisib (TGR-1202/ Ukoniq) is an oral, selective PI3Kδ/ CK-1ε inhibitor approved (February 2021) for marginal zone lymphoma and follicular lymphoma. Common side effects are low blood cell counts, decreased blood platelets, abdominal pain, pneumonia, urinary tract infection (UTI) and fever.

Duvelisib (IPI-145/CopiktraTM) is an approved (September 2018), oral dual inhibitor of PI3K-δ/γ. The most common side effect associated with Duvelisib are diarrhea, nausea, low blood cell counts, bone pain, muscle pain, fever, cough, tiredness or cold symptoms such as stuffy nose, sneezing, sore throat. Copanlisib (BAY 80–6946/AliqopaTM) is approved in September 2017. Copanlisib has many side effects such as burning, crawling, itching, numbness, prickling, cracked lips, diarrhea, difficulty in swallowing, lack or loss of strength swelling or inflammation of the mouth etc.

Dactolisib (NVP BEZ235) is an oral dual PI3K/mTOR inhibitor; it is in phase II clinical trials. Dactolisib has severe side effects such as elevated blood glucose level, ALT elevation, diarrhoea, hair loss, mucosal inflammation and skin rash. Apitolisib (GDC-0980/RG7422) and Gedatolisib (PF-05212384/PKI-587) are orally bioavailable dual inhibitor of PI3K and mTOR kinase, currently in phase II and phase III clinical development. Buparlisib (NVP-BKM120), Pilaralisib (XL-147) and Pictilisib (GDC-0941) are orally pan-class I, reversible inhibitors of PI3K.

Currently available PI3K inhibitors, especially PI3Kδ inhibitors have significantly improved the clinical situation, but exhibits adverse effects such as neutropenia, hypertriglyceridemia, hyperglycemia, diarrhea/colitis, skin rashes, pyrexia, or liver enzyme elevation with severe organ toxicity. Therefore due to undesired toxic parameter most of the PI3Kδ inhibitors are not targeted for RA treatment. So there is need to explore a new pharmacophore and strategies to design potent, selective and safe PI3Kδ inhibitor to overcome the side effects associated with existing inhibitors for the safe and effective treatment of RA treatment and chronic lymphocytic leukemia (CLL).

1.3. Objectives

Our objective is to design a novel, orally active, PI3Kδ selective inhibitor as anti-inflammatory agent for the treatment of RA and anti-cancer agent to treat B-cell CLL. Literature survey reveals that, PI3K isoform selectivity (particularly PI3Kδ) can be achieved by modulating pharmacophore structure or substituent on it. As a part of ongoing research activity, in the current investigation, we decided to optimize two standards compounds (Dactolisib and INK-654/666), wherein we mainly adopted bioisosteric replacement strategy to develop novel PI3Kδ inhibitors. In each section we have described:

a) Design of imidazo-quinoline derivative, benzofuran based pyrazolo-pyrimidine derivatives and 2,4-disubsitued quinoline pyrazolo-pyrimidine derivatives as PI3Kδ selective inhibitors.
b) Synthesis and characterization of designed PI3Kδ inhibitor
c) *In vitro* PI3Kδ inhibitory activity study
d) Isoform selectivity (α/β/γ/δ)
e) Pharmacokinetic study
f) *In vivo* study
g) Profiling study
h) Safety pharmacology study
i) Molecular modelling study

1.4. Design strategy

As discuss in the earlier section, knowing the potential side effects associated with the PI3K isoforms inhibitors. Recently, more efforts are directed towards the development of isoform selective inhibitors, particularly PI3Kδ selective inhibitors, for the effective treatment of autoimmune, inflammatory diseases, such as RA and CLL.

Berndt et al. reported PI3Kδ crystal structure in complex with IC-87114. PI3Kδ enzyme structure shows have four regions in the binding site; a) hinge region (ATP-adenine binging site), b) specificity pocket, c) affinity pocket and d) hydrophobic region **[Figure 5]**. Thus for potent PI3Kδ selective inhibition, compound must interact with the key amino acid residues in all four region. Thus, to achieve all the key interactions, molecule adopts “propeller shaped” orientation. Most of the PI3Kδ selective inhibitors either approved or in clinical development possess “propeller shaped” orientation.

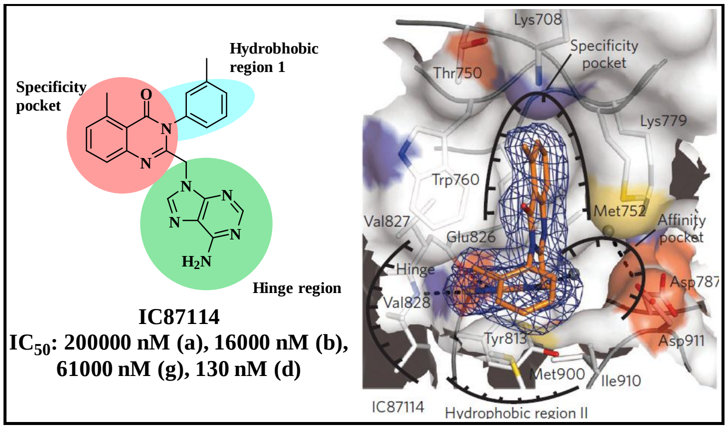


Figure 5.PI3Kδ crystal structure complexed with IC-87114

We aim to design, novel, potent and orally bioavailable PI3Kδ selective inhibitor mainly by favoring the suitable interaction of the designed molecule with PI3Kδ ATP binding pockets targeting hinge region, specificity pocket, affinity region and hydrophobic region.

In Chapter 2, we synthesized imidazo-quinoline based PI3Kδ inhibitor by introducing spacer in Dactolisib, so that newly designed compounds can fit in the ATP binding site of PI3Kδ protein **[Figure 6].** Initially, *in silico* modelling study of Dactolisib and new design compounds were done in PI3Kδ enzyme, which showed promising result. New designed compounds showed favorable interaction (hydrogen bonds) in hinge region, specificity pocket, hydrophobic region and affinity pocket. Hence, we expect that these newly designed, compounds with spacer would be novel, potent and orally bioavailable PI3Kδ selective inhibitors by retaining the key interactions of inhibitors, in the PI3Kδ ATP binding pocket.

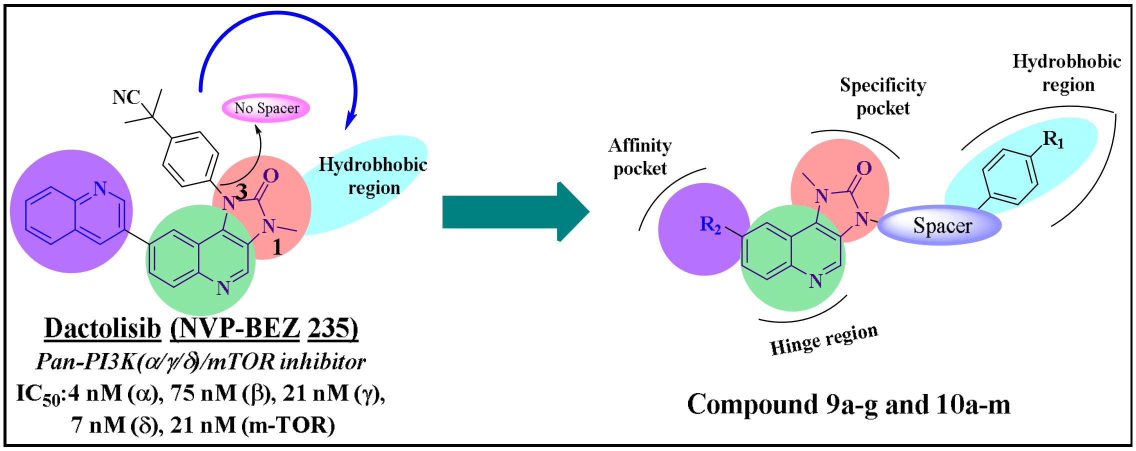


Figure 6: Imidazo-quinoline derivatives

We designed to two new scaffolds as PI3Kδ inhibitor by bioisosteric replacement of quinoline ring in INK-666 with benzofuran and 2,4-disubstituted quinoline ring.

Chapter 3 contains new scaffold, where 2,3-disubstituted quinoline ring of INK-666/INK-654 was bioisosterically replaced with benzofuran ring to get novel, potent and selective PI3Kδ inhibitors with benzofuran analogue **[Figure 7]**.

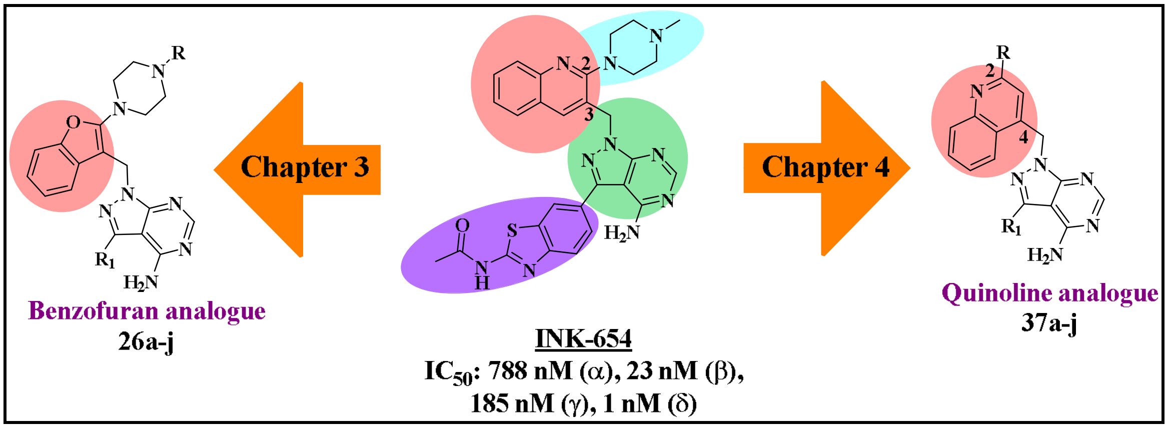


Figure 7: Benzofuran and 2,4-disustituted quinoline derivatives as PI3Kδ inhibitor

Literature around Intellikine scaffold (INK-666/INK-654) showed that the SAR is well established for 2,3-disubstitued quinoline ring where as its 2,4-disubstituted quinoline derivative are not discovered. Hence, in Chapter 4, we synthesized 2,4-disubstituted quinoline based PI3Kδ inhibitor to explain SAR **[Figure 7]**.

CHAPTER II

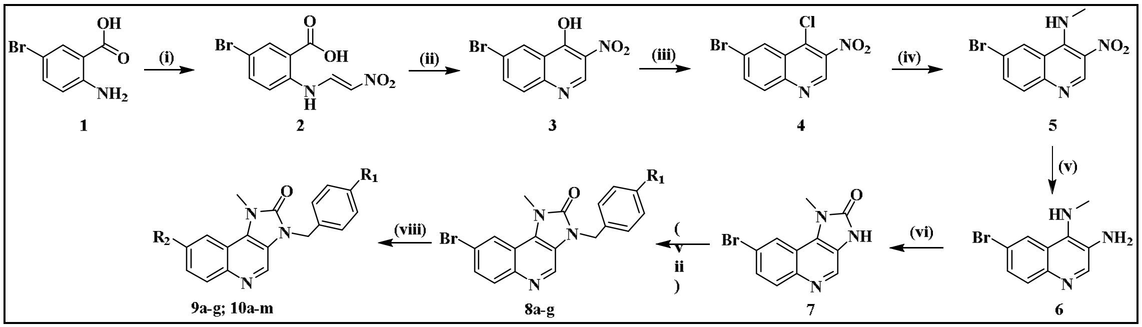
2. Designing, synthesis and biological evaluation of imidazo-quinoline derivatives as PI3Kδ selective inhibitors.

Chapter 2 is divided into two parts, In Part 1 synthesis of title compounds **9a-g** and **10a-l** using scheme 1 and boronate intermediates using scheme 2 is described. All the compounds were characterized by using different spectral techniques like ^{1}H, ^{13}C NMR, ESI-MS and UPLC analysis. In Part 2, *In vitro* PI3Kδ inhibitory activity, PI3K Isoform selectivity study, *In vivo* efficacy study, Anti-proliferative activity, CYP (Cytochrome) inhibition study, hERG inhibition study, Safety pharmacology study and Molecular modelling study of imidazo-quinoline derivatives is reported.

2.1. Synthesis of imidazo-quinoline based compounds 9a-g and 10a-l

Synthesis of imidazo-quinoline derivatives (**9a-g** and **10a-l**) was carried out as depicted in Scheme 1 following the modified literature procedure.

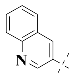
Scheme 1: Synthesis of compounds **9a-g** and **10a-l**.

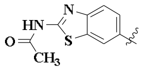
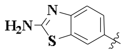


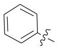
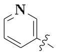
Reagents and conditions: i) conc. HCl, water, 26 ^{o}C, 6h, then nitro methane, NaOH, water, conc. HCl, 26 ^{o}C, 16h; ii) KOAc, acetic anhydride, 120-125 ^{o}C, 4h; iii) $POCl_3$, 120 ^{o}C, 4h; iv) Me-NH_2, TEA, DCM, 26 ^{o}C, 12h; v) $SnCl_2.2(H_2O)$, EtOAc, 26^{o}C; vi) Phosgene (20% in toluene), TEA, DCM, 0-26^{o}C; vii) R_1-Ar-CH_2-X (Cl or Br), NaH, THF, 0-26 ^{o}C; viii) R_2-$B(OH)_2$,$PdCl_2(PPh_3)_2$, $KHCO_3$, DMF, H_2O, 90-95 ^{o}C, 1.5 h.

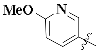
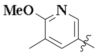
List of imidazo-quinoline derivatives **9a-g** and **10a-l**.

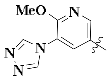
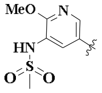
Compound	R_1
8a	$-C(CH_3)_2CN$
8b	$-CH(CH_3)_2$
8c	OCH_3
8d	CH_3
8e	NO_2
8f	F
8g	H

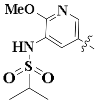
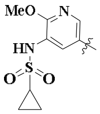
Compound	R_1	R_2
9a	$-C(CH_3)_2CN$	
9b	$-CH(CH_3)_2$	
9c	OCH_3	
9d	CH_3	
9e	NO_2	
9f	F	
9g	H	

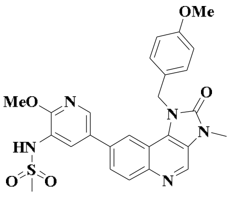
Compound	R_1	R_2
10a	OCH_3	
10b		

Compound	R_1	R_2
10c	OCH_3	
10d		

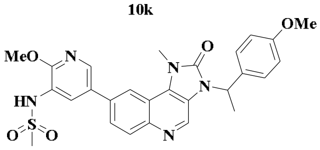
Compound	R_1	R_2
10e	OCH_3	
10f		

Compound	R_1	R_2
10g	OCH_3	
10h		

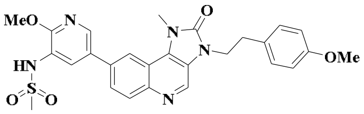
Compound	R_1	R_2
10i	OCH_3	
10j		



10k



10l

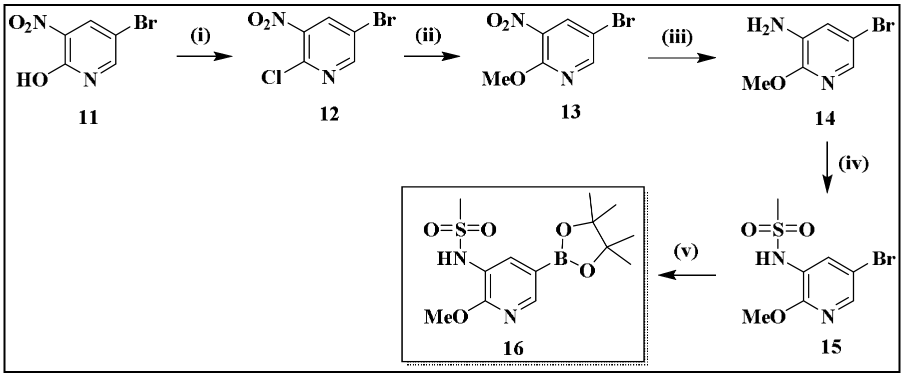


10m

2.1.1. Synthesis of 2-Methoxy-3-pyridyl boronate ester intermediate (16)

2-Methoxy-3-pyridyl boronate ester intermediate **16** was prepared using scheme **2** as shown below.

Scheme 2: Synthesis of 2-Methoxy-3-pyridyl boronate ester



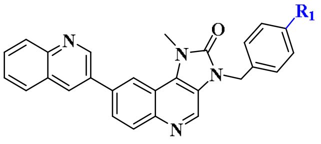
Reagents and conditions:(i) $POCl_3$, DIPEA, 80-85 °C, 4h; (ii) NaOMe, MeOH, 0-26 °C, 4 h; (iii) Fe, Conc. HCl, EtOH, 26 °C, 16h; (iv) Mesyl-Chloride, Pyridine, 26 °C, 24h; (v) Bis(pinacolato)diboron, $PdCl_2$(dppf), (1,4)-Dioxane, Reflux, 24h.

In imidazo-quinoline series 1, total 20 compounds were prepared (**9a-g** and **10a-m**) as PI3Kδ inhibitors. All the title compounds were prepared in good yield and characterized by various spectroscopic techniques. Assigned structures were found to be confirmed with spectral data.

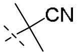
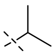
2.2. *In vitro* PI3Kδ inhibitory activity, selectivity and structure activity relationship (SAR)

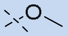
In vitro screening of compounds **9a-g** and **10a-m** was carried out using PI3K kinase activity assay kit (from Millipore) and the IC_{50} values are mentioned below.

Table 3: *In vitro* PI3Kδ inhibitory activity data of Imidazo-quinoline derivatives with modification **R_1** position (Set-1)



9a-g

Comp.	R_1	PI3Kδinhibition (%)$^{a, b}$	PI3Kδ IC_{50} (nM)^{c}
9a		56	ND
9b		73	28.3

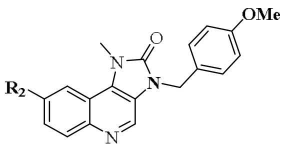
9c		**98**	**9.5**
9d	-CH_3	80	18.4
9e	-NO_2	62	ND
9f	-F	51	ND
9g	-H	22	ND
Dactolisib	-	92	8
Idelalisib	-	96	2.1

[a] All the data are shown as the mean for at least two experiments. [b] PI3Kδ inhibition at the concentration of 100 nM using PI3K Kinase Activity/Inhibitor assay Kit from Millipore. [c] The IC_{50} values for PI3Kδinhibition. ND: not detected

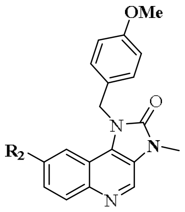
- Compound **9c** was most potent as compared to other compounds (**9c**, IC_{50}: 9.5 nM).

Further to improve PI3Kδ isoform selectivity, modelling studies were carried out, considering compound **9c** as primary lead. Based on these encouraging results of modelling study, 10 compounds (**10a-m**) were designed in Set-2 [Table 4] and subjected for *in vitro* screening and their respective results are as follows.

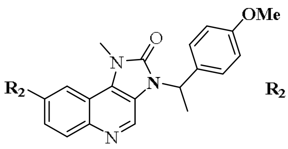
Table 4: Influence of modification at $\mathbf{R_2}$ position of quinoline moiety on PI3Kδ inhibitory activity and isoform selectivity (Set-2)



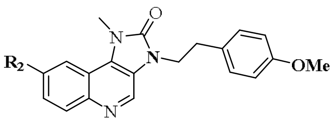
10a-j



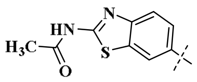
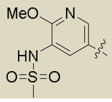
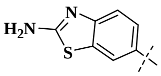
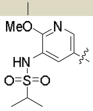
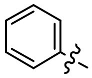
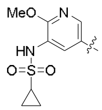
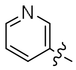
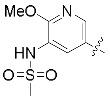
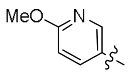
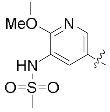
10k

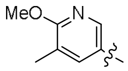
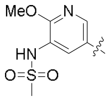
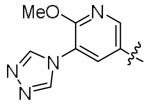


10l



10m

Comp.	**R_2**	**PI3Kδinhibition (%)[a,b]**	**PI3Kδ IC_{50} (nM)[c]**	**Comp.**	**R_2**	**PI3Kδinhibition (%)[a,b]**	**PI3Kδ IC_{50} (nM)[c]**
10a		52	ND	**10h**		**99**	**1.9**
10b		58	ND	**10i**		91	6.2
10c		72	29.4	**10j**		93	5.9
10d		81	20	**10k**		98	4.1
10e		87	11.4	**10l**		60	ND

10f		88	11.1	**10m**		56	ND
10g		62	ND	**Idelalisib**		96	2.1

[a]All the data are shown as the mean for at least two experiments. [b]PI3Kδ inhibition at the concentration of 100 nM using PI3K Kinase Activity/Inhibitor assay Kit from Millipore. [c]The IC_{50} values for PI3Kδ inhibition. ND: not detected.

- Compound **10h** with m-methanesulfonamide substitution was found to be the most potent (IC_{50}: 1.9 nM).
- Interchanging the substitution in **10h** on imidazole ring (methyl at N^3 and para-methoxy benzyl at N^1, **10k**), found to be two fold less active compared to **10h**.

2.2.1. Isoform selectivity study of 9c, 10h and 10k

Based on the preliminary PI3Kδ inhibitory activity results, most potent compounds (**9c**, **10h** and **10k**) were evaluated for their selectivity against PI3K isoforms (α, β and γ) and mTOR. Results are shown in below table 5.

Table 5: Isoform selectivity of compounds against PI3K (α, β, γ and δ) and mTOR activities

Comp.	**Biochemical IC_{50} [nM][a]**				
	PI3Kα[b]	**PI3Kβ[b]**	**PI3Kγ[b]**	**PI3Kδ[b]**	**mTOR[b] (p70S6K)**
9c	421	342	38	9.5	676
10h	**891**	**589**	**112**	**1.9**	**>1000**
10k	289	241	42	4.1	580
Dactolisib	5	79	6	8	14
Idelalisib	831	571	92	2.1	>1000

[a]The IC_{50} values are shown as the mean for at least two experiments.[b]PI3K inhibitory activity assay Kit (Millipore) was used to screen the test compounds.

- Compound **10h** (IC_{50}: 1.9 nM) demonstrated 469, 310, 59 and >500 fold selectivity over PI3Kα, β, γ and m-TOR respectively.

2.2.2. Anti-proliferative activities, CYP (Cytochrome) inhibition and hERG (human ether-a-go-go-related gene) liabilities for Compound 10h

- Compound **10h** was tested for its anti-proliferative activities against TMD-8 cell lines with Idelalisib as a reference compound. In anti-proliferative *in vitro* assay, **10h** and

Idelalisib exhibited potent anti-proliferative activity with an IC_{50} value of 340 and 795 nM respectively.

- Additional profiling studies of compound **10h** was carried out and it was found to be devoid of CYP (<10% CYP inhibition at 10 μM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and hERG liabilities (IC_{50}: > 30 μM).
- Highly potent and selective PI3Kδ inhibitor (**10h)** was selected for *in vitro* kinase profiling study at1 μM concentration, against 140 kinases and percent inhibition was found to <20% at 1 μM concentration, with **10h** across 140 kinase profiling panel.

2.2.3. *In vivo* pharmacokinetic (PK) and Pharmacodynamics (PD) studies of compound 10h

A comparative single dose PK profile of compounds **9h**, **10h** and Dactolisib was evaluated in male C57BL/6J mice and the various PK parameters were recorded **[Table 6]**.

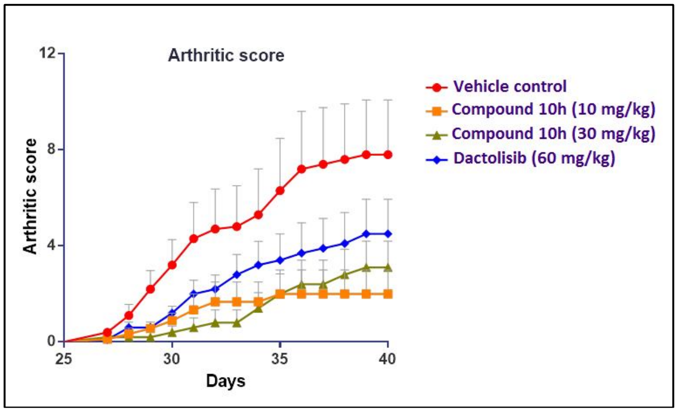
Table 6: Pharmacokinetic study parameters of **9c**, **10h** and **Dactolisib** in C57 mice

Comp	Tmax (h)	Cmax (μg/ml)	$t_{1/2}$ (h)	Cl (ml/min/kg), iv	AUC (0-α) h μg/ml	%F*
9c	0.38	127.61	2.31	22.9	329.08	14.77
10h	0.25	1278.49	3.48	8.24	3831.48	68.91
Dactolisib	0.21	273.83	1.88	72.5	243.64	37.82

[a] In male C57BL/6J mice (n=6), compounds were administered orally (p.o) at 3 mg/kgdose and plasma concentration was analysed by LC-MS, values indicate Mean ± SD.* Oral bioavailability (%F) was calculate wrt to iv AUC. Compounds **9c**, **10h** and Dactolisib administered at 1 mg/kg dose, iv AUC: 742.56, 1852.95 and 215.14 respectively.

In vivo anti-inflammatory activity of **10h** was checked in Collagen Induced Arthritis (CIA) mice model as shown in **Figure 8**.

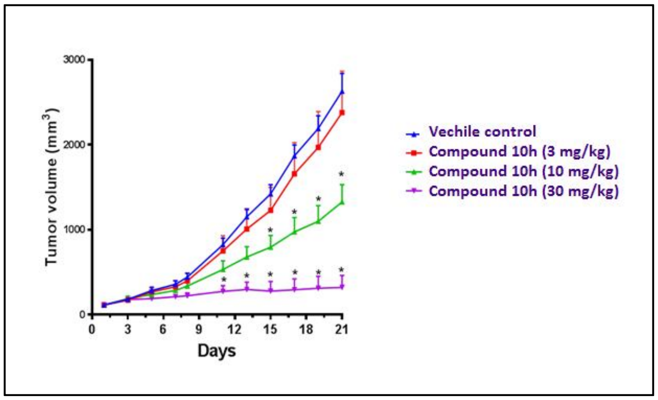
Figure 8: Effect of Compound **10h** and **Dactolisib** in CIA mice model



- As shown in the **Figure 8**, standard and **10h** showed significant reduction in the arthritic score, compared to vehicle control (untreated group).
- Body weights of the animals were also recorded 3 times a week as a measure of treatment related side effect and **10h** showed no significant reduction in the body weight.

In vivo anti-tumor activity of **10h** was checked in male SCID mice xenograft model (inoculated with TMD-8 cells) as shown in **Figure 9**.

Figure 9: *In vivo* anti-tumor activity of Compound **10h** in SCID mice xenograft model



- **10h** showed complete inhibition of tumor volume compared to vehicle control.
- **10h** showed no significant reduction in the body weight, even at 30 mg/kg dose.

2.2.4. Safety pharmacology study compound 10h

Daily oral administration of compounds **10h**, at 100 mg/kg over a period of 2 weeks did not affect the survival of Wistar rats, with no adverse effect related to gross pathology, clinical signs, body weight and food consumption were noticed as compared to the control group.

Compound **10h** treated groups showed no changes in the other key organs (brain, heart, kidney, spleen and liver) weights, **Table 7**.

Table 7: Relative organ weights (%) after 14 days repeat dose treatment with compound **10h**

Organs	**Compounds**	
	Control (Vehicle)	**10h^a (100 mg kg^{-1}, po, bid)**
Brain	0.680±0.025	0.665±0.02
Kidney	0.790±0.034	0.805±0.02
Heart	0.340±0.008	0.350±0.007
Spleen	0.260±0.006	0.255±0.01
Liver	3.580±0.15	3.610±0.078

^{a} Values expressed as mean ± SD: n=9, Male WR, dose 100 mg kg-1, po (bid), 14 days repeated dose toxicity study.

To assure hematological parameter and serum biochemistry, rats were anesthetized 24 h post treatment with compound **10h** and blood samples were collected. The whole blood was centrifuged at 3000 rpm, using a centrifuge at 37 ^{o}C for 15 mins and serum alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were assayed, using a diagnostic kit (Boehringer Mannheim). Total bilirubin (TBILI) was also analysed to check hepatotoxicity parameters.

Table 8: Hematological parameters and serum chemistry of compound **10h**

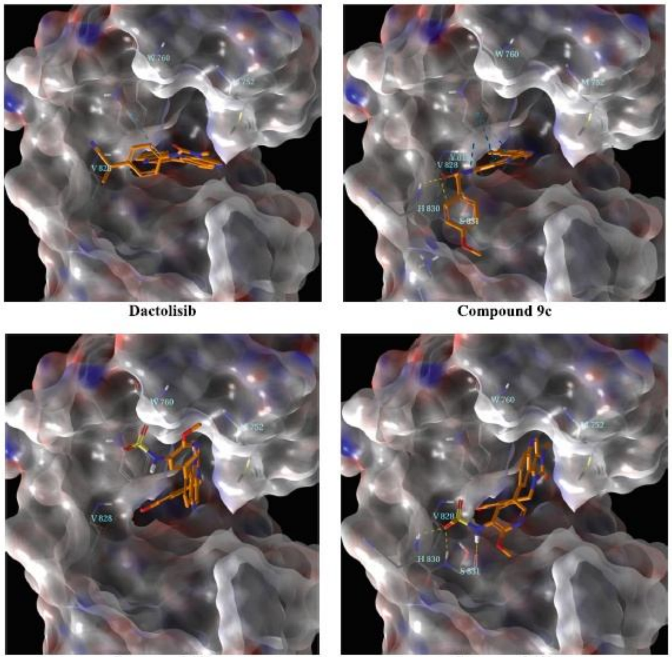
Parameters	**Compound**	
	Control	**10h^a**
RBC (10^6 μl^{-1})	7.15 ± 0.10	7.35 ± 0.28
WBC (10^3 μl^{-1})	9.21 ± 0.35	9.01 ± 0.28
TBILI (mg dL^{-1})	0.14 ± 0.10	0.15 ± 0.15
ALT (U L^{-1})	20.10 ± 0.80	21.05 ± 1.25
ALP (U L^{-1})	134.10 ± 6.10	122.10 ± 10.40
AST (U L^{-1})	145.10 ± 10.60	138 ± 1.20

^{a} Values expressed as mean ± SD: n=9, Male WR dose 100 mg kg-1, po (bid), 14 days repeated dose toxicity study

As shown in above table, **Table 8**, the hematological parameters white blood cell (WBC) and red blood cell (RBC) of compounds **10h** were found to be comparable to that of control animals. Similarly, compound 10h showed no significant changes in ALP, AST, ALT and TBILI as compared to the control group. Whereas Idelalisib has unscheduled death, hepatotoxicity, increased ALT, AST and ALP.

2.2.5. Molecular modelling study

The PI3Kδ crystal structure was retrieved from the RCSB Protein Data Bank (PDB: **4XE0**) was used to rationalised the potent and selective PI3Kδ inhibitory activity of **9c**, **10h**, **10k** and Dactolisib, **Figure 10**.



Dactolisib   Compound 9c

Compound 10h   Compound 10k

Figure 11: The Glide docking studies of Compounds **9c**, **10h**, **10k** and Dactolisib into site of PI3Kδ (PDB ID:**4XE0**). Compounds are shown as sticks. Hydrogen bonds are shown as yellow dash lines.

- As shown in the **Figure 10**, all the three **9c**, **10h** and **10k** compounds showed favourable interactions with the PI3Kδ ATP-binding pockets, imidazo-quinoline ring served as the hinge binder and it forms key hydrogen bonds with Val_{828} and Glu_{826}.
- Propeller-shaped conformation of **10h** favours to accommodate methanesulfonamide pyridyl ring of 10h, in the affinity pocket which strongly interacts with Asp_{911}.
- Additional hydrogen bonding of **10h** with Trp_{760} and propeller shape orientation contributes towards improved PI3Kδ selectivity and inhibitory activity.

2.3. Conclusion

In conclusion, we have synthesized and evaluated two sets of novel series of 1,3-dihydro-2*H*-imidazo[4,5-*c*]quinolin-2-one derivatives as selective PI3Kδ inhibitors. In first set, appropriate modifications were carried out in the imidazo-quinoline based pharmacophore, which led to an identification of a single digit nM potent PI3Kδ inhibitor (**9c**), with moderate isoform selectivity.

In set 2, further structure-activity relationship (SAR) studies on the 8th position of **9c** resulted in to the discovery of *N*-(2-methoxy-5-(3-(4-methoxybenzyl)-1-methyl-2-oxo-2,3-dihydro-1H-imidazo[4,5-*c*]quinolin-8-yl)pyridin-3-yl)methanesulfonamide (**10h**) that showed improved isoform selectivity, PK profile and good efficacy in a CIA and xenograft animal models.

In the repeat dose acute toxicity study, compound **10h** showed no adverse changes related to gross pathology, clinical signs and liver toxicity, hepatotoxicity, increased ALT, AST and ALP. The molecular docking study of compound **10h** indicated key hydrogen bonding interactions, which justifies its selective PI3Kδ inhibitory activity.

CHAPTER III

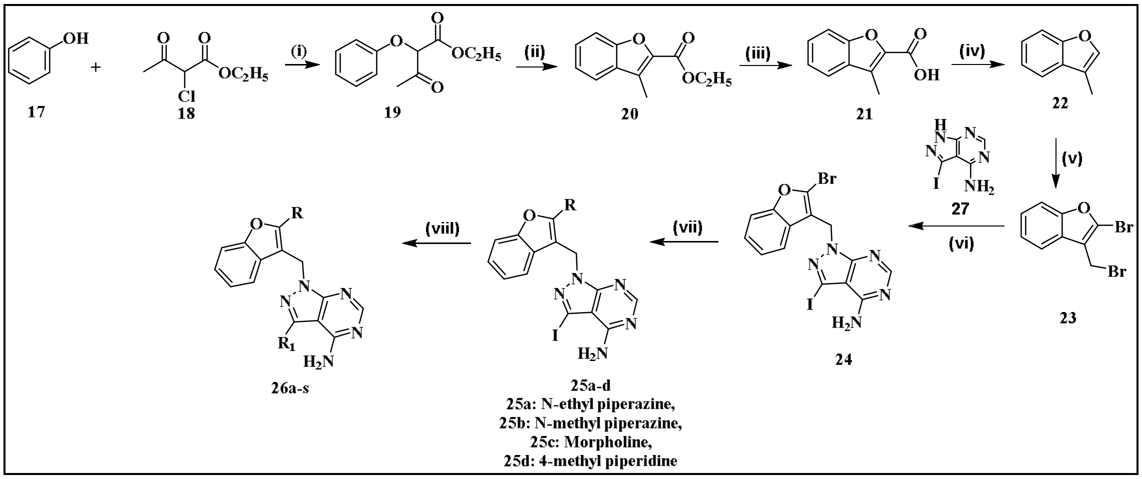
3. Design, synthesis and biological evaluation of benzofuran based pyrazolo -pyrimidine derivatives as PI3Kδ selective inhibitors.

Chapter 3 is divided into two parts, In Part 1 synthesis of title compounds **26a-s** using scheme 3 is described. All the compounds were characterized by using different spectral techniques like ^{1}H, ^{13}C NMR, ESI-MS and UPLC analysis. In Part 2, *In vitro* PI3Kδ inhibitory activity, PI3K Isoform selectivity study, CYP (Cytochrome) inhibition study, hERG inhibition study and Molecular modelling study of imidazo-quinoline derivatives are reported.

3.1. General procedure for the synthesis of benzofuran based compounds 26a-s

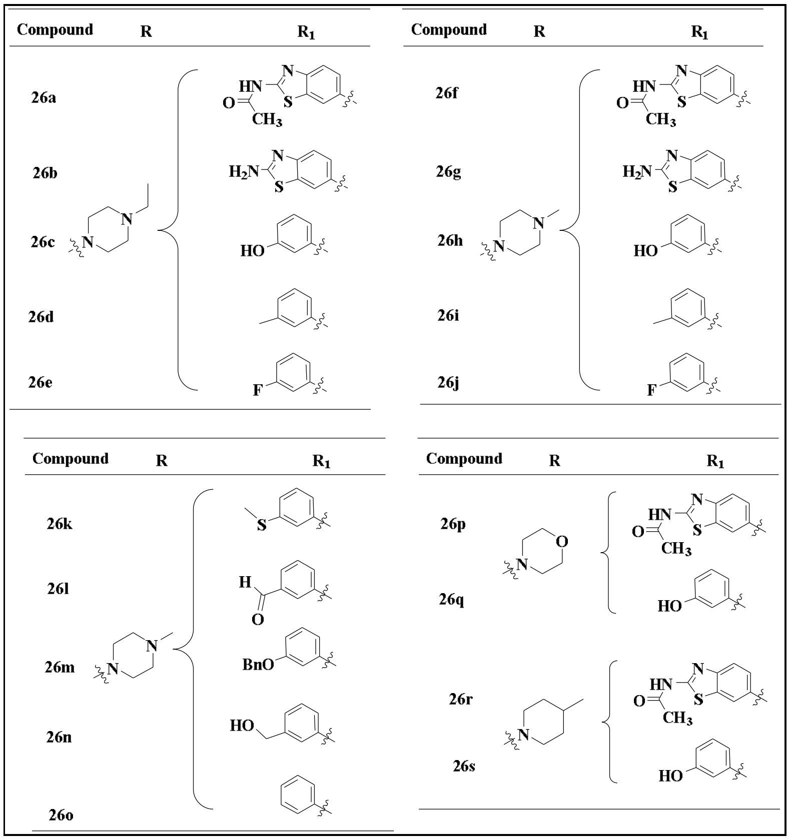
Synthesis of imidazo-quinoline derivatives (**26a-s)** was carried out as depicted in Scheme 1 following the modified literature procedure.

Scheme 3: Synthesis of Benzofuran pyrazolo pyrimidine compounds **26a-s**.



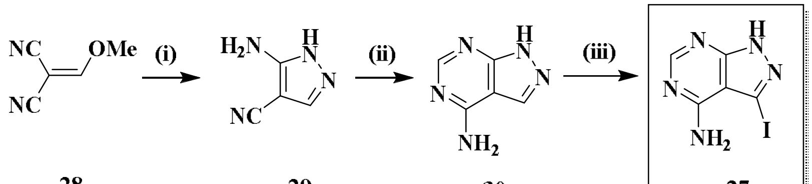
Reagents and conditions:(i) K_2CO_3, Acetone, Reflux, 5h; (ii) Conc.H_2SO_4, 0-5 °C, 4h; (iii) 10% KOH, 26 °C, 2h; (iv) 280 °C, 2h; (v) NBS, CCl_4, Reflux, 5h; (vi) t-BuOK, DMF, 26 °C, 15h; (vii) **R** (Secondary amine), (1,4)-Dioxane, Reflux, 12h; (viii) Aryl boronic acid (**R_1**), $PdCl_2$ $(PPh_3)_2$, $KHCO_3$, DMF: H_2O, 90 °C, 2h.

List of benzofuran pyrazolo pyrimidine compounds **26a-s**.



3.1.1. General procedure for the synthesis of 3-iodo-1H-pyrazolo [3, 4-d] pyrimidin-4-amine (27)

Scheme 4: Synthetic scheme of 3-iodo-1H-pyrazolo [3, 4-d] pyrimidin-4-amine **(27)**.



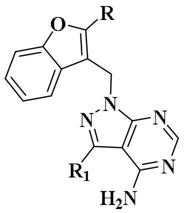
28 29 30 27

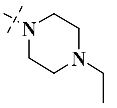
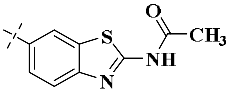
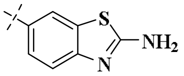
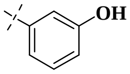
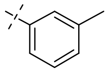
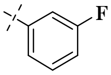
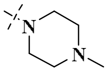
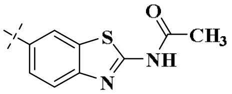
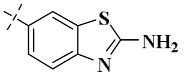
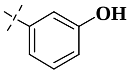
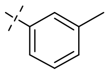
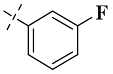
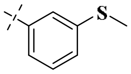
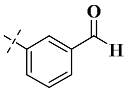
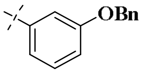
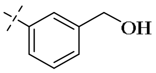
Reagents and conditions:(i) NH_2-NH_2, 80-85 °C, 5h; (ii) Formamide, 180-185 °C, 4h; (iii) NIS, DMF, 80-85 °C, 16 h.

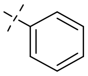
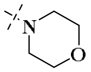
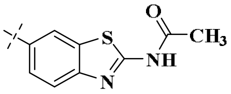
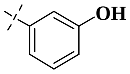
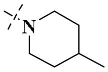
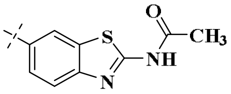
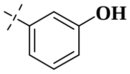
In Series-1, total **19** compounds were prepared (**26a-s)** as PI3Kδ inhibitors. All the title compounds were prepared in good yield and characterized by various spectroscopic techniques. Assigned structures were found to be confirmed with spectral data.

3.2. *In vitro* PI3Kδ inhibitory activity

Table 9: *In vitro* PI3Kδ inhibitory activity data Benzofuran pyrazolo pyrimidine derivatives:



Sr. No.	**R**	**R_1**	**PI3Kδ inhibition (%)$^{a, b}$**	**PI3Kδ IC$_{50}$ (nM)^c**
26a			**108**	**0.60**
26b			**112**	**0.58**
26c			**99**	**0.90**
26d			79	ND
26e			68	ND
26f			**102**	**0.68**
26g			**100**	**0.60**
26h			**99**	**0.95**
26i			78	10.1
26j			53	ND
26k			52	ND
26l			55	ND
26m			60	8.50
26n			50	ND

26o			45	ND
26p			65	7.20
26q			60	7.10
26r			60	6.62
26s			62	6.90
	INK-666		**110**	**0.50**

[a]All the data are shown as the mean for at least two experiments. [b]PI3Kδ inhibition at the concentration of 100 nM using PI3K Kinase Activity assay kit from Millipore. [c]The IC$_{50}$ values for PI3Kδ inhibition. ND: not detected.

- Compound **26a**, **26b** and **26c** showed excellent inhibitory activity with an IC$_{50}$: 0.60 nM, 0.58 nM and 0.90 nM respectively.
- Compound **26f**, **26g** and **26h** were found to be most potent with IC$_{50}$: 0.68 nM, 0.60 and 0.95 nM respectively.

3.2.1. Isoform selectivity study of selected compounds

Based on the above preliminary PI3Kδ inhibitory activity results, most potent compounds **26a**, **26b, 26c, 26f, 26g** and **26h** were evaluated for their selectivity against PI3K isoforms (α, β and γ) and mTOR.

Table 10: Isoform selectivity of compounds against PI3K (α, β, γ and δ) and mTOR activities:

Comp.	**Biochemical IC$_{50}$ [nM]^{a}**				
	PI3Kα^{b}	**PI3Kβ^{b}**	**PI3Kγ^{b}**	**PI3Kδ^{b}**	**mTOR^{b} (p70S6K)**
26a	320	10	90	0.60	>1000
26b	154	11	4	0.58	>1000
26c	**350**	**28**	**43**	**0.90**	**>1000**
26f	310	12	92	0.68	>1000
26g	150	10	4	0.60	>1000
26h	**350**	**28**	**45**	**0.95**	**>1000**
INK-666	**137**	**9**	**4**	**0.50**	**>1000**

Umbralisib	**>10000**	**1116**	**1065**	**22**	**>1000**

[a]The IC_{50} values are shown as the mean for at least two experiments.[b]PI3K inhibitory activity assay Kit (Millipore) was used to screen the test compounds.

- Compound **26c** and **26h** showed exceptional selectivity for PI3Kδ isoform against all other PI3K isoforms and m-TOR (>1000), 389, 31, 48 and 368, 29, 47 fold for PI3Kα, β, γ isoform respectively.

3.2.2. CYP (Cytochrome) inhibition and hERG (human ether-a-go-go-related gene) liabilities for Compound 26c and 26h

- Additional profiling studies of compound **26c** and **26h** was carried out and it was found to be devoid of CYP (<10% CYP inhibition at 10 μM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and hERG liabilities (IC_{50}: > 30 μM).

3.2.3. Molecular modelling study

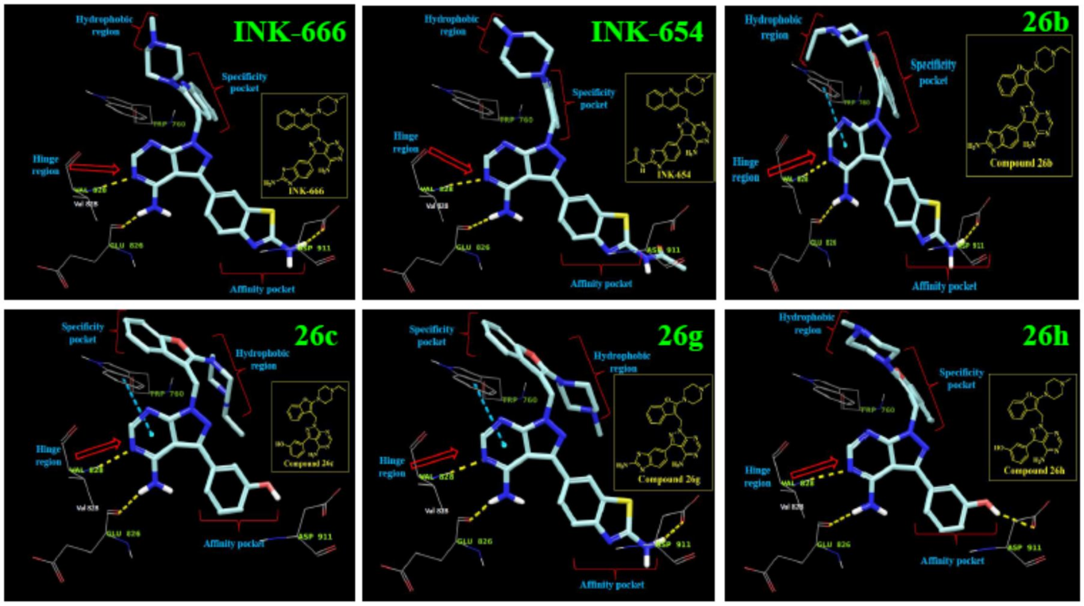


Figure 11: Docking of INK-666, INK-654, Compound **26b**, **26c**, **26g** and **26h**

3.3. Conclusion

- Compound **26c** and **26h** were found to be potent and selective PI3Kδ inhibitors over mTOR. *In vitro* results validate our hypothesis of designing benzofuran based novel, potent and selective PI3Kδ inhibitors as a bioisosteric replacement of quinoline ring of INK-666/654 with benzofuran ring system.

- Additional profiling studies of compound **26c** and **26h** was carried out and it was found to be devoid of CYP22 (<10% CYP inhibition at 10 µM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and showed no hERG liabilities (IC50:>30 µM), while INK 666 showed moderate CYP3A4 inhibition.
- Docking results of **26b**, **26c, 26g** and **26 h** correlates with its potent *in vitro* PI3Kδ activity.

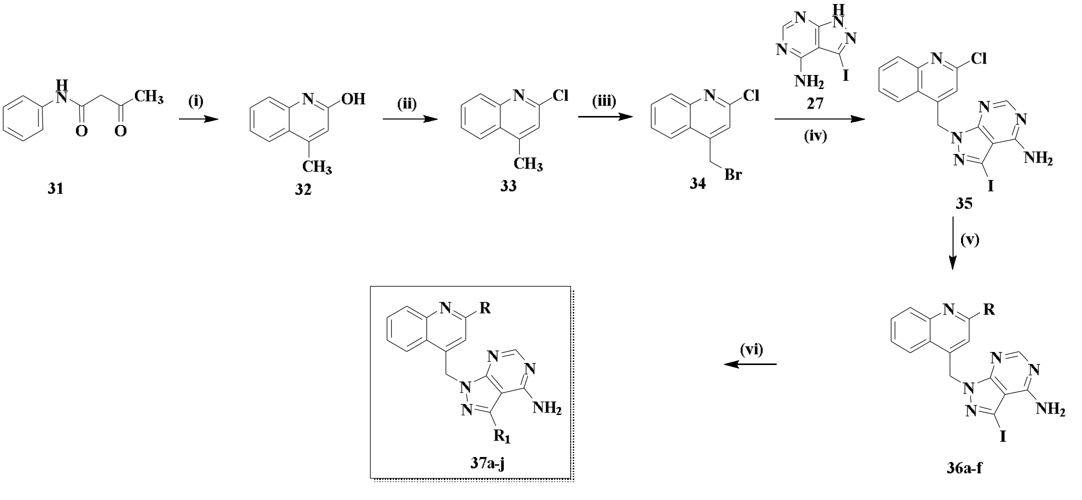
CHAPTER IV

4. Design, synthesis and biological evaluation of 2,4-disubsitued quinoline pyrazolo-pyrimidine derivatives as PI3Kδ selective inhibitors.

Chapter 4 is divided into two parts, In Part 1 synthesis of title compounds **37a-j** using scheme 5 is described. All the compounds were characterized by using different spectral techniques like ^{1}H, ^{13}C NMR, ESI-MS and UPLC analysis. In Part 2, *In vitro* PI3Kδ inhibitory activity, CYP (Cytochrome) inhibition study, hERG inhibition study and Molecular modelling study of imidazo-quinoline derivatives are reported.

4.1. General procedure of the synthesis of 2,4-disubstituted quinoline based compounds 37a-j

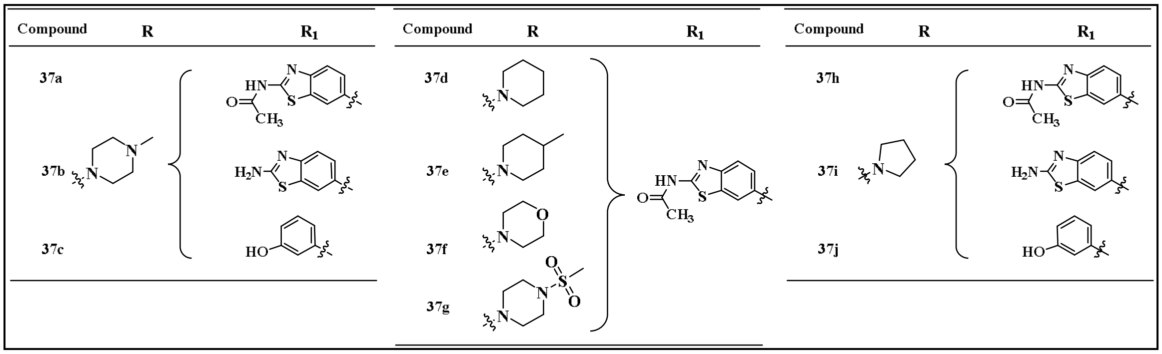
Scheme 5: Synthesis of 2,4- disubstituted quinoline derivatives **37a-j**.



36a: N-methylpiperazine, 36b: Piperidine, 36c: 4-methyl piperidine, 36d: Morpholine, 36e:1-(methylsulfonyl)piperazine, 36f: Pyrrolidine

Reagents and conditions:(i) Conc.H_2SO_4, 110 °C, 4h; (ii) $POCl_3$, 120 °C, 5h; (iii) NBS, Benzene, Reflux, 5h; (iv) t-BuOK, DMF, 26 °C, 15h; (v) Secondary amine (R), (1,4)-Dioxane, Reflux, 12h; (vi) Aryl boronic acid (R_1), $PdCl_2(PPh_3)_2$, $KHCO_3$, DMF:H_2O, 90 °C, 2h.

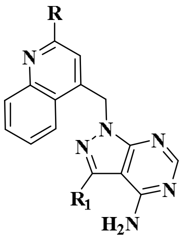
List of 2,4- disubstituted quinoline derivatives **37a-j**.

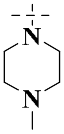
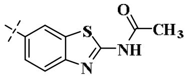
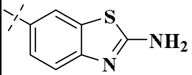
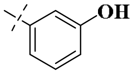
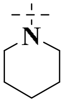
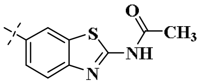
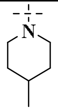
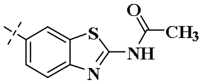


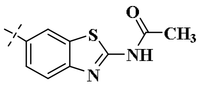
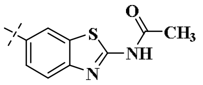
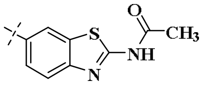
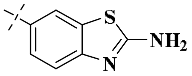
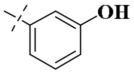
4.2. *In vitro* PI3Kδ inhibitory activity

All the synthesized compounds were assessed for their PI3Kδ inhibitory activity; INK-666 was employed as the positive control **[Table 11]**. It was found that the majority of 2,4-disubstituted quinoline (**37a-j**) analogues displayed varying degree of PI3Kδ inhibitory activity at 100 nM concentration.

Table 11: *In vitro* PI3Kδ inhibitory activity data 2,4-disubstituted quinoline pyrazolo pyrimidine derivatives



Sr. No.	R	R$_1$	PI3Kδ inhibition (%)$^{a, b}$	PI3Kδ IC$_{50}$ (nM)^{c}
37a			**110**	**0.60**
37b			**105**	**0.52**
37c			**94**	**0.82**
37d			60	ND
37e			52	ND

37f			70	9.85
37g			70	10.1
37h			**108**	**0.65**
37i			**105**	**0.60**
37j			**98**	**0.85**
INK-666			**110**	**0.50**

[a]All the data are shown as the mean for at least two experiments. [b]PI3Kδ inhibition at the concentration of 100 nM using PI3K Kinase Activity/Inhibitor assay Kit from Millipore. [c]The IC_{50} values for PI3Kδ inhibition. ND: not detected.

- Compound **37a**, **37b** and **37c** showed excellent inhibitory activity with an IC_{50}: 0.60 nM, 0.52 nM and 0.82 nM respectively. Compound **37d**, **37e** and **37f** were found to be less potent as compared to N-methyl piperazine derivative. Compound **37h**, **37i** and **37j** were most potent, showed IC_{50} of 0.65 nM, 0.60 and 0.85 nM respectively.

4.2.1. CYP (Cytochrome) inhibition and hERG (human ether-a-go-go-related gene) liabilities for Compound 37a-j

- Additional profiling studies of compound **37a**, **37b**, **37c**, **37h**, **37i** and **37j** was carried out and it was found to be devoid of CYP (<10% CYP inhibition at 10 μM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and hERG liabilities (IC_{50}: > 30 μM).

4.2.2. Molecular modelling study

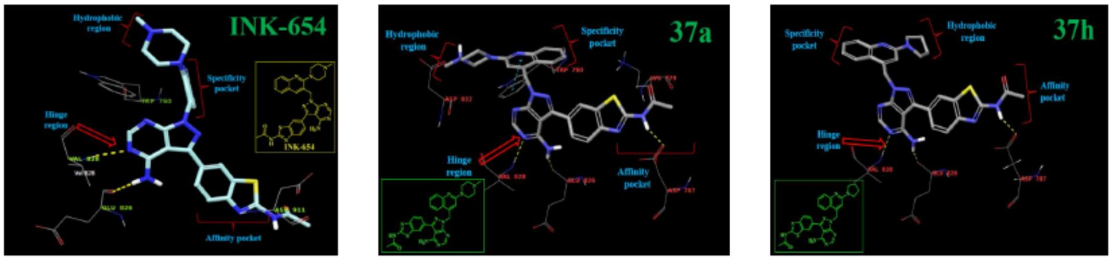


Figure 12: Docking image of INK-654, **37a** and **37h**

4.3. Conclusion

- Based on *in vitro* results, we can summarized that among twenty novel compounds tested for PI3Kδ activities, compound **37a**, **37b**, **37c**, **37h**, **37i** and **37j** were found to be potent PI3Kδ inhibitors. *In vitro* results validate our hypothesis of designing 2,4-disustituted quinoline based novel and potent PI3Kδ inhibitors.
- Additional profiling studies of selected compounds was carried out and it was found to be devoid of CYP22 (<10% CYP inhibition at 10 µM concentration, for CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) and showed no hERG liabilities (IC_{50} :> 30 µM), while INK 666 showed moderate CYP3A4 inhibition.
- Docking results of **37a** and **37h** correlates with its potent *in vitro* PI3Kδ activity.

CHAPTER V

5. Overall summary

In the present investigation altogether three series of PI3Kδ inhibitors were designed, synthesised and biologically evaluate. In the first series, imidazo-quinoline based PI3Kδ inhibitors, in the second series, benzofuran based PI3Kδ inhibitor and in the third series, 2,4-disubsitued quinoline based PI3Kδ inhibitors were prepared. Altogether, 49 compounds were synthesized, purified, characterized and subjected for *in vitro* PI3Kδ inhibitory activity. Most potent PI3Kδ inhibitors from each series were further subjected for the *in vitro* selectivity over other PI3K isoforms.

In the first series (imidazo-quinoline based PI3Kδ inhibitor) compound **9c** (IC_{50}: 9.5 nM) was identified as primary lead compound. Modification at 8th position on compound 9c resulted in to the discovery of compound **10h** (IC_{50}: 1.9 nM), with improved PI3Kδ inhibitory activity and isoform selectivity. **10h** showed no CYP inhibition up to 100µM concentration and hERG liabilities (IC50: > 30 µM).

In benzofuran based series, compounds **26a**, **26b**, **26c**, **26f**, **26g** and **26h** showed excellent PI3Kδ inhibitory activity and were found to be comparable with standard compound (INK-666). Compound **26c** and **26h** were more selective towards PI3Kδ isoform as compared to INK-666. Further, 26c and 26h showed no CYP inhibition up to 10 µM concentration and hERG liabilities (IC_{50}: > 30 µM).

In 2,4-disubstituted quinoline based series, compound **37a (**IC_{50}: 0.60 nM)**, 37b** (IC_{50}: 0.52 nM) and **37c** (IC_{50}: 0.82) nM showed excellent PI3kδ inhibitory activity. In second set, compound, **37h (**IC_{50}: 0.65 nM)**, 37i** (IC_{50}: 0.60 nM) and **37j** (IC_{50}: 0.85) nM were equally potent as compared to N-methyl piperazine derivative and reference compound INK-666.

Compounds **37a**, **37b**, **37c**, **37h**, **37i** and **37j** showed no CYP inhibition up to 10 μM concentration and hERG liabilities (IC_{50}: > 30 μM).

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