Synopsis of the thesis entitled

"Library of Small Molecules targeting Anticancer Activity"

To be submitted to

M. S. University of Baroda

For the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

By

Ms. Ishani I Sahay

Under the guidance of

Prof. Prasanna Ghalsasi

Department of Chemistry

Faculty of Science

M. S. University of Baroda

Vadodara, 390 002

India

July 2017

"Library of Small Molecules Targeting Anticancer Activity"

Chapter 1 presents the general introduction of cancer. Cancer in human is the result of a multi-step process. This process often involves the activation of oncogenes and/or the inactivation of tumor suppressor genes. These two steps arise not only due to mutations, but can also be the result of a translocation or an altered transcription rate. This made most of the anticancer drug molecules in the market, shown in Figure 1, targeted for stopping/arresting or killing genetic processes. On the other hand, recently, cancer is considered as a subject due to epigenetic alterations like promoter methylation (which may lead to tumor suppressor silencing) or decreased histone acetylation (which can result in the down regulation of proteins involved in apoptosis). This has changed targets to several enzymes, which are involved in epigenetic pathways leading to this dreadful disease. We realized Protein Arginine Methyl Transferases (PRMTs) to be our target of choice because of its effective epigenetic activity with less side effect[1].To activate/deactivate enzyme 'pharmacophore' remained a central theme. Therefore, this thesis work is directed towards generating designed pharmacophore for anticancer activity. This first chapter will introduce this concept in detail.

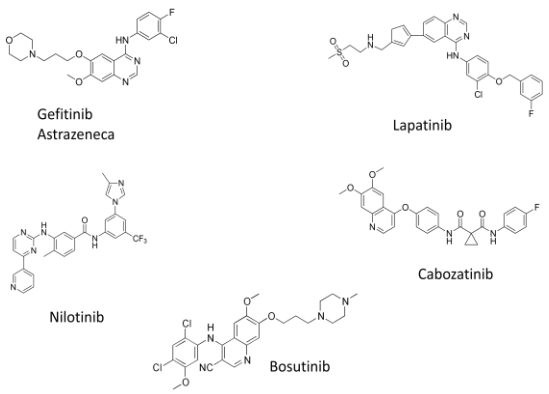


Fig 1.FDA approved marketed anti-cancer drugs

During the designing of these new pharmaphores we have kept Pan-Assay Interference Compounds (PAINS) in our mind. PAINS are getting attention due to ability of certain functional groups causing direct or indirect surge in activity across a range of platforms and against a range of proteins. The most common causes of PAINS activity realized are due to metal chelation, chemical aggregation, redox activity, compound fluorescence, or promiscuous binding[2].

Chapter 2: This chapter is focused on designing and synthesis of five different pharmacophores. The choice of pharmacophore is based on their presence in the core FDA approved marketed anti-cancer drugs. All the synthesized compounds were completely characterized using elemental analysis (CHN analysis), FT-IR, NMR and ESI-MS/HRMS. Biological activity was carried out using antiproliferative assay. The chapter is divided into five sections, because of five different pharmacophores.

Section 2.1: *Amidine-amide adducts as a pharmacophore*- Here synthesis of ten novel amidines has been achieved[3]. For investigation purpose this section is further divided into three parts, as shown in Figure 2. First part deals with the computational study of all the novel amidines, while second with synthesis and third with antiproliferative screening.

Preliminary docking studies were performed on ten amidine derivatives which were synthesized using schemes as shown below. In general the synthesis, the major part of this work, has been achieved in five steps.

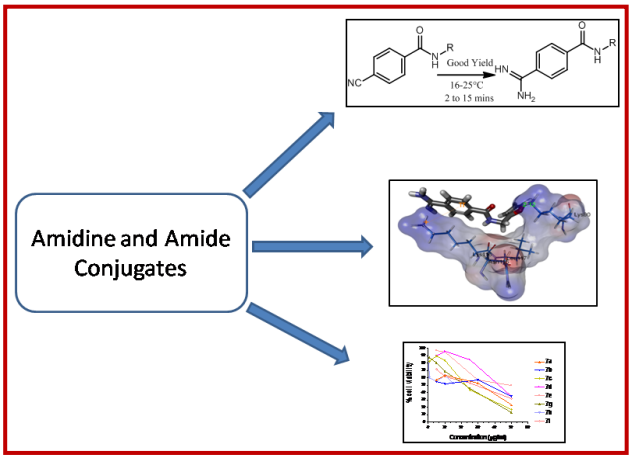
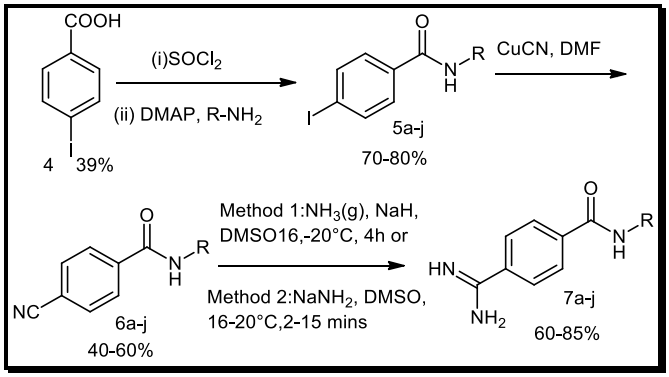


Fig 2: Showing the scheme, docking and biological evaluation of synthesized molecules

The synthesis starts with commercially available *para* amino benzoic acid, followed by protection of the carboxylic acid group. The protected carboxylic acid product was then diazotised and further iodinated, following the deprotection to give the compound 4, as shown in the scheme 1. The next step involves the conversion of carboxylic group into more reactive carbonyl chloride, followed by the amide formation (5a). Iodo group is converted to the cyano group (6a) using environment friendly reagent, copper cyanide. The next step, amidine formation from cyanide is the key step in this synthetic scheme. Unfortunately, commonly employed Pinner reaction for the conversion of cyano to amidine, didn't work for present substrates. Therefore, two different methodologies were developed to carry out this conversion for achieving high yields with less reaction time. The modified methods are shown in the Scheme 1. First method uses sodium hydride and dry ammonia gas, *in-situ* amine ion generation. Second method is based on the direct use of soda amide. Both the above method gave the comparable yields for ten different amidine derivatives.



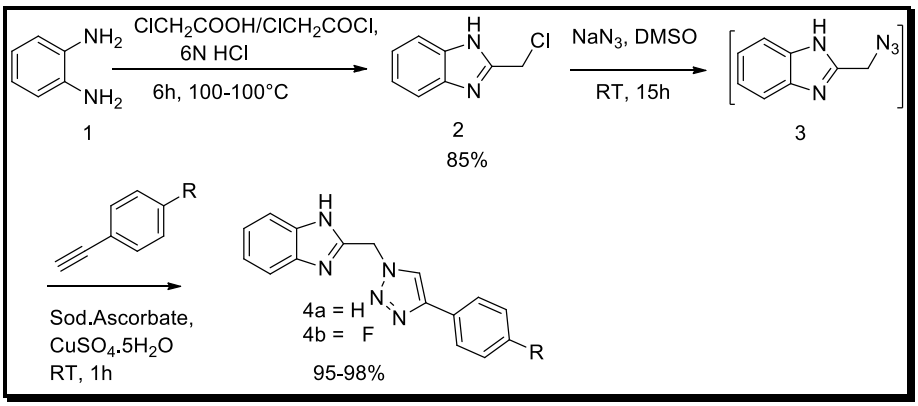
Scheme 1: Synthesis of 4-(aminoiminomethyl)-benzamide derivatives

Third part covers the biological survey of these compounds. In this initially all the newly synthesized molecules were tested on HeLa cell line. Selected compounds were screened for *in vitro* anti-proliferative activity using NCI (National Cancer Institute)-60-human-tumor-cell line-screening program.4-(aminoiminomethyl)-*N*-(3-pyridinylmethyl)benzamide shows 73.36% growth inhibition in HCT-116 colon cancer cell line (mean growth inhibition) at 10μM concentration.

Two heterocyclic structure containing derivatives of furan and picolyamine have been found to be most potent among all. These results are in well agreement with the docking data. Both these compounds have a heterocycle in conjugation with NH side of amide linkage, but also have flexible -CH_2bridge. Present study concludes that 4-(aminoiminomethyl)-*N*-(3-pyridinylmethyl) benzamide and 4-(aminoiminomethyl)-*N*-(2-furanylmethyl)benzamide can be investigated further for the development as new lead for anticancer activity.

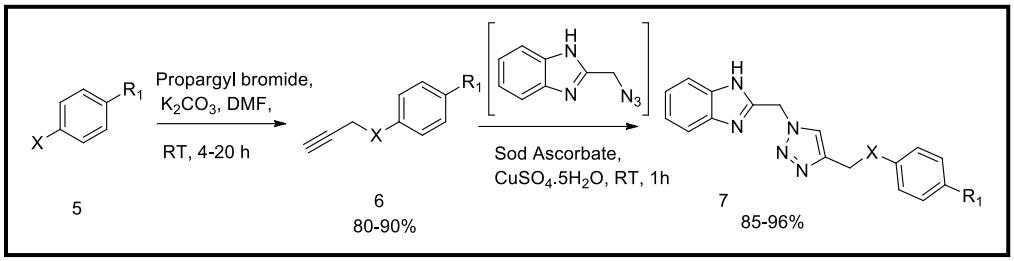
Section 2.2: *Triazole and benzimidazole as a pharmacophore*- Here one pot Click chemistry has been employed to link triazoleand benzimidazole pharmacophore to get N-((1-((1H-benzo[d]imidazol-2-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)aniline and its derivatives[4].

The designing of compounds was driven by three basic principles: 1) inserting flexible linker between benzimidazole and triazole pharmacophore: use of methylene bridge; 2). Derivatizing triazole at C_4 position with flexible group: CH_2-O/ CH_2-N; and 3). increasing solubility and/or bioavailability by derivatizing benzimidazole pharmacophore: N-ethylation. This design strategy forced us to employ three different synthetic routes, for synthesizing eleven new compounds, as discussed below.



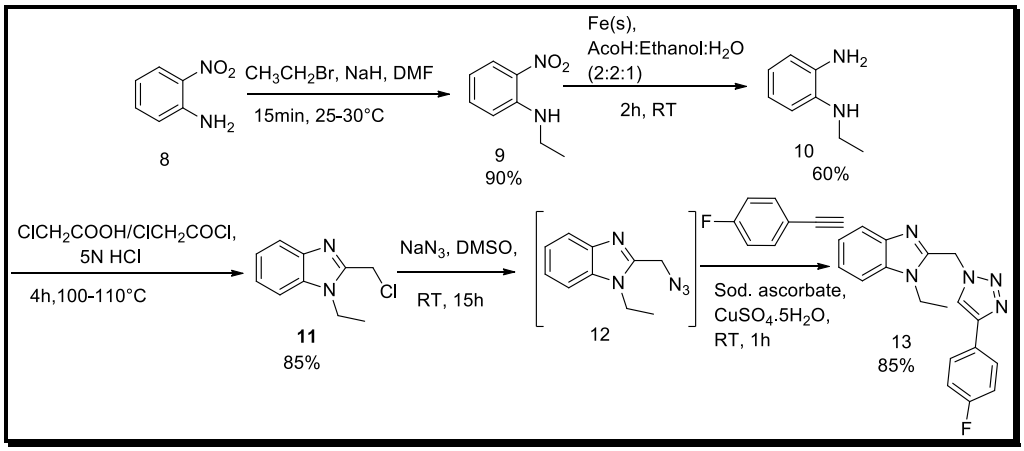
Scheme 2: Synthesis of 2-((4-phenyl-1H-1,2,3-triazol-1-yl)methyl)-1H-benzo[d]imidazole (4a and 4b)

Strategy 1: 2-Chloro benzimidazolewas synthesized by reacting *o*-phenylenediamine with two different reagents 1) 2-chloro acetyl chloride and 2) 2-chloro acetic acid. Both these reactions gave comparable yield. 2-cholrobenzimidazolewas transformed into 2-(azidomethyl)-1H-benzo[d]imidazole, **3**, using sodium azide in dry DMSO. Without isolating compound 3, *in situ*, alkyne derivative was added to obtain final compound **4**as shown in scheme 2.



Scheme 3: Synthesis ofN-((1-((1H-benzo[d]imidazol-2-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)aniline and its derivatives

Strategy 2: Our aim in this strategy is to derivatise triazole ring at C_4 position using the N-(prop-2-yn-1-yl) aniline/phenolic groups. Substituted phenol and aniline derivatives were allowed to react with propargyl bromide in the presence of K_2CO_3base and DMF as solvent (Scheme 3).

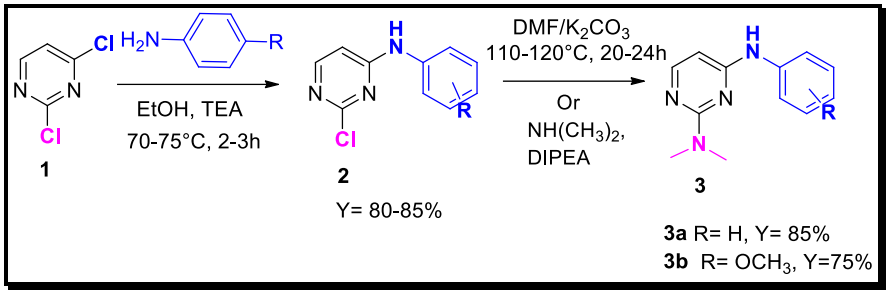


Scheme 4: Synthesis of 1-ethyl-2-((4-(4-fluorophenyl)-1H-1,2,3-triazol-1-yl)methyl)-1H-benzo[d]imidazole

Strategy 3: N-ethylation was carried out initially on *o*-nitro aniline as shown in Scheme 4. *o*-Nitro aniline was converted to N-ethyl derivative using ethyl iodide as per the reported procedure. Further, compound **9** was reduced to compound **10**, as shown in scheme 4, using iron powders. All the further steps were followed according to Scheme 2.

Selected compounds were screened for *in vitro* anti-proliferative activity using NCI (National Cancer Institute)-60-human-tumor-cell line-screening program. The most potent structure N-((1-((1H-benzo[d]imidazol-2-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)-4-chloroaniline(7e) showed 40% growth inhibition in renal cancer cell line (UO-31) at 10µM concentration. Present study concludes that, the structure of 7e can help in developing novel drug candidates for anticancer activity.

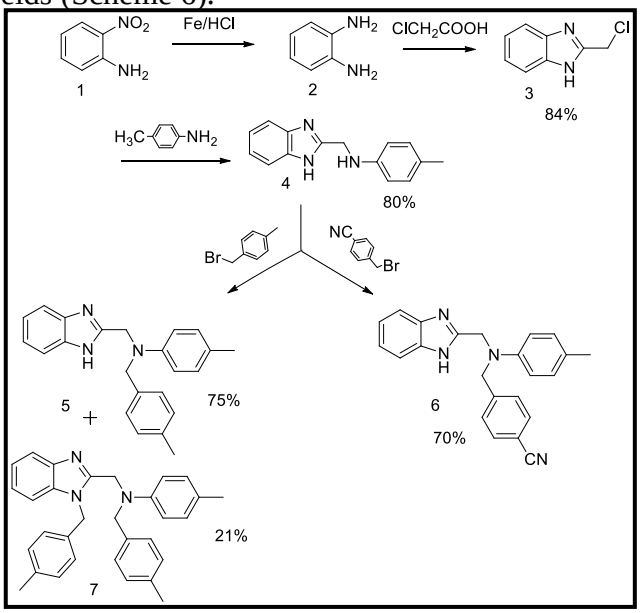
Section 2.3: *Pyrimidine as pharmacophore*- Here novel pyrimidine bearing compounds have been synthesized (Scheme 5). Pyrimidine drugs are used for the treatment of three main disease classes: anti-infective, cardiovascular, and oncological[5].The designing of compounds, was driven by three basic principles: 1) 2,4-linked pyrimidine derivatives with dimethyl amine group fixed at one position and 2) 4,6-linked pyrimidine derivatives with dimethyl amine fixed at one position and 3) Derivatising aniline derivatives in both the cases.



Scheme 5: Synthesis of N^2,N^2-dimethyl-N^4-phenylpyrimidine-2,4-diamine and its derivatives

This design strategy forced us to employ two different synthetic routes. First step is the reaction of 2,4 or 4,6 dichloropyrimidine with various aniline derivatives under ambient conditions. Second step being the second S_NAr reaction performed to insert dimethyl amine group, either by direct insertion or by *in situ* formation. Single crystal of one of the new derivative was obtained. Present study concludes that N^2,N^2-dimethyl-N^4-phenylpyrimidine-2,4-diamine and its derivatives can be synthesized in less time and with high yields using above strategy. The biological evaluation for anticancer screen is underway.

Section 2.4: *Benzimidazole as pharmacophore*- Here novel benzimidazole derivatives have been synthesized in good yields (Scheme 6).



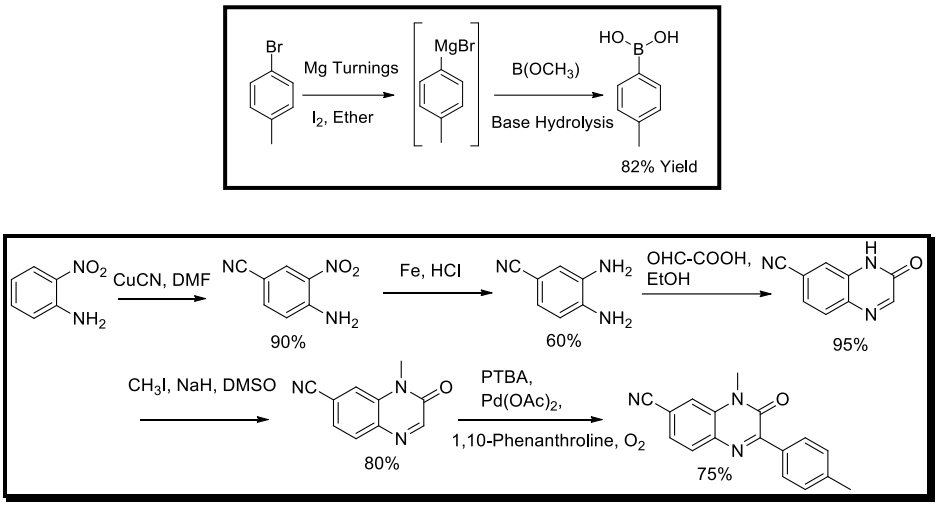
Scheme 6: Synthesis of N-((1H-benzo[d]imidazol-2-yl)methyl)-4-methyl-N-(4-methylbenzyl)aniline and its derivative

Synthesis starts with the cyclisation of respective diamine group and then followed by two substitution reactions to give the required product molecules. Synthesis of three novel molecules was achieved in multiple steps with good yields. Present study concludes that N-((1H-benzo[d]imidazol-2-yl)methyl)-4-methyl-N-(4-methylbenzyl)aniline and its derivatives can be

synthesized in less time, with high yields using above strategy and can help in developing novel drug candidates for anticancer activity.

Section 2.5: *Quinoxalones as pharmacophore*- Here quinoxalones have been considered as pharmacophores[6]. Synthesis of novel quinoxalone based compound is achieved in seven steps (Scheme 7).

As shown in the scheme 7, each step is designed in such a way as to get the maximum yield out of it. The first step was getting a Grignard reagent and then base hydrolysis to get the required boronic acid derivative. Then cyano group was introduced in the commercially available o-nitro aniline, followed by quinoxalinone formation. The intermediate was further C-C coupled to give the desired product.



Scheme 7: Synthesis of 4-methyl-3-oxo-2-(p-tolyl)-3,4-dihydroquinoxaline-6-carbonitrile

Present study concludes that 4-methyl-3-oxo-2-(p-tolyl)-3,4-dihydroquinoxaline-6-carbonitrilecan be synthesized in less time, with high yields using above strategy and can help in developing novel drug candidates for anticancer activity.

Chapter 3: This chapter deals with the un-usual behavior of benzimidazole and triazole adducts.

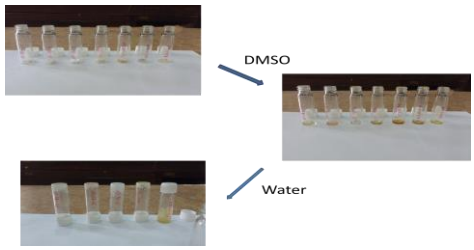
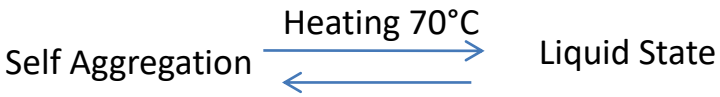


Figure 3: Experiment showing aggregation

When water was added in DMSO dissolved adducts clear solution we observed immediate conversion of liquid to solid phase, as shown in Figure 3. This interesting experimental fact

made us to further investigate this water induced aggregation phenomenon. On further analysis, we observed that aggregation can be possible in two ways: 1. Addition of water in clear DMSO solution and 2. Time duration for DMSO solution (concentration dependent). Transition from aggregated solid state to clear liquid state was observed at 70°C:



To get more insight into the mechanism, we have studied eight different structural analogues as shown in the figure 4.

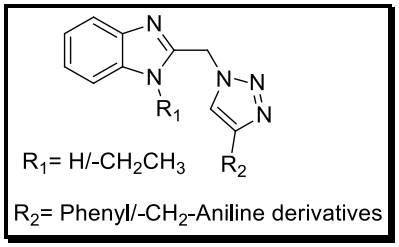


Figure 4: Schematic representation of molecules considered for this study

Five step objectives were formulated to investigate this aggregation phenomena were: 1) Labeling of protons using 2DNMR spectroscopy. 2) Systematic NMR studies of concentration dependent as well as water addition experiment. 3) Single crystal XRD analysis 4) TG-DTA, DSC 5) Microscopic imaging.

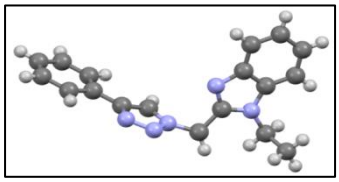


Figure 5: Single crystal of A4

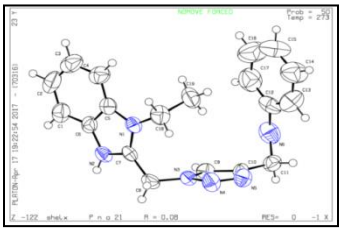


Figure 6: ORTEP View of A6

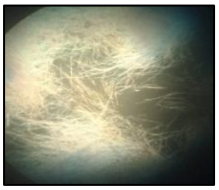


Figure 7: 10X Microscope image of A4

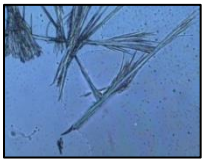
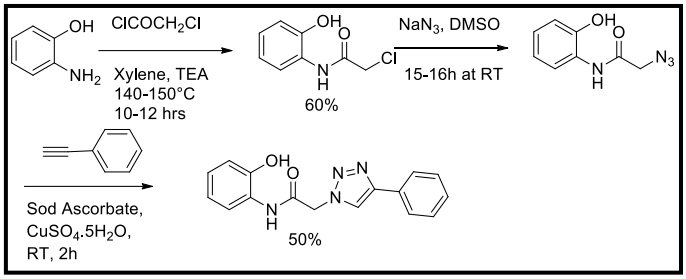


Figure 8: 20X POM image of A4

Eight molecules (Figure 4) investigated in this study, were having changes in ethyl group on Nitrogen of benzimidazole moiety, floro group and aniline group. Concentration dependent and water addition NMR experiments reveals the specific shifting of protons, such as methylene

bridges, *ortho* protons of the phenyl ring. This means that these protons are probable candidates for aggregation or self-aggregation behavior. Hydrogen bonding and close-contact in Single crystal XRD (Figure 5-6) studies and reversible thermal data supports our NMR conclusions. To observe magnitude of this aggregations microscopic images and Polarizing Optical Microscopic (POM) images were taken, as shown in Figure 7 and 8. In conclusion we observed aggregation for all the eight molecules at various concentrations. The fibers were stable at room temperature for days and their formation is thermoreversible. It is generally observed from our experiment that DMSO with time gives longer and thicker fibers as compared to water addition fibers. Detailed studies are presently in progress.

Annexure I: In this part of work we have considered triazole and *o*-hydroxy aniline adducts. Triazole possessing three nitrogen atoms in a five membered heterocyclic ring is an important pharmacophore.



Scheme 8: Synthesis of N-(2-hydroxyphenyl)-2-(4-phenyl-1H-1,2,3-triazol-1-yl)acetamide and its derivatives.

Synthetic strategy for novel triazole and *o*-hydoxy aniline adducts were designed and then synthesis was carried out(three in number, Scheme 8).Synthesis starts with condensation of chloro acetylchloride with *o*-hydroxy aniline. The next step is the azide formation followed by Click reaction to give the desired product. Overall yields of 30-40% were achieved. Single crystal of one of the intermediate compound was developed and solved. Present study concludes that N-(2-hydroxyphenyl)-2-(4-phenyl-1H-1,2,3-triazol-1-yl)acetamidecan be synthesized in less time, with high yields using above strategy and can help in developing novel drug candidates for anticancer activity.

Annexure II: In this part of work protein isolation was planned in collaboration. Knowing the protein-drug interaction gives good insights in designing the best possible drugs with minimum side effects.Having this in mindwe have developed a clone to give us the required protein (PRMT1; Protein Arginine Methyl Transferases). Isolation of this protein is underway.

In summary, shown in Figure 9, the thesis presents the overall synthesis of novel molecules with six different pharmacophores, normally observed in anti-cancer drugs: A. Amidine, B. Benzimidazole and triazole adducts, C. Pyrimidine, D. Benzimidazole, E. Quinoxazole F. Triazole and aniline adducts. The synthesis was designed to obtain over all good yields. Characterization of all the newly synthesized molecules was carried out using CHN, FT-IR, ^{1}H and ^{13}C NMR, ESI-MS/HRMS and where ever necessary by single crystal XRD analysis. The

docking studies have been performed for amidine derivatives. Synthesized molecules have been tested for their anti-proliferative activity using MTT assay and NCI-60 human cancer cell line screen. The observed IC_{50} values for the molecules shows the validity of the concept in designing and synthesis of novel molecules based on pharmacophore drug designing. An attempt to synthesize target protein (PRMT1: Protein Arginine Methyl Transferases) was also performed in collaboration. This thesis also uncovers (1) detailed investigation (using 2D NMR, DSC, microscopic imaging) of serendipitous assembly formation of triazole and benzimidazole adduct in presence of water; and (2) unconventional attempt to generate polymorphism in Letrozole, a standard anti-cancer drug.

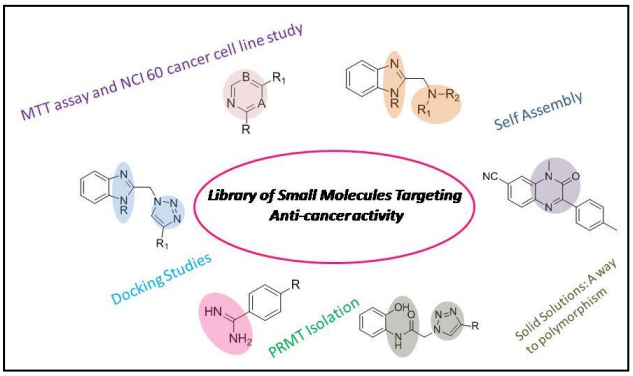


Figure 9: Summary of the research work done

References:

1. (a) Sudhakar A. *J. Cancer Sci.Ther*., **2009**,1, 1–4.(b) Spannhoff A.; Sippl, W.; Jung, M. *Int. J. Biochem. Cell Biol*., **2009**, 41, 4-11.
2. (a) Baell J. B.; Holloway, G. A., *J. Med. Chem.*, **2010**, 53, 2719-2740. (b) Gilberg E.; Jasial S.; Stumpfe D.; Dimova D.; Bajorath J. *J. Med. Chem.*, **2016**, 59, 10285-10290.
3. Sahay I. I.; Ghalsasi P. S.; Singh M.; Begum R. *Synth. Commun.*, **2017**, 47, 1400-1408.
4. Sahay I. I.; Ghalsasi P. S. *Synth. Commun.*, **2017**, 47, 825-834.
5. Vitaku E.; Smith D. T.;Njardarson J. T. *J. Med. Chem.*, **2014**, 57, 10257-10274.
6. Lawrence D. S.; Copper J. E.; Smith C. D. *J. Med. Chem.*, **2001**, 44, 594-601.

Ms. Ishani Sahay
(Ph.D student)

Prof. Prasanna Ghalsasi
(Guide)

Prof. N. D. Kulkarni
(Head)