Summary of the thesis entitled

"Library of Small Molecules targeting Anticancer Activity"

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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

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"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.

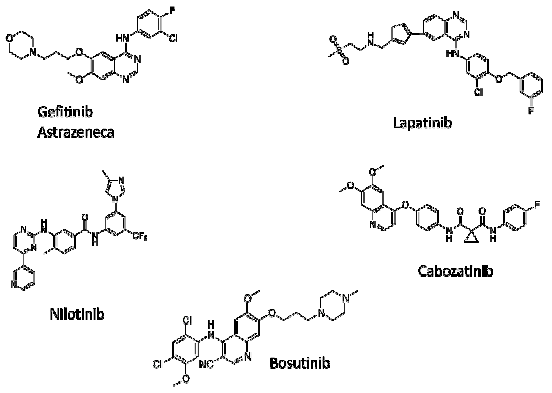


Fig 1.FDA approved marketed anti-cancer drugs

During the designing of these new pharmaphore bearing molecules 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.

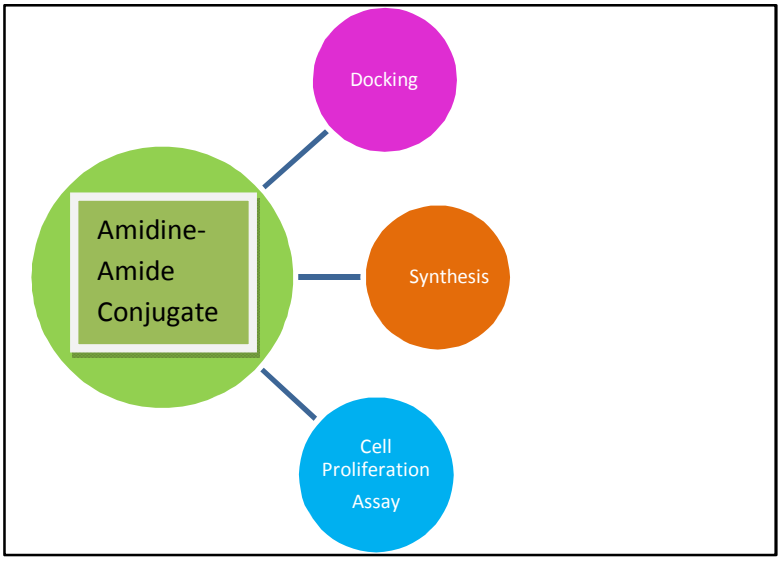
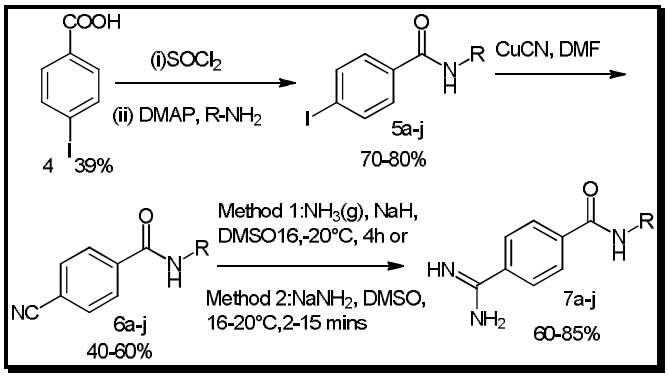


Figure 2: Showing the scheme, docking and biological evaluation of synthesized molecules

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 S*cheme 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.



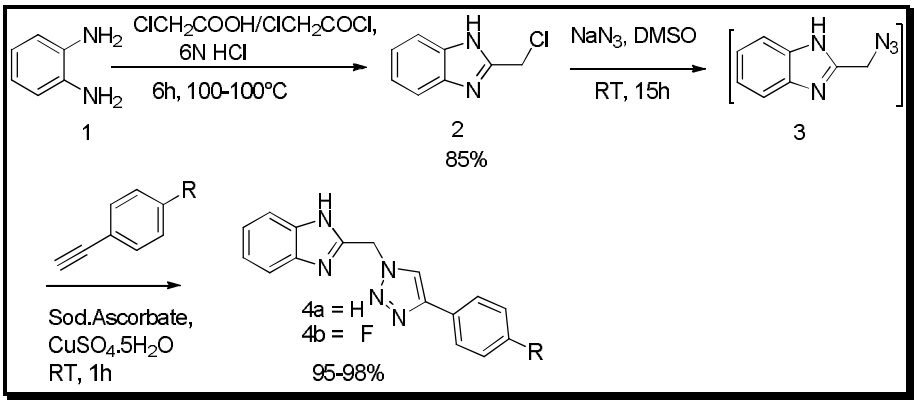
Scheme 1: Synthesis of 4-(aminoiminomethyl)-benzamide derivatives

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.

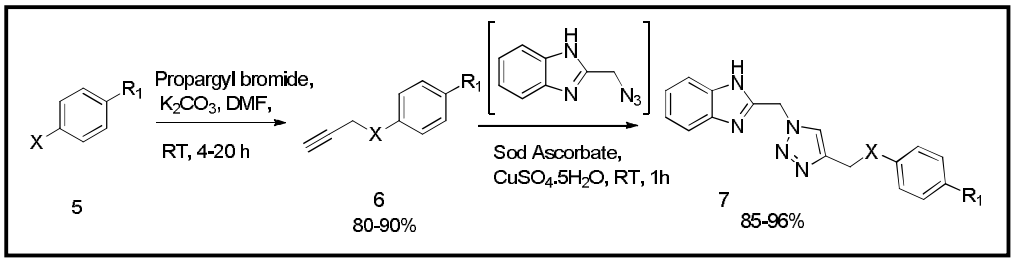
Section 2.2: *Triazole and benzimidazole adducts as a pharmacophore*- Here one pot Click chemistry has been employed to link triazole and benzimidazole pharmacophore to get N-((1-((1H-benzo[d]imidazol-2-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)aniline and its derivatives[4].

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.



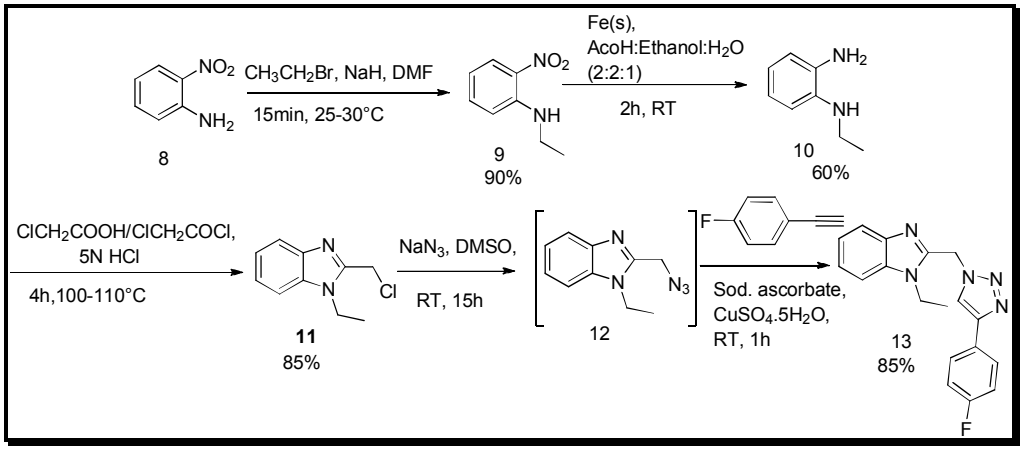
Scheme 2: Synthesis of 2-((4-phenyl-1H-1,2,3-triazol-1-yl)methyl)-1H-benzo[d]imidazole (4a and 4b)

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*).

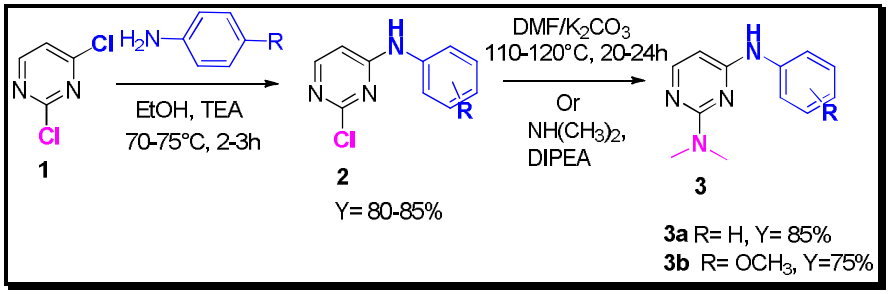


Scheme 4: Synthesis of 1-ethyl-2-((4-(4-fluorophenyl)-1H-1,2,3-triazol-1-yl)methyl)-1H-benzo[d]imidazole

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.

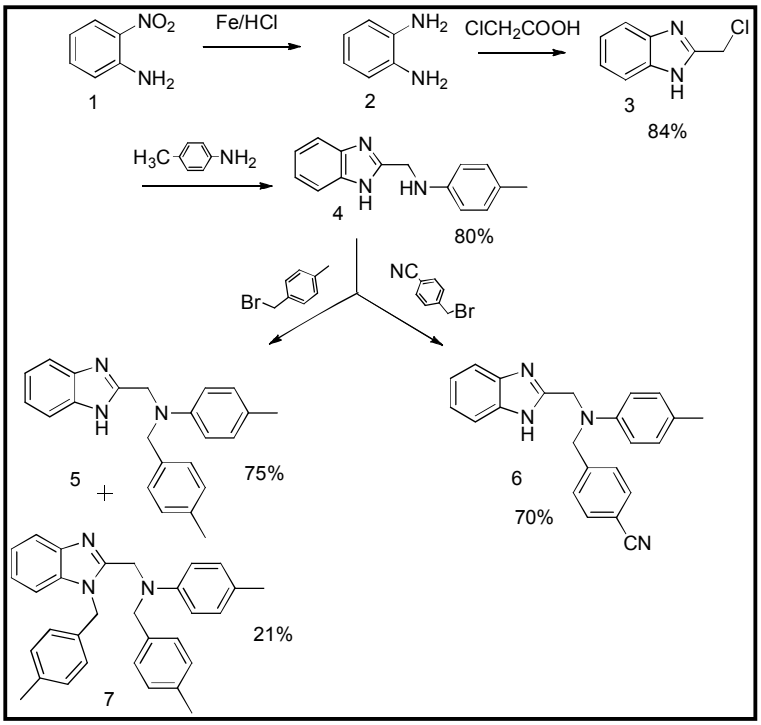
Section 2.3: *Pyrimidine and aniline adducts as pharmacophore*- Here novel pyrimidine bearing compounds have been synthesized (*Scheme 5*). Pyrimidine drugs are used for the treatment of three main disease classes: anti-infective, cardiovascular, and oncological[5].The designing of compounds, was driven 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 and aniline adducts as pharmacophore*- Here novel benzimidazole derivatives have been synthesized in good yields (*Scheme 6*).

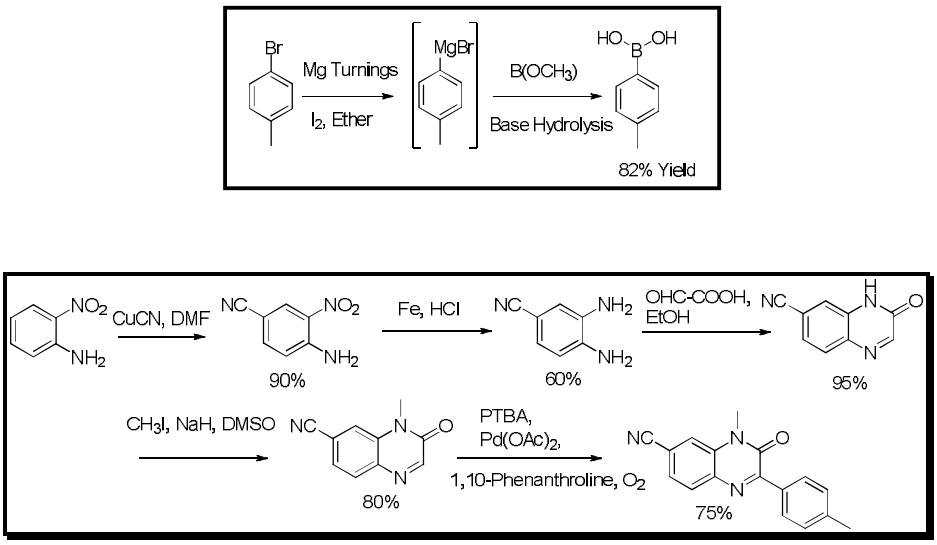


Scheme 6: Synthesis of N-((1H-benzo[d]imidazol-2-yl)methyl)-4-methyl-N-(4-methylbenzyl)aniline and its derivative

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

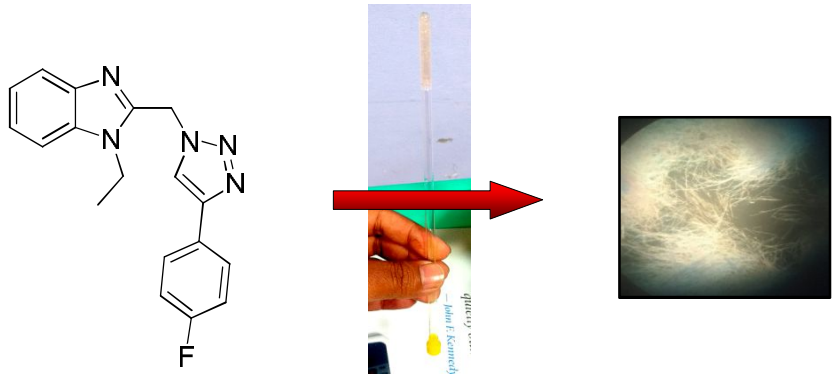
As shown in the *Scheme 7*, each step is designed in such a way as to get the maximum yield out of it. The first step was getting a Grignard reagent and then base hydrolysis to get the required boronic acid derivative. Then cyano group was introduced in the commercially available o-nitro aniline, followed 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-carbonitrile can be synthesized in less time, with high yields using above strategy and can help in developing novel drug candidates for anticancer activity.

Chapter 3: An interesting observation was made in chapter 2.2 (*Figure 3*). While recording NMR, the solution inside the NMR tube got solidified.



(a)

(b)

Figure 3 Serendipitous white solidification observed in NMR tube (a), for benzimidazole-triazole adducts (b) and under microscope (c)

Why did the compound, having benzimidazole linked triazole pharmacophore, remained in NMR tube after 12 hours and showed gel like or semisolid behavior? Some questions came up then. Is this a simple aggregation? Is it due to impurity? Is it common in literature? Can we see this behavior by changing other physical parameters, such as change in solvent system, temperature? Is it possible to trace 'molecular' origin for such behavior? Many such questions are answered in this chapter with the help of microscopic, thermal, single crystal and NMR studies. Designing of the molecules was based on three facts: 1. Check the effect of phenyl/aniline linkers 2. Check the effect of floro/non-florogroups 3. Check the effect of ethyl/non-ethyl group (*Figure 4*).

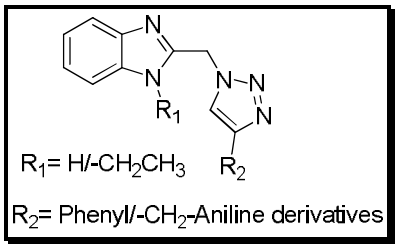
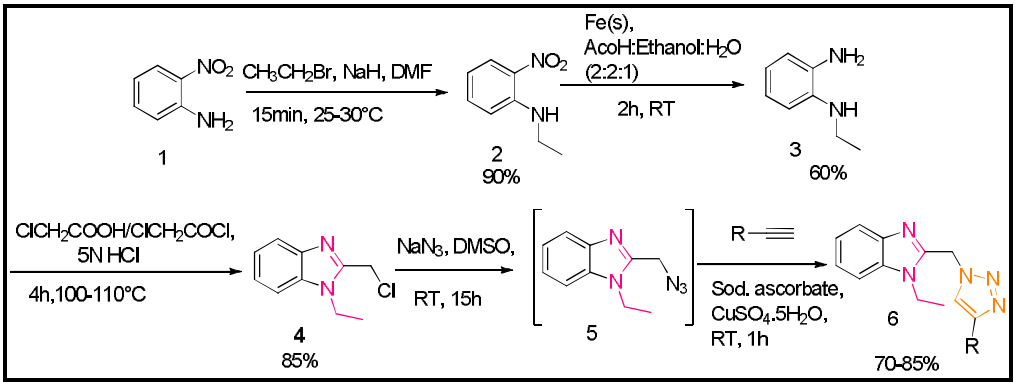


Figure 4: Schematic representation of molecules considered for this study

Three new benzimidazole-triazole adducts were synthesized by *in situ* click chemistry of an azide-containing substituted benzimidazole ring with phenylene or aniline carrying a triple bond functionality to give benzimidazole-triazole adducts (*Scheme 8*). The synthesis was carried out in

a similar fashion as was discussed in chapter 2.2. In totality eight molecules (*Figure 5*) were considered for the investigation, out of which five molecules were taken from the chapter 2.2 and the other three were new molecules synthesized using the same strategy as shown in *Scheme 8*.



Scheme 8: Synthesis of N-((1-((1-ethyl-1H-benzo[d]imidazol-2-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)aniline and its derivatives

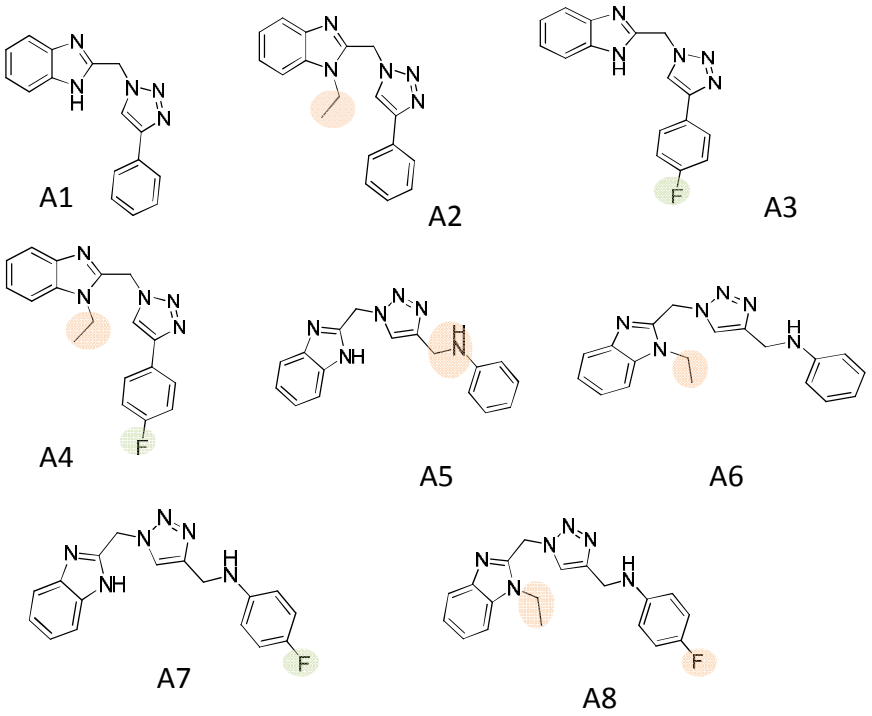


Figure 5: Benzimidazole-triazole adducts considered for the investigation

Firstly, the real time images were captured with polarizing optical microscopy for self aggregation (*Figure 6 and 7*). A microscopic study concluded that the growth of fibers is

sperulitical initially and then fibrillar with both tip branching and side branching. The fibers obtained from DMSO are much thicker as compared to water addition fibers.

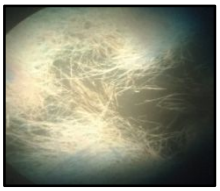


Figure 6: 10X Microscope image of A4

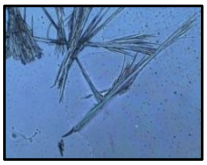


Figure 7: 20X POM image of A4

Thermal analyses TG-DTA and DSC were performed for understanding the stability and phase transitions, if any, for the molecules under consideration. We observed that the three representative molecules showed phase transitions at above 100°C. Successfully single crystal was obtained for two ethyl derivatives while for other derivatives we didn't get any (*Figure 8 and 9*). Single crystal XRD was performed for getting more insight to the various interactions in solid phase.

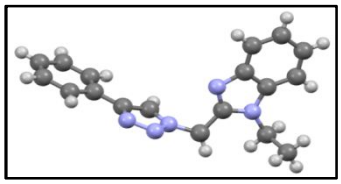


Figure 8: Single crystal of A2

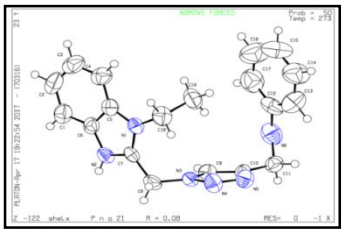
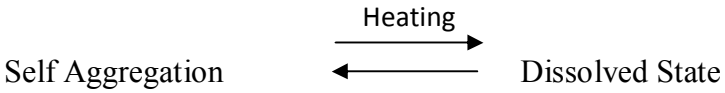


Figure 9: ORTEP View of A6

COSY and HSQC NMR measurements were used for the complete assignment of proton signals for all the newly synthesized eight benzimidazole linked triazole molecules in solution. Concentration dependent and titration with water, of proton chemical shifts of the molecules under consideration were measured. Concentration dependent and water addition NMR experiments revealed the specific shifting of protons, such as methylene bridges, *ortho* protons of the phenyl ring. Thus, these protons were probable candidates for aggregation or self-aggregation behavior. Close-contact in Single crystal XRD studies and thermal data supports our NMR conclusions. The fibers were stable at room temperature for more than a week's time and their formation is thermoreversible.

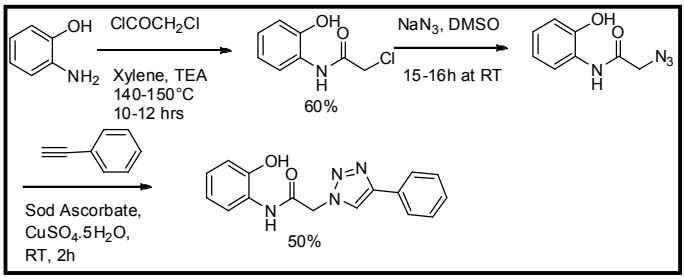


In conclusion, the self aggregation behavior of benzimidazole linked triazole adducts in NMR tube was observed. Systematic study were undertaken to unravel this behavior on eight designer derivatives. The synthesis of these molecules is carried out by modulating three facts: (i). flexibility effect of phenyl/aniline linkers; (ii). the effect of introducing 'floro' functionality; and (iii). addition of ethyl group on benzimidazole nitrogen. All these molecules showed long fiber like structure, which makes it semi-solid or gel like, with change in concentration in DMSO solvent and/or addition of water. Fibers obtained from DMSO are much thicker as compared to water addition fibers. A microscopic study concluded that the growth of fibers is sperulitical initially and then fibrillar with both tip branching and side branching. Thermal analyses TG-DTA and DSC showed phase transitions at above 100°C, for the three representative molecules. Single crystal X-ray study revealed absence of non-covalent interactions for two of the ethyl derivatives in solid phase. COSY and HSQC NMR measurements are used for the complete assignment of proton signals for all newly synthesized eight benzimidazole linked triazole molecules in DMSO. Concentration dependent and water addition proton NMR experiments revealed the specific shifting of peaks, such as methylene bridges and *ortho*-protons of the phenyl ring. Thus, these protons may be the probable candidates for aggregation or self-aggregation behavior. Close-contact in Single crystal XRD studies and thermal data supports our NMR conclusions. The fibers are stable at room temperature for more than a week's time and their formation is thermoreversible.

Chapter 4 reports crystallization of Letrozole, a well marketed drug for hormone receptor positive breast cancer. Letrozole molecule in-spite of having many pro-hydrogen-bonding sites and planar aromatic ring did not show their 'expression' in actual solid state crystal structure. Systematic proportions of hydroquinone and pyrogallol were planned for the same during slow crystal growth experiment. Detailed investigation of thus obtained products using DSC is performed, which showed interesting small but distinct shifting of endotherms. This result is well supported by single crystal data and powder XRD pattern of already reported forms reveals observation of new polymorph. New polymorph crystallizes in orthorhombic crystal system

with *C-1* space group. New polymorph can differ in solubility behavior, thermal stability, chemical inertness and hence bioavailability of the marked drug.

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 9: 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 9*). 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 presents efforts we put in protein isolation work which is performed in collaboration. Knowing the protein-drug interaction gives good insights in designing the best possible drugs with minimum side effects. Having this in mind we have developed a clone to give us the required protein PRMT1.

In summary, shown in *Figure 10*, 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. Designing of the novel molecules is done taking PAINS structures into consideration. The synthesis is designed to obtain over all good yields. Characterization of

all the newly synthesized molecules were 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 made in collaboration. This thesis also uncovers (1) detailed investigation (using microscopic imaging, TG-DTA, single crystal XRD and 2D NMR) of serendipitous assembly formation of triazole and benzimidazole adduct in presence of water; and (2) serendipitous discovery of polymorphism in Letrozole, a standard anti-cancer drug.

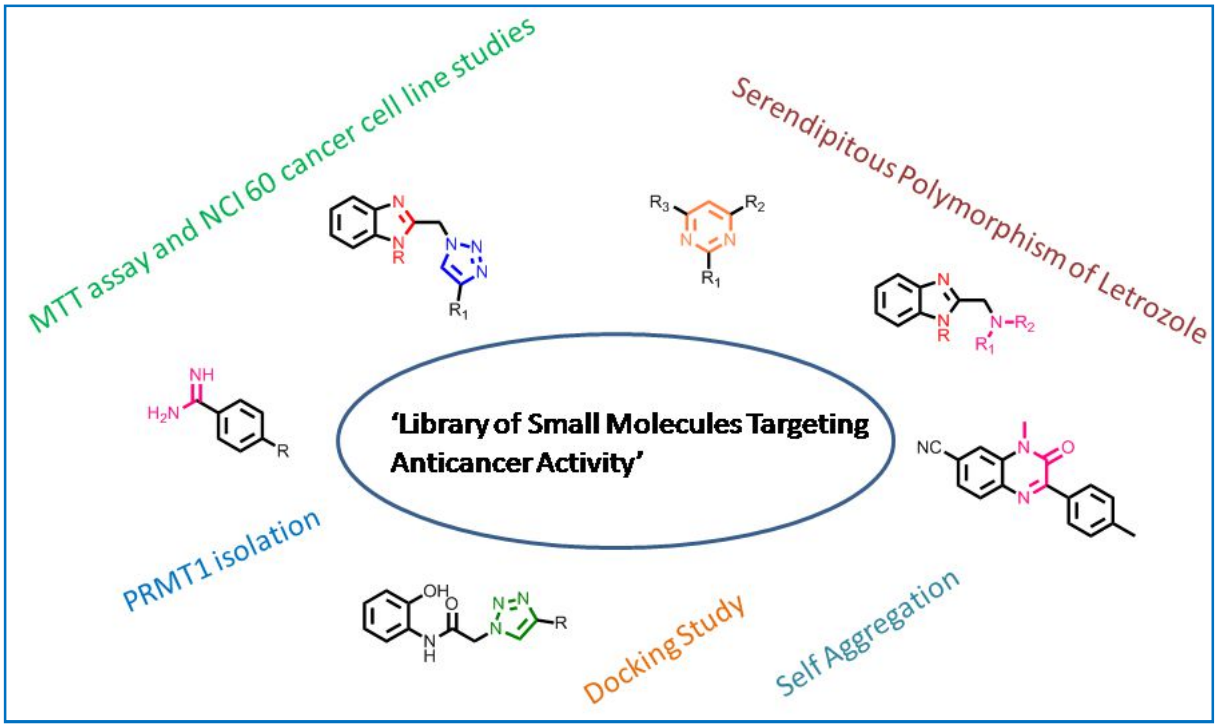


Figure 10: Summary of the research work done

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