SYNTHESIS AND *IN VITRO* EVALUATION OF HETEROCYCLIC CORE MOIETIES AS ANTICANCER AGENTS

Thesis submitted

by

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In fulfilment of the requirement

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Doctor of Philosophy

Under the supervision of

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Certificate

This is to certify that thesis entitled **"Synthesis and *in vitro* evaluation of heterocyclic core moieties as anticancer agents"**, being submitted by Meenakshi Verma in the fulfillment of the requirement for the award of Degree of Doctor of Philosophy to the School of Chemistry and Biochemistry, Thapar University, Patiala, is a record of candidate's own work carried out by her under my supervision and guidance. The matter presented in this thesis has not been submitted in part or full for the award of any degree in any other University or Institute.

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Candidate's Declaration

I, hereby declare that work presented in the thesis entitled **"Synthesis and *in vitro* evaluation of heterocyclic core moieties as anticancer agents"**, in fulfillment of the requirement for the award of Degree of Doctor of Philosophy, School of Chemistry and Biochemistry, Thapar University, Patiala, is an authentic record of my own work under the supervision of Dr. Kamaldeep Paul, Assistant Professor, School of Chemistry and Biochemistry, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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DEDICATED TO
MY
BELOVED FAMILY

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Table of Contents

Chapter	Section	Contents	Page No.
		Introduction	i-iv
		References	iv
1		**Review of Literature**	**1-22**
	1	Biological activity of 1,8-Naphthalimide based molecules	4
	2	Biological activity of Pyridine and triazole based molecules	14
		References	19
2		**Synthesis of naphthalimide-benzimidazole analogues as anticancer agents**	**23-54**
	2.1	Introduction	23
	2.2	Chemistry	24
	2.2.1	Synthesis of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one	24
	2.2.2	Synthesis of 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one	26
	2.3	Biology	30
	2.3.1	In vitro cancer screening with one dose	30
	2.3.2	*In vitro* anticancer screening with five dose concentration	34
	2.3.3	DNA binding studies	36
	2.4	ADME prediction	37
	2.5	Structure-Activity Relationships	38
	2.6	Molecular modelling studies	39
	2.6.1	Molecular modelling studies of **5d** with topoisomerase I, topoisomerase II and ribonucleotide reductase	39
	2.6.2	Molecular modelling studies of **5d** with DNA	41
	2.7	Conclusion	41

	2.8	Experimental Section	42
	2.8.1	Chemistry	42
	2.8.1.1	General procedure for synthesis of 3-bromo-benzo[de]benzo[4,5]imidazo[2,1-a]isoquinolin-7-one	43
	2.8.1.2	General procedure for synthesis of 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one	44
	2.8.2	*In vitro* anticancer screening assay	48
	2.8.3	DNA binding studies	50
	2.8.4	Molecular modelling	50
		References	51
3		**Synthesis of allylic-naphthalimide analogues as anticancer agents**	**54-98**
	3.1	Introduction	54
	3.2	Chemistry	55
	3.2.1	Synthesis of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione	55
	3.2.2	Synthesis of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione	57
	3.2.3	Synthesis of 2-allyl-benzo[*de*]isoquinoline-1,3-dione and 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione	61
	3.2.4	Synthesis of 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione and 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione	62
	3.3	Biology	63
	3.3.1	*In vitro* cancer screening with one dose	63
	3.3.2	*In vitro* anticancer screening with five doses	67
	3.3.3	MTT assay for determination of cytotoxicity	72
	3.3.4	Lactate dehydrogenase (LDH) release studies	73
	3.3.5	DNA binding studies	74
	3.3.6	Ethidium bromide (EtBr) displacement study	75
	3.4	ADME prediction	76
	3.5	Structure-activity relationship	77

	3.6	Molecular modelling studies	78
	3.6.1	Molecular modelling studies of 6b with topoisomerase I and II	79
	3.6.2	Molecular modelling studies of 6b with DNA	80
	3.7	Conclusion	81
	3.8	Experimental Section	81
	3.8.1	Chemistry	81
	3.8.1.1	General procedure for synthesis of 3-bromo-benzo[de]benzo[4,5]imidazo[2,1-a]isoquinolin-7-one	82
	3.8.1.2	General procedure for synthesis of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione	84
	3.8.1.3	Procedure for synthesis of 2-allyl-benzo[*de*]isoquinoline-1,3-dione	90
	3.8.1.4	Procedure for synthesis of 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione	91
	3.8.1.5	Procedure for synthesis of 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione	91
	3.8.1.6	Procedure for synthesis of 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione	92
	3.8.2	*In vitro* antitumor assay	92
	3.8.3	MTT assay	94
	3.8.4	LDH activity	94
	3.8.5	DNA binding studies	95
	3.8.6	Molecular modelling	95
		References	96
4		**Synthesis of pyridine-triazole based anticancer agents**	**98-129**
	4.1	Introduction	98
	4.2	Chemistry	99
	4.2.1	Synthesis of *N*-(3-hydroxy-pyridin-2-yl)benzamide and benzoic acid 2-amino-pyridin-3-yl ester	99
	4.2.2	Synthesis of *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)-benzamide	100
	4.2.3	Synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-(1,2,3)-triazol-4-yl)methoxy)pyridine-2-yl)-3-phenyl-acrylamide	101

4.2.4	Synthesis of *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide	102
4.2.5	Synthesis of 3-phenyl-*N*-prop-2-ynyl-*N*-(3-prop-2-ynyloxy-pyridin-2-yl)acrylamide and 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide	103
4.2.6	Synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-(1,2,3)-triazol-4-yl)methoxy) pyridine-2-yl)-3-phenyl-acrylamide	103
4.2.7	Synthesis of *N*-(3-allyloxy-pyridin-2-yl)-benzamide, benzoic acid 2-allylamino-pyridin-3-yl ester and *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide	107
4.3	Biology	109
4.4	ADME prediction	110
4.5	Structure activity relationship	111
4.6	Molecular modelling	111
4.7	Conclusion	113
4.8	Experimental Section	113
4.8.1	Chemistry	113
4.8.1.1	General procedure for synthesis of *N*-(3-hydroxy-pyridin-2-yl)benzamide and benzoic acid 2-amino-pyridin-3-yl ester	114
4.8.1.2	General procedure for synthesis of compound *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide	115
4.8.1.3	General procedure for synthesis of *N*-(3-((1-(2-substituted benzyl/allyl)-1*H*-1,2,3-triazol-4-yl)methoxy)pyridine-2-yl)benzamide	115
4.8.1.4	General procedure of synthesis of compound *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide	119
4.8.1.5	General method of synthesis of compounds3-phenyl-*N*-prop-2-ynyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide and 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide	119
4.8.1.6	General procedure for synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-(1,2,3)-triazol-4-yl)methoxy) pyridine-2-yl)-3-phenyl-acrylamide	120
4.8.1.7	Procedure for synthesis of *N*-(3-allyloxy-pyridin-2-yl)-benzamide	123
4.8.1.8	Procedure for synthesis of benzoic acid 2-allylamino-pyridin-3-yl ester	124

4.8.1.9	Procedure for synthesis of *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide	124
4.8.2	Biology	125
4.8.3	Molecular modelling	126
	References	127
	Summary	130
	List of Publications	141
	Papers presented at Conferences	142

INTRODUCTION

Cancer is not just one condition. Cancer occurs due to presence of abnormal cells in the body. There are many different types of cell in the body. Different parts of the body such as organs, bones, muscles, skin and blood are made up from different specialised cells. Cancer causing cells are abnormal and they multiply out of control. Nucleus of each cell contains genes, made up of DNA. These genes are the codes which control the functions of the cells. Normal cells in the body divide and multiply from time to time. As old cells wear out or become damaged, new cells are formed to replace them. Sometimes a cell becomes abnormal. This occurs if any gene in the cell get damaged or altered. The abnormal cell may then divide into two, then four and so on. A number of such abnormal cells may develop from the parent abnormal cell. If this multiplication of cells does not stop, it forms a clump of abnormal cells called a tumour. Tumours are divided into two types: benign and malignant. Benign tumours grow slowly, and do not spread. They are non-cancerous. Malignant tumours tend to grow quite quickly and sometimes spread over other tissues and organs. Most of the cancers are named for where they start. Symptoms and treatment of cancer depend upon various factors viz. its type, where it is located, where it has spread, how big the tumor is and how advanced it is. Treatment of cancer involves various different stages which may include surgery, radiation and/or chemotherapy. Some types of cancers can be felt or seen like - a lump on the skin can be an indicator of cancer in those particular locations. But sometimes the symptoms are not physically apparent like some brain tumors. Early detection of the disease can lessen the obstacles of successful treatment. Various anticancer drugs have been used to inhibit the growth of the tumor. Anticancer drugs ideally, are toxic to cancer cells and they either kill cancer cells or modify their growth. Sometimes they affect the genetic material of the cell directly. Discovery of anticancer agents started after 1940's. Most of the agents were discovered in 1950-1970. Some well known cytotoxic drugs are cisplatin, carboplatin, etoposide, topotecan, doxorubicin, epirubicin etc.

Various drugs used for cancer treatment are indulged with number of drawbacks which include low selectivity, unwanted side effects and more toxicity. Many challenges are on the way of successful treatment of this most fatal disease. Therefore, major progress in cancer treatment requires new drugs to overcome the factors which previously present drugs are lacking.[1-8] The finding of novel antitumor agents for cancer treatment is stimulated by searching biological targets and designing and synthesizing new molecules possessing no

side effects. These drugs, free from any toxicity and other drawbacks, are associated with the hope of millions of people worldwide.

Considering cancer cells the highly proliferative tissues, DNA becomes one of the most efficient biological targets for developing anticancer agents.[9] In last decades, great attention has been paid to design and synthesize promising DNA-targeted antitumor agents.[2,10-11] Therefore, design and synthesis of potent DNA-targeted compounds becomes one of the most important targets of chemistry, biology and medicine fields. Such compounds cover a wide area including DNA-alkylating agents, DNA groove binders and DNA intercalators. Possibly these molecules are intercalate into DNA base pairs via electron-deficient planar chromophores with flexible side chains. Aromatic heterocycles position at proper sites or interact with topoisomerases and therefore interfere with DNA replication and transcription.[12-15] Acridine, naphthodiazine and anthraquinone are some examples of famous DNA intercalating matrixes. Molecules that can bind with DNA, are of immense interest in the field of medicinal or clinical chemistry.[16,17] Being the genetic instructor, DNA is involved in gene transcription,[18] mutagenesis,[19] gene expression,[20,21] etc. and hence play very important role in development and functioning of living organisms.[22]

Number of efforts has been made in order to increase the DNA recognizing ability of DNA interacting agents. Dimerization of molecules is result of such efforts. A number of dimeric DNA binders are known out of which some exhibited significant cytotoxicity and *in vivo* antitumor activity compared to the monomers.[22]. Due to its direct approach for cancer treatment, DNA becomes the nucleus of present research. Study of type of the interactions in new molecules and DNA provide significant information for designing and synthesizing more efficient therapeutic agents capable of controlling various gene expression.[23,24]

Among the class of DNA intercalators, naphthalimide derivatives are one of those members which have gained considerable attention. Amonafide and mitonafide (Fig. 1) were the naphthalimide based drugs, first reached clinical trial stage. But due to unexpected central neurotoxicity and unpredictable side effects, amonafide failed to enter clinical phase III.[10] Mitonafide has been studied in clinical phase I and phase II where it exhibited good activity against solid tumors.[25] But, it also found to possessed unwanted toxicity effects. Two other compounds of the bis-naphthalimide series, LU 79553 and DMP 840, have been reported to be in clinical trials.[26–28] It is a well known fact that antitumor activity of naphthalimides is directly related to their ability to inhibit human DNA topoisomerase II.[29] Therefore their

importances increase as antitumor agents and also the urge to modify and synthesize novel naphthalimides.

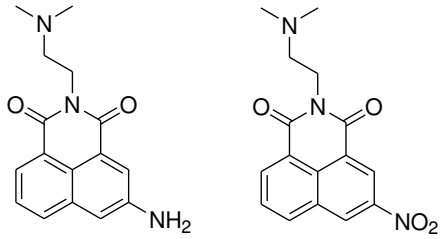

Fig. 1.

Pyridine is another class of nitrogen containing heterocycle, which also well known to medicinal field. Pyridine ring containing heterocyclic systems has gained significant importance by possessing anti-cancerous activities[30,31] and broad spectrum of biological and pharmacological properties. 1,2,3-Triazoles have also received wide attraction due to their special features like large dipole moment[32] stability towards metabolic degradation, capability of hydrogen bonding[33] and hence could react with protein in various different forms. In recent years, more attention has been paid towards their role as anti-tumour agents. Some of 1,2,3-triazoles have been reported which showed more activity than clinically used drug cis-platin[34] and are also used as DNA-alkylating and cross-linking agents[35] Reports have shown where introduction of 1,2,3-triazole to some other moiety exhibited promising DNA-binding affinity and anticancer activity in selected human cancer cell lines.[36]

Seeking the need and urgency of novel molecules and modifications of previously present drugs, novel naphthalimide analogues have been synthesized. Therefore, we have synthesized triazoles bearing pyridines, which could act more efficient anticancer agent. All the synthesized analogues have been tested for their antitumor activities. Role of their structural fragments have been studied on the basis of SAR studies.

Present work has been divided into following parts:

Chapter 1: Review of literature

Chapter 2: Synthesis of naphthalimide-benzimidazole analogues as anticancer agents

Chapter 3: Synthesis of allylic-naphthalimide analogues as anticancer agents

Chapter 4: Synthesis of pyridine-triazole based anticancer agents

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CHAPTER 1

REVIEW OF LITERATURE

Cancer may be assumed as major cause of death worldwide. Million of new cases are diagnosed every year. Cancer is basically out-of-control cell division. It is not only one disease but a group of more than 100 different and distinctive diseases. Cancer can involve any tissue of the body and have many different forms. Cancer, by definition, is a disease of gene. A gene is a small part of DNA (deoxyribonucleic acid), which is the master molecule of the cell. Genes make proteins, which are the main workers of the cells. These proteins allow our bodies to carry out our routine work. Many genes produce proteins that are involved in controlling the process of cell growth and division. An alteration to the DNA molecule can disrupt the genes and produce faulty proteins which cause the formation of abnormal cells. These abnormal cells begin to divide uncontrollably and form a new growth as tumour. Majority of cancers are caused by changes in cell's DNA because of damage due to the environment.

Cancer growth may be affected with following factors:

1. **Mitosis**: The most common form of cell division is called mitosis. It is used for growth and repair. Mitosis is closely controlled by the genes inside every cell. In case of cancerous cells, this control can go wrong. If that happens in just a single cell, it can replicate itself to make new cells that are also out of control. In this way cancer cells get multiply. They continue to replicate rapidly without the control systems that normal cells have.
2. **Enzymes:** Enzymes perform numerous functions during DNA replication, such as unraveling/winding of the DNA (helicase) to cutting DNA to adding nucleotides to newly developing DNA strands (polymerases). There are many other enzyme sub types involved in DNA replication however these are probably the major class of enzymes and their involvement.
3. **DNA:** Cells become cancer cells because of changes to their DNA. DNA is in every cell and it directs all its actions. In a normal cell, when DNA is damaged, the cell either repairs the damage or dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn't die like it should. Instead, the cell goes on making new cells that the body doesn't need. These new cells have the same damaged DNA as the first cell does.

Therefore, efficient anticancer agents are required that can stop the growth of the cancer cells without affecting the normal cells. Most commonly used anticancer agents are associated with following expected goals:

1. These are supposed to stop mitosis or the actual splitting of the parent cell into two new cells. Stopping mitosis actually stops cell division (replication) of the cancer and may ultimately stop the cancer progress.
2. These are coped with the expectation to inhibit the enzyme function at various steps to avoid the development of cancer cells.
3. These are expected to damage the DNA of the affected cancer cells which may inhibit the synthesis of new DNA strands to stop the cell from replicating, which is thought to be the reason of tumor growth.

Various drugs have been known which interfere with DNA synthesis. These are categorized as follows:

1. Alkylating agents are a class of drugs that bind to DNA and prevent proper DNA replication. They have chemical groups that can form permanent covalent bonds with nucleophilic substances in the DNA. These directly damage DNA (the genetic material in each cell) to keep the cell from reproducing. These drugs work in all phases of the cell cycle and are used to treat many different cancers including leukaemia as well as cancers of the lung, breast, and ovary.
2. The structure of groove binding molecules is characterized by several connected aromatic rings that allow freedom of movement and torsion. Binders usually have a characteristic curved shape compatible with the DNA groove. The formed complex is stabilised with hydrophobic interactions. Furthermore, DNA binders do not cause significant structural changes in the DNA molecule and no change to the DNA free energy structure.
3. DNA intercalators are the compounds having diverse structures and having ability to bind strongly but reversibly to DNA by intercalation of a flat, aromatic chromophore between the base pairs (Fig. 1). The forces that maintain the stability of the complex are; van der Waals, hydrogen bonding, hydrophobic effects, and/or charge transfer forces. Intercalation involves the insertion of a planar molecule between DNA base pairs, which results in a decrease in the DNA helical twist and hence lengthening of the DNA.

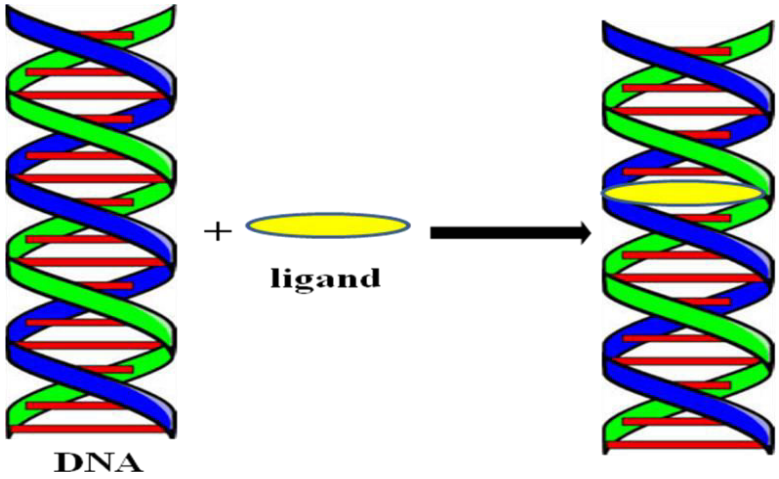


Fig. 1 Drug DNA Intercalation

Based on their mechanism of action, DNA interacting drugs have been classified into following categories.

1. Stop the synthesis of pre DNA molecule building blocks

These drugs may work in a number of ways. Folic acid, heterocyclic bases and nucleotides are known as DNA building blocks and these are made naturally within cells. These agents are supposed to block some step in the formation of nucleotides or deoxyribonucleotides which is necessary for making DNA; this leads to stop the synthesis of the nucleotides, which are the building blocks of DNA and RNA. Due to this, the cells cannot replicate because they cannot make DNA without the nucleotides. Some examples of such drugs are methotrexate, fluorouracil, hydroxyurea and mercaptopurine.

2. Damage the DNA directly in the nucleus of the cell

These type of drugs chemically damage DNA and RNA. They disrupt replication of the DNA and may totally stop replication. These type of drugs include cisplatin, antibiotics – daunorubicin, doxorubicin and etoposide.

3. Disrupting the formation of the mitotic spindles

When a cell starts to divide itself into two new cells, these spindles play very important role; they help to split the newly copied DNA in such a manner that a copy goes to each of the two new cells during cell division. But these categories of drugs breakdown the formation of these spindles and hence halt cell division. Some examples of such drugs include Vinblastine, Vincristine and Pacitaxel.

Keeping in mind the above discussion, it seems the necessity of the time to design highly efficient antitumor agents. In present scenario, the development of small molecules has received astronomical considerations which are capable of binding to deoxyribonucleic

acid (DNA) as well as exhibit anticancer activities.[1] 1,8-Naphthalimides are well known for their high anti-tumour activity towards various human and murine cells.[2] Starting from corresponding 1,8-naphthalic anhydride, variety of naphthalimide derivatives have been synthesized by reacting with different amines. Moreover, naphthalimide ring has also been substituted at 3- or 4-position by amino or nitro groups (eg. Amonafide, Mitonafide). It increases the probability of introduction of other functional groups at these positions, which may be further used for targeting biomolecules. This can affect the electronic properties of molecules that influencing the chemical, photochemical and spectroscopic properties of the molecule.

Ability to target biomolecules (particularly nucleic acids) is one of the attracting features of naphthalimides. Examples are present where naphthalimides have formed strong intermolecular complexes with mononucleotides. Due to their planar nature, the aromatic core of naphthalimides has been supposed to intercalate itself between the base-pairs of DNA. But, there are a number of ways in which these small molecules can bind to DNA (1) by binding to the grooves (major times the minor one) or (2) externally (if molecule shows a tendency for stacking). Binding mode may be dependent on DNA sequence. Binding to nucleic acids can be observed by some attractive techniques like UV-Visible absorption and fluorescence spectroscopy. Other methods that has been used for intercalation with DNA binding, are dichroism spectroscopies, hydrodynamic studies, e.g. viscometry, or biophysical measurements e.g. topoisomerisation.[3]

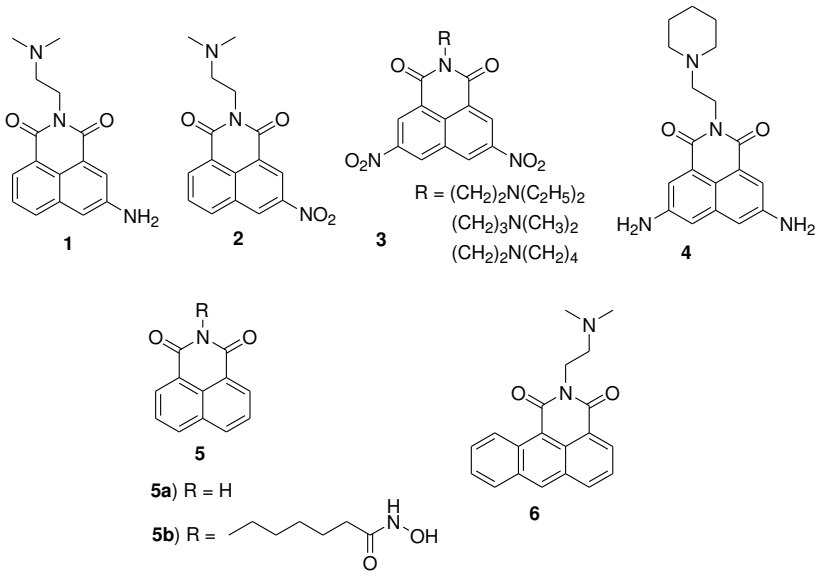
1. 1,8-Naphthalimides

Most of the naphthalimide based compounds have not reached the clinical trials due to poor therapeutic index and unpredictable toxicity. From the very first naphthalimide based compound, attention has been paid towards modification of naphthalimide skeleton. An overview of naphthalimide moiety has been concluded herein:

1.1. 1,8-Naphthalimides with nitrogen linkage

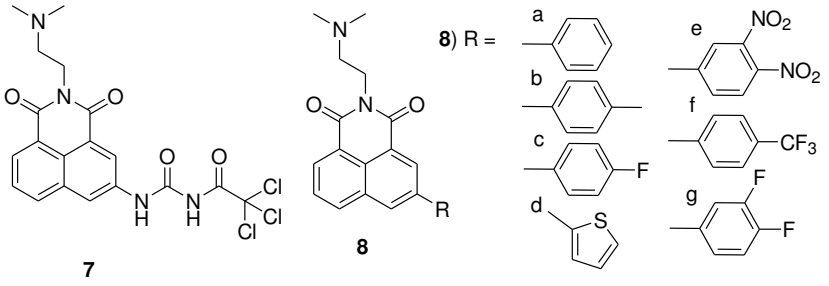
Amonafide (**1**) (3-amino-1,8-naphthlimide) and Mitonafide (**2**) (3-nitro-1,8-naphthalimide) proved two leading members of this family that entered into clinical trials (phase II) with IC_{50} values of 0.47 µM and 8.80 µM respectively, against HeLa cell lines. The dihydrochloride salt of amonafide[4] has also been successfully entered into phase II clinical trials for prostate cancer and have stabilised double strand DNA in denaturation experiments. Both, amonafide

and mitonafide exhibited DNA intercalation and hence inhibited topoisomerase II activity.[5,6]



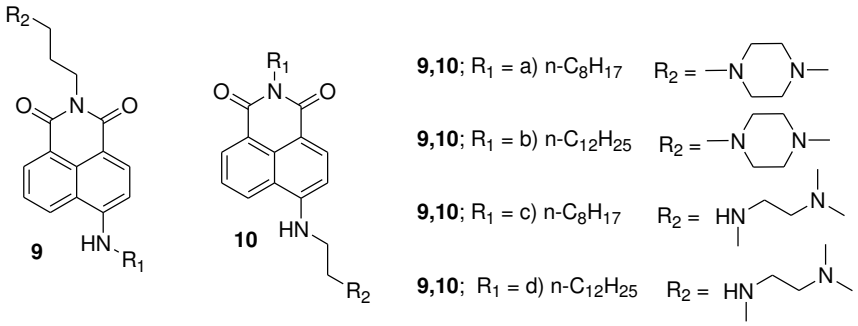
N-(Dialkylaminoethyl)-derivatives of 3,6-dinitro and 3,6-diamino-1,8-naphthalimides (**3-4**)[7] were reported by Zee-Cheng and Cheng, that showed high anticancer activity against leukemia (IC_{50} values of 0.036 and 0.33 μM respectively) and colon adenocarcinoma cell lines (IC_{50} values of 0.041 and 0.68 μM respectively). Bran˜a and co-workers developed a series of 3-amino-6-nitro-1,8-naphthalimide derivatives[8] which exhibited very high cytotoxicity than amonafide and mitonafide, against human CX-1 colon cancer and LX-1 lung cancer cell lines. Compound **5a** (Nafidimide) and **5b** (Scriptaid)[9] are the examples of unsubstituted naphthalimides. Compound **5a** was found to be cytotoxic against human myeloid leukemia cell lines KBM-3 and HL-6 while compound **5b** inhibited the histone deacetylase. Compound **6** (Azonafide) with polycyclic rings also showed significantly increase in antitumour activity compared to Amonafide. Thus, the length of side chains and size of substituents on naphthalimide have played important role in determining the anti-tumour activity of the naphthalimide analogues.[10,11]

Many other strategies have been followed to modify the naphthalimide chromophore, thereby improving its activity and lower the side effects. Analogues of Amonafide have been synthesised by Quaquebeke *et. al.* to overcome the drawbacks of basic moiety. These modifications involved incorporation of variety of functional groups like amide, urea, imine, amine and thiourea at 3-position of naphthalimide unit.[12] Amongst these, urea derivatives (**7**) proved to be better candidates showing improved *in vitro* and *in vivo* anticancer activity than others. This compound showed better antiproliferative agent than amonafide but have weaker DNA intercalations than amonafide.



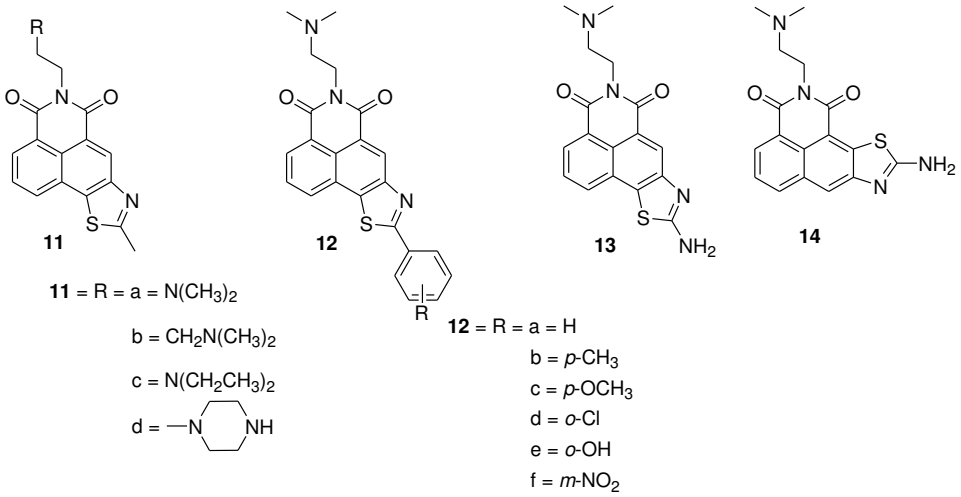
Modified 3-amino substituent of amonafide has also been reported (**8a–g**), in which the amino group has been replaced with phenyl group at 3-position.[13] Various studies have proved that these compounds intercalate with DNA and displayed better anticancer activities against HeLa and P388D1 cell lines than amonafide. This example clearly indicated the role of phenyl ring for improvement of antitumor activity.

Chen et al. has been designed the novel class of molecules by functionalization at imide nitrogen and 4-position of naphthalene ring using polyamines and alkyl chains (**9a–d** and **10a–d**).[14] These molecules showed the inhibitory activity against topoisomerase II and also showed better antitumor activity against variety of human cancer cell lines as compared to amonafide.

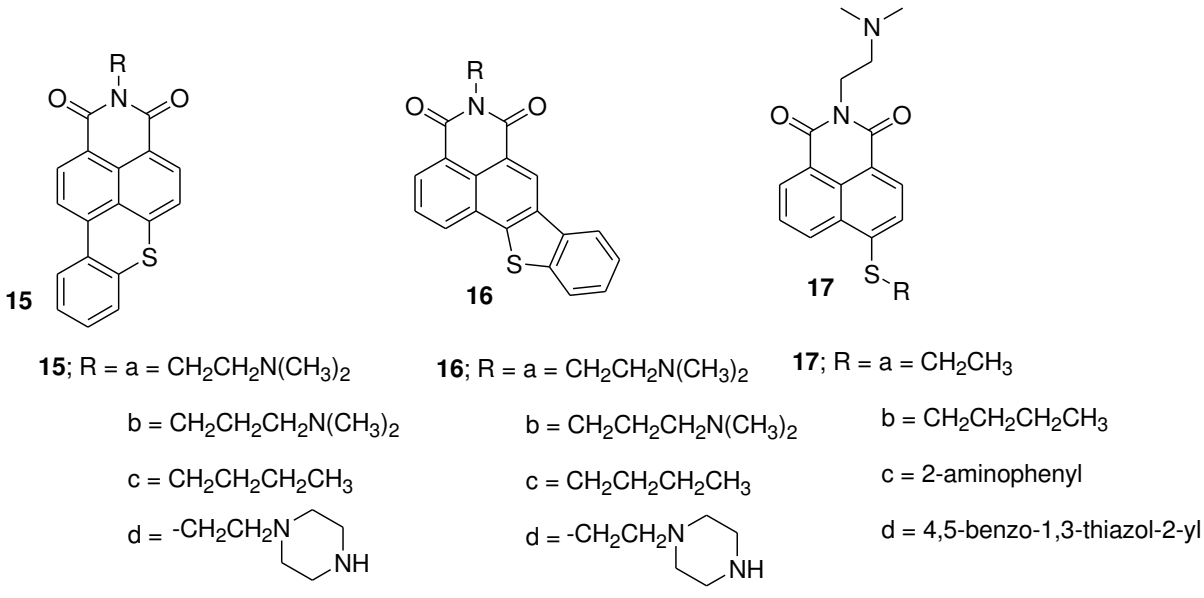


1.2. Napthalimides containing thio- group

Synthesis of 1,8-naphthalimide with thiazole or substituted thiazole groups at C4 position has been reported by Qian and co-workers (**11a-d**, **12a–f**).[15] It has been shown that the cytotoxicity of the molecules was not only depended on the ability of DNA binding and to penetrate the cell membrane, but it also included the basicity of the side chain of N atoms. Similarly, Li *et. al.* has been reported the development of 2-aminothiazole containing 1,8-naphthalimide (**13-14**) which not only showed moderate ct-DNA affinity but also exhibited photocleavage activity.[16]

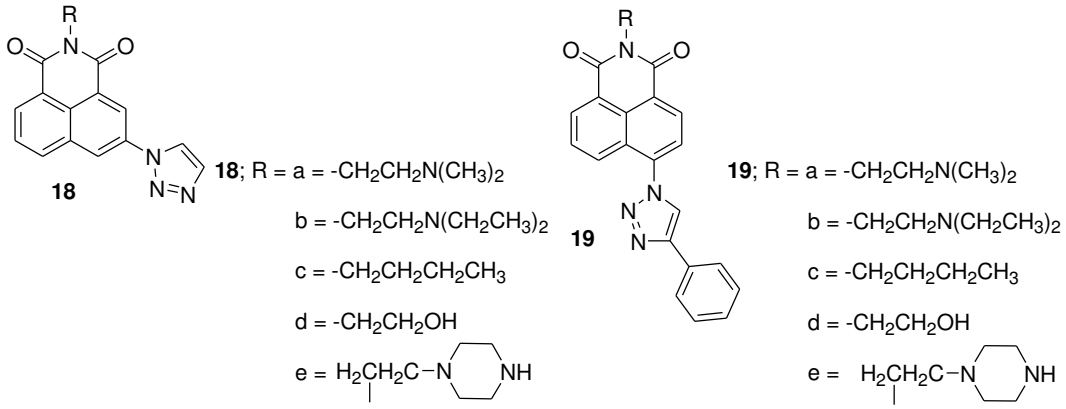


Similarly, Qian *et. al.* has reported a series of 1,8-naphthalimide analogous fused with six membered thio-heterocyclic ring **15a–d** and five membered thio-heterocyclic ring **16a–d**.[17] These compounds possessed high affinity for ct-DNA. Compound **16a** exhibited significantly higher cytotoxicity against a number of human cancer lines compared to amonafide. Further modifications have also performed keeping in mind the structural effects of **16a**, resulted in new derivatives **17a–d** which contain sulphur group attached to the alkyl chain at 4-position of naphthalene ring.[18] These molecules proved good photocytotoxic agents against human mammary cancer and colon cancer cell lines which may be due to their ability to inhibit the expression of topoisomerase II.



1.3. Naphthalimides with triazole ring

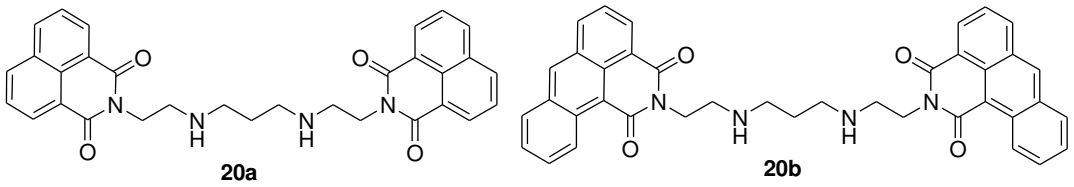
1,8-Naphthalimide bearing triazole moiety at its 3- or 4- position has been developed by Qian *et al.*[19] These derivatives (**18a–e** and **19a–e**) proved better cytotoxic agents than amonafide by showing good activities against several human cancer cell lines.



The improved cytotoxicity of these compounds has been explained on the basis of triazole ring at 3- or 4- position. However, it is noted that presence of side chains has also aided the effect to the cytotoxic activities of these molecules. Under specific conditions, these molecules also exhibited DNA cleavage.

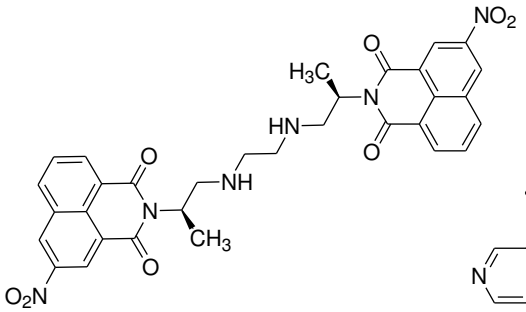
1.4. Bis-naphthalimides

In order to improve affinity for DNA and increase the cytotoxic potential, several bis-1,8-naphthalimide derivatives have been developed. Two 1,8-naphthalimide moieties, connected by a polyamine spacer constitute a class of bis-naphthalimides. Braña and co-workers first reported bis-naphthalimides in order to increase the antitumor activity and improved DNA binding.[20] Elinafide (LU79553) **20a**, exhibited high activity against a variety of human xenograft models such as LX-1 (lung), CX-1 (colon), and LOX (melanoma).[21] An anthracene derivative, **20b** (also known as Bibenoline), has also been developed.[22] that showed similar activity to **20a**. IC_{50} values for human colon cell lines HT-29 has been reported as 0.004 µM and 0.014 µM for **20a** and **20b** respectively.

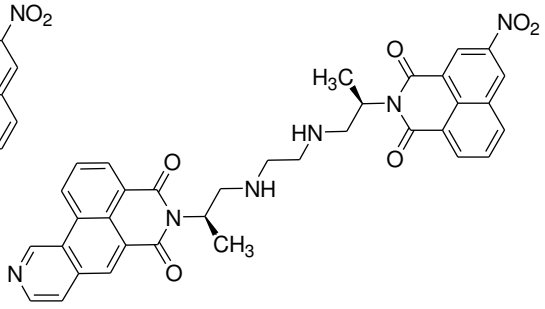


Chen and co-workers reported bis-naphthalimide DMP 840 (**21a**) that exhibited *in vitro* anticancer activity against leukemia and various solid tumours.[23] A series of compounds related to **21a** have been synthesised (**21b**), differing in the type of chromophores at one end of the molecule, but leaving one of the naphthalimides unchanged.[24] The compounds were evaluated *in vitro* for DNA binding that has been checked with ethidium bromide displacement and growth inhibition (L1210 murine leukaemia). The incorporation of a

phenanthrene chromophore was found to be an efficient DNA binder. However, it was established that electron donating and withdrawing groups at 6-position of the phenanthrene neither helped DNA binding nor inhibition. Compound **21b** was found to display excellent L1210 activity with IC_{50} value of 0.035 µM, which was similar to that reported for **20a** (IC_{50} = 0.034 µM).

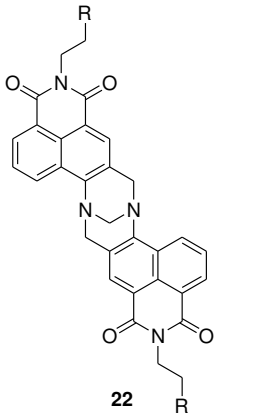


21a

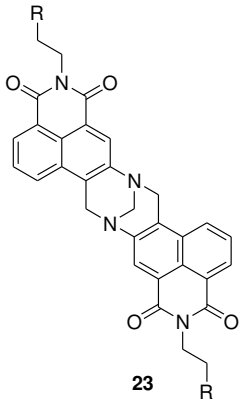


21b

Gunnlaugsson *et. al.* developed a series of bis-naphthalimides **22-24** linked by the Tröger's base (TB) moiety for DNA targeting.[25,26] Presence of terminal nitrogen atom in **22** and **23** was specifically designed to increase their water solubility. These molecules showed high affinity to bind with ct-DNA and stabilised ct-DNA in thermal denaturation experiment with $\Delta T_m > 15$ °C.[25]



22

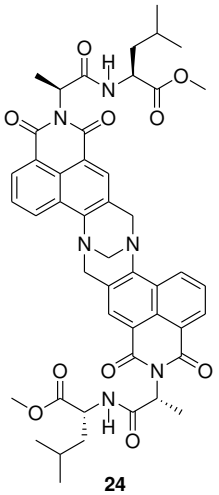


23

22; 23; a = R = $N(CH_3)_2$

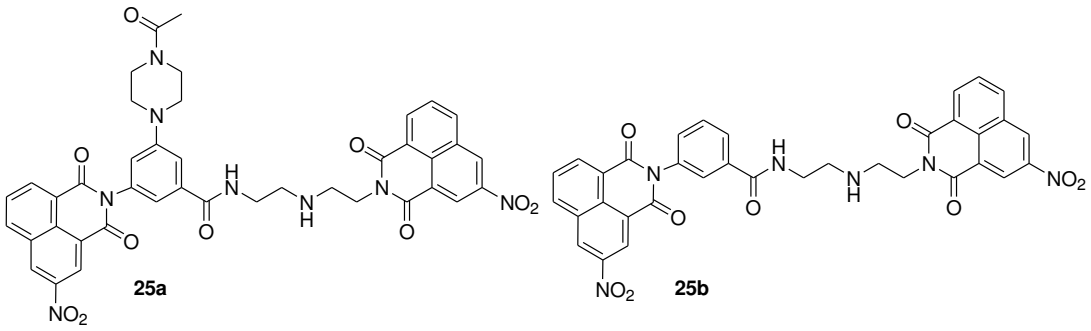
22; 23; b = R =

22; 23; c = R =



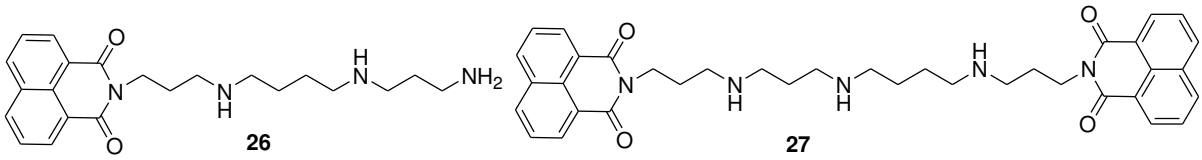
24

Furthermore, alternative bis-intercalators have also been developed where the two naphthalimide units were spaced by an alkyl or (poly)-aminoalkyl spacer. Conjugation via a peptide linkage to such amino acid derived structures was also undertaken that shown to be highly active and better DNA binders. Suzuki *et. al.* developed novel naphthalimido-benzamide derivatives **25a** and **25b**. The later exhibited significant anticancer activity and proved a very good DNA binder.



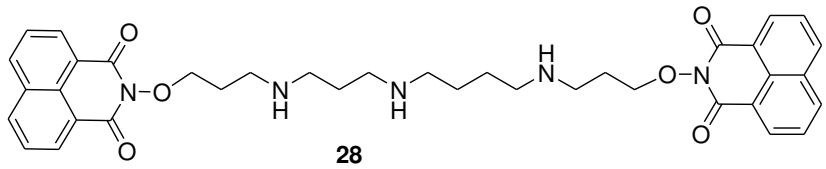
25a 25b

A series of bis-naphthalimide conjugates was designed by Lin and Pavlov in which spermine and spermidine were used as the linkers **26-27**.[27] Compound **27** exhibited high cytotoxic activity against colon cancer cell lines Caco-2 and HT29.



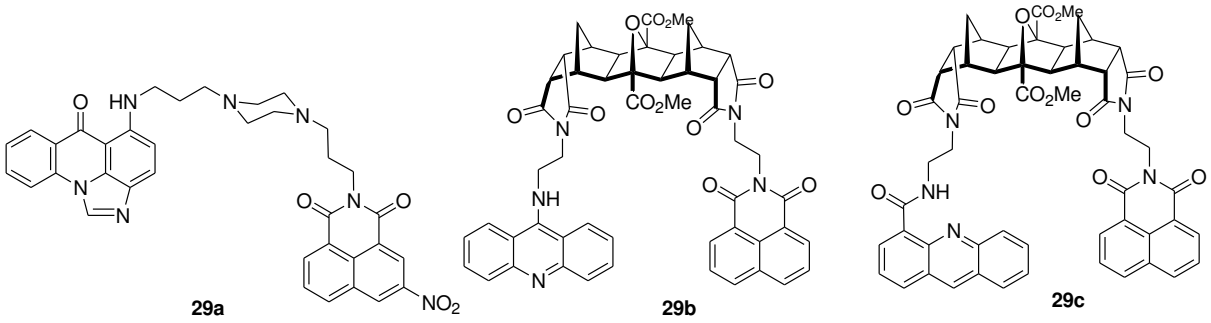
26 27

This moiety was further modified in case of bis-oxy-naphthalimidopolyamine by introducing oxygen atom to the *N*-naphthalimide (**28**) which showed intercalating behaviour with DNA but exhibited less cytotoxic activity than **27**.



28

Moreover, Cholody *et. al.*[28,29] have designed and synthesized asymmetrical bi-functional antitumour agents (**29a**). Later on, Waring and co-workers have reported bifunctional intercalators (**29b** and **29c**) which exhibited DNA unwinding properties and possessed Topo I mediated relaxation effect on covalently closed circular DNA (cccDNA).

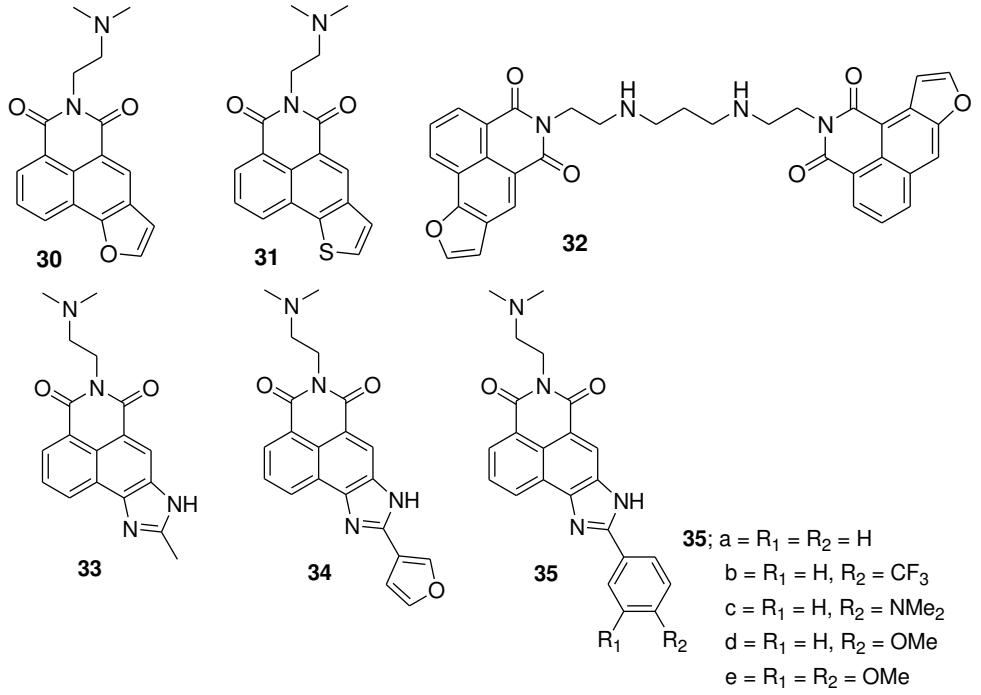


29a 29b 29c

1.5. Naphthalimides fused with other rings

A new class of 1,8-naphthalimides has been reported in which the basic naphthalene ring was fused with another heterocyclic ring. Branå *et. al.* has been reported the basic naphthalene

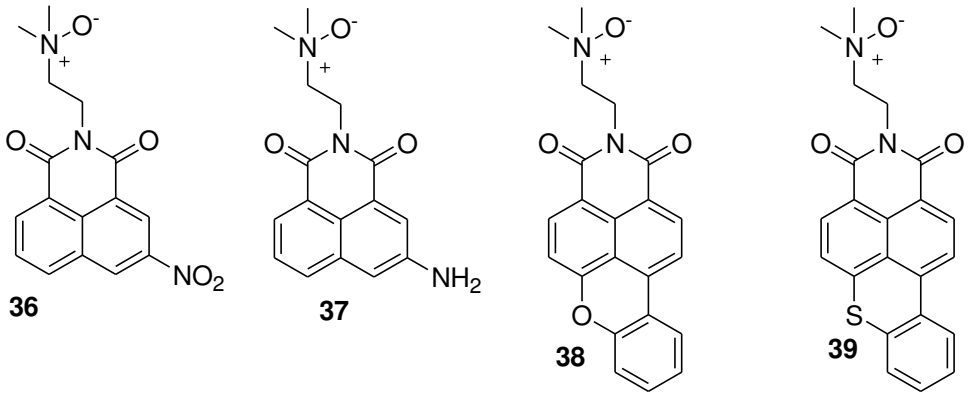
ring, fused with furan **30** or thiophene ring **31** and former showed higher acvtivity than later.[30] Dimerisation of furano-naphthalimides (**32**) enhanced the cytotoxicity of corresponding mononaphthalimides by more than 100 times, against CEM leukemia cell lines.[31] From these results, it has been suggested that furan ring plays an important role in such activities.



Mono imidazo-naphthalimides (**33**) showed better activity than amonafide. But dimerisation of imidazo-naphthalimides has shown contradictory results by showing no improvement in cytotoxic activity to the corresponding mono naphthalimides[32] which have been further explained on the basis of inadequate linker length to form stable complexes with DNA. Compound **34** and **35** bind to DNA via intercalation and stabilised DNA very effectively against thermal denaturation experiments.

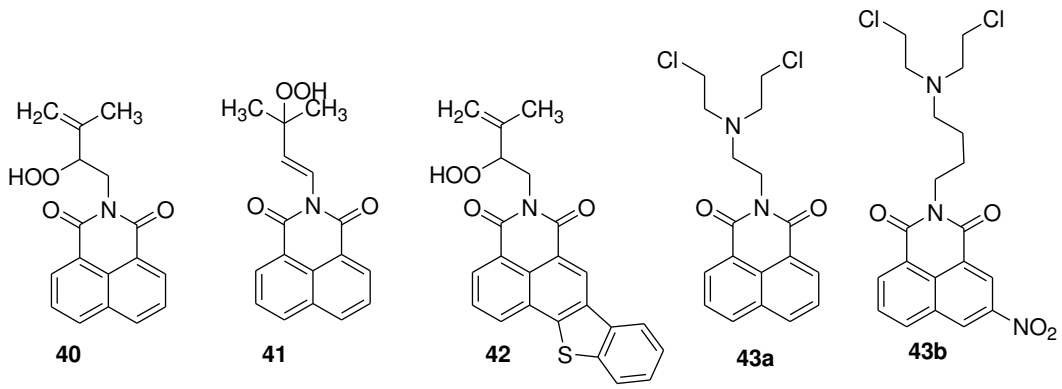
1.6. Naphthalimide prodrugs

Qian et al.[33] have reported tertiary amine N-oxides of naphthalimides as prodrug leads. All compounds showed potential against hypoxic A375 cells, which enabled them to be used as potential candidate of prodrug leads with hypoxic solid tumor preference **36-39**. It was also observed that ct-DNA binding affinities and cytotoxic activities of derivatives, containing tertiary amine *N*-oxide moiety in the side chain, against usual tumor cell lines were lower than their corresponding amines.



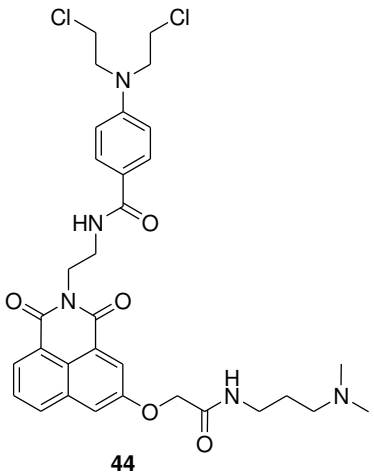
1.7. Other naphthalimide derivatives

Tao *et. al.*[34] has been synthesized 1,8-naphthalimide hydroperoxide derivatives **40-41** and studied their interactions with DNA. Similarly, **42** with large conjugated planar heterocyclic fused naphthalene ring also showed intercalation with DNA.[35] This compound possessed higher cleaving abilities than **40**.

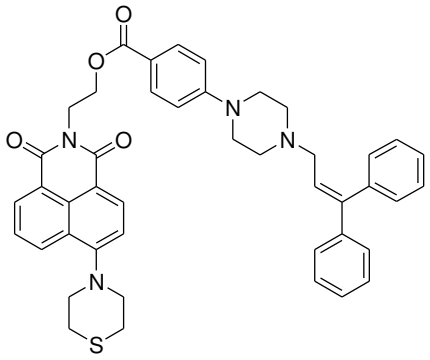


Xie *et. al.*[36] has been reported 3-nitro-naphthalimide and nitrogen mustard derivative **43a** by combining the naphthalimide unit with *N,N'*-bis(2-chloroethyl) amino group, which exhibited significant cytotoxicity against murine carcinoma cell lines. Further studies on 3-nitro-1,8- naphthalimide conjugated nitrogen mustard **43b** have shown very high anti-tumour activities and lower toxicity against hepatocellular carcinoma compared to amonafide.[37]

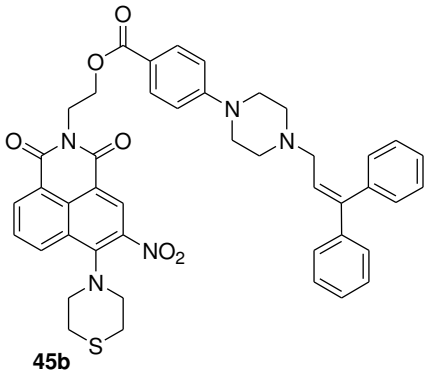
Similarly, Gupta *et. al.*[38] have developed 1,8-naphthalimide derivatives having nitrogen mustard functionalities at the imine position with amide linkage (**44**). These compounds have shown considerable cytotoxicity against various cancer cell lines. Qian *et. al.*[39] have reported some naphthalimide–benzoic acid conjugates (**45a–b** and **46a-b)** which have shown comparable cytotoxic activity as amonafide.



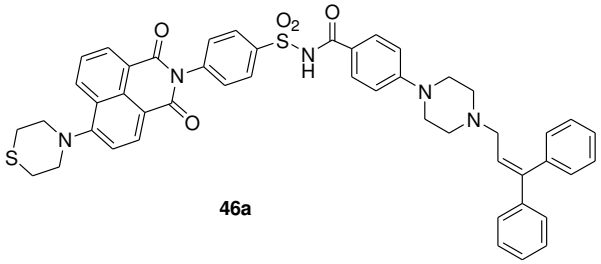
44



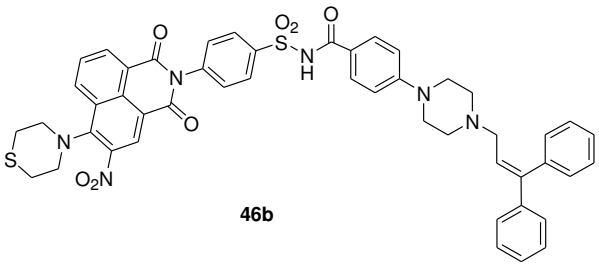
45a



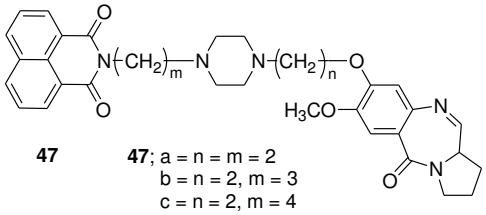
45b



46a



46b



47

47; a = n = m = 2
b = n = 2, m = 3
c = n = 2, m = 4

Using piperazine ring system, pyrrolo[2,1-*c*][1,4]benzodiazepines conjugated naphthalimide **47a–c** has been reported by Kamal *et. al.* and evaluated for their anticancer activities.[40] Compound **47b** and **47c** possessed highest binding affinities and hence increased the cytotoxic activity which clearly indicated the significance of length of alkane spacer between pyrrolo[2,1-*c*][1,4]benzodiazepines, piperazine and naphthalimide moieties.

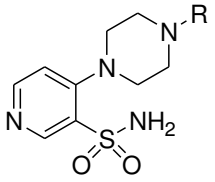
2. Pyridine and triazole analogues

Many organic chemists have been attracted with pyridine derivatives due to their biological and chemotherapeutic importance. The pyridine ring system has been proved to be an interesting class in heterocyclic chemistry and some of its derivatives are important as anticancer agents with low toxicity. On the other hand, number of triazole based compounds have been reported, possessing wide range of biological activities including antimicrobial,[41] analgesic,[42] anti-inflammatory,[43] local anesthetic,[44] antimalarial,[45] anticancer,[46] and anti-HIV

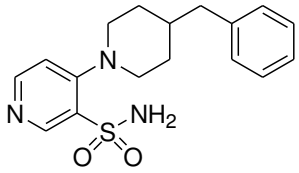
activity.[47] Many examples are reported in literature where pyridine and triazoles (individually, as well as in combination) have shown significant activities. A brief overview has been given as below:

2.1. Substituted pyridine-sulfonamides

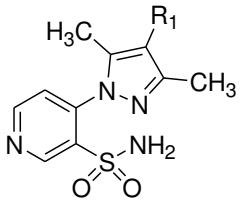
Sławinski *et. al.*[48] has been synthesized 4-substituted pyridine-3-sulfonamides (48-56) and evaluated for their anticancer activity at single dose of 10 µM against a panel of 60 human cancer cell lines at National Cancer Institute, USA. Compound **52** proved to be most active by possessing broad spectrum growth inhibition in the range of 25-89% over 26 cell lines representing all tumours subpanels.



48-52



53



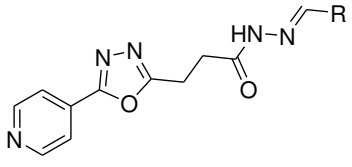
54-56

48; R = Ph
49; 4-ClPh
50; 4-FPh
51; 2-FPh
52; 3,4-diClPh

54; R_1 = H
55; R_1 = Me
56; R_1 = $CH_2CH_2CO_2Et$

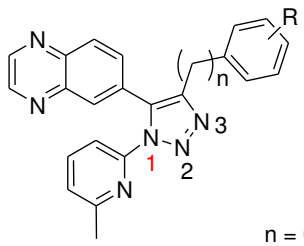
2.2. 1,3,4-Oxadiazole-pyridine derivatives

Zhang *et. al.*[49] have reported the synthesis of 1,3,4-oxadiazole derivatives having pyridine as its appended unit. On the basis of bioassay tests, compounds **57a-e** has been labelled as most active compounds of the series. These compounds showed broad-spectrum anticancer activity over cancer cell lines HEPG2, MCF7, SW1116 and BGC823 with IC_{50} values ranging from 0.76 to 9.59 µM. Compound **57d** also showed significant telomerase inhibitory activity with IC_{50} value of 0.76–1.54 µM against the tested cancer cell lines.



57a-e

57; a = R = 2-HO-C_6H_4
b = R = 2-HO-5-Cl-C_6H_3
c = R = 2-HO-3,5-2Cl-C_6H_2
d = R = 3,4-2HO-C_6H_3
e = R = 3-MeO-4-HO-C_6H_3



58

n = 0,1

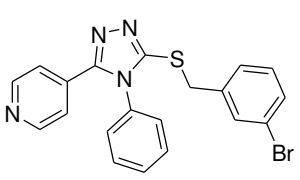
58; R = a = *n*-Butyl
b = Isopentyl
c = 4-Hydroxybutyl
d = 3,5-Dimethoxyphenyl
e = Phenyl
f = Benzyl

2.3. Triazole-pyridine analogues

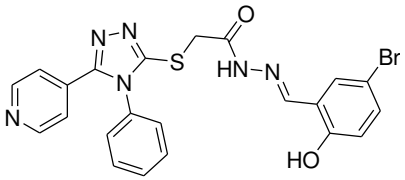
Transforming growth factor-β (TGF-β) participates over a range of biological processes and act as a multifunctional regulator. It also regulates build-up, distinction and activation of immune cells. Deregulation of TGF-β signalling may lead to cause various diseases like cancer, autoimmune disease, vascular disorder etc. ALK5 is type I (TbR-I) kinase receptors that transducers TGF-β signals. Inhibition of ALK5 may result in reduced TGF-β-induced excessive accumulation and would finally lead inhibit cancer and other disease.

1-(6-Methylpyridin-2-yl)-5-(quinoxalin-6-yl)-1,2,3-triazoles (**58a-f)** have been synthesized and evaluated for their ALK5 inhibitory activity by Li *et. al.*[50] Compound **58f** showed 80.8% ALK5 inhibition at 10 µM concentration with IC_{50} value of 4.69 µM. Other compounds exhibited moderate to significant ALK5 inhibitory activities.

Yang *et. al* [51] has been synthesized pyridine substituted 1,2,4-triazole derivatives and evaluated for their anti-cancer activities against MCF7 (human breast cancer), HCT116 (human colorectal cancer), and HepG2 (human hepatoma cells) cell lines. Compound **59** and **60** showed significant activity against the tested cell lines. Compound **60** possessed even better activity than the reference drug staurosporine against HCT116 cell line (IC_{50} = 1.99 µM for **60**, IC_{50} = 13.20 µM for staurosporine). Presence and position of halogen atom on benzene ring have positively influence on the activity.



59a

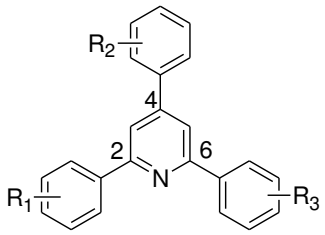


60a

2.4. Triphenyl pyridines as topoisomerase inhibitors

Karki *et. al.* has been reported dihydroxylated 2,4,6-triphenyl pyridines (**67-73**). The hydroxyl groups varied at ortho, meta or para positions of 2- and 6-phenyl, or 2- and 4-phenyl rings. These molecules have been evaluated for their cytotoxicity over various human cancer cell lines as well as for their topoisomerase I and II inhibition. It was generalized that dihydroxylated 2,4,6-triphenyl pyridines proved better cytotoxic agents as well as topoisomerase II inhibitors than monohydroxylated 2,4,6-triphenyl pyridines. Studies revealed that the presence of hydroxyl group enhanced only topo II inhibition and with increase in number of hydroxyl group, there is increase in topo II inhibition. When evaluated for their cytotoxicity on various human cancer cell lines (HCT15; human colorectal

adenocarcinoma cell line, K562; human myeloid leukemic tumor cell line, DU145; human prostate tumor cell line, MCF-7; human breast adenocarcinoma cell line and HeLa; human cervix tumor cell line), compounds **61** and **62** displayed strong cytotoxicity over most of the tested cell lines.[52]



61 = R_1 = *o*-OH, R_2 = H, R_3 = *p*-OH

62 = R_1 = *m*-OH, R_2 = H, R_3 = *p*-OH

63 = R_1 = *m*-OH, R_2 = *o*-OH, R_3 = H

64 = R_1 = *m*-OH, R_2 = *m*-OH, R_3 = H

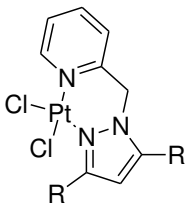
65 = R_1 = *m*-OH, R_2 = *p*-OH, R_3 = H

66 = R_1 = *p*-OH, R_2 = *o*-OH, R_3 = H

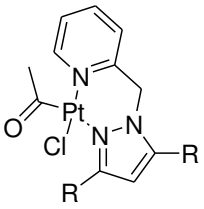
67 = R_1 = *p*-OH, R_2 = *p*-OH, R_3 = H

2.5. Pyridine metal complexes

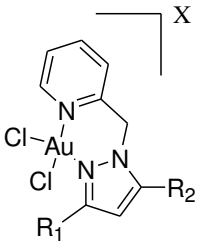
Metal complexes in medicinal chemistry have been extensively studied after the approval of chemotherapy drug cisplatin. The side effects associated with use of cisplatin have led to further improvements and finding of more efficient drugs. Segapelo *et. al.*[53] have synthesized pyridine based platinum (II) and gold (III) complexes which have evaluated as anticancer agents (**68-71**). Cytotoxicity studies of these complexes against HeLa cells revealed that Pt complexes (**68-69**) possessed better activity than Au complexes (**70-71**).



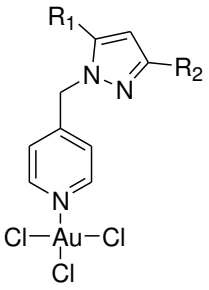
68; R = Me, Ph, *t*-Bu



69;R = Ph, *t*-Bu



70; R_1 = R_2 = Ph
R_1 = R_2 = *t*-Bu
R_1 = R_2 = Me
X = $[AuCl_4]^-$

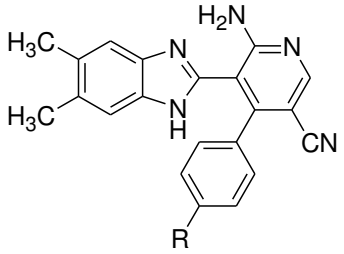


71; R_1 = R_2 = Me
R_1 = R_2 = Ph

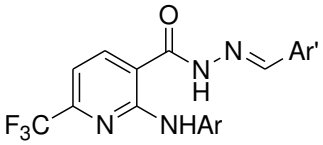
2.6. Benzimidazole-pyridine conjugates

S. A. Elzahabi and co-workers have been reported the synthesis of benzimidazole derivatives bearing pyridine nucleus (**72a-d**) and evaluated for their cytotoxic activity. Compounds **72b** (4-(*p*-chlorophenyl)pyridine) and **72d** (4-(*p*-methoxyphenyl)pyridine) possessed respective

broad and moderate antitumor activity over major cell lines of nine subpanels. These molecules have also been evaluated at five dose level screening at NCI, NIH, USA.[54]



72; R = a = H
b = Cl
c = OH
d = OCH_3



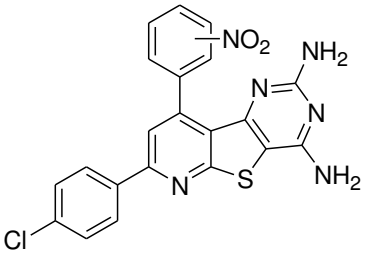
73; a = Ar = 2-Me-4-Cl-C_6H_3, Ar' = 2-Cl-C_6H_4
b = Ar = 2-Me-4-Cl-C_6H_3, Ar' = 2, 6-Cl_2-C_6H_3
c = Ar = 2-Me-5-Cl-C_6H_3, Ar' = 2, 6-Cl_2-C_6H_3
d = Ar = 3-CF_3-C_6H_4, Ar' = 2, 6-Cl_2-C_6H_3

2.7. 2-Arylamino-6-trifluoromethyl-3-(hydrazonocarbonyl)pyridines

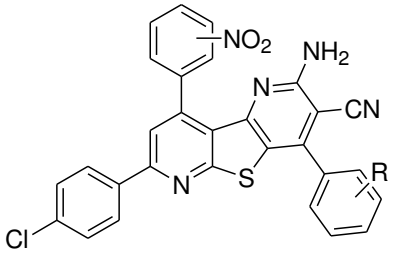
Onnis *et. al.* have been reported the synthesis of 2-arylamino-6-trifluoromethyl-3 (hydrazonocarbonyl)pyridines and evaluated for their anticancer activities. Compound **73b**, which is most active compound of this series, showed the inhibition of all tested cancer cell lines at nanomolar range with not showing any toxicity to normal cell lines. This compound has been selected by Biological Evaluation Committee of NCI and is under *in vivo* Hollow Fiber Assay studies. Compounds **73a** and **73c-d** also showed growth inhibition of all tested cancer cell lines with respective micro and submicromolar GI_{50} values. It has been observed that the presence of chloro group at 2- and 6- positions have significantly affected on the activity of the compound. Significant increase in antiproliferative activity has been shown on displacement of 3-amide group of 2-arylamino-6-trifluoromethylnicotinamides with hydrazono moiety.[55]

2.8. Fused pyridine ring system

Kadry *et. al.* have been synthesized fused pyridine ring system and evaluated for their anticancer activities against human breast adenocarcinoma MCF-7 and colon carcinoma cell line HCT116. Most of the compounds such as pyrido[3',2':4,5]thieno[3,2-*d*]pyrimidines (**74a**-**b**) and thieno[2,3-*b*:4,5-*b*'] dipyridine (**75a**-**c**) possessed good to moderate growth inhibition. Compound **75d** with 3-nitrophenyl group at both phenyl rings, proved to be the most potent (IC_{50} = 5.95 for MCF7 and 6.09 µM for HCT116) to reference drug doxorubicin (IC_{50} = 8.48 for MCF7 and 8.15 µM for HCT116). Among both the tested cell lines, it was observed all compounds showed potency against HCT-116 with IC_{50} values of 6.04 to 23.61 µM.[56]



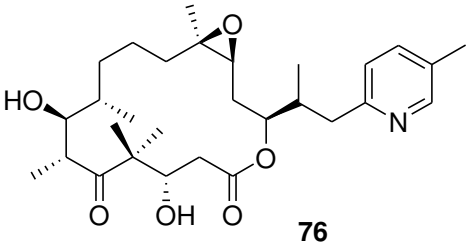
74; a = *o*-NO_2
b = *m*-NO_2



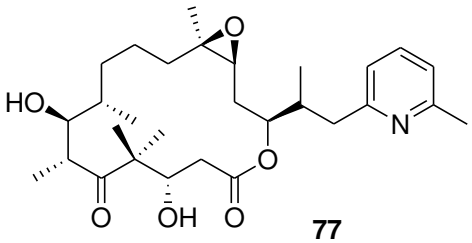
75; a = *o*-NO_2, R = H
b = *m*-NO_2, R = H
c = *o*-NO_2, R = *m*-NO_2
d = *m*-NO_2, R = *m*-NO_2

2.9. Pyridine epothilones

Nicolaou *et. al.*[57] has been synthesized pyridine epothilones (**76** and **77**) that exhibiting cytotoxicity against a number of human cancer cell lines. It has been observed that position of nitrogen and methyl group to substituted pyridine ring played an important role in the activity of the compounds.



76



77

On the basis of above discussion, it is clear that naphthalimides are playing a leading role as anticancer agents and DNA intercalators. There are large scope of modifications on napthalimide core to improve their antitumor activity of existing drugs. On the other hand, pyridine and triazoles are also playing important roles in bio-medicinal chemistry individually as well as collectively. It may be assumed that combination of these two moieties will result in higher activity. Therefore, naphthalimide and pyridine based novel derivatives has been synthesized keeping in mind their scope in future.

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CHAPTER 2
SYNTHESIS OF NAPHTHALIMIDE-BENZIMIDAZOLE ANALOGUES AS ANTICANCER AGENTS

2.1 Introduction

The design of highly efficient anti-tumour agents is of significant importance in organic and medicinal chemistry. Naphthalimide based drug constitute indispensable part in the development of anticancer drugs. Due to the presence of a coplanar fluorophore, л-deficient aromatic system and one or two basic side chain(s), naphthalimide constitutes an important class of drug in anti-tumour therapy and thus characterized by high cytotoxicity activity against a variety of tumour cells.[1-8] Naphthalimide compound is also an important class of DNA intercalator and has been demonstrated to inhibit Topo II.[9-10] Amonafide and mitonafide (**A** and **B**, Figure 1) were two of the most active naphthalimide-based Topo II inhibitors and had been tested in clinical trials. But, due to central neurotoxicity and limited efficacy in solid tumours, the clinical developments of these drugs were terminated.[11] To improve the efficacy and toxicological profiles, considerable efforts have been attempted to design more active naphthalimides,[12-14] such as elinafide and bisnafide (**C** and **D**, Figure 1) and most of the efforts were focused on the analogues with higher DNA-binding affinity.[15] Biological studies of these molecules showed that intercalating agents are capable of targeting DNA without inducing DNA damage and mutations in cellular genes. This phenomenon reduced side effects and the risk of developing secondary cancers, thereby supporting the discovery of nongenotoxic DNA-binding small molecules as anticancer therapies.[16]

Naphthalimide derivatives modified at different positions provided an effective way to improve the anticancer activity and decrease the toxic side effects.[17-19] Moreover, existing research results confirmed that the substituent at 5-position of the naphthalene ring is key to antitumour activity.[20-21] As the amino group at 5-position in case of amonafide showed extensive metabolism and the nitro-group in case of mitonafide was made responsible for toxic side effects, the modified derivatives were characterized by fused multi-aromatic ring system and one or two flexible basic side chains.[22-27] Thus, the fusion of one or more aromatic heterocycles with naphthalene core would be an efficient approach to enhance the antitumour activity. Although the significant results have been obtained in this way, there were some problems on the poor solubility and complicated synthesis of naphthalimide

derivatives. In this chapter, we have focused on fusion of benzimidazole with naphthalimide moiety and substitution with primary and secondary amines at 6-position of naphthalimide. These would reduce therapeutically problematic functions and lead to the discovery of several naphthalimides containing enlarged fused aromatic ring systems whose planar tri-cyclic ring system may also intercalate into DNA to increase medical or biological potential. Among the evaluated compounds, it has been significantly noted that especially sulphur-containing derivatives showed promising antiproliferative efficiency.[6a,15,21,27] One of the most active agent found within our study is thio heterocyclic naphthalimide **5d**, which exhibited superior selectivity towards several tumour cell lines and intercalating properties with ct-DNA.

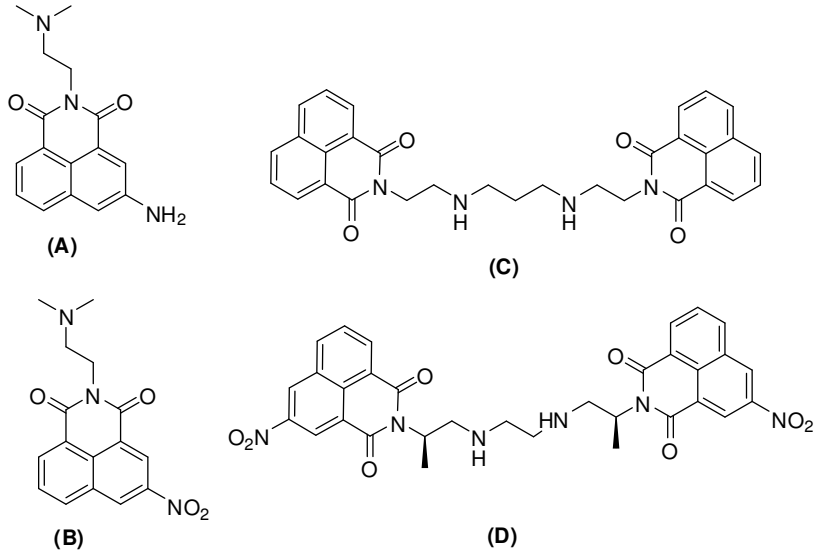


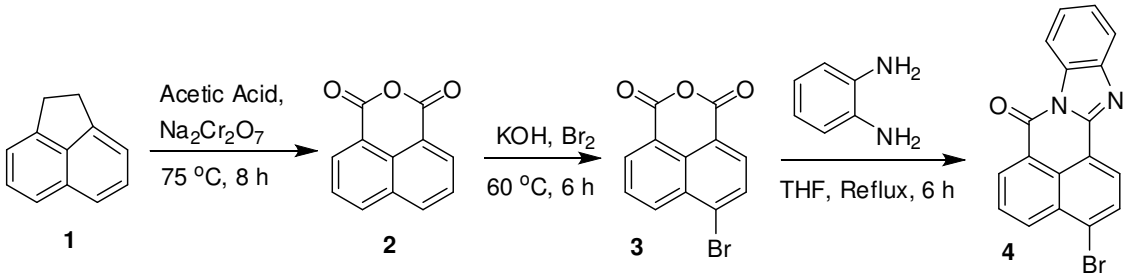
Figure 1. Chemical structures of Amonafide (**A**), Mitonafide (**B**), Elinafide (**C**) and Bisnafide (**D**).

Objective: Naphthalimides are known as a class of compounds with high antitumor activity upon a variety of murine and human tumor cells. These can bind to DNA by intercalation of the chromophore. Multi-disciplinary approaches including medicinal chemistry and early toxicology have resulted a new generation of promising naphthalimide derivatives. It is thus reasonable to expect that members of this class will be very useful in near future. A thorough study of literature revealed that no reports were present on combined naphthalimide-benzimidazoles. Therefore it was decided to synthesize naphthalimide-benzimidazole moieties and substitute various amines to the basic moieties.

2.2. Chemistry

2.2.1. Synthesis of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (4)

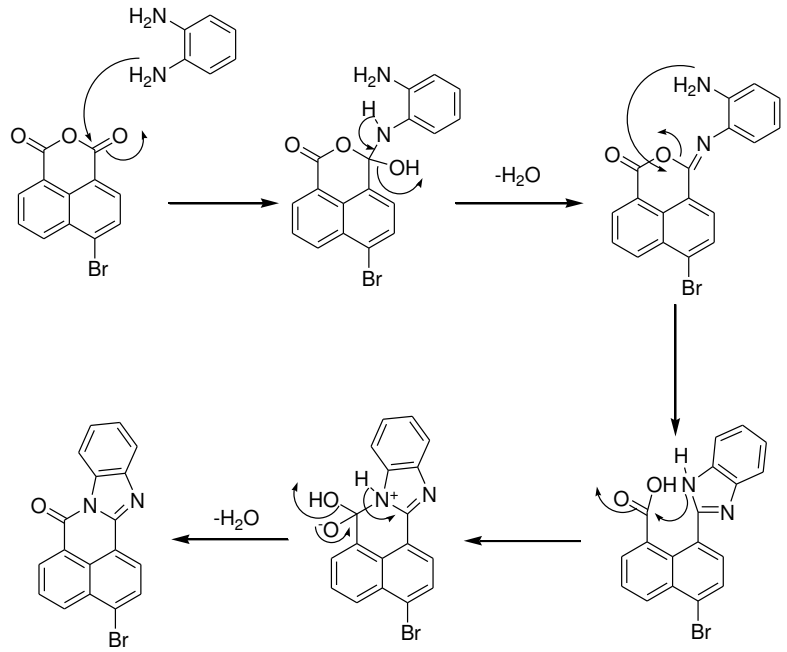
The target naphthalimide analogues were synthesized via multistep reactions from commercially available starting material acenaphthene **1** (Scheme 1). To a solution of acenaphthene (2.31 g, 15.0 mmol) and acetic acid (60 ml), sodium dichromate (11.17 g, 40.0 mmol) was added gradually in small fractions with continue stirring at room temperature. The reaction was highly exothermic and thus temperature was maintained at 25 ^{o}C using water bath. After stirring for 30 min at 25 ^{o}C, this suspension was heated to 75 ^{o}C and stirred for 8 h at same temperature. On completion of reaction, colour of the reaction mixture was changed to dark green. Ice cold water was added to the reaction mixture, resulted in precipitation of the crude product, filtered and washed with water a number of times to remove excess acid, till the dark green colour was totally washed away from the solid. Yellow product of 1,8-naphthalic anhydride (**2**) was separated out in 80% yield (m.p. 266-268 ^{o}C). 1,8-Naphthalic anhydride (**2**) was then completely dried over vacuum to remove any moisture. To a cooled solution of 1,8-naphthalic anhydride (1.98 g,10.0 mmol) in KOH (2.8 g in 12 ml water), 2 ml of bromine was added drop-wise with help of dropping funnel at room temperature. Any increase in temperature was controlled by water bath. The reaction mixture was continued to stir at room temperature for 30 min and then heated to 60 ^{o}C for 6 h. On the completion of reaction, yellow coloured precipitates were separated out. The reaction mixture was stirred at room temperature and acidified with concentrated hydrochloric acid (HCl). The solid residue was then filtered and heated with 5% NaOH solution, filtered it and filtrate was neutralized with HCl solution. Filtered and washed with cold water, dried to get yellow powder of 4-bromo-1,8-naphthalic anhydride (**3**) in 82% yield (m.p. 117-119 ^{o}C).



Scheme 1. Synthesis of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (**4**).

4-Bromo-1,8-naphthalic anhydride (**3**) (2.00 g, 5.0 mmol) and *o*-phenylenediamine (0.618 g, 5.0 mmol) in THF was heated to reflux for 6 h. Reaction was monitored by thin layer chromatography (TLC). After the completion of reaction, cooled the reaction mixture to get crude solid product, filtered and washed with THF to afford pure 3-bromo-

benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7- one (**4**) in 63 % yield (m.p. 190-192 ^{o}C), MS (EI) : m/z 350.1 (M^+ + 1).

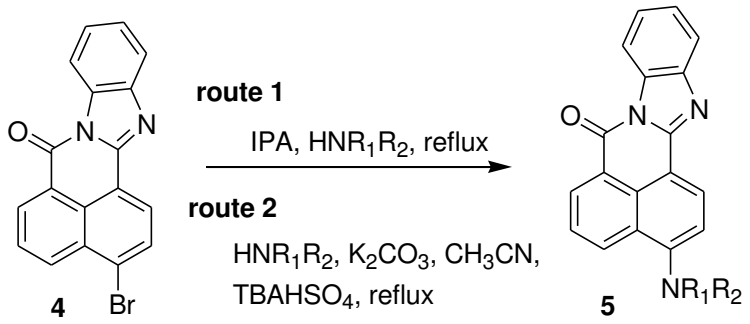


Scheme 2. Proposed mechanism for the synthesis of compound **4**.

^{1}H NMR spectrum of compound **4** showed three 1H double doublets at δ 8.90, δ 8.79 and δ 8.04, two 1H doublets at δ 8.53 and δ 8.24, 3H multiplet at δ 7.74 and 1H multiplet at δ 7.56 of aromatic-H. ^{13}C NMR spectrum of this compound showed –C=O signals at δ 165.7 and 136.5, 134.5, 134.4, 133.4, 131.9, 131.7, 131.6, 131.1, 130.9, 129.3, 128.6, 128.2, 127.2, 124.9, 121.0, 119.9 of aromatic C. The proposed mechanism for the synthesis of compound **4** has been shown in Scheme 2 where nucleophilic substitution of amine open the anhydride ring of naphthalimide followed by second substitution with another amino group to close the ring with formation of benzimidazole.

2.2.2. Synthesis of 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (5a-k)

3-Bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (**4**) was treated with different primary and secondary amines as depicted in Scheme 3. Two different approaches have been followed to synthesize the target compound **5a**. In the first case, a mixture of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (0.25 g, 0.7 mmol) and morpholine (0.84 mmol) was refluxed in the presence of isopropyl alcohol (IPA) for 15 h.



Scheme 3. Synthesis of 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (**5a-k**).

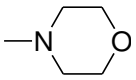
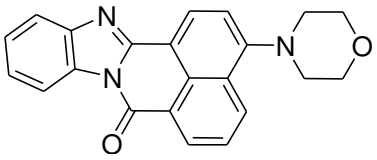
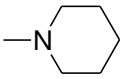
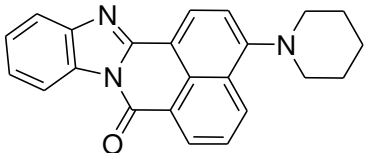
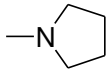
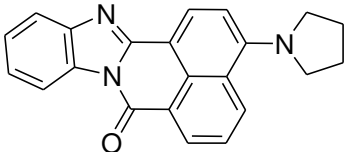
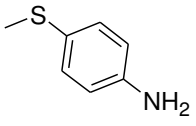
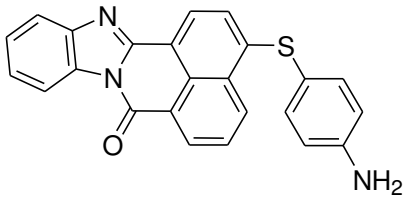
After completion of reaction (TLC), IPA was removed from the reaction mixture. The reaction mixture was extracted with chloroform and water, organic layer was collected and concentrated. The residue was dried over Na_2SO_4, resulted in solid crude product which was column chromatographed using hexane : ethylacetate (6:4) as eluents to get pure product **5a** in 45 % yield (m.p. 250-252 ^oC).

Alternatively, 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin- 7-one (**4**) (0.25 g, 0.7 mmol) was refluxed with morpholine (0.84 mmol) in the presence of potassium carbonate (0.059 g, 1.05 mmol) and $TBAHSO_4$ (catalytic amount) in acetonitrile (ACN) for 8 h. After the completion of reaction (TLC), reaction mixture was extracted with chloroform and water, separated the organic layer, dried over Na_2SO_4, filtered and concentrated to get crude product. The residue was purified with column chromatography on silica gel using hexane : ethylacetate (6:4) as eluents to afford pure orange coloured solid of **5a** in 66% yields (m.p. 250-252 ^oC), MS (EI) : m/z 356.1 (M^+ + 1). ^1H NMR spectrum of this compound showed four 1H double doublets at δ 8.85, δ 8.36, δ 7.77 and δ 7.33, 1H doublet at δ 8.72, two 1H multiplets at δ 8.58 and δ 7.89 and 2H multiplet at δ 7.50 of aromatic-H. Incorporation of morpholine group was also confirmed by two 4H triplets at δ 4.05 and δ 3.33 of O-CH_2 and N-CH_2 respectively. ^{13}C NMR spectrum of this compound showed signals at δ 159.7, 159.3(C=O) and 147.5, 138.2, 136.2, 134.7, 134.7, 132.3, 130.7, 129.9, 129.7, 129.4, 129.2, 129.0, 128.7, 127.9, 127.5, 126.0, 117.7, 117.5 (ArC), 66.9 (O-CH_2), 66.8 (O-CH_2), 53.6 (N-CH_2) and 53.5 (N-CH_2) of morpholine. On the basis of these spectral data, this compound has been assigned the structure of 3-morpholin-4-yl-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one **5a**.

Due to higher yields and lower reaction time, phase transfer catalyst (second approach) has been used for the synthesis of compounds **5b-k**. Under the similar reaction conditions, 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one was reacted with other

secondary amines viz., piperidine and pyrrolidine to give compounds **5b** (MS (EI) : m/z 354.1 (M^+ + 1)) and **5c** (MS (EI) : m/z 340.1 (M^+ + 1)) in 70 % and 67 % yields. Similarly, compound **4** was also treated with primary amines; 2-amino-thiophenol under the same reaction conditions to give compound **5d** in 56 % yield (MS (EI) : m/z 394.1 (M^++1)). ^{1}H NMR spectrum of this compound showed three 2H doublets at δ 8.70, δ 7.41 and δ 6.80, two 1H doublets at δ 8.37 and δ 7.27, 1H triplet at δ 7.83 and two 2H multiplets at δ 7.11 and δ 6.94 of aromatic-H which confirmed the formation of compound **5d**. ^{13}C NMR spectrum of this compound showed signal at δ 163.2, 163.0(C=O) and 150.1, 148.4, 144.6, 136.7, 130.7, 130.1, 129.1, 128.8, 128.4, 127.8, 126.2, 123.4, 122.3, 120.5, 119.0, 116.3, 115.7, 115.3, 111.7 for aromatic-C. On the basis of these spectral data, this compound has been assigned the structure of 3-(4-amino-phenylsulfanyl)-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one **5d**. Compound **4** was also treated with different primary amines and afforded compounds **5e-k** in 40-80 % yields as shown in Table 1.

Table 1. Synthesized compounds and their physical data

Compds.	-NR_1R_2	Product	Time (h)	Yield (%)	m.p. (^{o}C)	Colour
5a			8	66	250-252	orange
5b			8	70	200-202	yellow
5c			8	67	190-192	yellow
5d			10	56	137-139	pale brown

5e	10	80	240-242	brown	
5f	11	52	170-172	brown	
5g	10	45	220-222	brown	
5h	12	40	210-212	brown	
5i	10	61	137-139	brown	
5j	10	63	190-192	brown	
5k	12	58	220-222	brown	

2.3. Biology

2.3.1. *In vitro* cancer screening with one dose

The synthesized compounds **5a-k** were subjected to the National Cancer Institute (NCI) disease-oriented human cells screening panel assay for *in vitro* antitumor activity (Table 2, Figure 2).[28-31] Ten compounds have been selected for their single dose evaluation. These compounds (**5a, 5c-k**) were evaluated against 60 cell lines at a single dose of 10 µM which included nine tumour subpanels namely; leukemia, non-small cell lung, colon, central nervous syetem (CNS), melanoma, ovarian, renal, prostate, and breast cancer cells. The data reported as a mean graph of the percent growth of treated cells, and presented as percentage growth inhibition (GI %). All the compounds showed moderate to good inhibition values while compound **5d** exhibited significant growth inhibition and was evaluated for further 60 cell panels at five dose concentration levels.

Table 2 Percent growth inhibition of *in-vitro* subpanel tumour cell lines at 10 µM concentration of compounds **5a, 5c-5k**

Panel/Cell line	**5a**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**	**5i**	**5j**	**5k**	**5-FU**
Leukemia											
CCRF-CEM	-	46.30	**55.12**	-	-	-	24.98	27.47	48.24	34.56	57.1
HL-60(TB)	19.14	27.96	**81.83**	-	-	-	36.60	14.09	37.74	37.35	47.9
K-562	19.99	24.01	39.70	10.48	-	-	21.21	15.08	**53.46**	33.09	42.3
MOLT-4	-	**97.43**	**L**	10.14	-	-	30.59	25.87	38.16	**50.16**	43.1
RPMI-8226	18.35	24.26	**50.00**	42.60	28.57	43.83	19.50	24.64	**50.97**	24.36	41.4
SR	16.29	**79.81**	**83.87**	NT	NT	47.13	**84.42**	**78.49**	29.77	**98.89**	24.8
Non-Small Cell Lung Cancer											
A549/ATCC	-	**67.38**	**79.38**	-	14.97	-	23.35	17.60	29.13	33.46	34.2
EKVX	**61.72**	24.06	NT	NT	NT	NT	NT	NT	NT	NT	58.4
HOP-62	13.62	33.85	48.83	12.54	10.01	12.37	18.11	14.35	32.19	9.36	47.8
HOP-92	40.79	**59.33**	**71.29**	46.77	39.99	37.60	38.79	35.79	45.10	**51.55**	50.6
NCI-H226	-	44.68	**59.41**	-	17.31	-	26.30	24.92	30.35	28.02	69.5
NCI-H23	23.05	31.82	**62.45**	16.23	22.23	18.67	40.02	14.23	34.45	**54.00**	39.0
NCI-H322M	10.87	-	38.20	32.23	12.84	22.08	-	13.16	25.46	17.72	59.5
NCI-H460	11.58	**83.94**	**90.53**	22.14	42.76	13.43	**51.62**	27.34	47.30	**65.28**	13.0
NCI-H522	19.48	28.85	39.95	18.05	-	10.55	28.13	17.03	43.37	38.51	58.0
Colon Cancer											
COLO-205	12.36	36.39	49.22	15.52	-	11.81	14.60	15.07	29.00	24.42	40.2
HCC-2998	26.84	29.65	19.31	23.91	-	25.19	26.03	27.28	31.46	48.30	L

HCT-116	12.97	45.30	**53.20**	-	-	11.76	13.26	-	35.00	14.74	17.8
HCT-15	19.02	21.41	**59.54**	28.87	16.99	28.01	23.84	24.48	40.79	25.18	26.5
HT29	-	22.19	33.26	-	-	-	19.65	-	35.42	23.15	27.1
KM12	21.87	12.12	26.41	25.60	-	22.83	22.34	21.07	25.24	18.88	40.7
SW-620	-	40.47	**53.75**	11.98	-	-	13.59	-	22.93	19.86	50.1
CNS Cancer											
SF-268	-	-	40.60	-	-	-	-	-	12.77	-	59.0
SF-295	13.72	40.22	**88.03**	-	-	12.89	NT	NT	NT	NT	69.1
SF-539	-	-	**51.45**	16.19	-	-	-	-	14.29	-	L
SNB-19	-	24.00	**53.74**	22.44	-	14.11	-	-	10.13	10.59	65.9
SNB-75	20.90	26.97	**56.29**	29.25	18.47	**56.00**	26.56	20.05	21.44	7.02	65.9
U251	-	35.29	**60.10**	-	-	-	NT	NT	NT	NT	50.3
Melanoma											
LOX IMVI	15.18	48.31	**70.85**	16.42	14.77	12.81	24.11	13.07	30.38	26.63	30.4
MALME-3M	-	29.56	**65.97**	-	-	-	10.72	-	13.70	-	58.2
M14	-	-	**54.61**	-	-	-	-	-	-	13.32	NT
MDA-MB-435	-	-	19.78	-	-	-	-	-	19.48	-	36.6
SK-MEL-2	-	-	15.91	-	-	-	NT	NT	NT	NT	95.5
SK-MEL-28	-	-	26.26	-	-	27.07	-	-	-	-	NT
SK-MEL-5	26.55	35.45	**60.6**	29.60	20.19	23.78	24.48	18.17	48.16	33.91	33.7
UACC-257	-	-	28.99	13.83	-	-	NT	NT	NT	NT	19.5
UACC-62	37.86	37.45	**68.09**	32.24	23.56	15.17	14.09	13.13	35.70	17.25	39.7
Ovarian Cancer											
IGROV1	41.68	42.85	**65.49**	NT	**63.48**	51.13	**58.18**	46.61	43.37	**58.81**	51.2
OVCAR-3	-	1.79	22.24	-	10.12	-	-	-	30.01	13.22	47.4
OVCAR-4	30.08	21.16	26.02	24.01	-	46.26	14.24	17.14	30.92	19.91	59.4
OVCAR-5	-	-	14.18	15.22	-	-	-	-	16.68	-	44.3
OVCAR-8	-	33.96	**50.00**	10.73	-	-	-	-	24.58	-	NT
NCI/ADR-RES	-	16.96	43.42	-	-	-	-	-	25.40	-	47.6
SK-OV-3	-	20.92	46.12	-	-	-	-	10.94	10.94	-	77.5
Renal Cancer											
786-0	-	11.73	**62.66**	-	-	-	-	-	11.58	-	48.7
A498	-	**61.96**	41.22	33.84	32.85	-	-	-	-	-	L
ACHN	-	47.65	**77.92**	12.14	-	-	18.15	11.97	23.94	27.11	39.3
CAKI-1	30.37	48.66	94.10	20.13	10.13	16.10	**56.82**	38.00	48.16	**52.78**	39.4
RXF 393	-	-	27.44	1.98	-	-	-	-	-	-	34.3
SN12C	12.38	35.19	**80.78**	12.19	-	-	-	-	28.28	12.95	54.0
TK-10	28.19	12.36	21.42	24.68	-	21.91	29.84	NT	34.20	22.63	66.9
UO-31	34.11	43.81	**90.73**	**50.78**	38.32	47.89	41.38	49.37	**53.45**	**58.62**	41.3

Prostate Cancer											
PC-3	-	32.07	49.58	22.79	17.51	23.33	14.45	15.5	35.99	20.05	58.2
DU-145	-	21.86	**60.67**	-	-	-	-	-	-	-	35.5
Breast Cancer											
MCF7	**76.03**	**50.00**	**76.42**	**89.91**	**57.46**	**95.85**	**89.72**	**79.64**	**71.34**	**86.51**	11.5
MDA-MB-231/ATCC	18.00	22.86	45.60	29.13	18.51	33.82	20.17	17.90	29.40	17.68	78.1
HS 578T	**59.82**	32.80	40.37	26.39	15.28	18.80	-	-	-	-	L
BT-549	**-**	28.77	40.94	12.53	12.92	15.62	-	-	12.60	-	37.8
T-47D	**61.88**	27.85	**60.37**	**76.56**	31.68	**81.64**	**64.33**	47.34	**53.38**	**67.46**	56.7
MDA-MB-468	**L**	19.72	**56.48**	**L**	**81.08**	**L**	**L**	**L**	**L**	**L**	NT

Prominent GI values are bolded
-, GI < 10 %; L, compounds proved lethal to the cancer cell line; NT- Not Tested

In the present investigation, most of the tested compounds showed excellent antitumor activity. Compounds **5a**, **5e-g** and **5i** displayed modest potency against the tested tumour cell lines and considered to be the least effective member. Leukemia cancer cell line HL-60 (TB) proved to be sensitive towards compound **5d** with GI value of 81.83%, while leukemia cell line MOLT-4 proved to be sensitive towards compound **5c** with GI value of 97.43% and leukemia cell line SR showed sensitivity to compounds **5d**, **5h** and **5k** with GI values of 83.87%, 84.42% and 98.89% respectively.

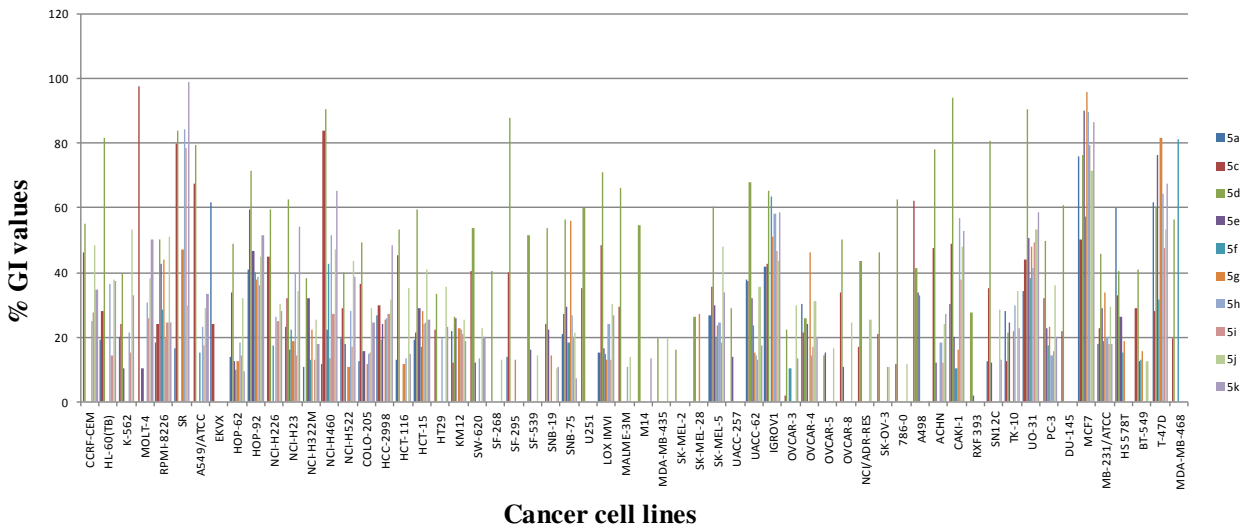


Figure 2. Growth inhibition of compounds **5a, 5c-k** over the full panel tumour cell lines at 10 µM concentration.

Compound **5d** has proved to be most active member of this series and hence its activity was tested at five different concentrations. Three response parameters, GI_{50}, TGI, and LC_{50} were

monitored for each cell line, using the known drug 5-fluorouracil (**5-FU**) as a positive control (Table 3). A comparison of values of MG-MID of GI_{50}, TGI, and LC_{50} for compounds **5d** and 5-fluorouracil conclude that compound **5d** is fivefold more active than 5-fluorouracil, with GI_{50} and TGI values of 5.05 µM and 38.71 µM respectively as compared to GI_{50} and TGI

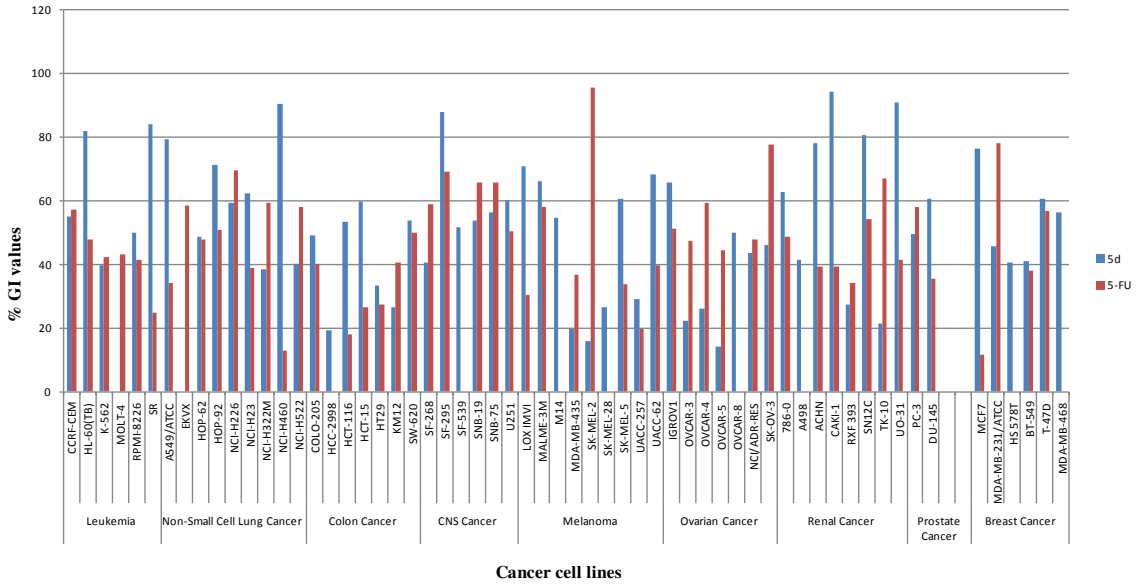


Figure 3. Growth inhibition of **5d** and **5-FU** over the full panel tumour cell lines at 10 µM concentration.

Compounds **5c** and **5d** exhibited sensitivity towards non-small cell lung cancer cell line NCI-H460 with GI values of 83.94% and 90.53% respectively. CNS cancer line SF-295, renal cancer cell lines SN-12C and UO-31 of compound **5d** showed sensitivity with GI values of 88.03%, 80.78%, and 90.73% respectively. Breast cancer cell line MCF7 exhibited sensitivity toward compounds **5e**, **5g**, **5h** and **5k** with GI values of 89.91%, 95.85%, 89.72% and 86.51% respectively. Compound **5d** proved to be lethal to leukemia cancer cell line MOLT-4 while breast cancer cell line MDA-MB-468 is lethal to compounds **5a**, **5e**, and **5g-k**. Compound **5d** exhibited more than 50% inhibition for most of the tumour cell lines and much better than 5-fluorouracil (positive control) in tested derivatives (Figure 3). From above results, it is clear that compound **5d** is the most active member of the series. This compound was tested against a panel of 60 different tumour cell lines at five dose concentration range.

2.3.2. *In vitro* anticancer screening with five dose concentration

values (22.6 µM and >100 µM) for 5-fluorouracil (Table 3, Figure 4). The obtained results revealed that compounds **5a**, **5i** and **5j** were devoid of any antitumour potency. Compounds

bearing pyrrolidine, 5-bromo-2-aminopyridine, butylamine and cyclohexylamine showed antitumour activity as exemplified by **5c, 5g, 5h** and **5k** respectively. Substitution with 4-aminothiophenol in **5d** showed broad spectrum of anti-tumour activity. Thus, thio group in naphthalimide derivative imparts a key role for the anticancer activity.

Table 3. Compounds **5d** and **5-FU** median growth inhibitory (GI_{50}, μM), total growth inhibitory (TGI, μM) and median lethal concentrations (LC_{50}, μM) of *in vitro* subpanel tumour cell lines.

Compds.	Activity	I	II	III	IV	V	VI	VII	VIII	IX	MG-MID[a]
5d	GI_{50}	2.9	3.94	6.58	4.09	6.93	6.97	3.30	3.23	5.20	5.05
	TGI	44.51	32.51	60.81	45.41	35.07	37.44	15.41	17.9	45.98	38.71
	LC_{50}	100	93.94	98.84	93.98	83.93	97.94	88.94	80.15	b	b
5-FU	GI_{50}	1.51	b	8.4	72.1	70.6	61.4	45.6	22.7	76.4	22.6
	TGI	b	b	b	b	b	b	b	B	b	b
	LC_{50}	b	b	b	b	b	b	b	B	b	b

I, leukemia; II, non-small cell lung cancer; III, colon cancer; IV, CNS cancer; V, melanoma; VI, ovarian cancer; VII, renal cancer; VIII, prostate cancer; IX, breast cancer.
[a] Full panel mean-graph midpoint (μM).
[b] Compounds showed values >100 μM.

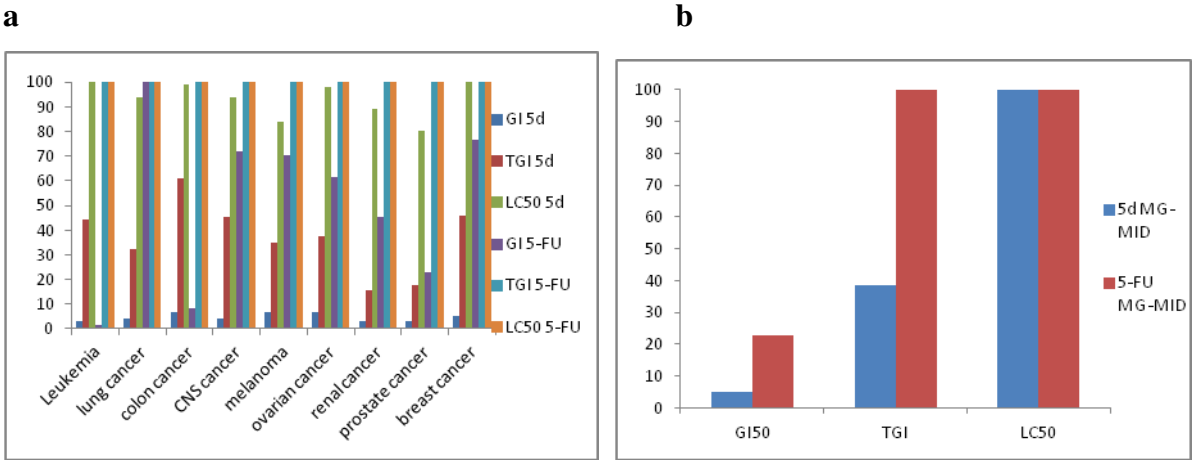


Figure 4. (a) Compounds **5d** and **5-FU** median growth inhibitory (GI_{50}, μM), total growth inhibitory (TGI, μM) and median lethal concentration (LC_{50}, μM) of *in vitro* subpanel tumour cell lines and (b) comparison of full panel mean-graph midpoint (μM) of compounds **5d** and **5-FU**.

2.3.3. DNA binding studies

Based on these *in vitro* anticancer studies, it has been revealed that the naphthalimide fused benzimidazole moiety improve the biological function. Due to the presence of the aromatic

ring systems and protonated amine sites in naphthalimide-benzimidazole analogues, DNA interaction or enzyme inhibition may participate for tumor growth inhibitory activities of these compounds. This has led us to check the interaction of these compounds with calf thymus (ct)-DNA. DNA binding properties of most active compound **5d** have been evaluated using both UV-Visible and fluorescence spectrophotometry along with thermal denaturation experiment in order to determine the interaction of compounds with ct-DNA. The addition of ct-DNA (4-100 µM) to the phosphate buffered solution of **5d** (50 µM) induced bathochromic shift (~20 nm) of absorption band from 410 nm to 430 nm with hypochromic effect (Figure 5a). The observed hypochromic shifts in absorption bands of **5d** upon addition of DNA results from the intercalation of **5d** with ct-DNA as suggested in the literature.[32] The intercalative nature of interaction of compound **5d** with ct-DNA has been additionally supported by thermal denaturation experiment. Intercalation of molecules into the double helix is known to stabilize the DNA against thermal strand separation and thus increases thermal melting temperature (Tm).[33] The derivative melting curve shows an increase of 8.2 ^{o}C in thermal melting temperature of ct-DNA (60 ^{o}C) upon addition of **5d** (68.2 ^{o}C). Thus, both UV-Visible titrations and thermal denaturation experiments indicated strong intercalative nature of interactions between compound **5d** and ct-DNA. Fluorescence titrations of **5d** were also performed with ct-DNA in phosphate buffered solution, showed decrease in the intensity of the emission band of **5d** at 500 nm (Figure 5b) upon addition of increasing concentration of ct-DNA.

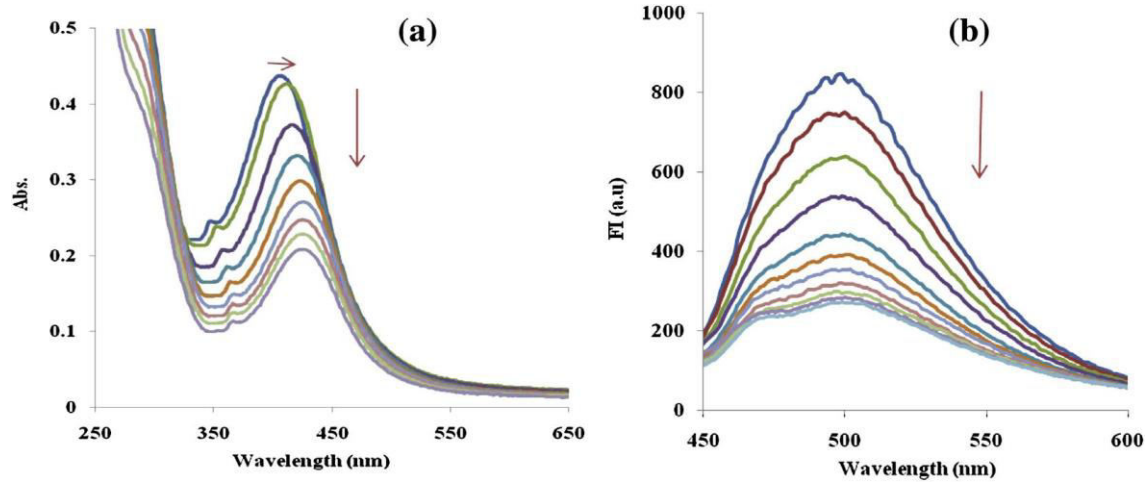


Figure 5. Effect of addition of ct-DNA on (a) absorption and (b) emission spectra of compound **5d** (50 mM, pH 7.0) in phosphate buffer.

Binding constant of **5d** with ct-DNA was log β = 5.32 calculated from titration data using Benesi-Hildebrand method. The apparent binding constant with ct-DNA was determined to

be log β = 5.15 for amonafide. The results clearly indicated that compound **5d** intercalates more strongly than amonafide.

2.4. ADME prediction

As a part of our study, the compliance of compounds **5a**, **5c-k** was also evaluated with different physico-chemical parameters; drug bioavailability parameters (Log P, Total Polar Surface Area) and other parameters such as molar refractivity, dipole moment, electron affinity and steric energy, determined by SciGress software.[34] The results disclosed in Table 4 showed that all the compounds exhibited good passive oral absorption and thus difference in their bioactivity cannot be attributed to this property.

Table 4. Calculated physicochemical parameters of compounds **5a**, **5c-k** and **5-FU**

Parameters	**5a**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**	**5i**	**5j**	**5k**	**5-FU**
Log P	3.9	4.5	4.9	4.9	5.1	5.4	4.7	5.5	4.6	5.1	-1.3
Polarizability	43.0	42.5	46.2	45.8	46.4	48.9	43.0	45.8	45.0	46.4	10.7
Molar refractivity	103	101	117	108	110	113	103	108	106	106	26.1
Dipole moment (Debye)	2.7	4.4	3.7	2.6	4.9	5.2	5.1	4.8	4.9	4.9	3.4
Solvent accessibility surface area (A m^2)	346	339	377	366	372	381	352	368	357	372	138
Conformation minimum energy (kcal/mole)	20.1	50.0	91.8	41.0	33.0	100	38.4	1.4	91.7	33.0	-107
Electron affinity (eV)	1.5	1.4	1.7	1.6	1.4	1.5	1.4	1.5	1.5	1.4	0.8
Steric energy (kcal/mole)	68.1	40.6	15.3	56.5	39.5	50.9	39.5	67.2	47.5	39.5	-13.4
Total polar surface area (TPSA)	46.8	37.6	66.4	46.4	60.6	59.2	46.4	46.4	59.2	46.4	65.7

Thus, compound **5d** showed more electronic affinity and less steric hindered for DNA intercalation. Comparison of these parameters with 5-fluorouracil also proved that compound

5d is superior in all aspects.

2.5. Structure-activity relationship

The structure-activity relationship (SAR) emerged from the anti-tumour activity results revealed that combination of naphthalimide and benzimidazole was well tolerated for their *in vitro* anticancer activity. We then turned our attention to exploring the SAR profile of the substitution on naphthalimide ring (**5a-k**) in hope of finding a permissive region; (i) The primary and secondary amines at C4-position of naphthalimide play a key role in varying the efficiency of antitumour activities. (ii) The primary amines positively influence in the antitumour effectiveness, inducing good activity against most of the cancer cell lines than secondary amines. (iii) The introduction of 4-amino-phenylsulfanyl group naphthalimide ring (**5d**) led to a broad spectrum of antitumour activity with respect to other primary amines. The role of sulphur atom in improving anti-tumour activities had also been reported in the literature.[15,21,27] (iv) Presence of 5-bromopyridine (**5g**) and 2,6-difluoroaniline (**5i**) slightly improved the activity. (v) Compounds with acyclic aliphatic amines (**5f** and **5h**) showed the least activity at 10 μM concentrations. (vi) Amongst the secondary amines, five membered ring, pyrrolidine (**5c**) has improved the anti-tumour activity than six membered ring, piperidine **(5b)**. (vii) Compound **5d** also showed stronger DNA intercalating properties than amonafide by having binding constant value more than amonafide (log β = 5.32 and 5.15 for **5d** and amonafide, respectively) which may be explained on the basis of substitutions on naphthalimide in case of **5d**. (viii) The hydrophilicity and steric parameters of the compounds may also play crucial role on the effectiveness of the compounds tested.

2.6. Molecular modelling studies

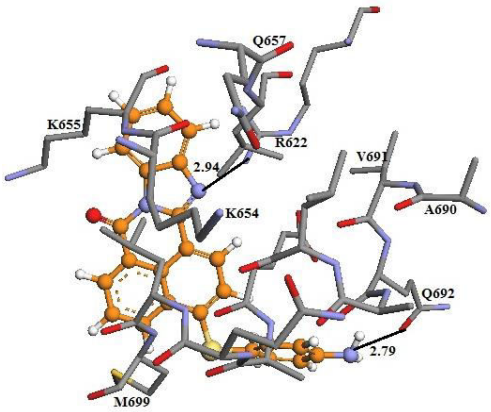
In order to predict the binding mode of the most active compound **6b** of this series, molecular modelling studies were carried out with topoisomerase I, topoisomerase II and ribonucleotide reductase as well as DNA.

2.6.1. Molecular modelling studies of 5d with topoisomerase I (Topo I), topoisomerase II (Topo II) and ribonucleotide reductase (RNR)

Molecular modelling studies were also carried out for compound **5d**, which has been proved to be most active member in this series. Although the cellular targets were not defined in the experimental anticancer investigation of these molecules, to look into the possible interactions at the enzymatic level, we have carried out the docking studies[35] of compound **5d**

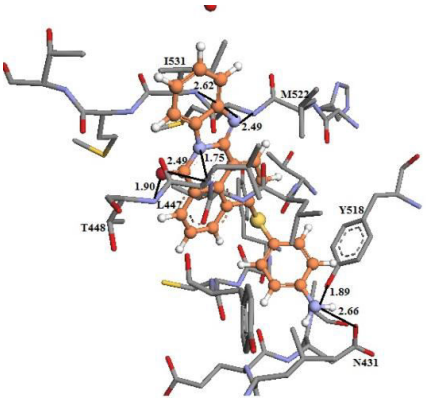
in the active sites of topoisomerase I (Topo I, pdb ID 1A36), topoisomerase II (Topo II, pdb ID 1BJT, enzymes responsible for DNA replication) and ribonucleotide reductase (RNR, pdb ID 4R1R). Docking of compound **5d** in the active site of Topo I (1A36) showed hydrogen bonding interaction between terminal NH group of naphthalimide with carbonyl of Gln 692 (d = 2.79 Å) amino acid residue. Nitrogen atom of imidazolone ring of **5d** showed hydrogen bonding with amine group of Arg 622 (d = 2.94 Å) amino acid residue. Aminothiophenol moiety was surrounded by amino acid residues Glu 695, Glu 696 and Leu 694 (Figure 6a).

a



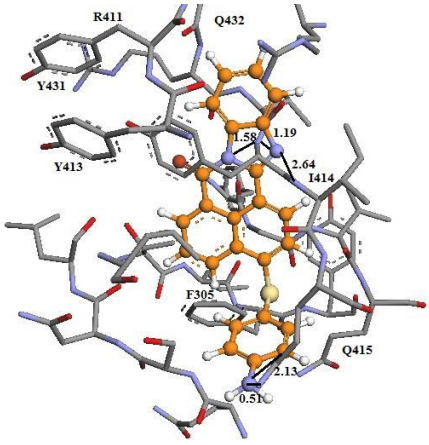
1A36_topoisomerase I

b



1BJT_topoisomerase II

c



4R1R_ ribonucleotide reductase

Figure 6. Compound **5d** docked in active site of Topo I (a), Topo II (b) and RNR (c). H-bonds of compound **5d** with different amino acids residues are visible. (Carbon atoms are given in different colour.)

Docking of compound **5d** with active site of Topo II (1BJT) showed hydrogen bonding interaction between terminal amine group of naphthalimide with Asn 431 (NH_2....O=C, d =

2.66 Å) and Tyr 518 (NH_2....O=C, *d* = 1.89 Å) amino acid residues. Carbonyl group of imidazolone (**5d**) showed hydrogen bonding interaction with amino group of Leu 447 (*d* = 2.49 Å) and Thr 448 (*d* = 1.90 Å) amino acid residues. Nitrogen atom of amide group of imidazolone also showed H-bonding with amino group of Leu 447 (*d* = 1.75 Å) amino acid residue. Imidazolone nitrogen atom of this compound showed hydrogen bonding interaction with amino group of Met 522 (*d* = 2.49 Å) and Ile 531 (*d* = 2.62 Å) amino acid residues. The aromatic rings of **5d** were surrounded by rings of Tyr 518, Tyr 472 and Tyr 469 amino acid residues (Figure 6b).

Molecular docking of terminal amine group of compound **5d** showed H-bonding interaction with Gln 415 (NH_2....O=C, *d* = 2.13 Å) amino acid residue of RNR (4R1R, another enzyme involved in the propagation of cancer, synthesis of raw material for replication of DNA). Imidazolone nitrogen atom of this compound showed hydrogen bonding with amino group of Ile 414 (*d* = 2.64Å) and Tyr 413 (*d* = 1.19 Å) amino acid residue. Imidazolone amide nitrogen atom also showed hydrogen bonding interaction with amino group of Tyr 413 (*d* = 1.58 Å) amino acid residue (Figure 6c). Compound **5d** has been surrounded by aromatic rings of residues Phe 305, Tyr 306 and Tyr 413.

Table 5. Hydrogen bonding of compound **5d** with topoisomerase I (1A36), topoisomerase II (1BJT) and ribonucleotide reductase (4RIR).

Groups of compound 5d	**1A36** **Amino acid residue**	**1BJT** **Amino acid residue**	**4RIR** **Amino acid residue**
Nitrogen atom of terminal amine group	Q692 (*d* = 2.79 Å)	N431 (*d* = 2.66 Å), Y518 (*d* = 1.89 Å)	Q415 (*d* = 2.13 Å)
Imidazolone carbonyl oxygen		L447 (*d* = 2.49 Å), T448 (*d* = 1.90 Å)	
Imidazolone nitrogen atom	R622 (*d* = 2.94 Å)	M522 (*d* = 2.49 Å), I531 (*d* = 2.62 Å)	I414 (*d* = 2.64 Å), T413 (*d* = 1.19 Å)
Imidazolone amide nitrogen atom		L447 (*d* = 1.75 Å)	T413 (*d* = 1.58 Å)

Therefore, docking of compound **5d** in the active site of these enzymes indicated the probable mode of action for anticancer activities. Remarkably, correlations between the experimental data and docking studies have proven useful for refining the structure of the molecules and improving their efficiencies (Table 5).

2.6.2. Molecular modelling studies of 5d with DNA

To explore most feasible binding site, interaction mode and binding affinity, docking studies have been performed on compound **5d** with DNA (PDB ID: 1BNA). Most stable binding conformation of **5d** and DNA revealed that it is stacked in the major groove via electrostatic interaction. The planarity of naphthalimide core is comfortable for strong л-л stacking interactions and fits inside the DNA strands by van der Waals interaction and hydrophobic

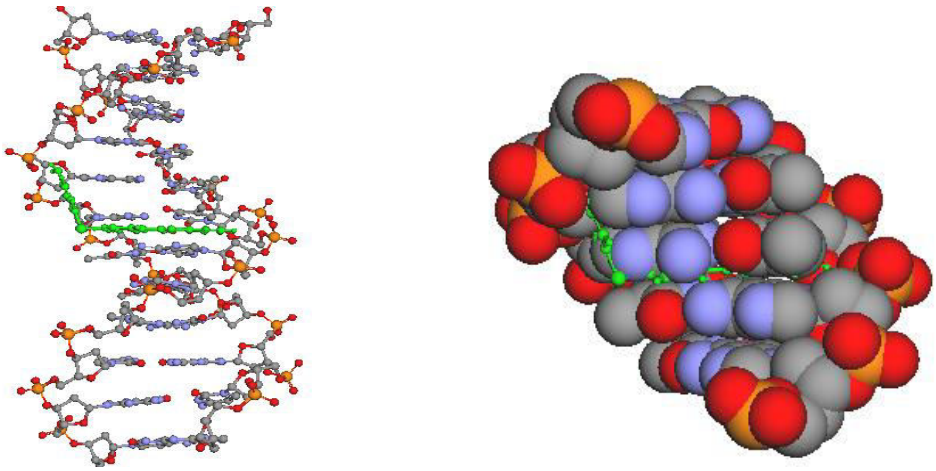


Figure 7 Molecular docked model of compound **5d** with DNA (pdb ID 1BNA)

contacts (Figure 7). These results are consistent with UV-Visible and fluorescence studies. CPK model also indicated the complete fitting of compound **5d** within DNA pocket. Therefore, docking of compound **5d** in the active site of these enzymes indicated the probable mode of action for anticancer activities.

2.7. Conclusion

The present work has led to the development of novel naphthalimide molecules. These molecules were well characterized by ^{1}H NMR and ^{13}C NMR as well as mass spectrometry. Some of these compounds shown promising anti-tumour activities and even more active than 5-fluorouracil at 10 µM concentration. Compound **5d** proved to be fivefold more active than **5-FU** in five dose concentration levels, with MG-MID GI_{50} and TGI values of 5.05 and 38.71 respectively. This compound also showed strong intercalating properties with ct-DNA. The experimental as well as theoretical studies clearly predicts that substitution at 4-position of naphthalimide and sulphur linkage is essential for the antitumour activity. The new naphthalimide compounds prepared in this work have good physical properties that qualify them to have good pharmacokinetic and drug bioavailability. Molecular modelling studies indicating considerable interactions of this compound in the active sites of topoisomerase I (Topo I, pdb ID 1A36), topoisomerase II (Topo II, pdb ID 1BJT) and ribonucleotide

reductase (RNR, pdb ID 4R1R) as well as DNA (pdb ID 1BNA), that also favoured the intercalation of synthesized compounds. These preliminary encouraging results of biological screening and docking of tested compounds could offer an excellent framework in this field and may lead to discovery of potent antitumour agents.

2.8 Experimental section

2.8.1. Chemistry

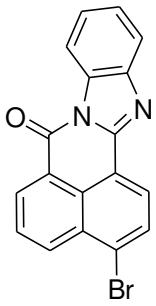
Melting points were determined in open capillaries and were uncorrected. Reactions were monitored by thin layer chromatography (TLC) with silica plate coated with silica gel GF 254. To prepare TLC plates, slurry of silica gel GF 254 was prepared with help of $CHCl_3$ and MeOH (4:1). TLC plates were dipped in the slurry, dried in an oven and used for comparing the reaction mixture and starting materials to check the progress of reaction. TLC plates were checked under UV lamp or were developed in iodine chambers. Column chromatography was performed with silica gel 60-120/100-200 mesh. Hexane/ ethyl acetate and chloroform/ methanol were adopted solvent systems. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400 MHz and Jeol ECS 400 NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvents. The chemical shifts were expressed in parts per million with TMS as internal reference and coupling constant (*J*) values are given in Hz. Spectral patterns are denoted as s = singlet; d = doublet; t = triplet; bs = broad singlet; dd = double doublet; dt = double triplet; q = quartet; m = multiplet. Mass Spectra of the synthesized compounds were recorded at Waters Micromass Q-Tof Micro. UV-Visible spectra were recorded using Champion UV/Vis spectrometer. Fluorescence measurements were performed on a Varian Cary Eclipse fluorescence spectrometer. Thermal denaturation experiments were performed on Shimadzu UV-2450.

Materials: Acenaphthene, potassium hydroxide, potassium carbonate, tetrabutylammonium hydrogensulfate and acetic acid were purchased from loba chemie. Sodium dichromate and bromine was purchased from spectrochemicals. THF used was of HPLC grade. Primary and secondary amines were purchased from sigma-Aldrich, loba chemie and spectrochemicals. Solvents like acetonitrile, chloroform, methanol, hexane and ethyl acetate were of LR grade and used after distillation.

2.8.1.1. General procedure for synthesis of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (4)

To a solution of acenaphthene (2.31 g, 15.0 mmol) and acetic acid (60 ml), sodium dichromate (11.17 g, 40.0 mmol) was added with stirring at room temperature. The suspension was heated to 75 ^{o}C for 8 h. After the completion of reaction, cold water was added to the reaction mixture to get solid, filtered and washed with water. Yellow solid product of 1,8-naphthalic anhydride (**2**) (2.6 g, 80%, m.p. 266-268 ^{o}C) was obtained. To a cooled solution of 1,8-naphthalic anhydride (1.98 g,10.0 mmol) in KOH (2.8 g in 12 ml water), bromine was charged with dropping funnel. The reaction mixture was heated to 60 ^{o}C for 6 h and then acidified with HCl solution. Solid was separated out, filtered to get crude product. The solid residue was heated with 5% NaOH solution, filtered and neutralized with HCl solution. Again filtered and washed with cold water, dried to get yellow powder of 4-bromo-1,8-naphthalic anhydride (**3**) (2.05 g, 82%, m.p. 117-119 ^{o}C). 4-Bromo-1,8-naphthalic anhydride (2.00 g, 5.0 mmol) and *o*-phenylenediamine (0.618 g, 5.0 mmol) in THF was refluxed for 6 h. After the completion of reaction (TLC), cooled the reaction mixture to get crude solid, filtered and washed with THF to afford pure 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7- one (**4**).

3-Bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7- one (**4**)



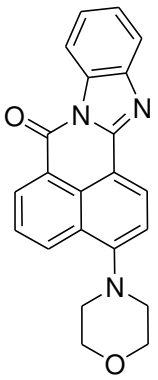
Brown powder (2.51g, 63%); m.p. 190-192 ^{o}C; ^{1}H NMR ($CDCl_3$+TFA): δ 8.90 (dd, ^{2}J = 8.48 Hz, ^{3}J = 0.68 Hz, 1H, ArH), 8.79 (dd, ^{2}J = 7.36 Hz, ^{3}J = 0.84 Hz, 1H, ArH), 8.53 (d, J = 7.92 Hz, 1H, ArH), 8.24 (d, J = 7.92 Hz, 1H, ArH), 8.04 (dd, ^{2}J = 8.48 Hz, ^{3}J = 7.48 Hz, 1H, ArH), 7.74 (m, 3H, ArH), 7.56 (m, 1H, ArH); ^{13}C NMR ($CDCl_3$+TFA): δ 165.7, 136.5, 134.5, 134.4, 133.4, 131.9, 131.7, 131.6, 131.1, 130.9, 129.3, 128.6, 128.2, 127.2, 124.9, 121.0, 119.9; MS (EI) : m/z 350.1 (M^{+}+1); Anal. Calc. for ($C_{18}H_9BrN_2O$): C, 61.91; H, 2.60; N, 8.08. Found: C, 61.76; H, 2.81; N, 8.45.

2.8.1.2. General procedure for synthesis of 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (5a-k)

A mixture of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (0.25 g, 0.7 mmol), potassium carbonate (0.059 g, 1.05 mmol), $TBAHSO_4$ (catalytic) and corresponding amine (0.84 mmol) in acetonitrile was heated to reflux for 8-12 h. After the completion of reaction, reaction mixture was extracted with chloroform, separated the organic layer, dried

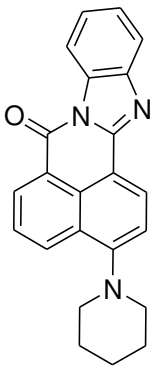
over Na_2SO_4, filtered and concentrated to get solid crude product. The residue was purified with column chromatography on silica gel using hexane: ethylacetate as eluents to afford 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one **(5a-k)**.

3-Morpholin-4-yl-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one (5a)



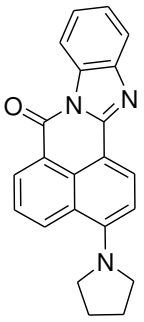
Orange powder (0.20g, 66%); m.p. 250-252 ^oC; ^1H NMR ($CDCl_3$): δ 8.85 (dd, ^2J = 7.28 Hz, ^3J = 0.96 Hz, 1H, ArH), 8.72 (d, J = 8.08 Hz, 1H, ArH), 8.58 (m, 1H, ArH), 8.36 (dd, ^2J = 8.44 Hz, ^3J = 1.00 Hz, 1H, ArH), 7.89 (m, 1H, ArH), 7.77 (dd, ^2J = 9.08 Hz, ^3J = 1.68 Hz, 1H, ArH), 7.50 (m, 2H, ArH), 7.33 (dd, ^2J = 17.12 Hz, ^3J = 8.08 Hz, 1H, ArH), 4.05 (t, J = 4.56 Hz, 4H, CH_2), 3.33 (t, J = 4.60 Hz, 4H, CH_2); ^{13}C NMR ($CDCl_3$+TFA): δ 159.7, 159.3, 147.5, 138.2, 136.2, 134.7, 134.7, 132.3, 130.7, 129.9, 129.7, 129.4, 129.2, 129.0, 128.7, 127.9, 127.5, 126.0, 117.7, 117.5, 66.9, 66.8, 53.6, 53.5; MS (EI) : m/z 356 (M^++1); Anal. Calc. for ($C_{22}H_{17}N_3O_2$): C, 74.35; H, 4.82; N, 11.82. Found: C, 74.03; H, 4.42; N, 11.45.

3-Piperidin-1-yl-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one (5b)



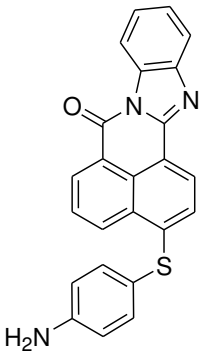
Yellow powder (0.17g, 70%); m.p. 200-202 ^oC; ^1H NMR ($CDCl_3$): δ 8.63 (dd, ^2J = 7.20 Hz, ^3J = 0.88 Hz, 1H, ArH), 8.55 (d, J = 8.12 Hz, 1H, ArH), 8.45 (dd, ^2J = 8.44 Hz, ^3J = 0.96 Hz, 1H, ArH), 7.72 (dd, ^2J = 8.28 Hz, ^3J = 7.44 Hz, 1H, ArH), 7.29 (dd, ^2J = 7.76 Hz, ^3J = 1.48 Hz, 1H, ArH), 7.21 (d, J = 8.16 Hz, 1H, ArH), 7.11 (dd, ^2J = 8.36 Hz, ^3J = 1.52 Hz, 1H, ArH), 6.94 (m, 2H, ArH), 3.27 (t, J = 4.96 Hz, 4H, CH_2), 1.90 (m, 4H, CH_2), 1.75 (t, J = 6.12 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6): δ 163.5, 163.0, 156.6, 144.9, 131.7, 130.1, 130.0, 130.0, 129.2, 128.6, 125.7, 125.1, 123.6, 120.8, 116.3, 116.0, 115.6, 114.4, 54.0, 25.7, 23.8; MS (EI) : m/z 354.1 (M^++1); Anal. Calc. for ($C_{23}H_{19}N_3O$): C, 78.16; H, 5.42; N, 11.89. Found: C, 78.32; H, 5.31; N, 11.45.

3-Pyrrolidin-1-yl-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one (5c)



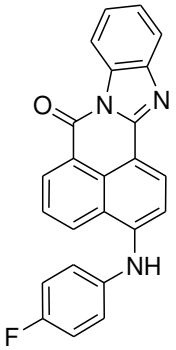
Yellow powder (0.16g, 67%); m.p. 190-192 ^{o}C; ^{1}H NMR ($CDCl_3$): δ 8.65 (t, *J* = 7.72 Hz, 2H, ArH), 8.48 (d, *J* = 8.68 Hz, 1H, ArH), 7.58 (dd, ^{2}J = 8.52 Hz, ^{3}J = 7.36 Hz, 1H, ArH), 7.28 (m, 1H, ArH), 7.12 (dd, ^{2}J = 8.12 Hz, ^{3}J = 1.52 Hz, 1H, ArH), 6.93 (m, 2H, ArH), 6.85 (d, *J* = 8.72 Hz, 1H, ArH), 3.83 (t, *J* = 6.44 Hz, 4H, CH_2), 2.13 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6): δ 163.7, 162.8, 152.1, 145.1, 132.5, 131.9, 131.3, 130.2, 129.4, 128.4, 122.8, 122.1, 121.2, 115.8, 115.5, 110.4, 108.1, 52.8, 25.5; MS (EI) : m/z 340.1 (M^{+}+1); Anal. Calc. for ($C_{22}H_{17}N_3O$): C, 77.86; H, 5.05; N, 12.38. Found: C, 77.61; H, 4.84; N, 12.45.

3-(4-Amino-phenylsulfanyl)-benzo[*de*]benzo[4,5]imidazo [2,1-*a*]isoquinolin-7-one (5d)



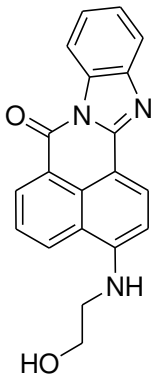
Pale brown powder (0.15g, 56%); m.p. 137-139 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 8.70 (d, *J* = 7.92 Hz, 2H, ArH), 8.37 (d, *J* = 8.00 Hz, 1H, ArH), 7.83 (t, *J* = 7.88 Hz, 1H, ArH), 7.41 (d, *J* = 6.60 Hz, 2H, ArH), 7.27 (d, *J* = 1.48 Hz, 1H, ArH), 7.11 (m, 2H, ArH), 6.94 (m, 2H, ArH), 6.80 (d, *J* = 6.60 Hz, 2H, ArH); ^{13}C NMR (DMSO-d_6): δ 163.2, 163.0, 150.1, 148.4, 144.6, 136.7, 130.7, 130.1, 129.1, 128.8, 128.4, 127.8, 126.2, 123.4, 122.3, 120.5, 119.0, 116.3, 115.7, 115.3, 111.7; MS (EI) : m/z 394.1 (M^{+}+1); Anal. Calc. for ($C_{24}H_{15}N_3OS$): C, 73.26; H, 3.84; N, 10.68; S, 8.15. Found: C, 73.55; H, 3.48; N, 10.45; S, 8.08.

3-(4-Fluoro-phenylamino)-benzo[*de*]benzo[4,5]imidazo[2,1- *a*]isoquinolin-7-one (5e)



Brown powder (0.21g, 80%); m.p. 240-242 ^{o}C; ^{1}H NMR ($CDCl_3$): δ 8.80 (m, 2H, ArH), 8.57 (d, *J* = 7.92 Hz, 2H, ArH), 8.50 (dd, ^{2}J = 7.28 Hz, ^{3}J = 4.00 Hz, 3H, ArH), 8.08 (dd, ^{2}J = 18.82 Hz, ^{3}J = 7.60 Hz, 1H, ArH), 7.87 (m, 4H, ArH); ^{13}C NMR (DMSO-d_6): δ 159.0, 158.8, 150.6, 150.4, 150.0, 140.6, 137.0, 134.2, 133.6, 129.7, 129.3, 127.7, 126.7, 126.8, 126.2, 123.6, 121.8, 121.2, 119.0, 116.2, 114.2, 113.7, 111.5, 106.9; MS (EI) : m/z 380.1 (M^{+}+1); Anal. Calc. for ($C_{24}H_{14}FN_3O$): C, 75.98; H, 3.72; N, 11.08; Found: C, 76.31; H, 4.03; N, 11.45.

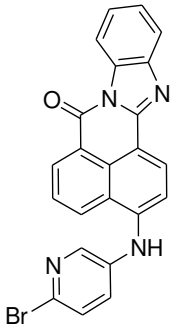
3-(2-Hydroxy-ethylamino)-benzo[*de*]benzo[4,5]imidazo[2,1- *a*]isoquinolin-7-one (5f)



Brown powder (0.12g, 52%); m.p. 170-172 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 8.78 (d, J = 7.80 Hz, 1H, ArH), 8.67 (d, J = 4.04 Hz, 1H, ArH), 8.61 (m, 1H, ArH), 8.53 (d, J = 8.80, 1H, ArH), 8.46 (d, J = 6.92 Hz, 1H, ArH), 8.30 (d, J = 8.12 Hz, 1H, ArH), 7.80 (d, J = 8.00 Hz, 1H, ArH), 7.70 (t, J = 6.72, 1H, ArH), 7.63 (s, 1H, ArH), 3.86 (m, 2H, CH_2), 3.56 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6): δ 164.8, 160.2, 158.1, 157.3, 147.8, 140.6, 134.1, 133.0, 131.2, 122.9, 121.1, 119.8, 118.6, 108.6, 60.8, 60.3;

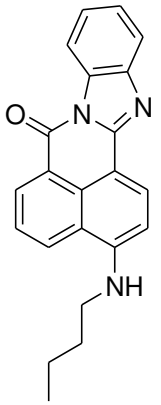
MS (EI) : m/z 330.1 (M^++1); Anal. Calc. for ($C_{20}H_{15}N_3O_2$): C, 72.94; H, 4.59; N, 12.76. Found: C, 72.78; H, 4.24; N, 12.45.

3-(5-Bromo-pyridin-2-ylamino)-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (5g) Brown powder (0.14g, 45%); m.p. 220-222^{o}C; ^{1}H NMR ($CDCl_3$): δ 8.86 (dd, ^{2}J = 7.32



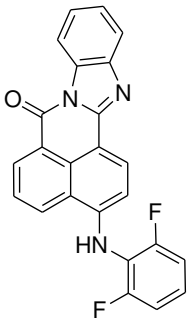
Hz, ^{3}J = 0.96 Hz, 1H, ArH), 8.82 (dd, ^{2}J = 7.32 Hz, ^{3}J = 1.08 Hz, 1H, ArH), 8.63 (d, J = 7.96 Hz, 1H, ArH), 8.57 (d, J = 7.92 Hz, 1H, ArH), 8.48 (m, 2H, ArH), 8.09 (dd, ^{2}J = 10.72 Hz, ^{3}J = 7.92 Hz, 2H, ArH), 7.87 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$): δ 157.3, 155.9, 152.1, 145.8, 142.2, 141.4, 139.4, 134.3, 131.9, 131.7, 118.1, 117.2, 117.0, 116.8, 116.7, 114.3, 109.8, 106.6; MS (EI) : m/z 442.1 (M^++1); Anal. Calc. for ($C_{23}H_{13}BrN_4O$): C, 62.60; H, 2.97; N, 12.70. Found: C, 62.55; H, 2.87; N, 12.45.

3-Butylamino-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one (5h)



Brown powder (0.1g, 40%); m.p. 210-212 ^{o}C; ^{1}H NMR ($CDCl_3$): δ 8.56 (d, J = 7.32 Hz, 1H, ArH), 8.45 (d, J = 8.68 Hz, 1H, ArH), 8.08 (d, J = 7.80 Hz, 1H, ArH), 7.57 (dd, ^{2}J = 8.28 Hz, ^{3}J = 7.36 Hz, 1H, ArH), 7.27 (m, 1H, ArH), 7.12 (dd, ^{2}J = 8.28 Hz, ^{3}J = 1.84 Hz, 1H, ArH), 6.93 (m, 2H, ArH), 6.70 (d, J = 8.24 Hz, 1H, ArH), 5.36 (t, J = 5.04 Hz, 2H, NH), 3.38 (q, J = 7.32 Hz, 2H, CH_2), 1.83 (m, 2H, CH_2), 1.58 (m, 2H, CH_2), 1.03 (t, J = 7.36 Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 164.3, 163.6, 149.7, 143.0, 135.0, 131.5, 130.3, 129.6, 129.5, 126.1, 124.6, 123.1, 122.4, 120.1, 119.4, 117.2, 109.8, 104.3, 43.4, 30.9, 20.3, 13.8; MS (EI) : m/z 342.1 (M^++1); Anal. Calc. for ($C_{22}H_{19}N_3O$): C, 77.40; H, 5.61; N, 12.31. Found: C, 77.80; H, 5.31; N, 12.30.

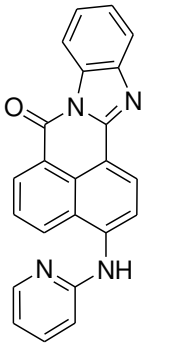
3-(2,6-Difluoro-phenylamino)-benzo[*de*]benzo[4,5]imidazo [2,1-*a*]isoquinolin-7-one (5i)



Brown powder (0.17g, 61%); m.p. 137-139 ^{o}C; ^{1}H NMR ($CDCl_3$): δ 8.81 (m, 1H, ArH), 8.78 (d, *J* = 6.88 Hz, 1H, ArH), 8.62 (d, *J* = 7.36 Hz, 2H, ArH), 8.59 (m, 2H, ArH), 8.25 (dd, ^{2}J = 11.0 Hz, ^{3}J = 8.24 Hz, 1H, ArH), 7.83 (m, 3H, ArH), 7.45 (m, 1H, ArH), 6.93 (t, *J* = 3.64 Hz, 1H, ArH); ^{13}C NMR ($CDCl_3$): δ 163.9, 160.8, 143.7, 143.0, 135.4, 134.4, 132.0, 131.8, 131.3, 131.2, 128.6, 127.5, 127.0, 125.9, 125.8, 125.5, 123.1, 122.7, 120.0, 119.6, 115.96; MS (EI) : m/z 398.1 (M^{+}+1);

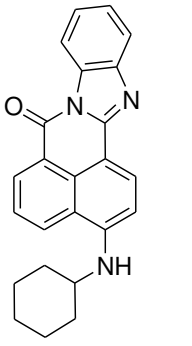
Anal. Calc. for ($C_{24}H_{13}F_2N_3O$): C, 72.54; H, 3.30; N, 10.57. Found: C, 72.33; H, 3.32; N, 10.55.

3-(Pyridin-2-ylamino)-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one (5j)



Brown powder (0.16g, 63%); m.p. 190-192 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 8.80 (d, *J* = 24.24 Hz, 1H, ArH), 8.60 (d, *J* = 8.28 Hz, 1H, ArH), 8.53 (dd, ^{2}J = 7.28 Hz, ^{3}J = 0.96 Hz, 2H, ArH), 8.38 (dd, ^{2}J = 8.28 Hz, ^{3}J = 0.80 Hz, 2H, ArH), 7.84 (t, *J* = 3.76 Hz, 2H, ArH), 7.45 (m, 1H, ArH), 7.24 (m, 1H, ArH), 7.01 (dd, ^{2}J = 7.76 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 6.90 (dd, ^{2}J = 8.04 Hz, ^{3}J = 1.00 Hz, 1H, ArH), 6.76 (t, *J* = 6.56 Hz, 1H, ArH); ^{13}C NMR (DMSO-d_6): δ 158.8, 152.8, 152.2, 139.3, 134.7, 132.3, 131.7, 126.6, 124.6, 124.3, 119.7, 117.1, 116.9, 116.3, 115.7, 113.4, 110.4, 109.3; MS (EI) : m/z 363.1 (M^{+}+1); Anal. Calc. for ($C_{23}H_{14}N_4O$): C, 76.23; H, 3.89; N, 15.46. Found: C, 76.21; H, 3.34; N, 15.45.

3-Cyclohexylamino-benzo[*de*]benzo[4,5]imidazo[2,1-*a*] isoquinolin-7-one (5k)



Brown powder (0.15g, 58%); m.p. 220-222 ^{o}C; ^{1}H NMR (DMSO-d_6): δ 8.22 (dd, ^{2}J = 23.32 Hz, ^{3}J = 8.04 Hz, 2H, ArH), 7.79 (m, 2H, ArH), 7.66 (dd, ^{2}J = 14.24 Hz, ^{3}J = 13.80 Hz, 2H, ArH), 7.44 (m, 1H, ArH), 7.38 (m, 1H, ArH), 7.20 (d, *J* = 8.08 Hz, 1H, ArH), 3.60 (m, 1H, CH), 1.78 (t, *J* = 5.08 Hz, 2H, CH_2), 1.67 (m, 2H, CH_2), 1.56 (m, 2H, CH_2), 1.31 (m, 4H, CH_2); ^{13}C NMR (DMSO-d_6): δ 157.3, 156.9, 151.7, 151.6, 142.0, 133.8, 131.9, 131.3, 131.1, 124.4, 121.9, 119.9, 117.0, 116.6, 111.5, 110.2, 108.2, 46.3, 45.3, 25.0, 13.2; MS (EI) : m/z 368.1 (M^{+}+1); Anal. Calc. for ($C_{24}H_{21}N_3O$): C, 78.45; H, 5.76; N, 11.44. Found: C, 78.93; H, 5.88; N, 11.42.

2.8.2. *In vitro* anticancer screening assay

The *in vitro* anticancer screening at NCI was a two-stage process, beginning with the evaluation of all compounds against the 60 cell lines at a single dose of 10 μM. The output from the single dose screen was reported as a mean graph and was available for analysis by the COMPARE program. Compounds which exhibited significant growth inhibition were evaluated against the 60 cell panel at five concentration levels. The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96 well microtiter plates in 100 mL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37^{o} C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide at 400 fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 h at 37 ^{o}C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 ^{o}C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test

growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as:

[(Ti - Tz)/(C-Tz)] × 100 for concentrations for which Ti>/= Tz

[(Ti – Tz)/Tz] × 100 for concentrations for which Ti < Tz:

Three dose response parameters were calculated for each experimental agent. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti-Tz)/(C-Tz)] ×100 = 50, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti =Tz. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from [(Ti-Tz) / Tz] ×100 = -50.

2.8.3. DNA binding studies

Stock solution of compound **5d** (1 mM) was prepared by dissolving **5d** in AR grade DMSO. The DNA binding experiments were carried out by making dilution of stock solution with phosphate buffer. Stock solution of ct-DNA was prepared by dissolving DNA in phosphate buffer (10 mM, pH 7.0). The DNA concentration was estimated from its absorbance intensity at 260 nm with a known molar absorption coefficient value of 6600 $dm^3M^{-1}cm^{-1}$. The purity of DNA was established from ratio of absorbance intensity at 260 nm and 280 nm.

UV-Vis and Fluorescence titrations: The titration experiments were performed by varying the concentration of ct-DNA and keeping the compound concentration constant (50 µM). All the UV spectra were recorded after equilibration of solution for 5 min. Fluorescence titrations were carried out on Cary Eclipse Fluorescence Spectrophotometer at ambient temperature. A slit width of 10 nm was used with λ_{ex} = 400 nm. The titration experiments were accomplished by varying the concentration of DNA in cuvette (0.5-150 µM).

DNA thermal denaturation: DNA melting experiments were carried out by observing the absorbance of ct-DNA at 260 nm at various temperature in the absence and presence of compound at 400 nm with a ramp rate of 0.5 ^oC/min in a phosphate buffer (pH 7.0) on a Shimadzu Spectrophotometer equipped with a Peltier thermo regulator.

2.8.4. Molecular modelling

Coordinates from the X-ray crystal structure of topoisomerase I (Topo I, pdb ID 1A36), topoisomerase II (Topo II, pdb ID 1BJT) and ribonucleotide reductase (RNR, pdb ID 4R1R) as well as DNA (pdb ID 1BNA) were taken from the RCSB Protein Data Bank. Compounds were constructed with the builder toolkit of the software package ArgusLab 4.0.1 (www.arguslab.com) and energy minimized using the semiempirical quantum mechanical method PM3. The monomeric structure of the enzyme was chosen, and the active site was defined around the ligand. The molecule to be docked in the enzyme active site was inserted into the work space carrying the structure of the enzyme. The docking program implements an efficient grid-based docking algorithm, which approximates an exhaustive search within the free volume of the binding site cavity. The conformational space was surveyed by the geometry optimization of the flexible ligand (rings are treated as rigid) in combination with the incremental construction of the ligand torsions. Thus, docking occurred between the flexible ligand parts of the compound and enzyme. The ligand orientation was determined by a shape scoring function based on Ascore and the final positions were ranked by lowest interaction energy values. Hydrogen bond between the compound and enzyme were explored by distance measurements.

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35. Compounds were constructed and docked with builder tool kit of software package ArgusLab 4.0.1 (www.arguslab.com).

CHAPTER 3

SYNTHESIS OF ALLYL-NAPHTHALIMIDE ANALOGUES AS ANTICANCER AGENTS

3.1 Introduction

In the area of anticancer research, the development of small molecules capable of interacting with deoxyribonucleic acid (DNA) and exhibiting anticancer activities has received enormous attention in recent years.[1] Amongst these, it has been found that 1,8-naphthalimides (benz[*de*]isoquinolin-1,3-diones) and its derivatives possess high anticancer activity towards various human and murine cells.[2-7] The development of functional 1,8-naphthalimide derivatives as anticancer agents and DNA targeting is a fast growing area and has resulted in several such derivatives like amonafide, mitonafide, elinafide and bisnafide that entered into clinical trials.[8] Additionally, the naphthalimide ring can also be substituted, for example, at the 3- or 4-position with amino, bromo or nitro groups. This not only allows the introduction of other active functional groups at these positions, which can be used for targeting biomolecules, but can have a major effect on the electronic properties with a consequent influence on the chemical, photochemical and spectroscopic properties. In literature, many examples are known where anticancer activities of naphthalimides have been significantly affected via fusing aromatic rings[9-10] or varying the position and size of side chains.[11] Qian and co-workers have synthesized a series of naphthalimides with the substitution of 1,2,3-triazole ring at 3 or 4 position. These newly synthesized compounds showed better cytotoxic activity against breast cancer cell line MCF-7 cells than amonafide.[12-13] Wang and co-workers have synthesized naphthalimide analogue substituted with amino acids and dichloroacetamide functionalizations at 3-position and evaluated their cytotoxic activities against Hela, A549 and K562 cancer cell lines.[14] Another example of such modification is UNBS5162, which proved to be a good anticancer agent and presently at clinical trials. It showed lesser toxic side effects which decreased CXCL chemokine expression in advanced solid tumors or lymphoma.[15] The probable mechanism of these anticancer activities of naphthalimide derivatives is supposed to be capable of either intercalation with base pairs or alkylation, or groove binding of DNA as the substituted naphthalimides are characterized by presence of planar chromophore portion.[16] Thus, naphthalimides not only interact with DNA, but members of this class also offer various sites for numerous modifications which provide a hope in the field of improvement of their antitumor activity.[17] Synthesis of such analogue is

the high priority for medicinal research because DNA is one of the important target for cancer treatment.[18] Inspired by its promising antitumor activities and keeping in mind for their possibility of DNA intercalation, we have synthesized the *N*-allyl naphthalimide analogue substituted with primary and secondary amines in order to improve their anticancer activities and selectivity profile. Calf thymus (ct)-DNA studies have also been performed with the most active compound of this series in order to observe its interaction with DNA.

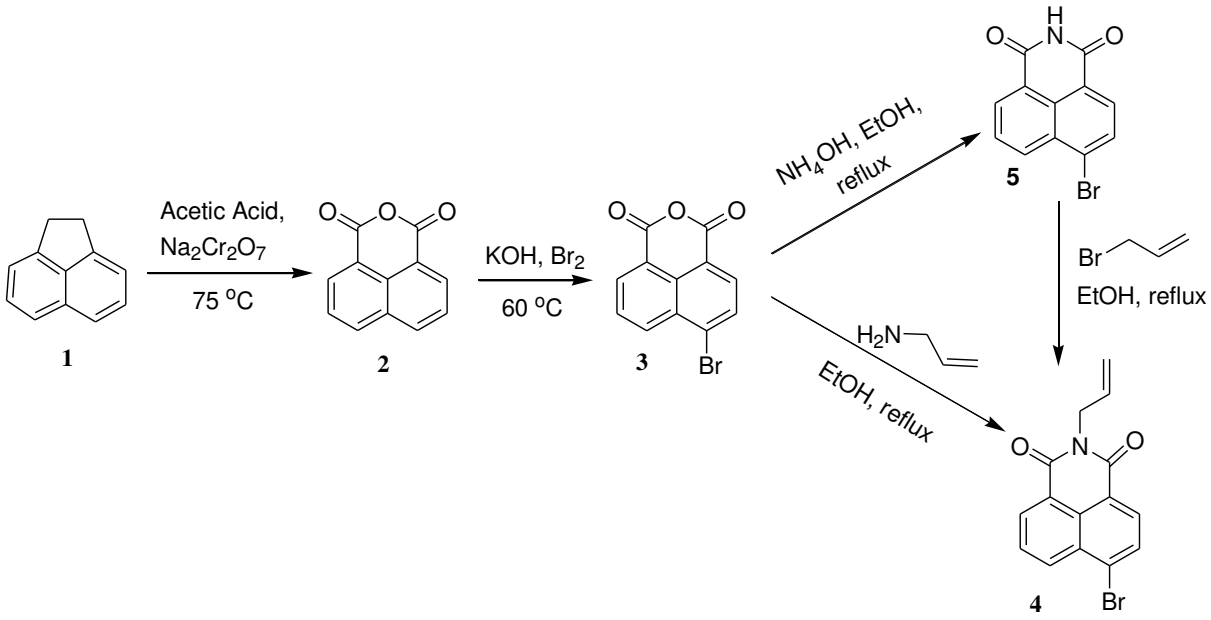
Objective: Numerous naphthalimide based anticancer analogues have been reported in literature having strong DNA interaction properties. But many of these drugs failed at various stages of studies due to side effects associated with them. As we have discussed our work on synthesis of naphthalimie analogues in the previous chapter, it was concluded that amine substituted analogues have possessed better anticancer activity as compared to standard drug 5-FU. Keeping in mind the previous results, we have synthesized novel naphthalimide analogues having allyl group at *N*-position and then substitution with various amines for anticancer activity. As naphthalimides are very good DNA intercalators, we have also studied DNA intercalation of these compounds.

3.2. Chemistry

3.2.1. Synthesis of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (4)

Target naphthalimide analogue **6a-n** has been prepared via multistep reactions using commercially available starting material acenaphthene (**1**) (Scheme 1 and 2). Sodium dichromate was added to a solution of acenaphthene and acetic acid in small fractions with continues stirring at room temperature, using a water bath to avoid any heat formation. This suspension was then heated to 75 ^{o}C for 8 h. On completion of reaction, cold water was added to the reaction mixture, resulted in precipitation of the crude product. After filtration and washing off with water, yellow coloured solid of 1,8-naphthalic anhydride (**2**) with 80% yield (m.p. 266-268 ^{o}C) was obtained. A solution of 1,8-naphthalic anhydride and hot KOH was prepared and cooled to room temperature. To this solution, bromine was added drop-wise with vigorous stirring. After complete addition, reaction mixture was heated to 60 ^{o}C for 6 h. The resulted solution was acidified with HCl. Brown coloured solid separated out and filtered. This solid residue was further heated with 60 ml of 5% NaOH solution, filtered and neutralized with HCl solution. Off white precipitates separated out, filtered and washed with cold water, dried to get white solid of 6-bromo-benzo[*de*]isochromene-1,3-dione (**3**) in 80%

yield (m.p. 117-119 ^{o}C). 6-Bromo-benzo[*de*]isochromene-1,3-dione (**3**) was then reacted with allylamine in ethanol at reflux temperature for 8 h. After the completion of reaction (TLC), cooled the reaction mixture to get crude solid, filtered and washed with ethanol to afford pure white compound of **4** with 80% yield (m.p. 128-130 ^{o}C).



Scheme 1 Synthesis of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (**4**)

An alternative method for synthesis of compound **4** has also been followed where 6-bromo-benzo[*de*]isochromene-1,3-dione (**3**) was refluxed with ammonium hydroxide and ethanol. The reaction mixture was then cooled and solid product was filtered to get 6-bromo-benzo[*de*]isoquinoline-1,3-dione (**5**) (m.p. 134-136 ^{o}C). Compound **5** was further refluxed with allylbromide in the presence of EtOH to yield crude product, washed with ethanol to get white solid of **4** in 65% overall yield (m.p. 128-130 ^{o}C). ^{1}H NMR spectrum of this compound showed three 1H double doublets at δ 8.66, δ 8.56, and δ 7.86, two 1H doublets at δ 8.41 and δ 8.04 of aromatic-H, 1H multiplet of CH at δ 6.04-5.95, two 1H double quartets of CH_2 at δ 5.35-5.30 and 5.24-5.20 (coupling constants were calculated as 17.40 Hz and 1.36 Hz; and 10.30 Hz and 1.36 Hz respectively) and 2H double triplet of N-CH_2 at δ 4.80-4.78 (coupling constant were calculated as 5.96 Hz and 1.36 Hz) which confirmed the incorporation of allyl group to naphthalimide core. ^{13}C NMR spectrum of this compound showed –C=O signal at δ 163.3 and 133.4, 132.2, 131.9, 131.4, 131.1, 130.6, 130.5, 129.0, 128.1, 123.0 (ArC), 122.1 (CH_2), 117.9 (CH) and 42.6 (N-CH_2). On the basis of these spectral data, this compound has assigned the structure of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione. Therefore,

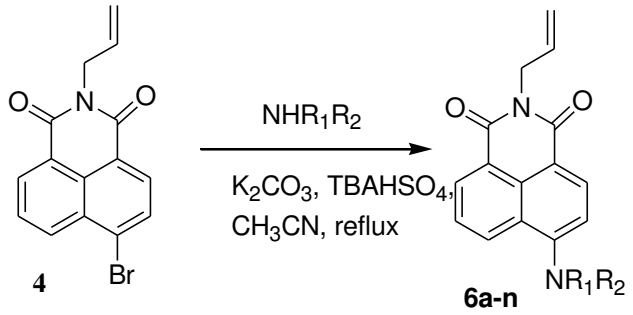
introduction of allyl group to 6-bromo-benzo[*de*]isochromene-1,3-dione has been confirmed with both the reaction conditions.

3.2.2. Synthesis of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione (6a-n)

A mixture of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione and piperidine was refluxed for 9 h in the presence of potassium carbonate and $TBAHSO_4$ (catalytic amount) in acetonitrile (Scheme 2). After completion of reaction, water was added to the reaction mixture and then extracted with chloroform, separated the organic layer, dried over Na_2SO_4, filtered and concentrated to get solid crude product which was purified using column chromatography on silica gel using hexane:ethylacetate (6:4) as eluents to give compound **6a** in 78 % yield (Table 1). Structure of this final compound was confirmed by ^1H and ^{13}C NMR as well as mass spectrometry. ^1H NMR spectrum of this compound showed three 1H double doublets at δ 8.59, δ 8.40 and δ 7.70, two 1H doublets at δ 8.51 and δ 7.19 of aromatic-H, 1H multiplet of CH at δ 6.03-5.95, two 1H double quartets of allylic-CH_2 at δ 5.32-5.27 and δ 5.20-5.17, 2H double triplet of N-CH_2 at δ 4.81-7.78, 4H triplet of pip-CH_2 at δ 3.24, 4H multiplet of pip-CH_2 at δ 1.91-1.86 and 2H multiplet of pip-CH_2 at δ 1.75-1.71 which confirmed the substitution of piperidine moiety to naphthalimide core. Protons at δ 5.32 showed coupling constant of 17.2 Hz and 1.40 Hz while protons at δ 5.20 showed coupling constant of 10.32 Hz and 1.36 Hz. Protons at δ 4.81 showed coupling constants of 5.52 Hz and 1.36 Hz. ^{13}C NMR spectrum of this compound showed two –C=O signals at δ 164.3 and 163.8, 157.3, 132.8, 132.4, 131.1, 130.7, 129.9, 126.2, 125.3, 122.9, 117.1, 115.7, 114.6 (ArC), 54.4 (pip-NCH_2), 42.1 (N-CH_2), 26.1 (pip-CH_2) and 24.3 (pip-CH_2). On the basis of these spectral data, this compound has been assigned the structure of 2-allyl-6-piperidin-1-yl-benzo[*de*]isoquinoline-1,3-dione (**6a**). Similarly, 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (**4**) was refluxed with morpholine in acetonitrile in the presence of K_2CO_3 and $TBAHSO_4$ gave yellow solid compound of **6b** (MS (EI) : m/z 323.1 (M^++1)) in 87 % yield which has been proved to be the most active compound for 60 human cancer cell lines for anticancer activity. ^1H NMR spectrum of this compound showed three 1H double doublets at δ 8.61, δ 8.44 and δ 7.73, 1H doublet at δ 8.55, 1H triplet at δ 7.27 of aromatic-H, 1H multiplet of CH at δ 6.04-5.94, two 1H double quartets of CH_2 at δ 5.33-5.28 and δ 5.21-5.18, 2H double triplet of N-CH_2 at δ 4.81-4.78, two 4H triplets of mor-CH_2 at δ 4.03 and δ 3.27 which confirmed the substitution of morpholine into naphthalimide core. Coupling constants for protons at δ 5.33 were calculated as 17.42 Hz and 1.36 Hz, for protons at δ 5.21 were

calculated as 10.08 Hz and 1.36 Hz and for protons at δ 4.81 showed coupling constant 5.96 Hz and 1.36 Hz. ^{13}C NMR spectrum of this compound showed two –C=O signals at δ 161.3, 160.8 and 152.9, 129.8, 129.5, 128.5, 127.3, 127.1, 123.3, 123.0, 120.4, 114.5, 114.2, 112.1 (ArC), 64.1 (mor-OCH_2), 50.6 (mor-NCH_2) and 39.5 (N-CH_2). On the basis of these spectral data, this compound has been assigned the structure of 2-allyl-6-morpholine-1-yl-benzo[*de*]isoquinoline-1,3-dione (**6b**). 2-Allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione was also treated with pyrrolidine in phase transfer reaction to give yellow solid of **6c** (MS (EI) : m/z 307.1 (M^++1)) in 76 % yield.

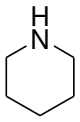
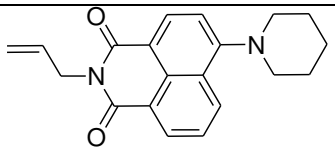
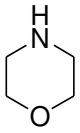
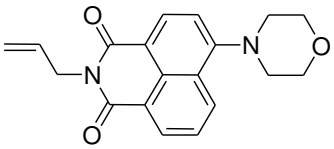
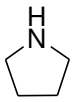
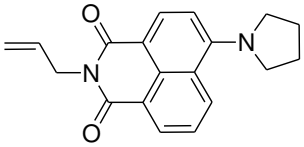
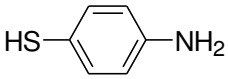
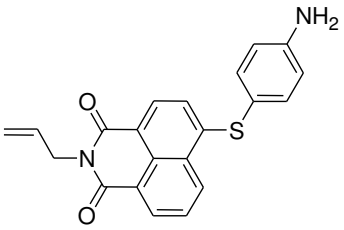
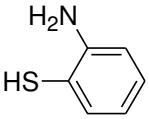
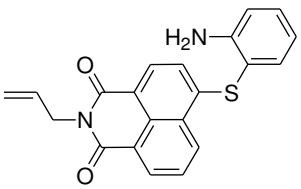
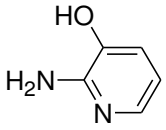
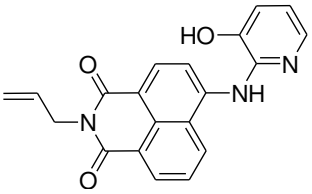
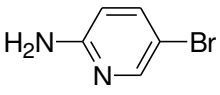
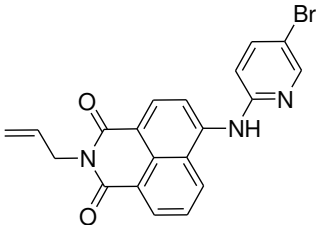
Under similar reaction conditions, **4** was also treated with primary amine, 4-aminothiophenol, in acetonitrile to give brownish-yellow solid of **6d** in 60 % yield (MS (EI) : m/z 361.1 (M^++1)). ^{1}H NMR spectrum of this compound showed 2H multiplet at δ 8.65-8.61, 1H doublet at δ 8.31, 1H double doublet at δ 7.79, two 2H doublets at δ 7.40 and δ 6.81 and 1H doublet at δ 7.06 of aromatic-H, 1H multiplet of CH at δ 6.01-5.95, two 1H double quartets of CH_2 at δ 5.32-5.26 and δ 5.21-5.17, 2H double triplet of N-CH_2 at δ 4.79-4.77 and 2H broad singlet of NH_2 at δ 4.01. ^{1}H NMR of this compound has also been done with D_2O exchange, which confirmed the signal at δ 4.01 of NH_2. ^{13}C NMR spectrum showed two –C=O signals at δ 163.8, 163.7 and 148.8, 148.3, 137.4, 132.1, 131.6, 131.0, 129.8, 128.3, 126.5, 122.9, 122.8, 118.5, 117.3, 116.3, 115.5 (ArC) and N-CH_2 signal at δ 42.3. On the basis of these NMR spectral data, this compound has been assigned the structure of 2-allyl-6-(4-amino-phenylsulfanyl)-benzo[*de*]isoquinoline-1,3-dione (**6d**). Other primary aromatic and aliphatic amines were reacted with 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (**4**) at same reaction conditions gave compounds **6e-n** in 49-65 % yields (Table-1).

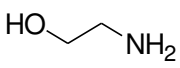
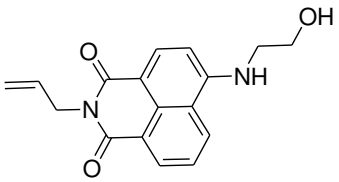
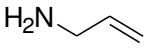
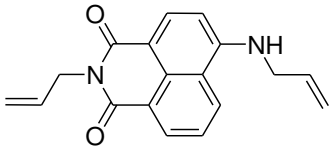
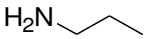
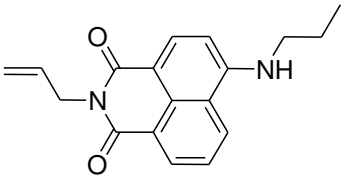
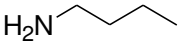
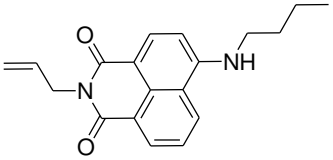
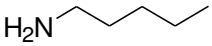
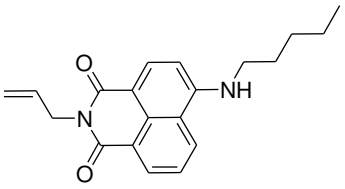
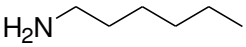
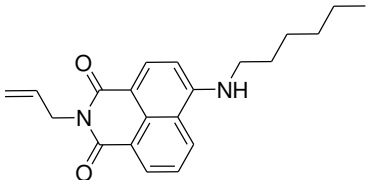
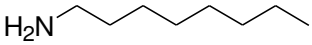
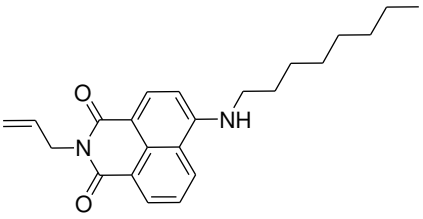


Scheme 2 Synthesis of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione

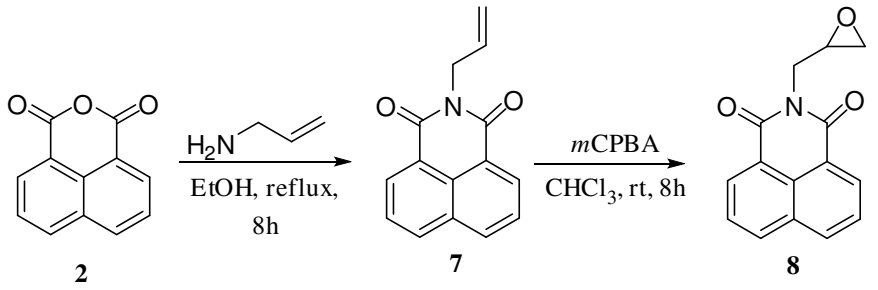
Table 1 Physical data of synthesized compounds

Entry	HNR_IR_2	Product	Time (h)	Yield (%)	m.p. (^{o}C)

1		 **6a**	9	78	115-117
2		 **6b**	8	87	173-174
3		 **6c**	9	76	155-158
4		 **6d**	11	60	185-188
5		 **6e**	12	59	205-208
6		 **6f**	10.5	63	210-212
7		 **6g**	10	53	205-207

8		 **6h**	8.5	57	-
9		 **6i**	9	50	-
10		 **6j**	8.5	65	-
11		 **6k**	8	62	-
12		 **6l**	8.5	59	-
13		 **6m**	9	51	-
14		 **6n**	8	49	-

3.2.3. Synthesis of 2-allyl-benzo[*de*]isoquinoline-1,3-dione (7) and 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (8)



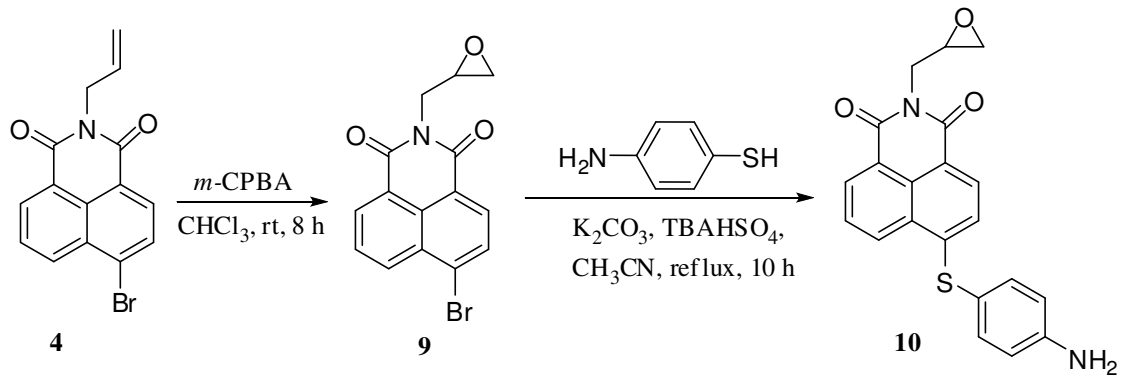
Scheme 3 Synthesis of 2-allyl-benzo[*de*]isoquinoline-1,3-dione (**7**) and 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**8**)

Epoxidation has also been done with naphthalimide derivatives to increase the polar interactions with DNA. Compound **2** was reacted with allyl amine in presence of ethanol at reflux conditions for 8 h. Reaction was monitored with TLC. On completion of reaction, reaction mixture was cooled to room temperature. Filtered the precipitate and washed with ethanol to remove any traces of allyl amine, resulted in pure compound **7** (m.p. 117-120 ^{o}C) with 71 % yield (Scheme-3). ^{1}H NMR spectrum of **7** showed two 2H doublets at δ 8.62 and δ 8.23, 2H triplet at δ 7.78 of aromatic-H, 1H multiplet of CH at δ 6.05-5.95, two 1H double triplets of CH_2 at δ 5.35-5.30 and δ 5.23-5.20, and 2H double doublet of CH_2 at δ 4.82 which confirmed the incorporation of allyl group to naphthalimide core. ^{13}C NMR spectrum of this compound showed –C=O signal at δ 163.9 and 134.0, 132.1, 131.5, 131.3, 128.1, 126.9, 122.5, 117.5, 117.5 (ArC), 42.4 (N-CH_2). On the basis of these spectral data, this compound has been assigned the structure of 2-allyl-benzo[*de*]isoquinoline-1,3-dione (**7**). 2-Allyl-benzo[*de*]isoquinoline-1,3-dione (**7**) was stirred in dry chloroform with *m*CPBA at room temperature for 8 h. After completion of reaction (TLC), reaction mixture was neutralized with $NaHCO_3$ and extracted with water. Organic layer was then dried over Na_2SO_4 and concentrated to get the crude product of **8** which was then purified by column chromatography using hexane : ethylacetate as eluents (59 %, m.p. 105-107 ^{o}C). ^{1}H NMR spectrum of this compound showed two 2H doublets at δ 8.64 and δ 8.28, 2H triplet at δ 7.81 of aromatic-H, 1H multiplet of CH at δ 4.49-4.37, 2H triplet of O-CH_2 at δ 4.12, 2H triplet of N-CH_2 at δ 3.65. The upfield shifting of allyl signals from δ 6.05-5.95 to δ 4.49-4.37 of multilplet CH, from δ 5.35-5.30 and δ 5.23-5.20 to δ 4.12 of CH_2, and from δ 4.82 to δ 3.65 of N-CH_2 proved the formation of epoxy ring in compound **8**. ^{13}C NMR spectrum of this

compound showed –C=O signal at δ 165.6 and 134.7, 132.0, 131.6, 128.2, 127.2, 122.1 (ArH), 70.7 (O-CH), 63.7 (O-CH_2), 42.7 (N-CH_2). On the basis of these spectral data, compound **8** has been assigned the structure of 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione.

3.2.4. Synthesis of 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (9) and 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (10)

Compound **4** was stirred with *m*-CPBA in the presence of dry chloroform at room temperature for 8 h. After completion of reaction (TLC), reaction mixture was neutralized with $NaHCO_3$ and extracted with water. Organic layer was separated, dried over Na_2SO_4 and concentrated to get the crude product of 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**9**) which was purified with column chromatography to remove any impurities using hexane : ethylacetate as eluents in 63 % yield (m.p. 110-113 ^oC).



4 **9** **10**

Scheme 4 Synthesis of compounds **9** and **10**

^1H NMR spectrum of this compound showed two 1H double doublets at δ 8.20 and δ 8.14, two 1H doublets at δ 7.96 and δ 7.64, 1H double doublet at δ 7.47 of aromatic-H, 1H multiplet of CH at δ 4.02-3.71, 2H triplet of O-CH_2 at δ 3.20 and 2H singlet of N-CH_2 at δ 2.73. Upfield shifting of proton signals from δ 6.01-5.98 to δ 4.02-3.71 of multiplet CH, from δ 5.32-5.26 and δ 5.21-5.17 to triplet at δ 3.20 of O-CH_2 and from δ 4.79-4.77 to δ 2.73 of N-CH_2 confirmed the formation of epoxy ring. ^{13}C NMR spectrum of this compound showed –C=O signal at δ 163.4 and 133.3, 132.1, 131.8, 131.4, 130.6, 130.1, 128.9, 122.8, 122.3, 117.8 (ArC), 69.3 (O-CH), 63.6 (O-CH_2), 42.5 (N-CH_2) which again confirmed the conversion of double bond into epoxy ring in compound **9**. On the basis of these spectral data, compound **9** has been assigned the structure of 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (Scheme-4).

6-Bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**9**) was then reacted with 4-amino-thiophenol in the presence of K_2CO_3 and $TBAHSO_4$ (catalytic amounts) using acetonitrile as solvent at reflux temperature for 10 h. On completion of reaction (TLC), solvent was removed under vacuum. After usual work up with water and chloroform, the organic layer was separated, dried over Na_2SO_4 and concentrated to get crude solid which was purified with column chromatography using hexane : ethylacetate as eluents to give yellow solid of compound **10** in 52 % yield (m.p. 145-146 ^oC). ^1H NMR spectrum of this compound showed 2H quartet at δ 8.67, 1H doublet at δ 8.31, 1H triplet at δ 7.81, 2H doublet at δ 7.40, 1H doublet at δ 7.07, 2H doublet at δ 6.81 of aromatic-H, 1H multiplet of CH at δ 4.45-4.34, 2H triplet of O-CH_2 at δ 4.09 and 2H doublet of N-CH_2 at δ 3.61. Addition of more aromatic protons confirmed the incorporation of phenyl ring to the compound **9**. ^{13}C NMR spectrum of this compound showed –C=O signal at δ 163.6 and 149.0, 148.5, 136.4, 130.6, 130.0, 128.8, 127.3, 127.1, 125.6, 121.8, 117.4, 115.1, 112.2 (ArC), 69.0 (O-CH), 63.6 (O-CH_2) and 41.8 (N-CH_2). On the basis of this spectral data, compound **10** has been assigned the structure of 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione.

3.3. Biology

3.3.1. *In vitro* cancer screening with one dose

All compounds were submitted to National Cancer Institute (NCI) for evaluation of their *in-vitro* antitumor activities.[19-21] Compounds **6b-c, 6f-i, 8** and **9** were evaluated against 60 human cell lines at single dose concentration of 10 μM which includes nine tumour subpanels and their outputs were reported as a mean graph of the percent growth of the treated cells and presented as percentage growth inhibition (GI %). Compound **6b** exhibited significant growth inhibition and evaluated as 60 cell panels at five dose concentration levels.

Preliminary *in vitro* antitumor screening revealed that only compounds belonging to secondary amines especially morpholine showed significant inhibition for almost all the cancer cell lines. The percentages of inhibition for these cancer cells were more than 60% of tested derivative while primary amines substituted **6f-i** showed weak activities with percentages of inhibition less than 40% except few cell lines (Table 2). These variations could be correlated to the difference in C6 substituents on the core naphthalimide moiety, in which the presence of morpholine moiety is an important factor affecting for antitumor activity. On the contrary; compound **6c** with pyrrolidine, showed potency towards non-small

lung cancer cell line EKVX and renal cancer cell line A498 with GI values of 40.31% and 33.55% respectively. In series of primary amines **6f-i**, significant growth inhibitions were observed for renal cancer cell (UO-31; 26.10–32.85%), lung cancer cell (HOP-92; 21.32–50.37%) and prostate cancer cell (PC-3; 23.41–39.40%). Compound **6i** with allylamine at C6 position also showed selectivity towards lung cancer cell line HOP 92 and breast cancer cell line MCF 7 with GI values of 50.37% and 66.05% respectively.

Table 2 Percentage growth inhibition (GI%) of the selected compounds over the most sensitive tumour cell lines at single concentration of 10 μM

Cell lines	**6c**	**6f**	**6g**	**6h**	**6i**	**8**	**9**
CCRF-CEM	-	-	-	-	-	48.90	-
MOLT-4	-	-	27.75	-	-	50.32	-
RPMI-8226	-	-	21.90	-	-	-	-
SR	22.00	25.51	20.73	-	25.25	22.58	-
EKVX	40.31	31.68	-	-	-	-	-
HOP-92	28.67	36.81	43.52	21.32	50.37	21.74	22.91
NCI-H522	24.02	-	-	-	-	-	-
HCT-116	-	25.75	22.83	-	-	24.60	-
SW-620	-	-	-	-	-	28.66	-
SNB-75	-	30.78	24.71	-	-	-	-
UACC-62	21.85	28.97	-	-	24.18	-	-
OVCAR-4	-	22.66	-	-	-	-	-
A498	33.55	-	-	-	-	-	23.56
CAKI-1	-	31.16	34.13	20.17	27.05	-	-
UO-31	-	26.10	32.85	27.36	28.50	-	17.69
LOX- IMVI	-	-	-	-	-	80.51	-
PC-3	23.41	39.40	28.38	25.20	30.66	-	-
MCF7	-	-	-	-	66.05	-	-
MDA-MB-231/ATCC	-	34.58	23.72	-	-	-	-
T-47D	25.27	29.71	-	-	-	-	-
MDA-MB-468	-	-	-	-	39.71	18.99	-

In the other series of compounds, **8** showed better activity as compared to compound **9**. Compound **8** proved to be sensitive towards leukemia cell lines CCRF-CEM, MOLT-4 and SR with GI value of 48.90, 50.32 and 22.58% respectively. Non-small cell lung cancer cell line HOP-92 showed sensitivity towards compound **8** with GI value of 21.74%. Colon cancer cell lines HCT-116 and SW-620 proved sensitive towards compound **8** with GI values of 24.60 and 28.66% respectively. Compound **8** proved to be most potent towards melanoma cancer cell line LOX IMVI with GI value of 80.51%. Breast cancer cell line MDA-MB-468 showed sensitivity towards compound **8** with GI value of 18.99%. Whereas compound **9** showed sensitivity towards non-small cell lung cancer cell line HOP-92 with GI value 22.91% and renal cancer cell lines A498 and UO-31 with GI values of 23.56 and 17.69% respectively.

On the other hand, naphthalimide analogue **6b** proved to be the most active member. It showed broad spectrum of activity against all nine subpanels of cancer cell lines at primary single dose concentration level (Table 3, Figure 1).

Table 3 Percentages of growth inhibition of compound **6b** over the full panel of tumour cell lines at single concentration of 10 μM

Subpanel Cell lines	Inhibition (%)	Subpanel Cell lines	Inhibition (%)
Leukemia Cancer		**Ovarian Cancer**	
CCRF-CEM	-7.18	IGROV1	-14.55
HL-60(TB)	-5.15	OVCAR-3	-66.42
K-562	90.22	OVCAR-4	85.64
MOLT-4	-21.21	OVCAR-5	78.58
RPMI-8226	-25.79	OVCAR-8	79.30
SR	-9.38	NCI/ADR-RES	93.94
Non-Small Cell Lung Cancer		SK-OV-3	86.12
A549/ATCC	96.95	**Renal Cancer**	
EKVX	-7.17	786-0	73.84
HOP-62	73.56	A498	-57.04
HOP-92	78.89	ACHN	81.42
NCI-H226	-2.43	CAKI-1	-39.46
NCI-H23	91.99	RFX 393	-29.91
NCI-H322M	-56.58	SN12C	83.04

NCI-H460	91.48	TK-10	-12.91
NCI-H522	-1.11	UO-31	-3.07
Colon Cancer		**Melanoma Cancer**	
COLO 205	-21.78	LOX IMVI	98.87
HCC-2998	-33.96	MALME-3M	-3.28
HCT-116	-25.42	M14	99.61
HCT-15	-9.11	MDA-MB-435	99.64
HT29	93.24	SK-MEL-2	-28.12
KM12	-56.35	SK-MEL-28	94.33
SW-620	92.76	SK-MEL-5	-62.71
CNS Cancer		UACC-257	-3.63
SF-268	-23.87	UACC-62	-24.66
SF-295	97.13	**Breast Cancer**	
SF-539	99.50	MCF7	-0.41
SNB-19	75.05	MDA-MB-231/ATCC	76.09
SNB-75	87.23	HS 578T	95.42
U251	94.63	BT-549	-47.77
Prostate Cancer		MDA-MB-468	-40.05
PC-3	-21.28	T-47D	-2.10
DU-145	89.72		

Leukemia cell line K-562 proved sensitive towards compound **6b** with GI value of 90.22%. Non-small cell lung cancer cell lines A549/ATCC, HOP-62, HOP-92, NCI-H23 and NCI-H460 showed sensitivity towards compound **6b** with GI values 96.95, 73.56, 78.89, 91.99 and 91.48% respectively. Colon cancer cell line HT29 showed sensitivity towards **6b** with GI value of 93.24%. CNS cancer cell lines SF-295, SF-539, SNB-19, SNB-75 and U251 showed sensitivity towards **6b** with GI values 97.13, 99.50, 75.05, 87.23 and 94.63% respectively. Prostate cancer cell line DU-145 showed sensitivity with GI value of 89.72%. Ovarian cancer cell lines OVCAR-4, OVCAR-5, OVCAR-8, NCI/ADR-RES and SK-OV-3 showed sensitivity towards **6b** with GI values 85.64, 78.58, 79.30, 93.94 and 86.12% respectively. Renal cancer cell lines 786-0, ACHN and SN12C proved sensitive towards **6b** with GI values of 73.84, 81.42 and 83.04% respectively. Melanoma cancer cell lines LOX IMVI, M14, MDA-MB-435 and SK-MEK-28 proved sensitive towards **6b** with GI values of 98.87, 99.61,

99.64 and 94.33% respectively. Breast cancer cell lines MDA-MB-231/ATCC and HS 578T with GI values 76.09 and 95.42% respectively.

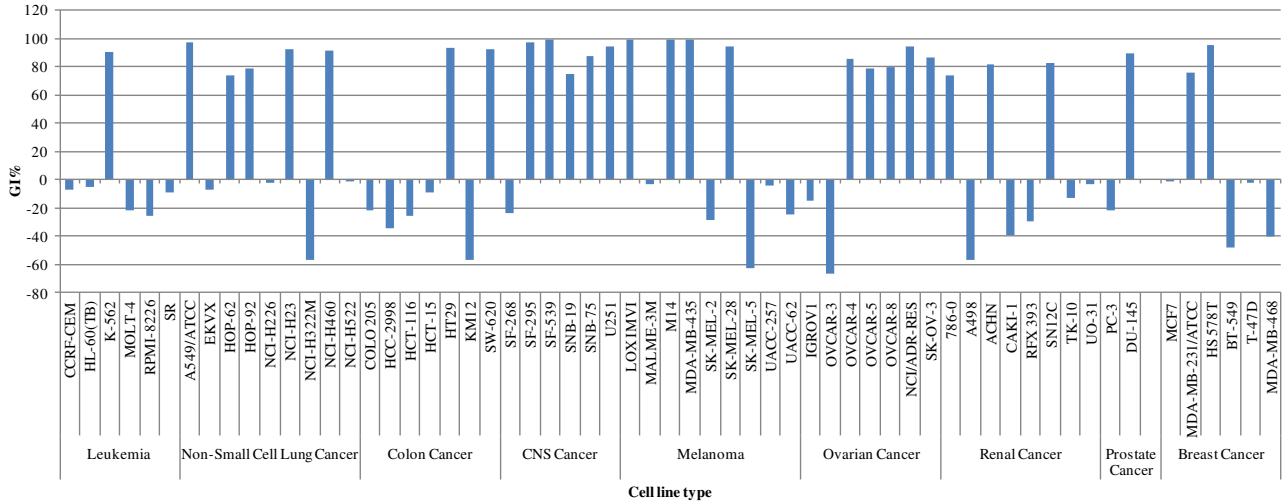


Figure 1 Percentage growth inhibition (GI%) of compound **6b** over the full panel cancer cell lines at concentration of 10 µM

3.3.2. *In vitro* anticancer screening with five dose concentration level

From the above data, it is clear that compound **6b** has proved to be the most active member of the series. Consequently, this active compound was carried over and tested against a panel of different tumor cell lines at five dose concentration level where it exhibited remarkable anticancer activity against non-small cell lung cancer cell line HOP-92, CNS cancer cell line SNB-75 and breast cancer cell line HS 578T with GI_{50} values in < 10 nM range. Compound **6b** also showed specificity towards central nervous system (CNS), melanoma, renal and breast cancer cell lines with GI_{50} values in the range of 18 nM-73 nM with excellent activity towards breast cancer cell lines. Compound **6b** also showed almost sixty fold more activity than naphthalimide derivative 3-(4-amino-phenylsulfanyl)-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one[10] with MG MID GI_{50}, TGI and LC_{50} values of 84.2 nM, 27.6 µM and 89.3 µM respectively (Table 4, Figures 2-5). Compound **6b** was also compared with approved chemotherapeutic drug, oxaliplatin[22] which also showed interaction with DNA. It was observed that compound **6b** showed almost one forty seven fold more activity than oxaliplatin (MG MID GI_{50}, TGI and LC_{50} values of 12.35 µM, 37.05 µM and 73.58 µM respectively). It is clear that replacement with morpholine group than other secondary or primary amines, and allyl group at N2 position leads to excellent increase in antitumor activity.

Table 4 Compound **6b**, naphthalimide analogue and oxaliplatin of median growth inhibitory (GI_{50}, μM), total growth inhibitory (TGI, μM) and median lethal concentrations (LC_{50}, μM) of *in vitro* subpanel tumor cell line

Compds.	Activity	I	II	III	IV	V	VI	VII	VIII	IX	MG-MID[a]
6b	GI_{50}	0.098	0.115	0.112	0.043	0.073	0.132	0.072	0.095	0.018	0.084
	TGI	[b]	21.3	17.04	10.53	15.61	40.18	33.42	7.174	3.962	27.69
	LC_{50}	[b]	97.35	[b]	92.41	87.55	80.14	89.48	[b]	[b]	89.38
Naphthalimide analogue	GI_{50}	2.90	3.94	6.58	4.09	6.93	6.97	3.30	3.23	5.20	5.05
	TGI	44.51	32.51	60.81	45.41	35.07	37.44	15.41	17.9	45.98	38.71
	LC_{50}	[b]	93.94	98.84	93.98	83.93	97.94	88.94	80.15	[b]	[b]
Oxaliplatin	GI_{50}	0.844	20.86	2.58	8.91	3.50	11.76	6.61	13.13	42.96	12.35
	TGI	15.84	31.11	44.17	55.87	38.18	42.12	40.24	59.62	6.30	37.05
	LC_{50}	[b]	79.40	73.96	63.10	72.07	[b]	79.40	[b]	[b]	73.58

I, leukemia; II, non-small cell lung cancer; III, colon cancer; IV, CNS cancer; V, melanoma; VI, ovarian cancer; VII, renal cancer; VIII, prostate cancer; IX, breast cancer.
[a] Full panel mean-graph midpoint (μM)
[b] Compounds showed values >100 μM

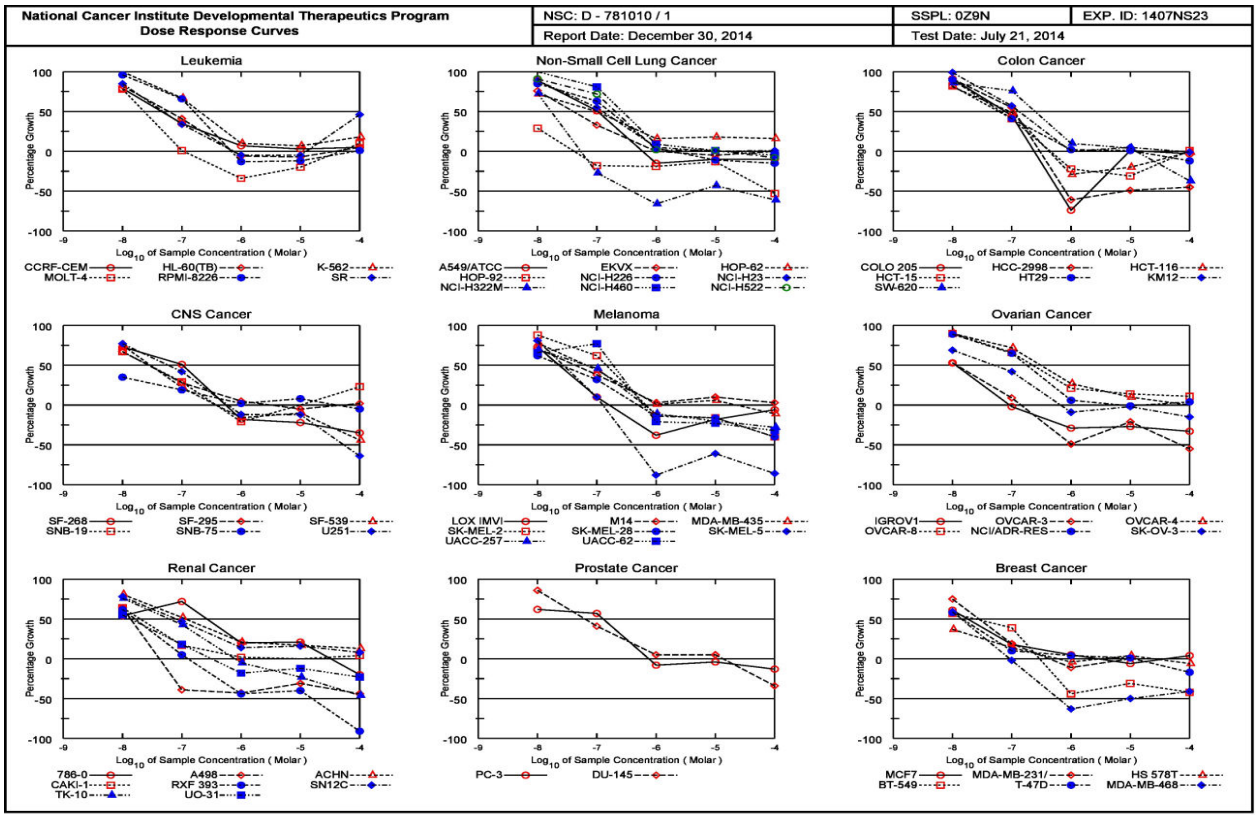


Figure 2 National Cancer Institute developmental therapeutics program *in-vitro* testing results of compound **6b** at five dose level in μM

National Cancer Institute Developmental Therapeutics Program
In-Vitro Testing Results

NSC : D - 781010 / 1	Experiment ID : 1407NS23	Test Type : 08	Units : Molar
Report Date : December 30, 2014	Test Date : July 21, 2014	QNS :	MC :
COMI : Meen-376	Stain Reagent : SRB Dual-Pass Related	SSPL : 0Z9N	

			Log10 Concentration											
	Time		Mean Optical Densities					Percent Growth						
Panel/Cell Line	Zero	Ctrl	-8.0	-7.0	-6.0	-5.0	-4.0	-8.0	-7.0	-6.0	-5.0	-4.0	GI50	TGI	LC50
Leukemia															
CCRF-CEM	0.621	2.635	2.203	1.325	0.756	0.675	0.730	79	35	7	3	5	4.52E-8	> 1.00E-4	> 1.00E-4
HL-60(TB)	1.063	3.285	2.894	1.981	1.003	0.988	1.171	82	41	-6	-7	5	6.14E-8	.	> 1.00E-4
K-562	0.344	2.397	2.469	1.729	0.556	0.488	0.720	104	67	10	7	18	2.02E-7	> 1.00E-4	> 1.00E-4
MOLT-4	0.679	2.824	2.343	0.708	0.447	0.541	0.902	78	1	-34	-20	10	2.30E-8	.	> 1.00E-4
RPMI-8226	1.053	2.754	2.691	2.179	0.920	0.928	1.078	96	66	-13	-12	1	1.61E-7	.	> 1.00E-4
SR	0.229	0.873	0.778	0.447	0.219	0.217	0.526	85	34	-5	-5	46	4.85E-8	.	> 1.00E-4
Non-Small Cell Lung Cancer															
A549/ATCC	0.335	2.046	1.866	1.202	0.286	0.301	0.301	89	51	-15	-10	-10	1.02E-7	5.95E-7	> 1.00E-4
EKVX	0.661	1.722	1.464	1.016	0.659	0.627	0.658	76	33	.	-5	.	4.05E-8	9.80E-7	> 1.00E-4
HOP-62	0.538	1.506	1.231	1.024	0.690	0.709	0.689	72	50	16	18	16	1.01E-7	> 1.00E-4	> 1.00E-4
HOP-92	1.253	1.576	1.346	1.032	1.010	1.096	0.589	29	-18	-19	-13	-53	< 1.00E-8	4.16E-8	8.41E-5
NCI-H226	0.671	1.642	1.499	1.286	0.705	0.598	0.571	85	63	4	-11	-15	1.67E-7	1.75E-6	> 1.00E-4
NCI-H23	0.657	2.289	2.113	1.554	0.734	0.659	0.676	89	55	5	.	1	1.26E-7	> 1.00E-4	> 1.00E-4
NCI-H322M	0.772	1.909	1.605	0.564	0.263	0.442	0.304	73	-27	-66	-43	-61	1.71E-8	5.38E-8	.
NCI-H460	0.287	2.054	2.112	1.722	0.453	0.298	0.273	103	81	9	1	-5	2.72E-7	1.27E-5	> 1.00E-4
NCI-H522	0.671	2.179	2.041	1.758	0.697	0.682	0.621	91	72	2	1	-8	2.06E-7	1.23E-5	> 1.00E-4
Colon Cancer															
COLO 205	0.463	1.550	1.448	0.957	0.119	0.469	0.449	91	45	-74	1	-3	7.93E-8	.	.
HCC-2998	1.104	3.223	3.042	2.268	0.435	0.567	0.607	91	55	-61	-49	-45	1.10E-7	2.99E-7	.
HCT-116	0.210	1.753	1.463	0.973	0.150	0.167	0.208	81	49	-29	-20	-1	9.61E-8	4.28E-7	> 1.00E-4
HCT-15	0.231	1.666	1.409	0.820	0.180	0.160	0.239	82	41	-22	-31	1	6.05E-8	.	> 1.00E-4
HT29	0.170	1.108	1.014	0.553	0.187	0.176	0.150	90	41	2	1	-12	6.50E-8	1.11E-5	> 1.00E-4
KM12	0.370	1.975	1.965	1.277	0.375	0.457	0.365	99	57	.	5	-1	1.31E-7	6.09E-5	> 1.00E-4
SW-620	0.325	1.388	1.237	1.137	0.428	0.368	0.204	86	76	10	4	-37	2.49E-7	1.25E-5	> 1.00E-4
CNS Cancer															
SF-268	0.423	1.384	1.129	0.914	0.347	0.329	0.274	73	51	-18	-22	-35	1.04E-7	5.48E-7	> 1.00E-4
SF-295	0.629	2.289	1.746	1.100	0.708	0.601	0.663	67	28	5	-5	2	2.78E-8	.	> 1.00E-4
SF-539	0.787	2.512	2.079	1.160	0.662	0.711	0.444	75	22	-16	-10	-44	2.93E-8	3.76E-7	> 1.00E-4
SNB-19	0.523	1.251	1.012	0.736	0.416	0.527	0.692	67	29	-21	.	23	2.84E-8	.	> 1.00E-4
SNB-75	0.831	1.524	1.076	0.963	0.845	0.886	0.789	35	19	2	8	-5	< 1.00E-8	4.06E-5	> 1.00E-4
U251	0.376	1.940	1.576	1.035	0.332	0.331	0.137	77	42	-12	-12	-64	5.92E-8	6.06E-7	5.45E-5
Melanoma															
LOX IMVI	0.449	2.717	2.103	0.668	0.279	0.370	0.423	73	10	-38	-18	-6	2.30E-8	1.59E-7	> 1.00E-4
M14	0.552	2.023	1.598	1.108	0.596	0.704	0.594	71	38	3	10	3	4.29E-8	> 1.00E-4	> 1.00E-4
MDA-MB-435	0.446	1.813	1.514	1.045	0.465	0.523	0.399	78	44	1	6	-11	6.59E-8	2.22E-5	> 1.00E-4
SK-MEL-2	0.832	2.306	2.137	1.750	0.726	0.697	0.502	88	62	-13	-16	-40	1.46E-7	6.75E-7	> 1.00E-4
SK-MEL-28	0.830	2.185	1.672	1.267	0.711	0.695	0.502	62	32	-14	-16	-40	2.54E-8	4.92E-7	> 1.00E-4
SK-MEL-5	0.664	2.733	2.341	0.876	0.082	0.260	0.094	81	10	-88	-61	-86	2.74E-8	1.27E-7	4.12E-7
UACC-257	1.031	2.442	2.014	1.682	0.918	0.822	0.747	70	46	-11	-20	-28	6.85E-8	6.42E-7	> 1.00E-4
UACC-62	0.885	2.648	2.053	2.238	0.702	0.679	0.600	66	77	-21	-23	-32	1.88E-7	6.13E-7	> 1.00E-4
Ovarian Cancer															
IGROV1	0.586	1.867	1.267	0.574	0.416	0.428	0.390	53	-2	-29	-27	-33	1.14E-8	9.15E-8	> 1.00E-4
OVCAR-3	0.650	1.898	1.314	0.762	0.329	0.511	0.293	53	9	-49	-21	-55	1.18E-8	1.42E-7	7.10E-5
OVCAR-4	0.702	1.397	1.327	1.201	0.893	0.771	0.705	90	72	27	10	.	3.10E-7	> 1.00E-4	> 1.00E-4
OVCAR-8	0.422	1.979	1.824	1.453	0.753	0.640	0.598	90	66	21	14	11	2.29E-7	> 1.00E-4	> 1.00E-4
NCI/ADR-RES	0.567	1.871	1.731	1.416	0.645	0.564	0.619	89	65	6	-1	4	1.80E-7	.	> 1.00E-4
SK-OV-3	0.766	1.584	1.334	1.106	0.698	0.748	0.650	69	42	-9	-2	-15	4.98E-8	6.67E-7	> 1.00E-4
Renal Cancer															
786-0	0.964	2.820	1.970	2.294	1.336	1.351	0.774	54	72	20	21	-20	2.63E-7	3.27E-5	> 1.00E-4
A498	1.357	1.742	1.605	0.831	0.774	0.932	0.758	64	-39	-43	-31	-44	1.38E-8	4.21E-8	> 1.00E-4
ACHN	0.528	2.155	1.840	1.367	0.875	0.798	0.740	81	52	21	17	13	1.13E-7	> 1.00E-4	> 1.00E-4
CAKI-1	0.473	2.045	1.474	0.735	0.503	0.471	0.534	64	17	2	.	4	1.95E-8	.	> 1.00E-4
RXF 393	0.594	1.182	0.958	0.626	0.333	0.359	0.054	62	5	-44	-40	-91	1.62E-8	1.28E-7	1.59E-5
SN12C	0.702	2.406	2.031	1.502	0.937	0.976	0.836	78	47	14	16	8	7.96E-8	> 1.00E-4	> 1.00E-4
TK-10	0.922	2.195	1.896	1.472	0.877	0.710	0.501	76	43	-5	-23	-46	6.23E-8	7.91E-7	> 1.00E-4
UO-31	0.641	1.896	1.326	0.873	0.527	0.562	0.494	55	18	-18	-12	-23	1.34E-8	3.22E-7	> 1.00E-4
Prostate Cancer															
PC-3	0.718	1.967	1.494	1.431	0.659	0.690	0.623	62	57	-8	-4	-13	1.28E-7	7.48E-7	> 1.00E-4
DU-145	0.444	1.501	1.354	0.880	0.502	0.499	0.293	86	41	5	5	-34	6.38E-8	1.36E-5	> 1.00E-4
Breast Cancer															
MCF7	0.310	1.731	1.177	0.565	0.374	0.292	0.363	61	18	5	-6	4	1.80E-8	.	> 1.00E-4
MDA-MB-231/ATCC	0.653	1.437	1.241	0.803	0.583	0.655	0.657	75	19	-11	.	.	2.80E-8	.	> 1.00E-4
HS 578T	1.127	1.924	1.419	1.238	1.088	1.157	1.062	37	14	-4	4	-6	< 1.00E-8	.	> 1.00E-4
BT-549	0.977	2.183	1.659	1.445	0.548	0.671	0.570	57	39	-44	-31	-42	2.34E-8	2.94E-7	> 1.00E-4
T-47D	0.533	1.021	0.817	0.584	0.552	0.539	0.441	58	10	4	1	-17	1.48E-8	1.15E-5	> 1.00E-4
MDA-MB-468	0.768	1.665	1.294	0.751	0.281	0.387	0.455	59	-2	-63	-50	-41	1.39E-8	9.20E-8	.

Figure 3 National Cancer Institute developmental therapeutics program *in-vitro* testing results of compound **6b** at five dose level in μM.

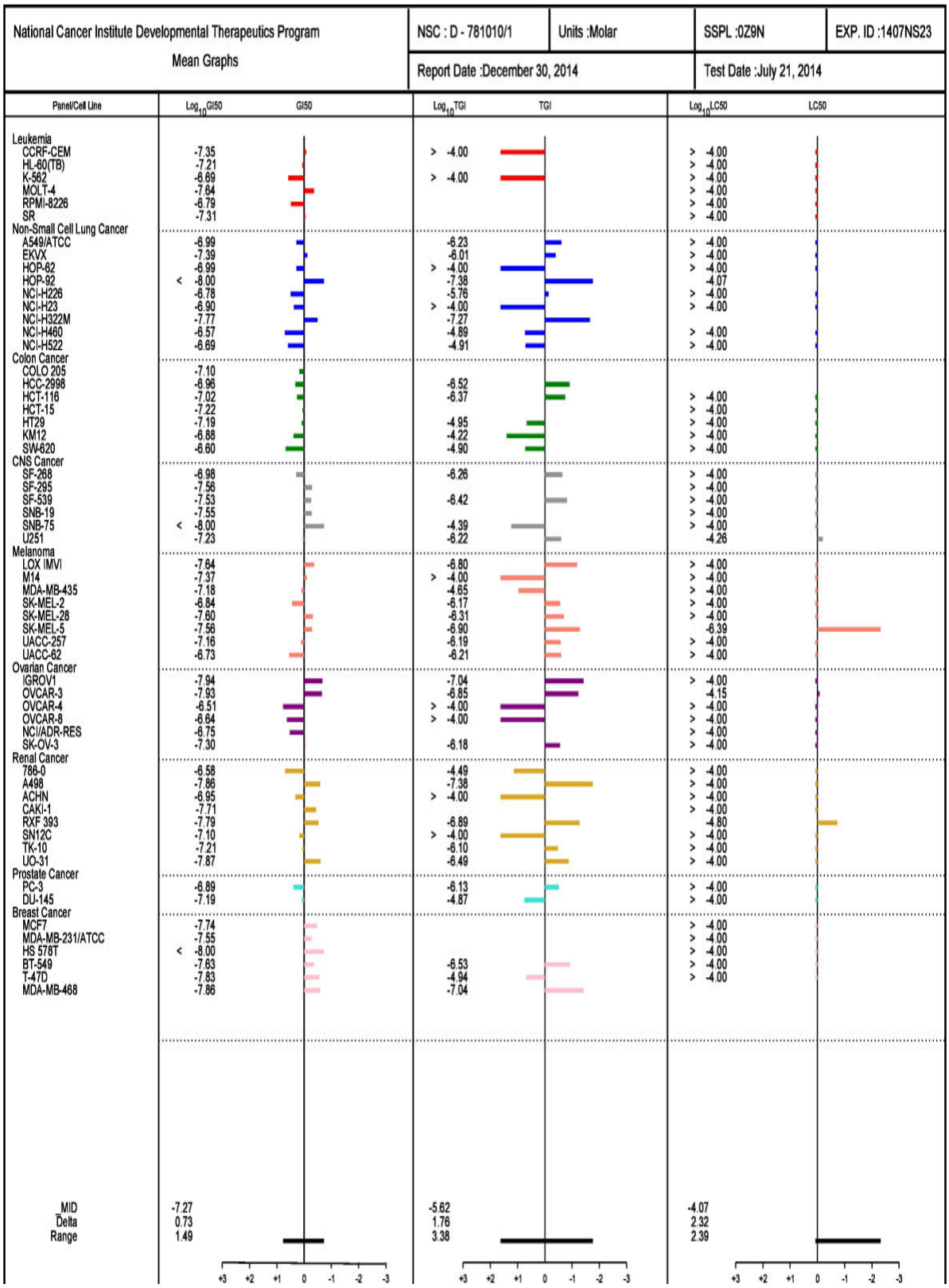


Figure 4 National Cancer Institute developmental therapeutics program *in-vitro* testing results of compound **6b** at five dose level in µM.

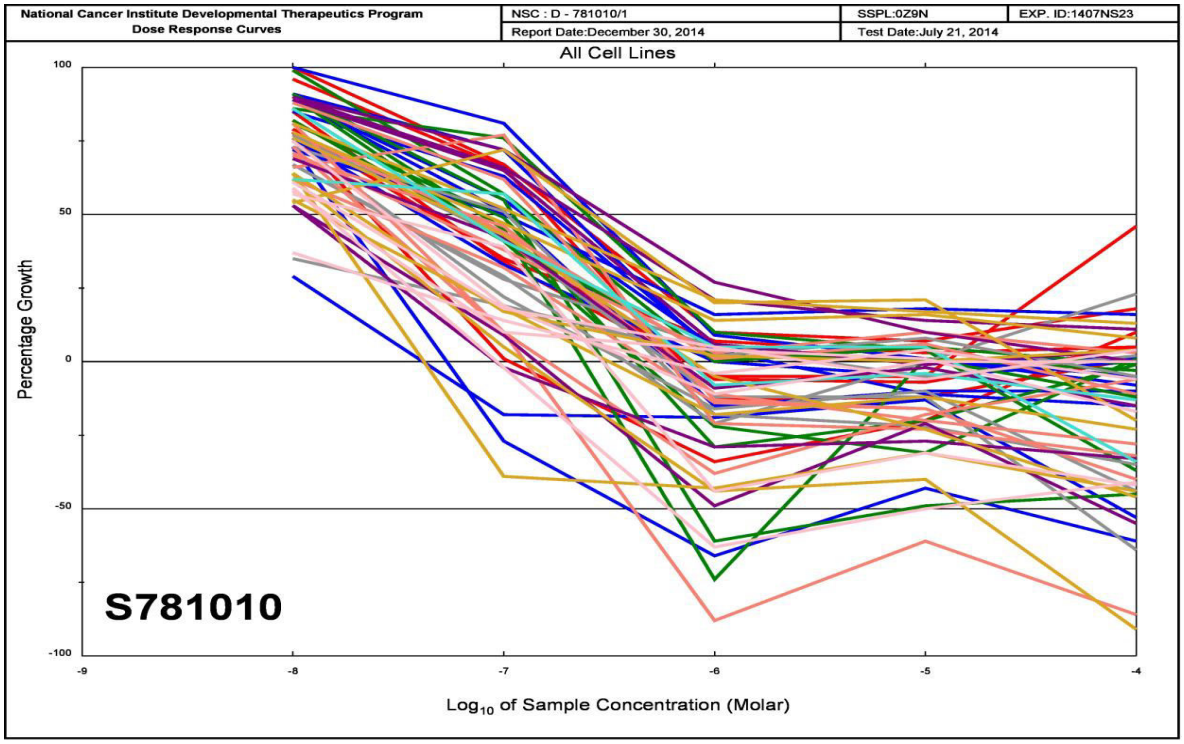


Figure 5 Five dose assay graph of compound **6b** against nine panel cancer cell line**.**

3.3.3. MTT assay for determination of cytotoxicity

Cytotoxicity of compound **6b** in normal human embryonic kidney cell line (Hek293) was performed by means of a colorimetric assay (MTT assay). The results showed that there was only 12%, 8.5%, 8%, 6.5% and 6% cytotoxicity of compound **6b** to human normal cell line Hek293 cells at 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M concentrations, respectively (Figure 6). Compound **6b** showed only 12% toxicity to Hek293 cells even at 100 µM. These results indicated that compound **6b** showed potent anticancer activity and low toxicity to normal cells.

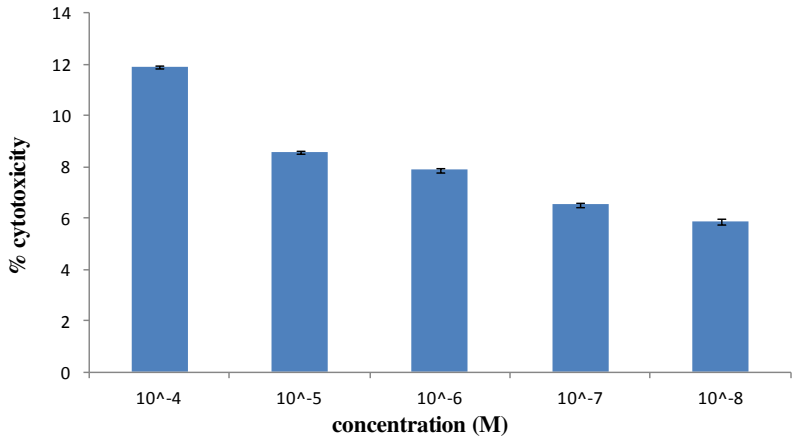


Figure 6 Effect of compound **6b** on Hek 293 cells

3.3.4. Lactate dehydrogenase (LDH) release studies

Lactate dehydrogenase (LDH) leakage is an indicator of cell membrane integrity.[23] When the cell membrane is disrupted, the membrane-bound LDH leaks into the medium and hence, LDH leakage is considered a hallmark of cytotoxicity. Cell death leads to a collapse in membrane integrity, thereby releasing LDH into the medium.

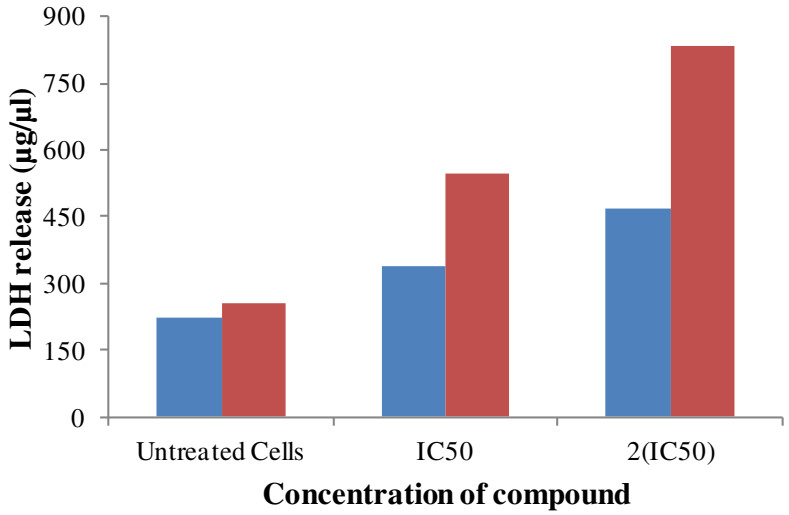


Figure 7 LDH release of compound **6b** at one and two doses after 12 h (blue bar) and 24 h (red bar).

In the present study, a significant level of LDH leakage was observed in the cell culture medium of the A549 cell line (human lung carcinoma) at one dose (IC_{50}) and two doses ($2xIC_{50}$), when they were treated with the respective IC_{50} concentrations of the compound for a period of 12 h and 24 h (Figure 7). It has been observed that compound **6b** exhibited significant membrane-damaging effects and showed selectivity towards cancer cells while exhibiting minimal/no toxicity towards normal cells.

3.3.5. DNA binding studies

Various naphthalimide derivatives have shown DNA-binding and exert potential anti cancer activity. DNA is one of the most promising biological target in treatment of cancer. The compounds capable of DNA-binding have attracted the researchers on the basis of their DNA-intercalating properties. Efforts have been made in order to increase the DNA recognizing ability of these compounds. Keeping in mind its importance, we have also performed DNA binding studies of the most active compound **6b** using both UV-Visible and fluorescence spectrophotometer in order to determine the interaction of compound with ct-

DNA.[24] The addition of ct-DNA (4-100 µM) to the phosphate buffered solution of **6b** (20 µM), caused decrease in absorption intensity at 405 nm (Figure 8).

Fluorescence titrations were also performed with ct-DNA in phosphate buffered solution of compound **6b**. The incremental addition of ct-DNA caused emission enhancement with concomitant blue shift of emission band from 555 nm to 520 nm. The apparent binding constant of **6b** with ct- DNA was determined to be log β = 5.03 calculated from titration data using Benesi-Hildebrand method which is comparable with amonafide having log β = 5.15.

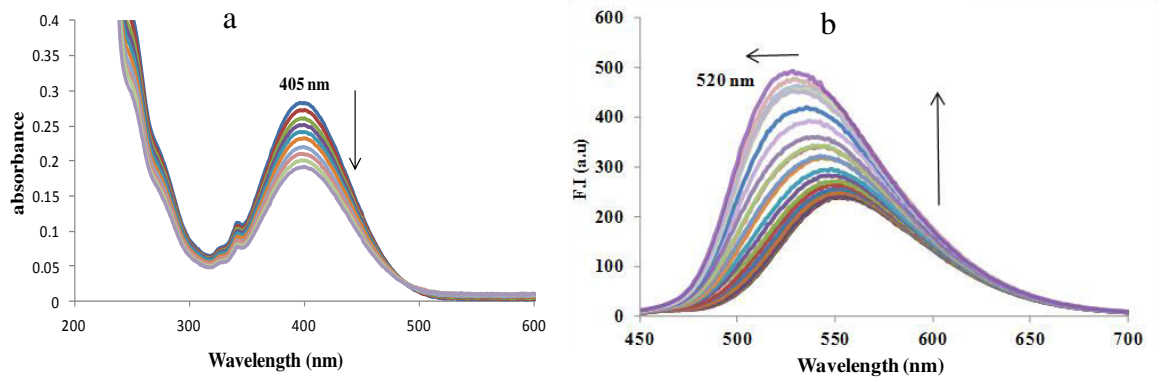


Figure 8 Effect of addition of ct-DNA (4-100 µM) on (a) absorption (b) emission spectra of compound **6b** (20 µM, pH 7.0) in phosphate buffer

The intercalative nature of compound **6b** with ct- DNA has been additionally supported by thermal denaturation experiment. Intercalation of molecules into the double helix is known to stabilize the DNA against thermal strand separation and thus increases thermal melting temperature (T_m).[25] The derivative melting curve shows an increase of 18.2 ^oC in thermal melting temperature of ct-DNA (60 ^oC) upon addition of **6b** (78.2 ^oC). Thus, both UV-Visible and fluorescence titrations as well as thermal denaturation experiments indicated intercalative nature of compound **6b** with ct-DNA.

3.3.6. Ethidium bromide (EtBr) displacement study

In order to prove the reversibility of **6b**:DNA complex, fluorescence quenching experiments of ethidium bromide:DNA were carried out by adding 0–20 µM of the compound to samples containing 10 µM EtBr, 10 µM DNA and a phosphate (pH = 7). Before the measurements, the system was shaken and incubated at room temperature for ~5 min. The emission was recorded at 100–750 nm. On addition of 100 µM solution of ethidium bromide to compound **6b:**DNA complex, the emission band at 520 nm disappeared with reappearance of the fluorescence maxima at 610 nm typically due to EtBr:DNA complex. It confirmed the reversible binding of ligand to DNA (Figure 9).

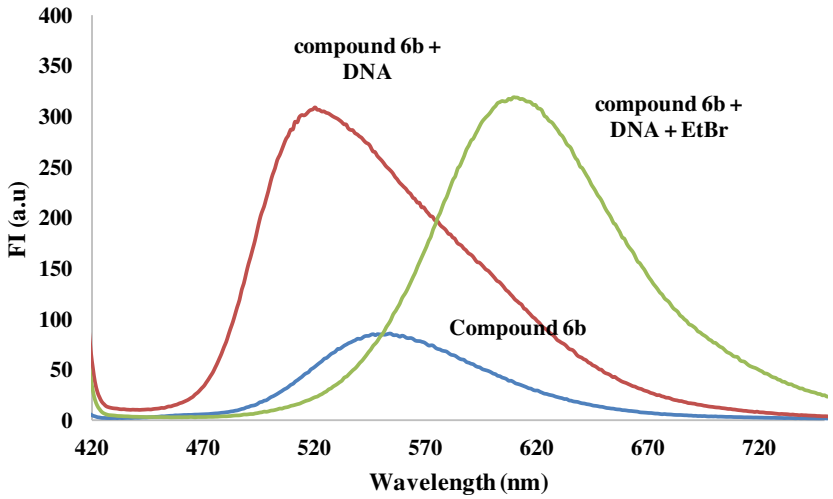


Figure 9 EtBr indicator displacement assay to show reversibility of compound **6b**:DNA binding

Competitive ethidium bromide binding studies were carried out in order to examine the binding mode of each complex with ct-DNA. The emission spectra of EtBr bound to ct-DNA in the absence and presence of each complex have been recorded at [EtBr] = 10 μM, [DNA] = 10 μM. The emission intensities of EtBr bound to ct-DNA at 610 nm enhanced indicating that they cannot effectively displace EtBr from the EtBr:DNA complex (Figure 10). This observation is often considered to be due to that they can bind weakly to the DNA, probably by electrostatic interactions. It is generally agreed that strong fluorescence enhancement accompanies intercalation of the dye into the double helix conformation of the nucleic acid, but there is also evidence for additional non-intercalative, less fluorescence-enhanced sites, which are presumed to involve electrostatic binding.[26]

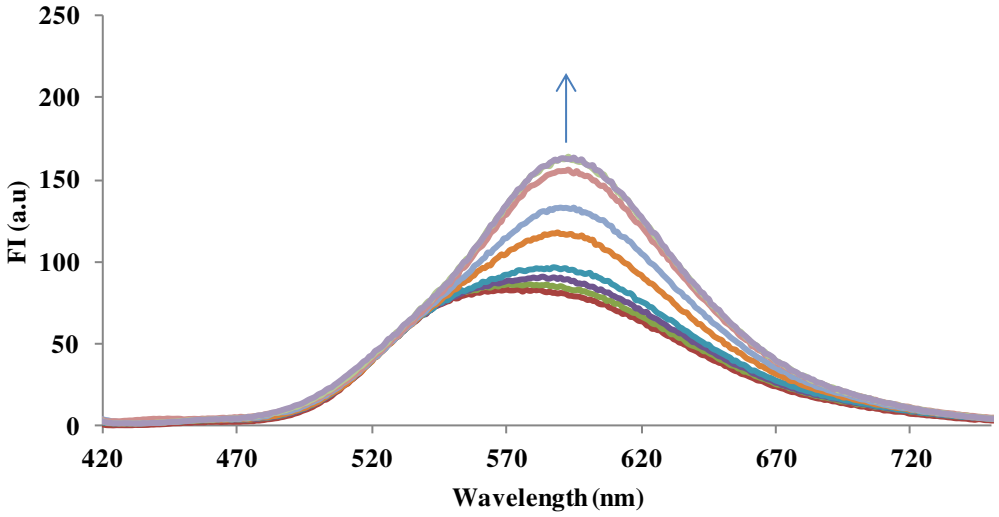


Figure 10 Effect of incremental addition of compound **6b** on EtBr:DNA complex

3.4. ADME prediction

In order to determine properties and the drug-like characteristics of compounds **6b-c**, **6f-i** and **8-9**, we carried out the calculation with Molinspiration software of the lipophilicity (expressed as the octanol/water partition coefficient and herein called log P), and the theoretical prediction of other ADME properties (molecular weight, TPSA, number of hydrogen donors and acceptors, and volume) for Lipinski's rule of five. Drug likeness seems to be a promising standard for the properties of a molecule which influences its pharmacodynamics and pharmacokinetics.[27] This rule is based upon the prediction that if a molecule have molecular weight ≤ 500, log P ≤ 5, ≤ 5 hydrogen bond donor sites and ≤ 10 hydrogen bond acceptor sites (N or O atoms), only then it can be accepted as a member of biological active drug family.[28]

Not considering this criteria may lead the poor absorption or permeation of the drug like molecule. Compounds selected for *in vitro* anticancer studies showed good physicochemical properties having no violation with any of the parameters. These results are concluded in table 5. A comparison among the values leave us with the result that higher activity of compound **6b** may be described on the basis of lower value of log P factor, which is an estimate of compound's overall lipophilicity (supported by low lipophilicity of amonafide). This parameter might be influence the factors like solubility and permeability through biological membrane.

Table 5 Structural properties of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione **6b-c**, **6f-i** and **8-9**, and the reference compounds.

Compds	m.wt.	LogP	TPSA	nON	nOHNH	nViolations	Volume
6b	**322.36**	**2.60**	**51.54**	**5**	**0**	**0**	**294.65**
6c	306.36	3.15	42.31	4	0	0	285.67
6f	345.35	3.47	84.22	6	2	0	300.15
6g	408.25	4.72	63.99	5	1	0	310.01
6h	296.32	1.87	71.33	5	2	0	266.50
6i	292.33	3.15	51.10	4	1	0	275.04
8	253.25	1.89	51.60	4	0	0	216.51
9	332.15	2.65	51.60	4	0	0	234.40

Amonafide	283.33	1.11	68.33	5	2	0	258.74
Naphthalimide analogue[10]	393.47	4.90	60.40	4	2	0	333.61

3.5. Structure-activity relationship

The structure-activity relationship emerged from the anti-tumour activity results clearly revealed that substitution of morpholine at C6 position, exceptionally showed very good activity in comparison to all other substituted amines. Considering all other amines we can conclude that:

1. Secondary cyclic amines influence the anti-tumour activity more effectively than the primary amines.
2. Among the secondary amines, morpholine group at C6 position of naphthalimide ring (**6b**) lead to broad spectrum of antitumour activity than the other secondary amines, piperidine and pyrrolidine.
3. Out of primary amines, presence of aromatic amines slightly improved the activity (mean values of growth percent 90.13 % and 91.39% for **6f** and **6g** respectively) as compared to aliphatic amine (mean value of growth percent 97.19% for **6h**).
4. Unsaturated primary amine showed intermediate activity when compared to primary aromatic and aliphatic amines (mean growth percent 94.15% for **6i**).
5. In case of epoxy compounds (**8**-**9**), compound **8** (mean value of growth percent 94.02%) proved to be more active than compound **9** (mean value of growth percent 100%). Thus, unsubstitution at C6 position as in case of compound **8** showed higher activity than bromine substitution (**9**).
6. Compound **8** showed activitiy comparable to that of compound **6c** with pyrrolidine at C6 position of naphthalimide, which may be explained on the basis of presence of epoxy ring at N-position in compound **8**. We can consider from the above result that presence of oxygen atom may play a key role in the biological activity of the compounds.
7. Thus, allyl group at N2 position and morpholine at C6 position required for the antitumor activity of the compound.
8. MTT assay and LDH release studies revealed that compound **6b** showed potent anticancer activity and is toxic for cancer cells and low toxicity to normal cells.

9. UV-Vis and fluorescence studies have revealed that compound **6b** strongly interacts with DNA. Also, ethidium bromide displacement studies confirmed the reversible binding of **6b** to DNA.
10. Also compound **6b** also followed Lipinski's rule of five and hence stands in the class of drug like molecules. It showed lower value of log P (lipophilicity factor) which counted for its higher activity.

3.6. Molecular modelling studies

In order to predict the binding mode of the most active compound **6b** of this series, molecular modelling studies were carried out with topoisomerase I and topoisomerase II as well as DNA.

3.6.1. Molecular modelling studies of 6b with topoisomerase I and II

Molecular docking provides useful information about drug receptor interactions and is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to predict the affinity and activity of the drug. To explore the most feasible binding site, interaction mode and binding affinity, docking studies have been performed on compound **6b** with topoisomerase I (Topo I, pdb ID 1A36) and topoisomerase II (Topo II, pdb ID 1BJT, enzymes responsible for DNA replication).[29-30] Carbonyl oxygen of compound **6b** in the active sites of topoisomerase I showed hydrogen bonding with amino groups of L658 (d = 1.99 Å) and V691 (d = 2.99 Å) amino acid residues. Morpholine

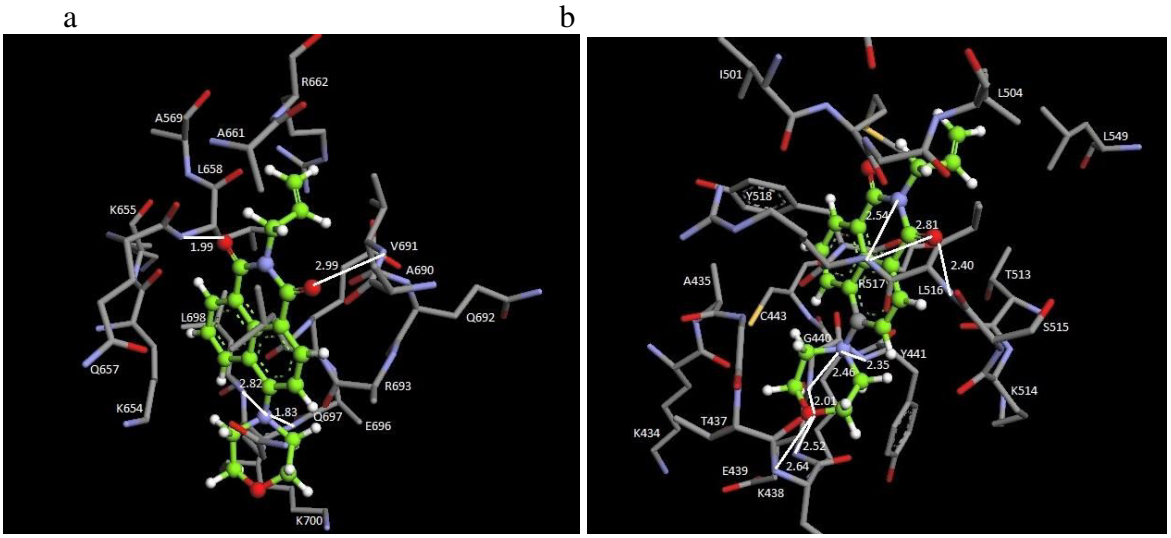


Figure 11 Compound **6b** docked in active sites of (a) topoisomerase I (Topo I, pdb ID 1A36) and (b) topoisomerase II (Topo II, pdb ID 1BJT)

nitrogen atom also showed hydrogen bonding with amino groups of Q697 (*d* = 1.83 Å) and L698 (*d* = 2.82 Å) amino acid residues. This molecule is surrounded with K655, E696, K700 and R693 amino acid residues (Figure 11a). Compound **6b** also fits in the active sites of topoisomerase II, showed hydrogen bonding between carbonyl group of naphthalimide with amino group of R517 (*d* = 2.81 Å) and L516 (*d* = 2.40 Å) amino acid residue (Figure 11b). Cyclopentadiene amide nitrogen atom showed hydrogen bonding with R517 (*d* = 2.54 Å) amino acid residue. Morpholine nitrogen atom of compound **6b** showed hydrogen bonding with amino groups of Y441 (*d* = 2.35 Å) and G440 (*d* = 2.46 Å) amino acid residues. Morpholine oxygen atom also showed hydrogen bonding with amino groups of G440 (*d* = 2.01 Å), K438 (*d* = 2.64 Å) and E439 (*d* = 2.52 Å) amino acid residues. Therefore, docking of compound **6b** in the active site of these enzymes indicated the probable mode of action for anticancer activities (Table 6).

Table 6 Hydrogen bonding of compound **6b** with topoisomerase I (1A36) and topoisomerase II (1BJT)

Groups	1A36	1BJT
	Amino acid residue	Amino acid residue
Carbonyl oxygen	L658 (d = 1.99 Å)	R517 (d = 2.81 Å)
	V691 (d = 2.99 Å)	L516 (d = 2.40 Å)
Cyclopentadiene amide nitrogen atom		R517 (d = 2.54 Å)
Morpholine nitrogen atom	Q697 (d = 1.83 Å)	Y441(d = 2.35 Å)
	L698 (d = 2.82 Å)	G440(d = 2.46 Å)
Morpholine oxygen atom		G440(d = 2.01 Å)
		K438 (d = 2.64 Å)
		E439 (d = 2.52 Å)

3.6.2. Molecular modelling studies of 6b with DNA (PDB ID: 1BNA)

To explore most feasible binding affinity with DNA, docking studies have also been performed on compound **6b** with DNA (PDB ID: 1BNA). Most stable binding conformation of **6b** and DNA revealed that it is stacked in the major groove via electrostatic interactions.

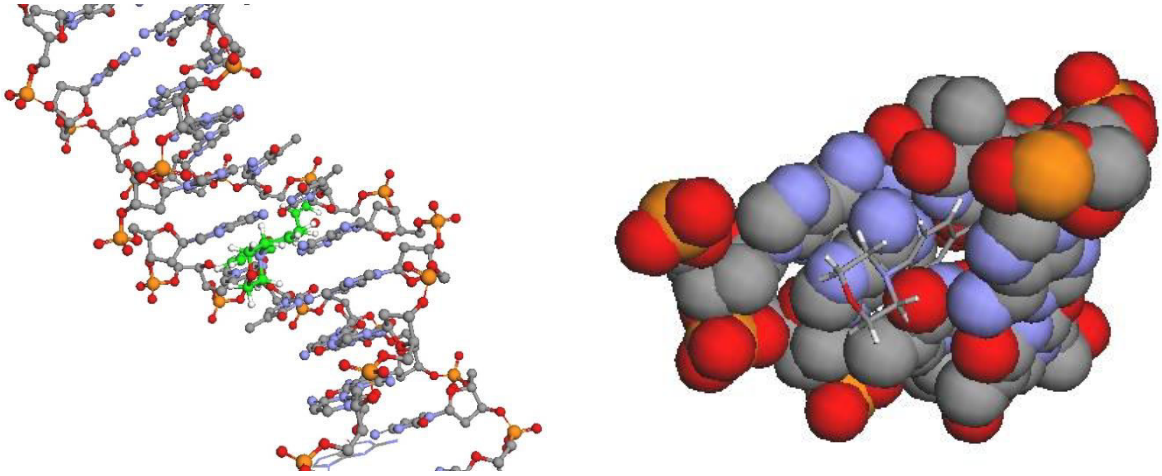


Figure 12 Molecular docked model of compound **6b** with DNA (pdb ID 1BNA)

The planarity of naphthalimide core is comfortable for strong л-л stacking interactions and fits inside the DNA strands by van der Waals interaction and hydrophobic contacts (Figure 12). These results are consistent with UV-Vis and fluorescence studies. CPK model of ligand and DNA explored that compound 6d was completely fit into the DNA pocket.

3.7. Conclusion

The present work has led to the development of novel *N*-allyl naphthalimide analogues with different substitution of primary and secondary amines at C6 position. These compounds were well characterized by ^{1}H and ^{13}C NMR as well as mass spectrometery. These compounds were evaluated towards 60 human tumor cell lines for their *in vitro* activities and some of which shown promising antitumor activities. Compound **6b** showed more active in most of the cancer cell lines at 10 μM concentration and showed broad spectrum of antitumor activity with mean value of -7.37 and MG MID GI_{50} value of 84 nM at five dose concentration levels. Compound **6b** showed almost one forty seven fold more activity than oxaliplatin, a known marketing drug. MTT assay and LDH release studies revealed that compound **6b** showed potent anticancer activity and is highly toxic to cancer cells and low toxicity to normal cells. Compound **6b** also showed strong intercalating properties with ct-DNA determined by UV-Visible and fluorescence spectroscopy as well as thermal denaturation experiment. In order to prove the reversibility of **6b**:DNA complex, fluorescence quenching experiments of ethidium bromide:DNA were carried out which confirmed the reversible binding of ligand to DNA. Molecular modelling studies indicated considerable interactions of these compounds in the active site of topoisomerase I (Topo I,

pdb ID 1A36) and topoisomerase II (Topo II, pdb ID 1BJT) as well as DNA (pdb ID 1BNA), that also favour the interaction of synthesized compounds. These preliminary encouraging results of biological screening could offer an excellent framework in synthetic and medicinal chemistry.

3.8. Experimental section

3.8.1. Chemistry

Melting points were determined in open capillaries and were uncorrected. Reactions were monitored by thin layer chromatography (TLC) with silica plate coated with silica gel GF 254. To prepare TLC plates, slurry of silica gel GF 254 was prepared with help of $CHCl_3$ and MeOH (4:1). TLC plates were dipped in the slurry, dried in an oven and used for comparing the reaction mixture and starting materials to check the progress of reaction. TLC plates were checked under UV lamp or were developed in iodine chambers. Column chromatography was performed with silica gel 60-120/100-200 mesh. Hexane/ ethyl acetate and chloroform/ methanol were adopted solvent systems. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 400 MHz and Jeol ECS 400 NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvents. The chemical shifts were expressed in parts per million with TMS as internal reference and coupling constant (*J*) values are given in Hz. Spectral patterns are denoted as s = singlet; d = doublet; t = triplet; bs = broad singlet; dd = double doublet; dt = double triplet; q = quartet; m = multiplet. Mass Spectra of the synthesized compounds were recorded at Waters Micromass Q-Tof Micro. UV-Visible spectra were recorded using Champion UV/Vis spectrometer. Fluorescence measurements were performed on a Varian Cary Eclipse fluorescence spectrometer. Thermal denaturation experiments were performed on Shimadzu UV-2450.

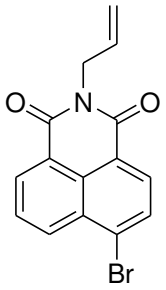
Materials: Acenaphthene, potassium hydroxide, potassium carbonate, tetrabutyl ammonium hydrogen sulphate, acetic acid and ammonium hydroxide were purchased from loba chemie. Sodium dichromate and bromine were purchased from spectrochemicals. Tetrahydrofuran was of HPLC grade. Primary and secondary amines were purchased from sigma-Aldrich, loba chemie and spectrochemicals. *m*-CPBA was purchased from Avra synthesis Pvt. Ltd. Solvents like acetonitrile, chloroform, methanol, hexane and ethyl acetate were of LR grade and used after distillation.

3.8.1.1. General procedure for synthesis of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (4)

Sodium dichromate (14.50 g, 55.34 mmol) was added to a solution of acenaphthene (3 g, 19.7 mmol) and acetic acid (60 ml) with continue stirring at room temperature. This suspension was then heated to 75 ^{o}C for 8 h. Reaction was monitored with TLC. On complete reaction, cold water was added to the reaction mixture, resulted in precipitation. After filtration and washing with water, off white solid of 1,8-naphthalic anhydride (3.09 g, 80%, m.p. 266-268 ^{o}C) **2** was collected and vacuum dried. A solution of 1,8-naphthalic anhydride (2.57 g, 12.9 mmol) and hot KOH (3.5 g in 15 ml water) was prepared and cooled to room temperature. To this solution, bromine was added drop-wise with vigorous stirring. After complete addition, reaction mixture was heated to 60 ^{o}C for 6 h. The resulted solution was acidified with HCl. Brown coloured solid was separated out and filtered. This solid residue was further heated with 60 ml of 5% NaOH solution, filtered and treated with HCl solution till complete neutralization. Yellow precipitates were separated out, filtered and washed with cold water, dried to get, off white solid of 6-bromo-benzo[*de*]isochromene-1,3-dione (**3**) (2.92 g, 82%, m.p. 117-119 ^{o}C). 6-Bromo-benzo[*de*]isochromene-1,3-dione (3.00 g, 10.8 mmol) was then reacted with allylamine (0.62 g, 10.8 mmol) in ethanol at reflux temperature for 8 h. After the completion of reaction (TLC), cooled the reaction mixture to get crude solid, filtered and washed with ethanol to afford pure white compound of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (2.4 g, 80%, m.p. 128-130 ^{o}C) (**4**).

An alternative method for synthesizing 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione has also been followed where 6-bromo-benzo[*de*]isochromene-1,3-dione (**3**) (2.00 g, 7.20 mmol) was refluxed with ammonium hydroxide (21.6 mmol) and ethanol. The reaction mixture was then cooled and solid product was filtered to get 6-bromo-benzo[*de*]isoquinoline-1,3-dione (**5**) (m.p. 134-136 ^{o}C). **5** (7.25 mmol) was further refluxed with allylbromide (7.50 mmol) in presence of EtOH to yield crude product, washed with ethanol to get white solid of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (**4**) (1.5 g, 65% overall, m.p. 128-130 ^{o}C).

2-Allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (4)

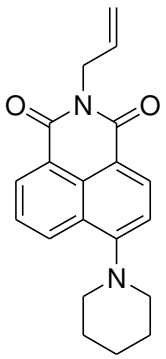


White solid (80 %); m.p. 128-130 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.66 (dd, ^{2}J = 7.32 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 8.56 (dd, ^{2}J = 8.68 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 8.41 (d, J = 7.80 Hz, 1H, ArH), 8.04 (d, J = 7.76 Hz, 1H, ArH), 7.86 (dd, ^{2}J = 8.24 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 6.04-5.94 (m, 1H, CH), 5.35-5.30 (dq, ^{2}J = 17.4 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.24-5.20 (dq, ^{2}J = 10.3 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.80-4.78 (dt, ^{2}J = 5.96 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.3 (C=O), 133.4, 132.2, 131.9, 131.4, 131.1, 130.6, 130.5, 129.0, 128.1, 123.0, 122.1, 117.9 (ArC), 42.6 (N-CH_2); MS (EI) : m/z 316.0 (M^++1). Anal. Calc. for $C_{15}H_{10}BrNO_2$: C, 56.99; H, 3.19; N, 4.43. Found: C, 56.71; H, 3.33; N, 4.59.

3.8.1.2. General procedure for synthesis of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione (6a-n)

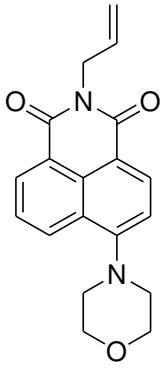
2-Allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (0.20 g, 0.65 mmol) was refluxed with primary and secondary amines (0.80 mmol) in the presence of K_2CO_3 (0.059 g, 1.05 mmol) in CH_3CN using $TBAHSO_4$ as catalyst for 8-12 h. On completion of reaction, solvent was evaporated under vaccum and residue was extracted with water and chloroform. Organic layer was collected, dried over Na_2CO_3 and concentrated to get the crude products of **6a-n**. The crude was purified by column chromatography using hexane:ethylacetate as eluents to afford pure target product of 2-allyl-6-substituted-benzo[*de*]isoquinoline-1,3-dione (**6a-n**).

2-Allyl-6-piperidin-1-yl-benzo[*de*]isoquinoline-1,3-dione (6a)[31]



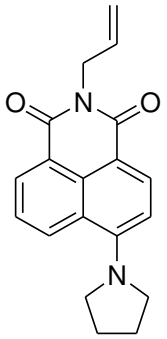
Yellow solid (78 %); m.p. 115-117 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.59 (dd, ^{2}J = 7.36 Hz, ^{3}J = 1.40 Hz, 1H, ArH), 8.51 (d, J = 8.24 Hz, 1H, ArH), 8.40 (dd, ^{2}J = 8.24 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.70 (dd, ^{2}J = 8.72 Hz, ^{3}J = 1.32 Hz, 1H, ArH), 7.19 (d, J = 8.24 Hz, 1H, ArH), 6.03-5.95 (m, 1H, CH), 5.32-5.27 (dq, ^{2}J = 17.2 Hz, ^{3}J = 1.40 Hz, 1H, CH_2), 5.20-5.17 (dq, ^{2}J = 10.32 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.81-4.78 (dt, ^{2}J = 5.52 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2), 3.24 (t, J = 5.04 Hz, 4H, pip-CH_2), 1.91-1.86 (m, 4H, pip-CH_2), 1.75-1.71 (m, 2H, pip-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.3 (C=O), 163.8 (C=O), 157.3, 132.8, 132.4, 131.1, 130.7, 129.9, 126.2, 125.3, 122.9, 117.1, 115.7, 114.6 (ArC), 54.4 (pip-NCH_2), 42.1 (N-CH_2), 26.1 (pip-CH_2), 24.3 (pip-CH_2); MS (EI) : m/z 321.1 (M^++1); Anal. Calc. for $C_{20}H_{20}N_2O_2$: C, 74.98; H, 6.29; N, 8.74. Found: C, 74.79; H, 6.43; N, 8.95.

2-Allyl-6-morpholin-4-yl-benzo[*de*]isoquinoline-1,3-dione (6b)[32]



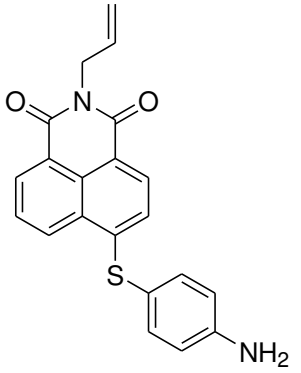
Yellow solid (87 %); m.p. 173-174 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.61 (dd, ^{2}J = 7.32 Hz, ^{3}J = 1.40 Hz, 1H, ArH), 8.55 (d, J = 8.24 Hz, 1H, ArH), 8.44 (dd, ^{2}J = 8.68 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.73 (dd, ^{2}J = 8.24 Hz, ^{3}J = 7.32 Hz, 1H, ArH), 7.27 (t, J = 9.36 Hz, 1H, ArH), 6.04-5.94 (m, 1H, CH), 5.33-5.28 (dq, ^{2}J = 17.42 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.21-5.18 (dq, ^{2}J = 10.08 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.81-4.78 (dt, ^{2}J = 5.96 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2), 4.03 (t, J = 4.12 Hz, 4H, mor-CH_2), 3.27 (t, J = 4.60 Hz, 4H, mor-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.3 (C=O), 160.8 (C=O), 152.9, 129.8, 129.5, 128.5, 127.3, 127.1, 123.3, 123.0, 120.4, 114.5, 114.2, 112.1 (ArC), 64.1 (mor-OCH_2), 50.6 (mor-NCH_2), 39.5 (N-CH_2); MS (EI) : m/z 323.1 (M^++1); Anal. Calc. for $C_{19}H_{18}N_2O_3$: C, 70.79; H, 5.63; N, 8.69. Found: C, 70.55; H, 5.89; N, 8.83.

2-Allyl-6-pyrrolidin-1-yl-benzo[*de*]isoquinoline-1,3-dione (6c)



Yellow solid (76 %); m.p. 155-158 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.59-8.55 (m, 2H, ArH), 8.42 (d, J = 8.68 Hz, 1H, ArH), 7.54 (dd, ^{2}J = 8.68 Hz, ^{3}J = 1.32 Hz, 1H, ArH), 6.80 (d, J = 8.68 Hz, 1H, ArH), 6.05-5.96 (m, 1H, CH), 5.31-5.26 (dq, ^{2}J = 16.96 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.19-5.16 (dq, ^{2}J = 10.54 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.81-4.79 (dt, ^{2}J = 5.48 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2), 3.78 (t, J = 6.44 Hz, 4H, pyrr-CH_2), 2.11-2.08 (m, 4H, pyrr-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4 (C=O), 163.6 (C=O), 152.5, 133.4, 132.6, 131.9, 131.0, 122.8, 122.3, 122.2, 116.8, 110.1, 108.3 (ArC), 53.0 (pyrr-CH_2), 42.0 (N-CH_2), 25.9 (pyrr-CH_2); MS (EI) : m/z 307.1 (M^++1); Anal. Calc. for $C_{19}H_{18}N_2O_2$: C, 74.49; H, 5.92; N, 9.14. Found: C, 74.78; H, 5.77; N, 9.36.

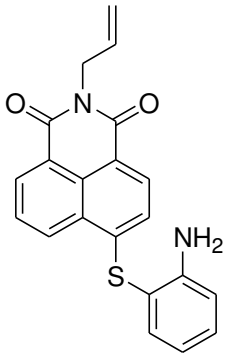
2-Allyl-6-(4-amino-phenylsulfanyl)-benzo[*de*]isoquinoline-1,3-dione (6d)



Brown solid (60 %); m.p. 185-188 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.65-8.61 (m, 2H, ArH), 8.31 (d, J = 8.24 Hz, 1H, ArH), 7.79 (dd, ^{2}J = 8.24 Hz, ^{3}J = 7.32 Hz, 1H, ArH), 7.40 (d, J = 8.72 Hz, 2H, ArH), 7.06 (d, J = 7.76 Hz, 1H, ArH), 6.81 (d, J = 8.68 Hz, 2H, ArH), 6.01-5.95 (m, 1H, CH), 5.32-5.26 (dq, ^{2}J = 17.42 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.21-5.17 (dq, ^{2}J = 10.08 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.79-4.77 (dt, ^{2}J = 5.48 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2),

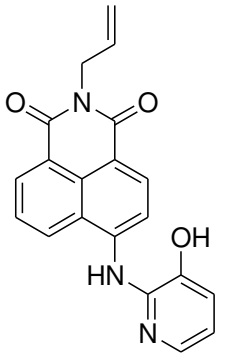
4.01 (bs, 2H, NH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.8 (C=O), 163.7 (C=O), 148.8, 148.3, 137.4, 132.1, 131.6, 131.0, 129.8, 128.3, 126.5, 122.9, 122.8, 118.5, 117.3, 116.3, 115.5 (ArC), 42.3 (N-CH_2); MS (EI) : m/z 361.1 (M^++1); Anal. Calc. for $C_{21}H_{16}N_2O_2S$: C, 69.98; H, 4.47; N, 7.77; S, 8.90. Found: C, 69.83; H, 4.69; N, 7.52; S, 8.73.

2-Allyl-6-(2-amino-phenylsulfanyl)-benzo[*de*]isoquinoline-1,3-dione (6e)



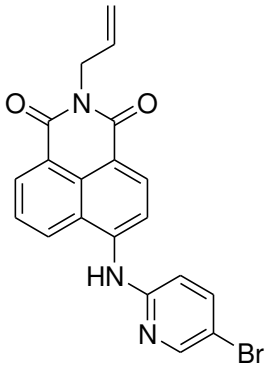
Yellow solid (59 %); m.p. 205-208 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.49 (q, *J* = 8.28 Hz, 2H, ArH), 8.11 (d, *J* = 7.80 Hz, 1H, ArH), 7.72-7.62 (m, 3H, ArH), 7.57 (d, *J* = 8.24 Hz, 1H, ArH), 7.47 (t, *J* = 7.76 Hz, 1H, ArH), 6.82 (d, *J* = 8.24 Hz, 1H, ArH), 5.90-5.82 (m, 1H, CH), 5.24 (d, *J* = 16.96, 1H, CH_2), 5.17 (d, *J* = 10.08 Hz, 1H, CH_2), 4.64 (d, *J* = 5.96 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.4 (C=O), 161.3 (C=O), 148.9, 143.1, 135.7, 130.7, 130.5, 129.7, 129.0, 128.2, 126.7, 126.4, 125.3, 120.8, 120.5, 116.8, 116.0, 115.0, 113.9, 106.6 (ArC), 42.3 (N-CH_2); MS (EI): m/z 361.1 (M^++1); Anal. Calc. for $C_{21}H_{16}N_2O_2S$: C, 69.98; H, 4.47; N, 7.77; S, 8.90. Found: C, 69.79; H, 4.64; N, 7.99; S, 8.65.

2-Allyl-6-(3-hydroxy-pyridin-2-ylamino)-benzo[*de*] isoquinoline-1,3-dione (6f)



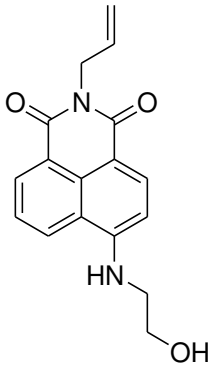
Yellow solid (63 %); m.p. 210-212 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.70 (d, *J* = 8.72 Hz, 1H, ArH), 8.65 (d, *J* = 7.36 Hz, 1H, ArH), 8.48 (dd, ^2J = 8.24 Hz,^3J = 0.92 Hz, 1H, ArH), 8.06 (d, *J* = 5.04 Hz, 1H, ArH), 7.82 (t, *J* = 7.56 Hz, 1H, ArH), 7.29-7.27 (m, 1H, ArH), 6.94 (dd, ^2J = 8.24 Hz, ^3J = 0.92 Hz, 1H, ArH), 6.79-6.75 (m, 1H, ArH), 6.02-5.94 (m, 1H, CH), 5.33-5.29 (dt, ^2J = 16.96 Hz, ^3J = 1.36 Hz, 1H, CH_2), 5.22-5.20 (dt, ^2J = 10.08 Hz, ^3J = 1.40 Hz, 1H, CH_2), 4.80-4.77 (m, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.8 (C=O), 163.2 (C=O), 158.1, 151.8, 145.0, 136.4, 132.9, 132.1, 129.6, 128.4, 128.1, 126.8, 123.5, 122.5, 117.5, 117.1, 114.5, 109.8 (ArC), 42.3 (N-CH_2); MS (EI) : m/z 346.1 (M^++1); Anal. Calc. for $C_{20}H_{15}N_3O_3$: C, 69.56; H, 4.38; N, 12.17. Found: C, 69.33; H, 4.54; N, 12.43.

2-Allyl-6-(5-bromo-pyridin-2-ylamino)-benzo[*de*]isoquinoline -1,3-dione (6g)



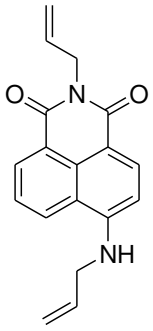
Brown solid (53 %); m.p. 205-207 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.75 (dd, ^{2}J = 8.68 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 8.67 (dd, ^{2}J = 7.32 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 8.45 (d, J = 8.24 Hz, 1H, ArH), 7.79 (dd, ^{2}J = 8.68 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.01 (d, J = 1.96 Hz, 2H, ArH), 6.86 (d, J = 8.24 Hz, 1H, ArH), 6.79 (d, J = 8.68 Hz, 1H, ArH), 6.04-5.95 (m, 1H, CH), 5.33-5.28 (dq, ^{2}J = 17.18 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.22-5.18 (dq, ^{2}J = 10.08 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.81-4.79 (dt, ^{2}J = 5.96 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.2 (C=O), 163.5 (C=O), 161.1, 146.1, 144.2, 133.1, 132.3, 131.9, 129.6, 128.8, 126.2, 123.6, 122.3, 122.0, 117.2, 116.3, 115.6, 109.3 (ArC), 42.2 (N-CH_2); MS (EI) : m/z 345.1 (M^{+}+1); Anal. Calc. for $C_{20}H_{14}BrN_3O_2$: C, 58.84; H, 3.46; N, 10.29. Found: C, 58.49; H, 3.65; N, 10.07.

2-Allyl-6-(2-hydroxy-ethylamino)-benzo[*de*]isoquinoline-1,3-dione (6h)



Brown liquid (57 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.56 (d, J = 7.32 Hz, 1H, ArH), 8.44 (d, J = 8.68 Hz, 1H, ArH), 8.35 (d, J = 8.28 Hz, 1H, ArH), 7.61 (t, J = 8.24 Hz, 1H, ArH), 6.70 (d, J = 8.72 Hz, 1H, ArH), 6.34 (t, J = 4.36 Hz, 1H, NH), 6.01-5.96 (m, 1H, CH), 5.31-5.25 (dq, ^{2}J = 17.4 Hz, ^{3}J = 1.40 Hz, 1H, CH_2), 5.19-5.16 (dq, ^{2}J = 10.3 Hz, ^{3}J = 1.40 Hz, 1H, CH_2), 4.79-4.77 (dt, ^{2}J = 5.48 Hz, ^{3}J = 1.84 Hz, 2H, N-CH_2), 4.21 (bs, 1H, OH), 4.00 (t, J = 4.60 Hz, 2H, CH_2), 3.53 (q, J = 5.04 Hz, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.3 (C=O), 163.8 (C=O), 163.9, 163.2, 158.0, 151.7, 149.4, 144.9, 136.4, 134.4, 132.9, 132.5, 132.1, 131.3, 129.7, 128.4, 128.1, 126.8, 126.1, 124.8, 123.5, 122.8, 122.5, 117.5, 117.2, 117.0, 114.5, 110.4, 109.9, 104.4 (ArC), 60.4 (O-CH_2), 45.2 (N-CH_2), 42.3 (N-CH_2); MS (EI) : m/z 297.0 (M^{+}+1); Anal. Calc. for $C_{17}H_{16}N_2O_3$: C, 68.91; H, 5.44; N, 9.45. Found: C, 68.76; H, 5.79; N, 9.22.

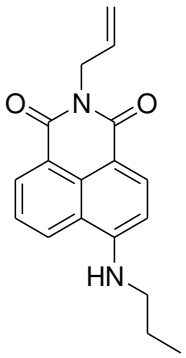
2-Allyl-6-allylamino-benzo[*de*]isoquinoline-1,3-dione (6i)



Brown liquid (50 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.59 (d, J = 7.32 Hz, 1H, ArH), 8.47 (d, J = 8.72 Hz, 1H, ArH), 8.14 (d, J = 8.72 Hz, 1H, ArH), 7.64 (t, J = 8.28 Hz, 1H, ArH), 6.73 (d, J = 8.72 Hz, 1H, ArH), 6.04-5.96 (m, 2H, CH), 5.49 (bs, 1H, NH), 5.41-5.16 (m, 4H, CH_2), 4.80 (dd, ^{2}J = 5.96 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2), 4.09 (t, J = 5.28 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4 (C=O), 163.8 (C=O), 149.0, 134.5,

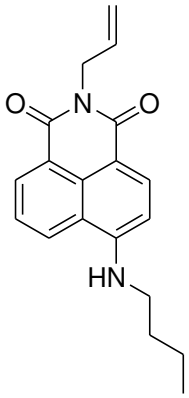
132.9, 132.5, 131.2, 118.0, 116.9, 104.9 (ArC), 46.0 (N-CH_2), 42.1 (N-CH_2); MS (EI) : m/z 293.1 (M^++1); Anal. Calc. for $C_{18}H_{16}N_2O_2$: C, 73.95; H, 5.52; N, 9.58. Found: C, 73.63; H, 5.70; N, 9.34.

2-Allyl-6-propylamino-benzo[*de*]isoquinoline-1,3-dione (6j)



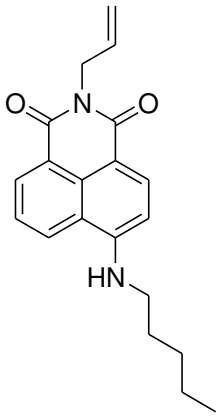
Yellow liquid (80 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (dd, ^2J = 7.32 Hz, ^3J = 0.92 Hz, 1H, ArH), 8.46 (d, J = 8.24 Hz, 1H, ArH), 8.11 (d, J = 7.80 Hz, 1H, ArH), 7.61 (dd, ^2J = 8.28 Hz, ^3J = 0.92 Hz, 1H, ArH), 6.72 (d, J = 8.72 Hz, 1H, ArH), 6.03-5.97 (m, 1H, CH), 5.36 (bs, 1H, NH), 5.31-5.26 (dq, ^2J = 17.16 Hz, ^3J = 1.40 Hz, 1H, CH_2), 5.19-5.16 (dq, ^2J = 10.52 Hz, ^3J = 1.40 Hz, 1H, CH_2), 4.80 (dt, ^2J = 5.48 Hz, ^3J = 1.36 Hz, 2H, N-CH_2), 3.40-3.35 (m, 2H, prop-NCH_2), 1.87-1.81 (m, 2H, prop-CH_2), 1.11 (t, J = 7.36 Hz, 3H, prop-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4 (C=O), 163.8 (C=O), 149.5, 134.6, 132.6, 131.2, 129.8, 125.8, 124.6, 122.9, 120.0, 116.9, 109.9, 104.3 (ArC), 45.4 (N-CH_2), 42.1 (N-CH_2), 22.1 (CH_2), 11.6 (CH_3); MS (EI) : m/z 295.1 (M^++1); Anal. Calc. for $C_{18}H_{18}N_2O_2$: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.72; H, 6.30; N, 9.31.

2-Allyl-6-butylamino-benzo[*de*]isoquinoline-1,3-dione (6k)



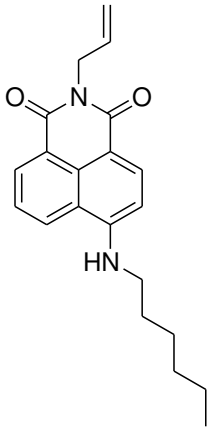
Yellow liquid (62 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (dd, ^2J = 7.32 Hz, ^3J = 0.92 Hz, 1H, ArH), 8.47 (d, J = 8.24 Hz, 1H, ArH), 8.10 (dd, ^2J = 8.24 Hz, ^3J = 0.92 Hz, 1H, ArH), 7.62 (dd, ^2J = 8.72 Hz, ^3J = 1.32 Hz, 1H, ArH), 6.72 (d, J = 8.68 Hz, 1H, ArH), 6.03-5.97 (m, 1H, CH), 5.31-5.26 (dq, ^2J = 17.18 Hz, ^3J = 1.36 Hz, 1H, CH_2), 5.19-5.16 (dq, ^2J = 10.08 Hz, ^3J = 1.40 Hz, 1H, CH_2), 4.80-4.78 (dt, ^2J = 5.96 Hz, ^3J = 1.36 Hz, 2H, N-CH_2), 3.43-3.38 (m, 2H, but-NCH_2), 1.84-1.75 (m, 2H, but-CH_2), 1.56- 1.50 (m, 2H, but-CH_2), 1.04 (t, J = 7.32 Hz, 3H, but-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4 (C=O), 163.8 (C=O), 149.5, 134.6, 132.6, 131.2, 129.8, 125.8, 124.6, 122.9, 120.0, 116.9, 109.9, 104.2 (ArC), 43.3 (but-NCH_2), 42.1 (N-CH_2), 30.9 (but-CH_2), 20.3 (but-CH_2), 13.8 (but-CH_3); MS (EI) : m/z 309.2 (M^++1); Anal. Calc. for $C_{19}H_{20}N_2O_2$: C, 74.00; H, 6.54; N, 9.08. Found: C, 74.33; H, 6.29; N, 9.37.

2-Allyl-6-pentylamino-benzo[*de*]isoquinoline-1,3-dione (6l)



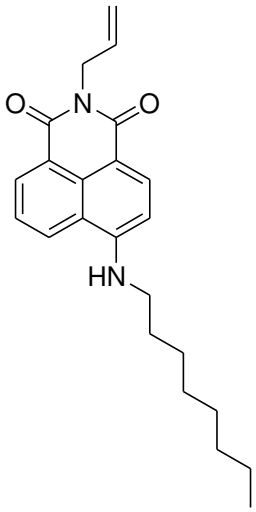
Yellow liquid (59 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.59 (dd, ^{2}J = 7.32 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 8.47 (d, J = 8.24 Hz, 1H, ArH), 8.10 (dd, ^{2}J = 8.24 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 7.63 (dd, ^{2}J = 8.68 Hz, ^{3}J = 7.32 Hz, 1H, ArH), 6.72 (d, J = 8.24 Hz, 1H, ArH), 6.03-5.97 (m, 1H, CH), 5.31-5.26 (dq, ^{2}J = 17.18 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.19-5.16 (dq, ^{2}J = 10.08 Hz, ^{3}J = 1.40 Hz, 1H, CH_2), 4.80-4.78 (dt, ^{2}J = 5.48 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2), 3.42-3.37 (m, 2H, pent-NCH_2), 1.85-1.78 (m, 2H, pent-CH_2), 1.50-1.38 (m, 4H, pent-CH_2), 0.97 (t, J = 7.36 Hz, 3H, pent-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4 (C=O), 163.8 (C=O), 149.5, 134.6, 132.6, 131.2, 129.8, 125.8, 124.6, 122.9, 120.1, 116.9, 109.9, 104.3 (ArC), 43.6 (pent-NCH_2), 42.1 (N-CH_2), 29.2 (pent-CH_2), 28.6 (pent-CH_2), 22.4 (pent-CH_2), 13.9 (pent-CH_3); MS (EI) : m/z 323.2 (M^++1); Anal. Calc. for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.68; H, 6.59; N, 8.57.

2-Allyl-6-hexylamino-benzo[*de*]isoquinoline-1,3-dione (6m)



Yellow liquid (51 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.61 (dd, ^{2}J = 7.76 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 8.49 (d, J = 8.24 Hz, 1H, ArH), 8.09 (d, J = 7.80 Hz, 1H, ArH), 7.65 (dd, ^{2}J = 8.24 Hz, ^{3}J = 1.32 Hz, 1H, ArH), 6.74 (d, J = 8.28 Hz, 1H, ArH), 6.04-5.95 (m, 1H, CH), 5.32-5.26 (dq, ^{2}J = 17.2 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 5.20-5.16 (dq, ^{2}J = 10.08 Hz, ^{3}J = 1.36 Hz, 1H, CH_2), 4.80-4.78 (dt, ^{2}J = 5.52 Hz, ^{3}J = 1.36 Hz, 2H, N-CH_2), 3.43-3.38 (m, 2H, hex-NCH_2), 1.85-1.78 (m, 2H, hex-CH_2), 1.52-1.46 (m, 2H, hex-CH_2), 1.38-1.36 (m, 4H, hex-CH_2), 0.94-0.91 (m, 3H, hex-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4 (C=O), 163.8 (C=O), 149.4, 134.6, 132.6, 131.2, 129.8, 125.8, 124.6, 123.0, 120.1, 116.9, 110.0, 104.3 (ArC), 43.7 (hex-NCH_2), 42.1 (N-CH_2), 31.5 (hex-CH_2), 28.9 (hex-CH_2), 26.8 (hex-CH_2), 22.5 (hex-CH_2), 14.0 (hex-CH_3); MS (EI) : m/z 337.2 (M^++1); Anal. Calc. for $C_{21}H_{24}N_2O_2$: C, 74.97; H, 7.19; N, 8.33. Found: C, 74.67; H, 7.32; N, 8.47.

2-Allyl-6-octylamino-benzo[*de*]isoquinoline-1,3-dione (6n)

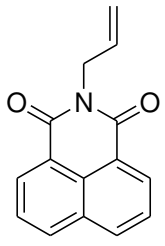


Yellow liquid (49 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.54 (d, *J* = 7.36 Hz, 1H, ArH), 8.43 (d, *J* = 8.24 Hz, 1H, ArH), 8.13 (d, *J* = 8.24 Hz, 1H, ArH), 7.57 (t, *J* = 8.04 Hz, 1H, ArH), 6.68 (d, *J* = 8.72 Hz, 1H, ArH), 6.03-5.96 (m, 1H, CH), 5.53 (t, *J* = 4.8 Hz, 1H, NH), 5.31-5.26 (dq, ^2J = 17.42 Hz, ^3J = 1.36 Hz, 1H, CH_2), 5.19-5.15 (dq, ^2J = 10.08 Hz, ^3J = 1.36 Hz, 1H, CH_2), 4.79 (d, *J* = 5.52 Hz, 2H, N-CH_2), 3.40-3.35 (m, 2H, octyl-NCH_2), 1.83-1.76 (m, 2H, octyl-CH_2), 1.51-1.44 (m, 2H, octyl-CH_3), 1.38-1.25 (m, 8H, octyl-CH_2), 0.89 (t, *J* = 6.88 Hz, 3H, octyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.3 (C=O), 163.7 (C=O), 149.6, 134.5, 132.5, 131.1, 129.7, 126.0, 124.4, 122.7, 120.0, 116.9, 109.6, 104.1 (ArC), 43.6 (octyl-NCH_2), 42.0 (N-CH_2), 31.7 (octyl-CH_2), 29.2 (octyl-CH_2), 29.1 (octyl-CH_2), 28.8 (octyl-CH_2), 27.1 (octyl-CH_2), 22.5 (octyl-CH_2), 14.0 (octyl-CH_3); MS (EI) : m/z 365.2 (M^++1); Anal. Calc. for $C_{23}H_{28}N_2O_2$: C, 75.79; H, 7.74; N, 7.69. Found: C, 75.95; H, 7.89; N, 7.53.

3.8.1.3. Procedure for synthesis of 2-allyl-benzo[*de*]isoquinoline-1,3-dione (7)

To a solution of benzo[*de*]isochromene-1,3-dione (**2**) (1.0 g, 5.04 mmol) in ethanol, allyl amine (5.5 mmol) was added at room temperature and refluxed for 8 h. On completion of reaction, reaction mixture was cooled to room temperature. The solid was separated out, filtered and washed with ethanol to remove any traces of allyl amine from the product and collected the pure product of 2-allyl-benzo[*de*]isoquinoline-1,3-dione.

2-Allyl-benzo[*de*]isoquinoline-1,3-dione (7)



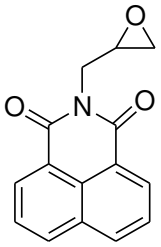
White solid (71 %); m.p. 117-120 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (d, *J* = 7.32 Hz, 2H, ArH), 8.23 (d, *J* = 8.72 Hz, 2H, ArH), 7.78 (t, *J* = 7.32 Hz, 2H, ArH), 6.05-5.95 (m, 1H, CH), 5.35-5.30 (dt, ^2J = 17.4 Hz, ^3J = 1.36 Hz, 1H, CH_2), 5.23-5.20 (dt, ^2J = 10.52 Hz, ^3J = 1.36 Hz, 1H, CH_2), 4.82 (dd, ^2J = 5.96 Hz, ^3J = 1.36 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.9 (C=O), 134.0, 132.1, 131.5, 131.3, 128.1, 126.9, 122.5, 117.5, 117.5 (ArH), 42.4 (N-CH_2); MS (EI) : m/z 238.2 (M^++1).

3.8.1.4. Procedure for synthesis of 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (8)

2-Allyl-benzo[*de*]isoquinoline-1,3-dione (**7**) (0.5 g, 2.10 mmol) was stirred with *m*-CPBA (1.09 g, 6.30 mmol) in dry chloroform at room temperature for 8 h. Reaction was monitored

with TLC. After completion of reaction, reaction mixture was neutralized with $NaHCO_3$ and extracted with water, dried over Na_2SO_4 and concentrated to get crude 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**8**) which was further purified by column chromatography to afford pure compound **8** using hexane : ethylacetate as eluents.

2-Oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (8)

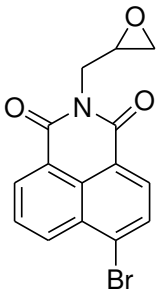


White solid (59 %); m.p. 105-107 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.64 (d, *J* = 7.36 Hz, 2H, ArH), 8.28 (d, *J* = 8.24 Hz, 2H, ArH), 7.81 (t, *J* = 7.80 Hz, 2H, ArH), 4.49-4.37 (m, 1H, CH), 4.12 (t, *J* = 4.60 Hz, 2H, O-CH_2), 3.65 (t, *J* = 4.12 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.6 (C=O), 134.7, 132.0, 131.6, 128.2, 127.2, 122.1 (ArH), 70.7 (O-CH), 63.7 (O-CH_2), 42.7 (N-CH_2); MS (EI) : m/z 254.2 (M^++1).

3.8.1.5. Procedure for synthesis of 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (9)

To a solution of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (**4**) (0.50 g, 1.58 mmol) in dry chloroform, *m*-CPBA (0.81 g, 4.75 mmol) was added. Reaction was stirred at room temperature for 8 h. On completion of reaction, reaction mixture was neutralized with saturated solution of $NaHCO_3$ and extracted. Dried the organic layer over Na_2SO_4 and concentrated to get the crude product which was purified with column chromatography using hexane: ethylacetate as eluents to afford pure 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**9**).

6-Bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (9)

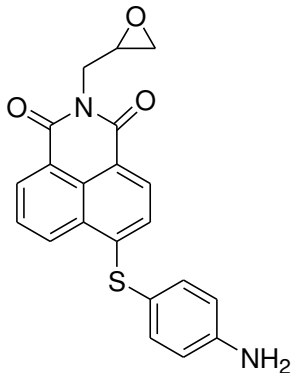


Light brown solid (63 %); m.p. 110-113 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.20 (dd, ^2J = 7.32 Hz, ^3J = 0.92 Hz, 1H, ArH), 8.14 (dd, ^2J = 8.68 Hz, ^3J = 0.92 Hz, 1H, ArH), 7.96 (d, *J* = 7.80 Hz, 1H, ArH), 7.64 (d, *J* = 7.80 Hz, 1H, ArH), 7.47 (dd, ^2J = 8.68 Hz, ^3J = 7.32 Hz, 1H, ArH), 4.02-3.71 (m, 1H, CH), 3.20 (t, *J* = 5.04 Hz, 2H, O-CH_2), 2.73 (s, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.4 (C=O), 133.3, 132.1, 131.8, 131.4, 130.6, 130.1, 128.9, 122.8, 122.3, 117.8 (ArC), 69.3 (O-CH), 63.6 (O-CH_2), 42.5 (N-CH_2); MS (EI) : m/z 333.1 (M^++1).

3.8.1.6. Procedure for synthesis of 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (10)

6-Bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**9**) (0.20 g, 0.60 mmol) was treated with 4-amino-thiophenol (0.07 g, 0.60 mmol), K_2CO_3 (0.10 g, 0.72 mmol) and $TBAHSO_4$ (catalytic amounts) in acetonitrile at reflux temperature for 10 h. On completion of reaction, solvent was removed under vacuum and residue underwent usual work up with water and chloroform, the organic layer was dried over Na_2SO_4 and concentrated to get crude solid which was purified with column chromatography using hexane: ethylacetate as eluents to afford pure yellow solid of 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**10**).

6-(4-Amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (10)



Yellow solid (52 %); m.p. 145-146 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.67 (q, *J* = 4.60 Hz, 2H, ArH), 8.31 (d, *J* = 7.80 Hz, 1H, ArH), 7.81 (t, *J* = 7.80 Hz, 1H, ArH), 7.40 (d, *J* = 8.24 Hz, 2H, ArH), 7.07 (d, *J* = 8.24 Hz, 1H, ArH), 6.81 (d, *J* = 8.24 Hz, 2H, ArH), 4.45-4.34 (m, 1H, CH), 4.09 (t, *J* = 4.60 Hz, 2H, O-CH_2), 3.61 (d, *J* = 3.68 Hz, 2H, N-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.6 (C=O), 149.0, 148.5, 136.4, 130.6, 130.0, 128.8, 127.3, 127.1, 125.6, 121.8, 117.4, 115.1, 112.2 (ArH), 63.6 (O-CH_2), 41.8 (N-CH_2); MS (EI) : m/z 377.4 (M^++1).

3.8.2. *In vitro* antitumor screening assay

In vitro anticancer screening at NCI was a two-stage process, beginning with the evaluation of all compounds against the 60 cell lines at a single dose of 10 μM. The output from the single dose screen was reported as a mean graph and was available for analysis by the COMPARE program. Compounds which exhibited significant growth inhibition were evaluated against the 60 cell panel at five concentration levels. The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96 well microtiter plates in 100 mL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37^o C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide at

400 fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Additional four, 10-fold or ½ log serial dilutions were made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 h at 37 ^{o}C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 ^{o}C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as:

$[(Ti - Tz)/(C-Tz)] \times 100$ for concentrations for which $Ti >= Tz$

$[(Ti – Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$:

Three dose response parameters were calculated for each experimental agent. Growth inhibition of 50% (GI_{50}) was calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from $Ti = Tz$. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from $[(Ti-Tz) / Tz] \times 100 = -50$.

3.8.3. MTT assay

Hek293 (Human embryonic kidney) cells, DMEM with 50mM glutamine, 10% FBS, 100 U/ml pencillin and 100 mg/ml streptomycin. The test was performed against Hek293 (Human embryonic kidney) cells. Cells were seeded in 96 well plates at the density of $1x10^{-5}$ cells/well in DMEM media supplemented with 10% FBS cells. Cells were incubated at 37 ^{o}C in 5% CO_2 incubator. Cells were treated with compound **6b** at five concentrations (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M) for 24 h at 37 ^{o}C. 10 µl of MTT (prepared in 1* PBS buffer) from 5 mg/ml stock was added in each well and incubated at 37 ^{o}C for 4 h in dark. The formazan crystals were dissolved using 100 µl of DMSO. Further, the amount of formazan crystal formation was measured as difference in absorbance by Bio-Red ELISA plate reader at 570 nm and 690 nm reference wavelength. The relative cell toxicity (%) related to control wells containing culture medium without test material was calculated by using formula:

$$\% \text{ Cell Toxicity} = 100 - \frac{\text{OD (Compound treated wells)}}{\text{OD (Untreated Wells)}} \text{ X } 100$$

3.8.4. LDH activity

Cell lines A549 ($5x10^{6}$cells/well) were seeded in 96 well plates with DMEM (Biological Industries) media contains 10% FBS (Sigma-Aldrich) and 1% PenStrep (Sigma-Aldrich), incubated at 37 ^{o}C in 5% CO_2 incubator. Cells were allowed to adhere. After 24 h, containing media was replaced by fresh media and treated with mentioned concentration of compound again following incubation for 12 and 24 h respectively in 5% CO_2 incubator at 37℃. Removal of aliquot of the culture medium form seeded cell followed by the addition of Lactate Dehydrogenase assay mixture (LDH assay substrate solution, LDH assay dye solution, and 1× LDH assay cofactor) into each sample in a volume equal to twice. Plates were covered with aluminum foil to protect from light and incubated at room temperature for 20–30 minutes. After incubation, reaction was terminated by the addition of 1/10 volume of 1 N HCl to each well. Absorbance measured at wavelength of 490 nm by ELISA reader. Measure absorbance of the blank was subtracted from this value.

3.8.5. DNA binding studies

Stock solution of compound **6b** (1 mM) was prepared by dissolving **6b** in AR grade DMSO. The DNA binding experiments were carried out by making dilution of stock solution with phosphate buffer. Stock solution of ct-DNA was prepared by dissolving DNA in phosphate

buffer (10 mM, pH 7.0). The DNA concentration was estimated from its absorbance intensity at 260 nm with a known molar absorption coefficient value of 6600 $dm^3M^{-1}cm^{-1}$. The purity of DNA was established from ratio of absorbance intensity at 260 nm and 280 nm.

UV-Vis and Fluorescence titrations: The titration experiments were performed by varying the concentration of ct-DNA and keeping the compound concentration constant (20 μM). All the UV spectra were recorded after equilibration of solution for 5 min. Fluorescence titrations were carried out on Cary Eclipse Fluorescence Spectrophotometer at ambient temperature. A slit width of 10 nm was used with λ_{ex} = 400 nm. The titration experiment were accomplished by varying the concentration of DNA in cuvette (0.5-150 μM).

DNA thermal denaturation: DNA melting experiments were carried out by observing the absorbance of ct-DNA at 260 nm at various temperature in the absence and presence of compound at 260 nm with a ramp rate of 0.5 ^oC/min in a phosphate buffer (pH 7.0) on a Shimadzu Spectrophotometer equipped with a Peltier thermo regulator.

3.8.6. Molecular modelling

Coordinates from the X-ray crystal structure of DNA (pdb ID 1BNA) and topoisomerase I (pdb ID 1A36) and topoisomerase II (pdb ID 1BJT) were taken from the RCSB Protein Data Bank. Compounds were constructed with the builder toolkit of the software package ArgusLab 4.0.1 (www.arguslab.com) and energy minimized using the semiempirical quantum mechanical method PM3. The monomeric structures of the enzymes were chosen, and the active site was defined around the ligand. The molecule to be docked in the enzyme's active sites was inserted into the work space carrying the structure of the enzyme. The docking program implements an efficient grid-based docking algorithm, which approximates an exhaustive search within the free volume of the binding site cavity. The conformational space was surveyed by the geometry optimization of the flexible ligand (rings are treated as rigid) in combination with the incremental construction of the ligand torsions. Thus, docking occurred between the flexible ligand parts of the compound and enzyme. The ligand orientation was determined by a shape scoring function based on Ascore and the final positions were ranked by lowest interaction energy values. Hydrogen bond between the compound and enzyme were explored by distance measurements.

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CHAPTER 4

SYNTHESIS OF PYRIDINE-TRIAZOLE BASED ANTICANCER AGENTS

4.1 Introduction

Cancer became the major health problem worldwide. Whether the improved medicines and advanced treatments have lessen the cancer deaths, but new diagnosed cases are still showing a rise curve. A number of cancers are still unable to meet the therapeutic needs. Moreover chemo resistance, side effects and safety hazards are the major obstacles in the way of successful treatment of cancer. Heterocyclic compounds gradually becoming more attractive targets in organic and medicinal chemistry due to their great chemical and biological importance. Amongst these, nitrogen containing rings have received great attention due to their broad application area in pharmacological as well as biological fields. There are many examples present in the literature where nitrogen containing pyridines are known for their anticancer,[1,2] antimicrobial,[3-5] anticonvulsant,[6] antiviral,[7] anti-HlV,[8] antifungal and antimycobacterial activities.[9] On the other hand, 1,2,3-triazoles (playing an important role as a member of heterocyclic family) are a subject of interest due to their importance in synthetic organic chemistry and biological activities[10] (figure 1). These have shown good activities as antituberculosis agents,[11,12] neuraminidase inhibitors,[13] fungicidal activity,[14-16] protein tyrosine phosphatase inhibitors,[17,18] anticancer,[19-22] antiviral[23-25] and analgesic compounds.[26] Some members of triazole family have also shown good anticancer activities against various human cancer cell lines.[27,28] Reports are also present in the literature where 1,2,3-triazoles along with some biological active moieties have significantly increased the activity of that molecule than the individual activity.[29] Narsaiah *et. al.* have reported pyridine and pyrimidine appended 1,2,3-triazoles as anti-biofilm and antimicrobials, synthesized via click reaction under Sharpless conditions.[30] Based on the importance of both the scaffolds, an idea has proposed to synthesize a single scaffold having 2,3-disubstituted pyridine with 1,2,3-triazole as its appended unit via click chemistry seeking towards future modifications onto their activities.

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (click chemistry) is widely utilized in the synthesis of 1,2,3-triazole.[31] Cu-promoted azidation and 1,3-dipolar [3+2] cycloaddition reaction between azides and terminal alkynes that can be carried out in one-pot synthesis also seems to be an attracting method. Herein, we demonstrated an efficient synthesis of novel 2,3-disubstituted pyridine molecules by performing one-pot copper-

catalyzed azidation and copper-catalyzed azide-alkyne cycloaddition reaction. Additionally, we have also evaluated the anticancer activity of these products, most of which exhibited moderate inhibition to cancer cell growth.

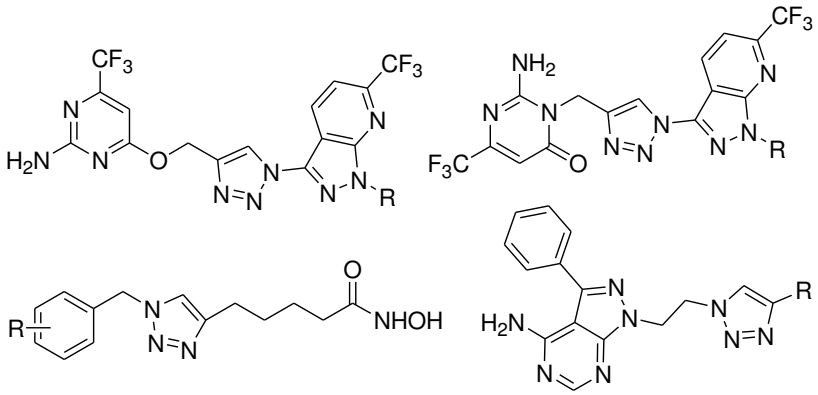
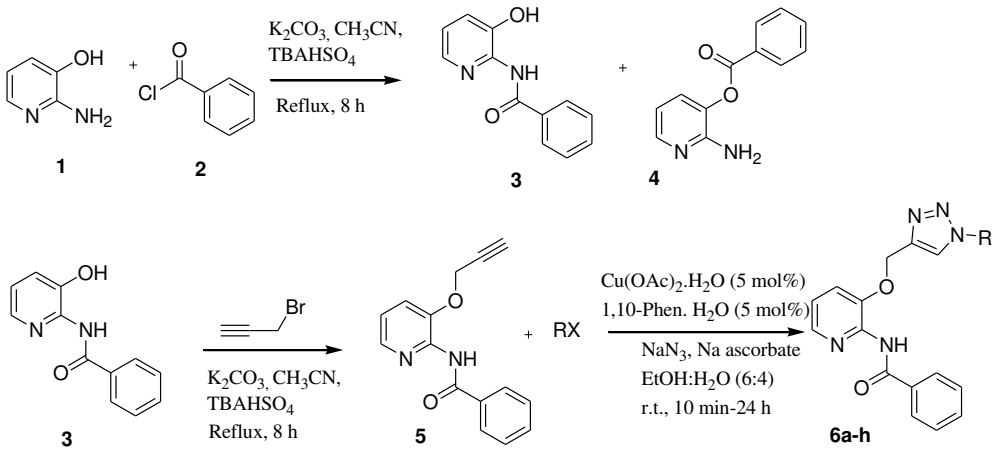


Figure 1. Biological active 1,2,3-triazole functionalized derivatives

Objective: Numerous pyridine based molecules are reported in literature with significant biological activities. Also, 1,2,3-triazoles are well known in literature having wide application area. But few reports are known for combination of these two heterocyclc moieties and no reports has been given with pyridine moiety. Therefore, it seemed to be interesting to synthesize 2,3-disubstituted pyridine analogues having 1,2,3-triazole as their appended unit and to study their anticancer activities and structure activity relationship. Click chemistry was adopted as convenient method to synthesize these target molecules.

4.2 Chemistry

4.2.1. Synthesis of *N*-(3-hydroxy-pyridin-2-yl)benzamide (3) and benzoic acid 2-amino-pyridin-3-yl ester (4)



Scheme 1. Synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-[1,2,3]-triazol-4-yl)methoxy) pyridine-2-yl)-3-phenyl-acrylamide

The precursor of *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**) was synthesized from easily available starting material 2-amino-3-hydroxy pyridine (**1**) (Scheme 1). 2-Amino-3-hydroxy pyridine (2.0 g, 18.17 mmol) has been electrophilic substituted with benzoyl chloride (**2**) (20.15 mmol) in the presence of potassium carbonate (3.0 g, 21.75 mmol) and acetonitrile using $TBAHSO_4$ as catalyst at reflux temperature for 8 h. TLC confirmed the formation of two expected products, **3** and **4**. After the completion of the reaction, reaction mixture was neutralized with $NaHCO_3$ and extracted with chloroform and water. The organic layer was dried over Na_2SO_4 and concentrated to give white solid of **3** (m.p. = 130-133 ^oC) and **4** (m.p. = 160-162 ^oC) in 65% and 10% yields respectively. ^1H NMR spectrum of compound **3** showed 1H broad singlet of NH at δ 8.62 (exchangeable with D_2O), three 1H double doublets at δ 8.34, δ 7.77 and δ 7.40, two 2H double doublets at δ 8.16 and δ 7.83, and 1H triplet at δ 7.60 of aromatic-H. ^1H NMR spectrum of compound **4** showed 2H broad singlet of NH_2 at δ 9.87 (exchangeable with D_2O), 2H double doublet at δ 8.02, two 1H triplets at δ 7.62 and δ 7.58, 2H triplet at δ 7.51 and two 1H double doublets at δ 7.41 and δ 7.11 of aromatic-H. Appearance of NH signal at δ 8.62 and NH_2 signal at δ 9.87 confirmed the benzoylation at NH_2 and OH respectively of 2-amino-3-hydroxy pyridine. On the basis of these spectral data, compounds **3** and **4** have been assigned the structures of *N*-(3-hydroxy-pyridin-2-yl)benzamide and benzoic acid 2-amino-pyridin-3-yl ester respectively. The crude **3** was > 95% pure according to ^1H NMR and was used in the next stage without further purification.

4.2.2. Synthesis of *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)-benzamide (5)

N-(3-Hydroxy-pyridin-2-yl)benzamide (**3**) (2.0 g, 10 mmol) was reacted with propargyl bromide (10 mmol) in the presence of potassium carbonate (2.0 g, 15 mmol), acetonitrile and $TBAHSO_4$ (catalytic amounts) at reflux temperature for 8 h. On completion of reaction (monitored with TLC), acetonitrile was removed under vacuum, extracted the residue with chloroform and water to yield crude product of **5** which was purified by column chromatography to obtain brown coloured liquid with 70% yield. ^1H NMR spectrum of this compound showed 1H broad singlet of NH at δ 8.72 (exchangeable with D_2O), 2H doublet at δ 7.94, 1H multiplet at δ 7.57-7.52, 2H multiplet at δ 7.50-7.46, two 1H double doublets at δ 7.39 and δ 7.11 of aromatic-H, 2H doublet of O-CH_2 at δ 4.80 and 1H triplet of ≡CH at δ 2.60. ^{13}C NMR spectrum of this compound showed δ 164.6 of C=O, 143.3, 140.7, 134.8, 131.9, 128.6, 127.3, 119.8, 119.6 (ArC), 59.0 (C), 58.9 (≡CH) and 56.5 (O-CH_2). Appearance

of propargyl signals at δ 4.80 of OCH_2 and δ 2.60 of ≡CH in ^{1}H NMR spectrum confirmed the formation of products.

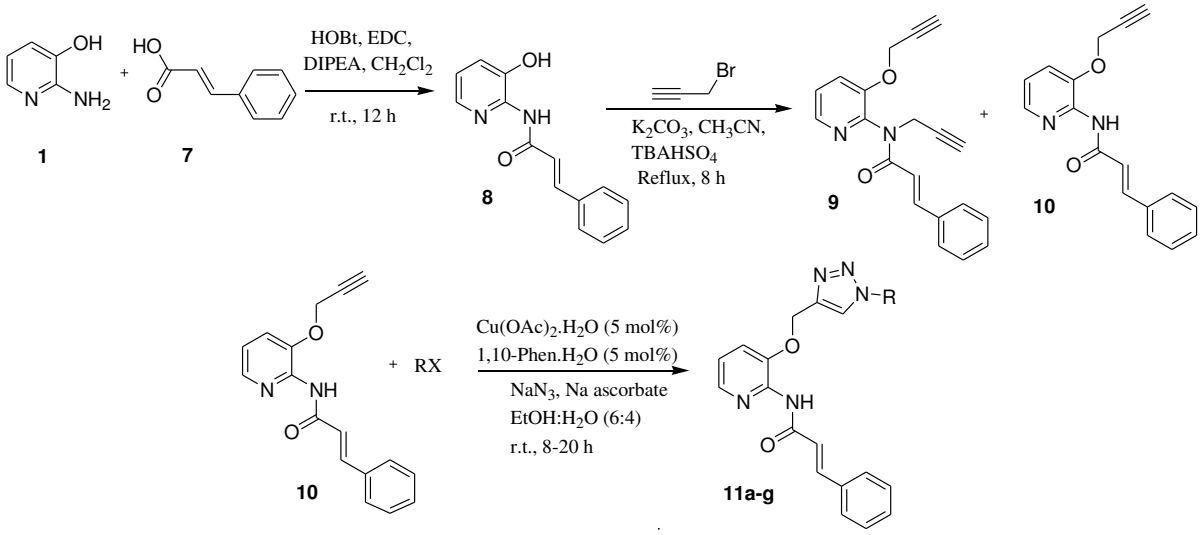
4.2.3. Synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-(1,2,3)-triazol-4-yl)methoxy)pyridine-2-yl)-3-phenyl-acrylamide (6a-h)

Target final step was the synthesis of pyridine analogues containing 1,2,3-triazole unit (**6a-h**) from *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**) and substituted benzyl halides. One pot Cu-catalyzed click reaction was first performed with *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**) and benzyl chloride using sodium azide, copper(II)acetate as catalyst, sodium ascorbate as reducing agent and 1,10-phenanthroline.H_2O as ligand in EtOH:H_2O (6:4) at room temperature for 24 h. *N*-(3-(Prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**) (100 mg, 0.40 mmol) was dissolved in 10 ml EtOH:H_2O (6:4) followed by addition of sodium azide, copper(II)acetate and sodium ascorbate (107 mg, 0.54 mmol), and stirred at room temperature for 10 min. Benzyl chloride (0.40 mmol) was added to this suspension and continued stirring at room temperature for 24 h. Reaction was monitored with TLC. On completion of reaction, cold water was added to the reaction mixture, resulted in the formation of white precipitates. Filtered it and washed with water. Dried the crude products over vacuum and then purified with column chromatography using ethylacetate: methanol (98:2) as eluents to afford pure **6a** in 55 % yield (Table 1). After washing with diethyl ether, the compound was subjected to dry under vacuum and its NMR data was recorded. ^{1}H NMR spectrum of **6a** showed 1H broad singlet of NH at δ 8.50, 1H double doublet at δ 8.13, three 2H multiplets at δ 7.89-7.87, δ 7.57-7.53 and δ 7.48-7.44, 1H double doublet at δ 7.42, 3H triplet at δ 7.35, 2H multiplet at δ 7.23-7.21 and 1H double doublet at δ 7.09 of aromatic-H, 2H singlet of O-CH_2 at δ 5.50 and 2H singlet of N-CH_2 at δ 5.29. ^{13}C NMR spectrum of this compound shown C=O signal at δ 164.8 and 144.6, 143.2, 142.1, 140.4, 134.6, 131.9, 129.1, 128.8, 128.6, 128.0, 127.4, 123.1, 120.4, 120.2 of aromatic-C, O-CH_2 at δ 62.7 and N-CH_2 at δ 54.2. Appearance of signals at δ 5.50 and δ 5.29 of respective OCH_2 and NCH_2 and disappearance of propargyl signals in ^{1}H NMR spectrum confirmed the formation of compound **6a**. Thus, on the basis of these NMR data, this compound has been assigned the structure of *N*-(3-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methoxy)pyridin-2-yl)benzamide. Similarly, compound **5** was also reacted with substituted benzyl halides and allyl bromide under the same reaction conditions to give compounds **6b-g** and **6h** respectively in 55-82%

yields. The reactions with *o*-fluorobenzyl and *p*-fluorobenzyl groups were faster than the other substituted halides.

4.2.4. Synthesis of *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (8)

Inspired by the excellent results obtained with benzoylation, we have further treated 2-amino-3-hydroxy pyridine (**1**) with cinnamic acid (**7**) in the presence of hydroxybenzotriazole (HOBt), (*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) (EDC) and diisopropylethylamine in dichloromethane at room temperature for 12 h to yield *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (**8**) (Scheme 2). Cinnamic acid (**7**) (2.6 g, 18 mmol) was dissolved in dichloromethane along with HOBt (5.4 g, 40 mmol), EDC (6.2 g, 40 mmol) and diisopropylethylamine (2.5 g, 20 mmol) at 0 ^{o}C and continued to stir at same temperature for 5 min. To this suspension, 2-amino-3-hydroxy pyridine (**1**) (2.0 g, 18 mmol) was added and reaction mixture was stirred at room temperature for 12 h. Progress of reaction was monitored with TLC. On completion of reaction, reaction mixture was extracted with chloroform and water. Organic layer was collected, dried over Na_2SO_4 and concentrated to give crude solid of **8** (m.p. = 145-147 ^{o}C) which was further purified with column chromatography to get yellow solid in 80% yield.



Scheme 2. Synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-(1,2,3)-triazol-4-yl)methoxy) pyridine-2-yl)-3-phenyl-acrylamide

^{1}H NMR spectrum of this compound showed 1H broad singlet of OH at δ 10.90 (exchangeable with D_2O), 1H broad singlet of NH at δ 10.20 (exchangeable with D_2O), two 1H doublets at δ 7.94 and δ 7.88, 2H multiplet at δ 7.52-7.50, 4H multiplet at δ 7.44-7.38, 1H double doublet at δ 7.19 and 1H doublet at δ 6.75 of aromatic-H which confirmed the

incorporation of cinnamic group with 2-amino-3-hydroxy pyridine. ^{13}C NMR spectrum of this compound showed –C=O peak at δ 166.3 and 145.4, 145.1, 140.5, 138.3, 138.2, 133.9, 131.0, 130.3, 129.3, 128.7, 128.6, 128.4, 128.0, 127.1, 123.4, 122.2, 118.8, 118.5, 117.4 of aromatic-C. On the basis of these spectral data, this compound has been assigned the structure of *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (**8**).

4.2.5. Synthesis of 3-phenyl-*N*-prop-2-ynyl-*N*-(3-prop-2-ynyloxy-pyridin-2-yl)acrylamide (9) and 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide (10)

N-(3-Hydroxy-pyridin-2-yl)-3-phenyl-acrylamide **8** (2.0 g, 10 mmol) was treated with propargyl bromide (1.17 g, 10 mmol) in the presence of potassium carbonate (2.0 g, 15 mmol) and acetonitrile using $TBAHSO_4$ as a catalyst. When this reaction was proceeded at room temperature for 8 h, it resulted in formation of 3-phenyl-*N*-prop-2-ynyl-*N*-(3-prop-2-ynyloxy-pyridin-2-yl)acrylamide (**9**) and 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide (**10**) (m.p. = 129-131 ^{o}C) in 10 % and 55 % yields respectively. When same reaction was preceded at reflux temperature, it resulted in formation of compound **9** in 60% yield along with traces of compound **10**. ^{1}H NMR spectrum of **9** showed 1H double doublet at δ 8.72, two 1H doublets at δ 7.74 and δ 7.53, 1H double doublet at δ 7.38, 2H double doublet at δ 7.34, 3H doublet at δ 7.28, 1H doublet at δ 6.23 of aromatic-H, 2H doublet of O-CH_2 at δ 4.74, 2H doublet of N-CH_2 at δ 4.71, 1H triplet of -CH at δ 2.40 and 1H doublet of -CH at δ 2.14. Appearance of 2H of OCH_2 at δ 4.74, 2H of NCH_2 at δ 4.71, 1H triplet at δ 2.40 and 1H doublet at δ 2.14 clearly indicated the substitution of propargyl group at both NH and OH. ^{1}H NMR spectrum of compound **10** showed 2H doublet at δ 8.10, 1H doublet at δ 7.85, 2H multiplet at δ 7.60-7.57, 4H multiplet at δ 7.41-7.34, two 1H double doublets at δ 7.32 and δ 7.05 of aromatic-H and NH, 2H doublet at δ 4.79 of O-CH_2 and 1H triplet at δ 2.60 of -CH. ^{13}C NMR spectrum of this compound showed –C=O peak at δ 164.8 and 143.3, 142.3, 142.2, 140.1, 134.8, 129.8, 128.7, 128.1, 120.6, 119.2, 119.1 (ArC), 56.3 (O-CH_2) which confirmed the single substitution of propargyl to *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (**8**).

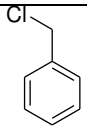
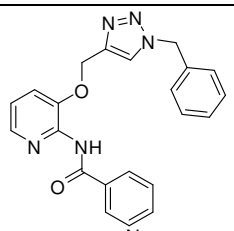
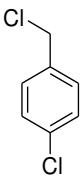
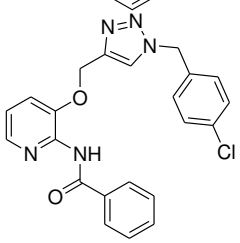
4.2.6. Synthesis of *N*-(3-(1-substituted benzyl/allyl-1*H*-(1,2,3)-triazol-4-yl)methoxy) pyridine-2-yl)-3-phenyl-acrylamide (11a-g)

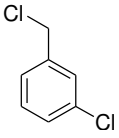
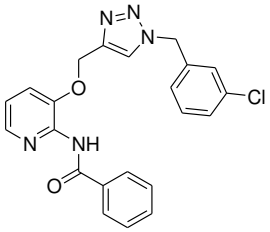
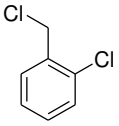
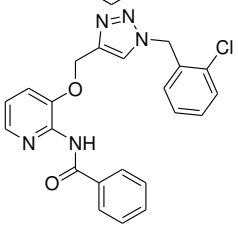
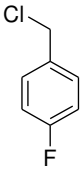
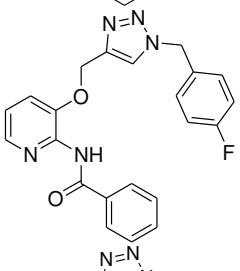
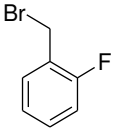
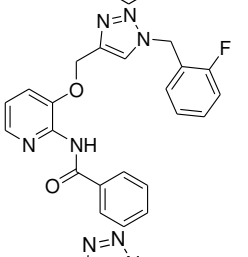
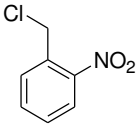
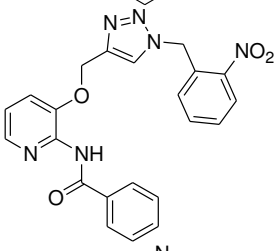
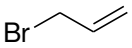
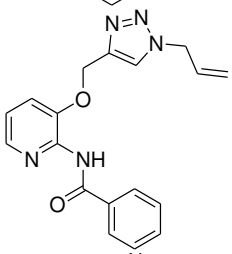
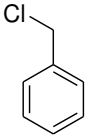
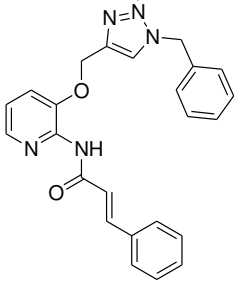
3-Phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide **10** (100 mg, 0.35 mmol) was underwent copper catalyzed click reaction with benzyl chloride using sodium azide in the

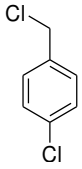
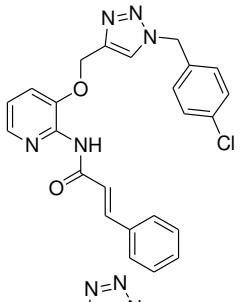
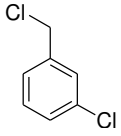
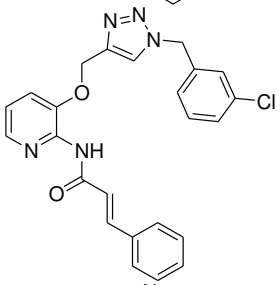
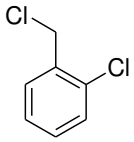
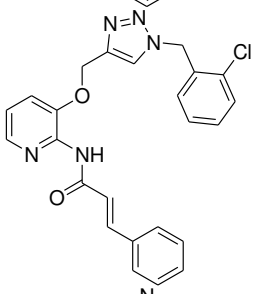
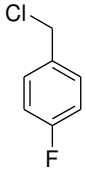
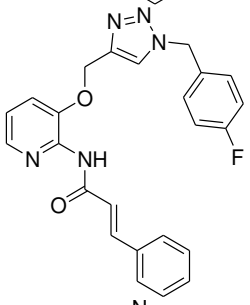
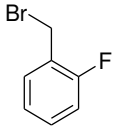
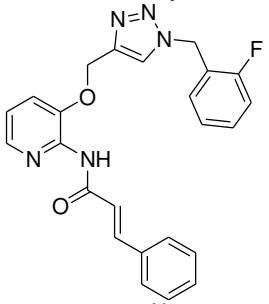
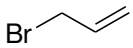
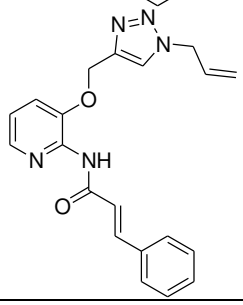
presence of $Cu(OAc)_2.H_2O$, sodium ascorbate and 1,10-phenanthroline.H_2O in EtOH:H_2O at room temperature for 20 h. 3-Phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)-acrylamide (**10**) was dissolved in 10 ml of EtOH:H_2O (6:4) along with sodium azide (76 mg, 1.02 mmol), $Cu(OAc)_2.H_2O$ (5 mg, 5 mol%), sodium ascorbate (107 mg, 0.47 mmol) and 1,10-phenanthroline.H_2O (5 mg, 5 mol%). Reaction mixture was stirred at room temperature for 10 min. Benzyl chloride (0.35 mmol) was added to this suspension and stirred for 20 h. Reaction was monitored with TLC. On completion of reaction, cold water was added to reaction mixture, resulted in formation of white precipitates of crude product. The solid was filtered and washed with water number of times to remove any salt. Vacuum dried the crude product and purified with column chromatography using ethylacetate: methanol (98:2) as eluents to get pure product of **11a** (MS (EI) : m/z 412.2 (M^++1)). ^{1}H NMR spectrum data of **11a** showed 1H broad singlet of NH at δ 8.11 (exchangeable with D_2O), 1H double doublet at δ 8.06, 1H doublet at δ 7.82, 3H multiplet at δ 7.59-7.57, two 4H multiplets at δ 7.41-7.37 and δ 7.36-7.32, 2H multiplet at δ 7.31-7.27, 1H double doublet at δ 7.03 of aromatic-H, two 2H singlets of CH_2 at δ 5.54 and δ 5.26. ^{13}C NMR spectrum of this compound has been showed –C=O signal at δ 164.8 and 143.2, 143.0, 142.1, 139.9, 134.9, 134.0, 129.9, 129.2, 128.9, 128.7, 128.1, 123.2, 120.6, 119.5 (ArC), 62.4 (O-CH_2) and 54.3 (N-CH_2). On the basis these spectral data, this compound has been assigned the structure of *N*-[3-(1-benzyl-1*H*-[1,2,3]triazol-4-ylmethoxy)-pyridin-2-yl]-3-phenyl-acrylamide.

Similarly, compound **10** was reacted with substituted benzyl halides and allyl bromide under the same reaction conditions to afford compounds **11b-11f** and **11g** respectively in 45-58% yields (Table 1).

Table 1 Physical data of compounds **6a–h** and **11a–g**

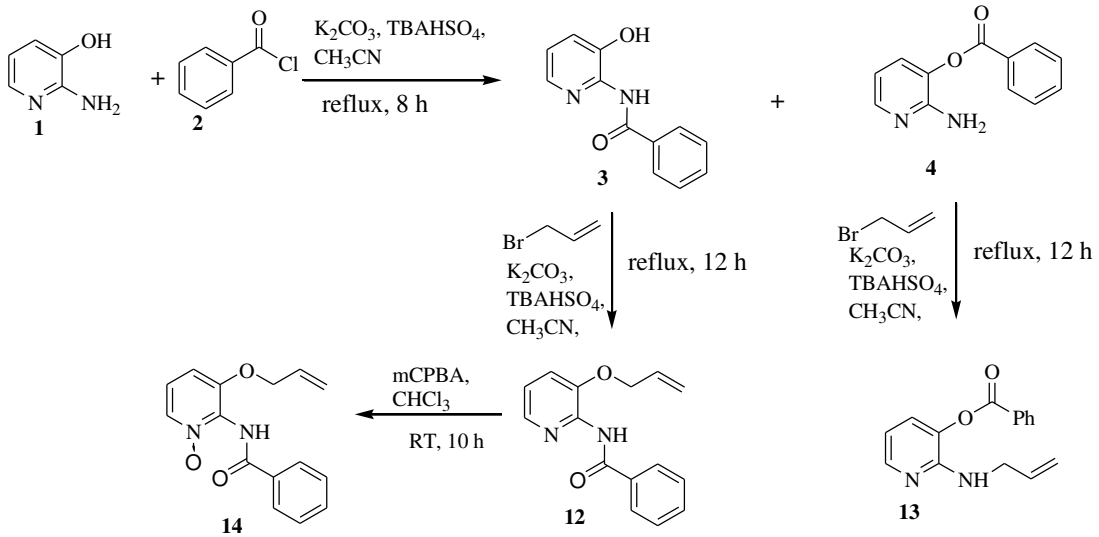
Entry	Compds.	RX	Product	Time	Yield (%)	m.pt. (°C)
1	**6a**			24 h	55	120 -122
2	**6b**			20 h	65	163 -164

3	**6c**			20 h	66	154 -157
4	**6d**			20 h	64	125-126
5	**6e**			10 min	82	165-168
6	**6f**			15 min	78	132 -134
7	**6g**			24 h	60	119 -123
8	**6h**			18 h	55	117 -120
9	**11a**			20 h	50	125-126

10	**11b**			18 h	58	160 -162
11	**11c**			20 h	57	161 -163
12	**11d**			18 h	56	128 -129
13	**11e**			8 h	58	169 -172
14	**11f**			8 h	55	138 -140
15	**11g**			20 h	45	120-122

4.2.7. Synthesis of *N*-(3-allyloxy-pyridin-2-yl)-benzamide (12), benzoic acid 2-allylamino-pyridin-3-yl ester (13) and *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide (14)

With continuation of 2,3-disubstitution of pyridine derivatives, we have also performed the allylation at 2- and 3- positions of 2-amino-3-hydroxypyridine (Scheme 3). The reaction was carried out with *N*-(3-hydroxy-pyridin-2-yl)-benzamide (**3**) (0.50 g, 2.30 mmol) and allyl-bromide (2.80 mmol) in the presence of K_2CO_3 (0.38 g, 2.76 mmol), $TBAHSO_4$ (catalytic amount) using acetonitrile as solvent at reflux temperature for 12 h. After completion of reaction, reaction mixture was extracted with chloroform and water. Organic layer was collected and dried over Na_2SO_4 and concentrated to get the crude product of compound **12** which was further purified with column chromatography using hexane:ethylacetate as eluents to obtain white solid (m.p. = 65-67 ^oC) in 68 % yield. ^1H NMR spectrum of compound **12** showed 1H broad singlet at δ 8.56 (exchangeable with D_2O), 1H doublet at δ 8.14, 2H doublet at δ 7.93, 3H multiplet at δ 7.58-7.47, 1H doublet at δ 7.21, 1H double doublet at δ 7.06 of aromatic-H, 1H multiplet at δ 6.09-5.98 of -CH, 2H multiplet at δ 5.39 of $-CH_2$ and 2H doublet at δ 4.66 of $O-CH_2$. Appearance of allyl signals at δ 6.09-5.98, 5.39 and 4.66 indicated the incorporation of allyl group to compound **3**. On the basis of ^1H NMR spectrum this compound has been assigned structure of *N*-(3-allyloxy-pyridin-2-yl)-benzamide.



Scheme 3. Synthesis of *N*-(3-allyloxy-pyridin-2-yl)-benzamide (**12**), benzoic acid 2-allylamino-pyridin-3-yl ester (**13**) and *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide (**14**)

Similarly, benzoic acid 2-amino-pyridin-3-yl ester (**4**) (0.10 g, 0.46 mmol) was treated with allyl bromide (0.56 mmol) in presence of K_2CO_3 (0.09 g, 0.56 mmol), $TBAHSO_4$ (catalytic

amounts) and acetonitrile at reflux temperature for 12 h. After usual workup, organic layer was collected and dried over Na_2SO_4 and concentrated to yield crude product of **13** which was then purified with column chromatography (hexane:ethylacetate) to yield white solid of compound **13** (m.p. = 73-75 ^oC) with 45% yield. ^1H NMR spectrum of this compound showed 1H broad singlet of NH at δ 9.20, 2H double doublet at δ 8.25, 1H doublet at δ 8.09, 2H multiplet at 7.51-7.39, three 1H double doublets at δ 7.36, δ 7.30 and δ 6.86 of aromatic-H, 1H multiplet at δ 6.14-6.04 of CH, 2H multiplet at δ 5.32-5.23 of CH_2 and 2H doublet at δ 5.10 of N-CH_2. Disappearance of NH_2 signal at δ 9.87 and appearance of new signal at δ 9.20 of NH confirmed the allylation at NH_2 group. On the basis of ^1H NMR spectrum, this compound has been assigned the structure of benzoic acid 2-allylamino-pyridin-3-yl ester.

N-(3-allyloxy-pyridin-2-yl)-benzamide (**12**) has further been used for the synthesis of *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide (**14**). *N*-(3-Allyloxy-pyridin-2-yl)-benzamide (0.10 g, 0.40 mmol) was reacted with *m*-CPBA (0.20 g, 1.18 mmol) in the presence of dry chloroform. Reaction was initiated at 0 ^oC. TLC confirmed the formation of new product but reaction rate was very slow. Seeking no further progress, reaction was shifted to room temperature and stirred the reaction for 10 h. After completion of reaction, reaction mixture was neutralized with saturated solution of $NaHCO_3$ and extracted with chloroform. Organic layer was collected and dried over Na_2SO_4 and concentrated to afford crude solid of compound **14**. The crude solid was then purified with column chromatography using ethylacetate as eluent to get pure product of brown solid (**14**) (m.p. = 69-71 ^oC) in 45% yield. ^1H NMR spectrum of this compound showed 1H broad singlet at δ 9.23 of NH (exchangeable with D_2O), 2H multiplet at δ 7.97-7.93, 1H triplet at δ 7.58, two 2H triplets at δ 7.48 and δ 7.04, 1H double doublet at δ 6.97 of aromatic-H, 1H multiplet at δ 6.02-5.93 of CH, two 1H double quartets at δ 5.42-5.36 and δ 5.26-5.22 of CH_2 and 2H double triplet at δ 4.68 of O-CH_2. No shifts in allyl signals, have denied the formation of epoxy ring of double bond. But a clear change in TLC of compound **14** from compound **12** confirmed the formation of new compound i.e. pyridyl-*N*-oxide. The formation of **14** was confirmed by mass spectrometry (MS (EI) : m/z 271.20 (M^++1)). ^{13}C NMR spectrum of this compound shown –C=O signal at δ 162.5 and 157.8, 152.1, 136.8, 133.7, 133.2, 125.7, 124.1, 121.6, 118.1, 117.1, 114.7, 111.7, 100.2 (ArC), 45.8 (O-CH_2). Based upon these spectral data, compound **14** has been assigned the structure of *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide.

4.3 Biology

***In vitro* cancer screening with one dose**

Six compounds (**6a**, **6b**, **6e**, **6h**, **11b** and **11c**) were selected by National Cancer Institute, Bethesda, Maryland, USA, on the basis of degree of the structure variation and computer modelling techniques for evaluation of their antitumor activity.[32-34] The selected compounds were subjected to *in vitro* anticancer assay against tumour cells in a full panel of 60-cell lines taken from nine different organs (lung, colon, breast, ovary, blood, kidney, skin, prostate and brain). These compounds were tested at a single dose concentration of 10 µM and the percentages of growth inhibition over sixty tested cell lines were determined.

Table 2 Percentage growth inhibition (GI%) of the selected compounds over the most sensitive tumor cell lines at single concentration of 10 µM

Compds.	CCRF-CEM	HL-60(TB)	SR	NCI-H460	HCT-15	SNB-75	SK-MEL-5	UO-31	PC-3	T-47D
6a	-	37.81	-	-	-	11.35	2.39	20.44	-	-
6b	-	6.38	53.94	-	3.57	20.17	4.71	20.45	4.34	6.05
6e	74.25	31.03	-	-	-	-	-	13.45	1.68	-
6h	-	11.07	-	-	7.87	4.10	5.91	6.20	-	-
11b	-	-	7.26	-	-	35.57	5.99	22.00	14.02	26.91
11c	8.29	-	22.15	33.75	55.56	-	33.75	27.64	36.96	17.60
5-FU	57.10	47.90	24.80	13.0	26.50	65.90	33.70	41.30	58.20	56.70

Preliminary *in vitro* antitumor screening was revealed that compound **6e** proved to be potent against leukemia cell line CCRF-CEM with GI value of 74.25% whereas leukemia cell line SR proved to be active towards **6b** with GI value of 53.94%. Leukemia cell line HL-60(TB) showed activity towards **6a** and **6e** with GI values of 37.81% and 31.03% respectively, whereas compound **11b** has found to be active towards CNS cancer cell line SNB-75 with GI value of 35.57%. Compound **11c** has shown activity towards colon cancer cell lines HCT-15 with GI value of 55.56%, non-small cell lung cancer cell line NCI-H460 with GI value of 33.75%, melanoma cancer cell line SK-MEL-5 with GI value of 33.75% and prostate cancer cell line PC-3 with GI value of 36.96%. These data revealed that compounds **6a**, **6b**, **6h**, **11b**

and **11c** have shown moderate inhibition, whereas compound **6e** exhibit good inhibition values against leukemia cancer cell lines (Table 2, Figure 2). Compounds **6b**, **6e**, **11c** possessed better activity than standard drug 5-fluorouracil (5-FU) at various cancer cell lines viz. CCRF-CEM, SR, NCI-H460, HCT-15, SK-MEL-5 etc.

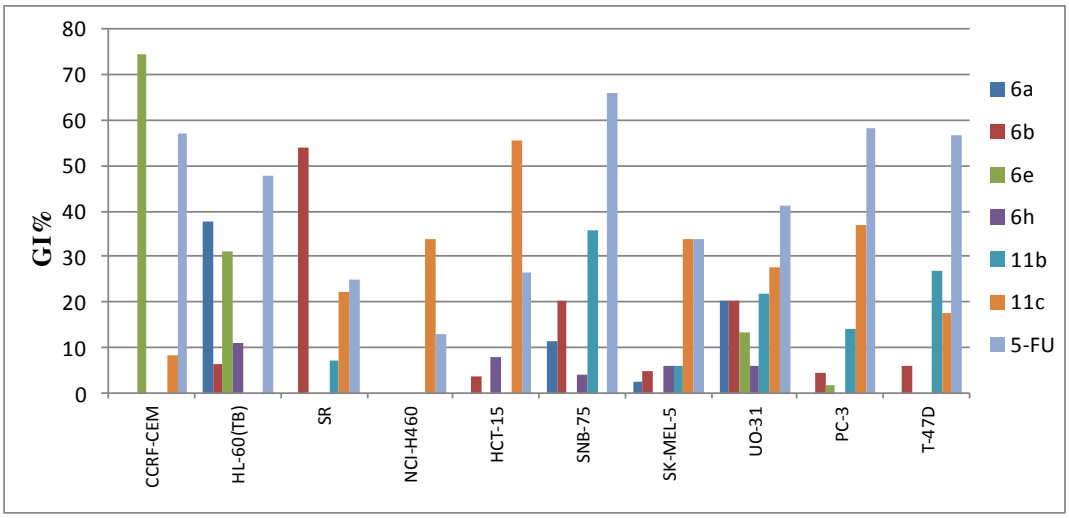


Figure 2. Percentage growth inhibition of the 6 selected compounds AND **5-FU** over the most sensitive tumor cell lines

4.4. ADME prediction

We have calculated the compliance to the Lipinski's rules of five for synthesized compounds, tested against 60 cancer cell lines at single dose concentration. It was observed that most of the biological active compounds have molecular weight (MW) ≤ 500 Da, a lipophilic factor (log P) ≤ 5, H-bond donors (HBD) ≤ 5 and H-bond acceptors (HBA) ≤ 10. All 6 compounds follow this rule with no violation in any of the above parameters (Table 3)

Table 3 Physicochemical parameters of compounds under biological investigation determined for Lipinski's rules of five

Comps	Log P	TPSA	MW	nON	nOHNH	nViolations
6a	3.2	81.9	385.4	7	1	0
6b	3.9	81.9	419.8	7	1	0
6e	3.4	81.9	403.4	7	1	0
6h	2.3	81.9	335.3	7	1	0
11b	4.5	81.9	445.9	7	1	0
11c	4.5	81.9	445.9	7	1	0

4.5. Structure activity relationship

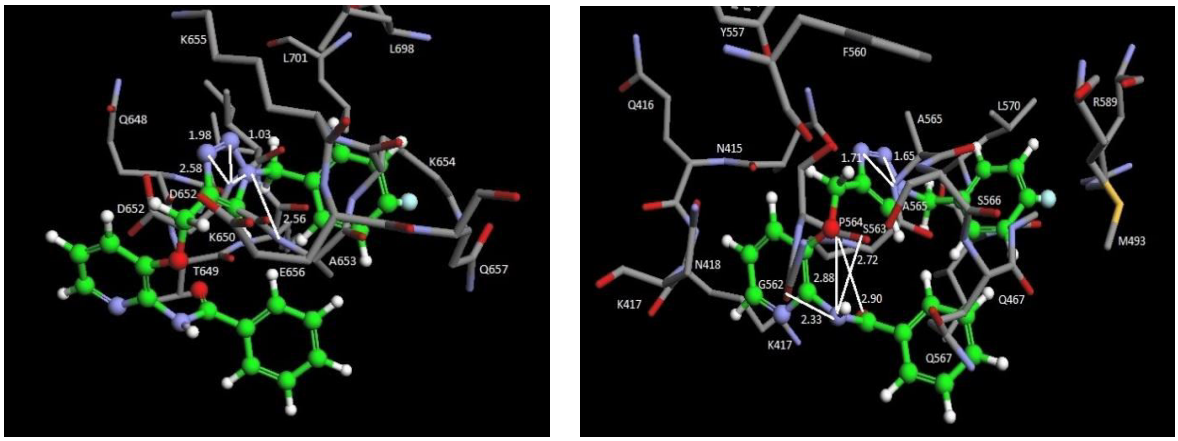
Compounds **6a**, **6b**, **6e**, **6h**, **11b** and **11c** have been tested for their anticancer activities at one dose concentration levels. Among all the selected compounds (**6a**, **6b**, **6e**, **6h**, **11b** and **11c**), **6e** has proved to be the most active member while compound **6h** proved to be least active compound of this series. Out of **11b** and **11c**, compound **11c** proved more active than **11b** with moderate activity over various cancer cell lines. We have concluded various factors as following:

1. Comparing the activities of **6b** and **6e**, higher activity of compound **6e** can be explained on the basis of fluoro group present at 4-position of phenyl ring, instead of chloro group as in case of **6b**. Hence, fluoro group is responsible for the highest activity of compound.
2. Considering the activities of **6a**, **6b** and **6e**, it clearly appeared that **6a** showed lower activity than **6b** and **6e**. It can be concluded that substituted benzyl group showed better activity than non-substituted benzyl when attached to triazole ring.
3. Comparing the activity of **6h** with any other compounds revealed that substitution with allyl bromide lead to the least active member of the series. It has been indicated that aromatic groups proved to be better substituents than aliphatic groups.
4. A comparison between the activities of **11b** and **11c** left us with the conclusion that variation in the position of chloro group had a significant effect on the activity of these compounds. Presence of chloro group at 4-position in case of **11b** showed moderate activity on single cancer cell line while varying the chloro group to 3-position in case of **11c** showed better activity than **11b** against various cancer cell lines.
5. Out of **6b** and **11b**, where both the molecules showed moderate activity, **6b** has better growth inhibition value. Here, both the molecules bear the same benzyl halide at same position. The difference in their activity may be explained on the basis of different substitution at 2 position of pyridine moiety. Where in case of **6b**, benzoyl group was substituted at 2 position of pyridine while in case of **11b**, cinnamic group was substituted. Hence, benzyl group proved to be a better substituent than cinnamic group.

4.6. Molecular modelling

In vitro evaluation of compound **6e** exhibited significant activity towards leukemia cancer cell line CCRF-CEM as shown in table 2. We have carried out docking[35] of compound **6e** in the active sites of toposimerase I (Topo I), (pdb ID 1A36) and ribonucleotide reductase

(RNR), (pdb ID 3K8T) that possesses potent chemotherapeutic efficancy against leukemia.[36] Docking of compound **6e** with active sites of Topo I (1A36) showed hydrogen bonding interactions of all the nitrogen atoms of triazole ring with amino group of D 652 (d = 2.58, 1.98 and 1.03 Å for N1, N2 and N3 respectively) amino acid residue. Moreover, nitrogen atom of triazole ring also showed hydrogen bonding with A 653 (d = 2.56 Å) amino acid residue (Table 4, Figure 3a).



a b

Figure 3. Compound **6e** docked in active site of Topo I (a) and RNR (b). H-bonds of compound **6e** with different amino acids residues are visible.

Table 4 Hydrogen bonding of compound **6e** with topoisomerase I (1A36) and ribonucleotide reductase (3K8T).

Groups of compound **6e**	1A36 Amino acid residue	3K8T Amino acid residue
Nitrogen atom 1 of triazole ring	D 652 (d = 2.58 Å)	A565 (d = 1.71 Å)
Nitrogen atom 2 of triazole ring	D 652 (d = 1.98 Å)	A565 (d = 1.65 Å)
Nitrogen atom 3 of triazole ring	D 652 (d = 1.03 Å) A653 (d = 2.56 Å)	
Nitrogen atom of amide group		G562 (d = 2.33 Å) P564 (d = 2.88 Å) S563 (d = 2.72 Å)
Carbonyl group of amide		P564 (d = 2.90 Å)

Docking of compound **6e** with active sites of RNR (3K8T) showed hydrogen bonding between nitrogen atom of amide group of **6e** with carbonyl groups of G 562 (d = 2.33 Å), P 564 (d = 2.88 Å) and S 563 (d = 2.72 Å) amino acid residues. Carbonyl group of amide of **6e**

also showed hydrogen bond interactions with P 564 (d = 2.90 Å) amino acid residue. Nitrogen atoms of triazole ring of **6e** also showed hydrogen bond interaction with amino group of A565 (d = 1.71 Å and 1.65 Å) amino acid residue (Table 4, Figure 3b).

4.7. Conclusion

A novel 2,3-disubstituted pyridines containing 1,2,3-triazole scaffold has been synthesized using one pot copper catalyzed azidation and CuAAC reaction in moderate to good yields. These compounds were well characterised by ^{1}H and ^{13}C NMR as well as mass spectrometry. All the synthesized compounds (**6a-h**, **11a-g**) showed good range of physicochemical properties. Out of these compounds **6a**, **6b**, **6e**, **6h**, **11b** and **11c** were evaluated for anticancer activity against 60 human cancer cell lines. Compound **6e** showed higher selectivity against leukemia cancer cell line CCRF-CEM. Molecular docking of this compound has also done in active site of topoisomerase I (Topo I, pdb ID 1A36) and ribonucleotide reductase (RNR, pdb ID 3K8T). These findings will be useful for the development of more potent antitumor agents in future.

4.8. Experimental section

4.8.1. Chemistry

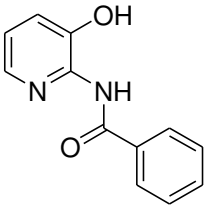
Melting points were determined in open capillaries and were uncorrected. Reactions were monitored by thin layer chromatography (TLC) with silica plate coated with silica gel GF 254. To prepare TLC plates, slurry of silica gel GF 254 was prepared with help of $CHCl_3$ and MeOH (4:1). TLC plates were dipped in the slurry, dried in an oven and used for comparing the reaction mixture and starting materials to check the progress of reaction. TLC plates were checked under UV lamp or were developed in iodine chambers. Column chromatography was performed with silica gel 60-120/100-200 mesh. Hexane/ ethyl acetate and chloroform/ methanol were adopted solvent systems. ^{1}H NMR and ^{13}C NMR spectra were recorded on Jeol ECS 400 NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvents. The chemical shifts were expressed in parts per million with TMS as internal reference and coupling constant (J) values are given in Hz. Spectral patterns are denoted as s = singlet; d = doublet; t = triplet; bs = broad singlet; dd = double doublet; dt = double triplet; q = quartet; m = multiplet. Mass Spectra of the synthesized compounds were recorded at Waters Micromass Q-Tof Micro.

Materials: 2-Amino-3-hydroxy pyridine, benzyl halides, *m*-CPBA, allyl bromide, cinnamic acid, hydroxybenzotriazole (HOBt) and (*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride) (EDC) were purchased from avra synthesis Pvt. Ltd., K_2CO_3, $TBAHSO_4$, $Cu(OAc)_2.H_2O$, sodium ascorbate, Na_2SO_4, $NaHCO_3$ were purchased from loba chemie. 1,10-Phenanthroline.H_2O was purchased from spectrochemicals. Propargyl bromide and DIPEA were purchased from sigma-Aldrich. Sodium azide was of merck grade. Solvents like chloroform, methanol, hexane and ethyl acetate were of LR grade and used after distillation. Acetonitrile was used of AR grade and purchased from sd-fine.

4.8.1.1. General procedure for synthesis of 3 and 4

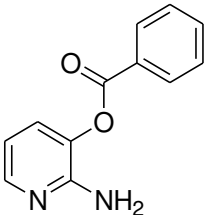
2-Amino-3-hydroxy pyridine (**1**) (2.0 g, 18.17 mmol) was dissolved in acetonitrile (50 mL), and K_2CO_3 (3 g, 21.75 mmol) and $TBAHSO_4$ (catalytic amounts) was added at room temperature. Benzoyl chloride (**2**) (20.15 mmol) was added to this suspension and heated to reflux for 8 h. Reaction was monitored with TLC. After completion of reaction, neutralized the reaction mixture with $NaHCO_3$ and extracted with chloroform. Washed the organic layer with H_2O, dried over Na_2SO_4 and concentrated, resulted in brown coloured solid of crude product. The crude residue was then purified by column chromatography using hexane: ethylacetate as eluents to give two products *N*-(3-hydroxy-pyridin-2-yl)benzamide (**3**) and benzoic acid 2-amino-pyridin-3-yl ester (**4**).

***N*-(3-Hydroxy-pyridin-2-yl)benzamide (3)**



Yield : 65 %; mp 130-133 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.62 (bs, 1H, NH), 8.34 (dd, ^2J = 4.80 Hz, ^3J = 1.48 Hz, 1H, ArH), 8.16 (dd, ^2J = 8.16 Hz, ^3J = 0.96 Hz, 2H, ArH), 7.83 (d, J = 7.04 Hz, 2H, ArH), 7.77 (dd, ^2J = 4.8 Hz, ^3J = 8.08 Hz, 1H, ArH), 7.60 (t, J = 1.16 Hz, 1H, ArH), 7.40 (dd, ^2J = 7.32 Hz, ^3J = 5.67 Hz, 1H, ArH).

Benzoic acid 2-amino-pyridin-3-yl ester (4)

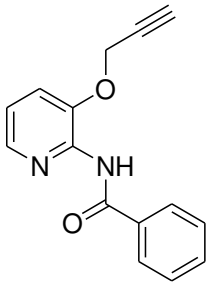


Yield : 10 %; mp 160-162 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 9.87 (bs, 2H, NH_2), 8.02 (dd, ^2J = 4.56 Hz, ^3J = 1.48 Hz, 2H, ArH), 7.62 (t, J = 1.20 Hz, 1H, ArH), 7.58 (t, J = 7.88 Hz, 1H, ArH), 7.51 (t, J = 7.88 Hz, 2H, ArH), 7.41 (dd, ^2J = 8.08 Hz, ^3J = 1.48 Hz, 1H, ArH), 7.11 (dd, ^2J = 8.08 Hz, ^3J = 4.64 Hz, 1H, ArH).

4.8.1.2. General procedure for synthesis of compound 5

N-(3-Hydroxy-pyridin-2-yl)benzamide (**3**) (2.0 g, 10 mmol) was refluxed with propargyl bromide (1.17 g, 10 mmol) in the presence of K_2CO_3 (2 g, 15 mmol) and $TBAHSO_4$ (catalytic amounts) using acetonitrile as solvent for 8 h. Acetonitrile was removed under vacuum and extracted with chloroform and water. Organic layer was separated, dried over Na_2SO_4, filtered and concentrated to get the crude product. The crude product was purified with column chromatography using ethylacetate: hexane (40:60) as eluents to give sticky brown liquid of *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**).

***N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (5)**

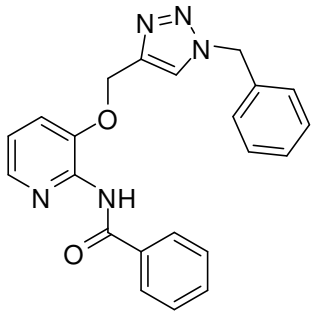


Yield : 70 %; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.72 (bs, 1H, NH), 8.16 (dd, ^2J = 4.60 Hz, ^3J = 1.36 Hz, 1H, ArH), 7.94 (d, J = 7.32 Hz, 2H, ArH), 7.57-7.52 (m, 1H, ArH), 7.50-7.46 (m, 2H, ArH), 7.39 (dd, ^2J = 8.24 Hz, ^3J = 1.36 Hz, 1H, ArH), 7.11 (dd, ^2J = 8.28 Hz, ^3J = 4.36 Hz, 1H, ArH), 4.80 (d, J = 2.28 Hz, 2H, O-CH_2), 2.60 (t, J = 2.72 Hz, 1H, ≡CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.6 (C=O), 143.3, 140.7, 134.8, 131.9, 128.6, 127.3, 119.8, 119.6 (ArC), 59.0 (C), 58.9 (≡ CH), 56.5 (O-CH_2); MS (EI) : m/z 253.2 (M^++1).

4.8.1.3. General procedure for synthesis of 6a-h

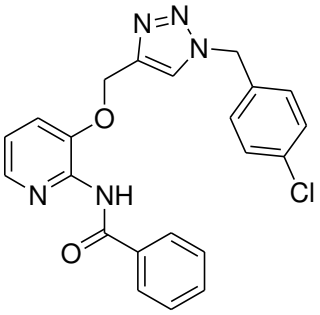
In a round bottom flask, *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**) (100 mg, 0.40 mmol), sodium azide (76 mg, 1.17 mmol), $Cu(OAc)_2.H_2O$ (5 mg, 5 mol%), 1,10-phenanthroline monohydrate (5 mg, 5 mol%) and sodium ascorbate (107 mg, 0.54 mmol) were added in EtOH:H2O (6:4, 10.0 ml) and stirred for 5 min at room temperature. Corresponding benzyl halide (0.40 mmol) was added to the reaction mixture and stirred at room temperature. Reaction time varied from 10 min to 24 h for various benzyl halides. After complete reaction (monitored by TLC), ice cold water was added to the reaction mixture till the product precipitate out, filtered it, and washed with cold water. Separated crude product was then dried over vacuum. The crude product was purified by column chromatography using ethylacetate: methanol (98:2) as eluents to get compounds **6a-h**.

***N*-(3-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methoxy)pyridin-2-yl)benzamide (6a)**



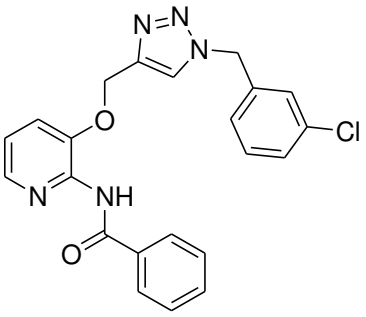
Yield : 55 %; mp 120-122 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.50 (bs, 1H, NH), 8.13 (dd, ^{2}J = 5.04 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.89-7.87 (m, 2H, ArH), 7.57-7.53 (m, 2H, ArH), 7.48-7.44 (m, 2H, ArH), 7.42 (dd, ^{2}J = 8.24 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.35 (t, J = 2.76 Hz, 3H, ArH), 7.23-7.21 (m, 2H, ArH), 7.09 (dd, ^{2}J = 8.24 Hz, ^{3}J = 5.04 Hz, 1H, ArH), 5.50 (s, 2H, CH_2), 5.29 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 144.6, 143.2, 142.1, 140.4, 134.6, 131.9, 129.1, 128.8, 128.6, 128.0, 127.4, 123.1, 120.4, 120.2 (ArC), 62.7 (O-CH_2), 54.2 (N-CH_2); MS (EI) : m/z 386.3 (M^{+}+1); Anal. Calc. for ($C_{22}H_{19}N_5O_2$): C, 68.56; H, 4.97; N, 18.17. Found: C, 68.73; H, 5.23; N, 18.34.

***N*-(3-((4-(4-Chlorobenzyl)-1*H*-1,2,3-triazol-1-yl)methoxy)pyridin-2-yl)benzamide (6b)**



Yield : 65%; mp 163-164 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.49 (bs, 1H, NH), 8.12 (d, J = 3.20 Hz, 1H, ArH), 7.88 (d, J = 7.32 Hz, 2H, ArH), 7.58 (s, 1H, ArH), 7.56 (d, J = 7.32 Hz, 1H, ArH), 7.48 (t, J = 7.32 Hz, 2H, ArH), 7.41 (d, J = 7.76 Hz, 1H, ArH), 7.30 (d, J = 8.28 Hz, 2H, ArH), 7.16 (d, J = 8.72 Hz, 2H, ArH), 7.09-7.06 (m, 1H, ArH), 5.46 (s, 2H, CH_2), 5.29 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.9 (C=O), 144.8, 143.5, 142.0, 134.8, 134.5, 132.6, 131.8, 129.9, 129.0, 128.7, 127.4, 123.2, 122.9, 120.7, 120.0 (ArC), 62.7 (O-CH_2), 53.5 (N-CH_2); MS (EI) : m/z 420.1 (M^{+}+1); Anal. Calc. for ($C_{22}H_{18}ClN_5O_2$): C, 62.93; H, 4.32; N, 16.68. Found: C, 62.79; H, 4.48; N, 16.59.

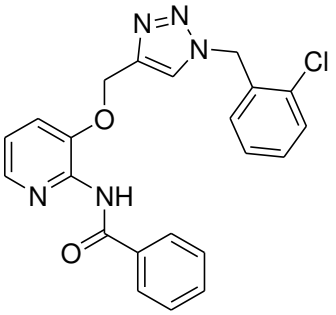
***N*-(3-((4-(3-Chlorobenzyl)-1*H*-1,2,3-triazol-1-yl)methoxy)pyridin-2-yl)benzamide (6c)**



Yield : 66%; mp 154-157 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.52 (bs, 1H, NH), 8.13 (dd, ^{2}J = 5.04 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.90 (d, J = 6.88 Hz, 2H, ArH), 7.61 (s, 1H, ArH), 7.57-7.54 (m, 1H, ArH), 7.48 (t, J = 7.36 Hz, 2H, ArH), 7.41 (dd, ^{2}J = 8.24 Hz, ^{3}J = 1.40 Hz, 1H, CH_2), 7.33-7.28 (m, 2H, ArH), 7.22 (t, J = 1.84 Hz, 1H, ArH), 7.10 (dd, ^{2}J = 8.24 Hz, ^{3}J = 5.04 Hz, 2H, ArH), 5.47 (s, 2H, CH_2), 5.31 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.9 (C=O), 144.8, 143.5, 142.1, 140.7, 140.3, 136.1, 134.9, 134.6, 131.8, 131.0, 129.0, 128.4, 127.4, 123.0, 121.2, 120.0 (ArC), 62.7 (O-CH_2), 53.5 (N-CH_2); MS (EI) : m/z 420.1

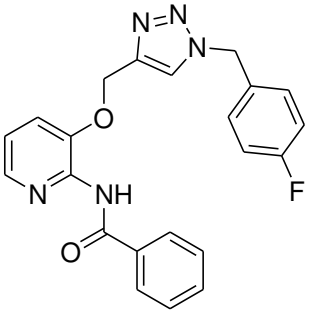
(M^++1); Anal. Calc. for ($C_{22}H_{18}ClN_5O_2$): C, 62.93; H, 4.32; N, 16.68. Found: C, 62.71; H, 4.53; N, 16.47.

***N*-(3-((4-(2-Chlorobenzyl)-1*H*-1,2,3-triazol-1-yl)methoxy)pyridin-2-yl)benzamide (6d)**



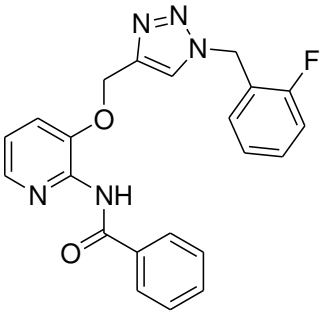
Yield : 64%; mp 125-126 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.53 (bs, 1H, NH), 8.13 (dd, ^2J = 5.04 Hz, ^3J = 1.40 Hz, 1H, ArH), 7.90 (d, J = 7.32 Hz, 2H, ArH), 7.66 (s, 1H, ArH), 7.57-7.54 (m, 1H, ArH), 7.48 (t, J = 2.76 Hz, 2H, ArH), 7.43-7.39 (m, 2H, ArH), 7.32-7.28 (m, 1H, ArH), 7.25-7.21 (m, 1H, ArH), 7.19 (dd, ^2J = 7.76 Hz, ^3J = 1.84 Hz, 1H, ArH), 7.09 (dd, ^2J = 8.24 Hz, ^3J = 5.04 Hz, 1H, ArH), 5.64 (s, 2H, CH_2), 5.30 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.9 (C=O), 144.6, 143.1, 142.1, 140.5, 134.6, 133.5, 131.9, 130.5, 130.4, 129.9, 128.6, 127.6, 127.4, 123.4, 120.4, 120.3 (ArC), 62.7 (O-CH_2), 51.5 (N-CH_2); MS (EI) : m/z 420.1 (M^++1); Anal. Calc. for ($C_{22}H_{18}ClN_5O_2$): C, 62.93; H, 4.32; N, 16.68. Found: C, 62.59; H, 4.57; N, 16.85.

***N*-(3-((4-(4-Fluorobenzyl)-1*H*-1,2,3-triazol-1-yl)methoxy)pyridin-2-yl)benzamide (6e)**



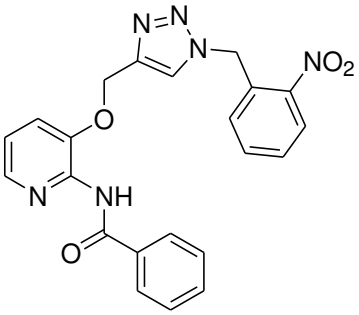
Yield : 82%; mp 165-168 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.52 (bs, 1H, NH), 8.12 (d, J = 4.56 Hz, 1H, ArH), 7.88 (d, J = 6.88 Hz, 2H, ArH), 7.57 (s, 1H, ArH), 7.56-7.54 (m, 1H, ArH), 7.47 (t, J = 7.32 Hz, 2H, ArH), 7.41 (dd, ^2J = 7.8 Hz, ^3J = 1.36 Hz, 1H, ArH), 7.22-7.19 (m, 2H, ArH), 7.09 (dd, ^2J = 8.28 Hz, ^3J = 5.04 Hz, 1H, ArH), 7.03-6.98 (m, 2H, ArH), 5.46 (s, 2H, CH_2), 5.29 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.9 (C=O), 144.8, 143.4, 142.0, 140.6, 140.3, 134.5, 131.8, 130.5, 130.0, 129.3, 128.9, 128.3, 127.4, 123.2, 122.8, 121.2, 120.7, 120.0, 119.9, 115.5, 115.3 (ArC), 62.7 (O-CH_2), 53.4 (N-CH_2); MS (EI) : m/z 404.2 (M^++1); Anal. Calc. for ($C_{22}H_{18}FN_5O_2$): C, 65.50; H, 4.50; N, 17.36. Found: C, 65.38; H, 4.33; N, 17.51.

***N*-(3-((4-(2-Fluorobenzyl)-1*H*-1,2,3-triazol-1-yl)methoxy)pyridin-2-yl)benzamide (6f)**



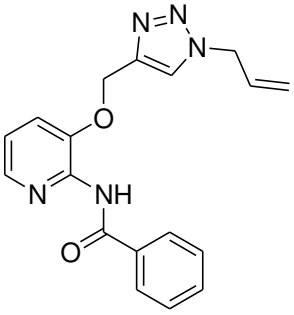
Yield : 78%; mp 132-134 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.53 (bs, 1H, NH), 8.13 (d, J = 4.56 Hz, 1H, ArH), 7.90 (d, J = 6.84 Hz, 2H, ArH), 7.66 (s, 1H, ArH), 7.57-7.53 (m, 1H, ArH), 7.48 (t, J = 7.32 Hz, 2H, ArH), 7.42 (dd, ^{2}J = 8.24 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.37-7.32 (m, 1H, ArH), 7.24 (dd, ^{2}J = 7.80 Hz, ^{3}J = 1.84 Hz, 1H, ArH), 7.13-7.05 (m, 3H, ArH), 5.56 (s, 2H, CH_2), 5.29 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 144.6, 143.2, 142.1, 140.7, 140.4, 134.7, 131.7, 129.9, 128.9, 128.8, 128.4, 127.4, 124.2, 123.2, 121.4, 121.0, 120.5, 119.8, 119.7 (ArC), 62.7 (O-CH_2), 47.8 (N-CH_2); MS (EI) : m/z 404.2 (M^++1); Anal. Calc. for ($C_{22}H_{18}FN_5O_2$): C, 65.50; H, 4.50; N, 17.36. Found: C, 65.33; H, 4.68; N, 17.18.

_N_-(3-((4-(2-Nitrobenzyl)-1_H_-1,2,3-triazol-1-yl)methoxy)pyridin-2-yl)benzamide (6g)



Yield : 60%; mp 119-123 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.56 (bs, 1H, NH), 8.14 (dd, ^{2}J = 7.76 Hz, ^{3}J = 1.32 Hz, 2H, ArH), 7.90 (d, J = 7.36 Hz, 2H, ArH), 7.84 (s, 1H, ArH), 7.58-7.50 (m, 3H, ArH), 7.48 (m, 3H, ArH), 7.10-7.05 (m, 2H, ArH), 5.90 (s, 2H, CH_2), 5.34 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 147.3, 144.6, 142.1, 134.5, 134.2, 131.8, 131.1, 130.0, 129.8, 129.0, 128.3, 127.4, 125.5, 125.2, 124.3, 120.1 (ArC), 62.7 (O-CH_2), 51.0 (N-CH_2); MS (EI) : m/z 431.4 (M^++1); Anal. Calc. for ($C_{22}H_{18}N_6O_4$): C, 61.39; H, 4.22; N, 19.53. Found: C, 61.47; H, 4.39; N, 19.76.

_N_-(3-((1-Allyl-1_H_-1,2,3-triazol-4-yl)methoxy)pyridin-2-yl)benzamide (6h)

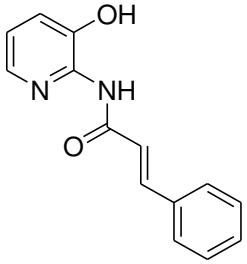


Yield : 55%; mp 117-120 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (bs, 1H, NH), 8.13 (d, J = 4.12 Hz, 1H, ArH), 7.92-7.90 (m, 2H, ArH), 7.65 (s, 1H, ArH), 7.57-7.53 (m, 1H, ArH), 7.49-7.46 (m, 2H, ArH), 7.43 (dd, ^{2}J = 7.80 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.09 (dd, ^{2}J = 8.28 Hz, ^{3}J = 4.56 Hz, 1H, ArH), 6.00-5.93 (m, 1H, CH), 5.33-5.24 (m, 4H, 2xCH_2), 4.96-4.94 (m, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.9 (C=O), 144.8, 143.1, 142.1, 140.5, 134.6, 131.9, 130.7, 128.6, 127.4, 123.0, 120.5, 120.2 (ArC), 62.7 (O-CH_2), 52.8 (N-CH_2); MS (EI) : m/z 336.3 (M^++1); Anal. Calc. for ($C_{18}H_{17}N_5O_2$): C, 64.47; H, 5.11; N, 20.88. Found: C, 64.39; H, 5.39; N, 20.65.

4.8.1.4. General procedure for synthesis of compound 8

In a 100 ml round bottom flask, cinnamic acid (2.6 g, 18 mmol) was stirred at 0 ^{o}C along with HOBt (5.4 g, 40 mmol), EDC (6.2 g, 40 mmol) and DIPEA (2.5 g, 20 mmol) in the presence of 50 ml CH_2Cl_2. 2-Amino-3-hydroxy pyridine (**1**) (2.0 g, 18 mmol) was added to above suspension and stirred at room temperature for 12 h. After completion of reaction, the reaction mixture was extracted with water. Organic layer was separated, dried over Na_2SO_4, filtered and concentrated to get crude product. The crude product was purified with column chromatography resulted into yellow solid of *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (**8**).

***N*-(3-Hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (8)**

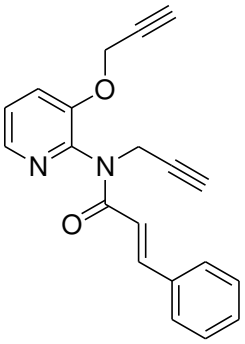


Yield : 80%; mp 145-147 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.90 (bs, 1H, OH), 10.20 (bs, 1H, NH), 7.94 (d, *J* = 4.12 Hz, 1H, ArH), 7.88 (d, *J* = 15.12 Hz, 1H, ArH), 7.52-7.50 (m, 2H, ArH), 7.44-7.38 (m, 4H, ArH), 7.19 (dd, ^{2}J = 7.80 Hz, ^{3}J = 4.56 Hz, 1H, ArH), 6.75 (d, *J* = 15.60 Hz, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.3 (C=O), 145.4, 145.1, 140.5, 138.3, 138.2, 133.9, 131.0, 130.3, 129.3, 128.7, 128.6, 128.4, 128.0, 127.1, 123.4, 122.2, 118.8, 118.5, 117.4 (ArC).

4.8.1.5. General method for synthesis of compounds 9 and 10

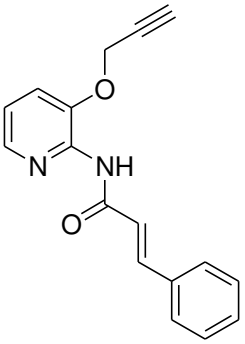
N-(3-Hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (**8**) (2 g, 10 mmol) was treated with propargyl bromide (1.17 g, 10 mmol) in the presence of K_2CO_3 (2 g, 15 mmol) and $TBAHSO_4$ (catalytic amounts) using acetonitrile as solvent. When this reaction was carried out at room temperature for 8 h, the resulted compounds were obtained as yellow solid of 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide (**10**) and 3-phenyl-*N*-prop-2-ynyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide (**9**) in 55% and 10% yields respectively. But when the same reaction was carried out at reflux temperature for 8 h, it resulted in formation of yellowish liquid of 3-phenyl-*N*-prop-2-ynyl-N-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide (**9**) in 60% yield with traces of compound **10**. Acetonitrile was removed under vacuum, extracted with $CHCl_3$ and water, separated the organic layer and dried over Na_2SO_4 to get the crude product. Column chromatography was done in order to get the corresponding compounds **9** and **10**.

3-Phenyl-*N*-prop-2-ynyl-*N*-(3-prop-2-ynyloxy-pyridin-2-yl)-acrylamide (9)



Yield : 60 %; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.72 (dd, 1H, ^{2}J = 4.60 Hz, ^{3}J = 1.40 Hz, ArH), 7.74 (d, 1H, J = 15.12 Hz, ArH), 7.53 (dd, 1H, ^{2}J = 8.24 Hz, ^{3}J = 1.36 Hz, ArH), 7.38 (dd, 1H, ^{2}J = 8.24 Hz, ^{3}J = 4.56 Hz, ArH), 7.34 (dd, 2H, ^{2}J = 5.96 Hz, ^{3}J = 2.28 Hz, ArH), 7.28 (d, 3H, J = 2.28 Hz, ArH), 6.23 (d, 1H, J = 15.12 Hz, ArH), 4.74 (d, 2H, J = 2.28 Hz, O-CH_2), 4.71 (d, 2H, J = 2.28 Hz, N-CH_2), 2.40 (t, 1H, J = 2.28 Hz, CH), 2.14 (d, 1H, J = 2.72 Hz, CH).

3-Phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)-acrylamide (10)

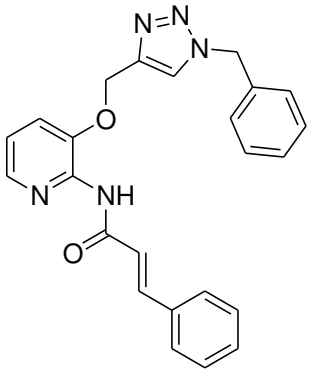


Yield : 55%; mp 129-131 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, J = 4.56 Hz, 2H, ArH, NH), 7.85 (d, J = 16.04 Hz, 1H, ArH), 7.60-7.57 (m, 2H, ArH), 7.41-7.34 (m, 4H, ArH), 7.32 (dd, ^{2}J = 8.24 Hz, ^{3}J = 1.36 Hz, 1H, ArH), 7.05 (dd, ^{2}J = 8.24 Hz, ^{3}J = 5.04 Hz, 1H, ArH), 4.79 (d, J = 2.28 Hz, 2H, O-CH_2), 2.60 (t, J = 2.28 Hz, 1H, CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 143.3, 142.3, 142.2, 140.1, 134.8, 129.8, 128.7, 128.1, 120.6, 119.2, 119.1 (ArC), 56.3 (O-CH_2); MS (EI) : m/z 279.3 (M^{+}+1).

4.8.1.6. General procedure for synthesis of 11a-g

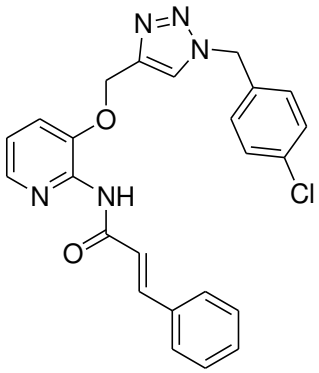
In a round bottom flask, 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)-acrylamide (100 mg, 0.35 mmol), sodium azide (76 mg, 1.02 mmol), $Cu(OAc)_2.H_2O$ (5 mg, 5 mol%), 1,10-phenanthroline monohydrate (5 mg, 5 mol%) and sodium ascorbate (107 mg, 0.47 mmol) were added in EtOH:H2O (6:4, 5 ml) and stirred for 5 min at room temperature. Corresponding benzyl halide (0.40 mmol) was added to the reaction mixture and stirred at room temperature. After complete reaction (monitored by TLC), ice cold water was added to reaction mixture till the product was precipitated out, filtered it, and washed with cold water. Separated crude product was then dried over vacuum. The crude product was purified with column chromatography (ethylacetate: methanol 98:2) to get compounds **11a-g**.

***N*-[3-(1-Benzyl-1*H*-[1,2,3]triazol-4-ylmethoxy)-pyridin-2-yl]-3-phenyl-acrylamide (11a)**

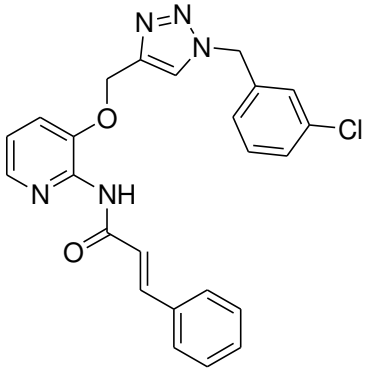


Yield : 50%; mp 125-126 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11 (bs, 1H, NH), 8.06 (dd, ^{2}J = 4.56 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 7.82 (d, J = 15.6 Hz, 1H, ArH), 7.59-7.57 (m, 3H, ArH), 7.41-7.37 (m, 4H, ArH), 7.36-7.32 (m, 4H, ArH), 7.31-7.27 (m, 2H, ArH), 7.03 (dd, ^{2}J = 8.28 Hz, ^{3}J = 5.04 Hz, 1H, ArH), 5.54 (s, 2H, CH_2), 5.26 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 143.2, 143.0, 142.1, 139.9, 134.9, 134.0, 129.9, 129.2, 128.9, 128.7, 128.1, 123.2, 120.6, 119.5 (ArC), 62.4 (O-CH_2), 54.3 (N-CH_2); MS (EI) : m/z 412.2 (M^{+}+1); Anal. Calc. for ($C_{24}H_{21}N_5O_2$): C, 70.06; H, 5.14; N, 17.02. Found: C, 70.27; H, 4.82; N, 17.37.

***N*-{3-[1-(4-Chloro-benzyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-pyridin-2-yl}-3-phenyl-acrylamide (11b)** Yield : 58%; mp 160-162 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (bs, 1H, NH), 8.06 (dd, ^{2}J = 4.80 Hz, ^{3}J = 1.60 Hz, 1H, ArH), 7.82 (d, J = 15.56 Hz, 1H, ArH), 7.60-7.59 (m, 2H, ArH), 7.57 (d, J = 1.80 Hz, 1H, ArH), 7.41-7.39 (m, 2H, ArH), 7.38 (d, J = 1.36 Hz, 2H, ArH), 7.37-7.34 (m, 2H, ArH), 7.33 (t, J = 2.28 Hz, 1H, ArH), 7.23-7.21 (m, 2H, ArH), 7.04 (dd, ^{2}J = 8.24 Hz, ^{3}J = 5.04 Hz, 1H, ArH), 5.51 (s, 2H, CH_2), 5.26 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 143.3, 143.2, 142.1, 140.0, 135.0, 134.9, 132.5, 129.9, 129.5, 129.4, 128.8, 128.1, 123.1, 120.5, 119.6, 119.5 (ArC), 62.4 (O-CH_2), 53.6 (N-CH_2); MS (EI) : m/z 446.3 (M^{+}+1); Anal. Calc. for ($C_{24}H_{20}ClN_5O_2$): C, 64.65; H, 4.52; N, 15.71. Found: C, 64.37; H, 4.73; N, 15.82.

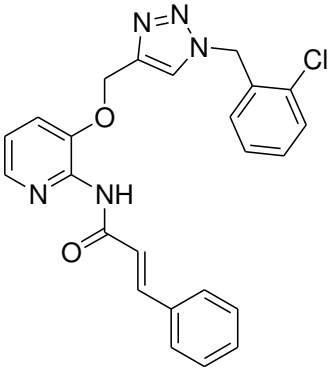


***N*-{3-[1-(3-Chloro-benzyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-pyridin-2-yl}-3-phenyl-acrylamide (11c)** Yield : 57%; mp 161-163 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (bs, 1H, NH), 8.06 (dd, ^{2}J = 5.04 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 7.82 (d, J = 16.04 Hz, 1H, ArH), 7.66 (s, 1H, ArH), 7.59-7.56 (m, 2H, ArH), 7.40-7.39 (m, 2H, ArH), 7.38 (d, J = 1.84 Hz, 3H, ArH), 7.32-7.30 (m, 2H, ArH), 7.28 (s, 1H, ArH), 7.15 (d, J = 6.88 Hz, 1H, ArH), 7.06 (dd, ^{2}J = 7.80 Hz, ^{3}J = 5.04 Hz, 1H, ArH), 5.51 (s, 2H, CH_2), 5.29 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 145.9, 143.6, 143.4, 143.3, 142.2,

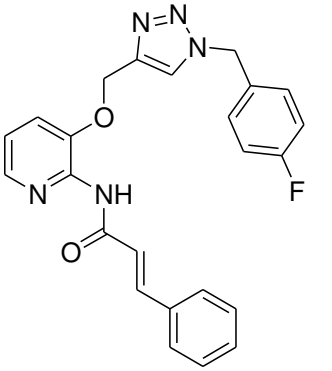


139.8, 136.0, 135.0, 134.8, 130.4, 129.9, 129.1, 128.8, 128.7, 128.2, 128.1, 126.1, 123.3, 120.5, 119.8 (ArC), 62.5 (O-CH_2), 53.6 (N-CH_2); MS (EI) : m/z 446.3 (M^++1); Anal. Calc. for ($C_{24}H_{20}ClN_5O_2$): C, 64.65; H, 4.52; N, 15.71. Found: C, 64.23; H, 4.29; N, 15.90.

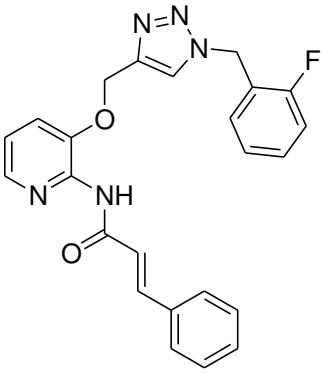
***N*-{3-[1-(2-Chloro-benzyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-pyridin-2-yl}-3-phenyl-acrylamide (11d)** Yield : 56%; mp 128-129 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.09 (bs, 1H, NH), 8.07 (dd, ^2J = 5.04 Hz, ^3J = 0.92 Hz, 1H, ArH), 7.59 (dd, ^2J = 7.8 Hz, ^3J = 1.36 Hz, 1H, ArH), 7.43 (d, J = 1.36 Hz, 1H, ArH), 7.41-7.39 (m, 2H, ArH), 7.38 (d, J = 1.36 Hz, 2H, ArH), 7.33 (dd, ^2J = 6.88 Hz, ^3J = 2.28 Hz, 1H, ArH), 7.29-7.27 (m, 2H, ArH), 7.27 (d, J = 1.36 Hz, 1H, ArH), 7.25-7.23 (m, 2H, ArH), 7.16-7.11 (m, 1H, ArH), 7.04 (dd, ^2J = 7.8 Hz, ^3J = 5.04 Hz, 1H, ArH), 5.68 (s, 2H, CH_2), 5.27 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.8 (C=O), 143.2, 142.9, 140.0, 134.9, 131.9, 130.5, 130.4, 130.0, 129.8, 129.2, 128.7, 128.1, 127.6, 123.5, 120.6, 119.5 (ArC), 62.4 (O-CH_2), 51.6 (N-CH_2); MS (EI) : m/z 446.3 (M^++1); Anal. Calc. for ($C_{24}H_{20}ClN_5O_2$): C, 64.65; H, 4.52; N, 15.71. Found: C, 64.73; H, 4.17; N, 15.33.



***N*-{3-[1-(4-Fluoro-benzyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-pyridin-2-yl}-3-phenyl-acrylamide (11e)** Yield : 58%; mp 169-172 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.20 (bs, 1H, ArH), 8.05 (d, J = 4.56 Hz, 1H, ArH), 7.81 (d, J = 15.56 Hz, 1H, ArH), 7.62 (s, 1H, ArH), 7.58-7.56 (m, 2H, ArH), 7.39 (d, J = 1.84 Hz, 2H, ArH), 7.38-7.36 (m, 2H, ArH), 7.29-7.27 (m, 3H, ArH), 7.07 (d, J = 8.68 Hz, 2H, ArH), 7.06 (d, J = 2.28 Hz, 1H, ArH), 5.50 (s, 2H, CH_2), 5.26 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.1 (C=O), 161.6, 143.4, 143.2, 143.1, 142.1, 139.8, 134.8, 130.1, 130.0, 129.9, 129.8, 128.7, 128.1, 126.5, 123.1, 120.6, 119.6, 116.3, 114.0 (ArC), 62.5 (O-CH_2), 53.5 (N-CH_2); MS (EI) : m/z 430.2 (M^++1); Anal. Calc. for ($C_{24}H_{20}FN_5O_2$): C, 67.12; H, 4.69; N, 16.31. Found: C, 66.83; H, 4.53; FN, 16.43.

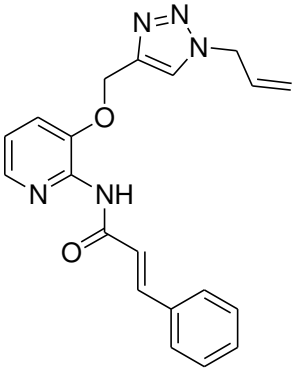


***N*-{3-[1-(2-Fluoro-benzyl)-1*H*-[1,2,3]triazol-4-ylmethoxy]-pyridin-2-yl}-3-phenyl-acrylamide (11f)** Yield : 55%; mp 138-140 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.16 (bs, 1H,



NH), 8.05 (s, 1H, ArH), 7.81 (d, *J* = 15.60 Hz, 1H, ArH), 7.70 (s, 1H, ArH), 7.58 (d, *J* = 2.40 Hz, 1H, ArH), 7.37-7.28 (m, 7H, ArH), 7.16-7.07 (m, 4H, ArH), 5.60 (s, 2H, CH_2), 5.26 (s, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.7 (C=O), 159.3, 143.1, 143.0, 134.9, 131.1, 131.0, 130.6, 129.9, 128.7, 128.1, 124.9, 123.4, 121.5, 121.3, 119.5, 116.0, 115.7 (ArC), 62.4 (O-CH_2), 47.9 (N-CH_2); MS (EI) : m/z 430.2 (M^++1); Anal. Calc. for ($C_{24}H_{20}FN_5O_2$): C, 67.12; H, 4.69; N, 16.31. Found: C, 67.43; H, 4.53; N, 16.56.

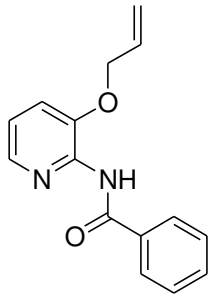
***N*-[3-(1-Allyl-1*H*-[1,2,3]triazol-4-ylmethoxy)-pyridin-2-yl]-3-phenyl-acrylamide (11g)**



Yield : 45%; mp 120-122 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.45 (bs, 1H, NH), 8.29 (dd, ^2J = 7.76 Hz, ^3J = 1.80 Hz, 1H, ArH), 8.12 (s, 1H, ArH), 8.07-8.03 (m, 1H, ArH), 7.82 (s, 1H, ArH), 7.81-7.77 (m, 3H, ArH), 7.68 (dd, ^2J = 8.28 Hz, ^3J = 4.60 Hz, 1H, ArH), 7.60-7.57 (m, 1H, ArH), 7.42-7.37 (m, 2H, ArH), 6.10-5.87 (m, 1H, CH), 5.57-5.42 (m, 1H, CH), 5.37-5.31 (m, 3H, CH, CH_2), 5.01-4.91 (m, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.9 (C=O), 159.4, 143.3, 143.1, 140.0, 135.0, 131.2, 130.8, 130.0, 128.8, 128.3, 125.0, 123.6, 121.6, 121.5, 120.8, 119.7, 116.1, 115.9 (ArC), 62.6 (O-CH_2), 48.0 (N-CH_2); MS (EI) : m/z 362.2 (M^++1). Anal. Calc. for ($C_{20}H_{19}N_5O_2$): C, 66.47; H, 5.30; N, 19.38. Found: C, 66.14; H, 5.11; N, 19.52.

4.8.1.7. Procedure for synthesis of *N*-(3-allyloxy-pyridin-2-yl)-benzamide (12) *N*-(3-Hydroxy-pyridin-2-yl)-benzamide (**3**) (0.50 g, 2.30 mmol) was dissolved in acetonitrile, K_2CO_3 (0.38 g, 2.76 mmol) and $TBAHSO_4$ (catalytic amount) were added to it at room temperature. Allyl bromide (2.80 mmol) was added to reaction mixture and reaction was shifted to reflux for 12h. After completion of reaction (TLC), acetonitrile was removed under vacuum, residue was extracted with chloroform and water to obtained crude product which was purified with column chromatography (hexane: ethylacetate), resulted white solid of pure *N*-(3-allyloxy-pyridin-2-yl)-benzamide (**12**).

***N*-(3-Allyloxy-pyridin-2-yl)-benzamide (12)**

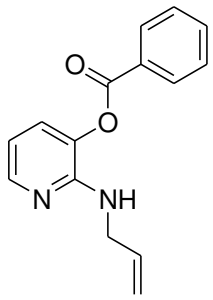


White solid (68 %); mp 65-67 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.56 (bs, 1H, NH), 8.14 (d, *J* = 6.80 Hz, 1H, ArH), 7.93 (d, *J* = 10.40 Hz, 2H, ArH), 7.58-7.47 (m, 3H, ArH), 7.21 (d, *J* = 10.4, 1H, ArH), 7.06 (dd, ^{2}J = 10.8 Hz, ^{3}J = 6.40 Hz, 1H, ArH), 6.09-5.98 (m, 1H, CH), 5.39 (m, 2H, CH_2), 4.66 (d, *J* = 5.60 Hz, 2H, O-CH_2); MS (EI) : m/z 255.2 (M^{+}+1).

4.8.1.8. Procedure for synthesis of benzoic acid 2-allylamino-pyridin-3-yl ester (13)

Benzoic acid 2-amino-pyridin-3-yl ester (**4**) (0.10 g, 0.46 mmol) was dissolved in acetonitrile in presence of K_2CO_3 (0.09 g, 0.56 mmol) and $TBAHSO_4$ (catalytic amounts). Allyl bromide (0.56 mmol) was added to this reaction mixture and was refluxed for 12 h. After completion of reaction (TLC), acetonitrile was removed under vacuum, residue was extracted with chloroform and water to get crude product which was further purified using column chromatography (hexane: ethylacetate), resulted in pure product of benzoic acid 2-allylamino-pyridin-3-yl ester (**13**).

Benzoic acid 2-allylamino-pyridin-3-yl ester (13)

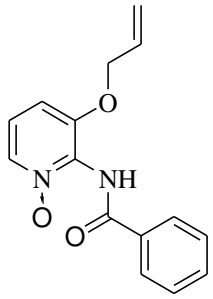


White solid (55 %); mp 73-75 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.20 (bs, 1H, NH), 8.25 (dd, ^{2}J = 10.20 Hz, ^{3}J = 2.36 Hz, 2H, ArH), 8.09 (d, *J* = 7.24 Hz, 1H, ArH), 7.51-7.39 (m, 2H, ArH), 7.36 (dd, ^{2}J = 6.28 Hz, ^{3}J = 1.40 Hz, 1H, ArH), 7.30 (dd, ^{2}J = 7.92 Hz, ^{3}J = 1.40 Hz, 1H, ArH), 6.86 (dd, ^{2}J = 7.92 Hz, ^{3}J = 6.40 Hz, 1H, ArH), 6.14-6.04 (m, 1H, CH), 5.32-5.23 (m, 2H, CH_2), 5.10 (d, *J* = 5.96 Hz, 2H, N-CH_2); MS (EI) : m/z 255.2 (M^{+}+1).

4.8.1.9. Procedure for synthesis of *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide (14)

N-(3-Allyloxy-pyridin-2-yl)-benzamide (**12**) (0.10 g, 0.40 mmol) was dissolved in dry chloroform. *m*-CPBA (0.20 g, 1.18 mmol) was added to this mixture with vigorous stirring at room temperature for 24 h. After completion of reaction, reaction mixture was neutralized with $NaHCO_3$, filtered and extracted with chloroform and water, resulted in crude solid. Crude product was purified using column chromatography using ethylacetate as eluent to afford brown solid of *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide (**14**).

***N*-(3-Allyloxy-1-oxy-pyridin-2-yl)-benzamide (14)**



Light brown solid (45 %); mp 69-71 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.23 (bs, 1H, NH), 7.97-7.93 (m, 2H, ArH), 7.58 (t, *J* = 7.36, 1H, ArH), 7.48 (t, *J* = 7.32 Hz, 2H, ArH), 7.04 (t, *J* = 7.32 Hz, 2H, ArH), 6.97 (dd, ^{2}J = 8.72 Hz, ^{3}J = 0.92 Hz, 1H, ArH), 6.02-5.93 (m, 1H, CH), 5.42-5.36 (dq, ^{2}J = 17.42 Hz, ^{3}J = 1.84 Hz, 1H, CH_2), 5.26-5.22 (dq, ^{2}J = 10.54 Hz, ^{3}J = 1.40 Hz, 1H, CH_2), 4.68 (dt, ^{2}J = 5.04 Hz, ^{3}J = 1.40 Hz, 2H, O-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.5 (C=O), 157.8, 152.1, 136.8, 133.7, 133.2, 125.7, 124.1, 121.6, 118.1, 117.1, 114.7, 111.7, 100.2 (ArC), 45.8 (O-CH_2); MS (EI) : m/z 271.2 (M^{+}+1).

4.8.2. Biology

In vitro anticancer screening at NCI was a two-stage process, beginning with the evaluation of all compounds against the 60 cell lines at a single dose of 10 μM. The output from the single dose screen was reported as a mean graph and was available for analysis by the COMPARE program. Compounds which exhibited significant growth inhibition were evaluated against the 60 cell panel at five concentration levels. The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated into 96 well microtiter plates in 100 mL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37^{o} C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs were solubilized in dimethyl sulfoxide at 400 fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/ml gentamicin. Additional four, 10-fold or ½ log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions were added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations.

Following drug addition, the plates were incubated for an additional 48 h at 37 ^{o}C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 ^{o}C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gently adding 50 µL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as:

$[(Ti - Tz)/(C-Tz)] \times 100$ for concentrations for which $Ti >= Tz$

$[(Ti – Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$:

Three dose response parameters were calculated for each experimental agent. Growth inhibition of 50% (GI50) was calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from $Ti = Tz$. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from $[(Ti-Tz) / Tz] \times 100 = -50$.

4.8.3. Molecular modelling

Coordinates from the X-ray crystal structure of topoisomerase I (Topo I, pdb ID 1A36), and ribonucleotide reductase (RNR, pdb ID 3K8T) were taken from the RCSB Protein Data Bank. Compounds were constructed with the builder toolkit of the software package ArgusLab 4.0.1 (www.arguslab.com) and energy minimized using the semiempirical

quantum mechanical method PM3. The monomeric structure of the enzyme was chosen, and the active site was defined around the ligand. The molecule to be docked in the enzyme active site was inserted into the work space carrying the structure of the enzyme. The docking program implements an efficient grid-based docking algorithm, which approximates an exhaustive search within the free volume of the binding site cavity. The conformational space was surveyed by the geometry optimization of the flexible ligand (rings are treated as rigid) in combination with the incremental construction of the ligand torsions. Thus, docking occurred between the flexible ligand parts of the compound and enzyme. The ligand orientation was determined by a shape scoring function based on Ascore and the final positions were ranked by lowest interaction energy values. Hydrogen bond between the compound and enzyme were explored by distance measurements.

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SUMMARY

Introduction

Cancer causing cells are abnormal and they multiply out of control. A number of such abnormal cells may develop from the parent abnormal cell. If this multiplication of cells does not stop, it forms a clump of abnormal cells called tumour. Anticancer drugs ideally, are toxic to cancer cells and they either kill cancer cells or modify their growth. Sometimes they affect the genetic material of the cell directly. Some well known cytotoxic drugs are cisplatin, carboplatin, etoposide, topotecan, doxorubicin, epirubicin etc.

Various drugs used for cancer treatment are indulged with number of drawbacks which include low selectivity, unwanted side effects and more toxicity. Therefore, major progress in cancer treatment requires new drugs to overcome the factors which previously present drugs are lacking.[1-8]

Considering cancer cells the highly proliferative tissues, DNA becomes one of the most efficient biological targets for developing anticancer agents.[9] In last decades, great attention has been paid to design and synthesize promising DNA-targeted antitumor agents.[2,10-11] Aromatic heterocycles position at proper sites or interact with topoisomerases and therefore interfere with DNA replication and transcription.[12-15] Molecules that can bind with DNA, are of immense interest in the field of medicinal or clinical chemistry.[16,17] Being the genetic instructor, DNA is involved in gene transcription,[18] mutagenesis,[19] gene expression,[20,21] etc. Study of type of the interactions in new molecules and DNA provide significant information for designing and synthesizing more efficient therapeutic agents capable of controlling various gene expression.[22,23] Naphthalimides are the most promising members of this class which interact with DNA and perform a tremendous role as anticancer agents. Nitrogen containing pyridines and 1,2,3-triazoles are also well known for their biological activities and played a vital role as anticancer agents.[24-26]

In present investigation, we have synthesized benzimidazole bearing naphthalimide analogues as well as *N*-allyl naphthalimides. These analogues were substituted with variety of primary and secondary amines. Keeping in mind, their DNA intercalating properties, UV-Visible and fluorescence spectrophotometry and thermal denaturation experiments were performed with the most active compounds. Similarly, 2,3-disubstituted pyridine analogue appended 1,2,3-triazole motifs were synthesized. Characterization of these molecules was done with different spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR and mass

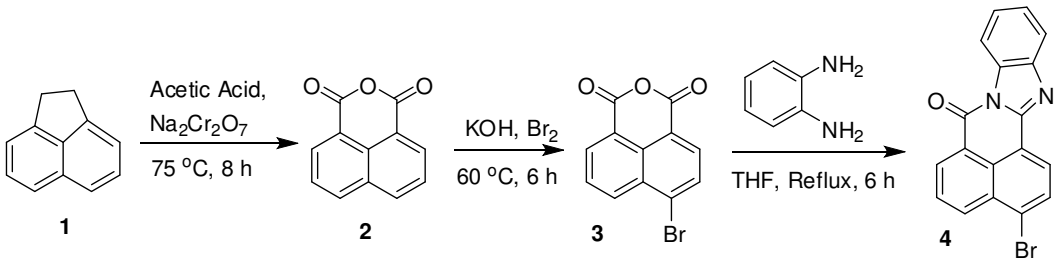
spectroscopy. The synthesized molecules were evaluated *in vitro* over 60 human cancer cell lines. ct-DNA studies have been done for finding the probable interaction with DNA. Structure-activity relationship studies were used to identify the structural features of these novel compounds. In order to have an insight into the molecular interactions of these compounds, their docking were also planned to scrutinize the mode of interaction of compound with amino acids in the active site of the enzyme,

Results and Discussion

Chapter 2: covers synthesis of benzimidazole bearing naphthalimide analogues, their characterization, *in vitro* evaluation, DNA studies, SAR studies and molecular modeling

2.1. Synthesis of 3-substituted-benzo[de]benzo[4,5]imidazo[2,1-a]isoquinolin-7-one

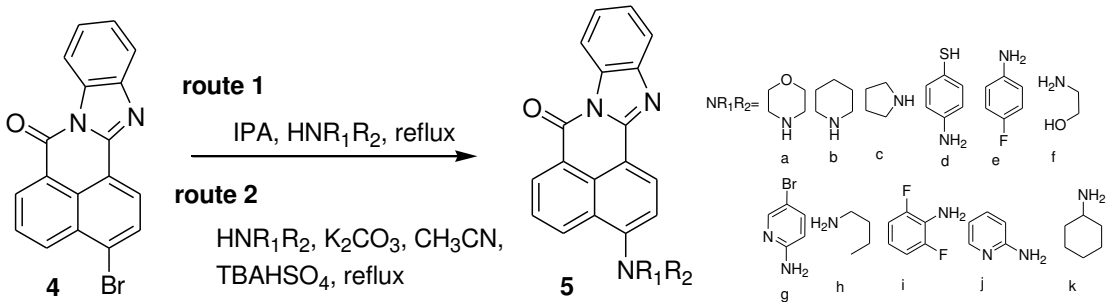
The present research work is based on the synthesis of naphthalimide analogues. To a solution of acenaphthene and acetic acid, sodium dichromate was added with stirring at room temperature. The suspension was heated to 75 ^{o}C for 8 h. After the completion of reaction, cold water was added to the reaction mixture to get solid, filtered and washed with water. Yellow solid product of 1,8-naphthalic anhydride (**2**) was obtained. To a cooled solution of 1,8-naphthalic anhydride in KOH bromine was charged with dropping funnel. The reaction mixture was heated to 60 ^{o}C for 6 h and then acidified with HCl solution. Solid was separated out, filtered to get crude product. The solid residue was heated with 5% NaOH solution, filtered and neutralized with HCl solution. Again filtered and washed with cold water, dried to get yellow powder of 4-bromo-1,8-naphthalic anhydride (**3**) (2.05 g, 82%, m.p. 117-119 ^{o}C). 4-Bromo-1,8-naphthalic anhydride (2.00 g, 5.0 mmol) and *o*-phenylenediamine (0.618 g, 5.0 mmol) in THF was refluxed for 6 h. After the completion of reaction (TLC), cooled the reaction mixture to get crude solid, filtered and washed with THF to afford pure 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (**4**) (Scheme 1).



Scheme 1

A mixture of 3-bromo-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one (0.25 g, 0.7 mmol), potassium carbonate, $TBAHSO_4$ (catalytic) and corresponding amine (0.84 mmol) in

acetonitrile was heated to reflux for 8-12 h. After the completion of reaction, reaction mixture was extracted with chloroform, separated the organic layer, dried over Na_2SO_4, filtered and concentrated to get solid crude product. The residue was column chromatographed on silica gel using hexane: ethylacetate as eluents to afford pure compounds of 3-substituted-benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one **(5a-k)** (Scheme 2).



Scheme 2

2.2. Characterization

All the synthesized compounds were characterized by ^1H and ^{13}C NMR spectra (Bruker FT-NMR 400 MHz and JEOL ECS-400 MHz spectrometers) and mass spectra (Waters QTOF Micro).

2.3. Biological Evaluation

(a) Compound **5a-k** was subjected to the National Cancer Institute (NCI) disease-oriented human cells screening panel assay for *in vitro* antitumor activity. Preliminary *in vitro* one dose anticancer assay was performed in full NCI 60 cell panel representing leukemia, melanoma, lung, colon, brain, breast, ovary, kidney and prostate cancer in accordance with protocol of NCI, USA. The data reported as a mean graph of the percent growth of treated cells, and presented as percentage growth inhibition (GI %). Among the selected compounds, **5d** has proved to be most active and its activity was tested at five different concentrations. Compound **5d** was fivefold more active than the known drug 5-fluorouracil (**5-FU**) with GI_{50} and TGI values of 5.05 and 38.71 respectively as compared to GI_{50} and TGI values (22.6 and >100) for 5-fluorouracil.

(b) DNA binding properties of most active compound **5d** has been evaluated using UV-Visible and fluorescence spectrophotometry with calf thymus (ct) DNA alongwith thermal denaturation experiment was performed in order to know nature of interaction of compound. The derivative melting curve shows an increase of 8.2 ^oC in thermal melting temperature of ct-DNA (60 ^oC) upon addition of **5d** (68.2 ^oC). Both UV-Visible titrations and thermal denaturation experiments indicated strong intercalative nature of interactions between

compound **5d** and ct-DNA. Binding constant of **5d** with ct-DNA was log β = 5.32 calculated from titration data using Benesi-Hildebrand method whereas log β = 5.15 for amonafide which clearly indicated that compound **5d** intercalates more strongly than amonafide.

2.4. ADME-T prediction

The compliance of compounds **5a**, **5c-k** were also evaluated with different physico-chemical parameters; drug bioavailability parameters (Log P, Total Polar Surface Area) and other parameters such as molar refractivity, dipole moment, electron affinity and steric energy, determined by SciGress software. All the compounds exhibited good passive oral absorption. It was observed that antitumour activity of compound **5d** (5-dose studies) increased with increase in molar refractivity (117.0), electron affinity (1.7 eV) and Total Polar Surface Area (66.4) and decreased in steric energy (15.3). Lipophilicity did not exert any effect on activity in all compounds but hydrophilicity produced an important parameter for the activity. Comparison of these parameters with 5-fluorouracil proved that compound **5d** is superior in all aspects.

2.5. Structure activity relationship

The structure-activity relationship (SAR) emerged from the activity results revealed that combination of naphthalimide and benzimidazole was well tolerated for *in vitro* anticancer activity. It was concluded that primary and secondary amines at C4-position of naphthalimide played a key role in varying the efficiency of antitumour activities. Introduction of 4-amino-phenylsulfanyl group at C4-position of naphthalimide ring (**5d**) led to a broad spectrum of antitumour activity with respect to other primary amines. Compound **5d** showed stronger DNA intercalating properties than amonafide which may be explained on the basis of substitutions on C4-position in case of **5d**.

2.6. Molecular modelling

Molecular modelling study of most active compound **5d** was carried out in the active sites of topoisomerase I, topoisomerase II and ribonucleotide reductase. Docking of compound **5d** in the active sites of these enzymes indicated the probable mode of action of this compound for anticancer activities.

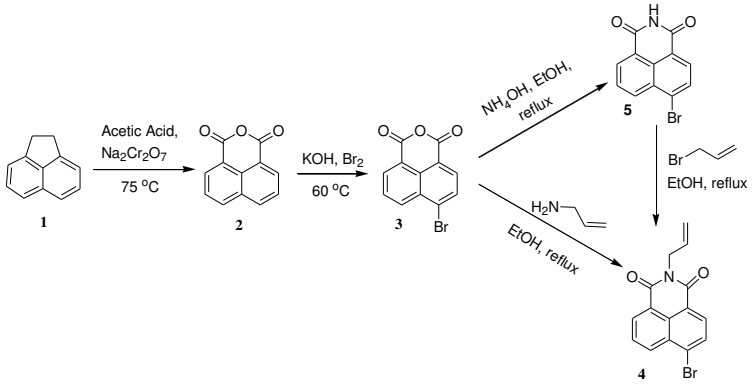
Chapter 3: covers synthesis of *N*-allyl naphthalimide analogues, their characterization, *in vitro* evaluation, DNA studies and molecular modeling

3.1. Synthesis of 2-allyl-6-substituted-benzo[de]isoquinoline-1,3-dione

Sodium dichromate was added to a solution of acenaphthene and acetic acid and heated to 75 ^{o}C for 8 h to obtain yellow coloured solid of 1,8-naphthalic anhydride. To a solution of 1,8-

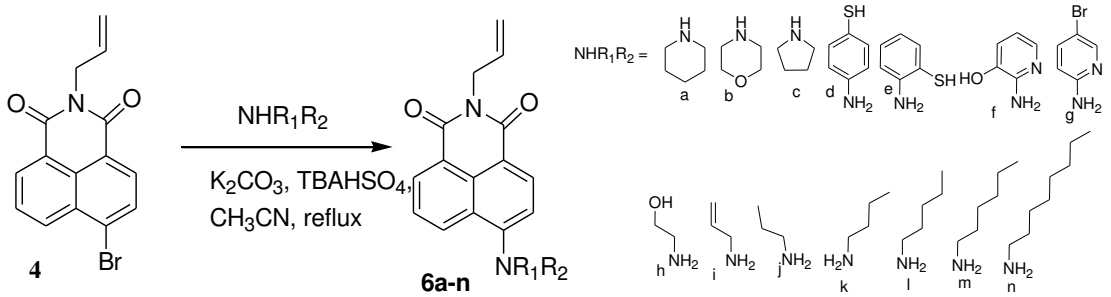
naphthalic anhydride and KOH, bromine was added drop-wise and heated to 60 ^{o}C for 6 h. After acidification with HCl, brown coloured solid was collected and heated with 60 ml of 5% NaOH solution, filtered and treated with HCl to get white solid of 4-bromo-1,8-naphthalic anhydride (**3**). Compound **3** was refluxed with allylamine in ethanol for 8 h which resulted in white compound of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (2.4 g, 80%, m.p. 128-130 ^{o}C) (**4**) (Scheme 3).

Alternatively, **3** was refluxed with ammonium hydroxide in ethanol to get 6-bromo-benzo[*de*]isoquinoline-1,3-dione (**5**) which was further refluxed with allylbromide in presence of EtOH to obtain white solid of 2-allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione (**4**).



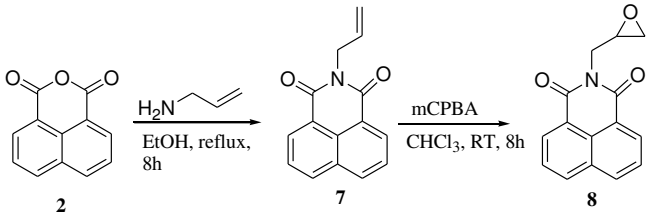
Scheme 3

2-Allyl-6-bromo-benzo[*de*]isoquinoline-1,3-dione was further refluxed with primary and secondary amines in the presence of K_2CO_3 in CH_3CN using $TBAHSO_4$ as catalyst for 8-12 h to obtain the crude product of **6a-n** (Scheme 4) which was then purified with column chromatography.



Scheme 4

3.2. Synthesis of 2-allyl-benzo[de]isoquinoline-1,3-dione and 2-oxiranylmethyl-benzo[de]isoquinoline-1,3-dione

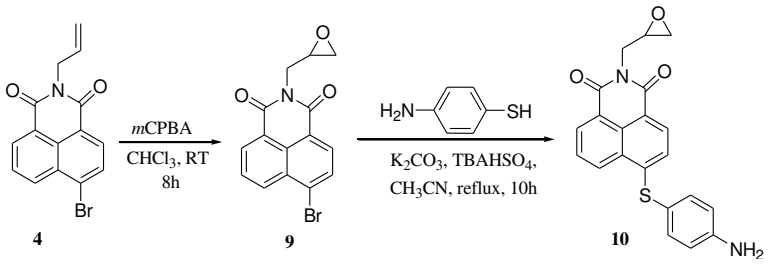


Scheme 5

Epoxidation has also been done to increase the polar interactions of compounds with DNA. Compound **2** was refluxed with allyl amine in presence of ethanol for 8 h to obtain 2-allyl-benzo[*de*]isoquinoline-1,3-dione. Reaction of **7** with *m*-CPBA in chloroform at room temperature resulted in 2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (Scheme 5).

3.3. Synthesis of 6-bromo-2-oxiranylmethyl-benzo[de]isoquinoline-1,3-dione and 6-(4-amino-phenylsulfanyl)-2-oxiranylmethyl-benzo[de]isoquinoline-1,3-dione

Compound **4** was reacted with *m*-CPBA in chloroform at room temperature for 8 h, results formation of crude 6-bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**9**) which was purified using column chromatography. 6-Bromo-2-oxiranylmethyl-benzo[*de*]isoquinoline-1,3-dione (**9**) was then refluxed with 4-amino-thiophenol in presence of K_2CO_3 and $TBAHSO_4$ using acetonitrile for 10 h to obtain crude solid of compound **10** (Scheme 6) which was then purified with column chromatography.



Scheme 6

3.4. Characterization

All the synthesized compounds were characterized by ^1H and ^{13}C NMR spectra (Bruker FT-NMR 400 MHz and JEOL ECS-400 MHz spectrometers) and Mass spectra (Waters QTOF Micro).

3.5. Biological evaluation

(a) Compounds **6b-c, 6f-i, 8** and **9** were submitted to the National Cancer Institute (NCI) for *in vitro* antitumor activity and were evaluated against 60 human cell lines at single dose concentration of 10 µM. Compound **6b** was evaluated at five dose concentration levels and proved 147 fold more active than oxaliplatin with MG MID GI_{50}, TGI and LC_{50} values of 84.2 nM, 27.6 µM and 89.3 µM respectively.

(b) Cytotoxicity of compound **6b** in human normal cell line (Hek293) was performed by means of a colorimetric assay (MTT assay). Compound **6b** showed only 12% toxicity to Hek293 cells even at 100 μM which indicated that compound **6b** showed potent anticancer activity and low toxicity to normal cells. Lactate dehydrogenase (LDH) release studies revealed that compound **6b** exhibited significant membrane-damaging effects showed selectivity towards cancer cells while exhibiting minimal/no toxicity towards normal cells.

(c) DNA binding studies of the most active compound **6b** has been evaluated using UV-Visible and fluorescence spectrophotometer and thermal denaturation experiment. UV-Visible, fluorescence titrations as well as thermal denaturation experiments indicated intercalative nature of compound **6b** with ct-DNA.

(d) Fluorescence quenching experiments have also been done with ethidium bromide:DNA and **6b**:DNA complex which strongly revealed the reversibility of results.

3.6. ADME-T prediction

Drug-like characteristics of compounds **6b-c** and **6f-i** were determined by calculation with Molinspiration software of the lipophilicity and the theoretical prediction of other ADME properties (molecular weight, TPSA, number of hydrogen donors and acceptors, and volume) for Lipinski's rule of five. All the compounds follow Lipinski's rule with no violation.

3.7. Structure activity relationship

The structure activity relationship emerged from the activity results clearly revealed that substitution of morpholine at C6 position, exceptionally showed very good activity in comparison to all other substituent amines. Secondary cyclic amines influence the antitumour more effectively than primary amines. Among primary amines, presence of aromatic amines has slightly improved the activity as compared to aliphatic amine.

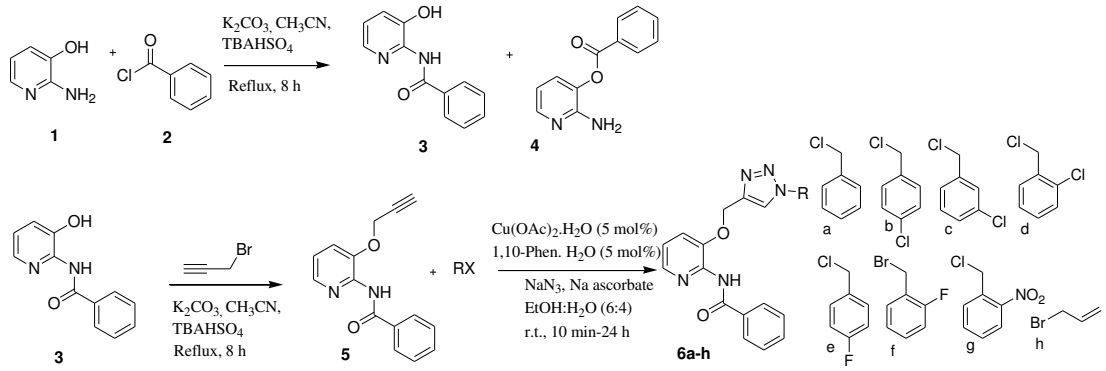
3.8. Molecular modelling

To explore the most feasible binding site, interaction mode and binding affinity, docking studies have been performed on most active compound **6b** with topoisomerase I and topoisomerase II and DNA. The planarity of naphthalimide core is comfortable for strong л-л stacking interactions and fits inside the DNA strands by van der Waals interaction and hydrophobic contacts.

Chapter 4: covers synthesis of 2,3-disubstituted pyridine analogue appended 1,2,3-triazole via click chemistry

4.1. Synthesis of N-(3-(1-substituted benzyl/allyl-1H-(1,2,3)-triazol-4-yl)methoxy)pyridine-2-yl)-3-phenyl-acrylamide (6a-h)

2-Amino-3-hydroxy pyridine was refluxed with benzoyl chloride in presence of K_2CO_3 and $TBAHSO_4$ in acetonitrile for 8 h, resulted in formation of two products *N*-(3-hydroxy-pyridin-2-yl)benzamide (**3**) and benzoic acid 2-amino-pyridin-3-yl ester (**4**) which were separated using column chromatography (Scheme 7).

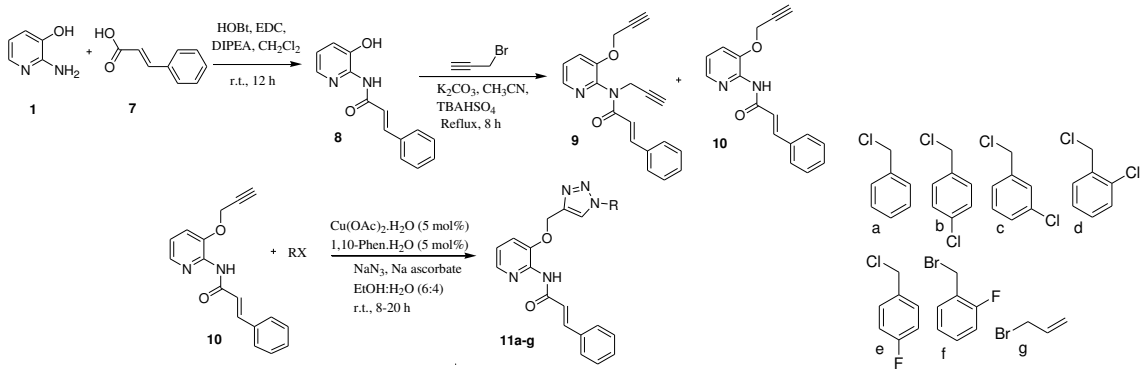


Scheme 7

N-(3-Hydroxy-pyridin-2-yl)benzamide (**3**) was refluxed with propargyl bromide in the presence of K_2CO_3 and $TBAHSO_4$ using acetonitrile as solvent for 8 h to obtain *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**). *N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)benzamide (**5**) was treated with various benzyl halides in presence of sodium azide, $Cu(OAc)_2.H_2O$, 1,10-phenanthroline monohydrate and sodium ascorbate in EtOH:H_2O (6:4) at room temperature to obtain **6a-h**.

4.2. Synthesis of N-(3-(1-substituted benzyl/allyl-1H-(1,2,3)-triazol-4-yl)methoxy)pyridine-2-yl)-3-phenyl-acrylamide (11a-g)

2-Amino-3-hydroxy pyridine was reacted with cinnamic acid in the presence of HOBt, EDC and diisopropylethylamine in dichloromethane at room temperature for 12 h to yield *N*-(3-hydroxy-pyridin-2-yl)-3-phenyl-acrylamide (**8**). While *N*-(3-Hydroxy-pyridin-2-yl)-3-phenyl-acrylamide was treated with propargyl bromide in the presence of potassium carbonate, $TBAHSO_4$ and acetonitrile at room temperature for 8 h, resulted in formation of 3-phenyl-*N*-prop-2-ynyl-*N*-(3-prop-2-ynyloxy-pyridin-2-yl)acrylamide (**9**) and 3-phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)acrylamide (**10**) in 10 % and 55 % yields respectively but at reflux temperature, same reaction resulted in formation of compound **9** in 60% yield along with traces of **10** (Scheme 8).

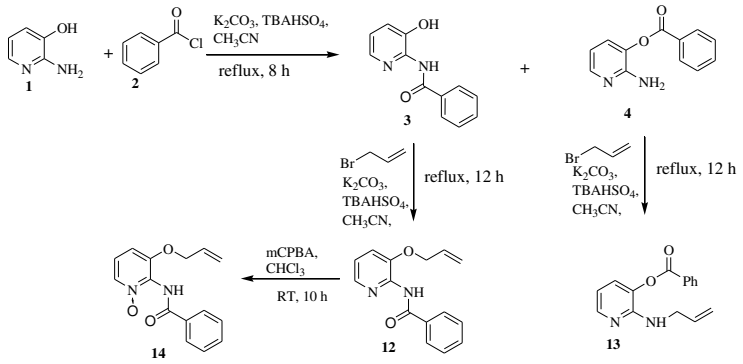


Scheme 8

3-Phenyl-*N*-(3-(prop-2-ynyloxy)-pyridin-2-yl)-acrylamide was reacted with various benzyl halides in presence of sodium azide, $Cu(OAc)_2.H_2O$, 1,10-phenanthroline monohydrate and sodium ascorbate in EtOH:H_2O (6:4) at room temperature resulted in compounds **11a-g** (Scheme 8).

4.3. Synthesis of N-(3-allyloxy-pyridin-2-yl)-benzamide, benzoic acid 2-allylamino-pyridin-3-yl ester and N-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide

N-(3-Hydroxy-pyridin-2-yl)-benzamide was refluxed with allyl bromide in the presence of K_2CO_3, $TBAHSO_4$ and acetonitrile for 12 h to obtain compound **12**. Benzoic acid 2-amino-pyridin-3-yl ester (**4**) was also refluxed with allyl bromide in the presence of K_2CO_3, $TBAHSO_4$ and acetonitrile for 12 h to obtain **13**. Compound **12** has further been treated with *m*-CPBA in chloroform for 10 h to obtain *N*-(3-allyloxy-1-oxy-pyridin-2-yl)-benzamide (**14**).



4.4. Characterization

All the synthesized compounds were characterized by ^1H and ^{13}C NMR spectra (Bruker FT-NMR 400 MHz and JEOL ECS-400 MHz spectrometers) and Mass spectra (Waters QTOF Micro).

4.5. Biological evaluation

Compounds **6a**, **6b**, **6e**, **6h**, **11b** and **11c** were submitted to the National Cancer Institute (NCI) for *in vitro* antitumor activity and were evaluated against 60 human cell lines at single dose concentration of 10 μM. All the compounds showed moderate activity against various cancer cell lines.

4.6. ADME- prediction

We have calculated the compliance to the Lipinski's rules of five for compounds **6a, 6b, 6e, 6h, 11b** and **11c**, tested against 60 cancer cell lines at a single dose concentration. All the compounds follow this rule with no violation in any of the parameters.

4.7. Structure activity relationship

Presence of halide group on phenyl ring showed better activity than the unsubstituted phenyl ring. It was observed that presence of fluoro group at 4-position of phenyl ring positively affected the activity of compound. Benzyl group has also proved to be a better substituent than cinnamic group.

4.8. Molecular modelling

To explore the most feasible binding site, interaction mode and binding affinity, docking studies have been performed on most active compound **6e** in the active site of ribonucleotide reductase (RNR) that possesses potent chemotherapeutic efficancy against leukemia.

Conclusion

We have successfully synthesized benzimidazole bearing naphthalimides and *N*-allyl naphthalimide analogues. 2,3-Disubstituted pyridine analogue appended 1,2,3-triazole were also synthesized by most convenient click chemistry approach. These novel molecules have been well characterized by ^{1}H NMR and ^{13}C NMR as well as mass spectrometry. These compounds were then evaluated *in vitro* towards 60 human cancer cell lines, predicted structure activity relationship. We have also calculated the compliance of all the compounds to the Lipinski's rules of five. Molecular modelling studies indicating considerable interactions of these compounds in the active sites of amino acids of Topo I, Topo II and RNR and as well as DNA.

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1. Vijay Luxami, Meenakshi Verma, Richa Rani, Kamaldeep Paul and Subodh Kumar, "FRET-based ratiometric detection of Hg^{2+} and biothiols usingnaphthalimide–rhodamine dyads", **Organic & Biomolecular Chemistry**, **2012**, *10*, 8076.
2. Meenakshi Verma, Vijay Luxami and Kamaldeep Paul, "Synthesis, *in vitro* evaluation and molecular modelling of naphthalimide analogue as anticancer agents", **European Journal of Medicial Chemistry**, **2013***, 68,* 352.
3. Meenakshi Verma, Vijay Luxami and Kamaldeep Paul, "Synthesis, *in vitro* evaluation and DNA interaction studies of N-allyl naphthalimide analogues as anticancer agents", **RSC advances**, **2015**, 5, 41803
4. Meenakshi Verma, Vijay Luxami and Kamaldeep Paul, "Synthesis of new 2,3-disubstituted pyridines containing a1,2,3-triazole in the side chain via one pot copper catalysed azide alkyne cycloaddition", **Arkivoc**, ***2015****,vii* , 28.
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1. Meenakshi and Kamaldeep Paul*, Designaing and synthesis of pyridine based prpafenone molecules: A novel scaffold for Multidrug Resistance Modulators, National conference on emerging trends in Chemistry-Biology interface (ETCBI-2011), 3-5 Nov. **2011**
2. Meenakshi , Vijay Luxami and Kamaldeep Paul*, A new Naphthalimide-fused benzimidazole scaffolds as anti-cancer agents, Chemical Constellation Cheminar-2012 [CCC-2012], NIT-Jalandhar , 10-12 Sept. **2012** (awarded best poster)
3. Meenakshi , Vijay Luxami and Kamaldeep Paul*, Synthesis and in-vitro evaluation of naphthalimide fused benzimidazole scaffolds as anti-cancer agents, National Conference on Recent Advances in Chemical & Environmental Sceinces (RACES), 31 Jan. **2013** (second prize in poster presentation)
4. Meenakshi , Vijay Luxami and Kamaldeep Paul*, Synthesis, antitumor activity and DNA intercalation of naphthalimide derivatives NCIMSF, Thapar University, Patiala, 26-28 Oct. **2013**
5. Meenakshi , Vijay Luxami and Kamaldeep Paul*, Synthesis, antitumor activity, DNA intercalation of naphthalimide and pyridine based derivatives, Drug discovery Chemistry, San Diego , CA, USA, 23-25 April, **2014**