Imidazole Based Compounds: Synthesis and *In Vitro* Anticancer Screening

Thesis submitted to the Central University of Punjab

For the award of

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In

Medicinal Chemistry

BY

Arvind Negi

Supervisor

Dr. Raj Kumar

Centre for Chemical and Pharmaceutical Sciences

School of Basic and Applied Sciences

Central University of Punjab, Bathinda

November, 2013

DECLARATION

I declare that the thesis entitled "Imidazole based compounds: Synthesis and in vitro anticancer screening" has been prepared by me under the guidance of Dr. Raj Kumar, Assistant Professor, Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

(Arvind Negi)
Centre for Chemical and Pharmaceutical Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda - 151001.

Date:

CERTIFICATE

I certify that ARVIND NEGI has prepared his thesis entitled “Imidazole based compounds: Synthesis and in vitro anticancer screening”, for the award of M.Pharm. (Medicinal Chemistry) degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab.

(Dr. Raj Kumar)
Assistant Professor
Centre for Chemical and Pharmaceutical Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda - 151001.

Date:

ABSTRACT

Imidazole based compounds: Synthesis and in vitro anticancer screening

Name of student	:	Arvind Negi
Registration Number	:	CUP/M.Pham-Ph.D/SBAS/CPS/2011-12/01
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Imidazole is an important five-membered aromatic heterocycle widely present in natural products and synthetic molecules. The unique structural feature of imidazole ring with desirable electron rich characteristic is beneficial for imidazole derivatives to readily bind with a variety of enzymes and receptors in biological systems through diverse weak interactions, thereby exhibiting broad bioactivities. Numerous imidazole-based compounds are in being used extensively in the clinics to treat various types of diseases. We have synthesized, designed and evaluated imidazole-based compounds for anti-proliferative activity against A-549 and Hep-G2 human cancer cell lines. Further the free radical scavenging activity of the selected compounds was performed in order to observe their antioxidant potential (if any). The combined results have shown advent of their first in vitro bioactivity as anticancer and antioxidant compounds and revealed their medicinal potential. The synthetics offer the scope for generation of a library of compounds and their evaluation against a panel of cancer cell lines, studies on structure activity relationship, tracing their molecular mechanism(s) in addition to their development at preclinical level in future.

(Arvind Negi) (Dr. Raj Kumar)

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviations
1.	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide	MTT
2.	Activin receptor-like kinase 5	ALK-5
3.	Adenosine triphosphate	ATP
4.	Alanine	Ala
5.	Arginine	Arg
6.	Bristol-Myers Squibb	BMS
7.	Coupling constant	J
8.	Cyclin dependent kinase	CDK
9.	Cytochrome complex	CYP
10.	Dihydro dichlorofluroscien diacetate	H_2DCFDA
11.	Dimethysulfoxide	DMSO
12.	Doublet	D
13.	Doublet of doublet	Dd
14.	Epidermal growth factor receptor	EGFR
15.	Fetal Bovine Serum	FBS
16.	Food and Drug Administration	FDA
17.	Fourier Transform Infrared	FT-IR
18.	Fransyltransferase	FTase
19.	Gastrointestinal tract	GIT
20.	G-Protein coupled receptor	GPCR

21.	Hertz	Hz
22.	Histidine	His
23.	Insulin like growth factor binding protein	IGFBP
24.	Insulin like growth factor-1 receptor	IGF-1R
25.	Insulin like growth factor-2	IGF-2
26.	Insulin receptors	IRs
27.	Melting Point	M.P.
28.	Micromolar	µM
29.	Milimolar	mmol
30.	Minimum Inhibitory Concentration	MIC
31.	Multiplet	M
32.	Namometer	Nm
33.	Nuclear Magnetic Resonance	NMR
34.	Parts per million	Ppm
35.	Phosphate Buffer Saline	PBS
36.	Rapidly Accelerated Fibrosarcoma	RAF
37.	Reactive Oxygen Species	ROS
38.	Red Blood Cells	RBC
39.	Singlet	S
40.	Small molecule inhibitors	SMI’s
41.	Sodium dodecyl sulphate	SDS
42.	Standard Deviation	S.D.
43.	Thin Layer Chromatography	TLC

44.	Threonine	Thr
45.	Topoisomerase	TOP
46.	Transforming Growth factor- β	TGF-β
47.	Ultraviolet	UV
48.	World Health Organization	WHO

Chapter 1
Introduction

Chapter 1

Introduction

According to World Health Organisation (WHO), cancer is a metabolic disorder which initiates uncontrolled growth of cell as it loses its basic framework and functionality (Organization, 1979). This proliferation of cells often invades surrounding tissues and metastasizes to distant sites of the body. In medical terms, Cancer is known as a malignant neoplasm, and encircles a broad group of various diseases, altogether consequently involved in unregulated cell growth, division, proliferation and migration and inhibits apoptosis (Fidler, 1978). Mainly it can follow lymphatic and blood stream route to spread to more distant parts of the body, attributed as malignancy of cancer cells whereas certain type of tumours which do not show uncontrolled growth, aggressive behaviour to invade into the neighbouring tissues and malignancy characteristics are called as benign tumours, e.g. moles (nevi) and uterine fibroids (leiomyomas) (Carmeliet, 2005; Carmeliet & Jain, 2000; Liotta, 2001).

Biologically the expression of many factors is altered and normal physiology of a cell is completely lost. Consequently cell divides, and increases its metabolic turn over which leads to the overgrowth and finally results in tumour formation. There are more than 200 different types of malignant cancers identified till now that afflicts humans. As far as the knowledge about malignancy was comprehended, it was assumed that it can only affect the somatic cells. But as the research in this context is progressed, scientific community world-wide unearthed that malignancy can affect germ cells also which is urging a high alert for the mankind as the disease severity is escalating tremendously at an alarming rate due to transmission of genetic information to the futuristic generations.

Chapter 2
Review of literature

Chapter 2

Review of literature

2.1 Cancer

Cancer is a metabolic disorder manifested by alteration of different levels of expressive factors, receptors and metabolites. There are total eight hallmarks which exhibit a typical characteristic of cancer cell (Hainaut & Plymoth, 2013). This includes sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. The development of each hallmark (**Figure 2.1**) involves multiple signalling pathways as indicated in **Figure 2.2**.

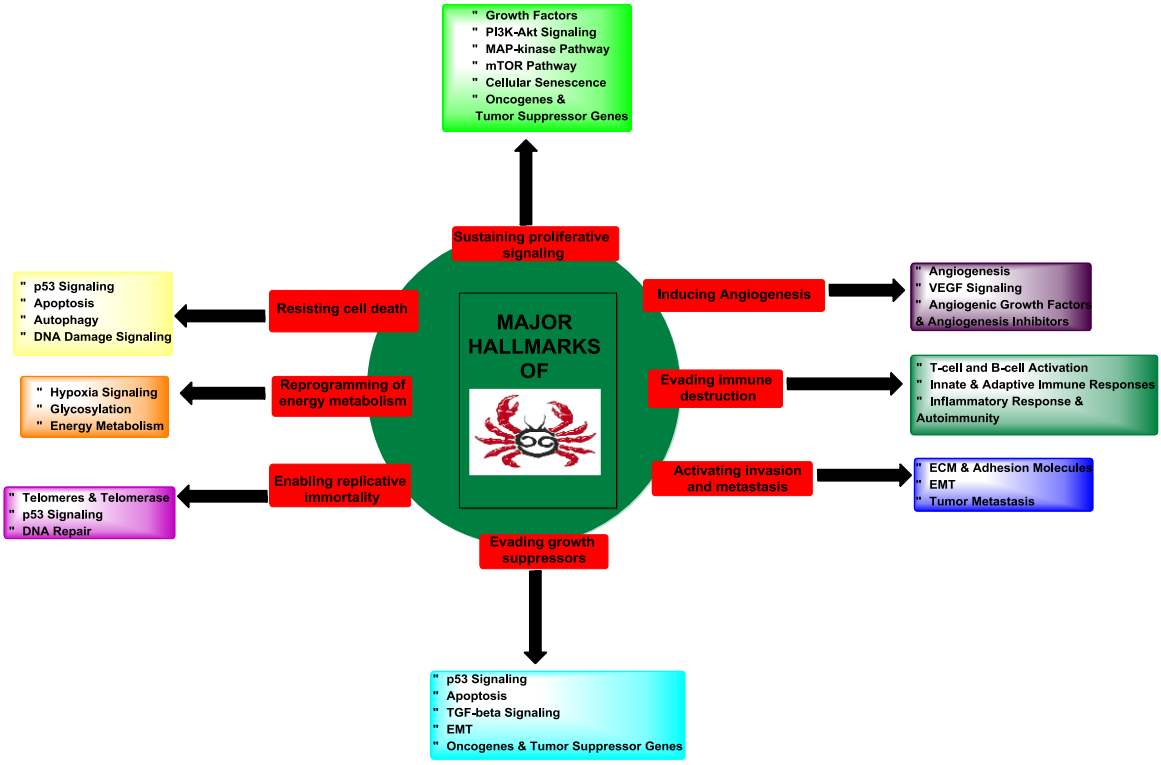


Figure 2.1 Hallmarks of cancer (Hananhan et al., 2011)

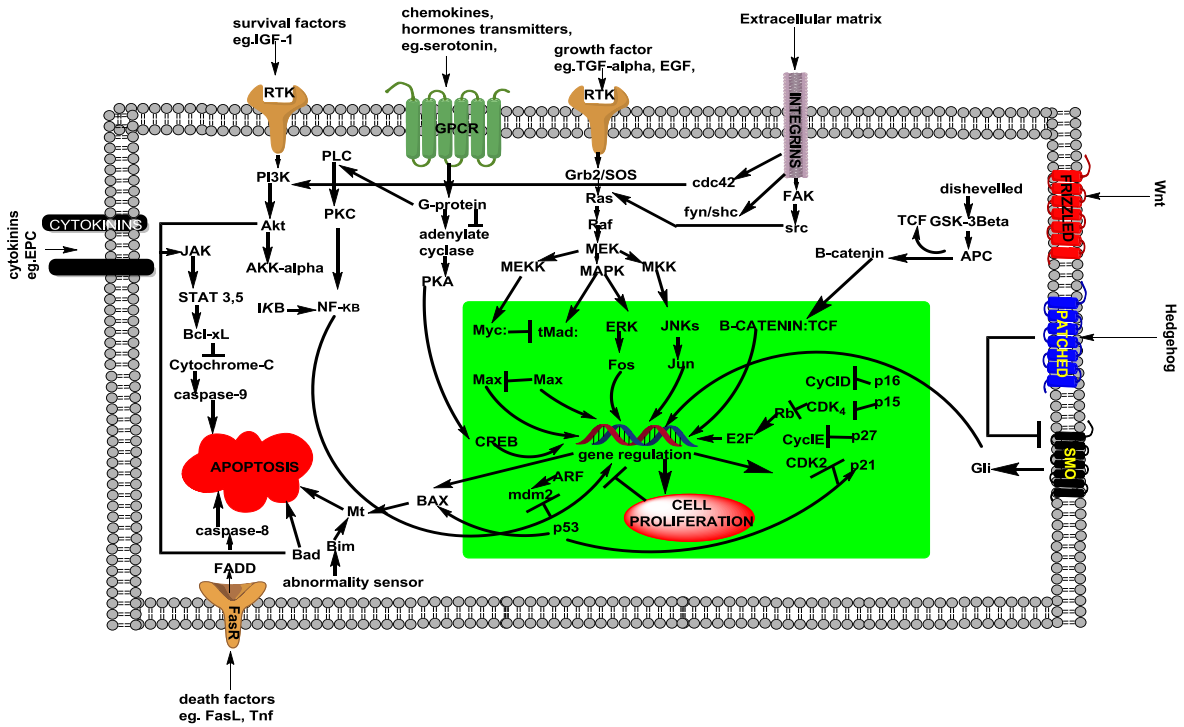


Figure 2.2 Factors that trigger cell signaling and cross talk in cancer. In this figure, the surface receptors (cytokinins, GPCRs (Dorsam & Gutkind, 2007), RTKs (Zwick et al ., 2001), Frizzled (Wang et al ., 2012), FasR, Integrins(Goodman & Picard, 2012), Patched, SMO etc) are influenced by the external factors. In cancer the concentration level of endogenous ligand precipitated or either intracellular linked enzyme or enzymatic domain expression increases abruptly. While cytoplasmic pathway run a phenomenon called as phosphorylation cascade which either activate or deactivate the successor accessory protein and this whole process is highly regulated via cross talk with other intracellular accessory protein possibly through the negative/ positive feedback mechanism. The green highlighted box designating the process which is directly involved in the genetic modification and alteration which further leads to the cancer progenesis (Cairns et al ., 2011; Eccleston & Dhand, 2006).

2.2 Impact of cancer

The impact of cancer in the world is depicted by WHO in its report disclosed in August 2012 (**Figure 2.3**, **Figure 2.4**) in which, it stated that the situation become worst in the developing countries may be due to the role of life style of the natives which is steeply transforming and accepting

the living pattern of western countries called as westernisation. The report also projected the estimated cancer cases for 2030 based on the human development index (HDI) and sex.

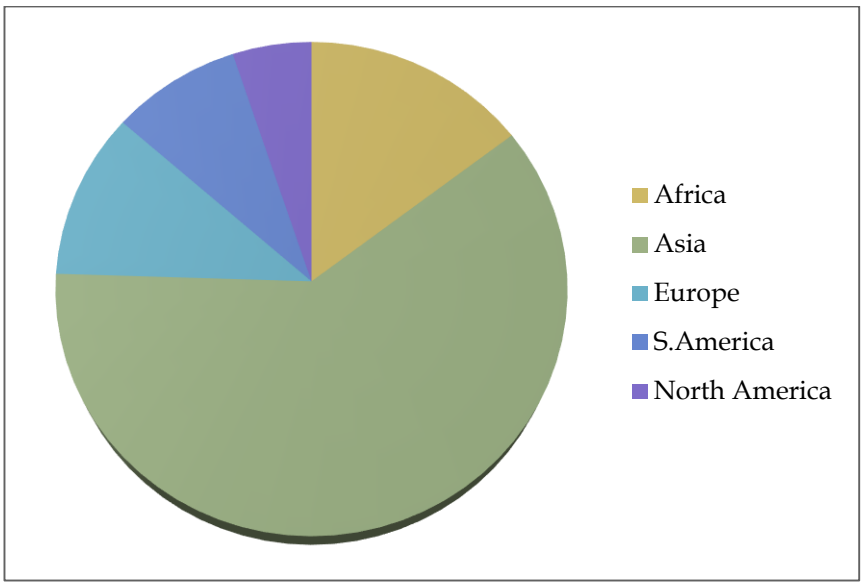


Figure 2.3 Impact of cancer in the different continents of world

Figure 2.4 Projection for 2030 on the basis of human developmental index and sex

Cancer is second notorious deadliest disease in the world. In order to find an effective cure against cancer, extensive research has been devoted to the development of anticancer therapeutics, which comprises surgical techniques, radiation therapy, and chemotherapy. Unfortunately there is no drug in the market which could cure the cancer completely. Still the exhaustive research is going on to resolve the mystery behind the

prominent mechanism of how the cancer progresses, so that a better therapeutic agent can be synthesized or targeted in a fashion to get the better clinical results. Although, the cancer chemotherapy has entered to greater extent with the development of new and effective agents but it still suffer from the problem like selectivity, toxicity and multiple drug resistance.

Food and Drug Administration (FDA) (Mullard, 2013) has approved 148 anticancer agents and specified tests in order to diagnosis and treat the cancer (Cairns et al ., 2011). The success rate for the small molecules, semi-synthetics and natural products is quite high, as indicated in **Figure 2.5**, **2.6**, **2.7** and **2.8**.

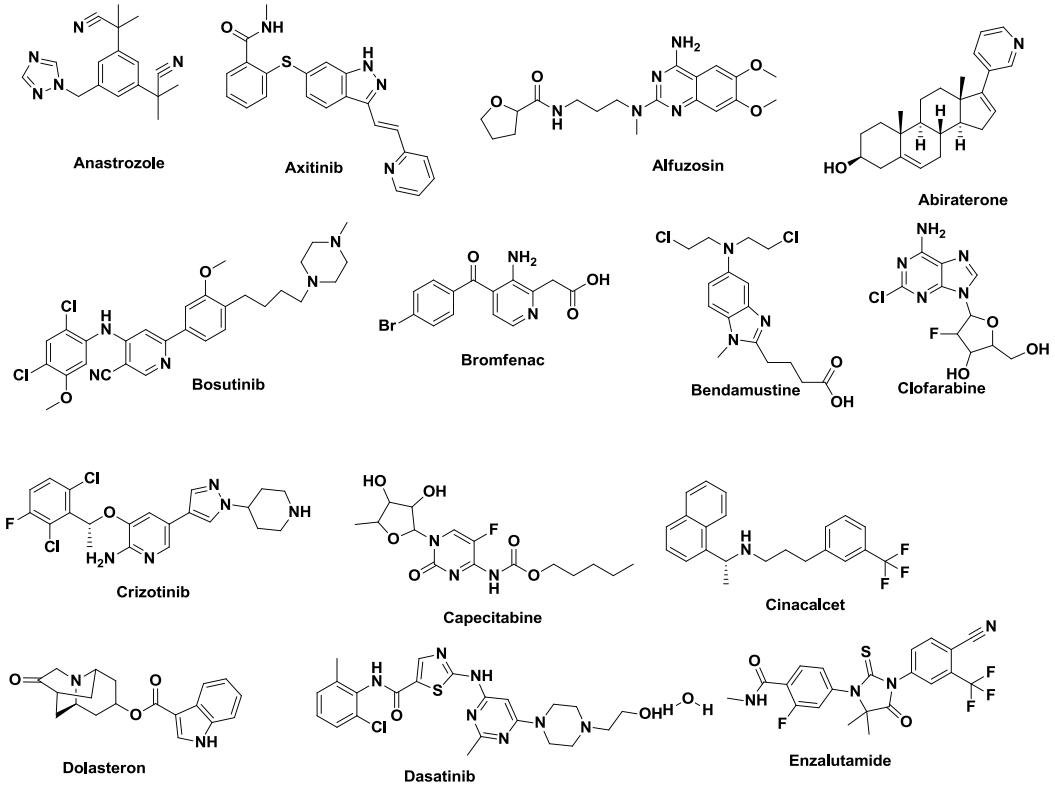


Figure 2.5 FDA approved small molecule as anticancer drugs

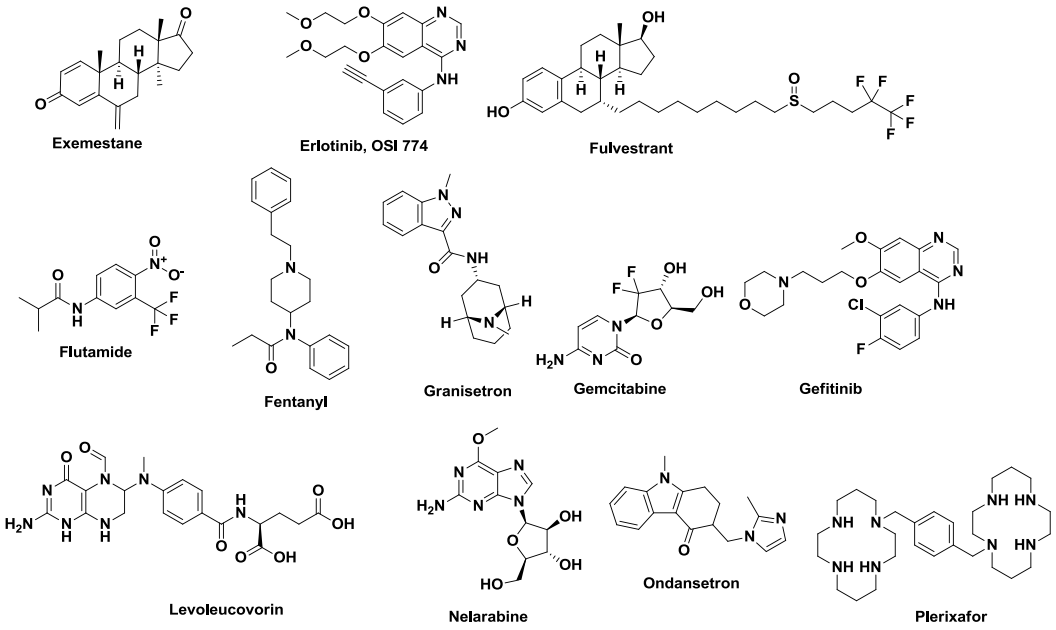


Figure 2.6 FDA approved small molecule as anticancer drugs

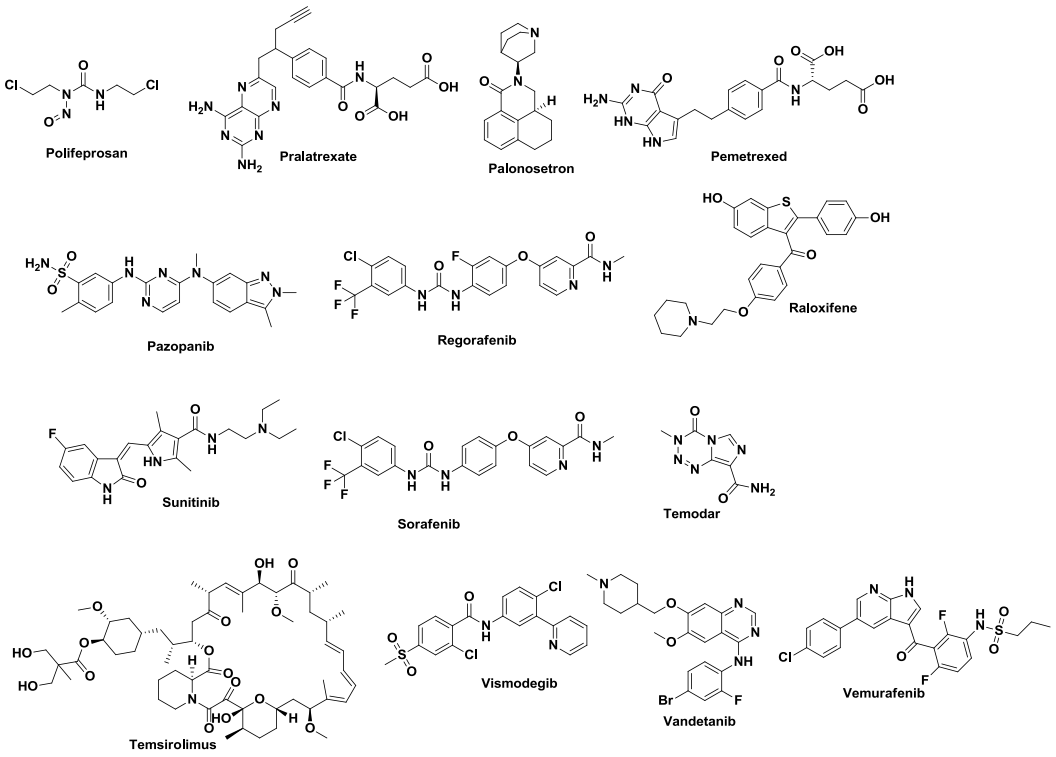


Figure 2.7 FDA approved small molecule as anticancer drugs

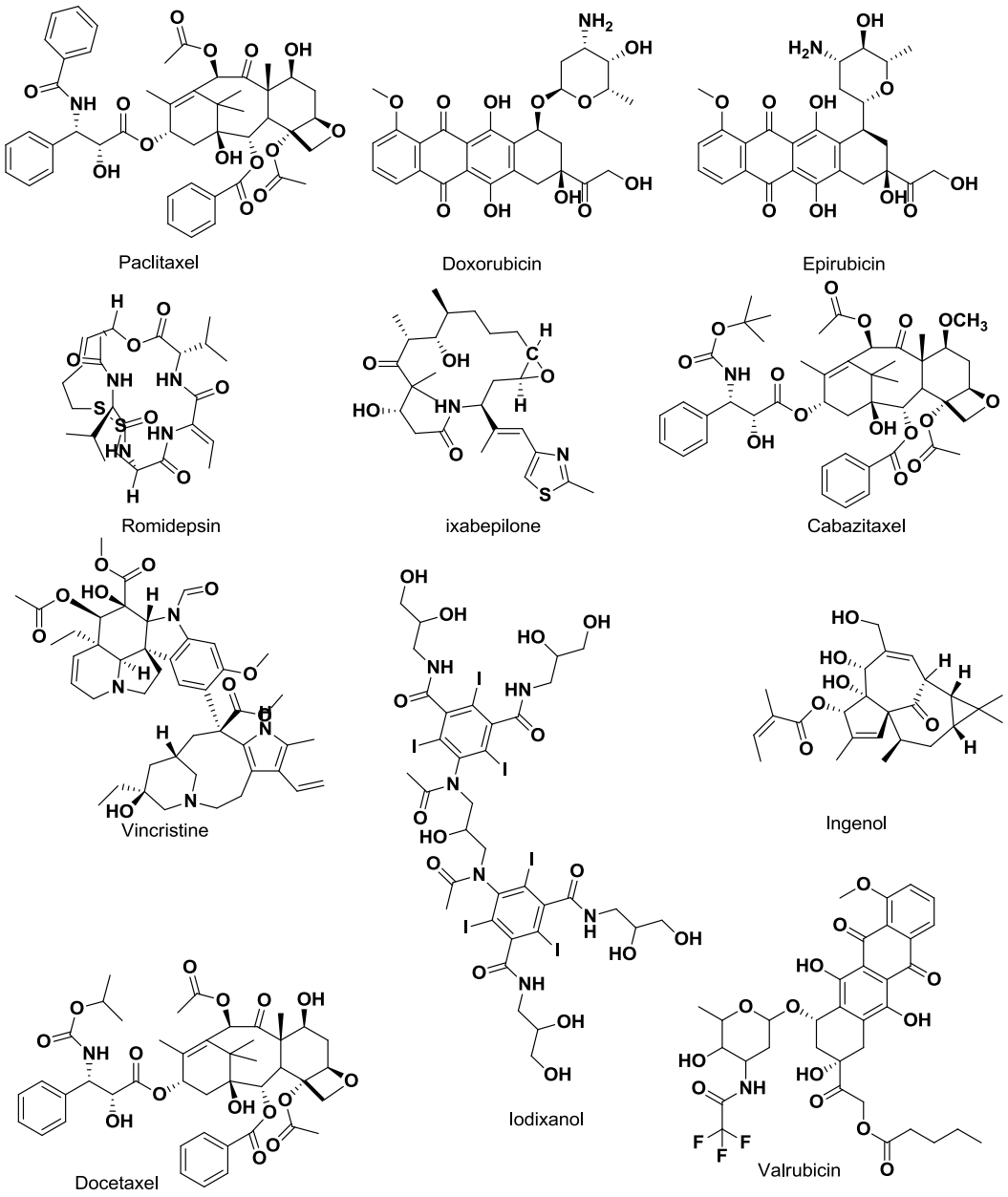


Figure 2.8 FDA approved natural and semi-synthetic anticancer drugs

2.3 Heterocyclics and their role in cancer

Heterocycles are the compounds which contain at least one element other than carbon in their ring and that element is called hetero atom e.g. Nitrogen, sulfur, phosphorus and oxygen. Heterocyclics are convoluted in the chemical control of biological litigates as the energy generators, metabolism, nerve impulse transmitters etc. Pozharskii et al defined them as molecular rings studded with jewels. The heterocyclic can vary in ring size, number of rings, type of heteroatom, position of heteroatom and number of heteroatoms. The diverse actions of heterocyclics i.e. electrophilic and nucleophilic action, oxidizing and reducing properties, acidic and basic attributes are associated with the electronic arrangements in heterocyclic molecules (Pozharskii et al ., 2012).

Imidazole is one of the main basic aromatic heterocyclic skeletons, significantly composed in the various endogenous biomolecules, such as histidine, and the related hormone like histamine. Even many drugs contain this basic skeleton as critical, for their activity such as antifungal, nitroimidazole, and the sedative midazolam.

Chemically, imidazole is a planar 5-membered ring (**Figure 2.9**). It exists in two equivalent tautomeric forms, because the proton can be located on either of the two nitrogen atoms. Imidazole is a highly polar compound, as evidenced by a calculated dipole of 3.61D. It is highly soluble in water. The compound is classified as aromatic due to the presence of a sextet of π-electrons, consisting of a pair of electrons from the protonated nitrogen atom and one from each of the remaining four atoms of the ring.

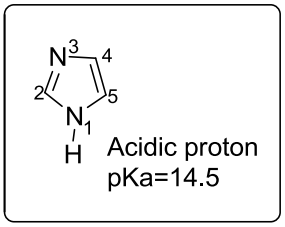


Figure 2.9 Chemical structure of Imidazole

Imidazole is amphoteric in nature. That is, it can function as both an acid and as a base. As an acid, the pKa of imidazole is 14.5, making it less acidic than carboxylic acids, phenols, and imides, but slightly more acidic than alcohols. As a base, the pKa of the conjugate acid (pK_{BH+}) is approximately 7, making imidazole approximately sixty times more basic than pyridine. The basic site is N-3. Protonation provides the imidazolium cation, which is symmetrical in nature.

2.4 Imidazoles as anticancer agents

In recent years much emphasis is given to the Imidazole derivatives as it revealed considerable promising versatility in incorporated functionality to a pharmacophore to make it as potential anticancer therapeutics (Zhang et al ., 2013). The possible biological interaction of imidazole could be through its interference with DNA synthesis via hydrogen bonds formation or binding to protein molecules. Some reports indicate that imidazole at high concentrations can directly inhibit the synthesis of essential components of cell membrane without interfering with sterols and sterol esters. Some of the imidazole derivatives had proved their valuability as clinical anticancer drugs that include a) dacarbazine b) zoledronic acid c) azathioprine d) tipifarnib e) nilotinib (shown in **Figure 2.10)**.

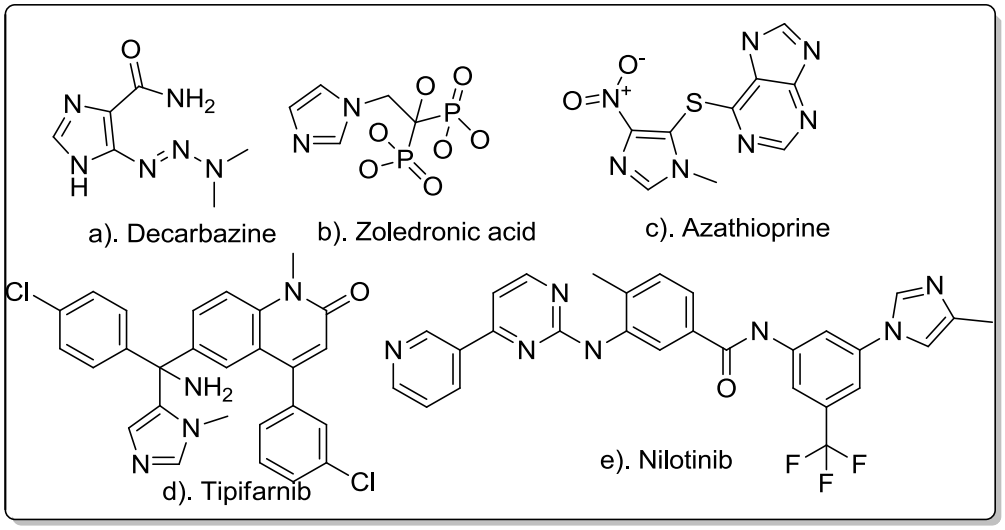


Figure 2.10 Clinically used imidazoline containing drugs

Recently, imidazole-based anticancer agents are found to interact with cancer precipitating factors like enzymes or receptors which abruptly altered their expression during the progression of the cancer, such as topoisomerases, microtubule, cytochrome P450 enzyme, rapidly accelerated fibrosarcoma (RAF) kinases, transforming growth factor-β etc.

2 .4.A. Imidazole as topoisomerase inhibitor

Topoisomerase (TOP) plays significant roles in cell progression, apoptosis, transcription, and other cellular homeostasis activities via preventing supercoiling of DNA. This target has gained significant importance in the previous decade since when its role in cancer has been found and later on recognized as a valuable and important target for chemotherapeutics (Kümler et al ., 2013). TOP interacts with DNA and form a complex to prevent supercoiling. Topotecan and irinotecan have been successfully developed as TOP inhibtors and used in the clinical practices. However these agents have suffered from certain shortcomings which include poor solubility, short action duration and high toxicity associated drug resistance which hinder their continuous use. Incorporation of imidazole into irinotecan ring influences the water solubility. Some of the structures for imidazoles as TOP inhibitors have been shown in **Figure 2.11**.

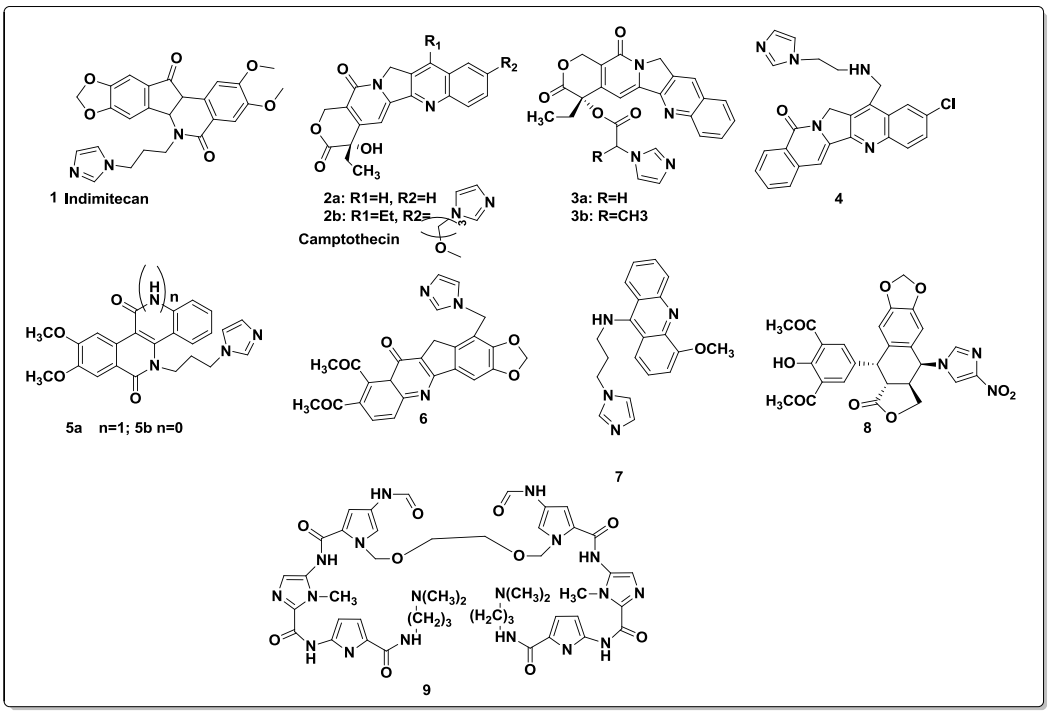


Figure 2.11 Imidazoles containing agents as topoisomerase inhibitors

Further, introduction of ethyl and imidazolyl groups into camptothecin (**2a**) at 7- and 10-position, respectively improved water solubility and potency of **2b** as compared to camptothecin.

2.4.2. Imidazoles as microtubule polymerization inhibitors

Microtubules are tubular polymers of tubulin, major components of cytoskeleton, are indispensable for the mitotic spindle, which are required for the separation of duplicated chromosomes during cell division. The abortive formation of microtubules can lead to cell arrest and finally apoptosis. Hence it is noteworthy anticancer strategy to design potential microtubule inhibitors. However taxanes, vinca alkaloids, and combretastatins are the successful tubulin-targeting compounds (Jordan et al ., 1998), used clinically in cancer but their use largely limited due to poor bioavailability and multidrug resistance. Cis-configuration of the double bond in combretastatin A-4 (**10**) is a critical for its antitubulin activity (Shan et al ., 2011). To confine the isomerization to only cis-form, bioisosteric replacement of the olefinic linker by a rigid imidazole ring was carried out,

which resulted into superior pharmacokinetic property and oral bioavailability. Some of the structures for imidazoles as microtubule polymerization inhibitors are shown in **Figure 2.12.**

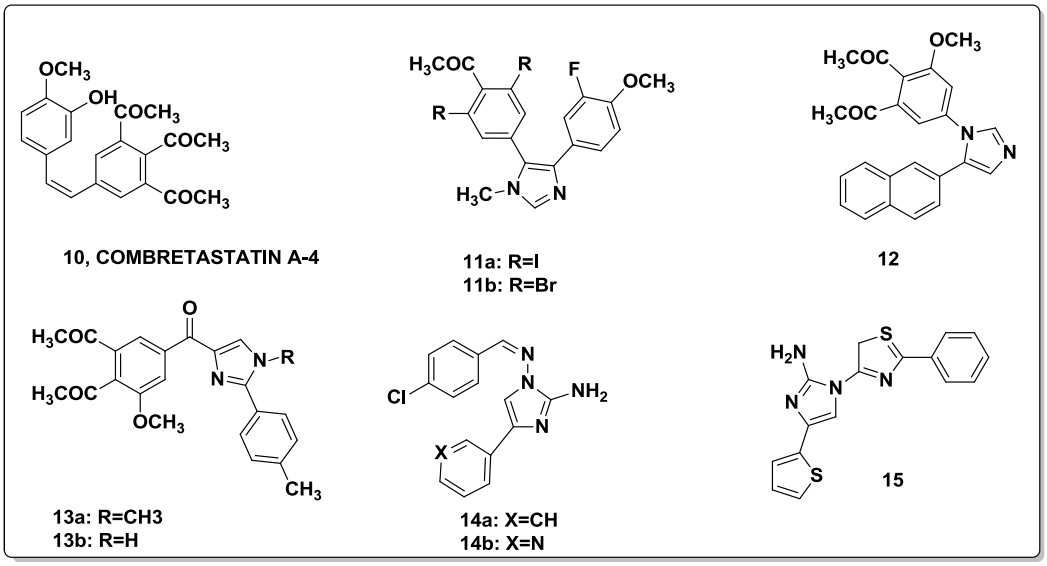


Figure 2.12 Imidazoles as microtubule inhibitors

2.4.3. Imidazoles as Cytochrome P450 Enzymes Inhibitors:

Cytochrome P450 (CYP) is a large diverse group of hemo-proteins present in hepatic cells. Majority of them are involved in metabolism like activities and some reports are also witnessed their role in activating the carcinogens which can lead to cancers (Lin & Lu, 1998). Azole-based CYP inhibitors such as fadrozole (**16**), YM-116 (**17**), and letrozole (**18a**) are the FDA recommolended first-line drugs in the adjunct therapy for breast cancer. These drugs inhibit the aromatase enzyme and abort the synthesis of active form of the estrogen (estradiol). Their inhibiting aromatization activity is largely characterized to their ability to coordinate with iron ion in aromatase heme through azole rings (Caporuscio et al ., 2011). However, CYP enzymes inhibitors also cause substantial systemic side effects due to the non-selectivity among CYP enzymes. Thus further investigation in designing of novel CYP inhibitors is necessarily required. Fortuitously, the discovery of YM-116 (**17**) bearing an imidazole ring provides a new hope in

the class of CYP inhibitors as anticancer drugs. Imidazoles containing cytochrome P450 enzymes inhibitors are shown in **Figure 2.13** (Bourrié et al ., 1996)**.**

Imidazole derivative (**20**, IC_{50} = 0.30μM) showed more potency than ketoconazole (IC_{50} = 0.52μM) towards human CYP24A1 hydroxylase.

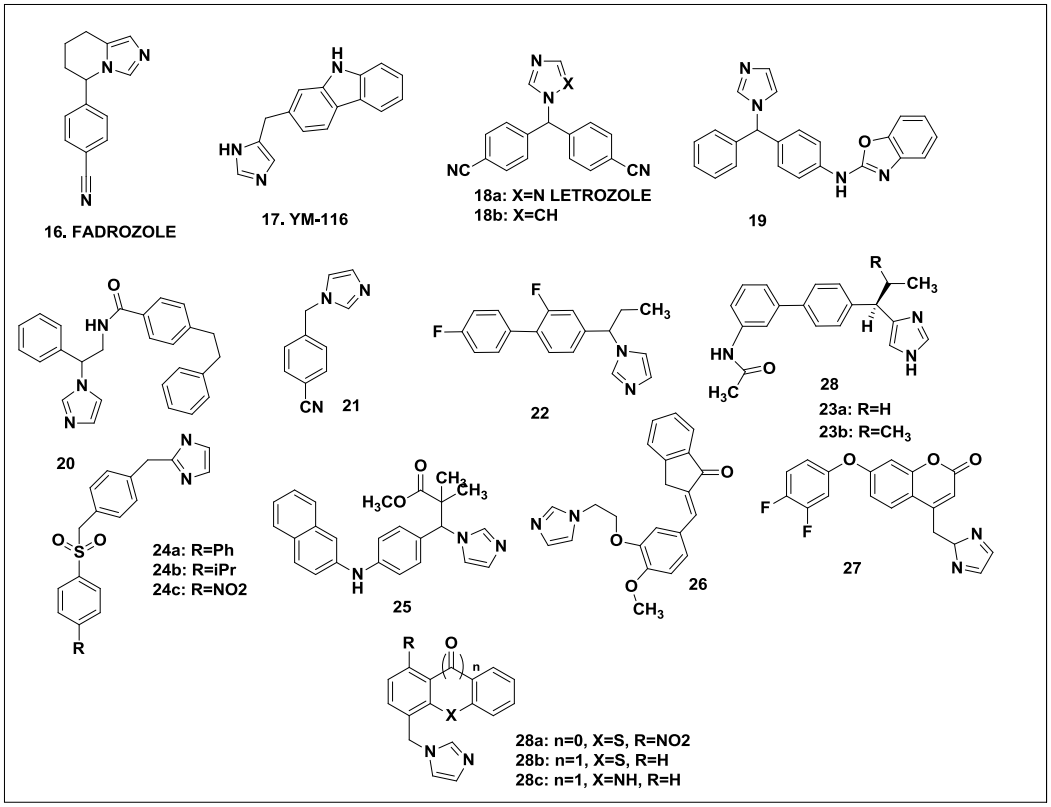


Figure 2.13 Imadazoles as cytochrome P450 inhibitors

2.4.D. Imidazoles as RAF Inhibitors

RAF kinases (A, B, and C) are actively participating in growth, differentiation, and proliferation of cell while their expression altered and deregulated in cancer, so can be drugable target for anticancer therapeutics (Takle et al ., 2006). Sorafenib is FDA approved RAF inhibitor, clinically used in renal cancer. Its imidazole derivative (**29a**) with amide linkage and (**29b**) bearing urea bridge were discovered as substantial C-

RAF inhibitory efficacy and improved anticancer activities with GI_{50} values of 0.62 and 0.65μM, respectively in comparison sorafenib (GI_{50} = 0.78μM) against melanoma cancer cell line WM3629. Some of RAF inhibitors which are bearing the imidazole in their structures are shown in **Figure 2.14** (Zhang et al ., 2013).

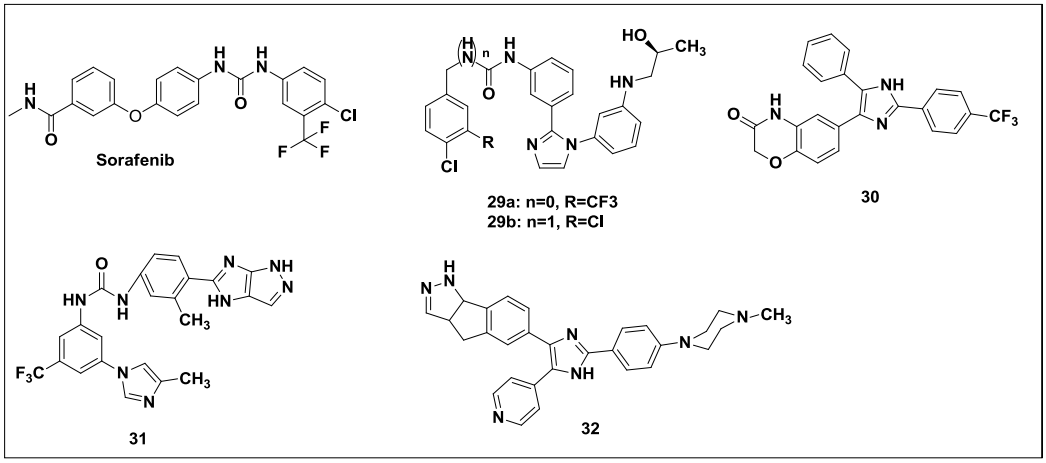


Figure 2.14 Imidazoles as rapidly accelerated fibrosarcoma kinase inhibitors

Imidazoles as TGF-β Inhibitors:

TGF-β regulates the cell proliferation and differentiation via activin receptor-like kinase 5 (ALK5). Many small molecules based on imidazole scaffold ALK5 inhibitors usually bearing pyridine ring as hinge-binding group are under preclinical development. It was also found that the pyridine and imidazole moiety were significant for ALK5 inhibitory activity. Recently, pyridine–imidazole hybrids also discovered as ALK5 inhibitors. Some of the Imidazoles containing TGF-β inhibitors are shown in **Figure 2.15** (Bonafoux & Lee, 2009; Callahan et al ., 2002; Zhang et al ., 2013).

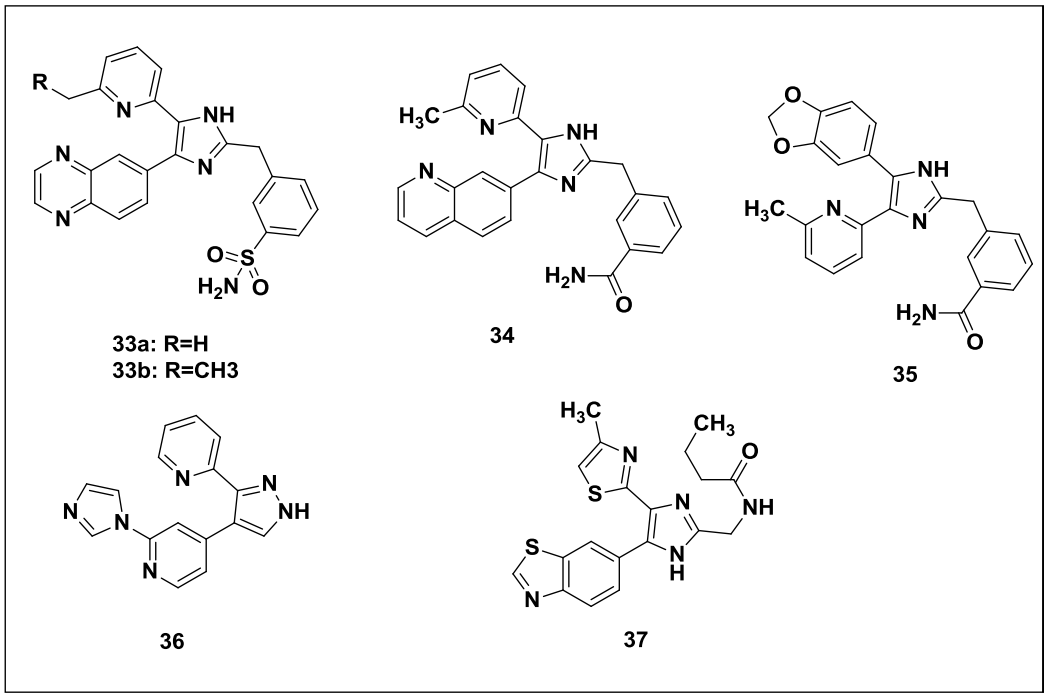


Figure 2.15 Imidazole as TGF-β inhibitors

2.4.7. Imidazoles as Farnesyltransferase Inhibitors

Farnesylation is a post-translational modification of proteins in which an isoprenyl group is added to a cysteine residue. It is a significant process to mediate protein-protein interactions and protein-membrane interactions resulting in intracellular signal transduction, cell proliferation, and apoptosis. Since the role of farnesyltransferase (FTase) has been discovered, it emerged as a potential anticancer target. Numerous FTase inhibitors have been developed as highly efficient and low toxicity profile anticancer agents. SAR studies revealed that Imidazole incorporation is necessary for biological activity of FTase inhibitors and possibly can interact with the cofactor, zinc ion present at the active site. The structures for imidazoles as farnesyltransferase inhibitors are presented in **Figure 2.16** (Luca, 2006; Tong et al ., 2003; Zhang et al ., 2013).

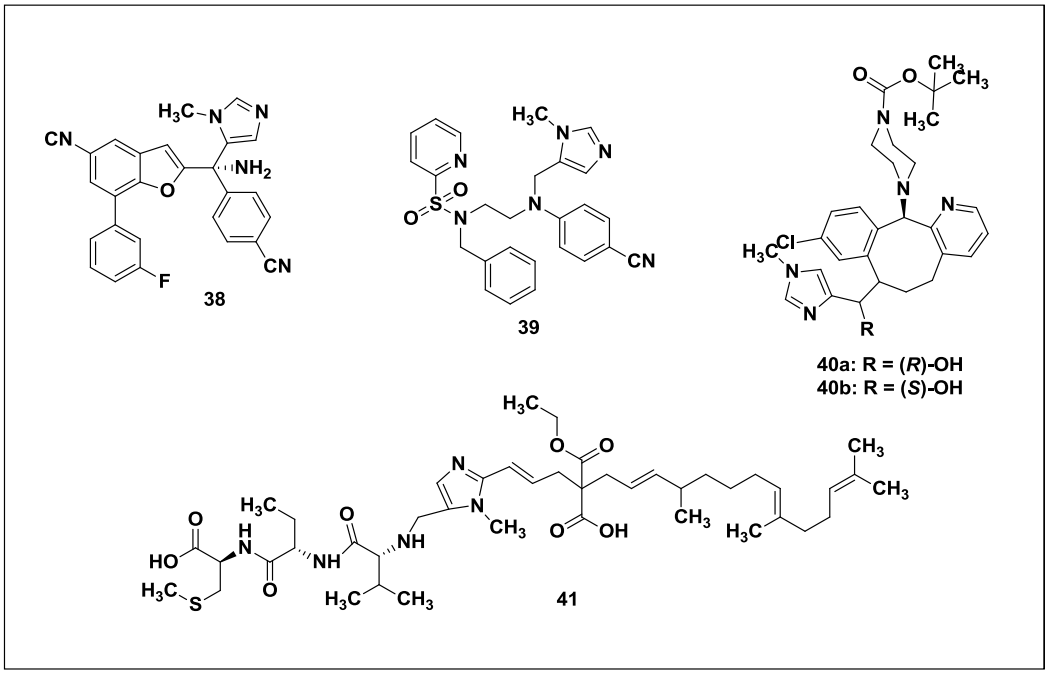


Figure.2.16 Imidazoles as Farnesyltransferase Inhibitors

2.4.F. Imidazole as IGF-1R tyrosine kinase inhibitors

Insulin like growth factor (IGF) system is composed of tyrosine kinases(Hubbard & Till, 2000)- insulin receptors (IRs) and insulin like growth factor–I receptor (IGF-1R) and non-tyrosine kinase- insulin like growth factor–2 receptor (IGF-2R); and their ligands i.e. insulin, IGF-I and IGF-2; and six IGF-binding proteins (IGFBPs). The system plays a dominant role in maintaining the development and metabolic homeostasis but any disruption causes pathological conditions such as diabetes and cancers (Baserga et al ., 2003; Belfiore & Malaguarnera, 2012; Buck & Mulvihill, 2011; Frasca et al ., 2008; Gallagher & LeRoith, 2011; Hofmann & García-Echeverría, 2005; Moschos & Mantzoros, 2002; Pollak, 2012; Samani et al ., 2007). Both IR and IGF-1R are tetrameric glycoproteins ($\alpha_2\beta_2$) i.e. 2-α and 2- β subunits linked by disulfide bonds. Each α-subunit i.e. ~130 kDa, contains ligand binding site on the cysteine-rich region in the extracellular α-subunit, whereas each β-subunit contains the tyrosine kinase domain of ~90-95kDa (Gustafson & Rutter, 1990; Benyoucef et al ., 2007). IGF-1R is quite homologous to IR, ranges from 45-65% in the ligand

binding domain to 60-85% in the kinase and substrate recruitment domains (ATP binding region), which were accessed by alanine scanning mutagenesis of insulin (Kristensen et al ., 1997) and alanine scanning mutagenesis of IGF-1R (Andersen et al ., 1995; Houten et al ., 1979; Kristensen et al ., 1997; Mynarcik et al ., 1997; Ullrich et al ., 1986; Whittaker et al ., 2001). The main difference between IGF-1R and IR occur in their enzymatic domain whereas a subtle difference has been observed in the hinge region (Thr 1053/ Arg 1054 in IGF-1R Vs Ala1080/His1081 in IR) (Favelyukis et al ., 2001). In addition the C-terminal of the intracellular β-subunits of IR is structurally and functionally different from IGF-1R, in tyrosine units as there are three in IGF-1R and only are two in IR (Faria et al ., 1994). Further, the unactivated, non-phosphorylated and fully activated, triply phosphorylated IGF-1R and IR differ structurally especially regarding the positions of the activation loop and αC-helix (W. Li et al ., 2004; Munshi et al ., 2002).

In spite of high homology between IR and IGF-1R, both receptors maintain a separate and distinct functions as IR exhibits its dominant role in metabolic homeostasis (preferably in glucose metabolism) (Matthews et al ., 1985; Patti & Kahn, 1998) while IGF-1R dominates in the cell development and proliferation processes (Baserga et al ., 2003; Larsson et al ., 2005). However, in certain cases especially cancer and diabetes, where IR in hybridization with IGF-1R (Belfiore, 2007), stimulates cell proliferation and differentiation (Belfiore & Malaguarnera, 2011; Blakesley et al ., 1997; Gallagher & LeRoith, 2010; Steller et al ., 1996). Involvement and overexpression of IGF-1R in various types of malignancies which include lung (Fidler et al ., 2012), breast (Curigliano et al ., 2012; Hankinson et al ., 1998; Karamouzis & Papavassiliou, 2012; LeRoith, 2012; Sachdev & Yee, 2001; Surmacz, 2000; Yee et al ., 1989; Yerushalmi et al ., 2012), prostate (Kimura et al ., 1996; Mantzoros et al ., 1997; Montgomery et al ., 2012; Rowlands et al ., 2012; Wolk et al ., 1998), GIT cancers (Adachi et al ., 2010) for e.g, colorectal (Guo et al ., 1992; Singh & Rubin, 1993; Wu et

al ., 2002), hepatocellular (Scharf & Braulke, 2003) and pancreatic carcinomas (Bergmann et al ., 1995; Korc, 1998; Moschos & Mantzoros, 2002; Samani et al ., 2007) at preclinical and clinical level is observed. Some of the imidazole based IGF-1R inhibitors are shown below (Negi et al ., 2013).

Imidazopyrazines/Imidazotriazines

OSI pharmaceuticals put efforts on the optimization of 8-amino-1,3-disubstituted–imidazo[1,5-a]pyrazine scaffolds (**42**) and led to the discovery of **43** as potent ATP-competitive IGF-1R inhibitors (**Figure 2.17**) which holds a key pharmocophoric donor/acceptor interactions with the kinase hinge region of IGF-1R (Mulvihill et al ., 2007). Moreover, imidazo[1,5-a]pyrazine core has more benefits in comparison to conventional pyrrolo[2,3-d]pyrimidine core which includes a little less in the values of polar surface area (PSA), log D and log P. Together all these parameters provide an edge by increasing the flexibility in attachment selection. Further lead optimization and DMPK profiling of **43** led to the discovery of novel molecules such as AQIP (Mulvihill et al ., 2008), PQIP (Ji et al ., 2007), OSI-906 (Mulvihill et al ., 2009), **44** (Jin et al ., 2011) and 4-aminoimidazo[5,1-f][1,2,4]triazine-derived, FQIT. Later on, FQIT was explored and disclosed as dual IGF-1R and IR inhibitor in the treatment of cancer (Jin et al ., 2012).

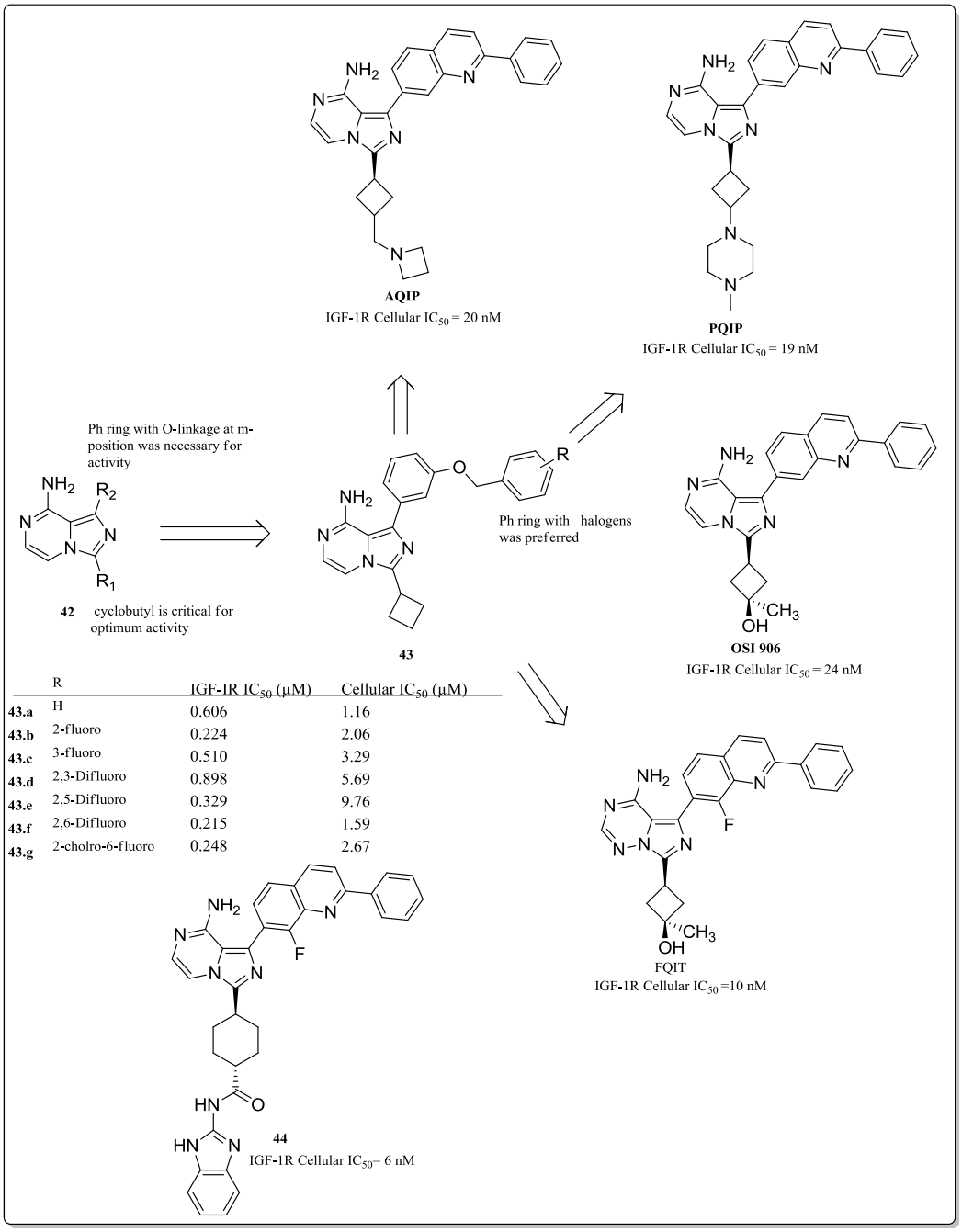


Figure 2.17 Lead optimization and SAR of imidazopyrazine/imidazotriazines

Imidazo[1, 2-a]pyridines

In 2009, Glaxo Smith Kline disclosed imidazo[1, 2-a]pyridines as IGF-1R inhibitors at nanomolar concentration (Emmitte et al ., 2009). During the development of SAR of the compounds, the following key points were observed as represented in **Figure 2.18** : (a) reversal orientation of the amide connectivity was critical for the activity (compare **45.a** with **45.b**), (b) substitutions at R_1 such as aniline (**45.c**) or its derivatives bearing 2-methoxy group (**45.f** and **45.g**-methoxy group enhances cellular potency and selectivity for IGF-1R), morpholine or bipiperidine moieties (**45.e-g**) were found to be important as compared to amino group (**45.c**) and (c) introduction of o-methoxy group at the ring A enhances IGF-1R inhibitory activity (**46**). The proposed binding mode of **46** dictates the various interactions such as (a) bidentate hinge H-bond between anilinopyrimidine moiety and Glu1050 and Met1052 and (b) piperazino-piperidine moiety occupying the solvent exposed region.

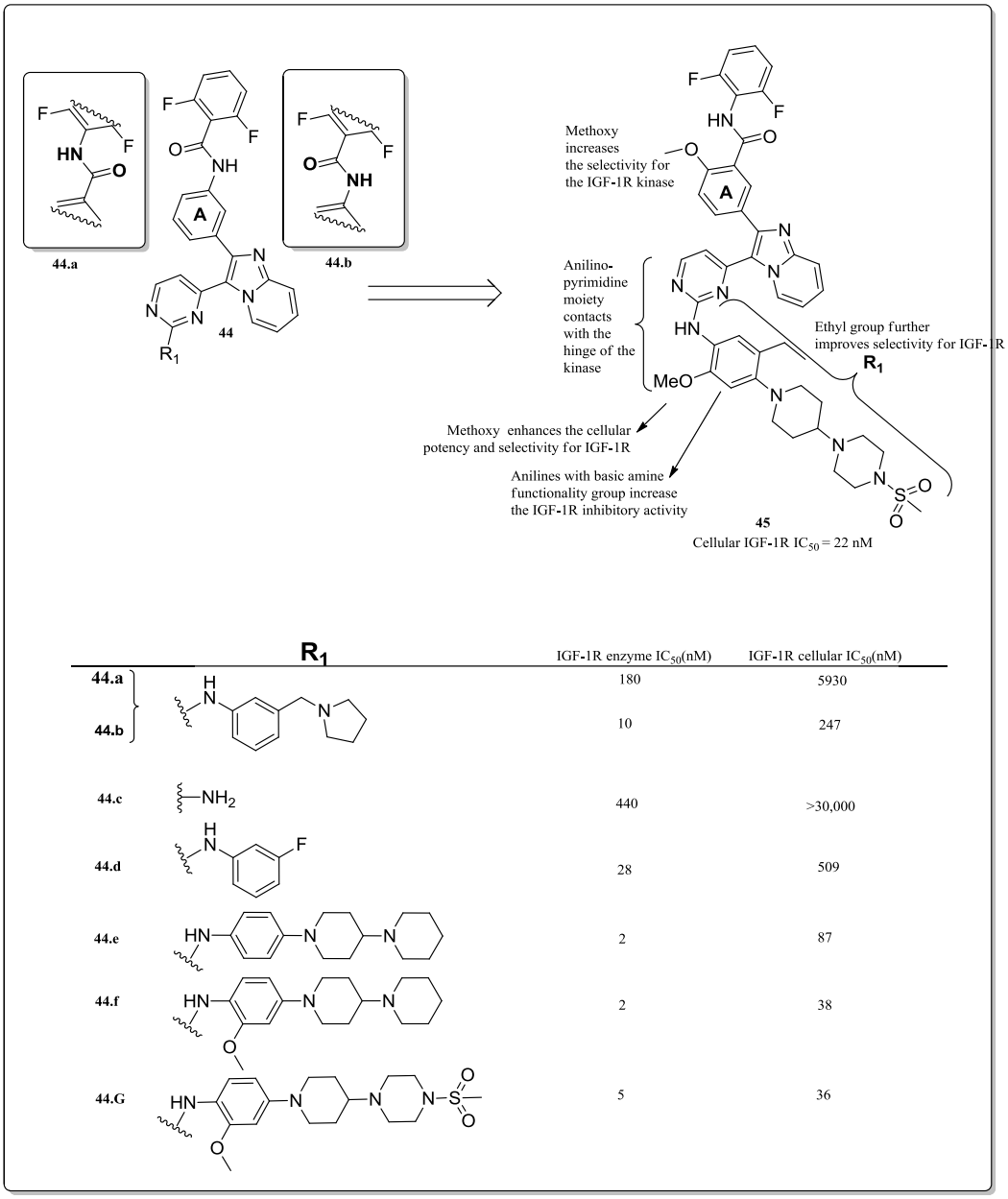
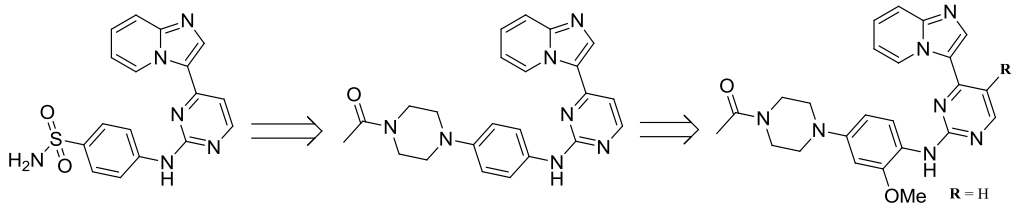


Figure 2.18 Imidazo[1, 2-a]pyridines and their SAR

In 2011, Astra Zeneca modified their earlier reported imidazo[1, 2-a]pyridines as cyclin dependent kinase (CDK) inhibitors(Anderson et al ., 2003; Byth et al ., 2004) and screened them through cellular and enzymatic IGF-1R high throughput screening protocol (Ducray et al ., 2011). Compound **46** was identified as hit molecule as shown in **Figure 2.19**. Lead

optimizations of **46** such as replacement of p-sulfonyl group with N-acetylpiperidine (**47**) led to the decrease in CDK and increase in IGF-1R inhibitory activities. Further, introduction of o-methoxy group imparted in enhancement in IGF-1R selectivity over CDK (**48**) due to its steric interaction with Phe-82 of CDK-2 (Anderson et al ., 2003). Exploitation of C5 position of the pyrimidine ring of **48** by putting electron withdrawing groups such as chloro and bromo (**49** and **50**, respectively), resulted further increase in the IGF-1R activity due to the lipophilic interactions of the halogens with the gatekeeper residue of IGF-1R. Various positions of the imidazopyridine ring of **49** were substituted with halogen, methyl, methoxy, amino or cyano groups, but none of the modification led to the enhancement of IGF-1R activity as shown in **Figure 2.20**. Compound **49** emerged as dual inhibitor of IGF-1R and IR (enzyme IC_{50} = 9 nM, Cellular IC_{50} = 12 nM). It was further optimized to get **50** as shown in **Figure 2.21** for increasing its oral absorption by replacement of N-acetyl piperazine ring and affinity for hERG ion channel to reduce cardiac arrhythmia in the patients (Ducray et al ., 2011).

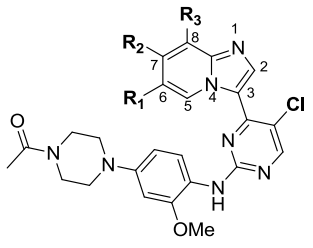


46 **47** **48**

	46	**47**	**48**
IGF-1R IC_{50} (μM)	1.26	0.27	0.45
CDK-2 IC_{50} (μM)	0.001	0.078	more than 100

	48.a	**48.b**	**48.c**	**48.d**
R	Cl	Br	Me	OMe
IGF-1R IC_{50} (μM)	0.010	0.003	0.047	0.090
CDK-2 IC_{50} (μM)	1.1	1.1	10	53
Cellular IC_{50} (μM)	0.017	0.009	0.092	0.19

Figure 2.19 Optimization of imidazo[1,2-a]pyridines



49

	Flouro			**Methyl**			**Methoxy**					
	49.a	**49.b**	**49.c**	**49.d**	**49.e**	**49.f**	**49.g**	**49.h**	**49.i**	**49.j**	**49.k**	**49.l**
R_1	**H**	**H**	**F**	**H**	**H**	**CH_3**	**H**	**H**	**OCH_3**	**NH_2**	**CH_2OH**	**CN**
R_2	**H**	**F**	**H**	**H**	**CH_3**	**H**	**H**	**OCH_3**	**H**	**H**	**H**	**H**
R_3	**F**	**H**	**H**	**CH_3**	**H**	**H**	**OCH_3**	**H**	**H**	**H**	**H**	**H**
IGF-1R IC_{50} (mM)	0.010	0.011	0.007	0.013	0.009	0.010	0.040	0.008	0.055	0.035	0.035	0.14
Cellular IGF-1R IC_{50} (mM)	0.022	0.025	0.013	0.038	0.026	0.013	0.11	0.043	0.066	0.054	0.11	0.77

Figure 2.20 Imidazo[1,2-a]pyridines with different substitutions

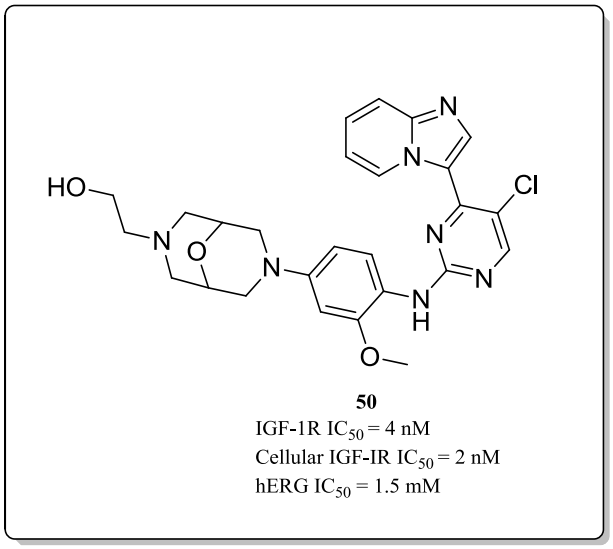


Figure 2.21 Compound **50** as IGF-1R inhibitor

Benzimidazole-pyridone

In 2005 Bristol-Myers Squibb (BMS) disclosed ATP-competitive inhibitors of IGF-1R kinase domain possessing benzimadazole-pyridone pharmacophore. The first hit of the class was **51** (M. Wittman et al ., 2005). X-ray structure of **51** in complex with kinase domain of IGF-1R, indicated the binding interactions and scope of substitution of methyl group of imidazole ring and substitution at C4 position of pyridone to further explore and access the open ribose binding pocket of the active site. Much attention was paid to substitute C4 position with hydroxy derivative of phenylethylamine to get more potent **52.** In order to maintain the optimum balance between CYP-450, IGF-1R inhibitory activity and oral bioavailability of **52**, its imidazole ring was replaced with morpholine and this resulted in two enantiomers **53** (BMS-536924; S-form; IC_{50} = 100 nM) and **54** (R-form; IC_{50} = 830 nM) (Velaparthi et al ., 2007a; Velaparthi et al., 2007b, Wittman, et al ., 2007a; M. D. Wittman et al ., 2007b). BMS-536924 was found better tolerated than alloxan-induced hypoinsulinemia and more effective than metformin in the treatment of experimental insulin-responsive breast cancer (Dool et al ., 2011). The same research group in 2006

reported **55** (BMS-554417) as the lead compound after substitution of the imidazole ring with piperazine of **52** (Haluska et al ., 2006). In the year 2008, **56** (BMS-695735) was disclosed with improved ADME properties including CYP 3A4 induction and inhibition, broad spectrum in vivo antitumor activity and minimal drug-drug interactions as compared to **53** (Velaparthi et al ., 2008). Further, BMS research group put major emphasis on structure activity and structure solubility studies of **53** that led to discovery of BMS-577098 (**57**) (Velaparthi et al ., 2010) (**Figure 2.22**).

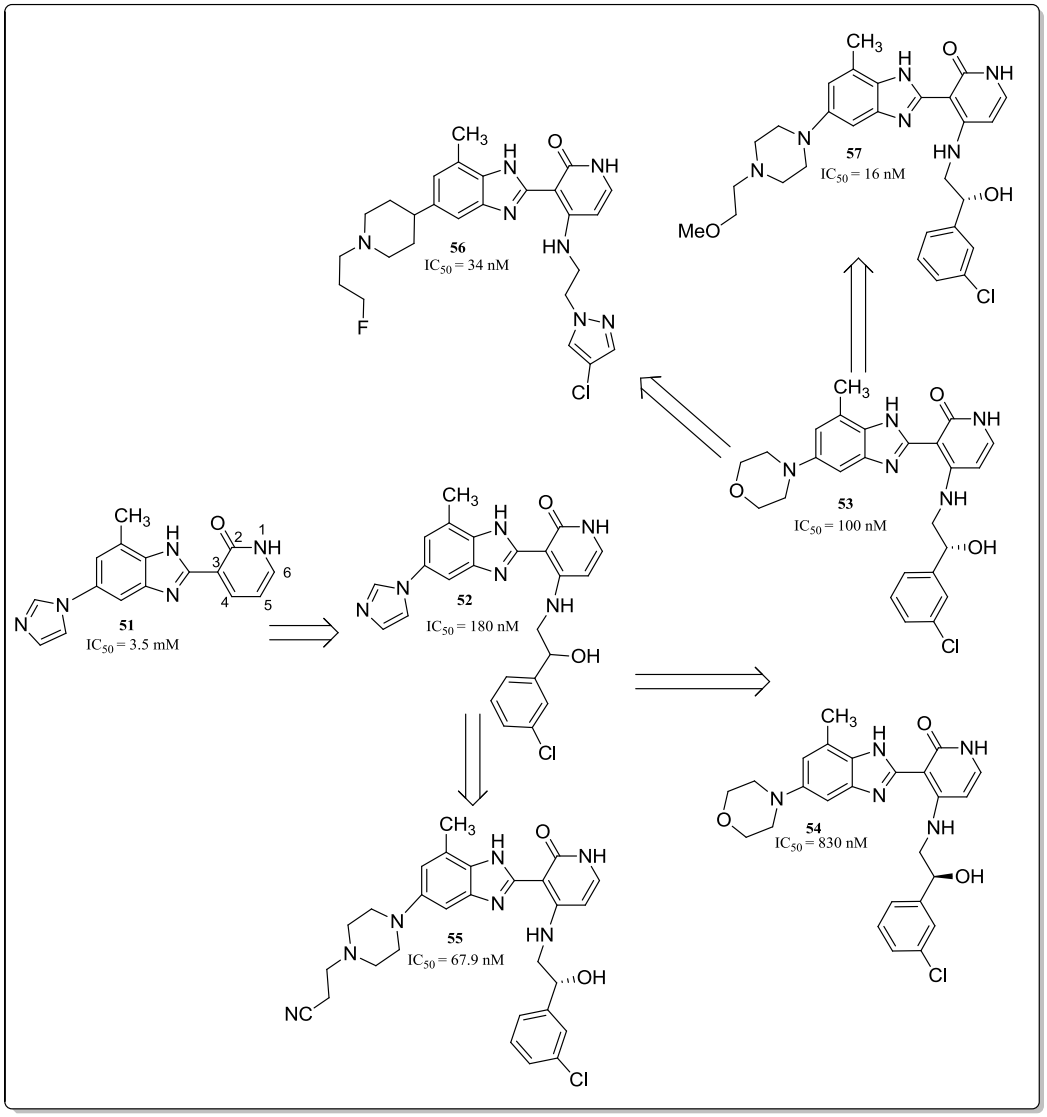


Figure 2.22 Discovery of Benzimidazole-pyridones as IGF-1R inhibitors and their SAR

Cyanoquinolines

Wyeth research group in 2009 reported 3-cyanoquinolines as IGF-1R inhibitors (**58; Figure 2.21**) (Miller et al ., 2009), which were effective at nanomolar concentration in cancer treatment and proved to be better agents as compared to a series of isoquinolinedione (Mayer et al ., 2008). SAR and X-ray crystal structure (**Figure 2.23**) highlighted the role of chloro at C3', 2-thio-imidazole head piece at C4' of aniline ring and basic amine (like N,N-dimethyl amino, piperazine, pyrrolidine, morpholine derivatives) at C7 position in governing the IGF-1R inhibitory activity. **59** emerged as the best obtained (Miller et al ., 2009), but unfortunately the selectivity ratio (IR-IC_{50} inhibition / IGF1R-IC_{50} inhibition) for **59** was 0.17.

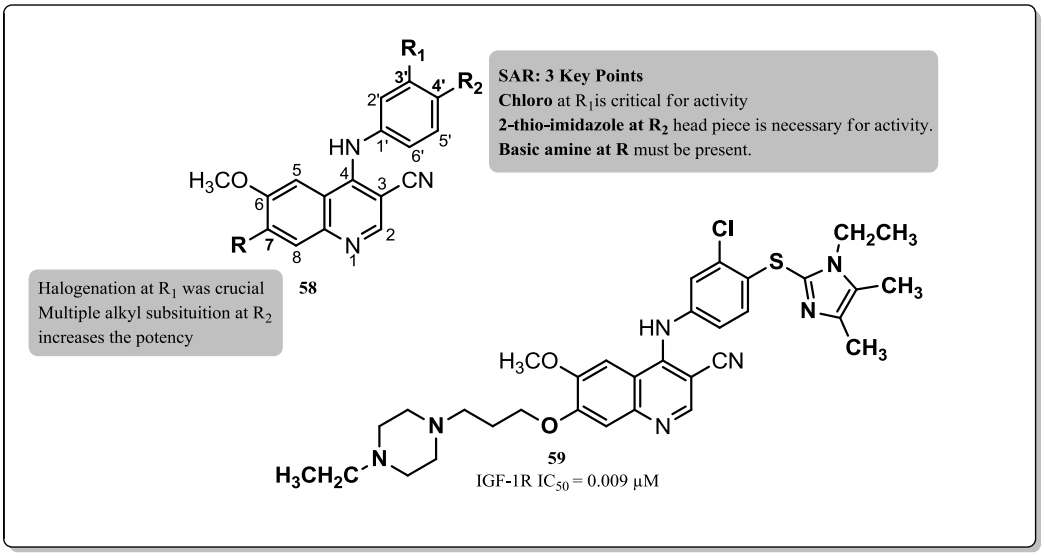


Figure 2.23 Cyanoquinolines and their SAR

Having seen the anticancer activity of a variety of compounds based on imidazole scaffold, we thought of designing compounds derived from imidazoles and tethering them with aromatic or heteraromatic moiety via an imine bond (Bhaviskar et al., 2013) and screening against some selected human cancer cell lines.

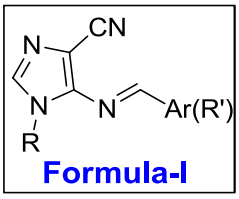
Chapter 3
Objectives

CHAPTER-3

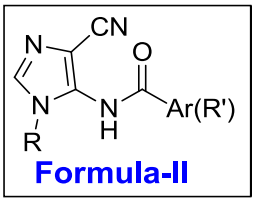
OBJECTIVES

The thorough literature survey fortified us to propose the research project with the following aims:

1. To design and synthesize imidazole based derivatives having the structure formulae shown below:



Formula-I



Formula-II

R, R`= Any alkyl or aryl alkyl group

Ar = Aromatic or hetroaromatics group

2. To evaluate the synthesized compounds for their in vitro anticancer activity using MTT assay against available human cancer cell lines.

Chapter 4

Material and Methods

CHAPTER 4

MATERIALS AND METHODS

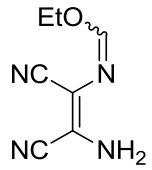
4.1 Synthesis

4.1.1 General

1. The reagents were purchased from Sigma-Aldrich, Loba - Chemie Pvt. Ltd., S.D. Fine Chemicals, Sisco Research Laboratory and used without further purification.
2. The progress of the reaction was determined by thin layer chromatography (TLC) carried out on pre-coated silica plates. Ethyl acetate : toluene (1:5), Ethyl acetate : n-hexane (1:1 and 2:3) were used commolonly as the eluents. Spots were then visualized under UV light.
3. Melting points were recorded on Stuart SMP - 30 melting point apparatus with open glass capillary tube and were uncorrected.
4. Infrared spectra of compounds were recorded on Bruker IR spectrophotometer at CUPB.
5. The ^{1}H and ^{13}C NMR of the compounds were recorded on Bruker Advance II instrument at 400 MHz and 100 MHz frequencies respectively, in $CDCl_3$, DMSO-d_6 and TMS ($\delta = 0$) as internal standard at SAIF, Panjab University, Chandigarh.
6. The solvent were evaporated on Ilmvac rotary evaporator with Julabo chiller under reduced pressure and traces were dried under IR lamp.

4.1.2B Synthesis and characterization of synthesized compounds

Ethyl-*N*-((*Z*)-2-amino-1,2-dicyanovinyl)formimidate (ARK-1)



ARK-1

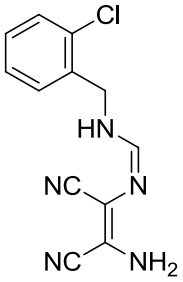
A mixture of 2,3-diaminomaleonitrile (6 g, 55.5 mmol, 1 equiv.) was refluxed with triethyl orthoformate (9.24 mL, 55.5 mmol, 1 equiv) in 1,4-dioxane (25mL) in a 100 ml round bottom flask at 85 - 95 °C for 6-8h. The reflux assembly overhead was fitted with silica guard tube cotton mouth closed. After refluxing, the reaction mixture was dried on rotary evaporatory and a dark brown mass was found. The dark brown mass was further extracted with diethyl ether (3 X 20 mL) which allowed to cool overnight to give **ARK-1** as light brown needles (2.87 g, 17.48 mmol).

55%, Brown solid, m.p. 195 – 197 °C

IR (KBr cm^{-1}): 3309 (N-H str.), 2247 (CN str.), 2207 (CN str.), 1636 (C=N str.), 1256 (C-O str.).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.95 (1H, s), 4.64 (D_2O exchangeable NH_2, 2H, bs), 4.23 (q, 2H, J = 7.3 Hz),1.33 (3H, t, J = 7.3 Hz).

Ethyl-*N*-((*Z*)-2-amino-1,2-dicyanovinyl)formimidate



ARK-2

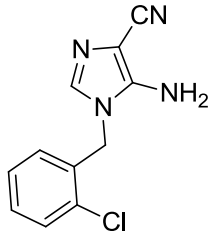
The o-chlorobenzylamine (1.01 g, 6.07 mmol) was added to a suspension of 1 (1.00 g, 6.09 mmol) in ethanol, and also added a pinch catalytic amount of anilinium chloride. The mixtures were stirred at room temperature until TLC (4 : 1 n-hexane : ethylacetate) showed that all the formimidate had disappeared (usually 10 -12h) . The precipitate from reaction mixture was washed with diethylether and dried on rota-vapour to remove the ethanol formed during the reaction and the product was crystallized in absolute ethanol to afford the pure compound (**ARK-2**).

60%, Light brown solid, m.p. 108–110 °C (decomp.).

IR (KBr cm^{-1}) : 3417 (N-H str.), 2212 (CN str.), 2191(CN str.) 1651 (C=N, str.), 1578 (N-H bend.), 741 (C-Cl str.).

^{1}H NMR (400 MHz, d_6-DMSO): δ = 8.16 (1H, d, J = 4.6 Hz), 7.74 (1H, d, J = 3.7 Hz), 7.42 - 7.44 (2H, m), 7.29 - 7.31 (2H, m), 4.57 (2H, d, J = 5.9 Hz), 6.10 (D_2O exchangeable NH_2, 2H, bs).

Ethyl-*N*-((*Z*)-2-amino-1,2-dicyanovinyl)formimidate



ARK-3

A suspension of the corresponding **ARK-2** (1.00 g) in potassium hydroxide solution (1 M, 10 mL) was stirred at room temperature for approximately 6-8h until TLC showed complete consumption of the starting material. The precipitated product was filtered off, washed with water (5 mL), followed by a mixture of dry diethyl ether/ethanol (10 : 1) and air-dried in the absence of light to give the desired products 3a-d. The yield of these reactions were 65 - 73%. Recrystallization of the product

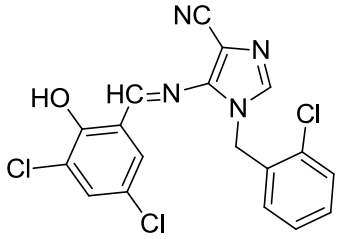
from dry diethyl ether/ethanol (1 : 1) and air-dried in the absence of light gave white crystals of **ARK-3** (0.76 g, 3.28 mmol).

65%, White solid

IR (KBr cm^{-1}) : 3357 (N-H str.), 2212 (CN str.), 1651 (C=N, str.), 1578 (N-H bend.), 741 (C-Cl str.).

^{1}H NMR (400 MHz, d_6-DMSO): δ = 7.50-7.52 (1H, m), 7.31-7.35 (2H, m), 7.19 (1H, s), 6.72-6.73 (1H, m), 6.32 (D_2O exchangeable NH_2, 2H, bs), 5.16 (2H, s).

(*E*)-1-(2-chlorobenzyl)-5-((3,5-dichloro-2-hydroxybenzylidene)amino)-1*H*-imidazole-4-carbonitrile (ARK-4)



ARK-4

To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 3,5-Dichlorosaliycaldehyde (99 mg, 0.51 mmol, 1.1 equiv.) by using methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 16-18 hrs. The TLC characterization supported the formation of the product.

Yellow solid, m.p. 183 - 187 °C

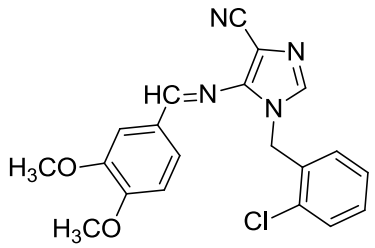
IR (KBr cm^{-1}): 2250 (CN str.), 1608 (C=N str.), 1581 (C=C aromatic, str.), 1206 (C-N str.), 731 (C-Cl str.).

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 11.35 (D_2O exchangeable OH, 1H, bs), 9.24 (1H, s), 7.9 (1H, s), 7.81 (1H, d, J = 2.6 Hz), 7.62 (1H, d, J = 2.56 Hz), 7.47 (1H, dd, J = 1.32 Hz), 7.27 - 7.36 (2H, m), 7.11 (1H, dd, J = 1.6 Hz), 5.45 (2H, s).

^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ: 161.73, 153.95, 145.19, 138.63, 133.26, 132.91, 132.21, 129.70, 129.51, 129.21, 128.19, 127.38, 123.79, 122.55, 122.49, 115.30, 99.85, 45.54.

MS (ESI): m/z = 406 $[M+1]^+$

(*E*)-1-(2-chlorobenzyl)-5-((3,4-dimethoxybenzylidene)amino)-1*H*-imidazole-4-carbonitrile (ARK-5)



ARK-5

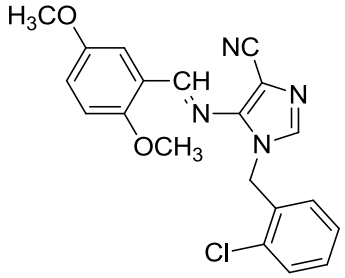
To the 100 mg suspension of ARK-3 (100 mg, 0.47 mmol, 1 equiv.), verataldehyde (86 mg, 0.51 mmol, 1.1 equiv.) by using methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 10-12h. The TLC characterization supported the formation of the product.

Pale white solid, m.p.192 – 198 °C

IR (KBr cm^{-1}): 2241 (CN str.), 1576 (C=N str.), 1571 (C=C aromatic, str.), 1261 (C-N str.), 731 (C-Cl str.).

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 8.97 (1H, s), 7.52 (1H, s), 7.50 (1H, d, J = 1.84 Hz), 7.41 - 7.45 (2H, m), 7.28 - 7.30 (1H, m), 7.20 – 7.24 (1H, m), 7.11 - 7.14 (1H, dd, J = 1.64 Hz), 6.95 (1H, d, J = 8.36 Hz), 5.37 (2H, s), 3.97 (3H, s), 3.95 (3H, s).

(*E*)-1-(2-chlorobenzyl)-5-((2,5-dimethoxybenzylidene)amino)-1*H*-imidazole-4-carbonitrile (ARK-6)



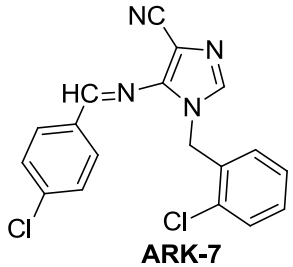
ARK-6

To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 2,5-Dimethoxybenzaldeyde (85.90 mg, 0.51 mmol, 1.1 quiv.) by using methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 12-14h. The TLC characterization supported the formation of the product.

White solid, m.p.169 – 175 °C

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 9.49 (D_2O exchangeable NH, 1H, bs), 7.57 (1H, d, J = 2.8 Hz), 7.52 (1H, s), 7.41 – 7.43 (1H, m), 7.33 (1H, d, J = 3.2 Hz), 7.28 (1H, d, J = 1.72 Hz), 7.07 - 7.15 (3H, m), 5.36 (2H, s), 3.80 (3H, s), 3.88 (3H, s).

(*E*)-5-((4-chlorobenzylidene)amino)-1-(2-chlorophenyl)-1*H*-imidazole-4-carbonitrile (ARK-7)



ARK-7

To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 4-chlorobenzaldeyde (71.69 mg, 0.51 mmol, 1.1 equiv.) by using methanol

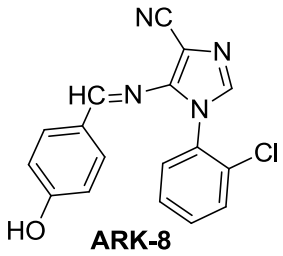
as solvent was added in 50mL RBF. The reaction mixture was then put on refluxing for 20-22h. The TLC characterization supported the formation of the product.

Yellow solid, m.p.167 -171 °C.

IR (KBr cm^{-1}): 2250 (CN str), 1608 (C=N str), 1581 (C=C aromatic, str.), 1206 (C-N str), 731(C-Cl str).

^1H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 9.06 (1H, s), 7.87 (2H, dd, J = 1.8 Hz), 7.54 (1H, s), 7.48 (2H, dd, J = 2.24 Hz), 7.42 – 7.44 (1H, m), 7.27-7.31 (1H, m), 7.21-7.25 (1H, m), 7.13 (1H, dd, J = 1.96 Hz).

(*E*)-1-(2-chlorophenyl)-5-((4-hydroxybenzylidene)amino)-1*H*-imidazole-4-carbonitrile (ARK-8)



ARK-8

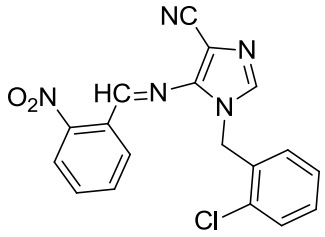
To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 4-hydroxybenzaldeyde (62.28 mg, 0.51 mmol, 1.1 equiv.) by using methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 18-20h. The TLC characterization supported the formation of the product.

Light Yellow solid, m.p. 201 – 204 °C

IR (KBr cm^{-1}): 2241 (CN str.), 1576 (C=N str.), 1571 (C=C aromatic str.), 1261 (C-N str.), 714 (C-Cl str.).

^1H NMR (400MHz, $CDCl_3$, TMS = 0) δ: 9.78 (1H, s), 8.80 (D_2O exchangeable OH, 1H, bs), 7.72 - 7.74 (4H, m), 7.28-7.34 (4H, m), 5.38 (2H, s).

(*E*)-1-(2-chlorophenyl)-5-((2-nitrobenzylidene)amino)-1*H*-imidazole-4-carbonitrile (ARK-9)



ARK-9

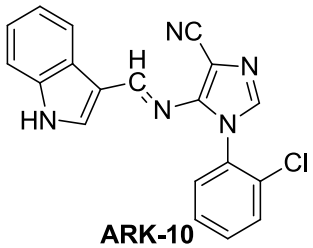
To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 2-nitrobenzaldeyde (77.07 mg, 0.51 mmol, 1.1 equiv.) by using methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 20-22h. The TLC characterization supported the formation of the product.

Pale yellow solid, m.p.184 – 189 °C

IR (KBr cm^{-1}): 2198 (CN str.), 1577 (C=N str.), 1448 (C=C aromatic str.), 1256 (C-N str.), 801(C-Cl str.).

^{1}H NMR (400MHz, $CDCl_3$, TMS = 0) δ: 9.54 (1H, s), 8.13 (1H, dd, J = 1.64 Hz), 8.08 (1H, dd, J = 1.32 Hz), 7.68 - 7.76 (2H, m), 7.56 (1H, s), 7.43-7.47 (2H, m), 7.12 (1H, dd, J = 1.64 Hz), 6.96 (1H, s, J = 1.80 Hz), 5.36 (2H, s)

(*E*)-5-(((1*H*-indol-2-yl)methylene)amino)-1-(2-chlorophenyl)-1*H*-imidazole-4-carbonitrile (ARK-10)



ARK-10

To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 3-indole-carboxaldeyde (74.03 mg, 0.51 mmol, 1.1 equiv.) by using

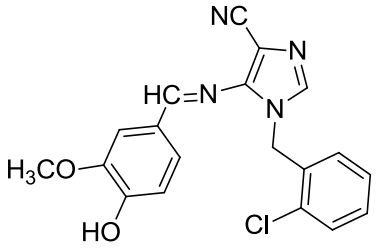
methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 14-16h. The TLC characterization supported the formation of the product.

Brownish yellow solid, m.p.167 – 170 °C

IR (KBr cm^{-1}): 2260 (CN str.), 1579 (C=N str.), 1559 (C=C aromatic, str.), 1198(C-N str.), 771(C-Cl str.).

^{1}H NMR (400MHz, $CDCl_3$, TMS = 0) δ: 12.08 (1H, s), 9.05 (1H, s), 8.22 (1H, s), 8.17 (1H, d, J = 3.04 Hz), 8.0 (1H, d, J = 7.8 Hz), 7.83 (1H, s), 7.46 - 7.51 (2H, m), 7.22 - 7.33 (2H, m), 7.08 - 7.12 (1H, m), 7.01 (1H, dd, J = 1.64 Hz), 5.43 (2H, s).

(*E*)-1-(2-chlorobenzyl)-5-((4-hydroxy-3-methoxybenzylidene)amino)-1*H*-imidazole-4-carbonitrile (ARK-11)



ARK-11

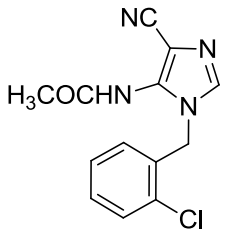
To the 100 mg suspension of **ARK-3** (100 mg, 0.47 mmol, 1 equiv.), 3-indole-carboxaldeyde (74.03 mg, 0.51 mmol, 1.1 equiv.) by using methanol as solvent was added in 50 mL RBF. The reaction mixture was then put on refluxing for 14-16h. The TLC characterization supported the formation of the product.

Dark Yellow solid, m.p.198 – 203 °C

IR (KBr cm^{-1}): 2277 (CN str.), 1589(C=N str.), 1481 (C=C aromatic, str.), 1301(C-N str.), 778(C-Cl str.).

^{1}H NMR (400MHz, $CDCl_3$, TMS = 0) δ: 9.83 (1H, s), 8.95 (1H, s), 7.51 (1H, s), 7.41 - 7.45 (5H, m), 7.04 (2H, d, J = 8.48 Hz), 5.36 (2H, s), 3.97 (3H, s).

(***N-(1-(2-chlorobenzyl)-4-cyano-1H-imidazol-5-yl)acetamide)*** (**ARK-12**)



ARK-12

To the 100 mg suspension of **ARK-4** (1.39 mmol, 1 equiv.), acetic anhydride was added in 50 mL RBF attached with reflux condenser on heating mantle for 6-8h. The TLC characterization supported the formation of the product.

White solid, m.p.192-194°C

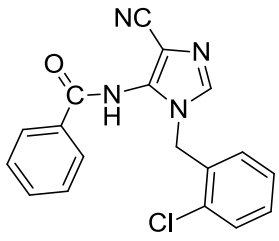
IR (KBr cm^{-1}): 2290 (CN str.), 1609 (C=N str.), 1341 (C=C aromatic, str.), 1213 (C-N str.), 691 (C-Cl str.).

^{1}H NMR (400 MHz, $CDCl_3$, TMS = 0) δ: 10.25 (D_2O exchangeable NH, 1H, bs), 7.76 (1H, s), 7.46 (2H, dd, J = 1.36 Hz), 7.29 - 7.37 (2H, m), 7.02 (1H, d, J = 7 Hz), 5.22 (2H, s), 2.04 (3H, s).

^{13}C NMR (100 MHz, $CDCl_3$, TMS = 0) δ: 169.18, 137.47, 135.06, 132.71, 132.30, 129.75, 129.47, 129.25, 127.39, 114.43, 107.12, 45.92, 22.36.

MS (ESI): m/z = 275 $[M+1]^+$

N-(1-(2-chlorobenzyl)-4-cyano-1H-imidazol-5-yl)benzamide (ARK-13)



ARK-513

To the 100 mg suspension of **ARK-3** (100 mg, 1.39 mmol, 1 equiv.), benzoyl chloride (1mL) as solvent was added in 50mL RBF. The reaction mixture was then put on refluxing for 18-20h. The TLC characterization supported the formation of the product.

Light yellow solid, m.p. 231 – 235 °C

IR (KBr cm^{-1}): 2244 (CN str.), 1609 (C=O str.), 1510 (C=C aromatic str.), 1293 (C-N str.), 817 (C-Cl str.).

^{1}H NMR (400MHz, $CDCl_3$, TMS = 0) δ: 8.30 (2H, d, J = 7.4 Hz), 7.82 (1H, s), 7.58 (1H, d, J = 7.28 Hz), 7.53 (2H, d, J = 7.84 HZ), 7.46 (2H, d, J = 4.32 Hz), 5.49 (2H, s).

4.2 Biological Studies

4.2.1 General

4.2.1.1 Chemicals

1. RPMI 1640 and DMEM, Penicillin/ Streptomycin antibiotic solution, phosphate buffer saline and fetal bovine serum media were used for culture of the cancer cell lines and were purchased from HiMedia.
2. MTT dye used for MTT assay was purchased from HiMedia.
3. DMSO, extrapure AR was purchased from SRL.

4.2.1.2 Instruments

Table 4.2.1.2 List of Instruments Used in Biological Evaluation

Instruments Used	**Company**	**Purpuse**
Automatic cell Counter	Invitrogen	For counting of cells
Incubator	Galaxy, New Brunswick	Incubation
Centrifuge 5430 R	Eppendorf, Germany	Centrifugation
laminar air flow	Macro Scientific Works	For aseptic condition
UV-VIS Spectrophotometer,	Shimadzu	Absorption studies
Inverted microscope	Magnus, Olympus	Visulization of the cancer cells

4.2.1.3 Cell lines under study

Two cancer cell lines– Hep-G2 and A-549 were used for evaluating anticancer assay and these were purchased from National Cell Repository at National Centre for Cell Sciences, Pune.

Hep-G2- It is a human hepatocellular carcinoma cell line which has been derived from a well-differentiated hepatocellular carcinoma. They secretes plasma proteins, e.g., albumin and fibrinogen, transferring. The cell lines have their use in the detection of hepatocarcinogenesis. (Holland et al ., 2001) (Aflatoonian & Moore, 2005).

A549- This is human lung carcinoma cell line which is adenocarcinoma human alveolar basal epithelial cells. These lung alveolar squamous cells contain desaturated fatty acids. The cell line is used for the drug metabolism as type II pulmonary epithelial cell. A549 cells has use of anti-cancer drug permeability and effectiveness analysis, cell ageing studies, apoptosis and profiling of cytokine expression. These cells are employed in the study of an array of molecular features of human tumours in culture (Jiang et al ., 2010; Lieber et al ., 1976).

4.2.1.4 Routine assay in cell culture laboratory

A. Culturing of the cell lines

Cancer cell lines were treated in the appropriate medium (DMEM). Trypsin is added to detach the cancer cell lines (trypsinization). Subsequently, trypsin was inactivated by the addition of media containing serum (1mL). Centrifugation was done on 1200 rpm at 37˚C for 10 mins for harvesting the cells. Further, supernatant was disposed and resuspension of the cell pellet was done using 2 mL of the media. The cell number was counted using automated cell counter. The cells were transferred to fresh media every three days.

B. Maintenance and sub-culturing of cell lines

The cancer cell lines were cultured. The maintenance of cultured cell lines was done in 25 cm^2 or 75 cm^2 flasks containing DMEM medium supplemented with 10% fetal Bovine serum (FBS), 1X antibiotic solution and afterward incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% humidity.

The cells were subcultured in 25 cm^2 flasks and become important when the cell lines have attained 70 - 80% growth. The reagents necessary for the procedure were placed in water bath maintained at 37˚C for 10 - 15 mins earlier to the sub-culturing. During sub-culturing, trypsin was added. After 5 mins, 1 mL of media containing serum was added for ceasing the action of trypsin. Cells were then transferred to 15 mL centrifuge tubes and centrifuged for 10 mins at 1200 rpm. The supernatant was cast aside and the pellet was again resuspended in complete media. The cell lines were transferred to fresh media every three days.

4.2.1.5 Evaluation of anticancer activity of the synthesized compounds (MTT Assay)

The assay was specified by Mosmann and is also known as cell viability assay. MTT is an in vitro colorimetric assay for the measurement of cell proliferation. The tetrazolium compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) when embarks in cells is reduced to an insoluble coloured formazan product by mitrochondrial succinate dehydrogenase. When it passes to the mitochondria it gets solubilised with DMSO (Figure 4.2.1). This is measured spectrophotometrically (Mirzayans, 2007; Mosmann, 1983).

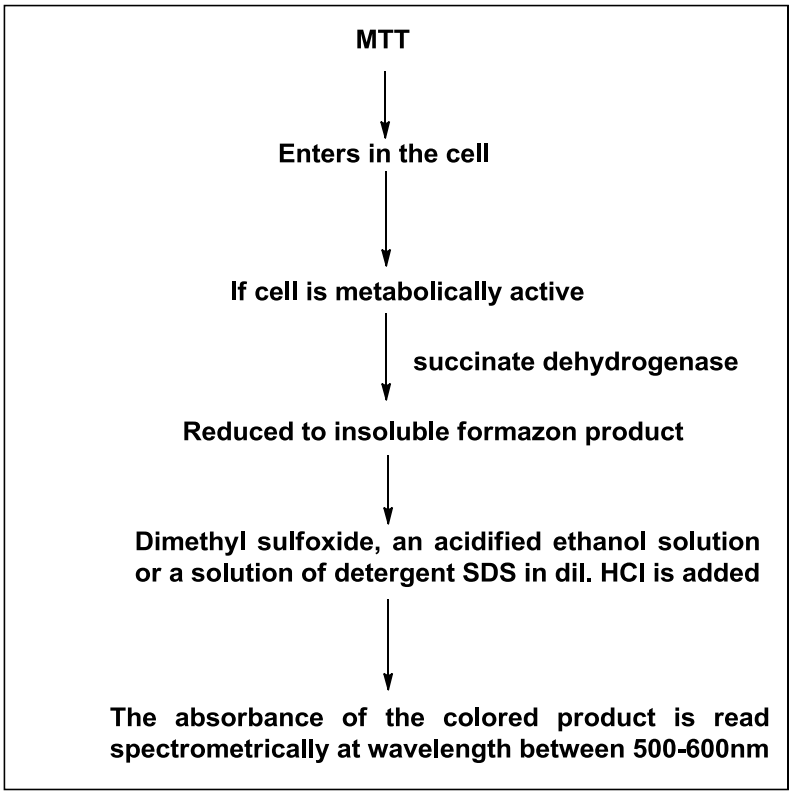


Figure 4.2.1.5: Principle of MTT Assay

Material: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), Phosphate buffer solution, DMSO (Dimethylsulfoxide)

Procedure: The cell from the cancer cell line – A-549 and Hep-G2 cells were counted on the automated cell counter. About 8,000-10,000 cells were seeded in each well of the 96 well plate. The plate was incubated at 37˚C with 5% CO_2 for 24 h. At the end of the 24 h, treatment was given to the cells in triplicate concentrations of 5 µM, 25 µM and 50 µM. The cells were further incubated for 48h. The media was removed from each well and MTT solution (5 mg/10mL) was added. This was incubated in the dark for 4 h. At the end of 4 h, the MTT solution was disposed from each well and the intracellular precipitate was dissolved in DMSO solution and the absorbance of the violet colour formed as consequence of DMSO addition is read spectrometrically at 570 nm.

4.2.1.6 Evaluation of antioxidant activity of the synthesized compounds (DPPH Assay)

Reactive oxygen species can harm various biomolecules including DNA and consequences are oxidative stress in the biological systems. Antioxidants are known to act via reducing the oxidative stress by scavenging of the free radicals and therefore, prevent many diseases including cancer.

Hence, we performed DPPH assay to study the antioxidant activity of our compounds in vitro. DPPH assay was used to determine the total antioxidant activity by scavenging of the stable DPPH free radical (Figure 4.2.1.6). Antioxidant activity was observed to escalate in the concentration dependent manner. Good antioxidant activity was observed. Thus these may be acting through free radical mechanism as anticancer agents.

Free radical scavenging activity was evaluated by measuring scavenging activity of the compounds on 2,2,-diphenyl-1-picrylhydrazyl according to the previously described method. The principle of the assay is the use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity (Musa et al ., 2013; Szabo et al ., 2007).

Briefly, 2 mL of the different concentrations of compounds in methanol were added to 2mL of 0.135 mM methanolic solution of DPPH. The mixture was shaken vigorously and was left to stand for 30 mins in the dark. Absorbance was recorded at 517 nm against the blank. Blank was prepared without the addition of DPPH. BHT was used as standard. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

Where, $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract/standard.

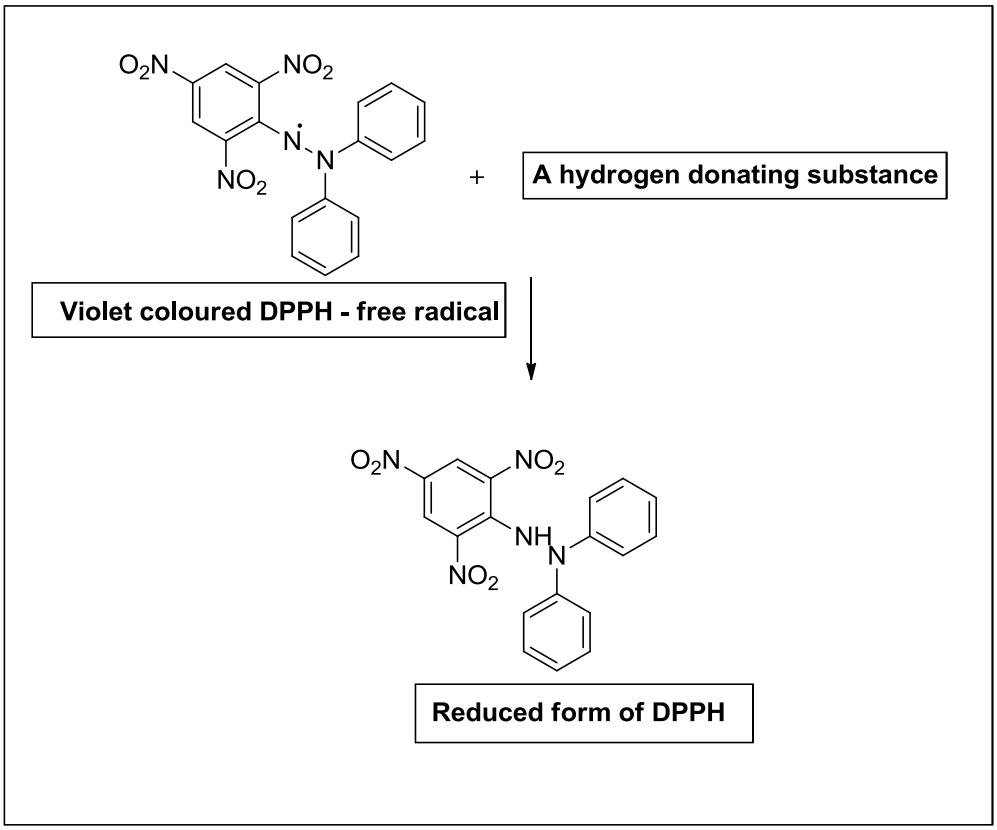


Figure 4.2.1.6: Mechanism of DPPH

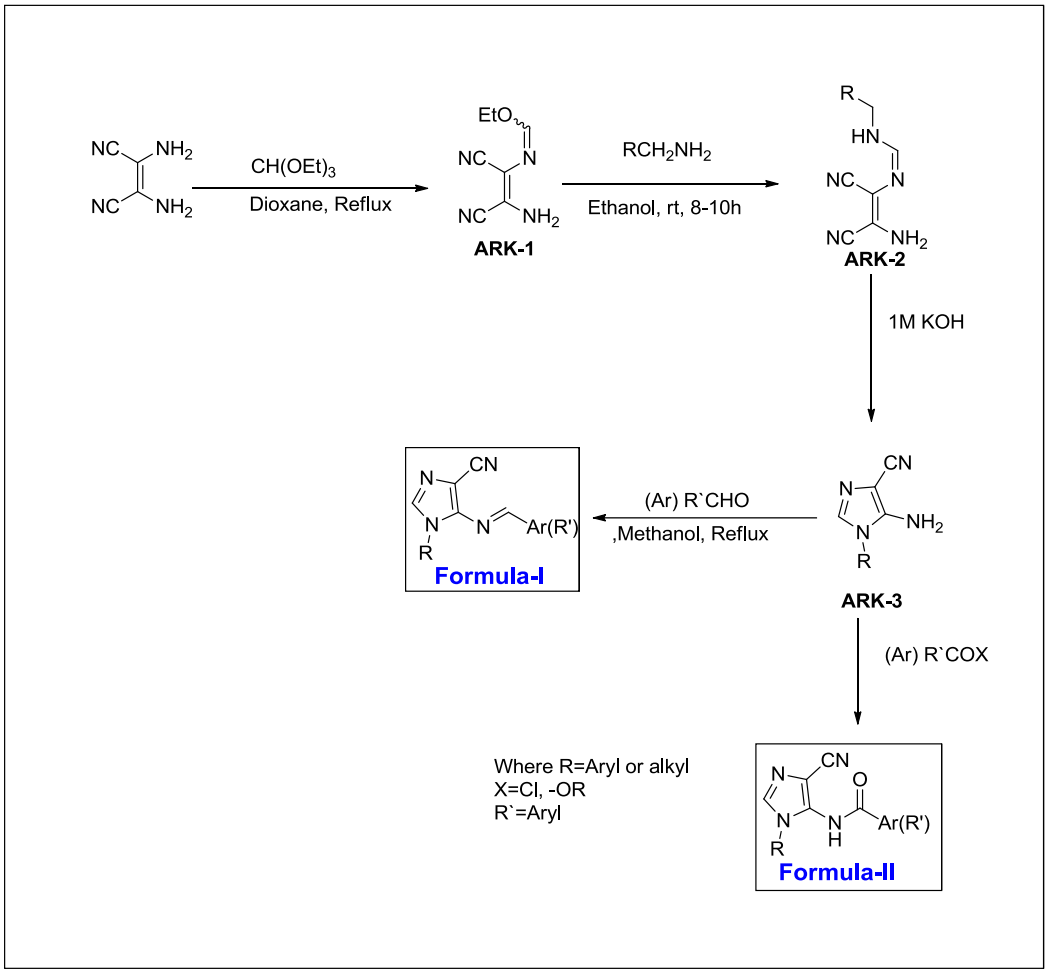
Chapter 5

Results and Discussion

Chapter 5

Results and Discussion

For the synthesis of the target compounds, proposed Scheme 5.1 was followed.

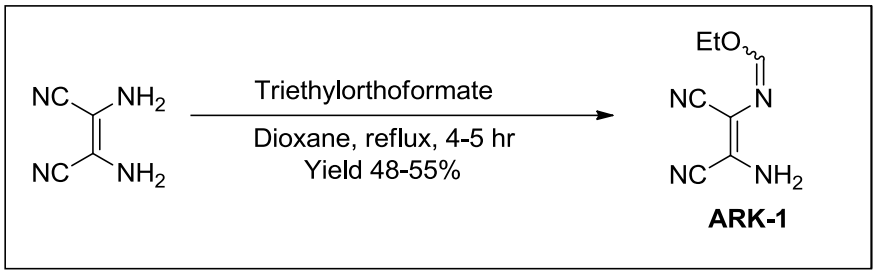


Scheme 5.1 Synthetic route for target compounds

In the step 1 (**Scheme 5.2**), the mixture of diaminomaleonitrile and triethyl orthoformate was refluxed in 1,4-dioxane as solvent to get Ethyl (Z)-N-(2-amino-1,2-dicyanovinyl)formimidate (**ARK-1**), the progress of the reaction was monitored by TLC.

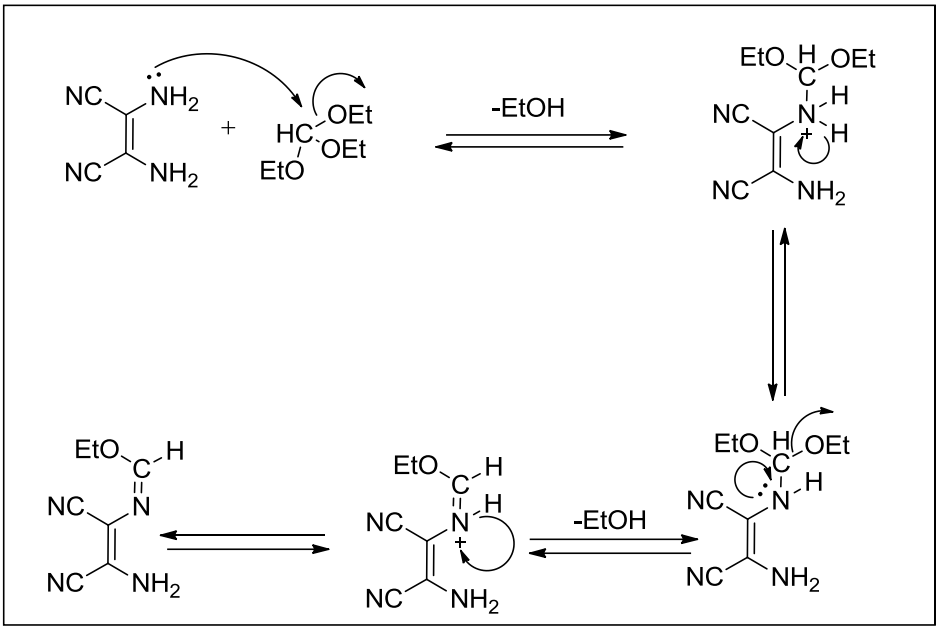
IR spectrum showed peaks at 3418 cm^{-1}, 2240 cm^{-1} and 1630 cm^{-1} confirming the presence of NH_2, CN and C=N respectively.

The proton NMR spectrum showed a characteristic triplet of three protons of CH_3 at *δ* 1.37. A quintet of two hydrogens of CH_2 appeared at *δ* 4.29 which was due to adjacent CH_3 group. A singlet of two hydrogens of NH_2 appeared at *δ* 4.69 which was D_2O-exchangable.



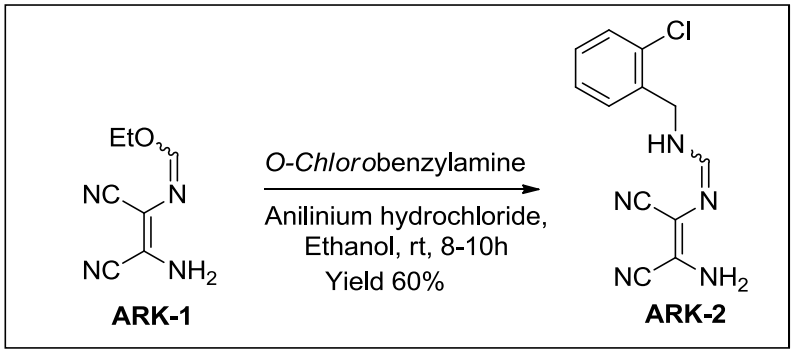
Scheme 5.2: Ethyl (Z)-N-(2-Amino-1,2-dicyanovinyl)formimidate (**ARK-1**).

Mechanistically, the above reaction involves the nucleophilic attack of one of nitrogen's lone pairs of **diaminomaleonitrile** on electrophilic carbon of triethyl-orthoformate leading to the formation of **ARK-1** via various intermediates as a shown in **scheme 5.3** with loss of two molecules of ethanol.



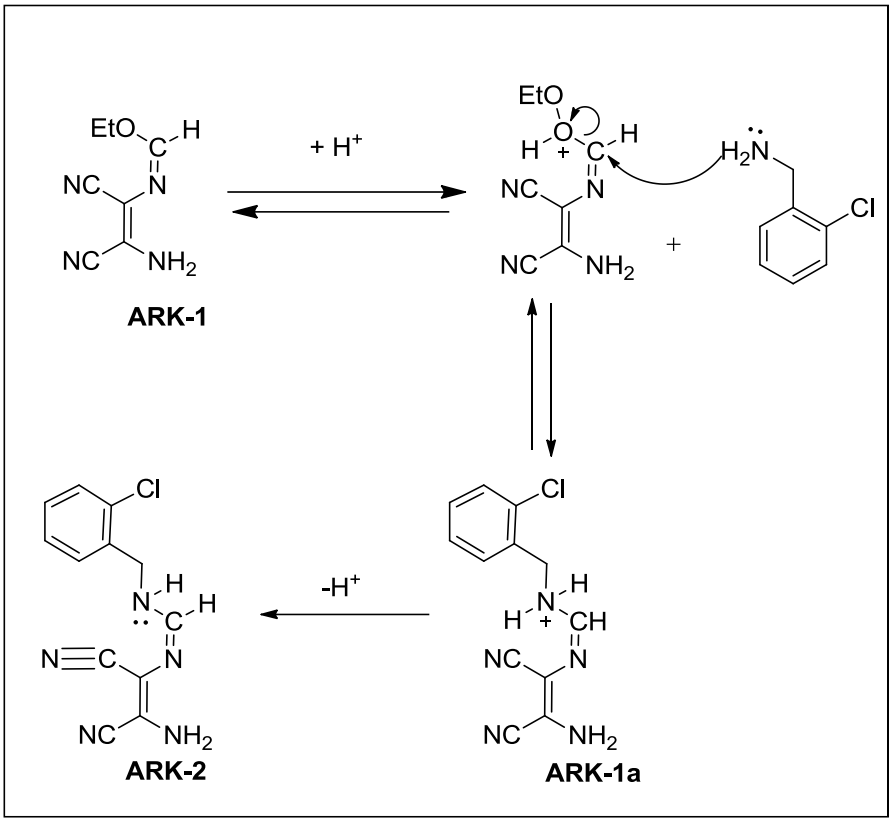
Scheme 5.3: Possible Mechanism of Formation of **ARK-1**

Then **ARK-1** was treated with o-chlrobenzylamine (OCB) in ethanol as a solvent which led to the formation of N'-((Z)-2-amino-1,2-dicyanovinyl)-N-(2-aminophenyl)formimida-mide (**ARK-2**; step-2; **Scheme 5.4**). The compound was used for next step without any further purification.



Scheme 5.4: N'-((Z)-2-amino-1,2-dicyanovinyl)-N-(2-aminophenyl)formimidamide (**ARK-2**)

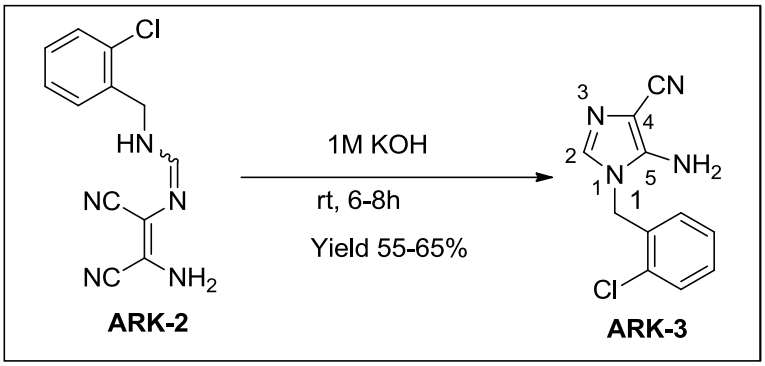
The formation of **ARK-2** can be mechanistically predicted as the nucleophilic attack nitrogen's lone pairs of OCB on electrophilic carbon atom of **ARK-1** via an intermediate **ARK-1a** as shown in **scheme 5.3** with the loss of one molecule of ethanol.



Scheme 5.5: Plausible Mechanism of Formation of (**ARK-2**)

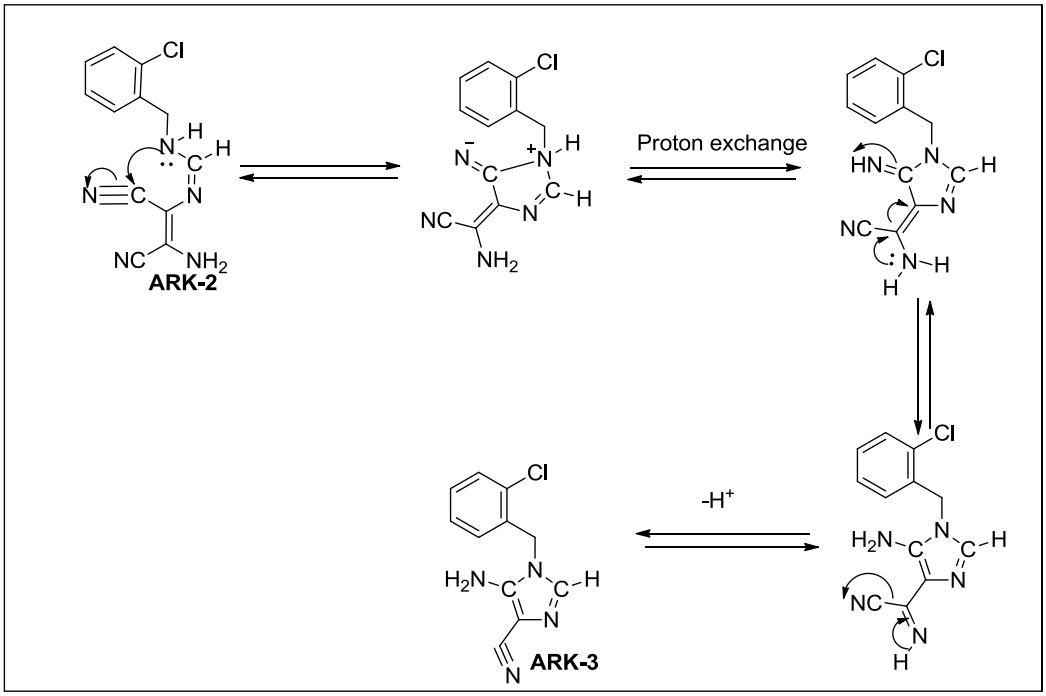
IR spectrum showed characteristic peaks of two cyano, NH and aromatic C=C at 2207, 2256 cm^{-1}, 3468 cm^{-1} and 1555 cm^{-1}, respectively. The sharp peak at 770 cm^{-1} indicated chloro-substituent, **ARK-2** formation.^{1}H NMR also showed a doublet at 4.57 ppm indicated the appearance of benzylic protons.

Further, stirring of **ARK-2** at room temperature with 1 M KOH afforded 5-amino-1-(2-chlorobenzyl)-1H-imidazole-4-carbonitrile (**ARK-3; Scheme 5.6**)



Scheme 5.6 5-amino-1-(2-chlorobenzyl)-1H-imidazole-4-carbonitrile (**ARK-3**)

The formation of **ARK-3** from **ARK-2** could be mechanistically outlined as shown in **scheme 5.7**.



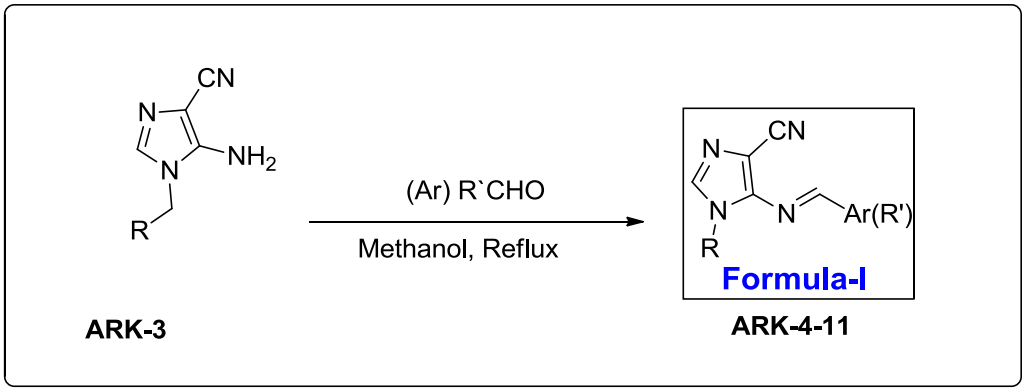
Scheme 5.7: Plausible Mechanism of Formation of **ARK-3**

IR spectrum shown cyanide, secondary amine stretching and bending vibration at 2230 cm^{-1}, 3361 cm^{-1}, and 1591 cm^{-1}, respectively. Disappearance of one cyanide peak (2256 cm^{-1}) also confirms **ARK-3**

formation. ^{1}H-NMR spectra showed appearance of singlet at 7.19 ppm which was due to imidazole proton.

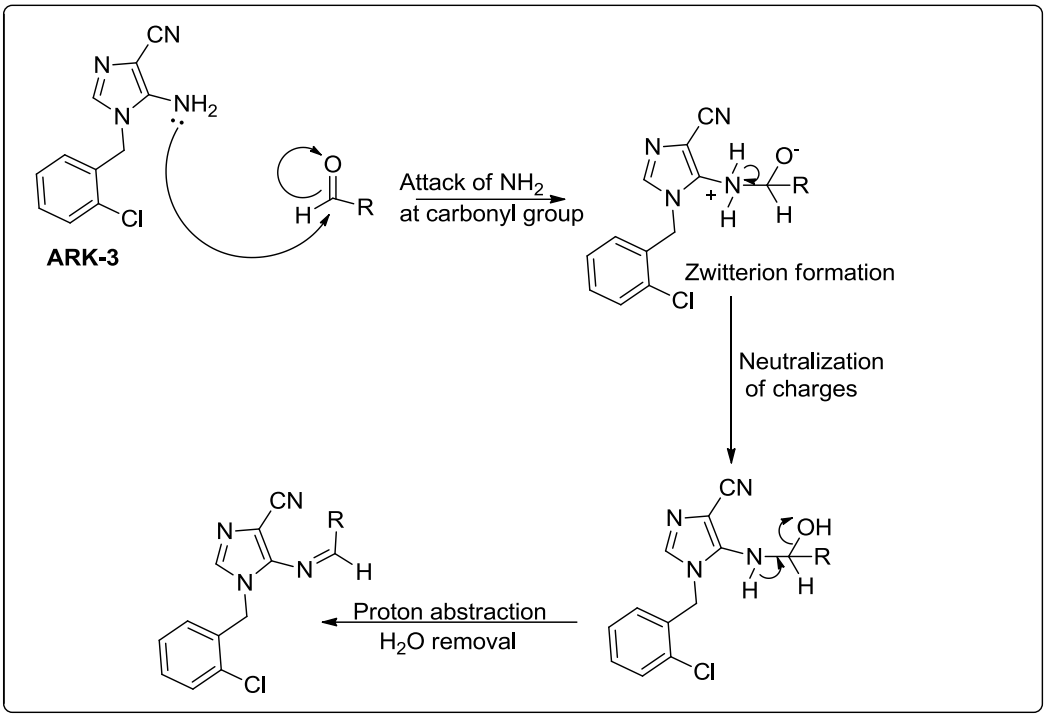
5.1 Synthesis of formula I compounds

The synthesis of imine derivatives was carried out by the reflux of **ARK-3** with aromatic aldehydes under methanol as shown in **Scheme 5.8** and **Figure 5.1**



Scheme 5.8 Synthesis of **ARK 4-11**

Mechanistically as represented in **Scheme 5.9**, nucleophilic attack of lone pair of NH_2 on the carbonyl group of aromatic aldehydes occur which leads to the formation of products via various intermediates. During the synthetic process, it was observed that the imine formation of those aldehydes having electron withdrawing group took shorter time than electron donating group containing aldehydes. It might be due to increase in the electrophilicity of carbonyl group because of presence of electron withdrawing groups on the ring rendering them more susceptible for nucleophilic attack.



Scheme 5.9 Plausible mechanism for formation of the compounds

IR spectra showed sharp peak in the range of 713 - 781 cm^{-1} predicting the presence of C-Cl bond stretching in the synthesized compounds. Sharp peak in the range of 1642 - 1688 cm^{-1} highlighted the formation of imine. Another peak in the range of 2210 - 2263 cm^{-1} confirmed the presence of cyano group.

^{1}H NMR spectrum characterization of the representative compound in **scheme 5.8** further confirmed the formation of the imine compounds as singlet at range 8.4 - 9.7 ppm due to –N=CH- group. The range of aromatic region is from 6.8 - 7.8 ppm. Methoxy containing compounds attributed chemical shift varying from 3.88 - 3.97 ppm (**ARK- 5**, **6, 11**). Phenol –OH appeared in the range of 8.80 – 11.35 ppm (**ARK**- **4**, **8**, **11**). Other characteristic peaks were also attained, as singlet at range 5.1 - 5.67 ppm for benzylic protons, peaks for 4 protons of chlorophenyl group in aromatic range at 6.76 - 7.91 ppm and a singlet of proton of C2 of imidazole moiety at 7.63 - 7.88 ppm were observed.

^{13}C spectra confirmed the product formation by indicating the peak for imine carbon at a downfield value of 161.73 ppm. Other characteristic peaks which appeared in the spectra were that of the aromatic ring carbons at a range of 122.39 - 139.11 ppm. The benzylic protons were shown at range of 45.11 - 45.65 ppm.

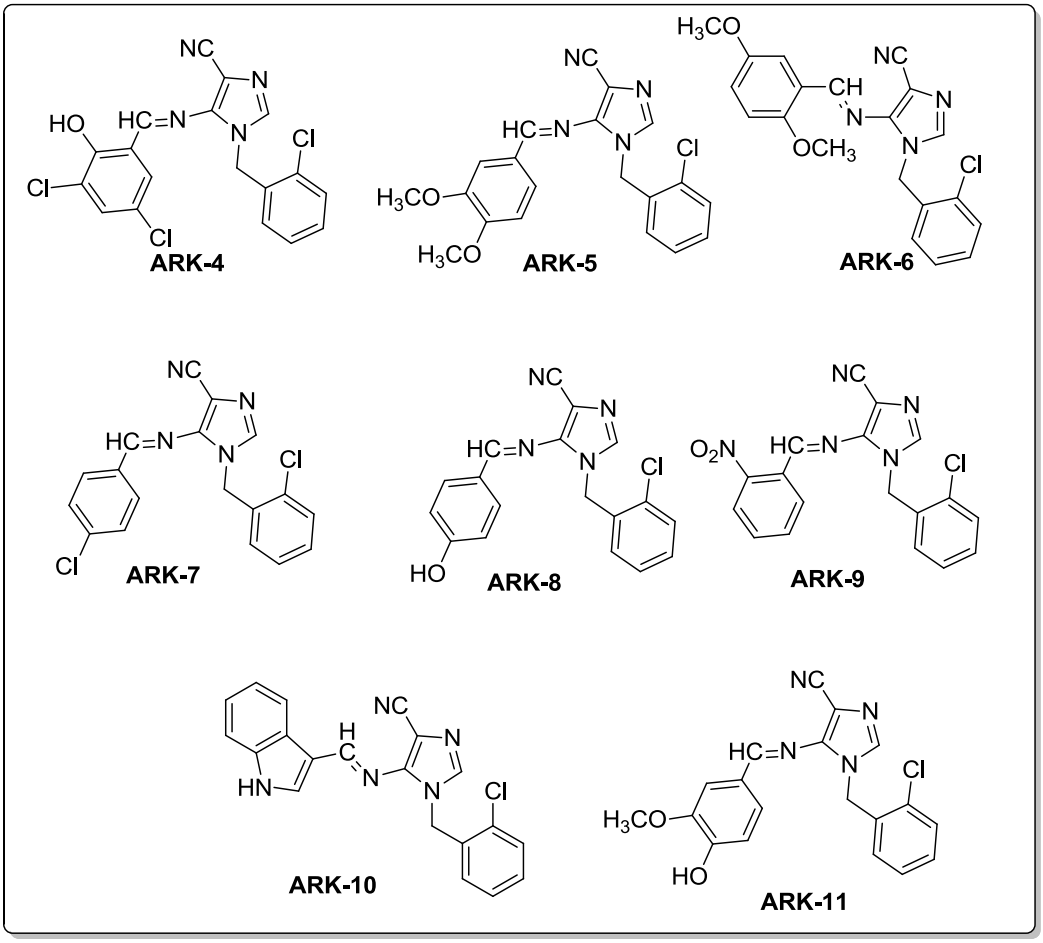
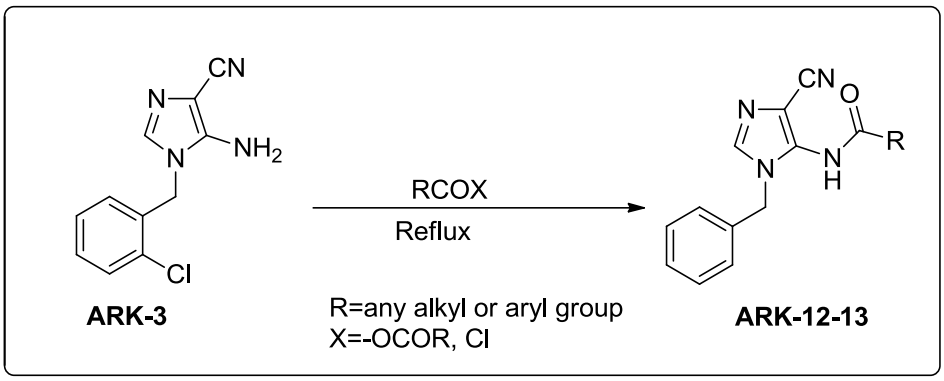


Figure 5.1 Chemical structures of Formula I

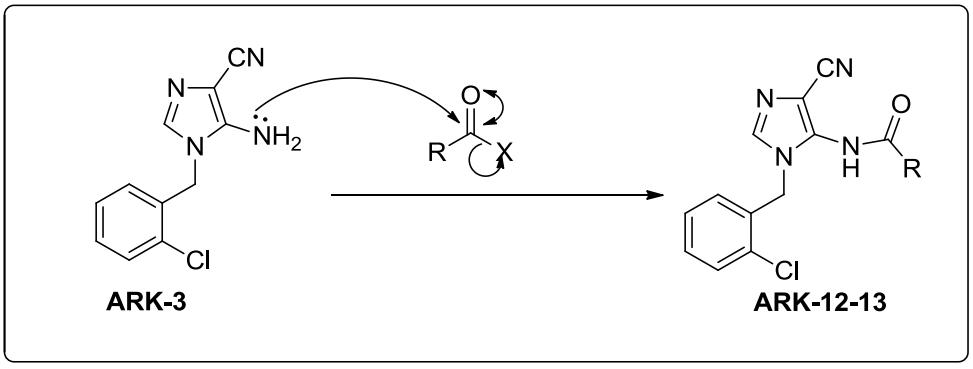
5.1 Synthesis of formula II compounds

Synthesis of **ARK-12** and **ARK-13**, via acetylation and benzylation was carried out at reflux using acetic anhydride and benzoyl chloride, respectively as shown in **Scheme 5.10** and in **Figure 5.2**



Scheme 5.10 Synthesis of formula II compounds

Reaction involves the nucleophile attack of NH_2 at electrophilic carbon of acid chlorides (Schotten Baumann reaction (J. J. Li, 2003)) or anhydride. During the synthetic process carried out, it was observed that the acetylation completed in 6-8h and needs optimum temperature of 45-55°C to selectively form **ARK-4**, but benzoylation takes much time, 14-16h to afford **ARK-5**.



Scheme 5.11 Plausible mechanism for formation for the formation of compounds

The compounds were characterized by their melting point values, FT-IR and NMR Spectra.

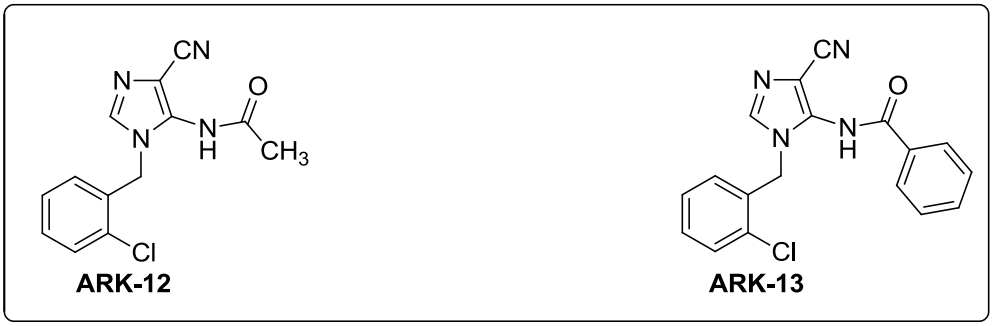


Figure 5.2 Chemical structures of synthetics of formula II

IR spectra showed peaks for carbonyl group at 1674 cm^{-1} and 1658 cm^{-1} for **ARK-12** and **ARK-13**. The reason behind the lower IR carbonyl stretching of amide in ARK-5 is possibly due to the additional aromatic-resonance with the carbonyl of the **ARK-13.** Peaks at 2865 cm^{-1} and 2937 cm^{-1} in **ARK-12** indicate the C-H stretching which were missing in ARK-13.

^{1}H NMR spectra confirmed the formation of the **ARK-12** and **ARK-13**. A singlet of three protons due to $–CH_3$ appeared at 2.04 ppm for **ARK-12** Two doublets at 8.30 ppm and 7.53 ppm and a singlet at 7.82 ppm, clearly indicated 5 protons of phenyl group of **ARK-13**. Although sp3 hybridised proton comes at 0.9 ppm but in **ARK-12**, because of diamagnetic anisotropy (Bothner-By & Pople, 1965) due to carbonyl deshielding effect the chemical shift value increases to 2.04 ppm. Other characteristic peaks were also obtained such as a singlet at range 5.22 - 5.49 ppm for benzylic protons, peaks of 4 protons of chlorophenyl group in aromatic range 7-7.8 ppm and singlet of proton of C2 of imidazole moiety at 7.76-7.82 ppm. ^{13}C spectra confirmed that the product formation by indicating the peak for the carbonyl carbon at a downfield value of 169.18 ppm. Other characteristic peaks which appeared in the spectra were that of the aromatic ring carbons at a range of 127.39-137.47 ppm. The benzylic carbon appeared at 45.92 ppm. The melting points for these compounds were found to be in the range of 192-194 ˚C (**ARK-12**) and 231-235˚C (for **ARK-13**).

5.2 Biological Studies

5.2.1 Evaluation of anticancer properties of the synthesized compounds:

Aiming to investigate the anticancer potential of the synthesized compounds (**ARK 4-12**), MTT assay was carried out with available lung (A-549) and hepatic (HepG) cancer cell lines. Approximately 8,000-10,000 cells were seeded per well of 96 well plate, overnight and treatments as indicated in the experimental design were given in triplicates. Almost all the compounds showed reasonable activity but **ARK-4**, **ARK-7**, **ARK-12**, **ARK-13** shown significant growth inhibition in these cancer cells A-549 and HepG.

These two cell lines- A-549, HepG were seeded in a 96 wells of microplate separately and subsequently treated with the compounds at different concentrations (5, 10, 25 µM) and was allowed to incubate overnight. The absorbance was measured spectrophotometrically at 570 nm. The values obtained were expressed as % inhibition (Mean ± S.D.) and graph was plotted.

All the compounds were tested against A-549 and HepG cancer cell lines

1. **Anticancer activity against A-549: Figure 5.2.1** depicts the plot, which highlights the inhibitory activity of the compounds against A-549. Compounds **ARK-8** and **ARK-9** showed commendable inhibitory potential at a low concentration of 5µM. Compounds **ARK-12**, **ARK-4**, **ARK-13** and **ARK-7** exhibit excellent activity albeit at a higher concentration of 50 µM. Compounds **ARK-6**, **ARK-7**, **ARK-8**, **ARK-9** also showed commendable activity against the cancer cell lines at a concentration of 25 µM.

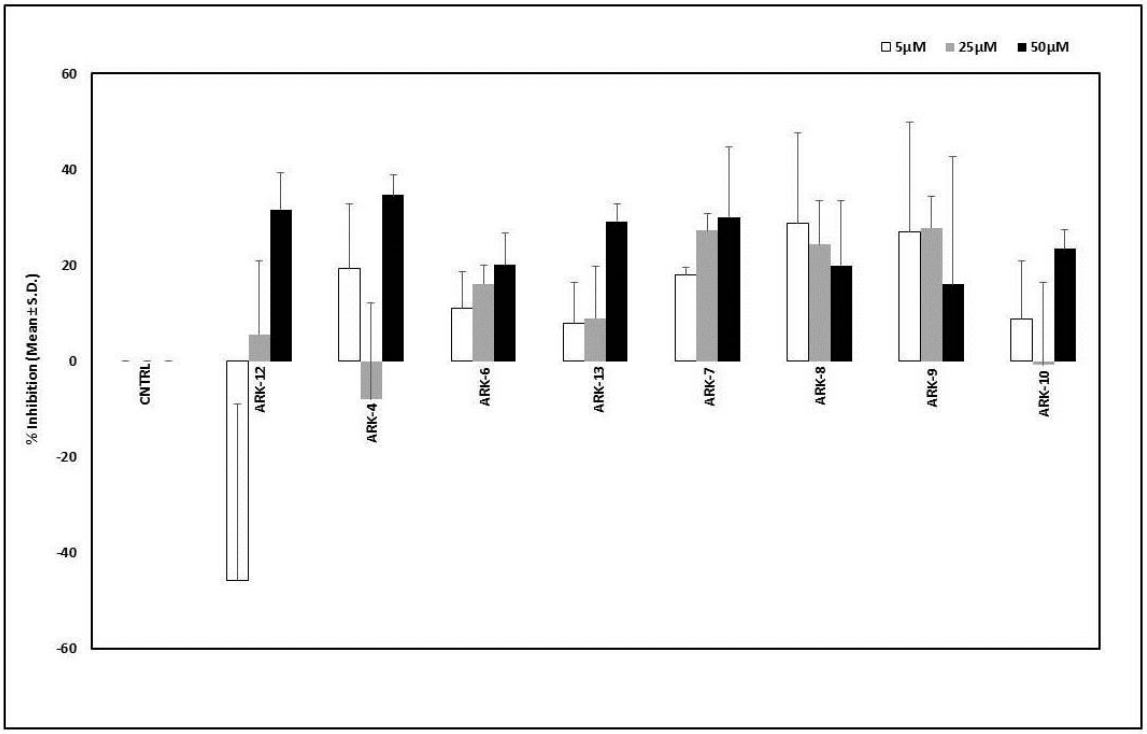


Figure 5.2.1 Percent inhibition of A-549 in response to treatment with synthesized compounds at concentrations of 5 µM, 25 µM and 50 µM for a time duration of 48 hrs. Data is expressed as mean values ± S.D. of three independent experiments.

2. **Anticancer activity against HepG: Figure 5.2.2** outlined the fact that the synthesized compounds showed excellent activity against HepG (hepatocellular cell line). All the synthesised compounds, except **ARK-6** and **ARK-8**, have shown significant anticancer activity against the cell line at concentration of 50 µM. These compounds also showed potent anticancer activity at concentration of 25 µM. At a concentration of 5 µM, the synthesised compounds exhibit very little or no activity.

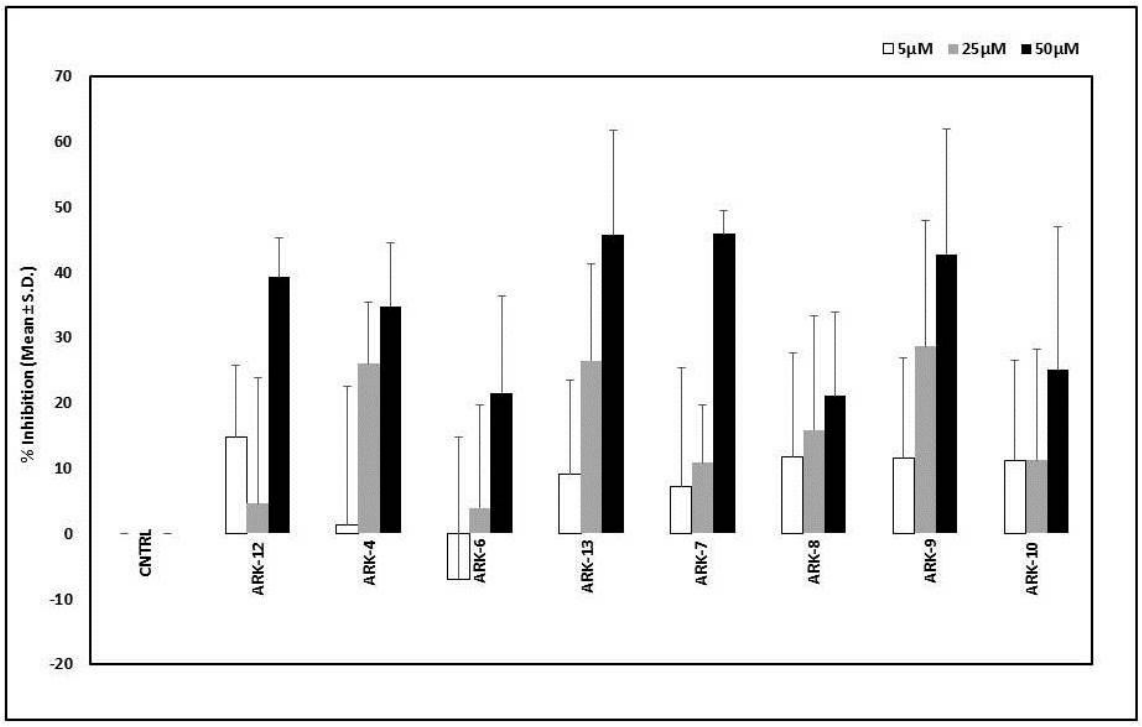


Figure 5.2.2 Percent inhibition of HepG2 in response to treatment with synthesized compounds at concentrations of 5 µM, 25 µM and 50 µM for a time duration of 48 hrs. Data is expressed as mean values ± S.D.

5.3 Evaluation of antioxidant properties of the compounds:

5.3.1 DPPH Assay

Being inspired from the anticancer activity of the synthetics, we thought of evaluating their potential as free radical scavengers which could be one of the mechanisms of observed anticancer activity. Some selected compounds **ARK-12**, **ARK-4, 6** and **ARK-7-9** were tested for their antioxidant potential at 4 mM concentration of which all were observed to possess excellent antioxidant activity (Table 5.3.1; Figure 5.3.1). All the synthesized compounds showed activity (> 95% inhibition) comparable to BHT (standard antioxidant used). The results proved the free radical scavenging potential of synthesised compounds.

Table 5.3.1 Antioxidant activity of the synthesized compounds[a]

[a]Compounds were screened at 4 mM concentration.

S.No	Compound	Absorbance	% Inhibition
Reference	**BHT (5mM)**	0.298	95%
1	**ARK-12**	0.182	99%
2	**ARK -8**	0.118	99%
3	**ARK-9**	0.114	99%
4	**ARK-4**	0.248	96%
5	**ARK -6**	0.205	96%
6	**ARK -7**	0.229	96%

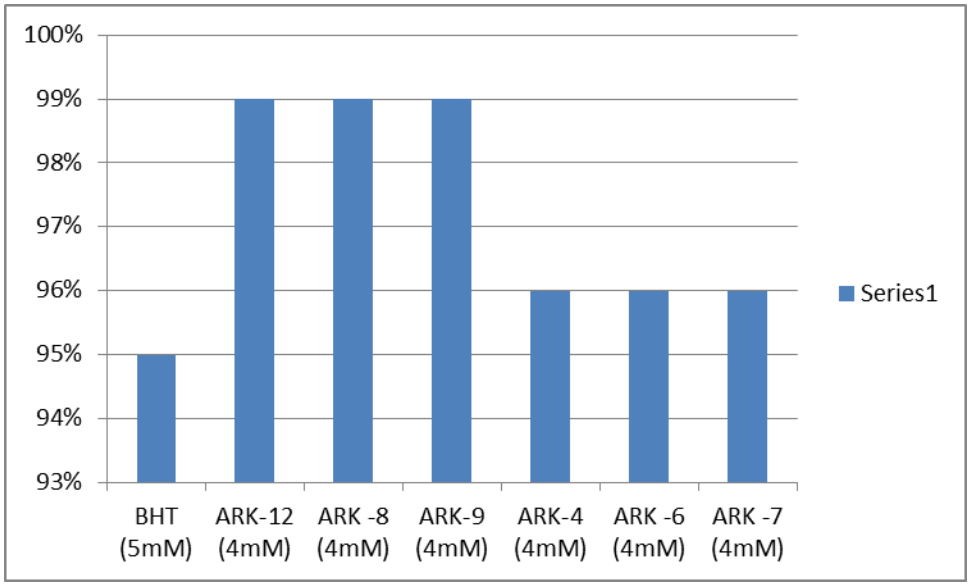


Figure 5.3.1 Free radical scavenging activity of the synthetics. Y axis denotes percentage inhibition of DPPH free radical.

Chapter 6

Conclusion

CHAPTER-6

CONCLUSION

Imidazole derived heterocycles have been found to play a vital role in all aspect such as biochemical, biophysical and significantly medicinal and this makes imidazole as indispensable scaffold. From all the activities reported, anticancer activity of the imidazole based compounds is extensively studied through multiple mechanisms involving inhibition of either insulin receptor kinases, topoisomerases etc. recently our group has reported the anticancer activity of imine based compounds.(Baviskar et al ., 2013). Based on the literature search and our own experiences, we designed and compounds having imidazoles in their pharmacophore and tethered them with aromatic or heteraromatic moiety via an imine bond. The synthesised compounds were characterised by various spectroscopic and spectrometric techniques. The synthetics were screened against available human cancer cell lines for their antiproliferative potential. Amonst all the screened compounds **ARK-8** and **ARK-9** showed commendable inhibitory potential at a low concentration of 5 µM against lung cancer cell line and **ARK-6** and **ARK-8**, showed significant anticancer activity against the hepatocellular cell line at concentration of 50 µM. Some selected compounds were further evaluated for their antioxidant potential and interestingly all of them exhibited excellent free radical scavenging activity at 4 mM concentration. The above combined results have shown advent of their first in vitro bioactivity as anticancer and antioxidant compounds and revealed their medicinal potential. The synthetics offer the scope for generation of a library of compounds and their evaluation against a panel of other cancer cell lines, studies on structure activity relationship, tracing their molecular mechanism(s) in addition to their development at preclinical level in future.

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SPECTRAL DATA

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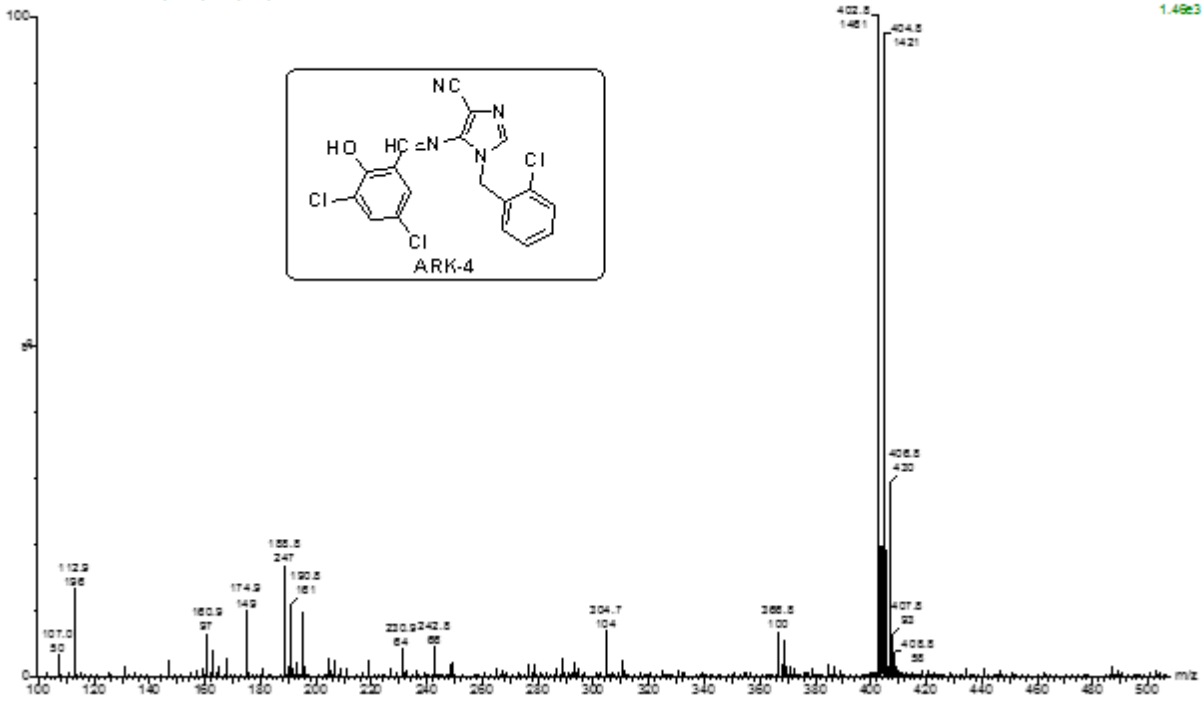

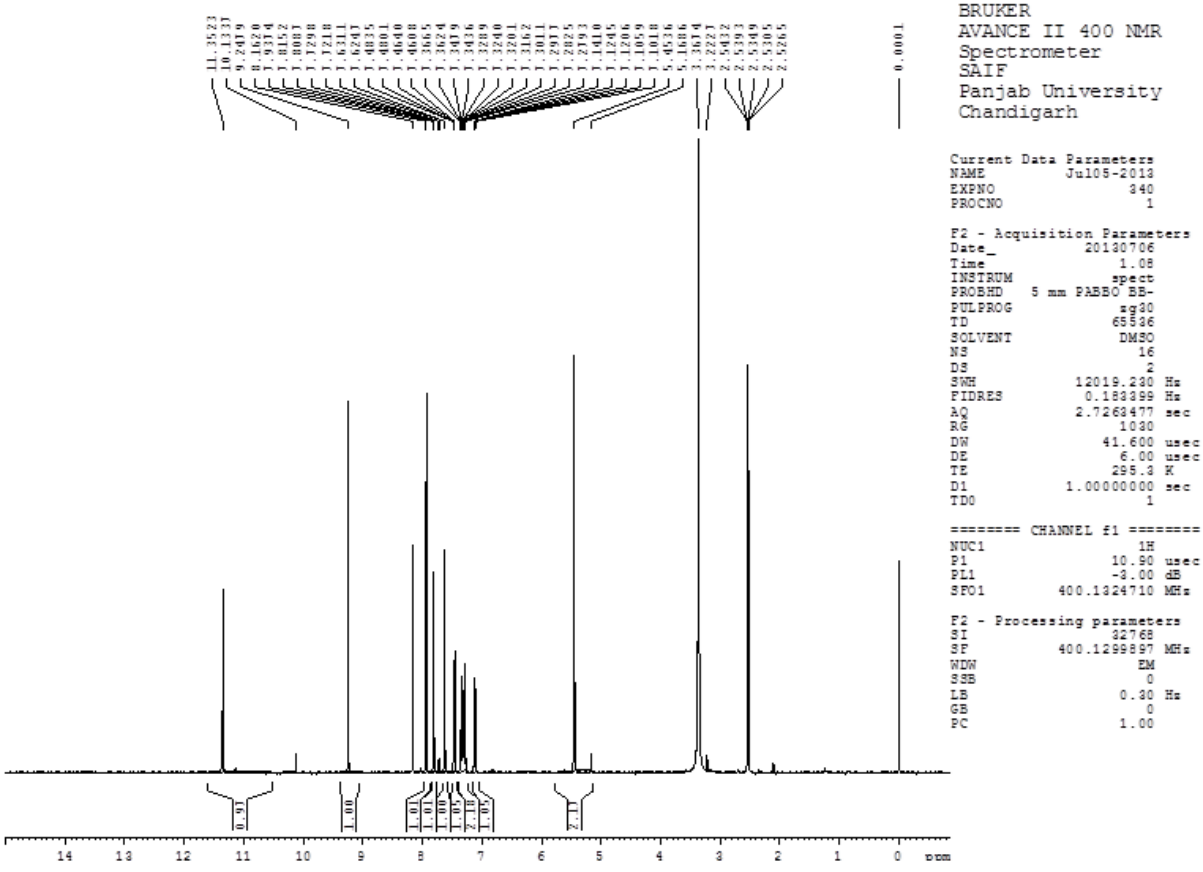

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AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

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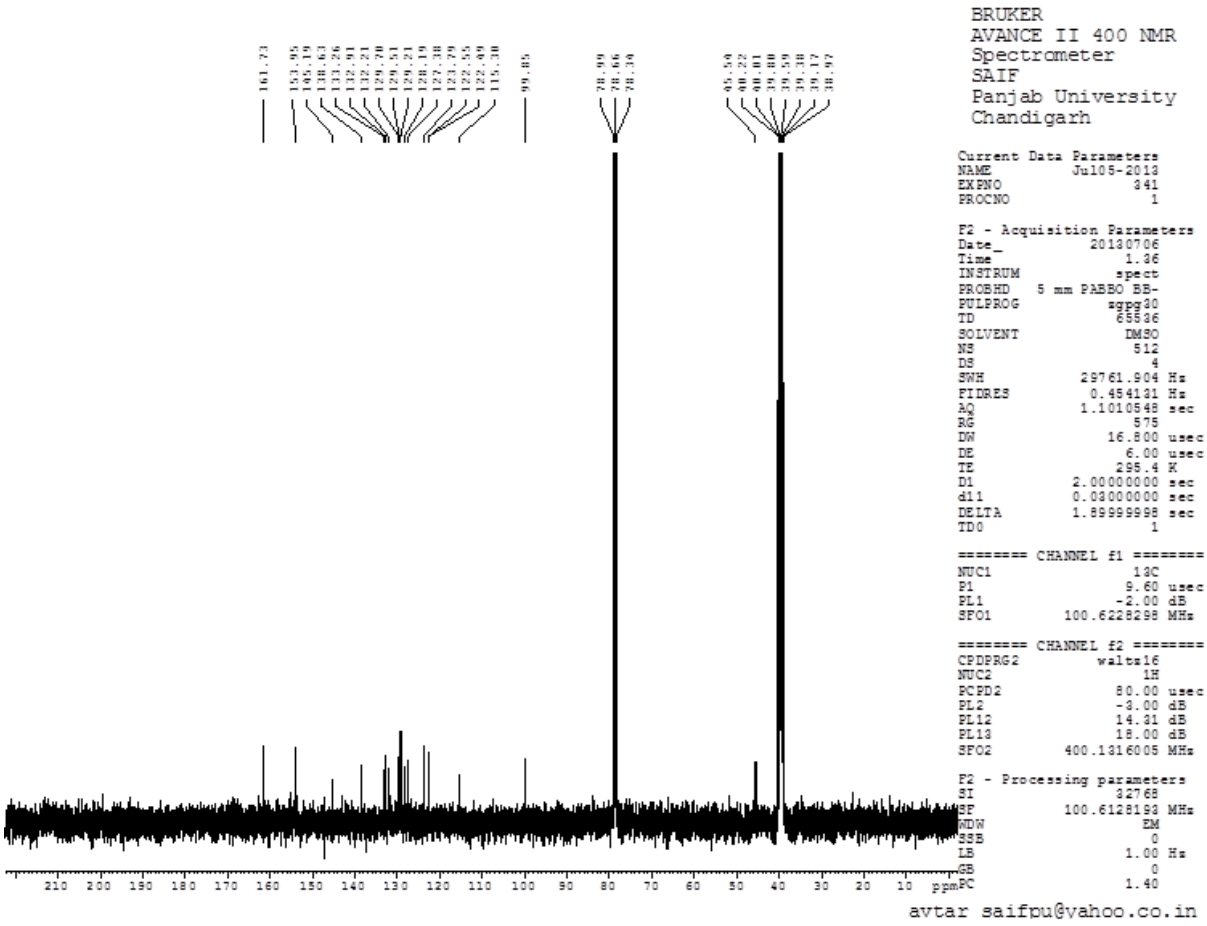
avtar saifpu@yahoo.co.in



BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh

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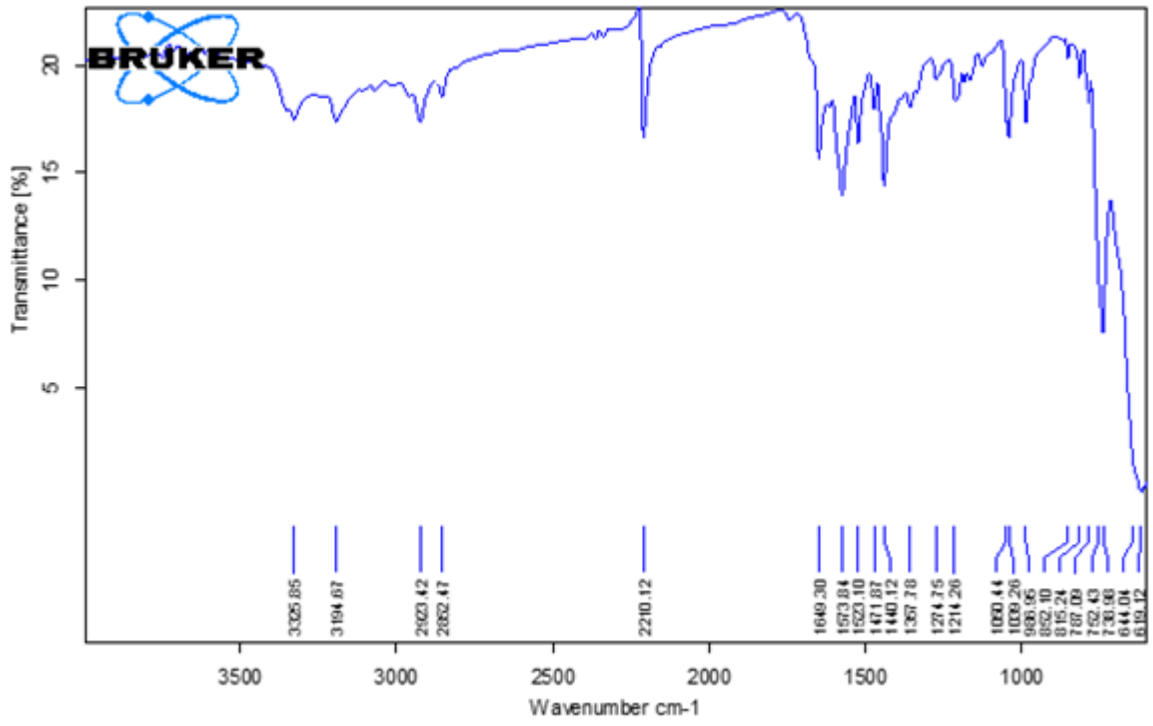
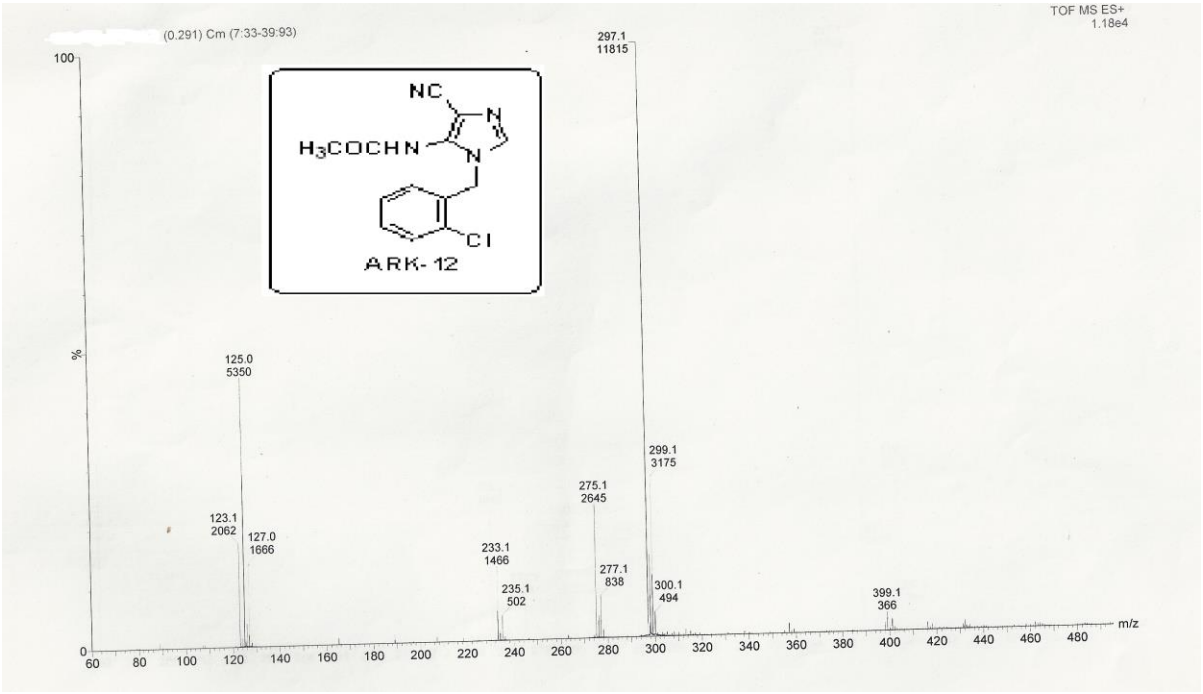
avtar saifpu@yahoo.co.in

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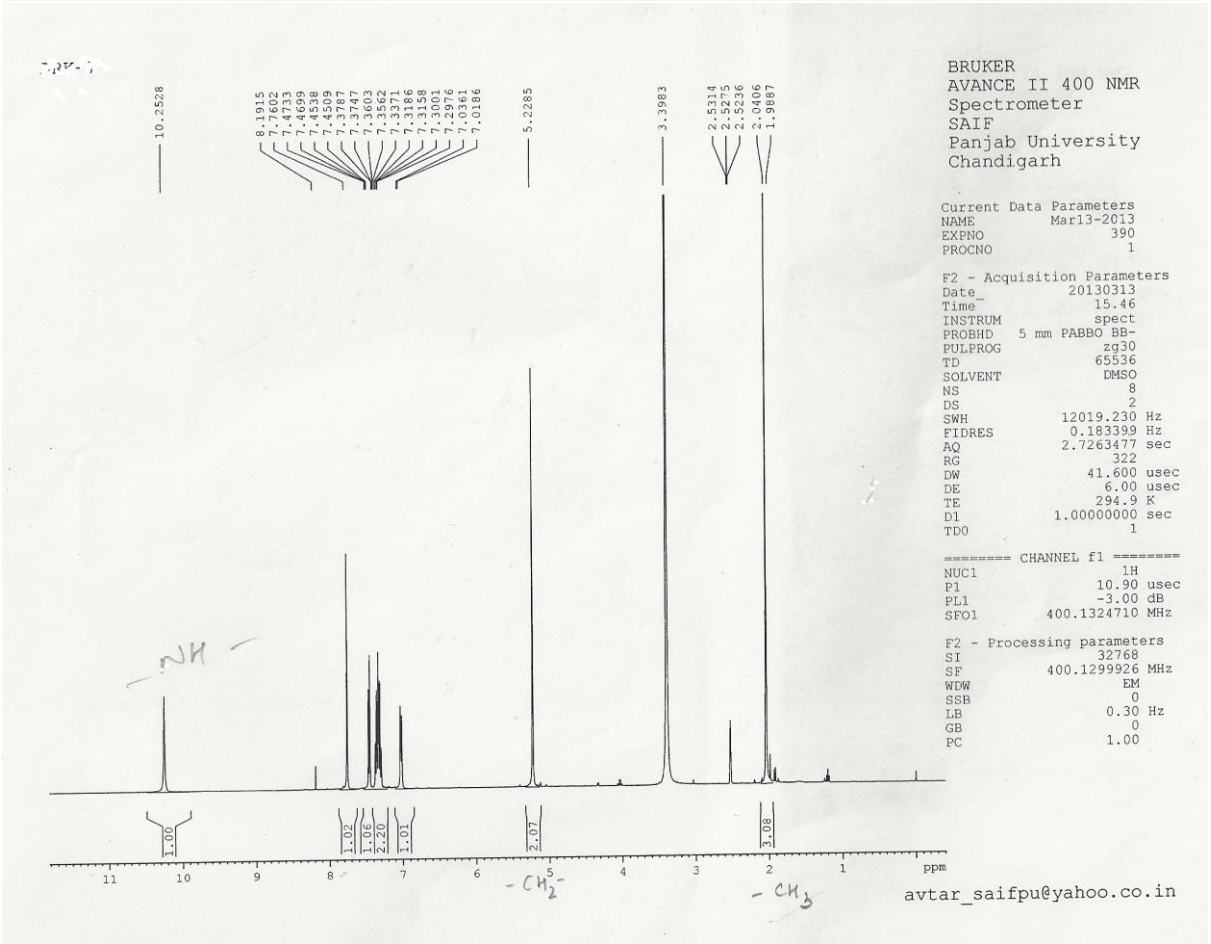
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BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh
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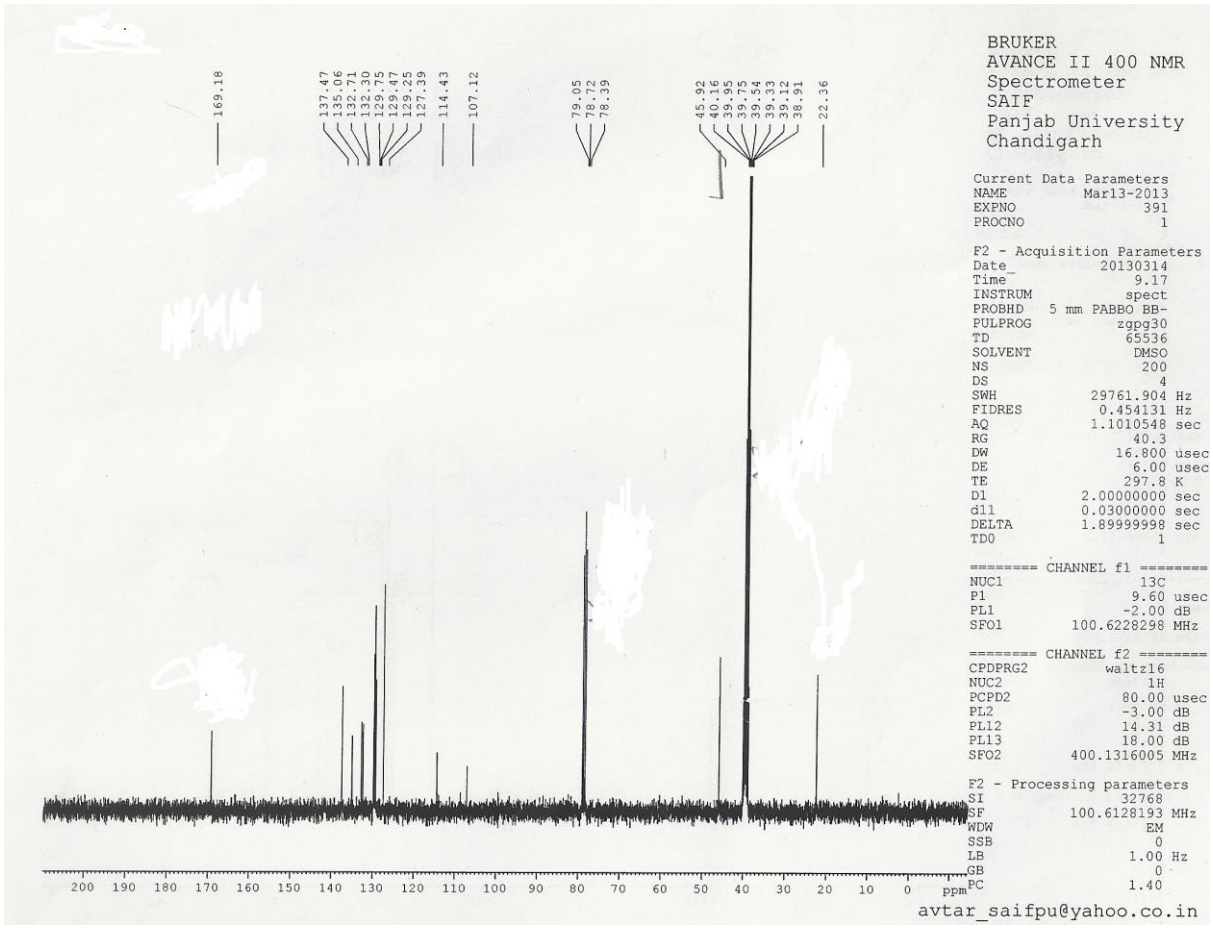
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BRUKER
AVANCE II 400 NMR
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Panjab University
Chandigarh
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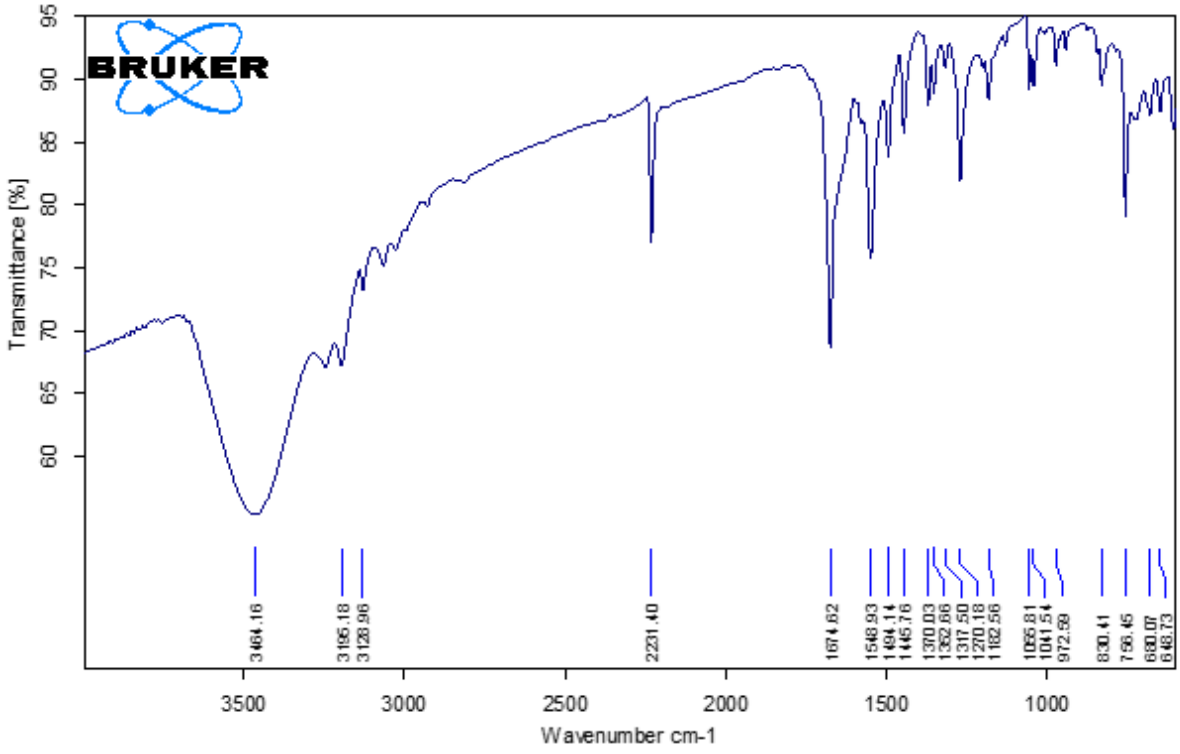
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