Chapter-2

Synthesis, characterization and biological activity of novel 2-aryl benzimidazole derivatives

There are number of class in an aromatic bicyclic organic compound. Benzimidazole is an aromatic bicyclic organic compound having nitrogen in its moiety. Heterocyclic compound benzimidazole is an important in its class. The fusion of benzene ring to the 4,5-position of imidazole ring as shown below generates benzimidazole[1,2,3]. Numbering of both benzimidazole and imidazole nucleus is shown in below **Figure 1**.

Figure 1.

Figure 1. Numbering of imidazole and benzimidazole nucleus

Due to electron attraction from the benzene ring in 1*H*-benzimidazole it decreases imidazole's electronic density which is of important role in chemical interaction and having varied biological activities and still of great scientific interest now a days[4] represented in **Figure 2**.

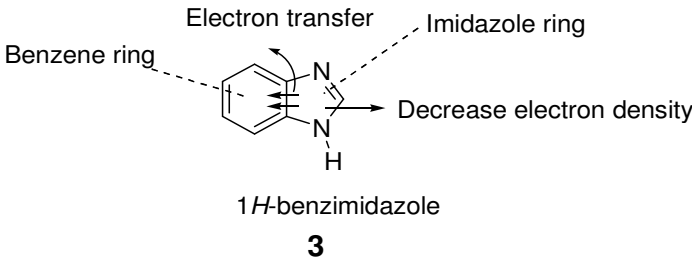
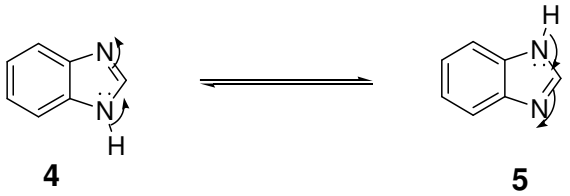


Figure 2. Electron distribution of benzimidazole nucleus

Hydrogen atom attached to nitrogen in the 1-position readily tautomerize in benzimidazoles. In the certain derivatives of benzimidazole due to this tautomerism in benzimidazole certain derivatives which appear at first to be isomers are in reality tautomers; although two nonequivalent structures can be written, only one compound is known[5]. Both structures represent the same compound as shown in **Figure 3**.

Figure 3



4 5

Figure 3. Tautomeric form of benzimidazole nucleus

From the sets of resonance structure the overall reactivity of imidazole and benzimidazole is defined in which the dipolar contributors have finite importance. In benzimidazole the nucleophilic attack is predicted at C-2. The reactivity of benzimidazole ion at the C-2 position with nucleophiles is enhanced as compared with the neutral molecule[6].

2.1 Physical Properties of Benzimidazole

Benzimidazole with the imide nitrogen (hydrogen in the 1-position) is more soluble in polar solvents and it is less soluble in organic solvents. Benzimidazole having nonpolar substituents in various positions of the benzimidazole ring, the solubility in nonpolar solvents is increased; for example, 2-methylbenzimidazole is easily soluble in ether. Distillation of benzimidazole is also possible and distills unchanged above 300°C. So benzimidazole is highly stable at higher temperature.

Benzimidazole is less basic than imidazoles. Benzimidazole is weakly basic. Accordingly, they are in general soluble in dilute acids. Benzimidazole is also sufficiently acidic to be generally soluble in aqueous alkali and form N-metallic compounds. The acidic properties of the benzimidazole, like those of the imidazoles[7], seem to be due to stabilization of the ion by resonance. The dipole moment of benzimidazole has been determined, the values that have been obtained being 3.93 D (in dioxane) and 4.08 D[8]. The infrared spectra of "pseudo bases" have been studied and indicate the presence of an amide linkage in these substances[9].

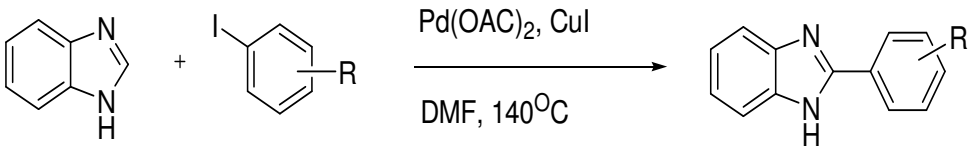
2.2 Chemical Properties of Benzimidazole

Benzimidazole ring is highly stable under most of the conditions. Benzimidazole, for example, is not affected by concentrated sulfuric acid when heated under pressure to 270°C, or by vigorous treatment with hot hydrochloric acid or with alkalis. Oxidation cleaves the benzene ring of benzimidazole only under vigorous conditions[10]. The benzimidazole ring is also quite resistant to reduction. So we can say that benzimidazole is stable under vigorous conditions.

2.3 Functionalization of Benzimidazole Nucleus

2.3.1 Direct coupling at the C-2 position

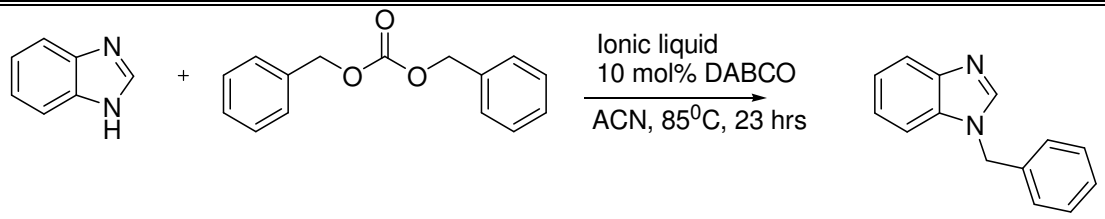
C-arylation is very much important synthetic reaction that is used to build important bioactive structures. Recently ligandless and base free conditions for the C-2-arylations of benzimidazole by using palladium and copper with aryl iodides have been described[11].



R = H, 4-OCH_3, 4-CF_3, 4-NO_2, 2-Me

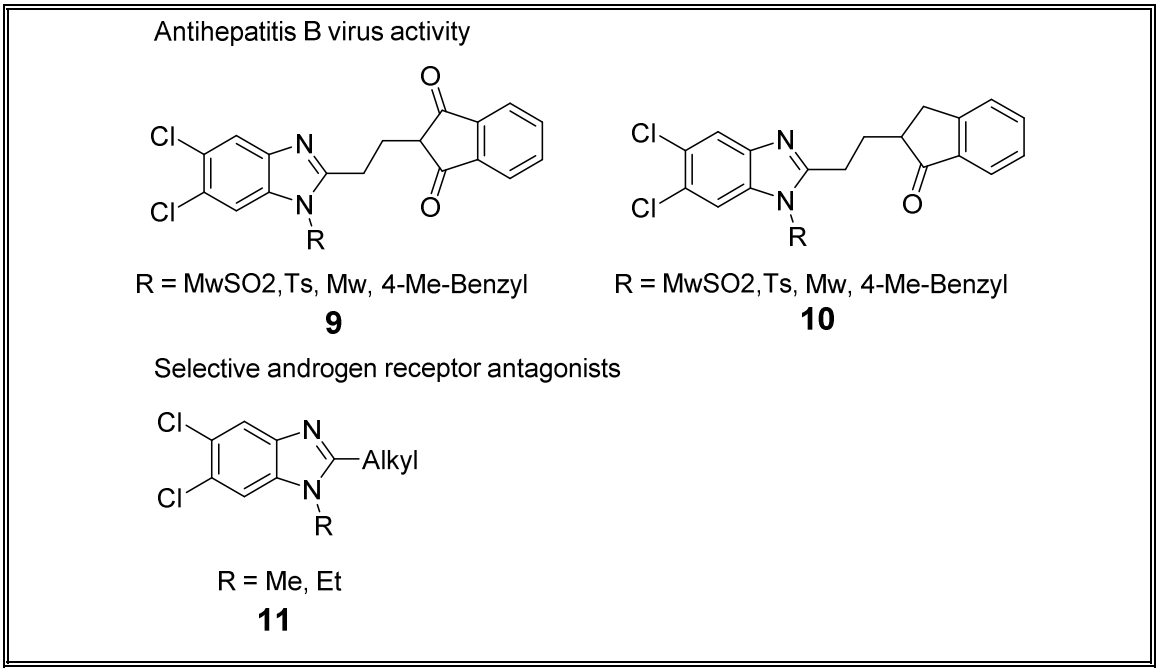
2.3.2 Substitution at the N-1 position

By using dibenzyl carbonate (**7**) as an alkylating reagent[12] in ionic liquids have been reported to slow *N*-benzylation reactions of benzimidazole (**6**). Microwave irradiation enhances the additional rate enhancement when applied in this reaction to afford the *N*-benzyl benzimidazole (**8**).



6 **7** **8**

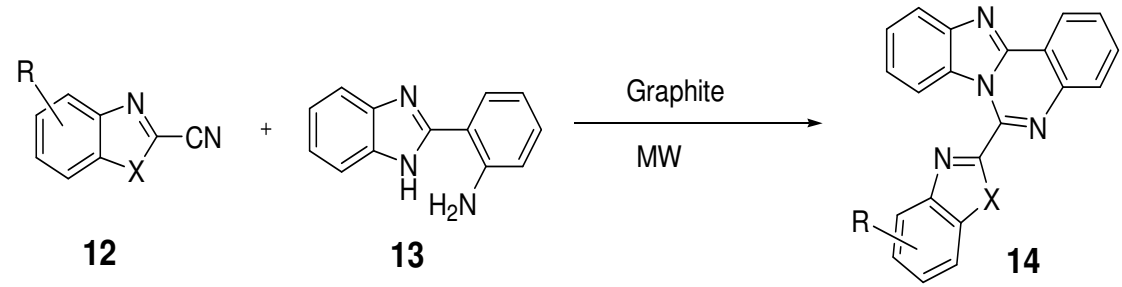
Li *et al*[13] reported several derivatives of *N*-substituted benzimidazole (**9**) and (**10**) as antihepatitis B virus activity (**9**, **10**) and several N-alkyl benzimidazole derivatives (**11**) behave as selective androgen receptor antagonists[14].



2.3.3 Cyclization between the N-1 and C-2 position

In order to build novel benzimidazole derivatives, first fusion can be done between the C-2 and *N*-1 positions. For example, Polyheterocyclic structures such as benzimidazoquinazolines made up of two fused heterocyclic rings often possess potent biological activity, like antiproliferative and DNA-intercalator activity[15], antifertility activity[16], anticonvulsant activity and myorelaxant activity[17]. These

benzimidazoquinazoline compounds (**14**) have been obtained by the condensation of 2-cyanobenzothiazoles or benzoxazoles (**12**) with 2-(2-aminophenyl) benzimidazole (**13**) under microwave conditions in the presence of graphite as a catalyst[18].

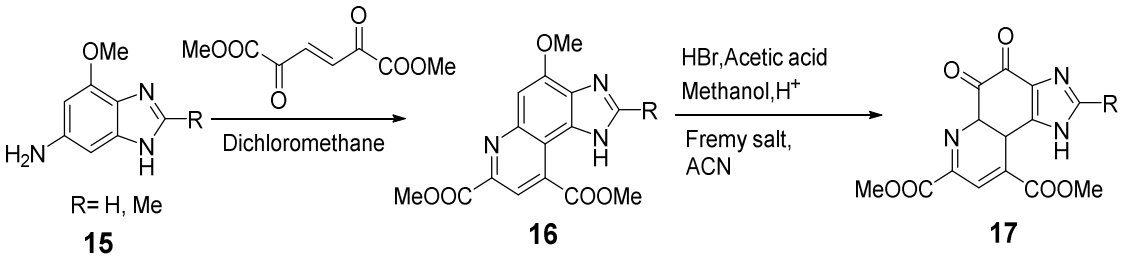


12 **13** **14**

X= S,O
R= H, 6-F, 6-Me, 6-OMe, 6-NO_2,
4,7-Me, 4,7-OMe

2.3.4 Doebner-Von Miller reaction at the C-7 position

Under Doebner-Von Miller reaction conditions in dichloromethane imidazoquinolines (**16**) have been synthesized in good yield by coupling benzimidazole (**15**) with dimethyl *trans*-2-ketoglutaconate. Then the demethylation of compound (**16**) is using hydrobromic acid in glacial acetic acid, re-esterification using methanolic hydrochloric acid and oxidized to afford benzimidazolequinone[19] (**17**).

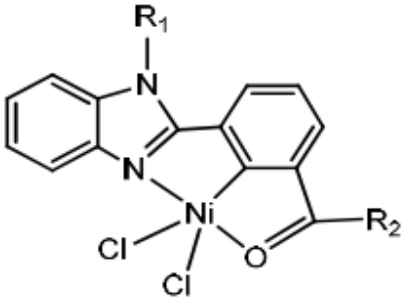


15 **16** **17**

2.4 Benzimidazole derived metal complexes

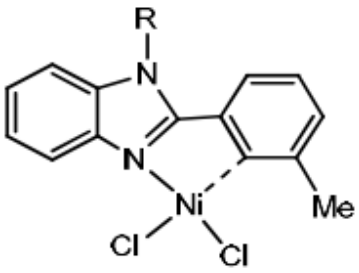
A number of nickel (II) complexes have been reported which shows good biological activities. The nickel (II) complexes (**18**) and (**19**) as shown below ligated by 2-(2-

benzimidazole)-pyridine derivatives having N and O donor atoms and nickel dichloride hexahydrate have been prepared[20]. Interestingly, benzimidazole derived copper (II) and nickel (II) complexes have revealed antibacterial, antifungal and DNA intercalator activities[21], whereas lanthanide (III) complexes exhibited seed germination inhibition activity[22].



R_1= H, Me; R_2= Me, EtO

18

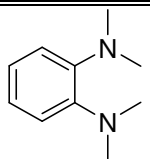


R = H, Me

19

2.5 Synthetic Approach for Benzimidazole Molecules

In all synthetic approach for the preparation of benzimidazole start with benzene derivatives having nitrogen containing functions *ortho* to each other, that is the starting material possess the functional unit designated by the given formula (**20**) as shown below.

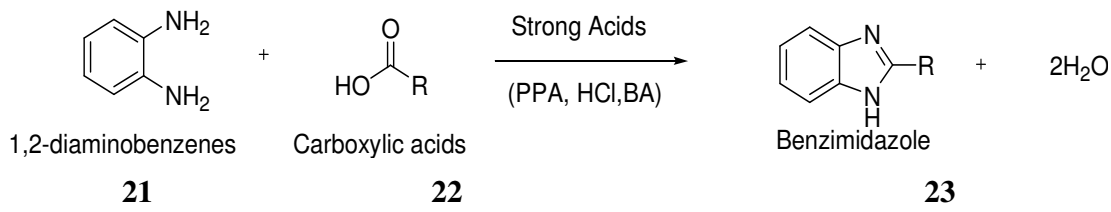


20

2.5.1 Method 1: General synthetic methodology

Traditionally, the most common synthetic method for the preparation of benzimidazole (**23**) have been reported from the reaction of 1,2-diaminobenzenes (**21**) with carboxylic

acids (**22**) under harsh dehydrating reaction conditions, utilizing strong acids such as polyphosphoric acid, hydrochloric acid, boric acid, or *p*-toluenesulfonic acid[23]. However, the use of milder reagents, particularly lewis acids[24], inorganic clays[25] or mineral acids has improved both the yield and purity of this reaction[26], example as given below.

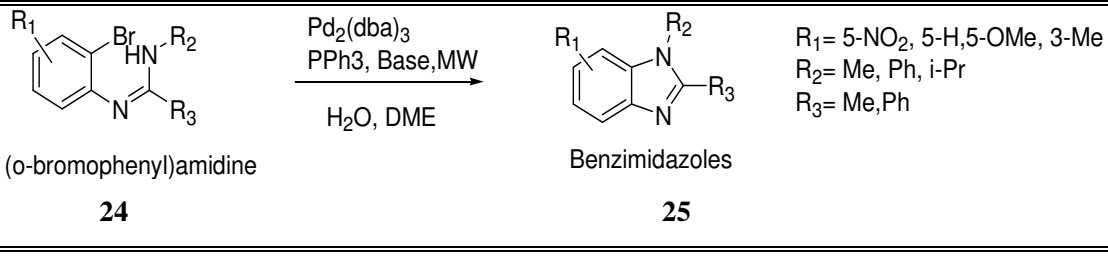


1,2-diaminobenzenes **21** + Carboxylic acids **22** → Benzimidazole **23** + $2H_2O$

On the other hand, the condensation reaction between 1,2-diaminobenzenes and aldehydes for the synthesis of benzimidazole requires an oxidative reagent. Number of oxidative reagents such as nitrobenzene, mercuric oxide, sodium metabisulfite, indium perfluorooctanesulfonates, lead tetraacetate, iodine, benzoquinone, copper (II) acetate, ytterbium perfluorooctane sulfonates and even air, have been employed for this purpose[27].

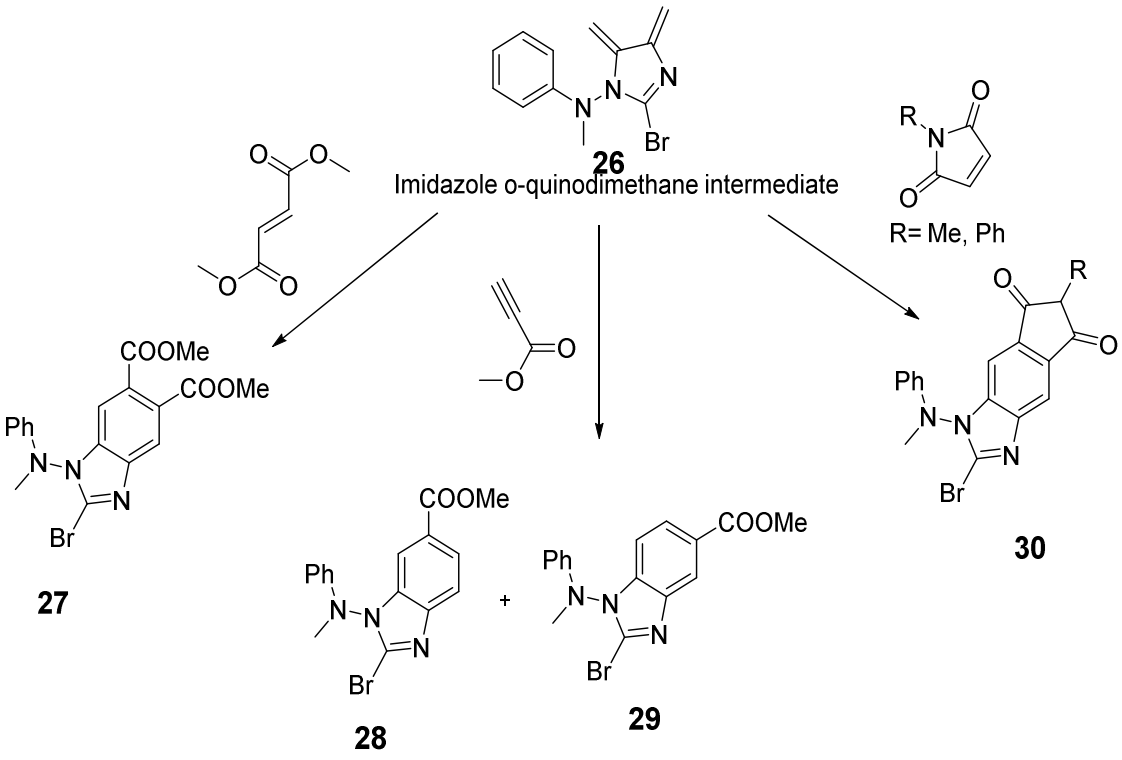
2.5.2 Method 2: Benzimidazole ring closure

In the presence titanium (IV)chloride as a catalyst number of substituted benzimidazole have been synthesized in very good yields in solvent free conditions from 1,2-diaminobenzene and aldehydes. The method is applicable to most aromatic, unsaturated and aliphatic aldehydes and to substituted 1,2-diaminobenzenes without significant differences[28]. A palladium catalyzed *N*- arylation reaction provided a novel synthesis of benzimidazole (**24**) from (*o*- bromophenyl)amidine (**25**) precursors under microwave irradiation. It has been found that 50% aqueous dimethyl ether (DME) is an optimal solvent for the reaction and that catalyst loading of palladium can be reduced to 1 mol.



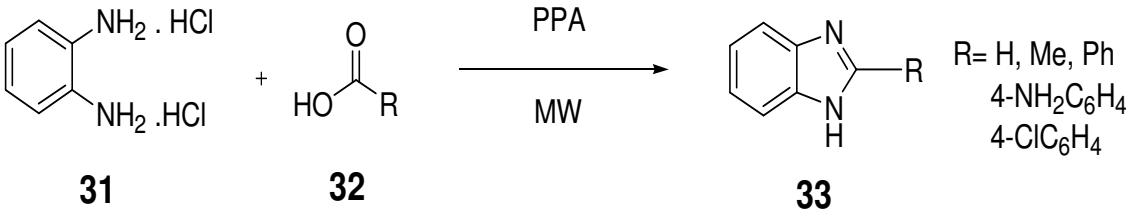
2.5.3 Method 3: Diels-Alder reaction

Imidazole *o*-quinodimethane intermediate (**26**), synthesized from 2-bromo-4,5-bis(bromo methyl) imidazole via *N*-bromosuccinimide mediated bromination of imidazole undergoes a Diels-Alder reaction with several symmetrically and asymmetrically substituted dienophiles to yield the benzimidazole derivatives (**27-30**) in moderate yields[29].



2.5.4 Method 4: Microwave assisted synthesis of benzimidazole

In the synthetic chemistry the use of microwave irradiation as a source of heat has been heralded as a promising method of increasing productivity, quality and reducing reaction time since its first use by **Gedye *et al***[30] in 1986. Recently, by using polyphosphoric acid under microwave assisted methods various derivatives of 2-alkyl and 2-aryl-substituted benzimidazole (**33**) have been synthesized from 1,2-diaminobenzene dihydrochloride (**31**) and its corresponding acids (**32**). The main advantage of this microwave assisted synthesis is in the reduction in the reaction time. As compared to the conventional methods, the reaction time required for the synthesis of benzimidazole derivatives was reduced to minutes. The conventional methods require up to four hours of heating completing the reaction. Furthermore, it was found that the application of microwave irradiation increased yields by 10-50% (**Table 1**).



31 **32** **33**

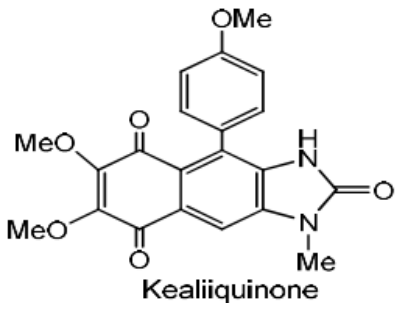
Table 1. Yield and reaction time for benzimidazole synthesis by using microwave irradiation

R	Time of Reaction	Yield (%)	MW	Yield (%)
H	2 hours	80	1 min 20 s	92
Me	45 mins	48	1 min 20 s	89
Ph	4 hours	34	4 min 30 s	84
4-$NH_2C_6H_4$	4 hours	57	5 min 20 s	95
4-ClC_6H_4	4 hours	43	4 min 30 s	89

MW- Microwave irradiation

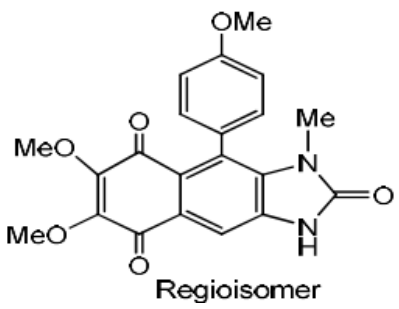
2.5.5 Method 5: Benzimidazole natural products

There are only few examples of benzimidazole derived alkaloids (natural products) can be found in the literature. They are rare in nature. On the other hand, the imidazole skeleton in various natural sources is quite common. From the yellow button like Micronesian sponge species of *Leucetta*[31] the benzimidazole alkaloid kealiiquinone (**34**) has been isolated. Recently, the regioisomer (**35**) of kealiiquinone has been successfully synthesized by **Nakamura *et al*** [32].



Kealiiquinone

34



Regioisomer

35

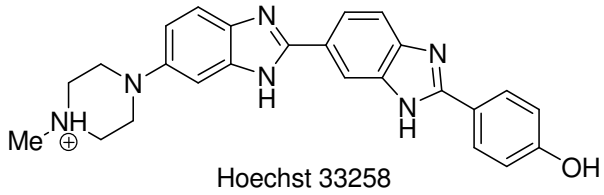
2.6 Benzimidazole as a Template for Various Biological Activities

Due to various modifications carried out in benzimidazole ring system many fruitful biological activities of the compounds have been found; some of these activities are described here.

2.6.1 Anti-tumor/Anticancer activity/Anti proliferative

In cancer chemotherapy there is currently much interest in the design of small molecules that bind to DNA with sequence selectivity and noncovalent interactions. A possible lead for this new class of compounds is Hoechst 33258 (**36**), which recognize adenine/thymine sequences in human DNA and is also an effective inhibitor of mammalian DNA topoisomerase[33]. Several structure-activity relationship studies have been performed on the Hoechst motif. The replacement of the terminal piperazine ring

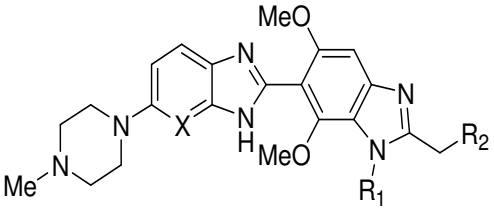
with an amidinium, an imidazoline or a tetrahydropyridinium group significantly reinforces the affinity of the drug for the adenine/ thymine stretches[34].



Hoechst 33258

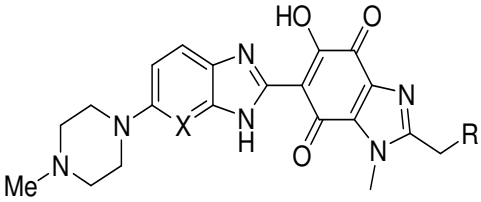
36

Many novel bisbenzimidazoles with a piperazine functional group with general formula (**37-40**) incorporating benzimidazole, pyridoimidazole and imidazoquinone moieties as one of the units of bisbenzimidazole have been synthesized[35]. The series of bisbenzimidazoles contains different leaving groups along with *p*-methoxy substituents. The latter may be expected to have some influence on the nitrogen lone pair and consequently on the binding characteristics of the ligand. These novel bisbenzimidazoles are found to be actively cytotoxic against many human cancer cell lines with GI_{50} values of between 0.01 and 100 μM, especially in the cases of renal cancer, CNS cancer, Colon cancer, Melanoma and Breast cancer cell lines. The pyridoimidazole compounds (**37**) and (**39**) are generally more potent.



37, X=N, R_1= H
38, X= CH, R_1 = H
39, X=N, R_1 = Me

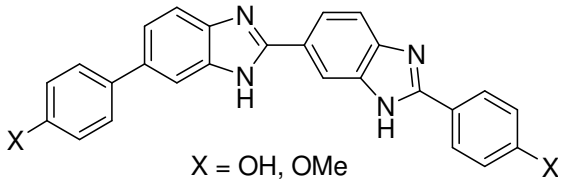
R_2 = OMe, OEt, OAc, OH



40, X= N, CH; R= OH,Cl

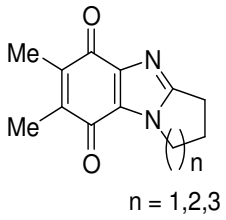
A novel head to head bisbenzimidazole compound (**41**) binds with high affinity to the minor groove of double stranded B-DNA with a strong preference for adenine/thymine rich regions. The bisbenzimidazole (**41**) showed potent growth inhibition in human

ovarian carcinoma cell lines (IC_{50} = 200-300 nM), with no significant cross resistance in two acquired cisplatin resistant cell lines and a low level of cross-resistance in the *p*-glycoprotein over expressing doxorubicin resistant cell line[36].

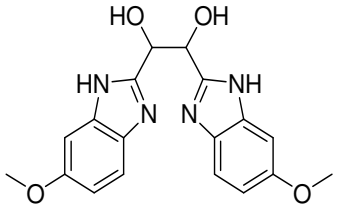


41

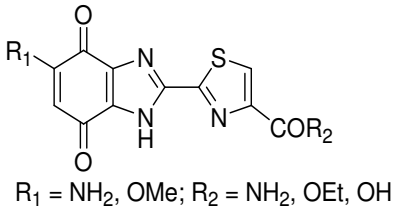
The benzimidazole-6,9-dione (**42**) has been found to be 300 times more cytotoxic towards the human skin fibroblast cell line in the MTT assay than the clinically used bioreductive drug, Mitomycin C. Attaching methyl substituents onto the quinone moiety increased reductive potential, decreased cytotoxicity and selectivity towards hypoxia[37]. In addition, the alkyl-linked bisbenzimidazole[38] (**43**) and thiazolylbenzimidazole-4,7-diones[39] (**44**) exhibited cytotoxic activity against tumor cell lines.



42

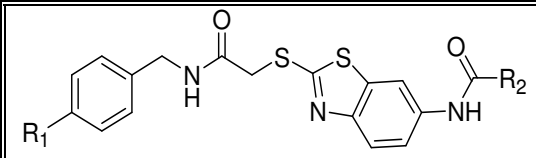


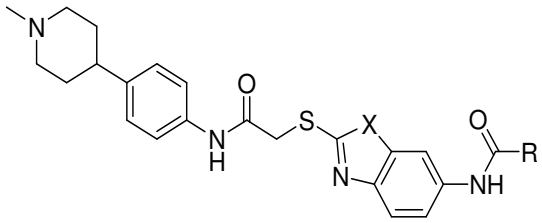
43



44

For lead optimization and their *in-vitro* antitumor activity **P. Xiang *et at***[40] synthesized five novel benzothiazole-2-thiol, benzimidazole-2-thiol and benzoxazole-2-thiol derivatives. To our delight, compounds (**46-48**) showed comparable antitumor activities and better solubility compared with the lead compound (**45**).





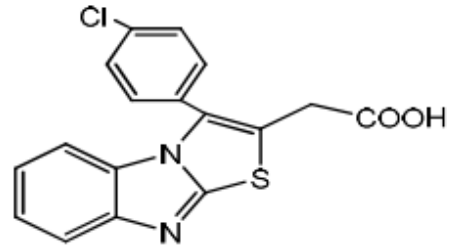
45, R_1 = Cl, R_2 = Methoxy phenyl

46, R_1 = H, R_2 = Chloro methyl

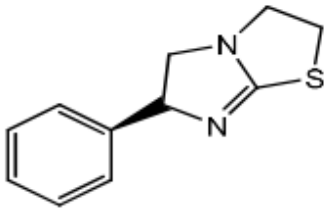
47, X= S, R = Chloro methyl

48, X= S, R = 2-Methoxy phenol

Tilomisole (WY-18, **49**) has been widely studied. It showed several potent activities, such as antinflammatory activity. It has been reported to possess remarkable anticancer activity since it could be considered an analog of Levamisole, a well known immune modulator which is used for the adjuvant treatment of the Colon cancer. However, Tilomisole (Wy18, **49**) has favorable biological response effects *in-vivo* and it is a suitable alternative to Levamisole in cancer treatments[41].



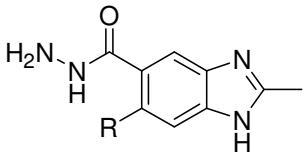
Tilomisole (Wy-18)



Levamisole

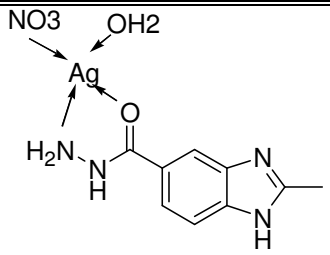
49

Metal complexes of 2-methyl-1*H*-benzimidazole-5-carboxylic acid hydrazide (**50, 51**) and its Schiff base 2-methyl-N-(propan-2-ylidene)-1*H*-benzimidazole-5-carbohydrazide with transition metal ions, for example copper, silver, nickel, iron and manganese were prepared. The antitumor activity of the synthesized compounds has been studied. The silver complex (**52**) was found to display cytotoxicity (IC_{50}¼ 2 mM) against both human Lung cancer cell line A549 and human breast cancer cell line MCF-7[42].



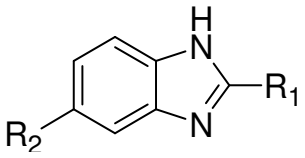
50, R=H

51, R= NO_2

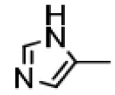
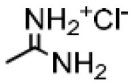
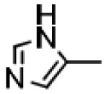
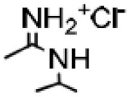
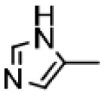
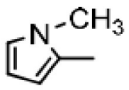
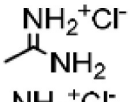
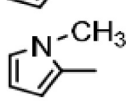
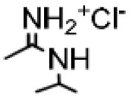


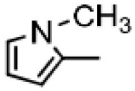
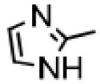
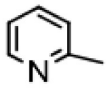
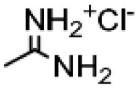
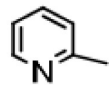
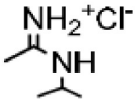
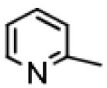
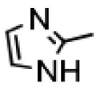
52

A set of heterocyclic benzimidazole derivatives have been prepared by **K. Starcevic *et al***[43] bearing amidino substituents at C-5 of benzimidazole ring by introducing various heterocyclic nuclei (pyridine, *N*-methyl-pyrrole or imidazole) at C-2 and evaluated their antitumor and antiviral activities (**53-61**). The compounds (**58**) and (**61**) have shown the most pronounced antitumor activity having imidazolinylamidino substituent. The compounds having pyridine ring at C-2 having the most distinct and selective antiviral activity toward coxsackie viruses and echoviruses.

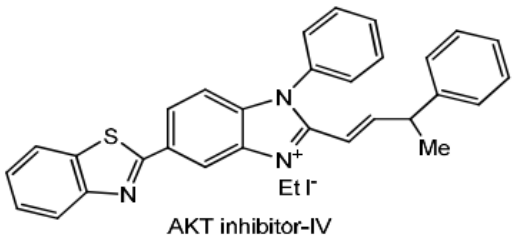


53-61

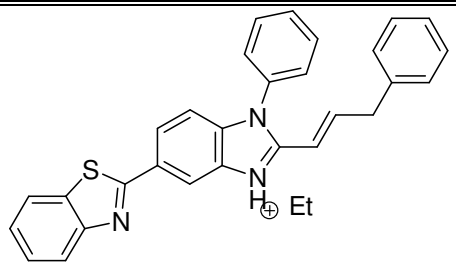
Compd.	R_1	R_2
53		
54		
55		
56		
57		

Compd.	R_1	R_2
58		
59		
60		
61		

Inhibitors of the PI3-kinase/AKT (protein kinase B) pathway are under investigation as anticancer and antiviral agents. The benzimidazole derivative AKT inhibitor-IV (Chem Bridge 5233705) affects this pathway and exhibits potent anticancer and antiviral activity. To probe its biological activity, **Q. Sun *et al***[44] synthesized AKT inhibitor-IV and (**62**) analogues using a novel six-step route based on ZrCl catalyzed cyclization of 1,2-arylenediamines with α, β-unsaturated aldehydes and examined their effects on viability of HeLa carcinoma cells, viability of normal human cells (NHBE), replication of recombinant parainfluenza virus-5 (PIV5) in HeLa cells and replication of the intracellular bacterium *M. fortuitum* in HeLa cells.

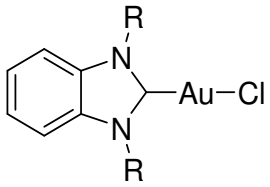


AKT inhibitor-IV



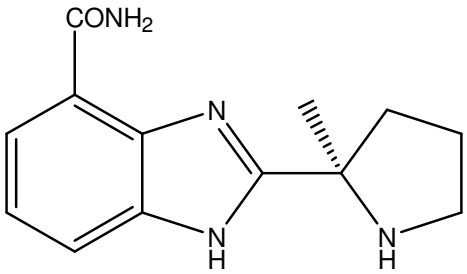
62

For decades to treat the symptoms of rheumatoid arthritis and have also demonstrated a significant potential as new anticancer drugs Gold (I) complexes such as auranofin have been used. The enzyme thioredoxin reductase (TrxR) is considered as the most relevant molecular target for these species. The investigated gold (I) complexes with benzimidazole derived *N*-heterocyclic carbene (NHC) ligands (**63-66**) represent a promising class of gold coordination compounds with a good stability against the thiolglutathione. TrxR was selectively inhibited by (**63-66**) in comparison to the closely related enzyme glutathione reductase and all complexes triggered significant antiproliferative effects in cultured tumor cells[45].



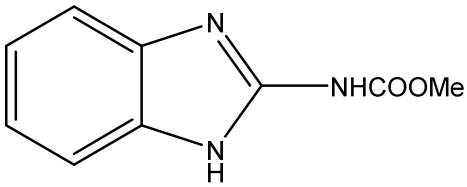
63-66

A series of cyclic amine containing benzimidazole carboxamide have been synthesized by **T. D. Penning et al**[46]. PARP inhibitors with a methyl substituted quaternary center at the point of attachment to the benzimidazole ring system. An excellent PARP enzyme potency as well as single digit nanomolar cellular potency has been exhibited by this compounds. Extensive efforts are made and the identified (**67**) which is (2-[(R)-2-methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide, ABT-888), currently in human phase-I clinical trials.



67

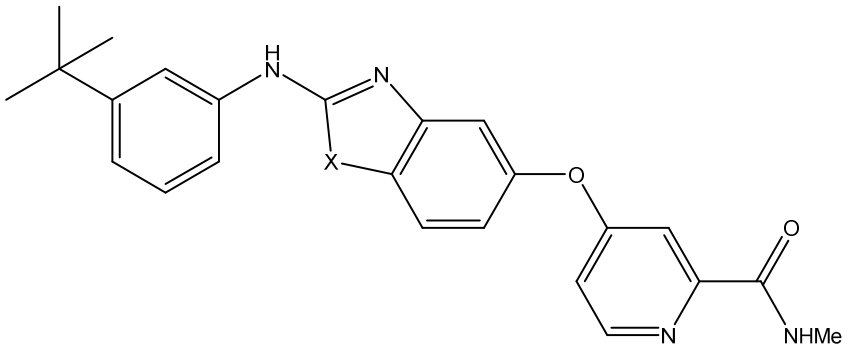
An anticancer agent Methyl-2-benzimidazolecarbamate (Carbendazim, FB642) (**68**) is an agent which induces apoptosis of cancer cells. In-vitro, FB642 demonstrated potent antitumor activity against both the murine B16 melanoma (IC_{50} = 8.5µm) and human HT-29 colon carcinoma (IC_{50} = 9.5µm) cell lines. FB642 was also highly active against both murine tumor models and human tumor xenografts at varying doses and schedules. Toxicity of FB642 in-vivo appeared to be dose-dependent. Lower doses in the range of 2000-3000 mg/kg were better tolerated, while still preserving antitumor activity. Evaluation of FB642 in phase I clinical trials of adult patients with advanced malignancies is currently ongoing[47].



FB642

68

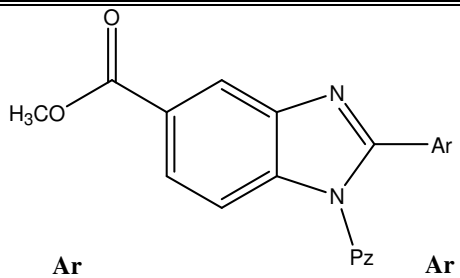
Two scaffolds based on 5,6-fused heterocyclic backbones were designed and synthesized as Raf kinase inhibitors (**69, 70 & 71**). The scaffolds were assessed for in-vitro pan-Raf inhibition, activity in cell proliferation, target modulation assays and pharmacokinetic parameters[48].



69-71

69, X=NMe, **70**, X=O, **71**, X=S

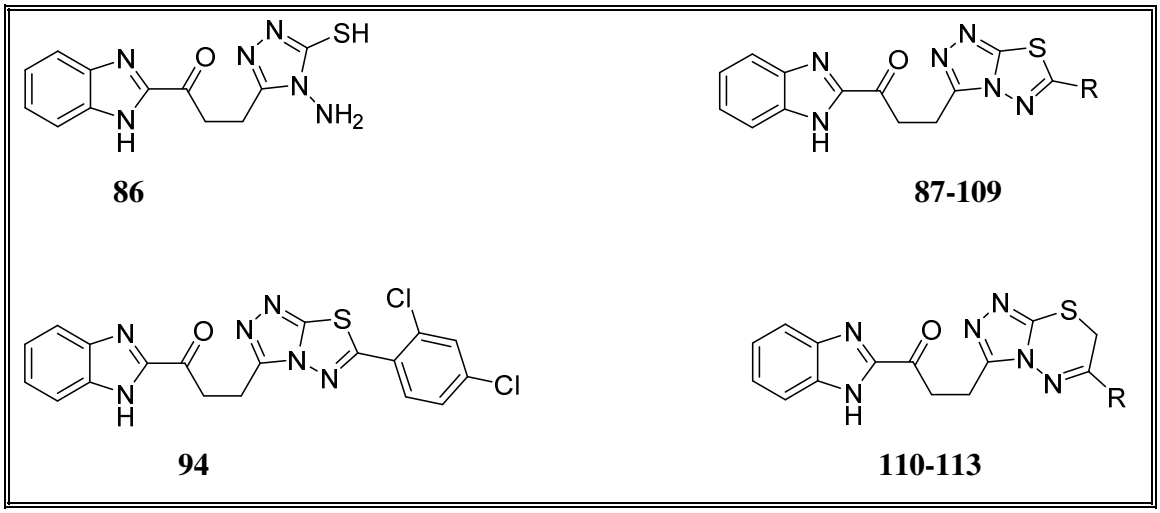
Some novel methyl-1-(5-tert-butyl-1H-pyrazol-3-yl)-2-(aryl)1H-benzo[d]imidazole-5-carboxylates (**72-85**) was synthesized by **R. Abonia et al**[49]. The obtained compounds were screened by the US National Cancer Institute (NCI) for their ability to inhibit 60 different human tumor cell lines. Compounds (**73**) and (**85**) exhibited the excellent activity against a range of cancer cell lines with remarkable values in panels of non-small cell Lung cancer, Melanoma and Leukemia, with GI_{50} range of 1.15-7.33 µM and 0.167-7.59 µM, respectively and suitable LC_{50} with values greater than 100 µM.



Ar	**Ar**
72 4-Br-C_6H_4	**79** 2-OH-4-$OCH_3C_6H_3$
73 4-Cl-C_6H_4	**80** 4-$(CH_3)_2NC_6H_4$
74 4-CH_3-C_6H_4	**81** 3,4-$(CH_3O)_2C_6H_3$
75 4-CH_3O-C_6H_4	**82** C_6H_5
76 4-F-C_6H_4	**83** 4-CF_3-C_6H_4
77 4,5-$(CH_3O)_3C_6H_2$	**84** 1,3-Benzodioxol-5-yl
78 2-OH-C_6H_4	**85** 2-Oxo-1,2-dihydroquinolin-3-yl

72-85

To produce promising anticancer agents two series of benzimidazole clubbed with triazolo-thiadiazoles **(87-109)** and triazolo- thiadiazines **(110-113)** were synthesized from 3-(4-amino-5-mercapto-4H-1,2,4-triazol-3- yl)-1-(1H-benzo[d]imidazol-2-yl) propan-1-one **(86)** by **A. Husain et al**[50]. In-vitro anticancer activities of synthesized compounds were investigated at the National Cancer Institute (NCI) against NCI 60 cell line panel; results showed good to remarkable broad-spectrum anticancer activity. Among them, one compound **94** (1-(1H-benzo[d]imidazol-2-yl)-3-(6-(2,4-dichlorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl)propan-1-one) exhibited significant growth inhibition with GI_{50} values ranging from 0.20 to 2.58μM and found superior selectivity for the leukemia cell lines and further screened at 10-fold dilutions of five different concentrations (0.01, 0.1, 1, 10 and 100 μM).

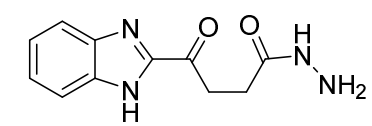


86 **87-109**

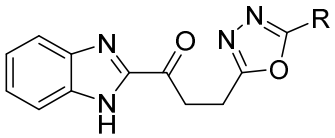
94 **110-113**

A series of benzimidazole bearing oxadiazole (**115-126**) and triazolothiadiazoles derivatives (**127-131**) from 4-(1H-benzo[d]imidazol-2-yl)-4-oxobutane hydrazide (**114**) synthesized by **A. Husain et al**[51] The synthesized compounds screened for their in-vitro anticancer activities at the National Cancer Institute (NCI), USA, against full NCI 60 human cell lines on nine cancer subpanel with significant results. Among them, compound **124** (3-(5-(4-amino-2,6-dibromophenyl)-1,3,4oxadiazol-2-yl)-1-(1H-benzo[d]imidazol-2-yl)propan-1-one) exhibited significant growth inhibition and further screened at 10-fold dilutions of five different concentrations (0.01, 0.1, 1, 10 and 100 μM) with GI_{50} values ranging from 0.49 to 48.0 μM and found superior for the non-small cell

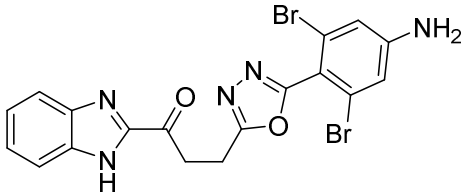
lung cancer cell lines like HOP-92 (GI_{50} 0.49, TGI 19.9, LC_{50}>100 and $Log_{10}GI_{50}$-6.30, Log_{10} TGI-4.70, $Log_{10}LC_{50}$> -4.00).



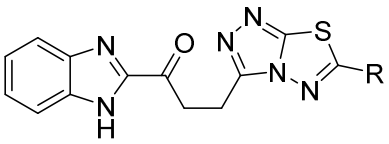
114



115-126

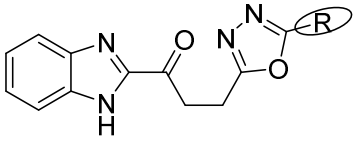


124

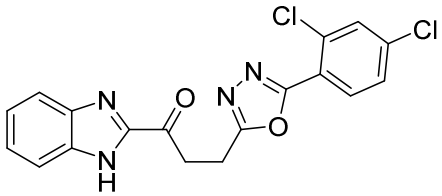


127-131

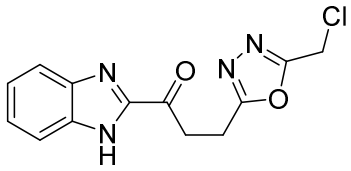
Twenty new compounds have been synthesized by **M. Rashid et al**[52]. The synthesis is carried out under microwave irradiation. The compounds 1-(1H-benzo[d]imidazol-2-yl)-3-(1,3,4-oxadiazol-5-substituted derivatives-2-yl)propan-1-ones (**132-151**) is obtained in good yields. Further, compound **132** was reacted with different secondary amines to produce novel 1-(1H-benzo[d]imidazol-2-yl)-3-(5(methylsubstituted)-1,3,4-oxadiazol-2-yl)propan-1-ones (**152-156**). The title compounds were screened for their in-vitro anticancer activity at National Cancer Institute (NCI), USA; at a single dose (10 µM) in NCI 60 cell line panel and results showed significant to good anticancer activity. One compound **146** (1-(1H-benzo[d]imidazol-2-yl)-3-(5-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2-yl)propan-1-one) emerged as lead compound; it was selected for five-dose level screening and found to have significant growth inhibition activity.



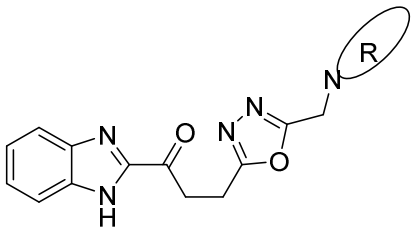
132-151



146



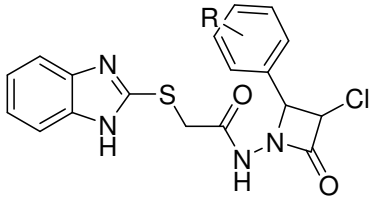
132



152-156

2.6.2 Antimicrobial activity

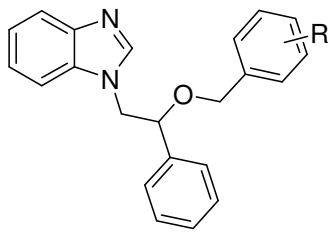
Recently, novel benzimidazole azetidin-2-ones (**157-166**) has been synthesized in an efficient and rapid way and screened for their antibacterial activity which revealed that all newly synthesized azetidin-2-ones exhibited potent antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli.* Among all of the compounds investigated (**165**) and (**166**) exhibited the greatest antibacterial activity against Gram-negative *E. coli* as compared to the antibiotic streptomycin[53].



157-166

Compd.	R	Compd.	R
157	4-NO	**162**	2-OMe
158	3,4,5-$(OMe)_3$	**163**	4-OMe
159	2-OH	**164**	2-Cl
160	3-OH	**165**	3-Cl
161	4-OH	**166**	4-Cl

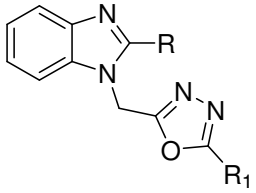
A series of Benzimidazole benzyl ethers (**167-174**) have been synthesized which shows good antibacterial activity against *S. aureus* and antifungal activity against *C. albicans* and *C. krusei*. The best antibacterial and antifungal activity have been found for the dichlorophenyl substituted benzimidazoles (**171, 172 & 174**). The biological activity shows promising for the antibacterial (MIC 3.12 µg/mL) and antifungal (MIC 12.5 µg/mL) for the synthesized compounds[54].



167-174

Compd.	R	Compd.	R
167	4-H	**171**	2,4-di-Cl
168	4-F	**172**	2,6-di-Cl
169	4-Cl	**173**	2,5-di-Cl
170	4-Br	**174**	3,4-di-Cl

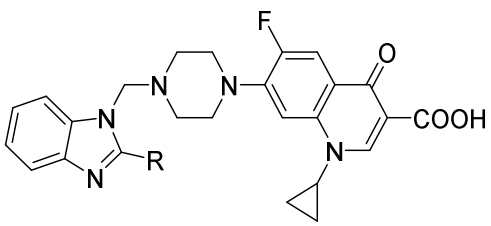
The oxadiazole nucleus containing benzimidazole nucleus is advantageous to design and synthesize some new benzimidazole derivatives bearing oxadiazole moiety and screen them for potential biological activities. Some benzimidazole derivative containing oxadiazole like, 1-{[5(alkyl/aryl)-1,3,4-oxadiazol-2-yl]methyl}-2-alkyl-1H-benzimidazoles (**175**) are synthesized and screened for their antimicrobial activities[55].



175

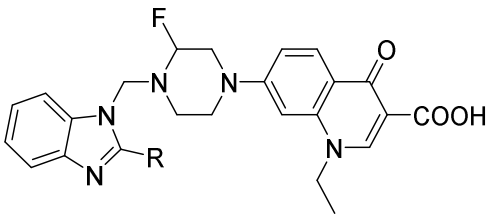
R= H, CH_3
R_1 = $-CH_3$, $-C_2H_5$, $-CH_2Cl$, $-CH_2CH_2Cl$, $-C_6H_5$
$-4-ClC_6H_4$, $2-OHC_6H_4$, $2-OCH_3C_6H_4$, $4-OCH_3C_6H_4$

From the microwave assisted synthesis of some fluroquinolones substituted benzimidazole derivatives have been reported. The derivatives of Ciprofloxacin (**176**) and Norfloxacin (**177**) have been synthesized and evaluated for their antimicrobial activity[56].



Ciprofloxacin derivatives
R=H, Ethyl, Propyl

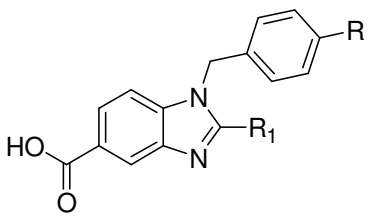
176



Norfloxacin derivatives
R=H, Ethyl, Propyl

177

For the in-vitro evaluation for antimicrobial activity against *S. aureus, E. coli* and *C. albicans* a series of 1,2-disubstituted benzimidazole-5(6)-carboxamides was prepared. Compounds which was investigated the best activity has been shown by compounds (**178-180**). The compound (**178**) which is Methyl benzimidazole-5-carboxylate derivatives showed potent activity comparable to that of fluconazole against *C. albicans*. The compound (**179**) showed best result against *S. aureus*[57].



178. R=H, R_1 =

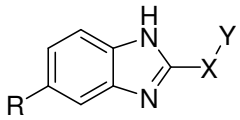
179. R = H, R_1 =

180. R = Cl, R_1 =

178-180

To determine antimicrobial activities and feasible structure-activity relationships a new series of 5-substituted benzimidazoles having cyclohexyl or cyclopentyl moieties at position-2 was synthesized. The synthesized compounds (**181-187**) are able to inhibit in-vitro growth of screened microorganisms, provided antibacterial activity against *S. aureus, S. faecalis* and *B. subtilis* possessing MIC values between 25-100 mg/mL, except the derivative 5-chloro-2-(2-cyclohexylethyl) benzimidazole (**187**), which was found

active at a MIC value of 12.5 mg/mL against each of the screened Gram-positive bacteria strains[58].

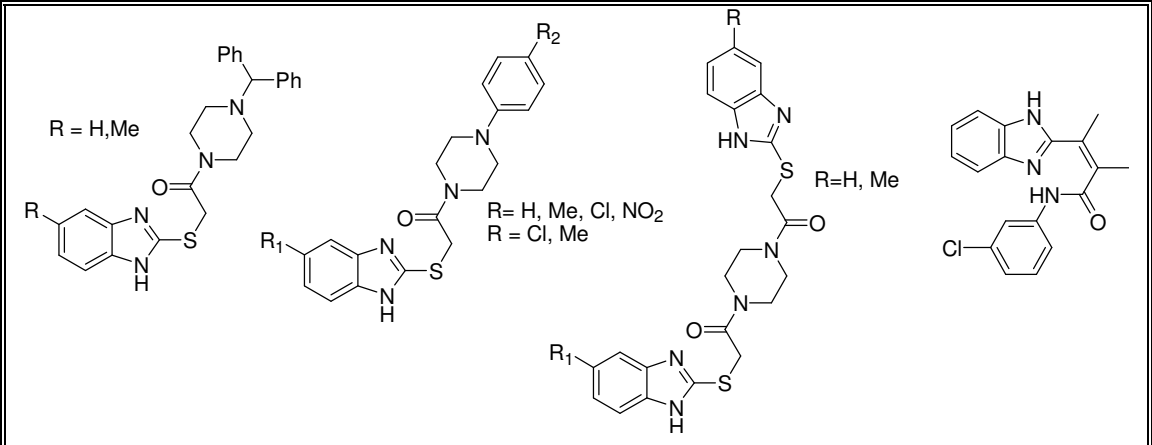


181-187

Compd.	R	X	Y
181	H	-	cyclopentyl
182	Cl	-	cyclopentyl
183	H	CH_2	cyclopentyl
184	Cl	CH_2	cyclopentyl
185	H	C_2H_4	cyclopentyl
186	Cl	C_2H_4	cyclopentyl
187	H	C_2H_4	cyclohexyl

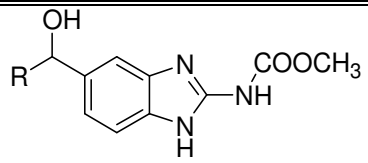
2.6.3 Anthelmintic activity

For the searching of the new anthelmintic drugs as comparison with the previously known benzimidazole anthelmintics (e.g., Albendazole, Mebendazole) is being actively pursued. Synthetic benzimidazole piperazine derivatives exhibited 50% anthelmintic activity in mice infected with *S.obvelata*[59]. Furthermore, piperazine derivatives of 5(6)-substituted-(1H-benzimidazol-2ylthio)acetic acids[60] (**188-191**) and benzimidazolyl crotonic acid anilide (**191**) have shown good anthelmintic activity[61].



188 **189** **190** **191**

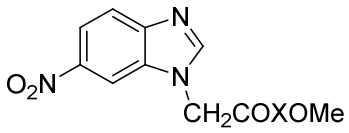
R. Sharma et al[62] studied the derivatives of certain methyl 5-(α-hydroxy-α-substituted-methyl)-1H-benzimidazole-2-carbomates (**192**) and this derivatives are screened for anthelmintic activity.



R = C_2H_5 (Mebendazole)
R = C_6H_4-NH_2, CH_3, C_2H_5, C_6H_4-NH_2

192

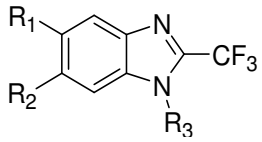
M. Himaja et al[63] prepared 6-nitrobenzimidazol-1-acetyl amino acid (**193**) and their anthelmintic activity has been evaluated and they found it as potent anthelmintic agent.



X = L-Tyr, L-Gly

193

H. Luis et al[64] synthesized and screen some new 2-(trifluoromethyl)-1H-benzimidazole derivatives (**194-196**) and in-vitro antiparasitic profile against *G.intestinalis, E. histolytica, T. vaginalis* and *T. spiralis* has been identified by the synthesized derivatives.

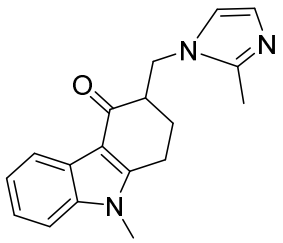


194: R_1= 2,3-$Cl_2C_6H_3O$, R_2 = H, R_3 - CH_3
195: R_1=H, R_2= 2,3$Cl_2C_6H_3O$, R_3 = CH_3
196: R_1=$C_{10}H_7O$, R_2 =Cl, R_3 = H

194-196

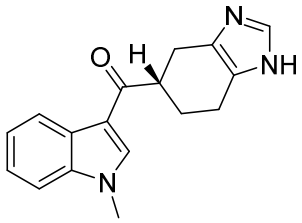
2.6.4 Antihypertensive activity

Toshiro et al[65] studied the supporting evidence for the pharmacokinetic properties of various serotonin-3(5-HT3) receptor antagonists, which include ondansetron (**197**) and ramosetron (**198**) especially their ability to induce or inhibit CYP450 isoenzymes in human liver microsomes. Ramosetron which is derivative of 1H-benzimidazole neither inhibited nor induced any of the metabolic activities, whereas ondansetron which is an aliphatic substituted imidazole derivative, competitively inhibited CYP1A2 and CYP2D6 activities.



Ondansetron

197

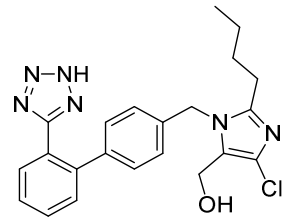


Ramosetron

198

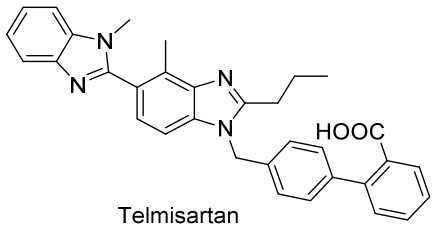
Wienen et al[66] studied the pharmacokinetics of angiotensin II-receptor antagonist class used widely as highly potent and selective antihypertensive, especially losartan (**199**) and Telmisartan (**200**). Based on their data under in-vitro and in-vivo conditions, telmisartan,

a 1H-benzimidazole derivative, did not induce or significantly inhibit CYP450 isoenzyme activity and was not metabolized by phase I reactions. **Wienen et al** pointed out that the medical use of telmisartan did not include the potential risk of drug interactions with compounds subject to CYP450-dependant biotransformation is low.



Losartan

199

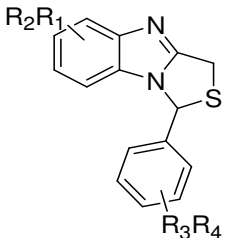


Telmisartan

200

2.6.5 Antiretroviral activity

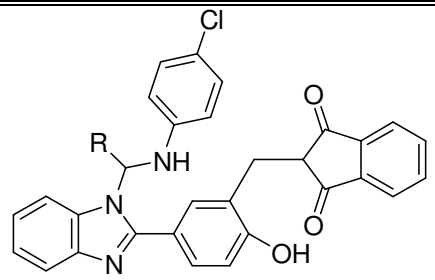
One of the key enzymes which play an essential and multifunctional role is reverse transcriptase. The enzyme play role in the replication of HIV-1 due to this benefit an attractive target for the development of new drugs that could be used in AIDS therapy has been constituted. Thiazolo-benzimidazole (**201**) proved to be a highly potent inhibitor of HIV-1 induced cytopathic effects. Based on the Structure activity relationship studies the substituents at C-1 in benzimidazole (**202-205**) significantly influence the interaction of the active compound with the receptor. Depending on the nature and position on the benzene fused ring influences the inhibitory potency, the methyl group present at C-3 is highly favorable to the pharmacological profile[67].



201-205

Compd.	R_1	R_2	R_3	R_4
201	H	H	2-F	6-F
202	H	H	3-NO_2	H
203	6-Me	7-Me	2-F	6-F
204	5-Me	H	2-F	6-F
205	8-Me	H	2-F	6-F

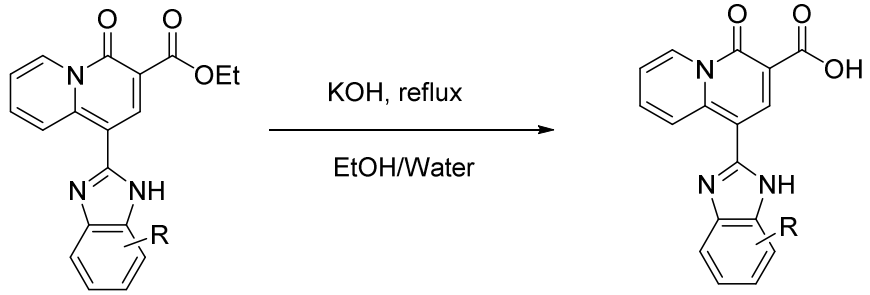
V. K. Pandey et al[68] synthesized 1,2-disubstituted benzimidazoles (**206**) and screened them for antiviral activity.



R= Phenyl. 4-OH Phenyl, CH_3, Phenyl

206

A series of 4-oxo-4H-quinolizine-3-carboxylic acid derivatives bearing sulfamido, carboxylamido, benzimidazole and benzothiazole substituents have been designed and synthesized by **Y. S. Xu et al**[69] and the synthetic compounds (**207-209**) were screened for possible HIV integrase inhibitory activity.



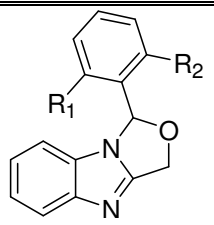
207: R=H

208: R= Cl

209: R= OCH_3

A. Chimirri et al[70] synthesized a new derivative of 1H,3H-oxazolo[3,4-α] benzimidazoles (OBZs) as HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTI) to extend the structure activity relationships observed for an early series of related 1H,3H-thiazolo[3,4-α] benzimidazole derivatives (TBZs). The new compounds (**210-212**)

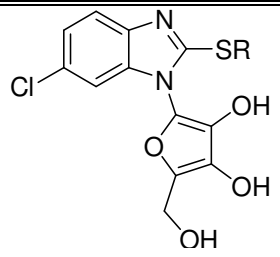
showed inhibitory activity against the replication of various HIV-1 strains, including NNRTI resistant strains. Testing of a representative OBZ derivative in an HPLC assay on biological fluids, indicated that the sulphur substitution appreciably improved the metabolic stability of the TBZ compound.



Compd.	R_1	R_2
210	H	H
211	Cl	F
212	F	F

210-212

R. Zou et al[71] synthesized a series of disubstituted benzimidazole ribonucleosides (**213-214**) and evaluated their antiviral activity.



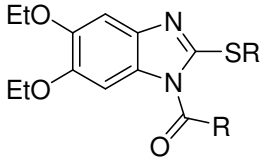
Compd.	R
213	CH_3
214	Bn

213-214

2.6.6 Antiinflammatory, analgesic and antiulcer activity

Based on the structure activity relationship studies of the synthesized 5,6-dialkoxy-2-thiobenzimidazole derivatives have revealed that compounds (**215-225**) possess significant antiinflammatory properties. Using carrageenan and bentonite model the activity has been determine the synthesized compounds. The carrageenan model suggests the most significant antiinflammatory effects were observed for compounds (**215, 218, 221, 222 & 223**). While using the bentonite model, the maximum activities were observed for compounds **219** and **222**. These results are promising which indicated that

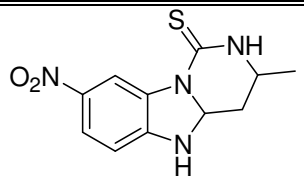
benzimidazoles derivatives are promising leads for the development of new anti-inflammatory agents[72].



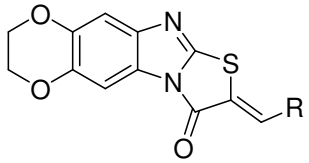
215-225

Compd.	R
215	Ph
216	4-MeC_6H_4
217	4-$MeOC_6H_4$
218	4-FC_6H_4
219	4-ClC_6H_4
220	3-ClC_6H_4
221	2-pyridyl
222	3-pyridyl
223	4-pyridyl
224	2-Thienyl
225	2-Furyl

The compounds Pyrimidobenzimidazole[73] (**226**) and dioxinobenzimidazothiazol-9-ones[74] (**227**) have been found antiinflammatory and analgesic activity, as evaluated by carrageenan induced rat paw edema and phenylquinone induced writhing tests. In addition, N-benzoyl and N-tosyl benzimidazole compounds (**228**) showed significant anti-inflammatory activity, as indicated by ear swelling induced by xylene in mice, and their ulcer indices were all lower than those of Aspirin[75].

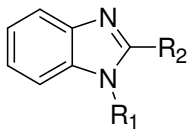


226



R= 4-FC_6H_4, 2-furyl, 3-pyridyl

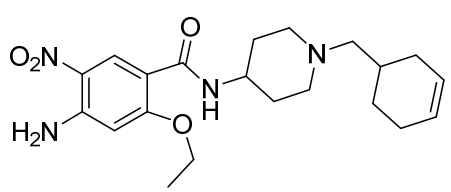
227



R_1 = Ts, COPh
R_2 = CCl_3, CH_2Ph

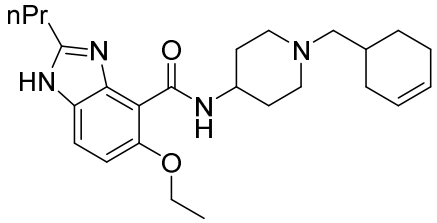
228

There are many proton pump inhibitors for the treatment of ulcer disease are commercially available. Despite the success of such commercial benzimidazole proton pump inhibitors for the treatment of ulcer disease, work is still in progress to discover new benzimidazole derived antiulcer drugs. The benzimidazole derivatives (**230**) which is related to Cinitapride (**229**) have been prepared and studied for their antiulcerative activity[76].



Cinitapride

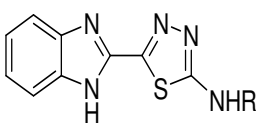
229



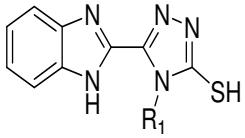
Cinitapride related benzimidazole

230

S. N. Sawhney et al[77] synthesized benzimidazole compound (**231**) and 2-(3-mercapto-4-substituted-4H-1,2,4-triazol-5-yl)-benzimidazole (**232**). The series of both of these types are screened for the potential antiinflammatory activity.



231

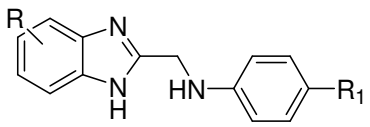


232

R=CH_3, C_2H_5, 4-$CH_3OC_6H_4$, 4-NO_2-C_6H_4
R_1 = CH_3, C_2H_5, CH_2=CH-CH_2, C_6H_5, 4-$CH_3OC_6H_4$

C. S. Kavitha et al[78] prepared a series of 2-methylaminobenzimidazole derivatives (**233**). The series have been prepared by the reaction of 2-(chloromethyl)-1H-benzimidazole derivatives with primary aromatic amines. The newly synthesized compounds were screened for analgesic and anti-inflammatory activities on the acetic acid induced writhing in mice and carrageenan induced paw oedema in rats. The synthesized compounds exhibited a potent analgesic (89% at 100 mg/kg b.w.) and antiinflammatory

(100% at 100 mg/kg b.w.) activities compared with standard drug Nimesulide (100% at 50 mg/kg b.w.), respectively.

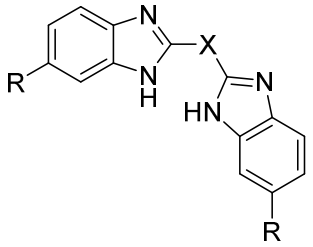


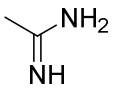
R = H, Br, NO_2
R_1 = H, Cl, Br, CH_3, OCH_3

233

2.6.7 Opportunistic infections

R. L. Lombardy et al[79] synthesized and study DNA interactions of benzimidazole dications (**234**) which shows activity against opportunistic infection.



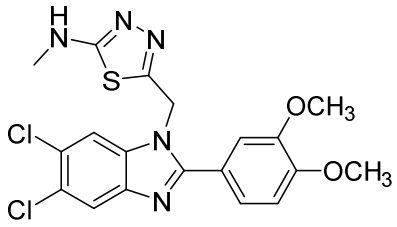
R = 

X = C=C

234

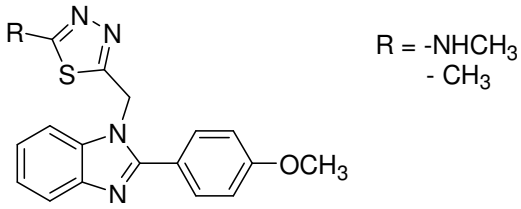
2.6.8 Antioxidant/Radio-protective activity

Some novel 5-[(2-(substitutedphenyl)-1H-benzimidazole-1-yl)methyl]methyl-1,3,4-thiadiazole-2-amines (**235**) were synthesized and tested for antioxidant properties by **Kus et al**[80] using various in-vitro systems.



235

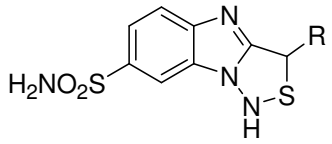
C. Kus et al[81] reported the antioxidant properties and synthesis of novel N-methyl-1,3,4-thiadiazol-2-amine and 4-methyl-2H-1,2,4-triazole-3(4H)-thione derivatives (**236**) of benzimidazole class.



236

2.6.9 Carbonic anhydrase inhibitors

A series of benzimidazo[1,2-c][1,2,3]thiadiazole-7-sulfonamides (**237-239**) were synthesized by **Daumantas et al**[82] as inhibitors of two isozymes of carbonic anhydrase, human carbonic anhydrase I (hCAI) and bovine carbonic anhydrase II (bCAII). The strongest binder to both isozymes of carbonic anhydrase was compound (**237**) with the observed K_d of about 0.04 lM. The most specific binder of hCAI was compound (**238**) that bound about four fold stronger to hCAI than to bCAII. The (**239**) compound bound three fold tighter to bCAII than to hCAI.

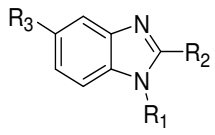


Compd.	R
237	Cl
238	SO_2CH_3
239	$N(CH_2CH_3)_2$

237-239

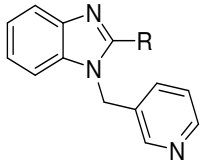
2.6.10 Anticonvulsant and antidepressant activity

A series of 1,2,5-trisubstituted benzimidazoles (**240-242**) derivatives have been synthesized and reported as potential anticonvulsant. The results of QSAR investigation and the study of various physicochemical properties indicates that the change in linker at position one (R_1) does not change the activity of the synthesized compounds and optimum chain length at position two (R_2) is responsible for the anticonvulsant activity[83].



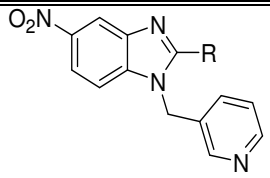
R_1 = picoline, R_2 = varying alkyl chain
R_3 = NO_2

240



R = H, CH_3, C_2H_5, C_3H_7, C_4H_9

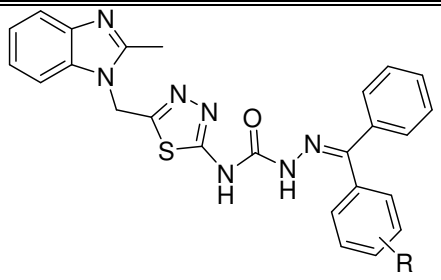
241



R = H, CH_3, C_2H_5, C_3H_7, C_4H_9

242

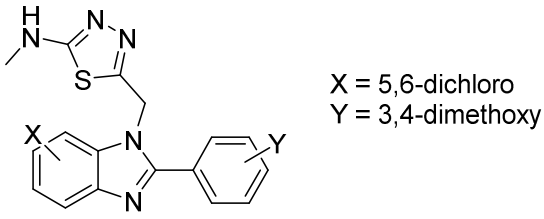
H. Rajak et al[84] synthesized novel 2,5-disubstituted-1,3,4-thiadiazoles (**243**) as potential anticonvulsant activity using standard drug Carbamazepine and Phenytoin at concentration 30 and 100 mg/kg.



R = H, 4-OH, 4-NO_2, 4-OCH_3

243

Pattanayak et al[85] synthesized the new benzimidazole derivatives of 2-amino-5-sulfanyl-1,3,4-thiadiazole (**244**) and evaluated their antidepressant activity. The synthetic compounds exhibited good to remarkable antidepressant activity in comparison to reference drugs.



244

2.7 Current work

Recent medicinal chemistry applications of benzimidazole analogs include antibacterial and antifungal agents, anthelmintic agents, HIV-1-induced cytopathic inhibitor, anti-inflammatory and antiulcer agents, cytotoxic and antitumor agents, anticancer, DNA binding agents, enzyme and receptor agonists or antagonists. Other applications of benzimidazoles include their use as organic ligands, fluorescent whitening agent dyes and functional materials. Therefore, the construction of these heterocycles has always been of great interest to organic and medicinal chemists and has consequently received much attention.

In order to develop eco-friendly green chemistry we have decided to use air as oxidant and alcoholic solvents as the solvent media at ambient temperature to obtain novel benzimidazoles (**Scheme 2**).

To optimize reaction conditions equal molar amounts of 3-[(3-Amino-4-methylamino-benzoyl)-pyridin-2-yl-amino]-propionic acid ethyl ester **(1)** and aldehyde (3-hydroxy benzaldehyde) **(2)** was dissolved in different alcoholic solvents at ambient temperature in the presence of air with/without catalyst to obtain novel benzimidazole compound **(mpd1)**

(**Scheme 1**). Effect of different alcoholic solvents and catalysts in the synthesis of novel benzimidazole compound (**mpd1**) by using air as oxidant are presented in **Table 2**.

3-[(3-Amino-4-methylamino-benzoyl)-pyridin-2-yl-amino]-propionic acid ethyl ester **(1)** [CAS No.: 212322-56-0] is commercially available and intermediate of the drug Dabigatran, which can be synthesized as below **Scheme 1**.

2.8 Reaction Scheme

2.8.1 Scheme 1: Synthesis of 3-[(3-Amino-4-methylamino-benzoyl)-pyridin-2-yl-amino]-propionic acid ethyl ester **(1)** and **(mpd1)**

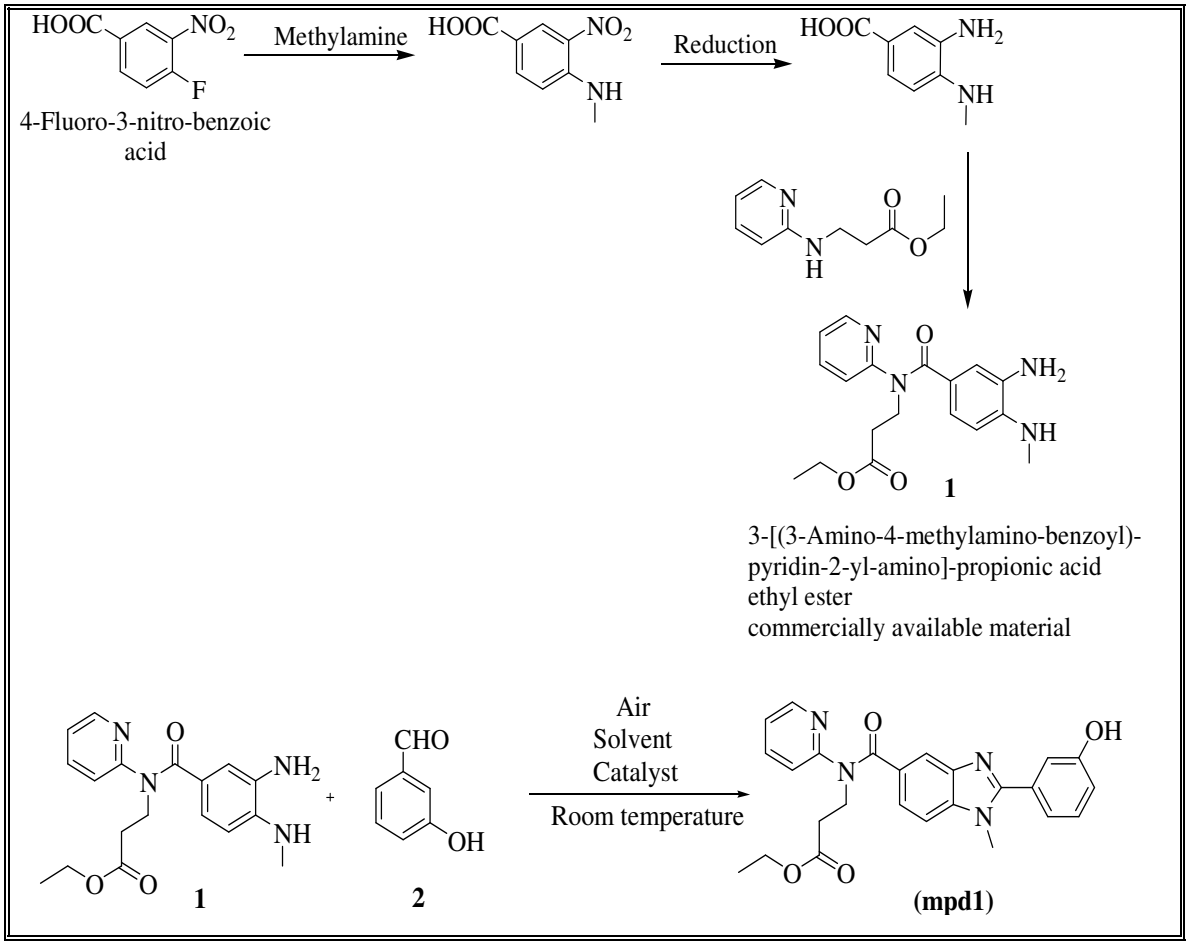


Table 2. Effect of different alcoholic solvents and catalysts in the synthesis of novel benzimidazole compound (**mpd1**) by using air as oxidant

Sr.No	Solvent	Catalyst (Mol %)	Temperature	Yield (%)
1	Ethanol	p-TsOH (5)	25˚C	10
2	Methanol	p-TsOH (5)	25˚C	15
3	Isopropyl alcohol	p-TsOH (5)	25˚C	18
4	Ethanol	p-TsOH (10)	25˚C	14
5	Methanol	p-TsOH (10)	25˚C	22
6	Isopropyl alcohol	p-TsOH (10)	25˚C	26
7	Ethanol	Acetic acid (5)	25˚C	56
8	Methanol	Acetic acid (5)	25˚C	63
9	Isopropyl alcohol	Acetic acid (5)	25˚C	69
10	Ethanol	Acetic acid (10)	25˚C	67
11	Methanol	Acetic acid (10)	25˚C	76
12	**Isopropyl alcohol**	**Acetic acid (10)**	**25˚C**	**92**
13	Isopropyl alcohol	None	25˚C	13

As **Table 2** conditions indicates presence of acetic acid (10mol %) and isopropyl alcohol (Sr. No. 12) gave us excellent yield, easy isolation (only by filtration of the reaction mass, no further purification is required). All the other compounds **(mpd 1-12 and BI 1-6)** are prepared and characterized according to the optimized conditions i.e. acetic acid (10mol %) and isopropyl alcohol by using air as oxidant at ambient temperature (**Scheme 2**).

Equal molar amount of di-amino compound **(1)** and differently substituted aryl aldehydes were reacted at room temperature in isopropyl alcohol in the presence of acetic acid as a catalyst and air as the oxidant to get novel benzimidazole compounds **(mpd 1-12 and BI 1-6)**. Moderate to high yields were obtained. Generally reaction completed within ca.16 hours, and in many cases reaction time is less than 10 hours (**Table 2**). As indicated by the TLC all the reactions **(mpd 1-12 and BI 1-6)** were clean, and the isolation of product from the reaction mass was done by direct filtration, so no need to remove/distill solvent from the reaction mass and no further purification is required. Thus the procedure indicated herein is safe, economical and eco-friendly green chemistry.

2.8.2 Scheme 2. General scheme of novel benzimidazole compounds (**mpd 1-12 and BI 1-6**)

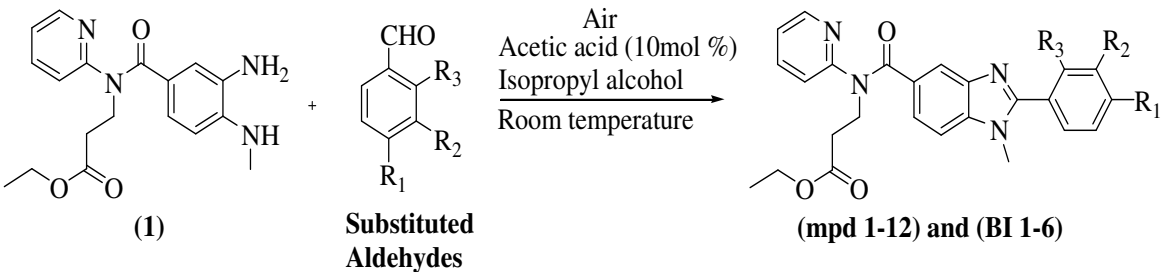
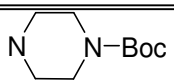
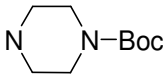
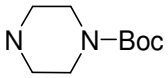
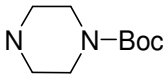


Table 3. Reaction time and yield data of novel benzimidazole compounds **(mpd 1-12 and BI 1-6)**

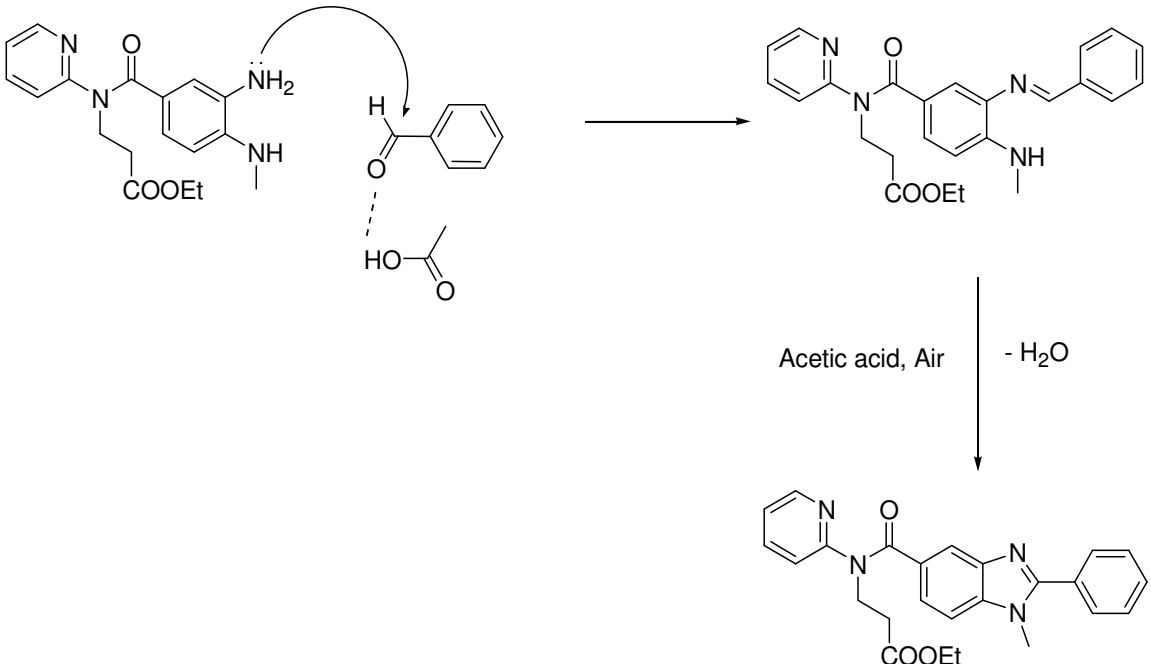
Compd.	R_1	R_2	R_3	Reaction time (hrs)	Yield (%)
mpd 1	H	OH	H	16	92
mpd 2	OH	Br	H	12	86
mpd 3	Cl	H	H	11	87
mpd 4	F	H	H	12	86
mpd 5	OH	H	H	10	81
mpd 5-isobutyl	H	$OCH_2CH(CH_3)_2$	H	15	90
mpd 6	$OCH_2CH(CH_3)_2$	Br	H	14	88
mpd 7	H	OCH_2CH_3	H	13	80
mpd 8	H	H	OH	10	83
mpd 9	H	$O(CH_2)_2CH_3$	H	10	79
mpd 10	H	$OCH(CH_3)_2$	H	15	84
mpd 11	$O(CH_2)_2CH_3$	H	H	12	78
mpd 12	Br	H	H	12	85
BI-01	OCH_2Ph	H	H	12	90
BI-02	H	H	OCH_2Ph	12	91

BI-03		H	OCH_2COOEt	10	89
BI-04		H	$OCH_2CH(CH_3)_2$	12	88
BI-05		H	$O(CH_2)_2CH_3$	10	86
BI-06		H	OH	12	92

2.9 Reaction mechanism

Diamino compound first form schiff's base with aldehyde in the presence of acetic acid, then cyclization takes place with the removal of water to generate benzimidazole moiety. The general mechanism with benzaldehyde is represented in the **Scheme 3.**

Scheme 3: Reaction mechanism for the formation of benzimidazole



2.10 Experimental

General Procedures. All the reagents were obtained commercially and used with further purification. All melting points were taken in open capillaries and are uncorrected. The

monitoring of the progress of all reactions and homogeneity of the synthesized compounds was carried out by TLC. TLC was run using TLC aluminum sheets silica gel $60F_{254}$ (Merck). Elemental analysis (% C, H, N) was carried out by EURO EA 3000 CHN elemental analyzer. IR spectra were recorded on a shimadzu FTIR 8401 spectrophotometer in KBR. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using solvent peak as internal standard. Mass spectra were scanned on a shimadzu LCMS 2010 spectrometer.

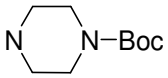
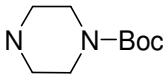
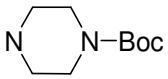
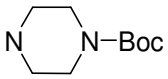
General procedure for synthesis of novel benzimidazole compounds (mpd 1-12 and BI 1-6)

Di-amino compound **(1)** (1 mole), substituted aryl aldehydes (1 mole), acetic acid (10 mol %) and isopropyl alcohol were charged in round bottom flask with mechanical stirrer, air purging and condenser. The reaction mixture was stirred for the time indicated in **Table 3**. After completion of the reaction, as indicated by TLC, the reaction mixture was filtered under suction, washed with isopropyl alcohol to afford novel benzimidazole compounds **(mpd 1-12 and BI 1-6)**.

2.11 Physical data

2.11.1 Physical data of novel benzimidazole compounds **(mpd 1-12 and BI 1-6)**

Compd.	**R_1**	**R_2**	**R_3**	**m.p.** (°C)	**Yield (%)**
mpd 1	H	OH	H	233-234	92
mpd 2	OH	Br	H	256-258	86
mpd 3	Cl	H	H	242-243	87
mpd 4	F	H	H	269-271	86
mpd 5	OH	H	H	227-229	81
mpd 5-isobutyl	H	$OCH_2CH(CH_3)_2$	H	237-239	90
mpd 6	$OCH_2CH(CH_3)_2$	Br	H	246-248	88
mpd 7	H	OCH_2CH_3	H	210-211	80

mpd 8	H	H	OH	227-228	83
mpd 9	H	$O(CH_2)_2CH_3$	H	198-199	79
mpd 10	H	$OCH(CH_3)_2$	H	176-177	84
mpd 11	$O(CH_2)_2CH_3$	H	H	188-189	78
mpd 12	Br	H	H	213-214	85
BI-01	OCH_2Ph	H	H	155-156	90
BI-02	H	H	OCH_2Ph	146-147	91
BI-03		H	OCH_2COOEt	208-209	89
BI-04		H	$OCH_2CH(CH_3)_2$	222-223	88
BI-05		H	$O(CH_2)_2CH_3$	231-232	86
BI-06		H	OH	216-217	92

TLC solvent system: Compounds (EA:Hexane) 3:7

MPs were taken in open capillary and are not corrected

2.12 Spectral discussion

2.12.1 Mass spectral study

Mass spectra were recorded on Shimadzu GC-MS-QP-2010 model using Direct Injection Probe technique. The molecular ion peak was found in agreement with molecular weight of the respective compound. Characteristic M^{+2} ion peaks with one-third intensity of molecular ion peak were observed in case of compounds having chlorine atom. The compounds having chlorine atom showed this characteristic peak. Fragmentation pattern can be observed to be particular for this kind of compounds and the characteristic peaks obtained for each compound. Various characteristic peaks obtained for each compound in this series can be discussed as below.

2.12.2 IR spectral study

Various functional groups present in molecule were identified by characteristic frequency obtained for them. Presence of carbonyl group can be confirmed by IR spectra because carbonyl stretching frequency was observed for carbonyl group present in the moiety. C=O group (N-CO-) and C=O groups of ester were observed between 1650-1750 cm^{-1}. Peaks were identified for aromatic and alkyl group as per their characteristics. In case of compounds having different substations on aromatic ring, characteristic frequencies were observed depending on the functional group present i.e. hydroxyl, chloro, fluoro etc.

2.12.3 ^{1}H NMR spectral study

Numbers of proton identified from NMR spectrum and their chemical shift (ppm) were in agreement of structure of molecule. Methyl protons of ethyl ester (-$OCH_2\underline{CH_3}$) were observed at around 1.1-1.3 ppm as triplet and methylene protons (-$O\underline{CH_2}CH_3$) were observed at around 3.9-4.1δ ppm as quartet. Protons of N-CH_3 were observed at around 3.81δ ppm as singlet. Aromatic protons were observed between 6.7-8.5δ ppm. J values were calculated to identify ortho and meta coupling. In some cases, aromatic protons were obtained as multiplet.

2.12.4 Elemental analysis

Elemental analysis showed calculated and found percentage values of carbon, hydrogen and nitrogen in support of structure of synthesized compounds. The spectral and elemental analysis data are given below for individual compounds.

2.12.5 Spectral data of synthesized compounds (mpd 1-12 and BI 1-6)

3-{[2-(3-Hydroxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 1). Off-white solid, (92 %), m.p. 233-234 °C, Anal.Calcd for $C_{25}H_{24}N_4O_4$: C 67.55, H 5.44, N 12.60% Found: C 67.79, H 5.55, N

12.88%. IR (KBr, cm^{-1}): 3325 (OH), 1726 (C=O), 1650 (N-C=O). ^{1}H NMR (400 MHz, $DMSOd_6$): δ 1.11-1.15 (t, 3H, CH_3), 2.68-2.72 (t, 2H, CH_2), 3.81 (s, 3H, N-CH_3), 3.95-4.01 (m, 2H, CH_2), 4.22-4.26 (t, 2H, N-CH_2), 6.93-8.42 (m, 11H, Ar-H), 9.78 (s, 1H, OH), ^{13}C NMR (400 MHz, $DMSOd_6$) δ: 14.25 (CH_3), 31.68 (N-CH_3), 33.35 (CH_2COO), 43.78 (N-CH_2), 60.25 ($COOCH_2$), 109.21, 116.12, 116.31, 121.01, 121.10, 122.01, 123.43, 123.94, 130.24, 131.32, 131.33, 138.46, 138.79, 141.74, 149.43, 154.56, 156.77, 159.32 (Ar-C), 171.04 (N-C=O), 171.95 (C=O), MS: (M+1) 445.2, (M+Na) 467.3

3-{[2-(3-Bromo-4-hydroxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 2). White solid, (86 %), m.p. 256-258°C, Anal.Calcd for $C_{25}H_{23}BrN_4O_4$: C 57.37, H 4.43, N 10.70% Found: C 57.03, H 4.75, N 10.81%. IR (KBr, cm^{-1}): 3310 (OH), 1715 (C=O), 1645 (N-C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.19-1.29 (t, 3H, CH_3), 2.79-2.84 (t, 2H, CH_2), 3.80 (s, 3H, N-CH_3), 4.04-4.14 (m, 2H, CH_2), 4.41-4.46 (t, 2H, N-CH_2), 6.73-8.43 (m, 10H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.31 (CH_3), 32.08 (N-CH_3), 33.45 (CH_2COO), 44.96 (N-CH_2), 60.76 ($COOCH_2$), 109.62, 110.95, 116.72, 120.37, 120.95, 121.38, 122.75, 124.28, 129.87, 130.65, 133.88, 137.44, 137.65, 141.01, 149.15, 154.09, 156.24, 156.33 (Ar-C), 171.10 (N-C=O), 171.96 (C=O)

3-{[2-(4-Chloro-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 3). Yellow solid, (87 %), m.p. 242-243°C, Anal.Calcd for $C_{25}H_{23}ClN_4O_3$: C 64.86, H 5.01, N 12.10% Found: C 64.75, H 5.15, N 12.01%. IR (KBr, cm^{-1}): 1719 (C=O), 1655 (N-C=O), 755 (C-Cl). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.17-1.26 (t, 3H, CH_3), 2.78-2.85 (t, 2H, CH_2), 3.81 (s, 3H, N-CH_3), 3.95-4.00 (m, 2H, CH_2), 4.21-4.25 (t, 2H, N-CH_2), 6.93-8.42 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.29 (CH_3), 31.99 (N-CH_3), 33.46 (CH_2COO), 44.86 (N-CH_2), 60.64 ($COOCH_2$), 109.49, 121.09, 121.19, 122.56, 124.29, 128.21, 129.19, 130.47, 130.77, 136.44, 137.42, 137.95, 142.11, 149.09, 154.01, 156.41 (Ar-C), 171.08 (N-C=O), 171.89 (C=O), MS: (M+1) 463.1, (M+2) 465.1

3-{[2-(4-Fluoro-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 4). Off-white solid, (86 %), m.p. 269-271 °C, Anal.Calcd for $C_{25}H_{23}FN_4O_3$: C 67.25, H 5.19, N 12.55% Found: C 67.14, H 5.25, N 12.62%. IR (KBr, cm^{-1}): 1746 (C=O), 1642 (N-C=O), 1200 (C-F). ^1H NMR (400 MHz, $CDCl_3$): δ 1.17-1.26 (t, 3H, CH_3), 2.68-2.72 (t, 2H, CH_2), 3.83 (s, 3H, N-CH_3), 3.96-4.00 (m, 2H, CH_2), 4.22-4.26 (t, 2H, N-CH_2), 6.93-8.42 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.31 (CH_3), 31.96 (N-CH_3), 33.49 (CH_2COO), 44.88 (N-CH_2), 60.66 ($COOCH_2$), 109.45, 116.00, 116.29, 121.09, 121.18, 122.59, 124.22, 125.95, 130.44, 131.50, 131.61, 137.42, 137.94, 142.14, 149.11, 154.27, 156.47, 162.22, 165.54 (Ar-C), 171.15 (N-C=O), 171.93 (C=O), MS: (M+1) 447.2, (M+Na) 469.2

3-{[2-(4-Hydroxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 5). White solid, (81 %), m.p. 227-229 °C, Anal.Calcd for $C_{25}H_{24}N_4O_4$: C 67.55, H 5.44, N 12.60% Found: C 67.75, H 5.51, N 12.68%. IR (KBr, cm^{-1}): 3322(OH), 1720 (C=O), 1644 (N-C=O). ^1H NMR (400 MHz, $DMSOd_6$): δ 1.12-1.17 (t, 3H, CH_3), 2.67-2.72 (t, 2H, CH_2), 3.82 (s, 3H, N-CH_3), 3.93-4.00 (m, 2H, CH_2), 4.22-4.26 (t, 2H, N-CH_2), 6.93-8.42 (m, 11H, Ar-H), 9.77 (s, 1H, OH), ^{13}C NMR (400 MHz, $DMSOd_6$) δ: 14.24 (CH_3), 31.66 (N-CH_3), 33.32 (CH_2COO), 43.79 (N-CH_2), 60.23 ($COOCH_2$), 109.21, 116.12, 116.31, 121.01, 121.10, 122.01, 123.43, 123.94, 130.24, 131.32, 131.33, 138.46, 138.79, 141.74, 149.43, 154.56, 156.77, 159.32 (Ar-C), 171.04 (N-C=O), 171.95 (C=O), MS: (M+1) 445.1, (M+Na) 467.2

3-{[2-(3-Isobutoxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd-5 isobutyl). Off-white solid, (90 %), m.p. 237-239 °C, Anal.Calcd for $C_{29}H_{32}N_4O_4$: C 69.58, H 6.44, N 11.19% Found: C 69.65, H 6.72, N 11.36%. IR (KBr, cm^{-1}): 1735 (C=O), 1670 (N-C=O). ^1H NMR (400 MHz, $CDCl_3$): δ 1.10-1.12 (d, 6H, CH_3), 1.21-1.25 (t, 3H, CH_3), 2.18-2.24 (m, 1H, CH), 2.82-2.85 (d, 2H, CH_2), 3.84 (s, 3H, N-CH_3), 3.87-3.88 (d, 2H, CH_2), 4.07-4.12 (q, 2H, CH_2), 4.43-4.47 (t, 2H, N-CH_2), 6.76-8.44 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.33 (CH_3), 19.35 (CH_3), 28.44 (CH), 32.03 (N-CH_3), 33.52 (CH_2COO), 44.89 (N-CH_2), 60.69 ($COOCH_2$), 75.62 (CH), 109.39, 112.64, 112.76, 121.08, 122.61, 123.01, 124.15, 129.83,

130.38, 134.20, 137.43, 138.00, 142.15, 149.12, 153.86, 156.49, 157.05 (Ar-C), 171.22 (N-C=O), 171.97 (C=O), MS: (M+1) 501.2, (M+Na) 523.4, (M+K) 539.2

3-{[2-(3-Bromo-4-isobutoxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 6). White solid, (88 %), m.p. 246-248 °C, Anal.Calcd for $C_{29}H_{31}BrN_4O_4$: C 60.11, H 5.39, N 9.67 % Found: C 60.19, H 5.51, N 9.83 %. IR (KBr, cm^{-1}): 1738 (C=O), 1660 (N-C=O). ^1H NMR (400 MHz, $CDCl_3$): δ 1.10-1.12 (d, 6H, CH_3), 1.21-1.25 (t, 3H, CH_3), 2.18-2.24 (m, 1H, CH), 2.82-2.85 (d, 2H, CH_2), 3.84 (s, 3H, N-CH_3), 3.87-3.88 (d, 2H, CH_2), 4.07-4.12 (q, 2H, CH_2), 4.43-4.47 (t, 2H, N-CH_2), 6.76-8.44 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.33 (CH_3), 19.35 (CH_3), 28.44 (CH), 32.03 (N-CH_3), 33.52 (CH_2COO), 44.89 (N-CH_2), 60.69 ($COOCH_2$), 75.62 (CH), 109.39, 112.64, 112.76, 121.08, 122.61, 123.01, 124.15, 129.83, 130.38, 134.20, 137.43, 138.00, 142.15, 149.12, 153.86, 156.49, 157.05 (Ar-C), 171.22 (N-C=O), 171.97 (C=O), MS: (M+) 579.2, (M+Na) 603.1

3-{[2-(3-Ethoxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 7). Off-white solid, (80 %), m.p. 210-211 °C, Anal.Calcd for $C_{27}H_{28}N_4O_4$: C 68.63, H 5.97, N 11.86% Found: C 68.79, H 5.95, N 11.88%. IR (KBr, cm^{-1}): 1726 (C=O), 1650 (N-C=O). ^1H NMR (400 MHz, $CDCl_3$): δ 1.10-1.13 (t, 3H, CH_3), 1.21-1.24 (t, 3H, CH_3), 2.82-2.84 (d, 2H, CH_2), 3.84 (s, 3H, N-CH_3), 4.07-4.12 (q, 2H, CH_2), 4.43-4.47 (t, 2H, N-CH_2), 6.76-8.44 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.33 (CH_3), 32.03 (N-CH_3), 33.52 (CH_2COO), 44.89 (N-CH_2), 60.69 ($COOCH_2$), 109.39, 112.64, 112.76, 121.08, 122.61, 123.01, 124.15, 129.83, 130.38, 134.20, 137.43, 138.00, 142.15, 149.12, 153.86, 156.49, 157.05 (Ar-C), 171.22 (N-C=O), 171.97 (C=O), MS: (M+1) 473.2, (M+Na) 495.2, (M+K) 511.2

3-{[2-(2-Hydroxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 8). Off-white solid, (83 %), m.p. 227-228 °C, Anal.Calcd for $C_{25}H_{24}N_4O_4$: C 67.55, H 5.44, N 12.60% Found: C 67.79, H 5.55, N 12.88%. IR (KBr, cm^{-1}): 3330 (OH), 1722 (C=O), 1658 (N-C=O), MS: (M+1) 445.2, (M+Na) 467.3

3-{[1-Methyl-2-(3-propoxy-phenyl)-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 9). White solid, (79 %), m.p. 198-199 °C, Anal.Calcd for $C_{28}H_{30}N_4O_4$: C 69.12, H 6.21, N 11.51 % Found: C 69.15, H 6.34, N 11.68%. IR (KBr, cm^{-1}): 1740 (C=O), 1644 (N-C=O). ^1H NMR (400 MHz, $CDCl_3$): δ 1.01-1.03 (t, 3H, CH_3), 1.11-1.14 (t, 3H, CH_3), 1.76-1.79 (m, 2H, CH_2), 2.80-2.82 (t, 2H, CH_2), 3.84 (s, 3H, N-CH_3), 4.07-4.12 (m, 4H, CH_2), 4.40-4.45 (t, 2H, N-CH_2), 6.70-8.39 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 13.33 (CH_3), 13.7 (CH_3), 23.4 (CH_2), 32.09 (N-CH_3), 33.42 (CH_2COO), 44.81 (N-CH_2), 60.60 (COOCH_2), 74.5 (CH_2), 109.30, 112.60, 112.79, 121.09, 122.60, 123.00, 124.19, 129.83, 130.35, 134.20, 137.45, 138.00, 142.18, 149.10, 153.89, 156.49, 157.05 (Ar-C), 171.20 (N-C=O), 171.90 (C=O)

3-{[2-(3-Isopropoxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 10). Light yellow solid, (84 %), m.p. 176-177 °C, Anal.Calcd for $C_{28}H_{30}N_4O_4$: C 69.12, H 6.21, N 11.51 % Found: C 69.18, H 6.33, N 11.61%. IR (KBr, cm^{-1}): 1742 (C=O), 1639 (N-C=O). ^1H NMR (400 MHz, $CDCl_3$): δ 1.21-1.22 (t, 3H, CH_3), 1.33-1.35 (d, 6H, CH_3), 2.84-2.85 (d, 2H, CH_2), 3.87 (s, 3H, N-CH_3), 3.88-3.89 (d, 2H, CH_2), 4.06-4.11 (m, 3H, CH & CH_2), 4.42-4.49 (t, 2H, N-CH_2), 6.79-8.49 (m, 11H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.31 (CH_3), 22.35 (CH_3), 32.05 (N-CH_3), 33.57 (CH_2COO), 44.81 (N-CH_2), 60.63 (COOCH_2), 75.62 (CH), 109.30, 112.69, 112.71, 121.00, 122.69, 123.05, 124.15, 129.88, 130.33, 134.26, 137.48, 138.00, 142.10, 149.14, 153.89, 156.44, 157.07 (Ar-C), 171.24 (N-C=O), 171.95 (C=O)

3-{[1-Methyl-2-(4-propoxy-phenyl)-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 11). Off-white solid, (78%), m.p.188-189°C, Anal.Calcd for $C_{28}H_{30}N_4O_4$: C 69.12, H 6.21, N 11.51 % Found: C 69.18, H 6.24, N 11.63%. IR (KBr, cm^{-1}): 1741 (C=O), 1642 (N-C=O).

3-{[2-(4-Bromo-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (mpd 12). White soilid, (85%), m.p.213-214,

Anal.Calcd for $C_{25}H_{23}BrN_4O_3$: C 59.18, H 4.57, N 11.04 % Found: C 59.28, H 4.44, N 11.12%. IR (KBr, cm^{-1}): 1737 (C=O), 1652 (N-C=O).

3-{[2-(4-Benzyloxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (BI-1). Off-white solid, (90%), m.p.155-156, Anal.Calcd for $C_{32}H_{30}N_4O_4$: C 71.89, H 5.66, N 10.48 % Found: C 71.70, H 5.54, N 10.32%.

3-{[2-(2-Benzyloxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester (BI-2). Off-white solid, (91%), m.p.146-147, Anal.Calcd for $C_{32}H_{30}N_4O_4$: C 71.89, H 5.66, N 10.48 % Found: C 71.75, H 5.57, N 10.35%. IR (KBr, cm^{-1}): 1747 (C=O), 1644 (N-C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.19-1.24 (t, 3H, CH_3), 1.98 (bs, HOD of $CDCl_3$), 2.80-2.85 (t, 2H, CH_2), 3.79 (s, 3H, N-CH_3), 4.04-4.11 (q, 2H, OCH_2), 4.42-4.47 (t, 2H, N-CH_2), 5.13 (s, 2H, OCH_2Ph), 6.72-8.43 (m, 16H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.31 (CH_3), 31.99 (N-CH_3), 33.52 (CH_2COO), 44.85 (N-CH_2), 60.64 ($COOCH_2$), 70.23 (CH_2Ph), 109.28,115.22, 120.97, 122.33, 122.59, 123.89, 127.66, 128.32, 128.81, 130.17, 130.98, 136.51, 137.37, 138.05, 142.25, 149.06, 155.18, 156.52, 160.26 (Ar-C), 171.27 (N-C=O), 171.94 (C=O).

4-(4-{5-[(2-Ethoxycarbonyl-ethyl)-pyridin-2-yl-carbamoyl]-1-methyl-1H-benzoimidazol-2-yl}-3-ethoxycarbonylmethoxy-phenyl)-piperazine-1-carboxylic acid tert-butylester (BI-3). Off-white solid, (89%), m.p.208-209 IR (KBr, cm^{-1}): 1740 (C=O), 1636 (N-C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.19-1.26 (m, 6H, CH_3), 1.47 (s, 9H, Boc protons,$C(CH_3)_3$), 1.73 (bs, HOD of $CDCl_3$), 2.80-2.85 (t, 2H, CH_2), 3.07-3.09 (bt, 4H, CH_2 of piperazine ring), 3.53-3.56 (bt, 4H, CH_2 of piperazine ring), 3.71 (s, 3H, N-CH_3), 4.04-4.11 (q, 2H, OCH_2), 4.15-4.23 (q, 2H, OCH_2), 4.42-4.47 (t, 2H, N-CH_2), 4.52 (s, 2H, OCH_2COO), 6.74-8.45 (m, 10H, Ar-H), ^{13}C NMR (400 MHz, $CDCl_3$) δ: 14.29, 14.34 (CH_3), 28.59 (Boc. CH_3), 31.53 (N-CH_3), 33.56 (CH_2COO), 44.93 (N-CH_2), 50.26 (CH_2 of piperazine ring), 60.68 (CH_2 of piperazine ring), 61.57 ($COOCH_2$), 66.10 ($\underline{C}(CH_3)_3$), 80.14 ($O\underline{CH_2}CO$), 109.31,113.25, 120.37, 120.46, 121.04, 121.32, 122.73, 124.01, 129.88,

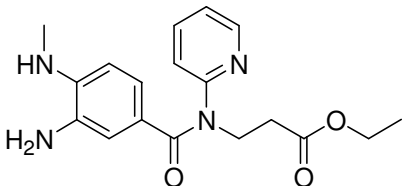
137.45, 142.44, 146.65, 149.12, 149.74, 153.51, 154.89(Ar-C), 156.59 (Boc-CO), 168.86 (C=O ester group), 171.31 (N-C=O), 172.01 (C=O).

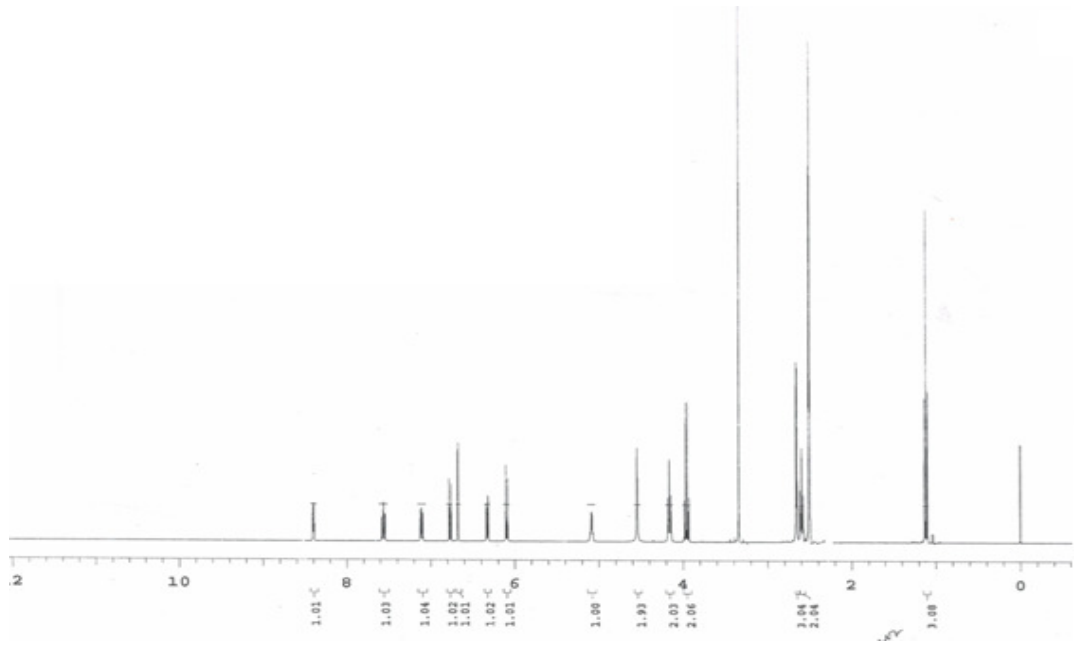
4-(4-{5-[(2-Ethoxycarbonyl-ethyl)-pyridin-2-yl-carbamoyl]-1-methyl-1H-benzoimidazol-2-yl}-3-isobutoxy-phenyl)-piperazine-1-carboxylic acid tert-butyl ester (BI-4). Off-white solid, (88%), m.p.222-223, Anal.Calcd for $C_{38}H_{48}N_6O_6$: C 66.65, H 7.06, N 12.27 % Found: C 66.55, H 7.12, N 12.35%. IR (KBr, cm-1): 1739 (C=O), 1644 (N-C=O).

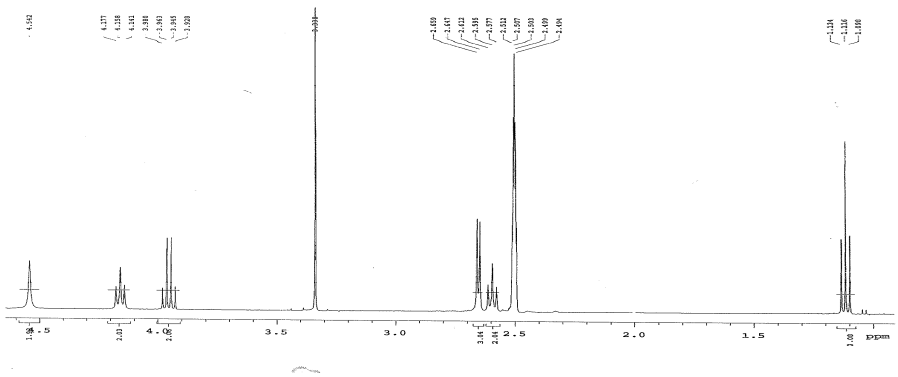
4-(4-{5-[(2-Ethoxycarbonyl-ethyl)-pyridin-2-yl-carbamoyl]-1-methyl-1H-benzoimidazol-2-yl}-3-propoxy-phenyl)-piperazine-1-carboxylic acid tert-butyl ester (BI-5). White soilid, (86%), m.p.231-232, Anal.Calcd for $C_{37}H_{46}N_6O_6$: C 66.25, H 6.91, N 12.53 % Found: C 66.35, H 6.81, N 12.60%. IR (KBr, cm^{-1}): 1747 (C=O), 1635 (N-C=O), MS: (M+1) 671.4, (M+Na) 693.4.

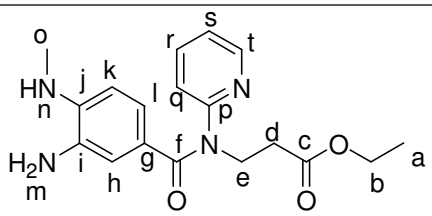
.**4-(4-{5-[(2-Ethoxycarbonyl-ethyl)-pyridin-2-yl-carbamoyl]-1-methyl-1H-benzoimidazol-2-yl}-3-hydroxy-phenyl)-piperazine-1-carboxylic acid tert-butyl ester (BI-6).** White soilid, (92%), m.p.216-217, Anal.Calcd for $C_{34}H_{40}N_6O_6$: C 64.95, H 6.41, N 13.37 % Found: C 64.89, H 6.50, N 13.39%. IR (KBr, cm^{-1}): 3329 (OH), 1738 (C=O), 1650 (N-C=O).

2.12.5.1 ^{1}H NMR spectrum of 1


HN
H2N
N
N
O
O
O







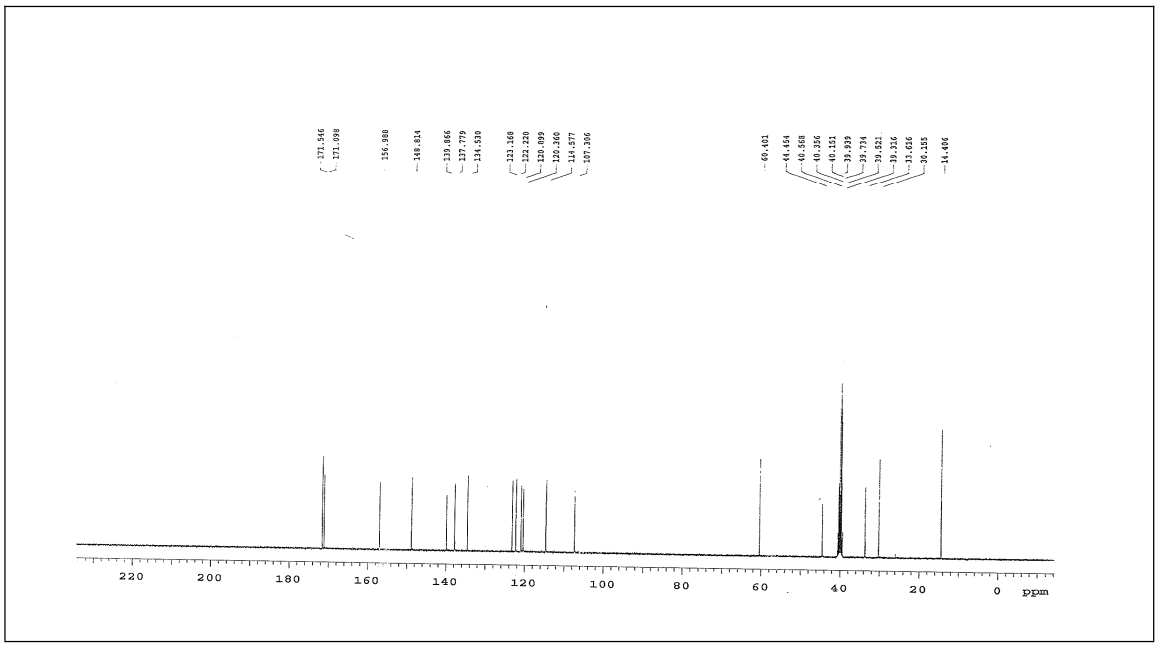
3-[(3-Amino-4-methylamino-benzoyl)-pyridin-2-yl-amino]-propionic acid ethyl ester

Compound 1

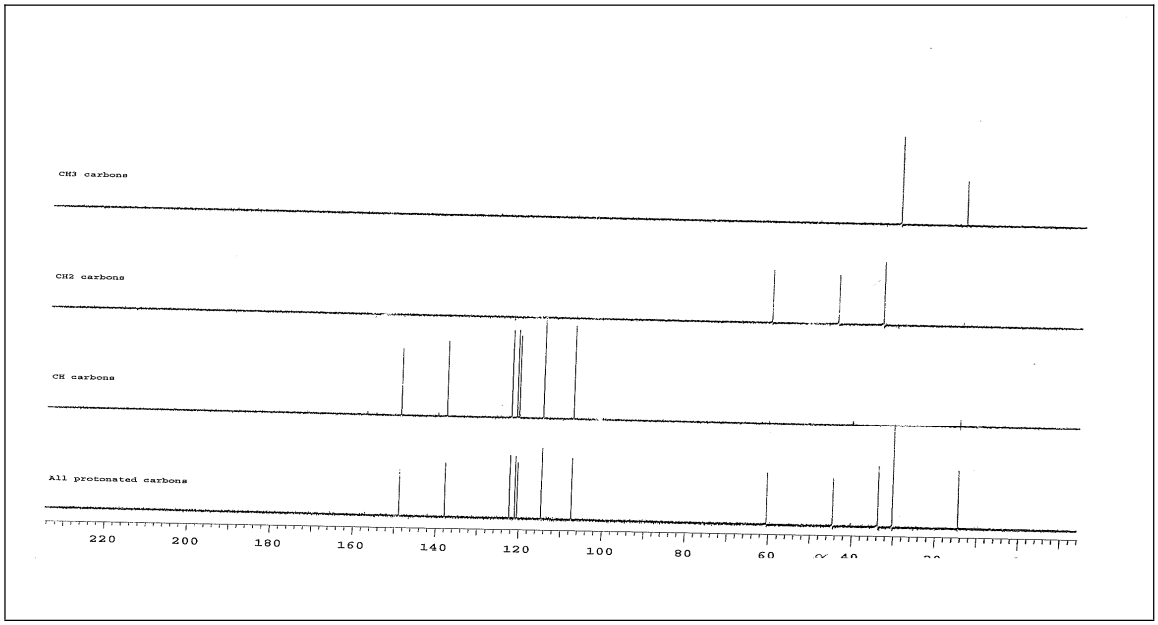
Assignment of ^{1}H NMR of compound 1:

Sr.No.	Signal (δppm)	No. of Protons	Multiplicity	Assignment
1	1.09-1.13	3H	triplet	a
2	2.57-2.61	2H	triplet	d
3	2.64-2.65	3H	doublet	o
4	3.92-3.98	2H	quartet	b
5	4.14-4.17	2H	triplet	e
6	4.54	2H	broad singlet	m
7	5.06-5.10	1H	quartet	n
8	6.08-6.10	1H	doublet (J= 8.0 Hz)	k
9	6.31-6.33	1H	dd ($J_{1,2}$= 2.0 Hz) ($J_{1,3}$= 8.0 Hz)	s
10	6.66-6.67	1H	doublet	h
11	6.75-6.77	1H	doublet (J= 8.0 Hz)	l
12	7.08-7.12	1H	multiplet	q
13	7.53-7.58	1H	multiplet	s
14	8.38-8.40	1H	multiplet	t

2.12.5.2 Carbon NMR spectrum of 1



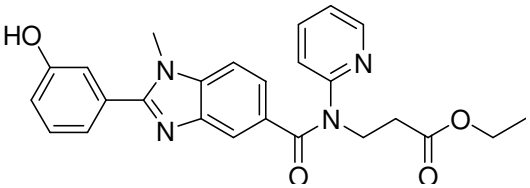
2.12.5.3 DEPT spectrum of 1

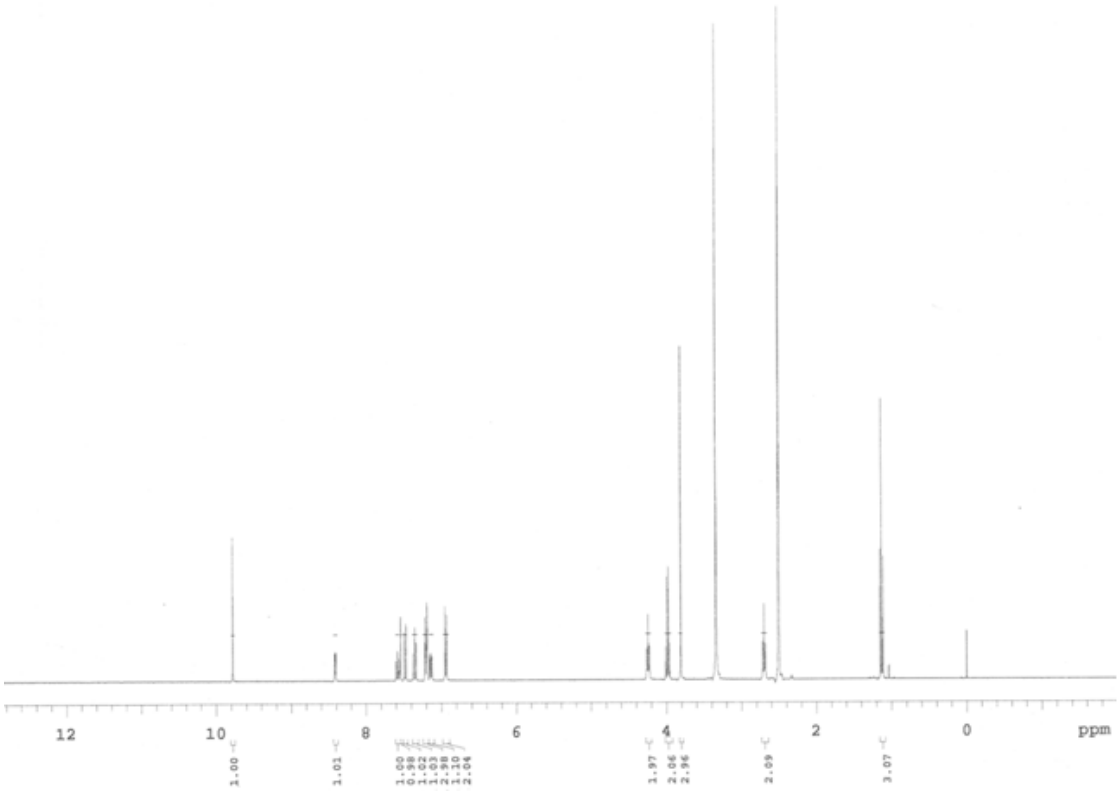


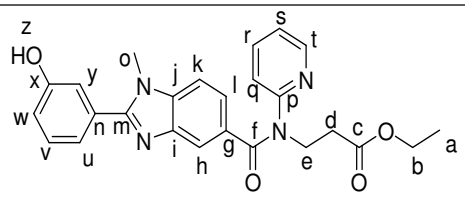
Assignment of ^{13}C NMR:

Sr.No.	Signal (δppm)	Assignment of carbon
1	14.40	a
2	30.15	o
3	33.61	d
4	44.45	e
5	60.40	b
6	107.30	q
7	114.57	s
8	120.36	h
9	120.89	l
10	122.22	k
11	123.16	g
12	134.53	i
13	137.77	j
14	139.86	r
15	148.81	t
16	156.98	p
17	171.09	f
18	171.54	c

2.12.5.4 ^{1}H NMR spectrum of mpd 1


HO
N
N
N
N
O
O


12
10
8
6
4
2
0
ppm
1.00
1.01
1.00
0.98
1.03
1.03
2.98
1.10
2.04
1.97
2.06
2.96
2.09
3.07



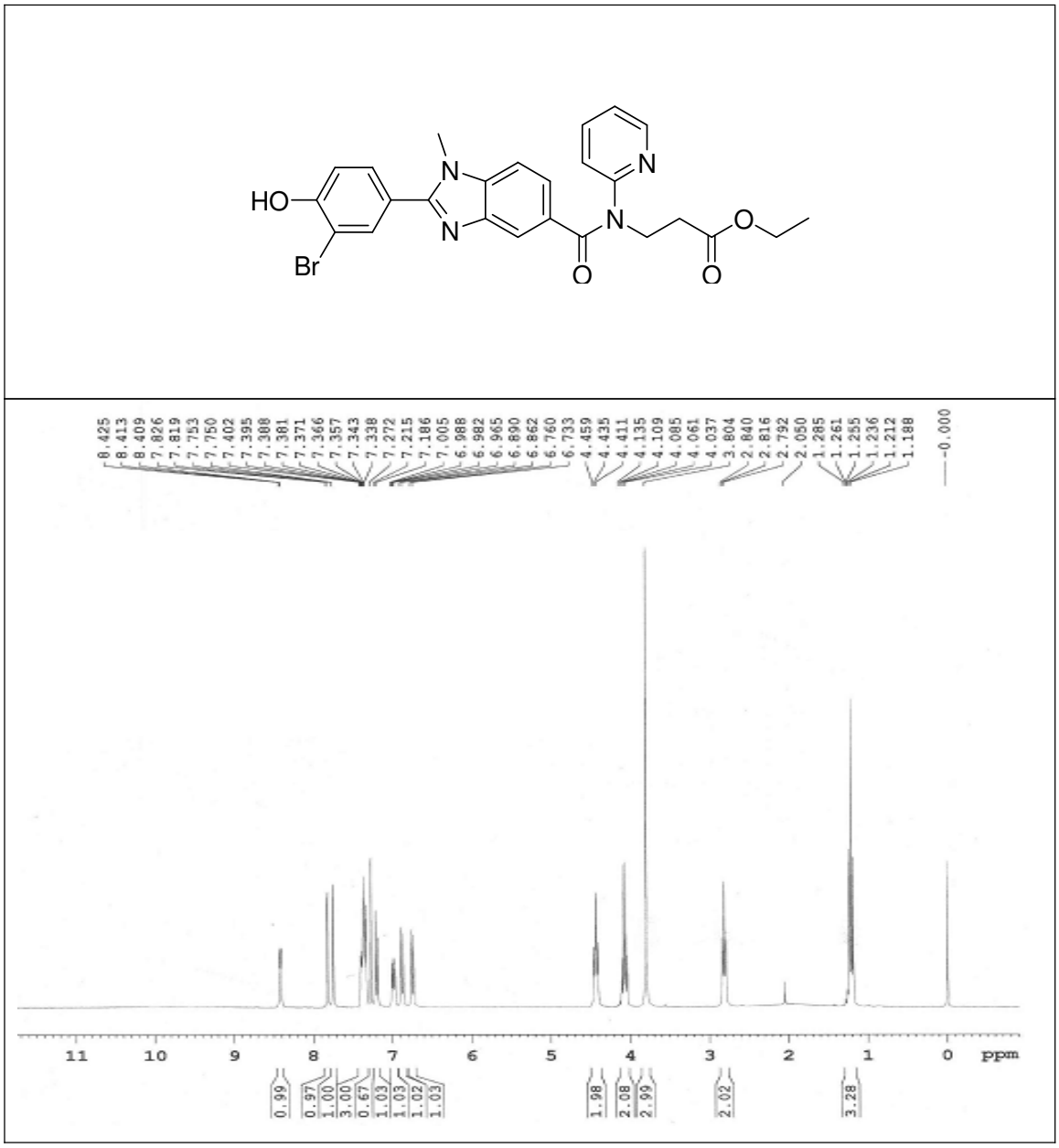
3-{[2-(3-Hydroxy-phenyl)-1-methyl-1H-benzoimidazole-5-carbonyl]-pyridin-2-yl-amino}-propionic acid ethyl ester

mpd 1

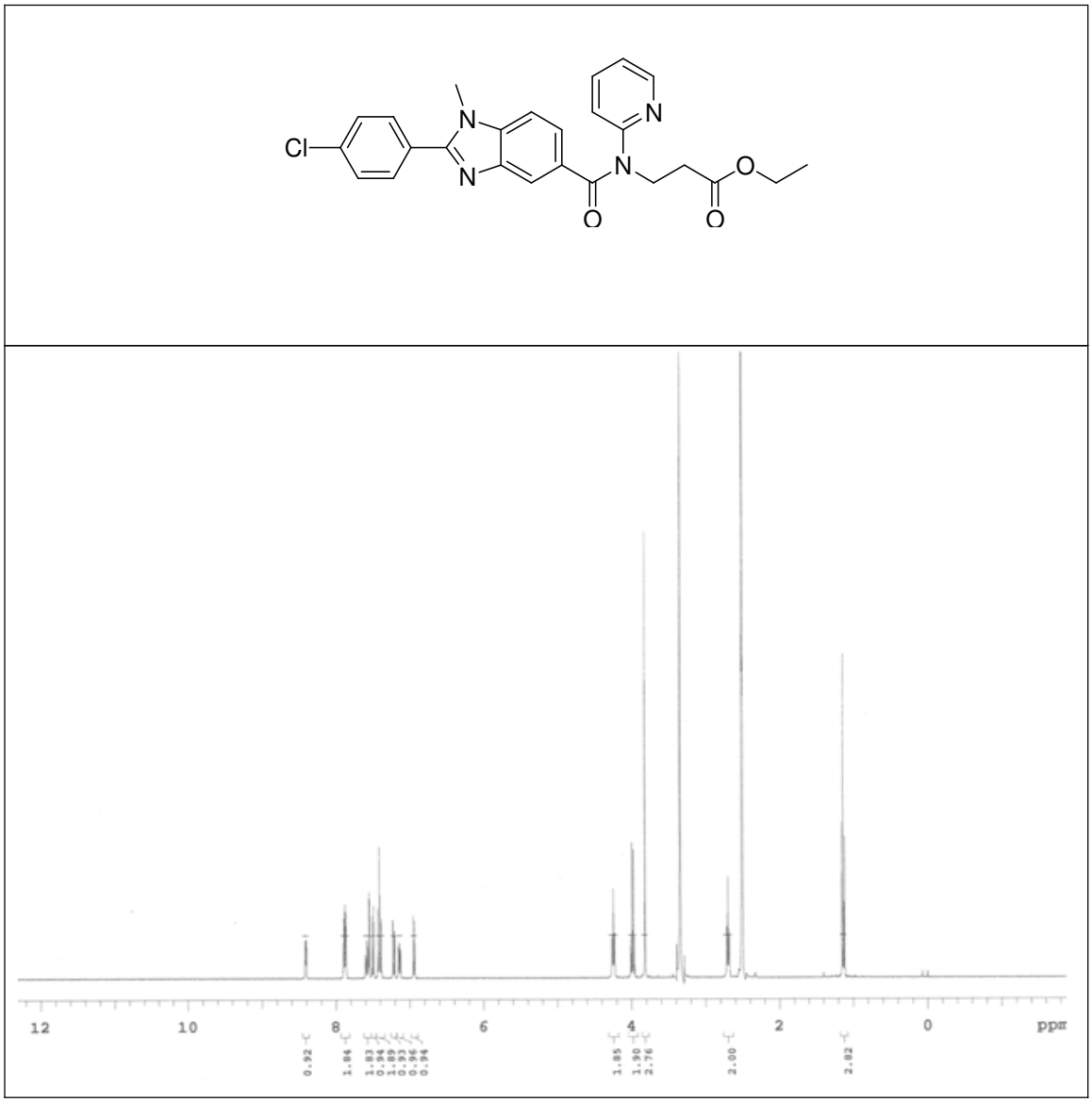
Assignment of ^{1}H NMR of mpd 1:

Sr.No.	Signal (δppm)	No. of Protons	Multiplicity	Assignment
1	1.11-1.15	3H	triplet	a
2	2.68-2.72	2H	triplet	d
3	3.81	3H	singlet	o
4	3.95-4.01	2H	multiplet	b
5	4.22-4.26	2H	triplet	e
6	6.93-8.42	11H	multiplet	Ar-H
7	9.78	1H	singlet	z

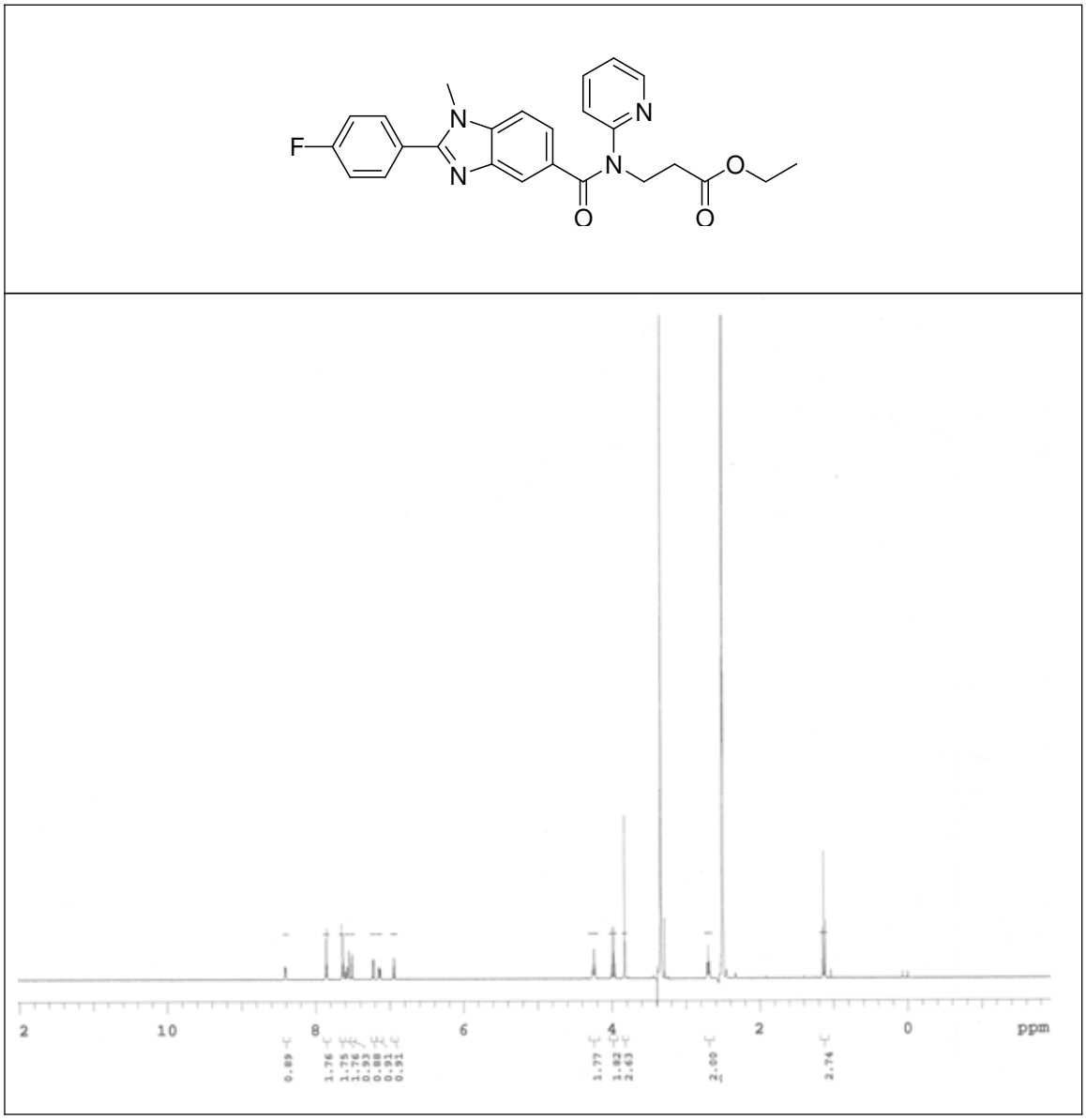
2.12.5.5 ^{1}H NMR spectrum of mpd 2



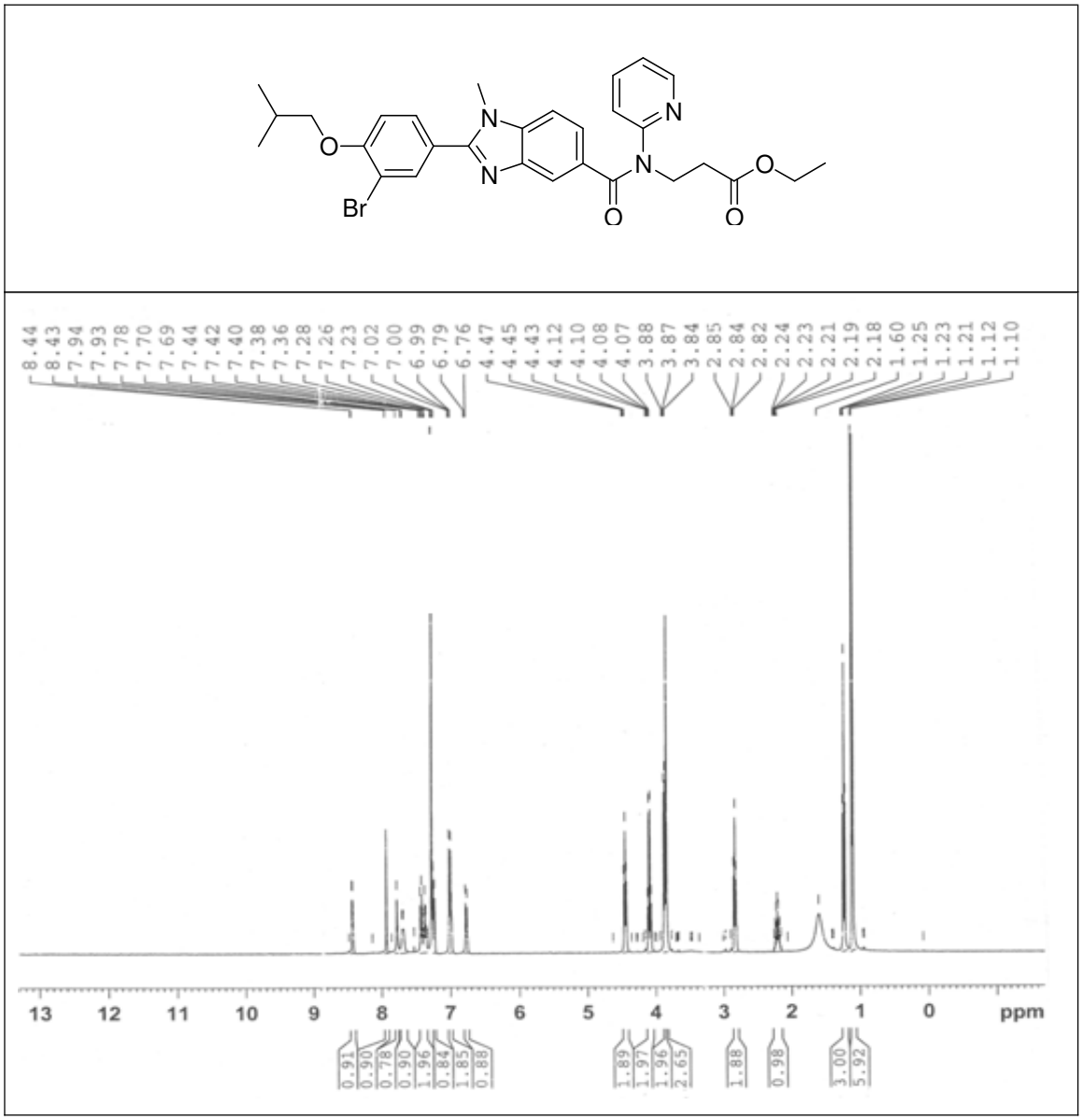
2.12.5.6 ^{1}H NMR spectrum of mpd 3


Cl
N
N
N
N
O
O
O
12
10
8
6
4
2
0
ppm
0.92
1.84
1.83
0.94
1.89
0.92
0.96
0.94
1.85
1.90
2.76
2.00
2.82

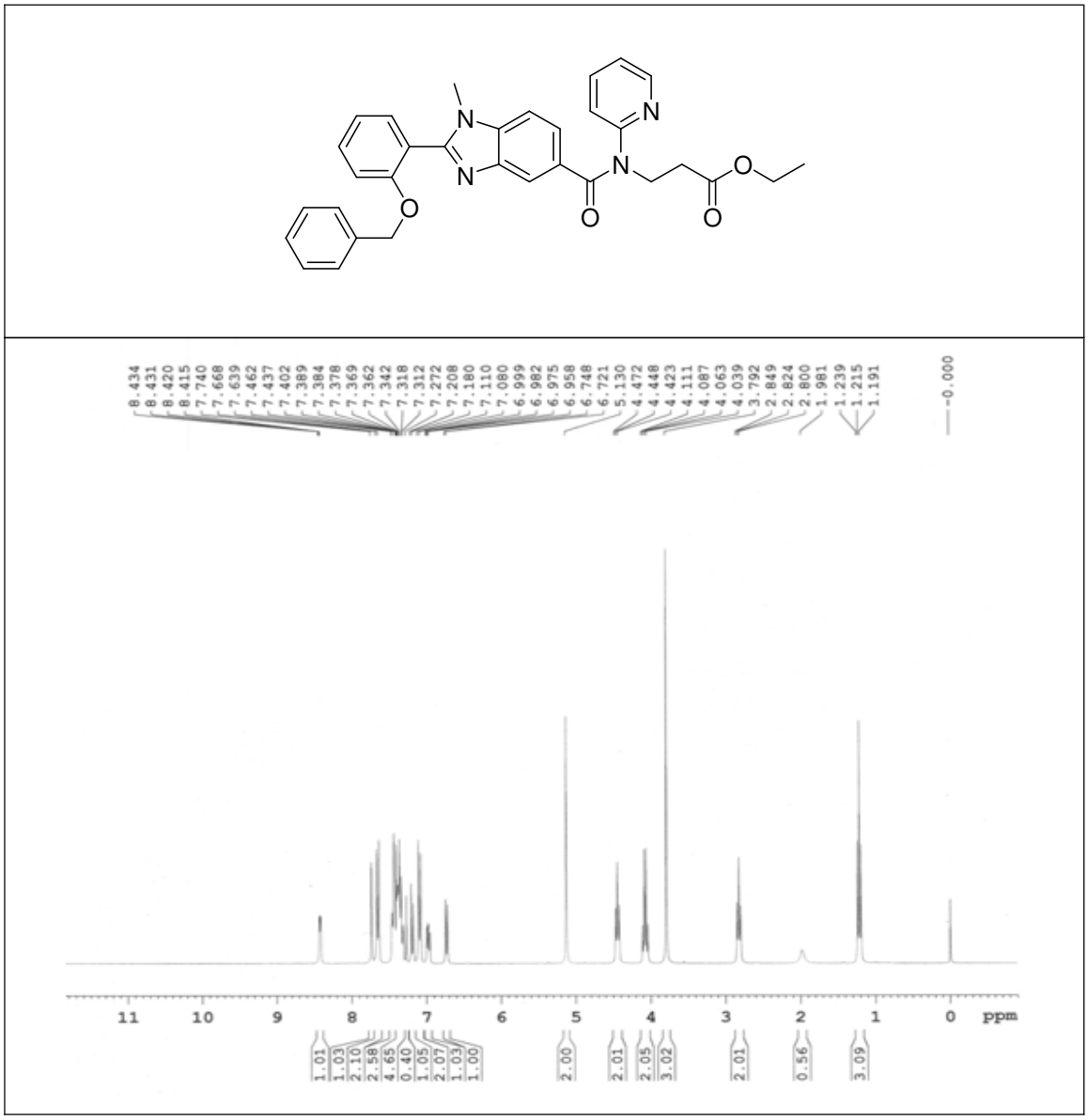
2.12.5.7 ^{1}H NMR spectrum of mpd 4



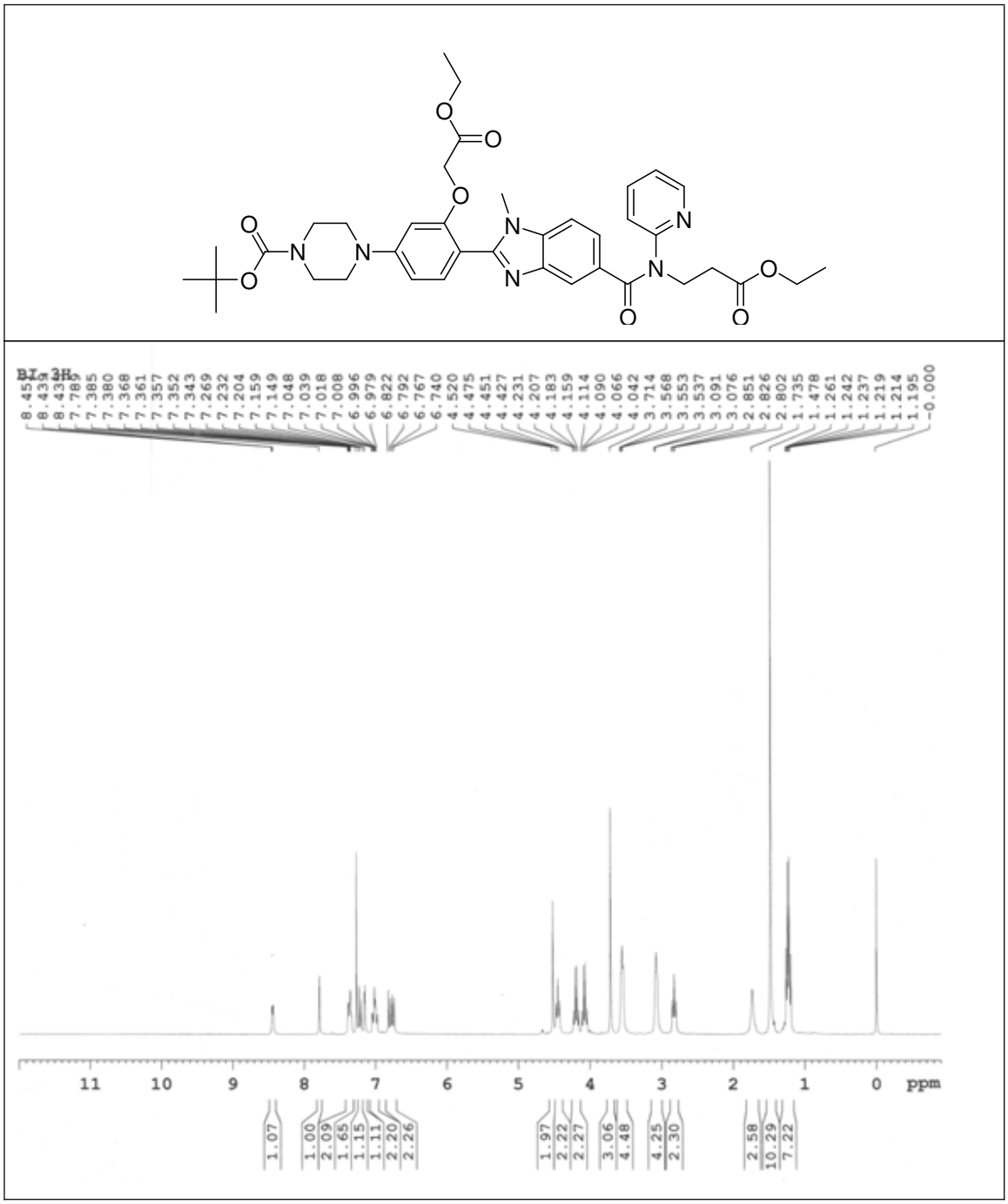
2.12.5.8 ^{1}H NMR spectrum of mpd 6



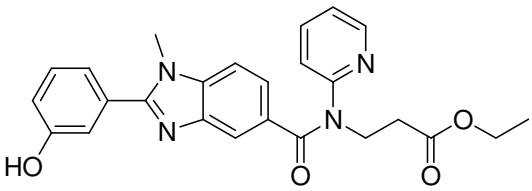
2.12.5.9 ^{1}H NMR spectrum of BI-02



2.12.5.10 ^{1}H NMR spectrum of BI-3

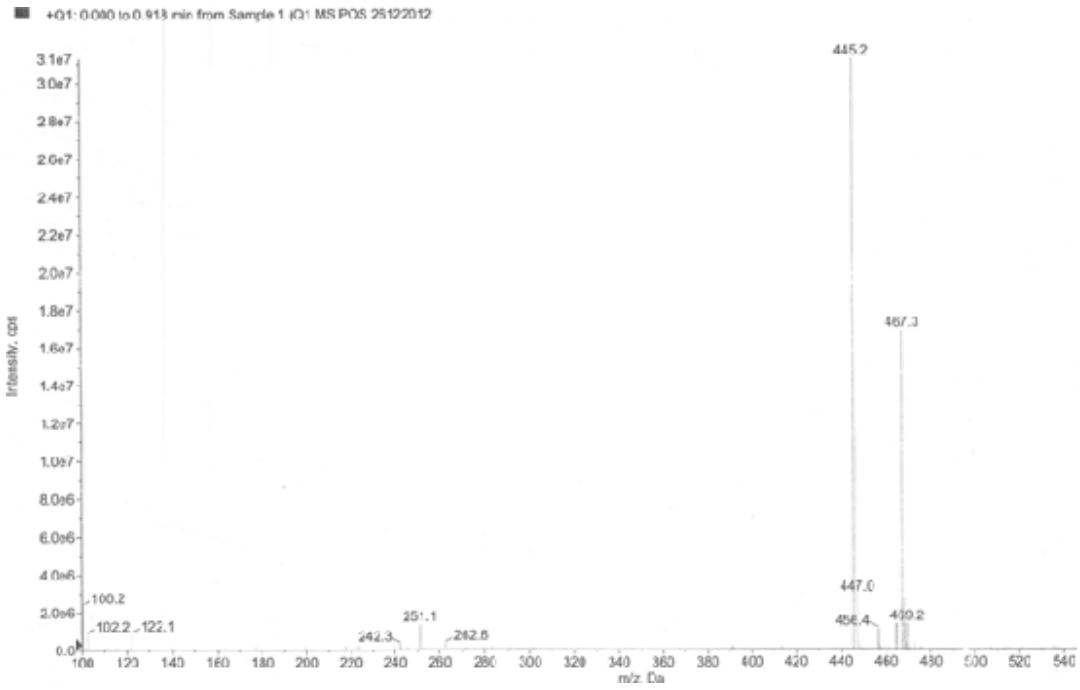


2.12.5.11 Mass spectrum of mpd 1

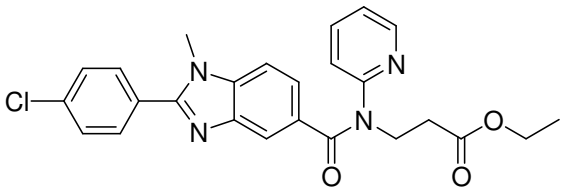


$C_{25}H_{24}N_4O_4$
m/e: 444.18

Mass (m/z): 445.2 (M+1)

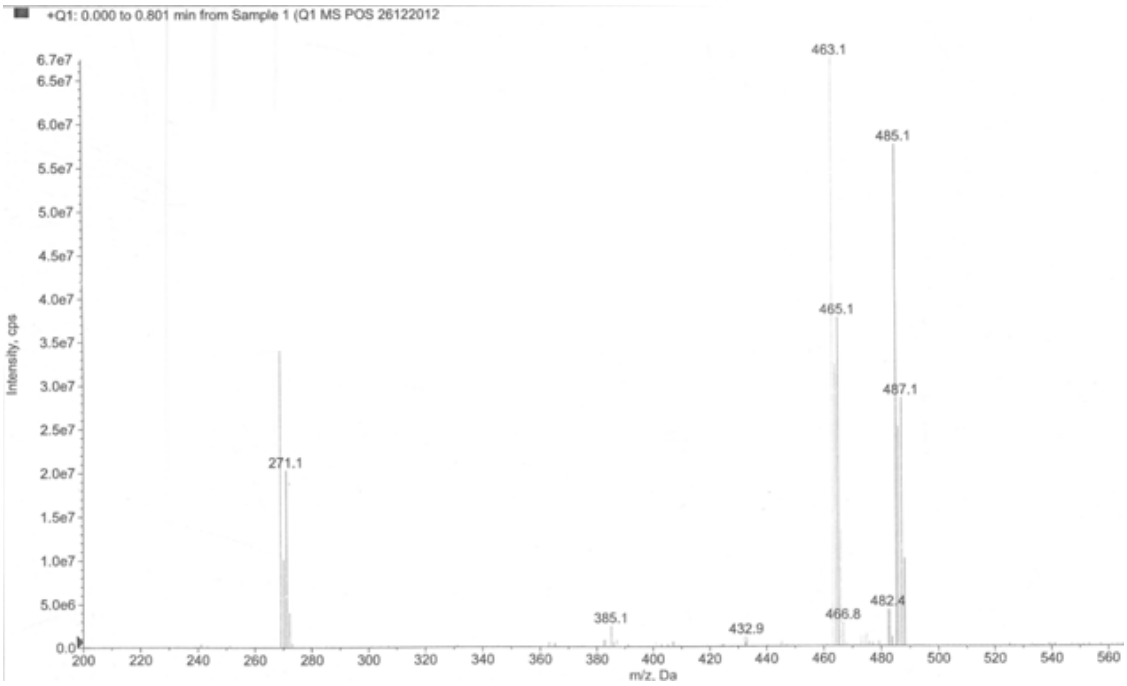


2.12.5.12 Mass spectrum of mpd 3

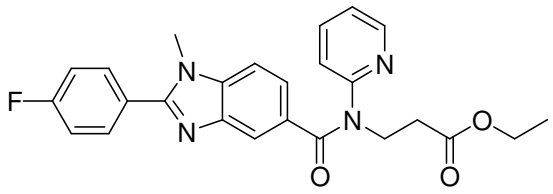


$C_{25}H_{23}ClN_4O_3$
m/e: 462.15

Mass (m/z): 463.1 (M+1) , 465.1 (M+2)

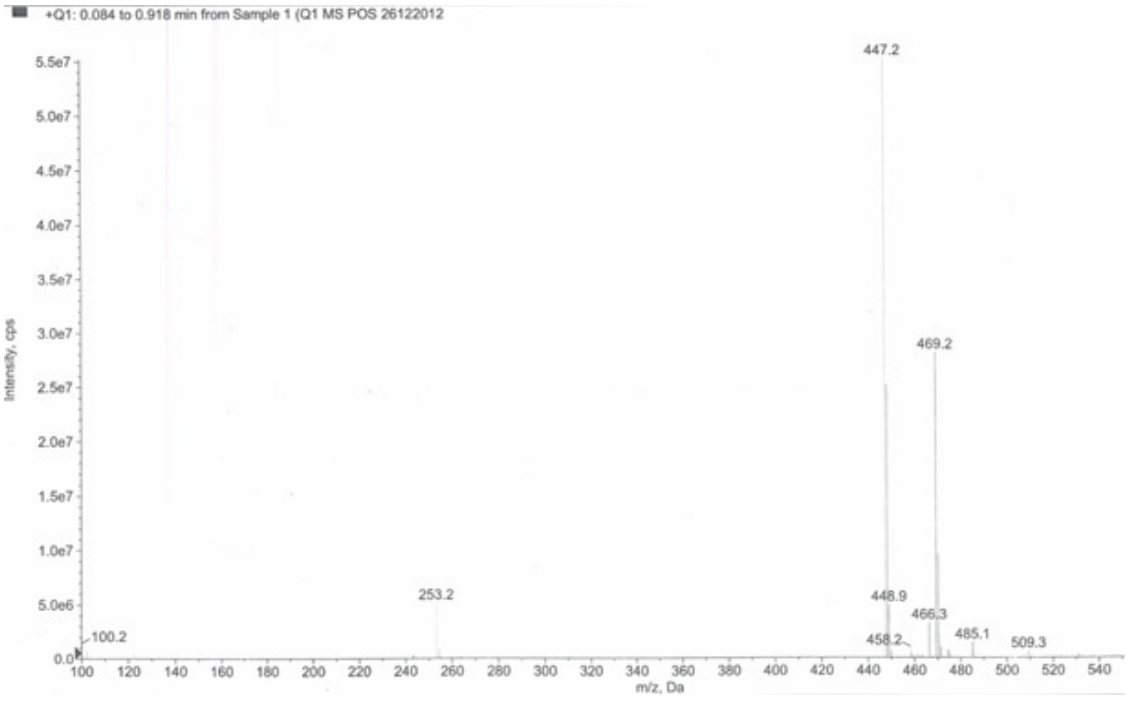


2.12.5.13 Mass spectrum of mpd 4

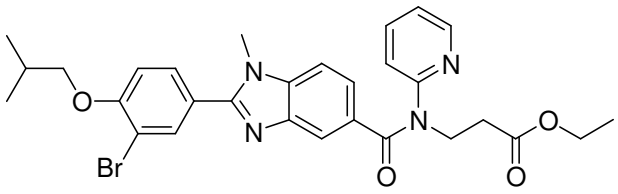


$C_{25}H_{23}FN_4O_3$
m/e: 446.18

Mass (m/z): 447.2 (M+1)

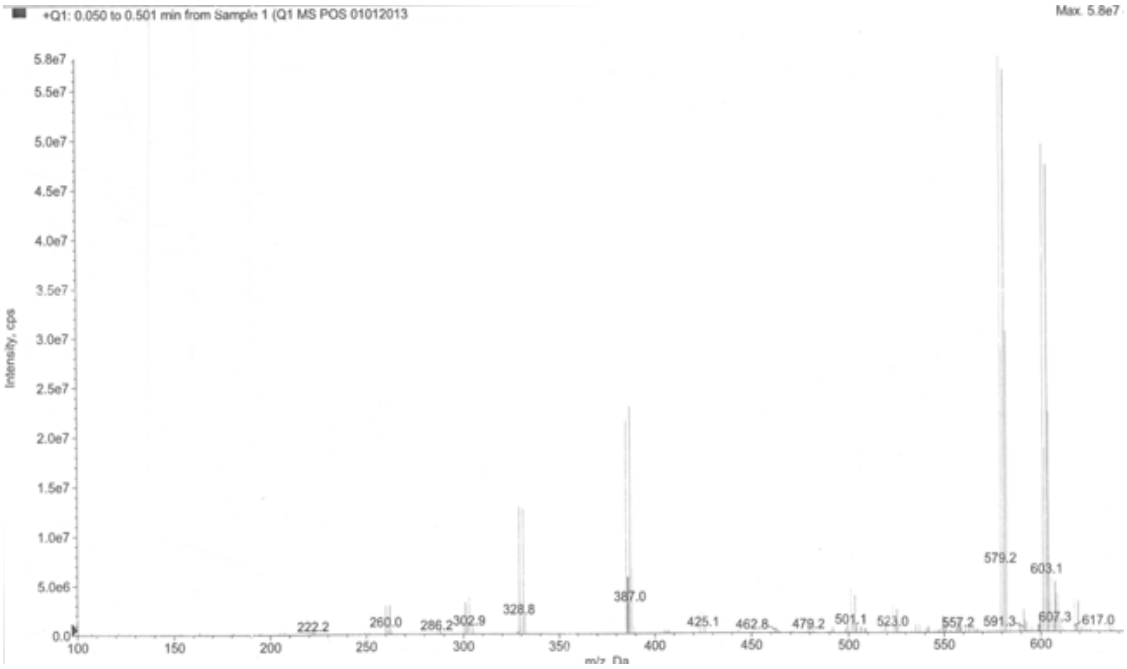


2.12.5.14 Mass spectrum of mpd 6

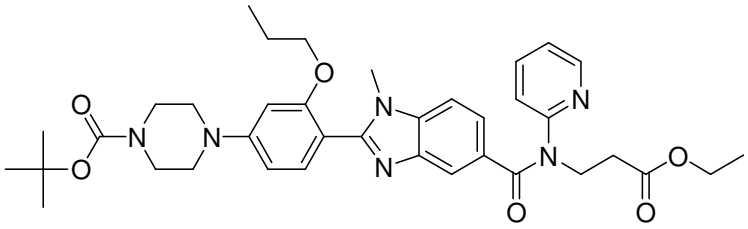


$C_{29}H_{31}BrN_4O_4$
m/e.: 578.15

Mass (m/z): 579.2 (M+1)

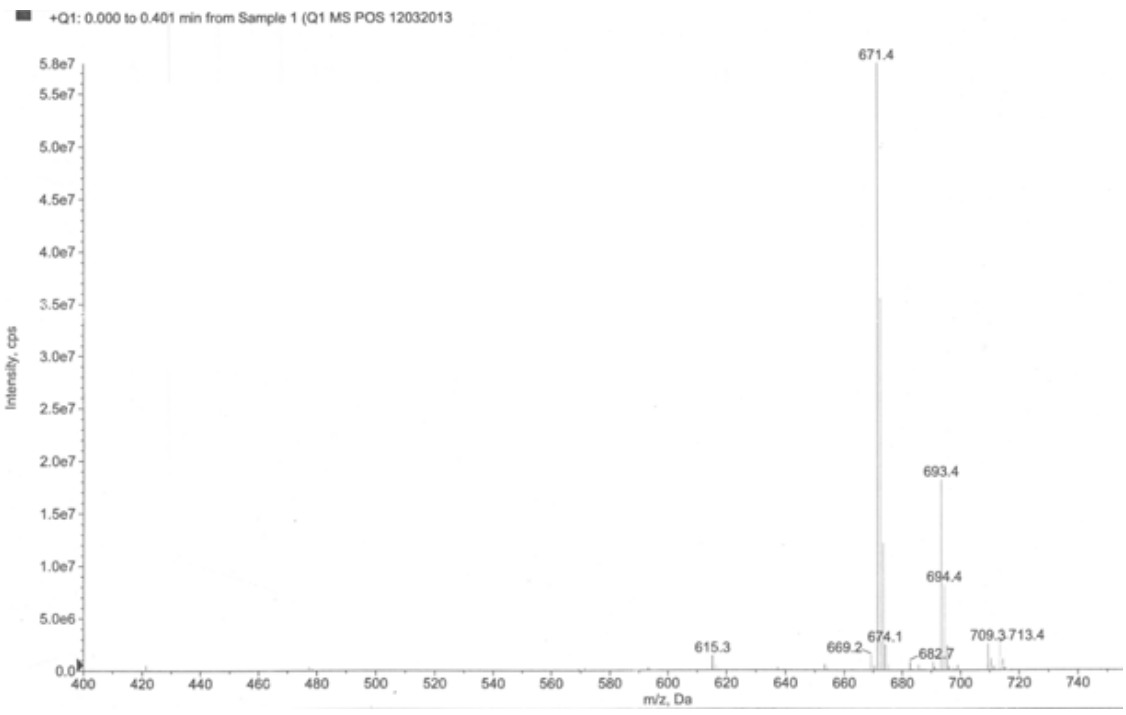


2.12.5.15 Mass spectrum of BI-5

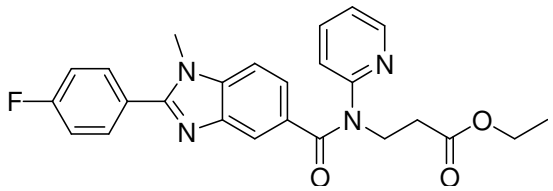


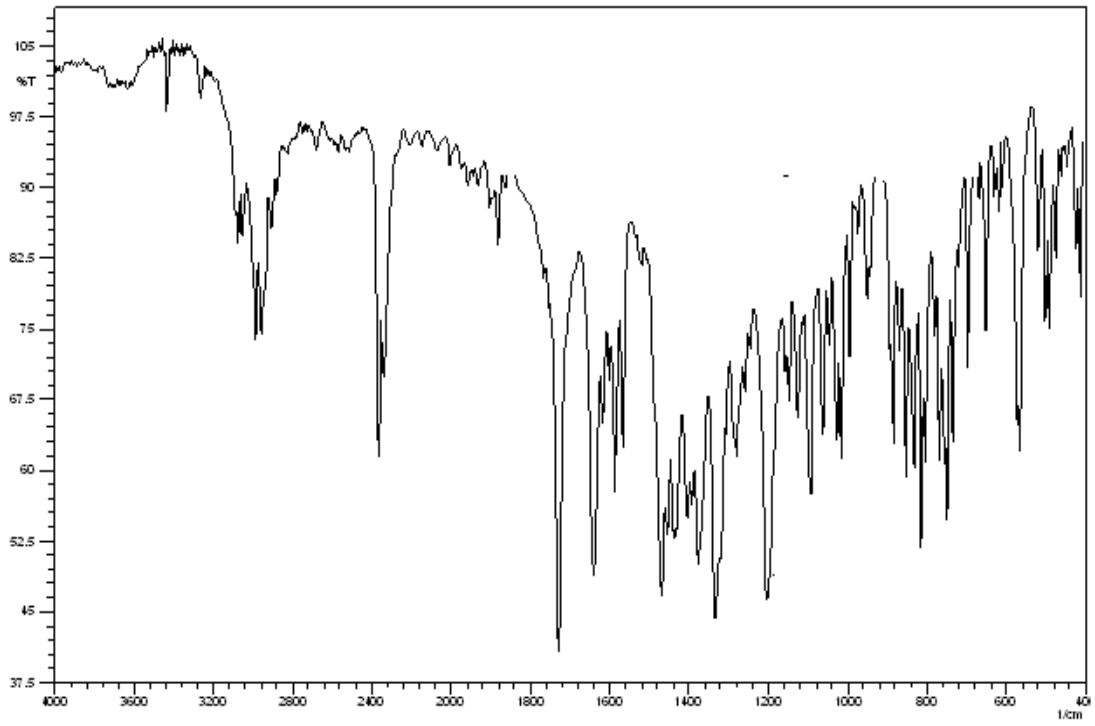
$C_{37}H_{46}N_6O_6$
m/e: 670.35

Mass (m/z): 671.4 (M+1)



2.12.5.16 IR spectrum of mpd 4



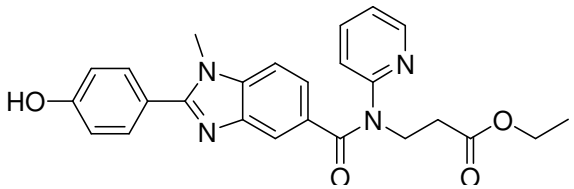


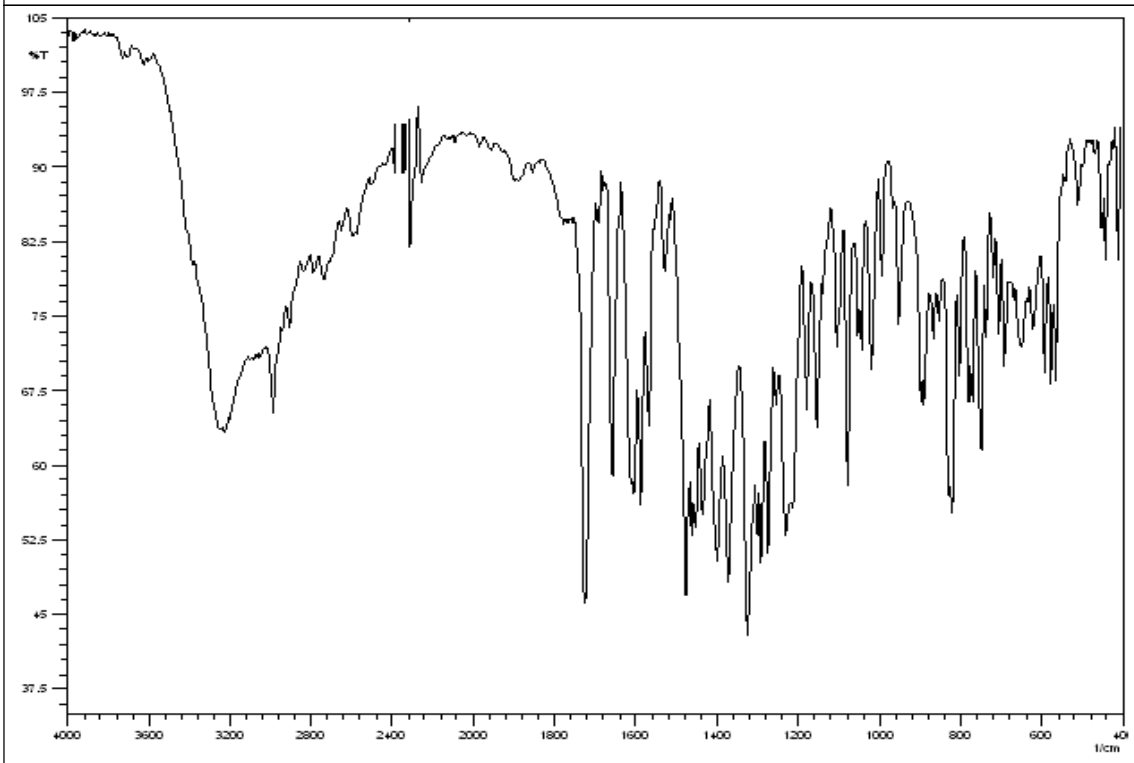
Instrument: SHIMADZU FTIR 8400 spectrophotometer,

Frequence range: 4000-400 cm^{-1} (KBr disc).

IR (KBr, cm^{-1}): 1746 (C=O), 1642 (N-C=O), 1200 (C-F)

2.12.5.17 IR spectrum of mpd 5





Instrument: SHIMADZU FTIR 8400 spectrophotometer,

Frequence range: 4000-400 cm^{-1} (KBr disc).

IR (KBr, cm^{-1}): 3322(OH), 1720 (C=O), 1644 (N-C=O)

2.13 Biological activity

***In-vitro* cytotoxicity screening method**

Cell line

Specific cells that can grow indefinitely given the appropriate medium and conditions i.e. living cells those are maintained *in-vitro* in artificial media of serum and nutrients for the study and growth of certain strains, experiments in controlling diseases, or study of the reaction to certain drugs or agents. Human tumor cell line panels combined with rapid high throughput cytotoxicity testing have proven to be valuable tools for drug screening and early drug evaluation and investigation of drug resistance mechanisms.

The National Cancer Institute (NCI) pioneered the utilization of large human tumor cell line panels for drug screens, after phasing out their previously used animal models. The disease-oriented cell line panel used by NCI consists of 60 different cell lines, which consists of seven sub panels representing common solid tumors, leukemia and lymphomas. To date, more than 100,000 compounds and a large number of natural product extracts have been tested in their short-term growth inhibition assay.[86] Typically, compounds are applied to the cell lines in a wide concentration range, and concentrations that inhibit / kill e.g. 50 % of the cells (GI_{50}/ IC_{50}) are determined. The IC_{50} concentrations for a drug in many cell lines provide a drug specific profile, which can be compared to profiles from other drugs. This approach has successfully been used for drug mechanism classification of standard drugs, and assignment of drug action to investigational drugs and discovery of new classes of chemotherapeutic compounds.

Types of mammalian cell culture

a. Primary Cultures

Primary cultures are derived directly from excised, normal animal tissue and cultured either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. Such cultures are initially heterogeneous but later become dominated by fibroblasts. The preparation of primary cultures is labor intensive and they can be

maintained *in-vitro* only for a limited period of time. During their relatively limited life span primary cells usually retain many of the differentiated characteristics of the cell *in-vivo*.

b. Continuous Cultures

Continuous cultures are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. Cell lines of a finite life are usually diploid and maintain some degree of differentiation. The fact for these types of cell cultures, senescence after approximately thirty cycles of division. Means it is essential to establish a system of Master and Working banks in order to maintain such lines for long periods. Continuous cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumor cells. Tumor cell lines are often derived from actual clinical tumors, but transformation may also be induced using viral oncogenes or by chemical treatments. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original *in-vivo* characteristics.

Isolation of cells

Cells can be isolated from tissues for *ex-vivo* culture in several ways. Cells can be easily purified from blood; however only the white cells are capable of growth in culture. Mononuclear cells can be released from soft tissues by *enzymatic digestion* with enzymes such as collagenase, trypsin, or pronase, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed in growth media, and the cells that grow out are available for culture. This method is known as *explant culture.*

Cells that are cultured directly from a subject are known as *primary cells*. With the exception of some derived from tumors, most primary cell cultures have limited lifespan.

After a certain number of population doublings cells undergo the process of senescence and stop dividing, while generally retaining viability.

An established or immortalized cell line acquire the ability to proliferate indefinitely

either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. There are numerous well established cell lines representative of particular cell types.

Maintenance of cells in culture

Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37°C, 5 % CO_2 and 95 % Relative Humidity) in a cell incubator. Culture conditions vary widely for each cell type and variation of conditions for a particular cell type can result in different phenotypes being expressed.

Aside from temperature and gas mixture, the most commonly varied factor in culture system is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrient components. The growth factors used to supplement media are often derived from animal blood, such as calf serum. These blood-derived ingredients pose the potential for contamination of derived pharmaceutical products with viruses or prions. Current practice is to minimize or eliminate the use of these ingredients where possible**.**

Some cells naturally live without attaching to a surface, such as cells that exist in the blood-stream. Others require a surface, such as most cells derived from solid tissues. Cells grown unattached to a surface are referred to as *suspension cultures* for example, HL60 etc. Other *adherent cultures* cells can be grown on tissue culture plastic, which may be coated with extracellular matrix components (e.g. collagen or fibronectin) to increase its adhesion properties and provide other signals needed for growth. Examples of adherent cell lines are NCI-H23, HEK-293T, MCF-7 etc.

Manipulation of cultured cells

As cells generally continue to divide in culture, they generally grow to fill the available area or volume. This can generate several issues:

- Nutrient depletion in the growth media.

- Accumulation of apoptotic/necrotic (dead) cells.
- Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing known as contact inhibition.
- Cell-to-cell contact can stimulate promiscuous and unwanted cellular differentiation.

These issues can be dealt with using tissue culture methods that rely on sterile technique. These methods aim to avoid contamination with bacteria or yeast that will compete with mammalian cells for nutrients and/or cause cell infection and cell death. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms. Antibiotics can also be added to the growth media.[87, 88] Amongst the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells.

a. Media changes

The purpose of media changes is to replenish nutrients and avoid the build up of potentially harmful metabolic byproducts and dead cells. In the case of suspension cultures, cells can be separated from the media by centrifugation and re-suspended in fresh media. In the case of adherent cultures, the media can be removed directly by aspiration and replaced.

b. Passaging cells

Passaging or sub-culturing cell culture involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached; this was historically done with a mixture of trypsin-EDTA; however other enzyme mixes are now available for this purpose. A small number of detached cells can then be used to seed a new culture.[89]

Description of cell lines used in the cytotoxicity study

Figure 4: MCF-7 cell line

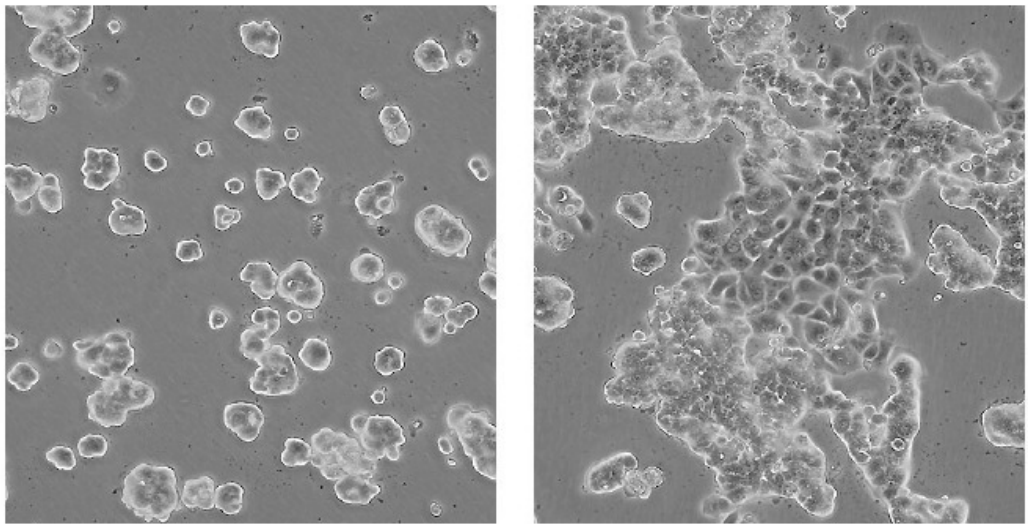


Figure 4: MCF-7 cell line (link 5)

Organism: *Homo sapiens* (human)

Tissue: mammary gland; breast Adenocarcinoma; derived from metastatic site: pleural effusion

Morphology: epithelial

Growth Properties: adherent

Medium

1. Culture medium: Eagle's Minimum Essential Medium, 0.01mg/ml bovine insulin, fetal bovine serum to a final concentration of 10% .
2. Freeze medium: Eagle's Minimum Essential Medium, 0.01mg/ml bovine insulin, fetal bovine serum to a final concentration of 10%, 5% V/V DMSO

The MCF7 line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. Growth of MCF7 cells is inhibited by tumor necrosis factor

alpha (TNF alpha). Secretion of IGFBP's can be modulated by treatment with antiestrogens.(Link 6)

MCF-7 was isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers (Soule, HD *et al.,*1973). Prior to MCF-7, it was not possible for cancer researchers to obtain a mammary cell line that was capable of living longer than a few months (Glodek, Cass., 1990). The patient, whose name is unknown to the vast majority of cancer researchers, died in 1970. Her cells were the source of much of current knowledge about breast cancer (Soule, HD *et al.,* 1973). Her name was Frances Mallon and, at the time of sampling, she was a nun in the convent of the Immaculate Heart of Mary (Monroe, Michigan) under the name of Sister Catherine Frances.

Figure 5: MDA-MB-231

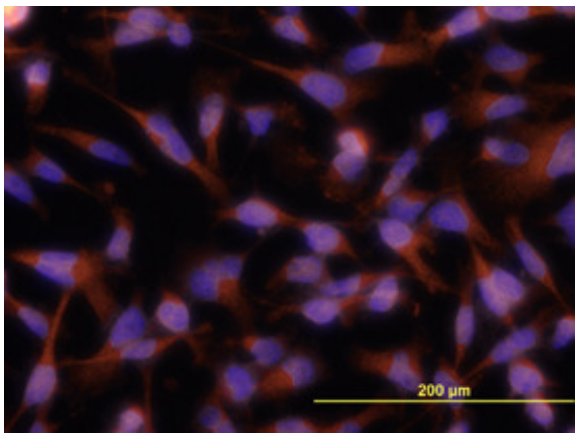


Figure 5: MDA-MB-231 Cells

The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Center. With epithelial-like morphology, the MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells. In vitro, the MDA-MB-231 cell line has an invasive phenotype. It has abundant activity in both the Boyden chamber chemoinvasion and chemotaxis assay. The MDA-MB-231 cell line is also able to grow on agarose, an indicator of transformation and tumorigenicity, and displays a relatively high colony forming efficiency. In vivo, the MDA-MB-231 cells form mammary fat pad tumors in nude mice. IV injection of cells into the tail vein of nude mice has been shown to produce experimental metastasis.

Types of *In-vitro* cytotoxicity assays

To study cytotoxic activity of a compound, cytotoxic assays are carried out. It is now well-documented that apoptosis or Programmed cell death is the key mechanism by which chemotherapeutic agents exert their cytotoxicity.

These assays are principally of two types:

I. **Radioactive and non-radioactive assays** that measure increases in plasma membrane permeability, since dying cells become leaky.

II. **Colorimetric assays** that measure reduction in the metabolic activity of mitochondria; mitochondria in dead cells cannot metabolize dyes, while mitochondria in live cells can metabolize it and so are distinguished.

Depending on the knowledge of physiological events occurring in cell cycle and death, assay type is chosen and used. A number of methods have now been developed to study apoptosis in cell populations.

Cytotoxicity tests measure the concentration of the substance that damages components, structures or cellular biochemical pathways, and they also allow direct extrapolation of quantitative data to similar *in-vivo* situations. This refers to the *in-vitro* assessment of material to determine whether or not it releases toxic chemicals in sufficient quantities to kill cells either directly or indirectly through the inhibition of cell metabolic pathways.

a. Common Basic Steps of *in-vitro* Assays

Although the techniques for testing drug sensitivities of tumor cells differ, each employ four common basic steps:

i. Isolation of cells,

ii. Incubation of cells with drugs,

iii. Assessment of cell survival, and

iv. Interpretation of the result. (Brown and Markman, 1996)

b. Ideal characteristics of *in-vitro* methods

- An ideal *in-vitro* screening method should be simple economical, reproducible, rapid and sensitive.
- The assay should be applicable to large number of tumor types and test compounds.
- The choice of cell lines should be representative of clinical situation as close as possible.
- The range of drug concentration used *in-vitro* should be comparable to that expected for *in-vivo* treatments.
- The assay should be able to process a large number of samples quickly and in automated fashion.
- Data acquisition should be simple, easily interpreted and applied.

c. Advantages of *in-vitro* methods:

The development of *in-vitro* cytotoxicity assays has been driven by the need

- To rapidly evaluate the potential toxicity of large numbers of compounds,
- To limit animal experimentation whenever possible, and
- To carry out tests with small quantities of compound.
- Most cost effective and easier to manage.

The most promising advantage of *in-vitro* methods over *in-vivo* method is, here culture can be cultivated under a controlled environment (pH, temperature, humidity, oxygen carbon-dioxide balance etc.) resulting in homogeneous batches of cells and thus minimizing experimental errors.

d. Limitation of *in-vitro* methods:

- They often furnish false positive results (compounds show no activity *in-vivo*) and false negative results (compounds show no activity *in-vitro* but show activity *in-vivo* as they need to be bio-transformed *in-vivo* to pharmacologically active compounds).

- A second pitfall is that role of pharmacokinetic in determining drug effects cannot be evaluated *in-vitro*.
- Geometry of solid tumors *in-vivo* is very different from that of cells growing *in-vitro* in suspension or monolayer culture.

Table 4: *In-vitro* cytotoxicity assays and theirs principle

Sr. no.	**Category of viability assay**	**Type of assay**	**Principles**
1.	Membrane integrity assay	1. Trypan blue dye exclusion assay 2. Fluorescent dyes assay 3. LDH leakage assay	The determination of membrane integrity via dye exclusion from live cells
2.	Functional assay	1. MTT, XTT assay 2. Crystal violet /Acid phosphatase (AP) assay 3. Alamar Blue oxidation reduction assay 4. Neutral red assay 5. [3H]-thymidin / BrdU Incorporation	Examining metabolic components that are necessary for cell growth.

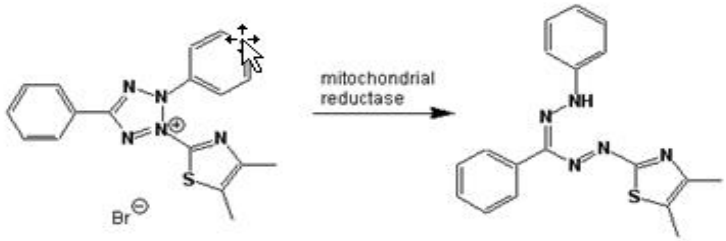
3.	Protein assay	1. SRB assay	Based on measurement of total protein content.
4.	DNA labeling assay	1. Fluorescent conjugates	Simultaneous cell selection and viability assay
5.	Morphological Assay	1. Microscopic observation	Determination of morphological change
6.	Reproductive assay	1. Colony formation assay	Determination of growth rate

MTT assay

It is a laboratory test and a standard colorimetric assay for measuring cellular growth. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials.

This assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in range of cell lines.[90]

Figure 6: Principle of MTT



MTT | formazan

Figure 6: Principle of MTT assay (link 27)

a. Applications

MTT used for the non-radioactive, spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well-plate format. It can be used for:

- Measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients.
- Analysis of cytotoxic and cytostatic compounds, such as anti-cancer drugs and other pharmaceutical compounds.
- Assessment of growth-inhibitory antibodies and physiological mediators.

b. Advantages

- Rapid, versatile, quantitative and highly reproducible
- Adaptable to large-scale screening; relevant for most cells
- MTT reduction correlates to indices of cellular protein and earlier cell number
- More sensitive and earlier predictor of toxicity than classical LDH or neutral red measurements

c. Disadvantages

- Production of the MTT product is dependent on the MTT concentration in the medium. The kinetics and degree of saturation are dependent on cell type.
- Assay is less effective in the absence of cell proliferation.
- MTT cannot distinguish between cytostatic and cytocidal effect.
- Individual cell numbers are not quantitated and results are expressed as a percentage of control absorbance.
- Test is less effective if cells have been cultured in the same media that has supported growth for a few days, which leads to under estimation of control and untreated samples.[91]

To summarize, the design of a screening assay is an array of multiple choices, all of which have significant impacts on the outcome of the overall drug discovery process. Most importantly, the correct selection of the target and assay format, detailed optimization and miniaturization as well as the choice of appropriate detection technology for each individual assay can lead to savings in time, money and labor along with improved data quality in all stages of the drug discovery process.

The following materials were procured for the project work and were maintained at appropriate temperature as recommended by the manufacturer.

Material

a. Reagents

1. Trypan blue Dye (Hyclone, Lot No: 029K2358, 100 ml)
2. Triton X100 (MP Biomedicals, Lot No: 8009H, 100 ml)
3. DMSO cell culture grade (Bioworld, Lot No: 1388B230, 500 ml)
4. Sodium bicarbonate (Bioworld, Lot No: 1775B29)
5. Amphotericin B (Himedia, Lot No: 1397893, 100 ml)
6. Penicillin and Streptomycin solution stabilized (Sigma, Lot No: 1208029, 100

ml)

7. EDTA (MP Biomedicals, Lot No: YY02022B207Y)

8. DPBS / modified 1X (Dulbecoo's phosphate buffer saline without Ca^+ and Mg^+) (Himedia, Lot No: LW537, 100 ml)

9. Trypsin 1X Gamma irradiated (SAFC Bioscience, Lot No: 8NO535, 500 ml)

10. Methotrexate (MP Biomedicals, Cat no. 102299, Lot no. R27204)

11. Triton – X 100 (Bioworld, Cat no. 730208, Lot no. 1 8278075)

12. Iso Propanol (Finar Chemicals, Cat no. 11390, Lot no. 19075330)

b. Media:

1. DMEM (Dulbecoos Modified Eagels medium, low glucose with glutamine) (MP Biomedical, Lot No: C1478),

2. FBS (Fetal Bovine Serum, South American origin, 500 ml) (Quaditive, Lot No: 103128, 500 ml),

3. Tryptone Soya broth (TSB) (Himedia, Lot No: YH031).

c. Glass wares and plastic wares

1. 96-well microtiter plate (Flat Bottom, U Bottom, V Bottom),

2. Tissue culture flasks (75 cm^2 T Flask vented and 150 cm^2 T Flask vented),

3. Falcon tubes (15 ml, 50 ml), Cryotubes (2ml), Cell scrapper,

5. Reagent bottles (100 ml, 250 ml, 500 ml, 1000 ml),

6. Haemocytometer cell counting chamber.

d. Equipments

1. Fluorescence inverted microscope (Leica DM IL, Germany),

2. Biosafety cabinet class-II (Esco, Singapore),

3. Cytotoxic safety cabinet (Esco, Singapore),

4. CO_2 incubator (RS Biotech, mini galaxy A, Scotland),

5. Deep freezer (Dairei, Denmark),
6. ELISA plate reader (Thermo, USA),
7. Micropipettes (Eppendorff, Germany),
8. RO water system (Millipore, USA),
9. Electronic water bath (Genei, India).

e. Cell proliferation kit/reagent

1. MTT Dye Powder

Methods

Preparation of compound dilution:

a. Preparation of Stock solution of Test compounds:

Stock solution has been prepared as per the standard procedure.

b. Dilution of Test Compounds:

10 µl of 10 mg conc. of test compound was added in to 900 µl of complete media and as a result 100 µg conc. of test sample was obtained.

Than 1:2 dilution of test sample was done as shown in Table. It was done by mixing 50 µl of test compound with 100 µl of complete media. For this, initially 100 µl of complete media was added in to well no. 1 – 9. Well 10 contained 150 µl test substance only, from that 50 µl was pipette out and added in to well no. 9 which already contain 100 µl of complete media, which lead to 1:2 dilution of test sample. Same procedure was repeated 9 times in order to get final conc. of test Sample as per test design.

Figure 7: MDA-MB-231 cells

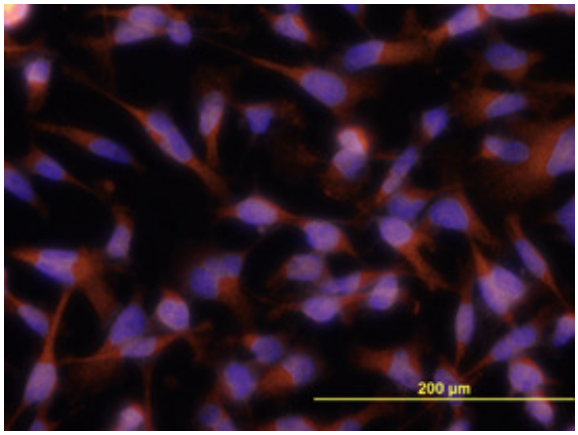
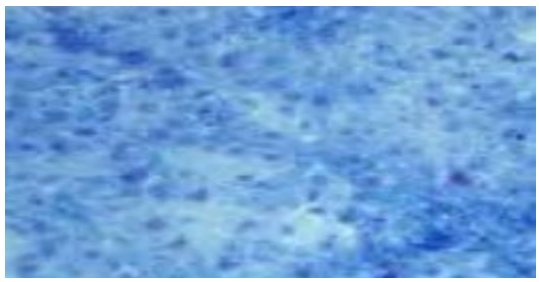


Figure 7: MDA-MB-231 Cells

The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Center. With epithelial-like morphology, the MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells. In vitro, the MDA-MB-231 cell line has an invasive phenotype. It has abundant activity in both the Boyden chamber chemoinvasion and chemotaxis assay. The MDA-MB-231 cell line is also able to grow on agarose, an indicator of transformation and tumorigenicity, and displays a relatively high colony forming efficiency. *In vivo*, the MDA-MB-231 cells form mammary fat pad tumors in nude mice. IV injection of cells into the tail vein of nude mice has been shown to produce experimental metastasis.

Vero

The *Vero* lineage was isolated from kidney epithelial cells extracted from an African green monkey (*Cercopithecus aethiops*). The Vero cell lineage is continuous and aneuploid. It is highly sensitive normal cells line.



Vero Cells

Preparation of compound dilution

Stock solutions of compounds were prepared in 2% DMSO at concentration of 100µM and culture medium with various concentrations of samples was used in the assay. The final concentration of DMSO (2%) used does not interfere cell viability

Compounds	**MW**	**1M=xmg/ml**	**100 µM=xmg/ml**
MPD-1	444.48	444.48	0.044
MPD-2	523.98	523.98	0.052
MPD-3	462.93	462.93	0.046
MPD-4	446.47	446.47	0.044
MPD-5	448.48	448.48	0.044
MPD-5-ISO	500.59	500.59	0.050
MPD-6	579.48	579.48	0.058
MPD-7	472.48	472.48	0.047
MPD-9	486.59	486.59	0.049
BI-02	534.61	534.61	0.053
Methotrexate	454.44	454.44	0.045

Table 5: (1:3) dilution of test compound used in the assay

Well no. 1-9 contain complete media 100µl										
Well No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Compound dilution	50 µl mixture	50 µl mixture	50 µl mixture	50 µl mixture	50 µl mixture	50 µl mixture	50 µl mixture	50 µl mixture	50 µl T.C	150 µl

	from well 2	from well 3	from well 4	from well 5	from well 6	from well 7	from well 8	from well 9	. from well 10	T.C.
Final con. (µM)	0.005	0.015	0.045	0.13	0.41	1.23	3.7	11.1	33.3	100

Where, T.C.= Test compound

PLATE ASSIGNMENT

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	Sample 1
B	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	
C	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	Sample 2
D	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	
E	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	Sample 3
F	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	
G	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	Sample 4
H	0.005 µg/ml	0.01 µg/ml	0.04 µg/ml	0.13 µg/ml	0.41 µg/ml	1.23 µg/ml	3.7 µg/ml	11.11 µg/ml	33.33 µg/ml	100 µg/ml	NC	PC	

Where, NC-Negative control-Only Media

PC-Positive control-Media+Cell+DMSO (without test compound)

100 µM of 1mM conc. of test compounds was added in to 900 µl of culture media and as a result 100 µM conc. of test compound was obtained.

Than 1:3 dilution of test compound was done as shown in table. It was done by mixing 50 µl of test compound with 100 µl of complete media. For this initially 100 µl of complete

media was added into well no. 1-9. Well no. 10 contained 150 µl test substance only, from that 50 µl was pipette out and added into well 9 which already contain 100 µl of complete midia, which lead to 1:3 dilution of test compound. Same procedure was repeated 9 times in order to get final conc. of test compounds upto 0.005 µM.

Reference substances:

Methotrexate, a cytotoxic anticancer substance used in antineoplastic therapy also allowing us to classify the thiazole derivatives according to their relative toxicity. MW of doxorubicin is 454.44 stock solution of it was prepared with 1 ml of DMSO, which produces stock solution of doxorubicin of 10mM conc. Stock solution was further diluted for 10 times using DMSO to obtain 1mM solution.

Experimental setup

Cell Lines and Culture Medium:

MDA-MB-231 and Vero cell cultures were used in these experiments were derived from National Centre for Cell Science (NCCS), Pune. Stock cells of these cell line was cultured in DMEM, supplemented with 10% FBS (fetal bovine serum). Along with media cells were also supplemented with 5 % HBSS, penicillin, streptomycin and Amphotericin – B, in a humidified atmosphere of 5 % CO_2 at 37 °C until confluence reached. The cells were dissociated with 0.2 % trypsin, 0.02 % EDTA in phosphate buffer saline solution. The stock cultures were grown initially in 25 cm^2 tissue culture flasks, than in 75 cm^2 and finally in 150 cm^2 tissue culture flask and all cytotoxicity experiments were carried out in 96 microtitre well- plates. 2×10^4 cells/well was added in to each well of 96 well-plates. It was calculated as follow.

Calculation for number of cells in 96 well plates:

For this we need to calculate for no. of cells required for 100 wells ≈ 96 well,

No. of cells / well × 100

$= 2 \times 10^4 \times 100$

$= 2 \times 10^6$ cells / plate

Total volume of media for 100 wells

= volume of media / well × 100

= 100 µl × 100

= 10 ml

Therefore, we need a total of 2×10^6 cells in 10 ml of medium, then aliquot the required volume of cell suspension in to each wells.

Design of experiment:

Cell lines in exponential growth phase were washed, trypsinized and re-suspended in complete culture media. Cells were seeded at 2×10^4 cells / well in 96 well microtitre plate and incubated for 24 hrs during which a partial monolayer forms. The cells were then exposed to various concentrations of the test compounds (as indicated in plate assignment) and standard doxorubicin. Control wells were received only maintenance medium. The plates were incubated at 37 °C in a humidified incubator with 5 % CO_2, 75 % Relative Humidity for a period of 24 hrs. Morphological changes of drug treated cells were examined using an inverted microscope at different time intervals and compared with the cells serving as control. At the end of 24 hrs, cellular viability was determined using MTT assay.

Screening of test compound by MTT assay

Protocol

- Cells were preincubated at a concentration of $1 \times 1\ 0^6$ cells / ml in culture medium for 3 hrs at 37°C and 6.5 % CO_2, 75 % Relative Humidity.
- Cells were seeded at a concentration of 5×10^4 cells / well in 100 µl culture medium and various amounts of compound (final concentration e.g. 100 µM/ml – 0.05 µM/ml) were added into microplates (tissue culture grade, 96 wells, flat bottom).
- Cell cultures were incubated for 24 hrs at 37 °C and 6.5% CO_2.
- 10 µl MTT labeling mixture was added and incubate for 4 hrs at 37 °C and 6.5 % CO_2, 75 % Relative Humidity.
- 100 µl of solubilization solution was added to each well and incubate for overnight.
- Absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 540 and 600 nm according to the filters available for the ELISA reader, used. (The reference

wavelength should be more than 650 nm).

Data Interpretation

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

After 24 hrs, the cytotoxicity data was evaluated by determining absorbance and calculating the correspondent chemical concentrations. Linear regression analysis with 95 % confidence limit and R^2 were used to define dose-response curves and to compute the concentration of chemical agents needed to reduce absorbance of the formazan by 50 % (IC_{50}).

Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

$$\textbf{\% Viability} = (A_T - A_B) / (A_C - A_B) \times 100 \ldots \ldots \ldots (1)$$

Where,

A_T = Absorbance of treated cells (drug)

A_B = Absorbance of blank (only media)

A_C = Absorbance of control (untreated)

There by,

$$\textbf{Cytotoxicity} = 100 - \textbf{\% cell survival} \ldots \ldots \ldots (2)$$

Determination of IC_{50} value

According to the FDA, IC_{50} represents the concentration of a drug that is required for 50 % inhibition *in-vitro*. In our study, IC_{50} is a concentration of drug at which 50 % of cell population die.

For primary screening, we use a threshold of 50 % cell growth inhibition as a cut off for compound toxicity against cell lines. IC_{50} determined from plot of Dose Response curve

between log of compound concentration and percentage growth inhibition. IC_{50} value has been derived using curve fitting methods with *GraphPad Prism* as statically software (Ver. 5.02).

IC_{50} values were calculated using the nonlinear regression program Origin The average of two (duplicates manner) were taken in determination.

Graph was plotted by keeping log concentration of drug on X axis and % cell growth inhibition or % cytotoxicity Y axis. IC_{50} was estimated as a concentration of drug at 50 % position on Y axis.

The relationship should be sigmoidal, log drug concentration on the X axis and 'response / measurement' of the Y axis. The prism web site has some good guides for this.

Result and discussion

Characterization of cell lines and culture media

Characterization of cell lines was performed for detection of microbial and cross contamination. Cell lines used in our experiments were free from any kind of microbial or fungal contamination (Table No. 6), which in essential in order to continue our screening experiments.

Table 6: Results for Characterization of cell lines

cell line	**%Viability**		**PDT(hrs)**	**Microbial contamination**	**Cross contamination**	**pH**
	stock	**after**				
MDA-MB-231	31.05	79.35	34.72	No Contamination	No	7.5
Vero	62.16	86.20	21.50	No Contamination	No	7.1

Culture media were also tested for microbial contaminations. To prevent microbial contamination, 2.5 % Amphotericin B (µg/ml) was supplemented to media which act as working concentration. Bacterial contamination was prevented by addition of 1 % of Antibiotic, 100 X (10000 U/ml Penicillin G, 10000 µ g/ml Streptomycin) into culture medial. All subculturing activities were done under class – II Biosafety cabinet. (Esco, Singapore)

Cross contamination of cell line was tested by direct observation of particular cell line under inverted microscope. PDT for specific cell line was determined. From viability studies and PDT, we have concluded that the cell lines derived from ATCC were initially free from cross contamination.

To prevent the cross contamination of cell lines during our experiments work, separate pipettes and plastic tips were used for individual cell line. Along with that, particular cell line was used at the time under Class – II Bio safety cabinet. These were proving to be valid steps to prevent cross contamination of cell lines throughout the experiment.

RESULTS OF CYTOTOXICITY STUDY

MDA MB 231 Cell line [Human Breast cancer]

Table 7: % Cell inhibition of MDA-MB-231 cell by tests compounds at different concentration

Conc. µg/ml	**Log con.**	**% Cell Inhibition**						
		MPD1	**MPD2**	**MPD3**	**MPD4**	**MPD5**	**MPD5-Isobutyl**	**Std.**
0.01	-2.29	-0.10	0.0	0.35	0.30	-0.38	0.10	-0.10
0.02	-1.82	0.73	-0.31	0.89	0.99	0.42	0.02	0.53
0.05	-1.34	1.32	0.89	2.39	0.35	0.85	0.78	1.55
0.14	-0.86	4.75	1.70	1.75	1.85	2.35	0.96	4.45
0.41	-0.39	11.79	1.88	7.76	2.96	10.55	1.53	11.88
1.23	0.09	9.77	3.75	13.53	9.74	18.69	1.86	9.97
3.70	0.57	27.35	12.70	27.83	12.39	21.76	2.65	26.12
11.11	1.05	38.58	27.56	45.47	10.45	25.64	7.19	38.58

33.33	1.52	46.10	40.88	57.88	21.36	47.82	13.85	46.12
100.00	2.00	66.78	71.88	78.52	42.28	58.64	28.76	66.88
IC_{50} (µM/ml)		**7.868**	**35.90**	**8.826**	**87.88**	**11.75**	**105.1**	**8.394**
R^2		**0.9658**	**0.9886**	**0.9888**	**0.9373**	**0.9427**	**0.9964**	**0.9677**

Figure 8: Dose Response Curve of various compounds against MDA-MB-231 cell line

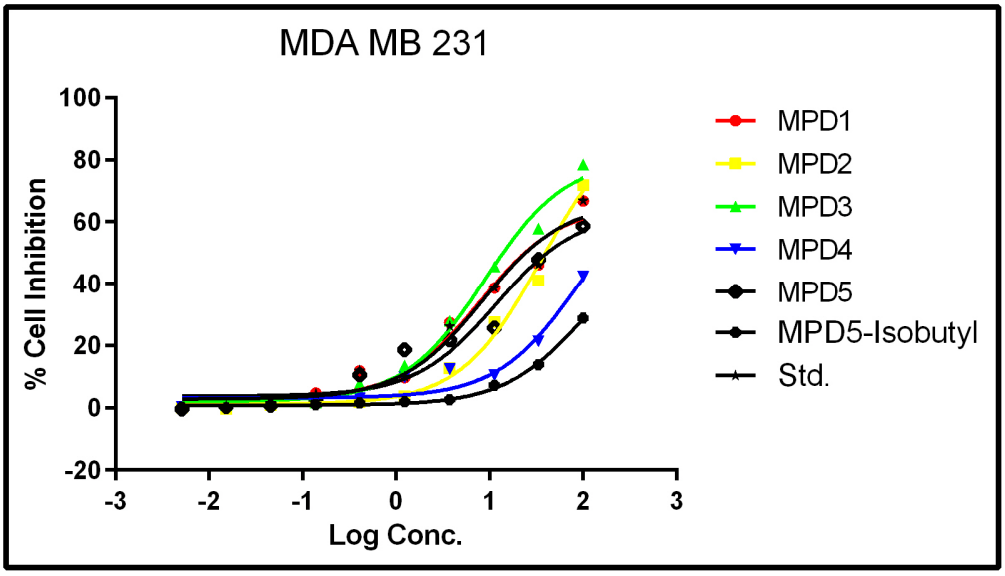


Figure 8: Dose Response Curve of various compounds against MDA-MB-231 cell line

Table 8: % Cell inhibition of **MDA-MB-231 cell** by tests compounds at different conc.

Conc. µg/ml	**Log con.**	**% Cell Inhibition**				
		MPD6	**MPD7**	**MPD9**	**BI-02**	**Std.**
0.01	-2.29	0.20	-0.02	0.10	0.20	-0.10
0.02	-1.82	1.30	0.80	0.31	1.60	0.53

0.05	-1.34	2.30	0.78	0.96	0.97	1.55
0.14	-0.86	3.98	1.30	1.30	1.30	4.45
0.41	-0.39	9.30	2.80	2.12	1.80	11.88
1.23	0.09	14.20	7.90	5.64	2.30	9.97
3.70	0.57	28.60	15.30	17.82	4.30	26.12
11.11	1.05	48.70	31.70	33.64	5.30	38.58
33.33	1.52	61.40	58.70	58.64	15.30	46.12
100.00	2.00	78.20	69.40	67.54	32.70	66.88
IC_{50} (µM/ml)		**7.743**	**16.17**	**13.40**	**179.7**	**8.394**
R^2		**0.9934**	**0.9965**	**0.9971**	**0.9950**	**0.9677**

Figure 9: Dose Response Curve of various compounds against MDA-MB-231 cell line

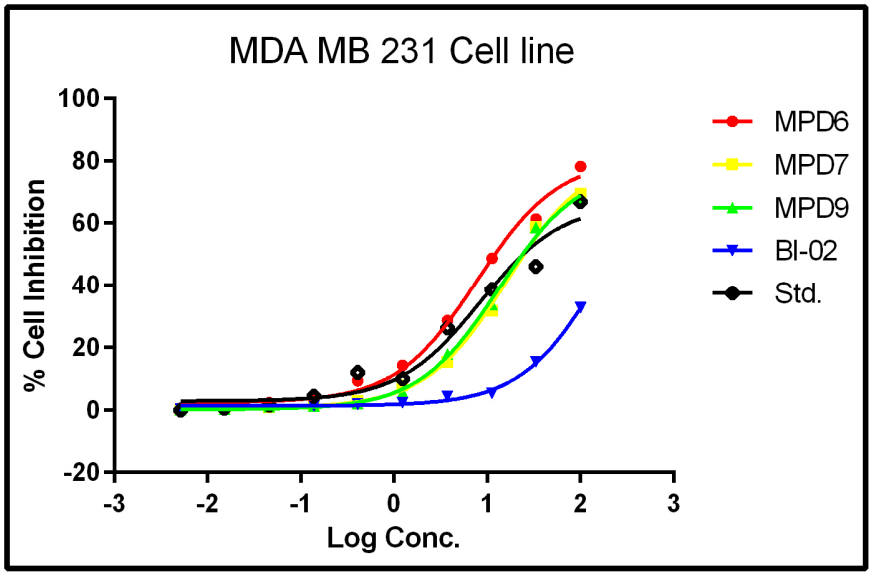


Figure 9: Dose Response Curve of various compounds against MDA-MB-231 cell line VERO Cell line [Normal cell line]

Table 9: % Cell inhibition of **VERO Cell** by tests compounds at different concentration

Conc. µg/ml	**Log con.**	**% Cell Inhibition**						
		MDP1	**MDP2**	**MDP3**	**MDP4**	**MDP5**	**MDP5-Isobutyl**	**Std.**
0.01	-2.29	-0.12	-0.32	-0.32	-0.18	-0.32	0.02	0.0

0.02	-1.82	0.05	-0.13	0.12	-0.24	0.13	0.56	0.02
0.05	-1.34	0.35	0.05	0.65	-0.35	0.859	0.896	0.785
0.14	-0.86	0.68	0.12	1.45	0.87	1.145	1.15	0.963
0.41	-0.39	1.22	0.06	2.06	2.06	1.985	1.96	1.23
1.23	0.09	2.56	4.05	3.98	2.98	2.321	2.45	1.56
3.70	0.57	2.05	5.02	5.11	4.35	4.785	6.85	2.45
11.11	1.05	6.16	8.34	7.34	5.31	11.35	16.75	7.89
33.33	1.52	8.62	11.31	11.04	8.83	21.09	35.86	13.45
100.00	2.00	10.31	15.34	25.32	12.34	18.76	67.42	28.96
IC_{50} (µM/ml)		**151.68**	**208.564**	**127.7**	**104.12**	**110.41**	**74.28**	**105.7**
R^2		**0.9768**	**0.9739**	**0.9651**	**0.9488**	**0.9629**	**0.9995**	**0.9935**

Figure 10: Dose Response Curve of various compounds against Normal cell line

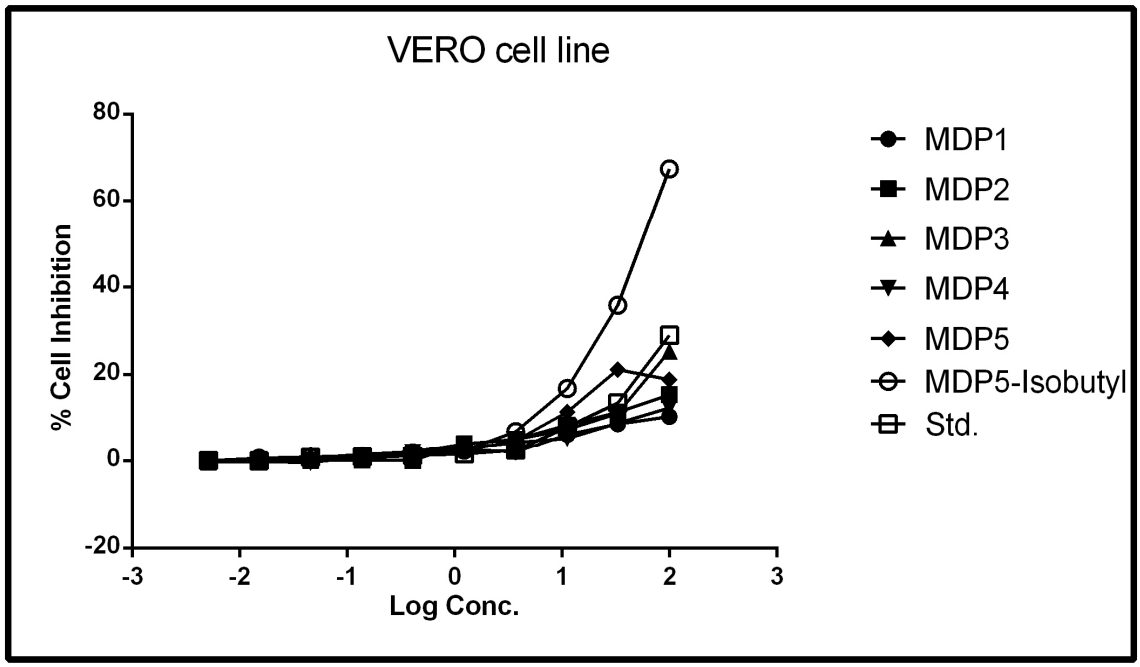


Figure 10: Dose Response Curve of various compounds against Normal cell line

Table 10: % Cell inhibition of **VERO Cell** by tests compounds at different concentration

Conc. µg/ml	**Log con.**	**% Cell Inhibition**				
		MPD6	**MPD7**	**MPD9**	**BI-02**	**Std.**
0.01	-2.29	0.11	0.32	0.05	-0.37	0.0

0.02	-1.82	0.45	0.96	0.12	0.36	0.02
0.05	-1.34	0.987	1.52	0.08	0.97	0.785
0.14	-0.86	1.05	1.69	1.06	1.12	0.963
0.41	-0.39	1.687	2.15	1.22	1.86	1.23
1.23	0.09	2.011	4.65	1.65	3.46	1.56
3.70	0.57	2.456	8.10	2.98	9.37	2.45
11.11	1.05	3.52	18.96	3.56	17.42	7.89
33.33	1.52	9.75	35.25	10.24	27.72	13.45
100.00	2.00	36.22	62.34	35.22	54.24	28.96
IC_{50} (µM/ml)		**>100**	**54.42**	**>100**	**59.49**	**>100**
R^2		**0.9909**	**0.9978**	**0.9938**	**0.9875**	**0.9935**

Figure 11: Dose Response Curve of various compounds against Normal cell line

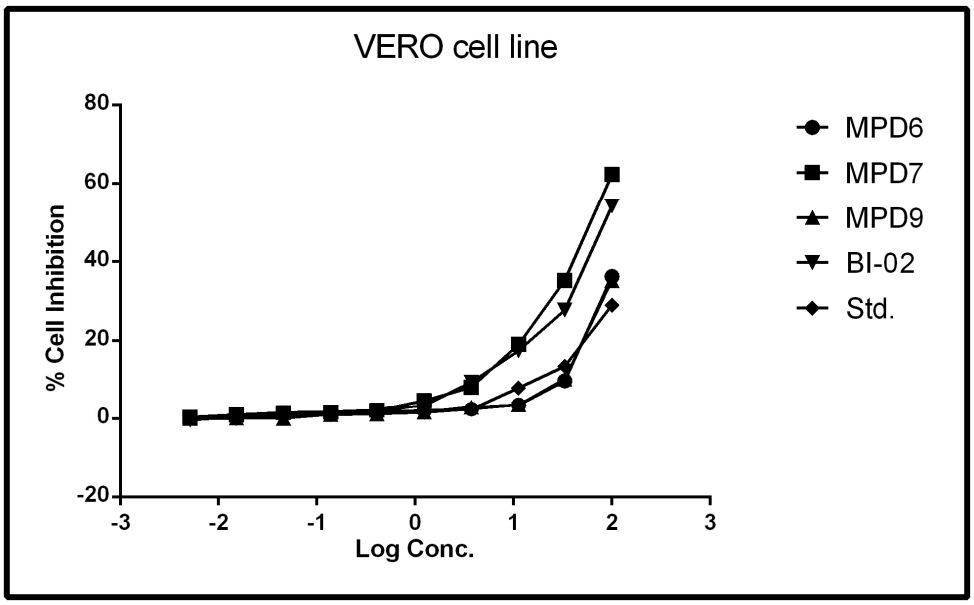


Figure 11: Dose Response Curve of various compounds against Normal cell line

In the present investigation, all the compounds were evaluated against various cell line named K-562 and Normal cell line Vero cell line for each tested compound as well as Std. anticancer drug Methotrexate, Dose Response Curve (DRC) against all cell lines was plotted with 10 analysis point i.e. with 10 different drug concentrations. The

concentration causing 50% cell growth inhibition (IC_{50}) was determined from DRC using *GraphPad Prism* software (Ver. 5.04) (GraphPad Software, Inc., USA) and Micorsoft Excel 2007 (Microsoft Corporation, USA) application.

Amongst all the tested compounds MPD6 (7.7743µM/ml), MPD1 (7.886µM/ml) and MPD-3(8.826µM/ml) gave highest potential effect on MDA-MB-231 cell line compare to Std. Methotrexate drug having IC_{50} value (8.394µM/ml).

Compounds MPD5, MPD9 and MPD7 have good activity on MDA-MB-231 cell line. While, MPD2, MPD5-Isobutyl were less active or almost not effective against tested cell line. So, further the activity of all effective compounds series were tested against normal cell line [VERO cell line] and it was concluded that compounds MPD-7, MPD5-isobutyl and BI-02 were found to be toxic for normal cell. On other side excepting MPD-7, others were not effective to inhibit tested cells.

Conclusion

From above all the results, it can be concluded that compounds MPD6, MPD1 and MPD-3 gives good cytotoxic activity on MDA-MB-231 cell line. Compounds MPD5, MPD9 and MPD7 have good activity but less than std. drug. While compound MPD2, MPD5-isobutyl and BI-02 were found to be not effective.

2.14 Reference

1 R. C. Elderfield, *Heterocyclic compounds,* Wiley, New York, **1957**, 5

2 M. Achesonr, F. E. King, C. Spensleyp, *Nature*, **1947**, 53, 160.

3 M. R. Grimmett, *Advan. Heterocycl. Chem.,* **1970**, 12, 103.

4 N. A. Sharif, S. X. Su, J. M. Yanni, *J. Ocul. Pharmacol*. **1994**, 10, 653.

5 A. R. Day, *Electronic Mechanisms* of *Organic Reactions,* American Book, **1983**, 242.

6 Kartaraski, *Comp. Hetro, Chem.* **1984**, 5, 345.

7 A. E. Remick, *Electronic Interpretations* of *Organic Chemistry,* John Wiley and Sons, Inc., New York, **1949**.

8 K. A. Jensen, Fridioehr, K. D. V. Selskab, *Math. fys.Medd*.**1943**, 20, 1; *Chem.Abstracts*, **1945**, 39, 2068.

9 W. Smithc, S. Rasmussern, A. Ballars*d*, *J. Am. Chem. Soc.* **1949**,7(1), 1082.

10 O. Fischer, *Ber.* **1889**, 23*,* 645.

11 F. Bellina, C. Calandri, S. Cauteruccio, R. Rossi, *Tetrahedron,* **2007**, 63, 1970.

12 W. C .Shieh, M. Lozanov, O. Repic, *Tetrahedron Lett.* **2003**, 44, 6943.

13 A. P. Combs, W. Zhu, M. L. Crawley, B. Glass, P. Polam, R. B. Sparks, D. Modi, A. Takvorian, E. Mc-Laughlin, E. W. Yue, Z. Wasserman, M. Bower, M. Wei, M.

Rupar, P. J. Ala, B. M. Reid, D. Ellis, L. Gonneville, T. Emm, N. Taylor, S. Yeleswaram, Y. Li, R. Wynn, T. C. Burn, G. Hollis, P. C.C. Liu, B. Metcalf, *J. Med. Chem.* **2006**, 49, 3774.

14 R. A. Ng, J. Guan, V. C. Alford, J. C. Lanter, G. F. Allan, T. Sbriscia, O. Linton, S. G. Lundeen, Z. Sui, *Bioorg. Med. Chem. Lett.* **2007**, 17, 784.

15 V. L. Dalla, O. Gia, S. M. Magno, A. D. Settimo, A. M. Marini, G. Primofiore, F. D. Settimo, S. Salerno, *II Farmaco* **2001**, 56, 159.

16 V. K. Pandey, N. Raj, U. K. Srivastava, *Acta Pharm. Jugoslavica*, **1986**, 36, 281.

17 L. N .Vostrova, T. A .Voronina, T. L. Karaseva, S. A. Gernega, E. I. Ivanov, A. M. Kirichenko, M. Y. Totrova, *Khim-Farm Zh* **1986**, 20, 690.

18 S. Frere, V. Thiery, C. Bailly, T. Besson, *Tetrahedron* **2003**, 59, 773.

19 D. M. D. Fouchard, L. M. V. Tillekeratne, R. A. Hudson, *J. Org. Chem.* **2004**, 69, 2626.

20 P. Hao, S. Zhang, W. H. Sun, Q. Shi, S. Adewuyi, X. Lu, P. Li, *Organometallics* **2007**, 26, 2439.

21 F. Arjmand, B. Mohani, S. Ahmad, *Eur. J. Med. Chem.* **2005**, 40, 1103.

22 K. B. Gudasi, R.V. Shenoy, R. S. Vadavi, M. S. Patil, S. A. Patil, R. R. Hanchinal, S. A. Desai, H. Lohithaswa, *Bioinorg. Chem. Appl.,* **2006**, 9, 1.

23 X. Jing, Q. Zhu, F. Xu, X. Ren, D. Li, C.Yan, *Synth. Commun.* **2006**, 36, 2597.

24 V. K. Tandon, M. Kumar, *Tetrahedron Lett.* **2004**, 45, 4185.

25 K. Bougrin, A. Loupy, A. Petit, B. Daou, M. Soufiaoui, *Tetrahedron* **2001**, 57, 163.

26 D. S. Vanvliet, P. Gillespie, J. J. Scicinski, *Tetrahedron Lett*. **2005**, 46, 6741.

27 A. Hegedus, Z. Hell, A. Potor, *Synth. Commun.* **2006**, 36, 3625.

28 R. R. Nagawade, D. B. Shinde, *Ind. J. Chem. B*, **2007**, 46B, 349.

29 C. Neochoritis, D. Livadiotou, S. J. Stephanidou, C. A. Tsoleridis, *Tetrahedron Lett*. **2007**, 48, 2275.

30 R. Gedye, F. Smith, K. Westaway, H. Ali, L. Baldisera, L. Laberge, J. Rousell, *Tetrahedron Lett.* **1986**, 27, 279.

31 D. J. Faulkner, *Nat. Prod. Rep.* **1992**, 9, 323.

32 S. Nakamura, N. Tsuno, M. Yamashita, I. Kawasaki, S. Ohta, Y. Ohishi, *J.*

Chem.Soc. Perkin. Trans. **2001**, 1, 429.

33 P. G. Baraldi, A. Bovero, F. Fruttarolo, D. Preti, M. A. Tabrizi, M. G. Pavani, R.Romagnoli, *Med. Res. Rev.* **2004***,* 24, 475.

34 B. S. P. Reddy, S. K. Sharma, J. W. Lown, *Curr. Med. Chem.* **2001***,* 8, 475.

35 A. K. Singh, J. W. Lown, *Anticancer Drug Des*. **2000**, 15, 265.

36 A. Joubert, X. W. Sun, E. Johansson, C. Bailly, J. Mann, S. Neidle, *Biochemistry,* **2003**, 42, 5984.

37 M. Lynch, S. Hehir, P. Kavanagh, D. Leech, J. O. Shaughnessy, M. P. Carty, F. Aldabbagh, *Chem. Eur. J.,* **2007**, 13, 3218.

38 Y. Kubota, H. Fujii, J. Liu, S. Tani, *Nucleic Acids Res. Suppl.,* **2001**, 101.

39 L. Garuti, M. Roberti, A. Pession, E. Leoncini, S. Hrelia, *Bioorg. Med. Chem. Lett*., **2001**, 11, 3147.

40 P. Xiang, T. Zhou, L. Wang, C. Y. Sun, J. Hu, Y. L. Zhao, L. Yang, *Molecules,* **2012**, 17, 873.

41 R. O. Dillman, K. P. Ryan, J. B. Dillman, D. L. Shawler, R. Maguire, *Mol. Biother.,* **1992***,* 4, 10.

42 S. A. Galal, K. H. Hegab, A. S. Kassab, M. L. Rodriguez, S. M. Kerwin, A. M. A. E. Khamry, H. I. E. Diwani, *Euro. J. Med. Chem.*, **2009**, 44, 1500.

43 K. Starcevic, M. Kralj, K. Ester, I. Sabol, M. Grce, K. Pavelic. G. K. Zamola, *Bioorg. Med. Chem.,* **2007***,* 15, 4419.

44 Q. Sun, W. Runzhi, C. Sutang, Y. Lin, L. Sellers, K. Sakamoto, B. He, B. R. Peterson, *J. Med. Chem.*, **2011**, 54, 1126.

45 R. Rubbiani, I. Kitanovic, H. Alborzinia, S. Can, A. Kitanovic, L. A. Onambele, *J.Med. Chem.,* **2010**, 53, 8608.

46 T. D. Penning, G. D. Zhu, V. B. Gandhi, J. Gong, X. Liu, Y. Shi, V. Klinghofer, E. F. Johnson, C. K. Donawho, D. J. Frost, V. B. Diaz, J. J. Bouska, D. J. Osterling, *J. Med.Chem.* **2009**, 52, 514.

47 D. Hao, J. D. Rizzo, S. Stringer, R. V. Moore, J. Marty, D. L. Dexter, G. L. Mangold, J. B. Camden, D. D. Von-Hoff, S. D. Weitman, *Investi. New Drugs,* **2002***,* 20, 261.

48 S. Ramurthy, M. Aikawa, P. Amiri, A. Costales, A. Hashash, J. M. Jansen, S. Lin, S. Mab, P. A. Renhowe, C. M. Shafer, S. Subramanian, L. Sung, J. Verhagen, *Bioorg. Med. Chem. Lett*. **2011**, 21, 3286.

49 R. Abonia, E. Cortés, B. Insuasty, J. Quiroga, M. Nogueras, J. Cobo, *Eur. J. Med.Chem.* **2011**, 46, 4062.

50 A. Husain, M. Rashid, A. A. Siddiqui, R. Mishra, *Eur. J. Med. Chem.* **2013**, 62, 785.

51 A. Husain, M. Rashid, R. Mishra, S. Parveen, D. S. Shin, D. Kumar, *Bioorg. Med. Chem. Lett*. **2012**, 22, 5438.

52 M. Rashid, A. Husain, R. Mishra, *Eur. J. Med. Chem.* **2012**, 54, 855.

53 K. G. Desai, K. R. Desai, *Bioorg. Med. Chem.* **2006**, 14, 8271.

54 O. O. Guven, T. Erdogan, H. Goeker, S. Yildiz, *Bioorg. Med. Chem. Lett*. **2007**, 17, 2233.

55 K. F. Ansari, C. Lal, *Eur. J. Med. Chem.* **2009**, 44, 2294.

56 S. Jubie, R. Rajeshkumar, R. B. Yella, G. Siddhartha, M. Sandeep, K. Surendrareddy, H. S. Dushyatha, K. Elango, *J. Pharm. Sci. Res.* **2010**, 2(2), 69.

57 H. Giiker, M. Tuqbilek, G. Ayhan, N. Altanlar, *II Farmaco,* **1998***,* 53, 415.

58 I. Orena, O. Temiza, I. Yalcýna, E. Senera, N. Altanlarb, *Eur. J. Pharm. Sci.* **1998**, 7, 153.

59 K. K. Anichina, D. I. Vuchev, A. T. Mavrova, *Probl. Infect. Parasit. Dis.***2006**, 34, 19.

60 A. T. Mavrova, K. K. Anichina, D. I. Vuchev, J. A. Tsenov, P. S. Denkova, M. S. Kondeva, M. K. Micheva, *Eur. J. Med. Chem.* **2006***,* 41, 1412.

61 N. M. Gaur, S. V. Patil, V. K. Mourya, S. B. Wagh, *Ind. J. Heterocycl.Chem.***2000***,* 9, 227.

62 S. Rashmi, U. V. S. Jujher, D. S. Bhanuki, *Ind. J. Chem.* **1989**, 28B, 702.

63 M. H. Rajiv. M. V. Ramana, *Ind. J. Heterocycl. Chem.* **2002**, 2, 121.

64 L. F. Hernández, C. A. Hernández, R. Castillo, V. G. Navarrete, A. O. Soria, H. M. Hernández, M. L. Yépez *Eur. J. Med. Chem.* **2010**, 45(7), 3135.

65 N. Toshiro, Y. Sachiko, S. Miho, O. Naoto, I. Kaoru, N. Yasuo, T. Akira,

Biol.Pharm. Bull. **2006**, 29(9), 1931.

66 W. Wienen, M. Entzeroth, J. C. A. Van-Meel, J. Stangier, U. Busch, T. Ebner, J. Schmid, H. Lehmann, K. Matzek, J. K. Rawson, V. Gladigau, N. Hauel, *Cardio. Drug Reviews* **2000**, 18(2), 127.

67 M. L. Barreca, A. Chimirri, E. D. Clercq, L. D. Luca, A. M. Monforte, P. Monforte, A. Rao, M. Zappala, *II Farmaco* **2003**, 58, 259.

68 V. K. Pandey, V. D. Gupta, D. N. Tiwari, *Ind. J. Heterocycl. Chem.,* **2005***,* 14, 217.

69 Y. S. Xu,C. C. Zeng, Z. G. Jiao,L. M. Hu, R. G. Zhong, *Molecules,* **2009**, 14, 868.

70 A. Chimirri, P. Monforte, A. Rao, M. Zappalà, A. M. Monforte, G. D. Sarro, C. Pannecouque, M. Witvrouw, J. Balzarini, E. D. Clercq, *Antiviral Chem. Chemo,* **2001**, 12, 169.

71 R. Zou, K. R. Ayres, J. C. Drach, L. B. Townsend, *J. Med. Chem.,***1996***,* 39, 3477.

72 L. K. Labanauskas, A. B. Brukstus, P. G. Gaidelis, V. A. Buchinskaite, E. B. Udrenait, V. K. Dauksas, *Pharm. Chem. J.,* **2000***,* 34, 353.

73 S. M. Sondhi, S. Rajvanshi, M. Johar, N. Bharti, A. Azam, A. K. Singh, *Eur. J. Med.Chem.,***2002***,* 37, 835.

74 L. Labanauskas, A. Brukstus, E. Udrenaite, P. Gaidelis, V. Bucinskaite, V. Dauksas, *Chemija.,***2000**, 11, 211.

75 A. Puratchikody, V. Sivajothi, A. Jaswanth, K. Ruckmani, M. Nallu, *Ind. J. Heterocycl. Chem.,* **2002***,* 11, 241.

76 G. Srinivasulu, P. P. Reddy, P. Hegde, R. Chakrabarty, *Heterocycl. Commun.* **2005***,* 11, 23.

77 S. N. Sawhney, G. Asha, *Ind. J. Chem.* **1991**, 30B, 407.

78 C. S. Kavitha, Achar, K. M. Hosamani, H. R. Seetharamareddy, *Eur. J. Med. Chem.,* **2010***,* 45, 2048.

79 R. L. Lombardy, F. A. Tanious *J. Med. Chem.* **1996**, 39, 1452.

80 C. Kus, G. A. Kilcigil, S. Ozbey, F. B. Kaynak, M. Kaya, T. Coban, B. C. Eke, *Bioorg. Med. Chem.,* **2008***,* 16, 4294.

81 S. P. Vikrant, J. M. Amey, S. R. Shukla, *Eur. J. Med. Chem.* **2009**, 44, 5112.

82 V. Dudutien, L. Baranauskien, D. Matulis, *Bioorg. Med. Chem. Lett*. **2007**, 17,

3335.

83 J. Singh, P. Grover, D. P. Pathak, *Acta Pharma. Scien.* **2010**, 52, 511.

84 H. Rajak, N. Aggarwal, S. Kashaw, M. D. Kharya, P. Mishra, *J. Korean Chem. Soc.* **2010**, 54(1), 158.

85 P. Pattanayak, R. Sharma, P. K. Sahoo, *Med. Chem. Res.* **2009**, 18(5), 351.

86 Bussey KJ, *Mol. Cancer Ther.* **2006**, 5, 853

87 Cabrera CM, *Cytotechnology*, **2006**, 51, 45

88 Bruce A. Chabner, *Journal of the National cancer institute*, **1990**, 82,13

89 Chatterjee R., *Science*, **2007**, 315, 928

90 Corey AH, Owen TC, Barltrop JA, Corey JG, *Cancer communications*, **1991**, 2(7), 207

91 Barnas T, Dobrucki, *J. Cytometry*, **2002**, 1, 47(4), 236.