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Synthesis of imidazothiadiazole–benzimidazole conjugates as mitochondrial apoptosis inducers

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A series of imidazothiadiazole–benzimidazole conjugates **3a–z** were synthesized and evaluated for their cytotoxic activity against a set of four selected human cancer cell lines. Amongst them, compounds **3b** and **3y** exhibited significant antiproliferative activity in ME-180 (cervical) cell line. Flow cytometric analysis showed that these two compounds arrested the cell cycle in the Go/G1 phase leading to the loss of mitochondrial membrane potential followed by apoptotic cell death. Further, Hoechst 33258 staining, DNA fragmentation assay, Annexin-V staining assay and caspase-3 also suggested that **3b** and **3y** induced the cell death by apoptosis. Docking studies revealed that compound **3b** binds to the Gly142, Phe101, Asn140 and Arg143 on B-cell lymphoma 2 (Bcl-2) proteins and inhibition of Bcl-2 protein could be the possible mechanism for these compounds.

Introduction

Cancer is one of the major global health problems, in which cell or a group of cells exhibit uncontrolled growth and invasion, indicating the second-leading cause of death in the Western world.[1] According to the World Health Organization (WHO), it is estimated that 12 million deaths could occur due to cancer in 2030. The discovery and development of new, effective and more selective compounds is the most important task for the treatment of cancer. It is credible that the future of tumor therapy is in the development of molecularly targeted drugs that particularly block key mechanisms that are involved in development and progression of specific types of tumor.[2] The search for such potential anticancer agents have directed to the discovery of small synthetic molecules with promising anti-carcinogenic activity. Nitrogen-bridgehead fused heterocyclics containing an imidazole ring is a very common structural moiety in many pharmacologically active molecules that exhibit a wide range of activities for diverse number of targets.[3–8]

Amongst the most widely used heterocyclic system is imidazo[2,1-*b*][1,3,4]thiadiazole. Imidazothiadiazoles show a wide spectrum of biological properties like antibacterial,[9] antifungal,[10] antitubercular[11] and anticancer.[12-16] Imidazothiadiazoles display different type of molecular mechanisms in cancer chemotherapy.[13-18] Previous report on 2-amino-1,3,4-thiadiazole analogues deal with the activity of these compounds against numerous transplanted animal tumours.[18] Terzioglu and co-workers reported the hydrazone derivatives of 2,6-dimethylimidazo[2,1-*b*][1,3,4]-thiadiazole-5-carbohydrazide as promising anticancer agents against ovarian cancer cell line (OVCAR),[13] this finding prompted to further investigate the antitumor effects of imidazo[2,1-*b*][1,3,4]-thiadiazoles.[14–17]

On the other hand, benzimidazole moiety is structurally related to purine bases and is extensively found in a variety of natural products. Several promising anticancer agents were known to contain this heterocycle. The ring system of benzimidazole can be considered as a new device for the target specific transcription aspect at the binding sites related to biological systems.[19,20] The cytotoxic benzimidazoles act mainly as topoisomerase inhibitors, alkylating agents, DNA binding agents and also as poly(ADP-ribose)polymerase-1 (PARP-1) inhibitors.[21–28] Some representative imidazo[2,1-*b*][1,3,4]thiadiazoles and benzimidazole derivatives are shown in Figure 1.

Our earlier efforts toward the discovery of new synthetic small molecules led to the development of a number of hybrids/conjugates as potent cytotoxic agents.[29–32] In continuation of these efforts, an attempt has been made, considering the significant bioactivities of conjugates with an imidazo[2,1-*b*][1,3,4]thiadiazole as the core moiety. As reported from the literature, imidazothiadiazoles exhibit tubulin polymerization inhibition and show the G_2/M cell cycle arrest[17] whereas benzimidazoles mostly act as topoisomerase I and DNA binding agents and show the G_1/G_0 cell cycle arrest.[25] We designed imidazothaidiazole-benzimidazole conjugates with a view to develop effective cytotoxic agents and to investigate their cytotoxic pathway on mammalian cells. Therefore, in the present study we synthesized some imidazothiadiazoles that are

linked to the benzimidazole scaffold. The promising activity exhibited by some of these conjugates prompted us to examine their role in the cell proliferation and apoptosis of the human cervical cancer cell line (ME-180). The biological assays on these imidazothaidiazole-benzimidazole conjugates show the G_0/G_1 cell cycle arrest and leading to the mitochondrial apoptotic cell death.

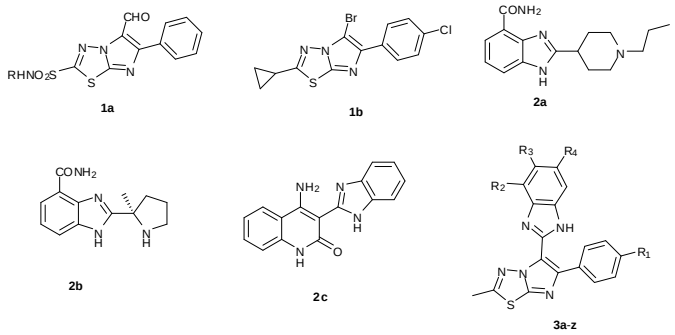
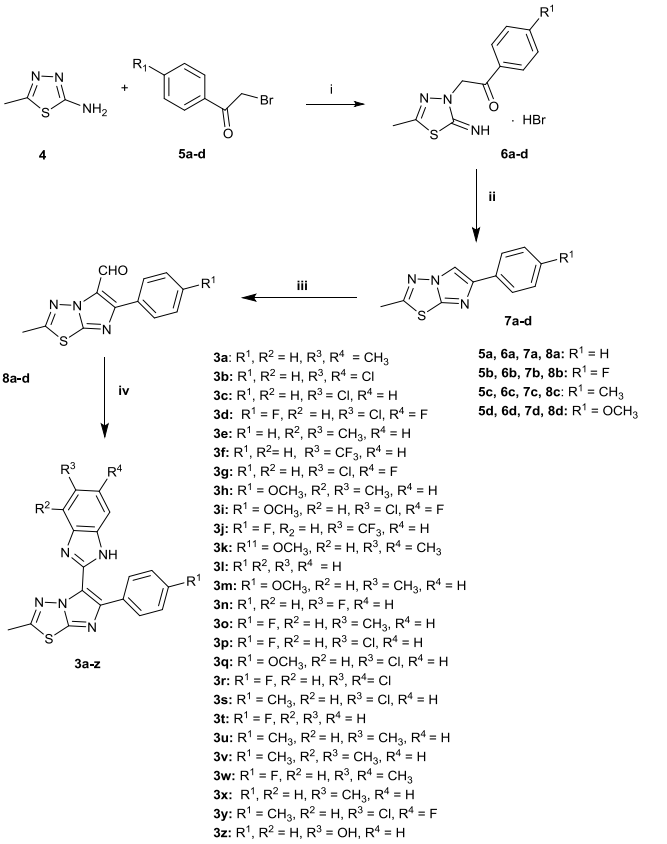


Fig. 1 Structures of some representative imidazo[2,1-*b*][1,3,4]thiadiazole and benzimidazole derivatives

Results and discussion

Chemistry

The synthesis of imidazothiadiazole–benzimidazole conjugates (**3a**–**z**) was carried out with the commercially available 5-methyl-1,3,4-thiadiazol-2-amine (**4**) and bromoketones (**5a**–**d**) as the starting materials as shown in Scheme 1. The reaction between 5-methyl-1,3,4-thiadiazol-2-amine (**4**) and appropriate bromoketones (**5a**–**d)** in acetone produced the intermediate compounds **6a**–**d**, which were isolated and used in the successive step without any further purification to generate imidazo[2,1-*b*][1,3,4]thiadiazoles **7a**–**d**. The imidazo[2,1-b][1,3,4]thiadiazole aldehydes **8a**–**d** were obtained by employing Vilsmeier reaction with the corresponding imidazo[2,1-*b*][1,3,4]thiadiazoles **7a**–**d**.[33] The oxidative cyclization of the appropriately substituted *o*-phenylenediamine and imidazo[2,1-*b*]thiadiazole aldehydes **8a**–**d** in the presence of $Na_2S_2O_5$ in ethanol, afforded the desired imidazothiadiazole–benzimidazole conjugates (**3a**–**z**) with good yields.[34]



Scheme 1 *Reagents and conditions*: i) acetone, reflux, 6–8 h; ii) 2N HCl, reflux, 1 h, 79–85%; iii) $POCl_3$, DMF, reflux, 1 h, 70–76%; iv) Substituted *o*-phenelenediamine, $Na_2S_2O_5$, EtOH, reflux, 4 h, 65–79%.

Biological evaluation

Anticancer activity. These conjugates **3a**–**z** were screened for their anticancer activity against a set of selected human cancer cell lines of ME-180 (cervical), A375 (melanoma), DU145 (prostrate) and A549 (lung) by using MTT assay[35] and the results obtained are illustrated in Table 1. All these conjugates exhibited cytotoxic activity with IC_{50} values ranging from 0.5 to >30 μM, while doxorubicin and nocodazole were used as the positive controls. Amongst these two conjugates **3b** and **3y** showed significant cytotoxic activity in ME-180 (IC_{50} values 0.5 and 0.7 μM) and A375 (IC_{50} values 2.8 and 1.04 μM) cell lines whereas other conjugates showed promising to moderate activity in different cancer cell lines.

In a view to study the structure–activity relationship (SAR), we varied the substitution pattern of the phenyl rings on imidazothiadiazole and benzimidazole with electron donating as well as withdrawing substituents while keeping the core moiety imidazothiadiazole–benzimidazole unchanged. Some interesting trends have been observed in the SAR studies of these newly synthesized compounds. The substitution pattern of R^1 on the phenyl ring of the imidazothiadiazole unit has showed some impact on the antiproliferative activity of these compounds. From the cell line assays, it appears that a number of compounds with electron-donating substituents like H, methyl and methoxy at R^1 position (**3b**, **3c**, **3i**, **3q**, **3s**, **3u** and **3y**) exhibited considerable cytotoxic activity (IC_{50} values 0.5 to 5.8 μM and 1.04 to 7.7 μM against ME-180 and A375, respectively). Whereas in case of electron-withdrawing substituents like fluoro at R^1 position (**3d**, **3j**, **3o**, **3r**, **3t** and **3w**) on the phenyl ring of the imidazothiadiazole unit, showed comparatively reduced cytotoxicity (IC_{50} values 5.8 to 13.9 μM and 7.3 to 22.2 μM against ME-180 and A375, respectively). The substitution pattern (R^2, R^3 and R^4) on the fused phenyl ring of the benzimidazole subunit considerably affects the cytotoxic activity of these conjugates. Amongst the synthesized compounds, most of the electron-withdrawing halo substituted compounds such as **3b**, **3c**, **3i**, **3q**, **3s** and **3y** displayed higher potency than other substitution patterns on the phenyl ring of

the benzimidazole subunit. Especially, chloro substitution at R^3 and R^4 played an important role in the overall activity profile of these compounds (**3b**, **3p**, **3q** and **3y**) with IC_{50} values in the range of 0.5 to 5.8 µM and 1.04 to 4.8 µM against ME-180 and A375, respectively.

Table 1 Cytotoxic activitya of imidazothiadiazole–benzimidazole derivatives (**3a-z**) expressed in µM

Compd	ME-180[b]	A375[c]	DU145[d]	A549[e]
3a	19.9	22.5	19.8	>30
3b	0.5	2.8	19.1	13.6
3c	5.4	7.7	25.1	16.9
3d	13.9	17.7	>30	>30
3e	15.8	22.9	>30	18.4
3f	3.9	5.2	19.6	13.6
3g	16.9	20.8	>30	16.4
3h	3.7	5.8	14.2	16.2
3i	4.6	6.8	>30	17.9
3j	5.8	7.3	15.8	12.7
3k	12.3	12.5	23.3	16.5
3l	16.9	19.9	>30	16.6
3m	8.7	10.9	25.1	18.4
3n	15.7	18.1	16.9	>30
3o	6.6	7.7	19.9	11.7
3p	1.9	4.8	14.4	10.2
3q	2.6	4.3	15.8	7.9
3r	10	14.2	>30	>30
3s	3.6	5.01	7.3	4.4
3t	18.1	22.2	>30	>30
3u	4.5	6.1	10.7	9.8
3v	9.7	11.4	12.5	10.3
3w	6.1	7.6	>30	>30
3x	15.8	20.4	14.4	12.5
3y	0.7	1.04	11.8	10.3
3z	16.4	19.07	20.6	19.4
Doxorubicin	0.5	1.2	2.4	1.1
Nocodazole	1.7	0.2	2.1	2.5

[a] IC_{50} or 50% inhibitory concentration and the values are average of three individual experiments. [b]Cervical cancer,[c]Melanoma,[d]Prostate cancer, [e]Lung cancer.

These observations reveal that, halo substitution on the fused phenyl ring of the benzimidazole subunit and electron-donating groups on the phenyl ring of the imidazothiadiazole unit are important for maintaining antiproliferative activity. Therefore, the conjugates **3b** and **3y** with 6-chloro and 5-chloro/fluoro on the phenyl ring of the benzimidazole subunit and unsubstituted/methyl substituted phenyl ring of the imidazothiadiazole unit exhibited higher cytotoxicity in ME-180 (IC_{50} values 0.5 and 0.7 µM) and A375 (IC_{50} values 2.8 and 1.04 µM) cell lines as compared with other substitution pattern of these conjugates. Hence, the SAR studies based on the cell viability values obtained in ME-180 cells encouraged us to investigate the influence of conjugates **3b** and **3y** on cell cycle.

Cell cycle analysis. To study the mechanism underlying the cytotoxic activity of **3b** and **3y**, cell cycle disruption and apoptotic ability of these conjugates were performed in ME-180 cells. The effect on cell cycle distribution was examined by fluorescence activated cell sorting (FACS) analysis.[36] ME-180 cells were exposed to 0.5 and 1 µM of **3b** and **3y** for 48 h, compound **3b** has shown 74% G_0/G_1 at 0.5 µM, upon increasing the concentration to 1µM resulted with 88.7% where as **3y** with 80.7% at 0.5 µM and 87.3% at 1µM (Table 2). These results showed that the accumulation of cells were in the G_0/G_1 phase and was comparable to doxorubicin at 1µM. Doxorubicin at lower concentration arrests G_2/M phase (data not shown), however upon increasing the concentration to 1µM, it shows G_0/G_1 arrest. Interestingly, this was also accompanied by compensatory decrease in G_2 phase of cells. Conjugates **3b** and **3y** caused a G_0/G_1 phase arrest, as the majority of accumulation effect was detected by exposure for 48 h. These results suggested that both the conjugates (**3b** and **3y**) inhibited the cellular proliferation via G_0/G_1 phase arrest in a dose dependent manner.

Table 2 Cell cycle phase distribution of ME-180 cells following treatment with compounds **3b**, **3y** and Doxorubicin

Sample	Sub-G_1	G_1	S	G_2/M
Control	7	59.9	19.3	13.2
3b-0.5µM	5.4	68.1	15.7	10.4
3b-1µM	16.3	72.4	9.3	1.6
3y-0.5µM	14.1	66.6	12.3	6.7
3y-1µM	11.6	75.7	10.1	2.3
DOXO-1µM	7.7	64.8	16.1	10.9

Hochest staining. Apoptosis is one of the major pathways that lead to the process of cell death. The classic characteristics of apoptosis are chromatin condensation and fragmented nuclei. Therefore it was considered of interest to investigate the apoptotic inducing effect of these two potential conjugates (**3b** and **3y**) by Hoechst staining (H 33258) in the cervical cancer cell line (ME-180). In this assay, cells were treated with **3b** and **3y** at 1µM for 24 h. Manual field quantification of apoptotic cells based on cytoplasmic condensation, presence of apoptotic bodies, nuclear fragmentation and relative fluorescence of the test compounds (**3b** and **3y**), represented these compounds induce cell death by apoptosis (Fig. 2).

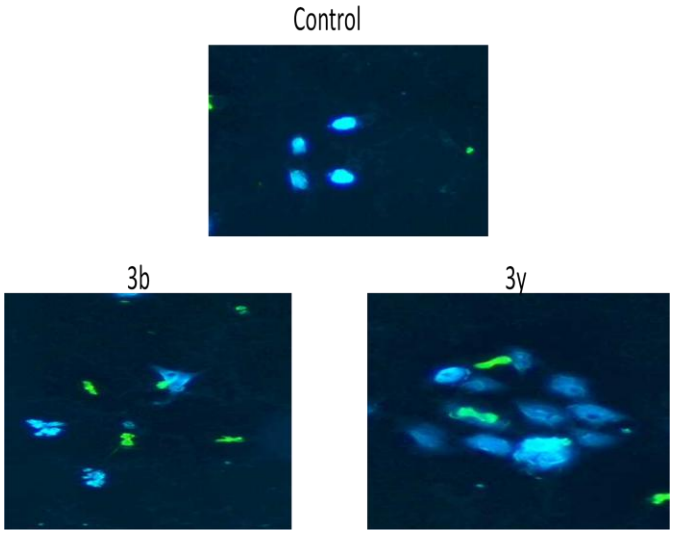


Fig. 2. Hoechst staining of the conjugates (**3b** and **3y**) at 1µM in cervical cancer cells (ME-180).

DNA fragmentation assay. It is well known and a typical biochemical hallmark of apoptotic cell death. From the cytotoxicity studies, it was observed that compounds **3b** and **3y** considerably inhibits the growth of human cervical cancer cell line ME-180. Therefore it was of interest to determine the mechanism of cell death in the same cell line.

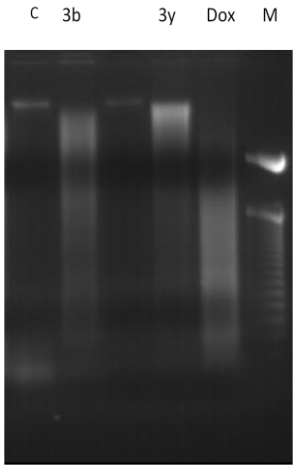


Fig. 3 DNA fragmentation of the conjugates (**3b** and **3y**) in ME-180 cancer cells.

The DNA fragmentation analysis[37] revealed that compounds **3b** and **3y** induced a discrete ladder pattern in ME-180 cell line at 1 µM after 48 h of incubation thereby showing significant fragmentation in a similar pattern with that of doxorubicin, which was used as a positive control, however, no such effect was observed in untreated cells (Fig. 3).

Annexin V FITC dual staining assay. The apoptotic effect of these conjugates (**3b** and **3y**) was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[38-41] to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis. In this assay, ME-180 cells were treated with **3b** and **3y** for 48 h at 2 µM concentration to study the apoptotic effect. It was observed that these compounds showed promising apoptotic effect against ME-180 cells as shown in Figure 4 and the results indicated that **3b** and **3y** showed 22.6% and 16.5% of apoptosis, respectively. In the untreated cells (control) 5% of apoptosis was observed and the standard doxorubicin showed 15.1% of apoptosis at 2 µM concentration. Thereby, these results suggest that these conjugates induce apoptosis in ME-180 cells. Moreover these studies clearly indicated that the cell cycle arrest at G_0/G_1 phase takes place by the induction of cellular apoptosis.

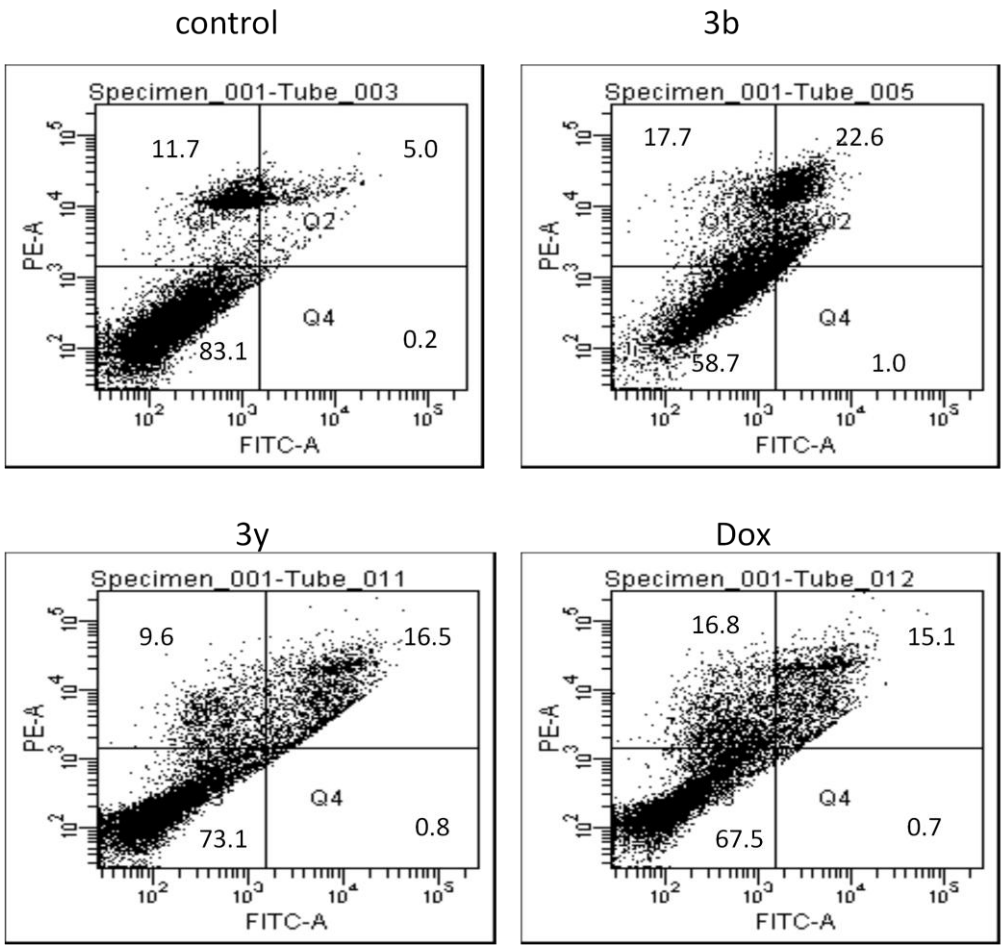


Fig. 4 Annexine V - FITC, control cells of ME-180, ME-180 cells treated with **3b**, **3y** and doxorubicin for 24 hrs at 1µM. Q2 (late apoptotic); Q4 (early apoptotic).

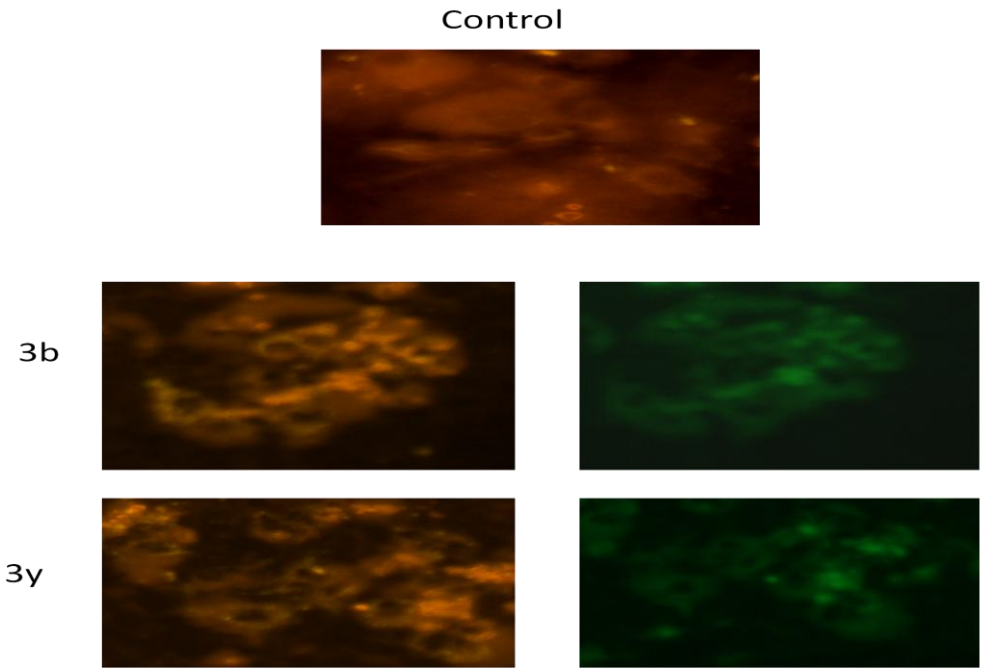


Fig. 5 The loss of mitochondrial membrane potential was monitored by JC-1 stain. High $\Delta\Psi m$ in control mitochondria causes association with the dye in an aggregated state leading to red-orange fluorescence emission. Compounds **3b** and **3y** lead to dissipation of $\Delta\Psi m$, and the dye is now partitioned into cytosol, resulting in green fluorescence.

Loss of mitochondrial membrane potential using JC-1 dye. The loss of mitochondrial membrane potential is a major characteristic of apoptosis.[42] Measurement of mitochondrial membrane potential (DYm) serves as a marker to estimate the overall function of mitochondria during the induction of apoptosis. Therefore, in this study we examined the effect of **3b** and **3y** on DYm. ME-180 cells were treated with compounds **3b** and **3y** at 1 µM concentration for 24 h and stained with JC-1 dye. In the control cells, the dye was concentrated in the mitochondrial matrix where it forms red fluorescent aggregates (J-aggregates) because of the electrochemical potential gradient. Where as in the cells treated with **3b** and **3y**, apoptosis was induced and the mitochondrial membrane is depolarized thus preventing the accumulation of the JC-1 dye in the mitochondria. Therefore, the dye in the monomeric form is dispersed throughout the entire cell leading to a shift in the colour from orange to green fluorescence (JC-1 monomers). The apoptotic cells with green fluorescence are easily differentiated from the healthy cells with orange fluorescence. Therefore it was quite evident that these conjugates (**3b** and **3y**) significantly depolarize the mitochondrial membrane potential (Fig. 5) thereby inducing cell death in ME-180 cells.

Activation of caspase induction. Caspase-3 is activated in the apoptotic cell, both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways.[43] In intrinsic activation, cytochrome-c from the mitochondria works in combination with caspase-9, apoptosis-activating factor 1 (Apaf-1), and ATP to process procaspase-3.[44] These molecules are sufficient to activate caspase-3 *in vitro*, but other regulatory proteins are necessary for *in vivo*. Hence it was considered worthwhile to examine the cytotoxic effect of **3b** and **3y** by virtue of apoptotic cell death. This prompted us to treat ME-180 cells with compounds **3b** and **3y** to examine the activation of caspase-3,[45] with doxorubicin as a positive control. These results indicate about 3 to 5 fold induction in caspase-3 activity in cells treated with 1 µM concentration by these compounds (Fig. 6).

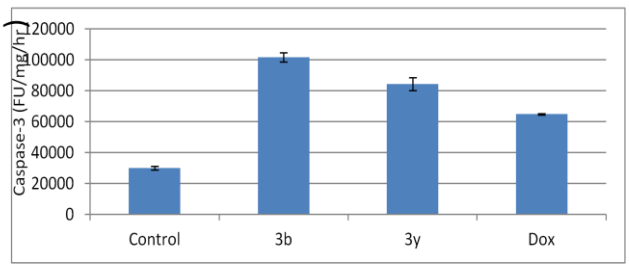


Fig. 6 Effect of the compounds **3b**, **3y** and doxorubicin on caspase-3 activity: ME-180 cells were treated for 48 h with 1 µM concentration. Doxorubicin is used as a positive control. Values indicated are the mean_SD of two different experiments performed in triplicates.

However, under identical conditions, doxorubicin showed the caspase activity about 3 to 4 fold increase as compared to the control. These results are in line with the prediction of apoptosis induction by the activation of caspase-3 in ME-180 cells, treated with **3b** and **3y**.

Molecular Docking studies. Biological studies have shown that these molecules induce cell death by mitochondrial

apoptotic pathway. After the treatment of cancer cells with these molecules, change in membrane potential was observed. To know the possible mechanism, docking studies were carried out with compounds **3b** and **3y** on Bcl-2 protein. Coordinates of protein structure were obtained from Protein Data Bank (PDB ID 2O22).[46] Geometry of the molecules were optimized by Gaussian 09 using PM3 semi-empirical method.[47] Docking studies were performed using AutoDock 4.2 docking software.[48] For docking protocol validation purpose, docking of reference ligand was performed. Docking results showed the similar docking pose to the pose of co-crystal ligand with RMSD 1.19 Å which is in the acceptable range. Therefore, docking studies were performed on most active molecules **3b** and **3y** which are showing similar docking score to that of the reference ligand. Docking pose for **3b** showed that –NH of imidazole ring is forming hydrogen bonding interactions with Gly142 and imidazole ring is forming π-π stacking with Phe101. Besides this, **3b** is forming hydrophobic interactions with Ala97, Arg104, Val145, Ala146 amino acids. Imidazothiadiazole forming hydrophobic interactions with Tyr105, Asp108 amino acids and phenyl ring is forming interactions with Asn140, Arg143 amino acids. Docking studies of these compounds on Bcl-2 protein suggest that Bcl-2 inhibitions could be the possible mechanism for these compounds. The binding pose for the molecule **3b** in Bcl-2 protein was shown in Fig. 7.

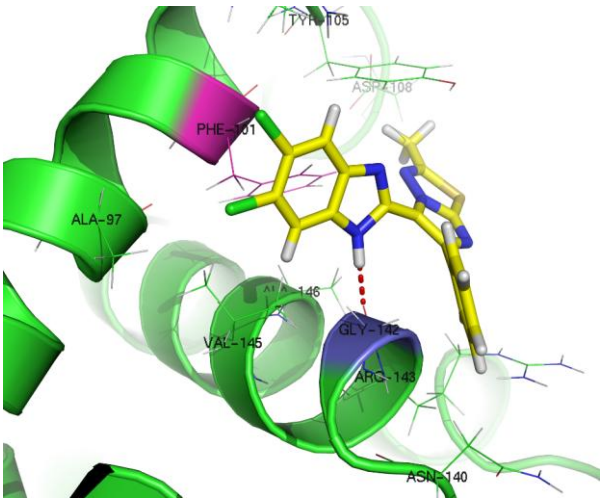


Fig.7 Docking pose for the compound **3b.** Compound is shown in yellow color and the protein is shown in green color. Hydrogen bond is shown in red dotted line. Residues forming hydrogen bonding interactions and π-π interactions are highlighted in different colors.

Conclusion

In summary, a series of imidazothiadiazole–benzimidazole conjugates (**3a**–**z**) were synthesized, evaluated for their anticancer potential against some representative human cancer cell lines (ME-180, A375, DU145 and A549) and showed promising cytotoxicity at micromolar (μM) concentration, with IC_{50} values ranging from 0.5 to >30 μM. Interestingly, two conjugates (**3b** and **3y**) exhibited significant antiproliferative activity (IC_{50}, 0.5 and 0.7 μM respectively) against ME-180 cancer cell line. The loss of mitochondrial membrane potential assay proved that these conjugates induce cell death by mitochondrial apoptosis. Flow cytometric analysis revealed that the cell cycle arrest is in the G_0/G_1 phase. Further, Hoechst 33258 staining, DNA fragmentation assay, Annexin staining and activation of caspase-3 also recommended that they caused cell death by apoptosis. Docking studies of **3b** and **3y** on Bcl-2 protein suggested that Bcl-2 inhibitions could be the possible mechanism for cytotoxic activity of these compounds. Moreover, the overall results indicate that these conjugates based on the imidazothiadiazole–benzimidazole scaffold have the potential to be exploited for their anticancer property and could be considered as an interesting lead for the efficient treatment against cervical cancer.

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Electronic Supplementary Information (ESI) available: Experimental procedures, Spectral data of all the final compounds. See DOI: 10.1039/b000000x/

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