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Isoxazole derivatives of 6-fluoro-*N*-(6-methoxybenzo[*d*]thiazol-2-yl)benzo[*d*]thiazol-2-amine and *N*-(pyrimidin-2-yl)benzo[*d*]thiazol-2-amine: regulation of cell cycle and apoptosis by p53 activation via mitochondrial-dependent pathways

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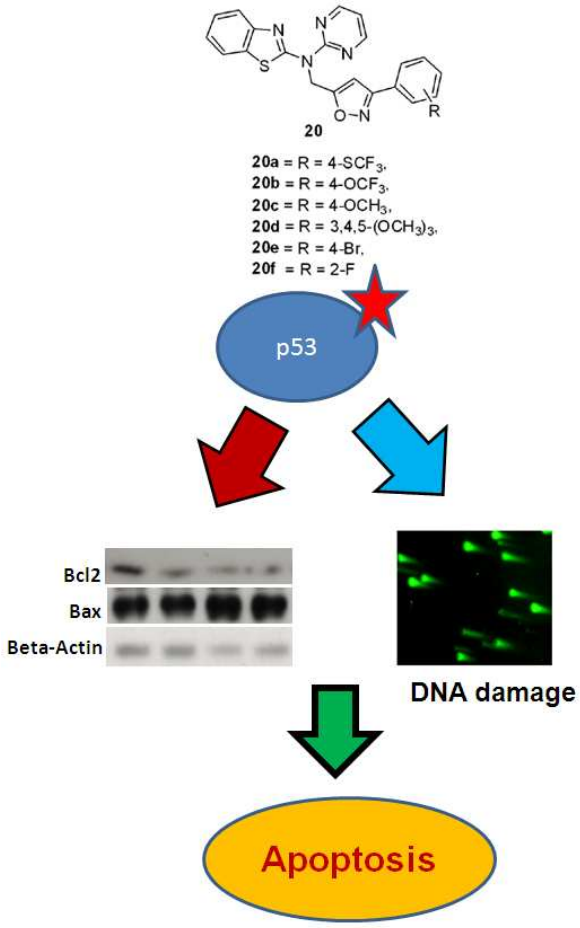

Graphical abstract: Compounds induce DNA damage and activate p53 protein which in turn activates Bax and decreases the levels of Bcl2 protein. These events resulted in apoptosis in colo-205 cells.

Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

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A series of isoxazole derivatives of 6-fluoro-*N*-(6-methoxybenzo[*d*]thiazol-2-yl)benzo[*d*]thiazol-2-amine and *N*-(pyrimidin-2-yl)benzo[*d*]thiazol-2-amine were synthesized and evaluated for their cytotoxicity. These compounds exhibited anti-cancer activity against Colo205, U937, MCF7 and A549 cancer cell lines and were effective in Colo205 cell line than in other cell lines with IC_{50} values ranging from 5.04-13 μM. The detailed biological aspects of one of the promising compound **20c** on the Colo205 cell line were studied. Interestingly, compound **20c** induced G2 /M cell cycle arrest. The levels of p53 increased tremendously in **20c** treated Colo205 cells. The balance in levels of key mitochondrial proteins such as Bcl-2 and Bax was altered which resulted in apoptosis by accelerating the expression of caspases. Thus, Compound **20c** can be considered as a potential small molecule activator of p53 which regulates the equilibrium between rapid cell proliferation and apoptosis and can be considered as a potential plausible candidate for further biological testing in *in vivo* colon cancer models.

Introduction

Colorectal cancer is one of the major leading cause of cancer related death in both men and women[1-3] and is more frequent in the developed countries. Existing data indicates that the chemotherapeutic drugs for colorectal cancer include fluorouracil, capecitabine, UFT, leucovorin, irinotecan or oxaliplatin which can be used alone or in combination with surgery as adjuvant therapy.[4-7]

The emergence of resistance, side effects and limited or transient response associated with current therapies instigate chemists, biologists and the oncologists to explore novel chemotherapeutic molecules for treatment of colorectal cancer. Therefore, successful chemoprevention largely depends on the ability to hunt for promising small molecules which trigger cell death in tumor cells by acting on one or several targets.

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† Electronic Supplementary Information (ESI) available: [Spectral data of Compounds **13a–f, 20a–f,** and experimental procedures for synthesis and biological evaluations]. See DOI: 10.1039/b000000x/

In this scenario, it is interesting to note that p53 is a tumor suppressor that maintains genome integrity to prevent cells from inappropriate growth and division. It also regulates diverse processes such as cell cycle arrest, apoptotic cell death, senescence or DNA repair, depending on the cell type and cellular stress. Therefore, it is an attractive target meant for chemotherapy for the oncologists and the chemists.[8-10] Depending on the strength of DNA damage, p53 preferentially modulates transcription of either pro arrest or proapoptotic target genes.[11]

In this respect, it is noteworthy to uncover that the benzothiazoles offer highly privileged promising structures displaying significant pharmacophoric behaviour with diverse range of biological activities including antitumor activity to form important scaffold of drugs, rendering this molecule and its derivatives as potent chemotherapeutic agents.[12-14] Studies carried out so far indicate that the imidazo benzothiazoles, polymerized benzothiazoles and other substituted benzothiazoles such as 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (PMX 610) **1** exhibit potent and selective *in vitro* antitumor properties in human cancer cell lines (e.g., colon, non small cell lung and breast subpanels).[15,16] Also, effective ligands for the arylhydrocarbon receptor (AhR) which translocate with the drug to cell nuclei have been well studied in the benzothiazoles which include 2-(4-amino-3-methylphenyl) benzothiazole (DF 203) **2** and the 5-fluoro analogue (5F 203) **3**. These subsequently lead to induction of cytochrome p450 CYP1A1 resulting in generation of a reactive chemical intermediate (or intermediates) that selectively

generates DNA adducts only in sensitive tumor types (e.g. mammary and ovarian tumor cell lines). [17-20] A class of antitumor benzothiazoles i.e., benzothiazole substituted 4-hydroxycyclohexadieneone (AW 464) **4** was obtained originally from the oxidation of 2-(4-hydroxyphenyl) benzothiazole with hypervalent iodine oxidants. It is the prototype of a new series of "quinols" with potent antitumor activity against renal and colon cancer cell lines which affects cell signalling events downstream of the redox regulatory protein thioredoxin.[21,22] Another trifluoromethyl substituted series with triazole and oxazole containing nucleus is Mubritinib (TAK-165) **5**. It is known as potent inhibitor of human epidermal growth factor receptor 2 (HER2) tyrosine kinase which was under development by Takeda for the treatment of cancer.[23] Combretastatin A-4 (CA-4), a naturally occurring stilbene showed most potent cytotoxicity against a variety of human cancer cell lines including multiple drug-resistant cancer cell lines. In a recent study, cis-restricted analogues of CA-4 (2-methoxy-5-(5-(3,4,5-trimethoxyphenyl)isoxazol-3-yl)phenol) **6**, were reported to possess potent apoptosis-inducing activity.[24,25]

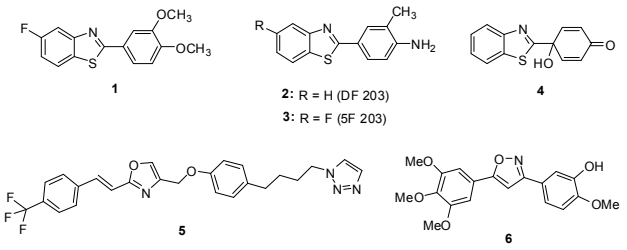


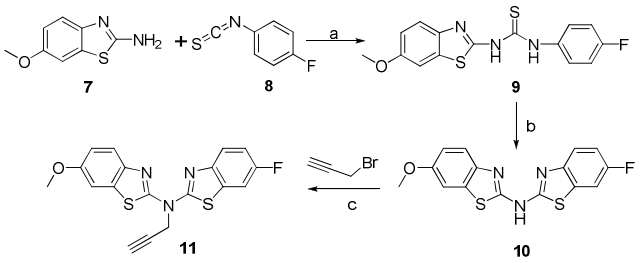
Figure 1. Chemical structure of biologically active anti-tumor agents.

Our recent studies on antitumor activity [26-29] and realizing the importance of benzothiazoles, their derivatives in chemotherapy, and in continuation of our ongoing efforts on the design of novel antitumor agents it was planned to synthesize and perform biological evaluation of certain novel isoxazole linked bis (benzothiazole) and pyrimidine derivatives. A random screen of these analogues on a panel of four selected cancer cell lines displayed higher antitumor activity on colo205 cell line, than others. These compounds caused DNA damage that resulted in cell cycle arrest and apoptosis by p53 dependent pathway involving mitochondria as a key target, the details of which are described herein in the present study.

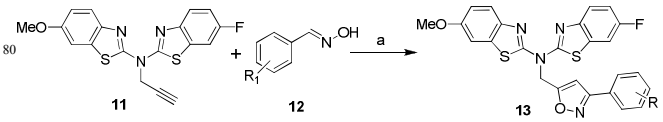
Results and discussion

Chemistry

All the present isoxazole derivatives were synthesized from appropriate aromatic aldoximes **12** and **19** and alkynes **11** and **18** as shown in scheme 2 and 4 using methods described previously in detail by us.[26]First, we designed the compounds **10** and **17** *via* chemoselective oxidative cyclization of thiourea **9** and **16** as per our previously reported method[30] using the readily available starting material **7** and **14**, as given in scheme 1 and scheme 3. Slight structural modification of compound **10** is conceivable to create more attractive compound by replacing one of the core moiety of benzothiazole with pyrimidine which may influence the activity. The compounds **11** and **18** were synthesized by alkylation of compounds **10** and **17** using two equivalents of inorganic base in DMF solvent at ambient temperature. Following reaction with various aromatic aldoximes dissolved in dichloromethane, triethylamine was added simultaneously dropwise to a dichloromethane/aqueous sodium hypochlorite biphasic mixture containing a 10-fold excess of the alkyne dipolarophile. The *in situ* generated nitrile oxide underwent 1,3-dipolar cycloaddition and lead to isoxazole formation.

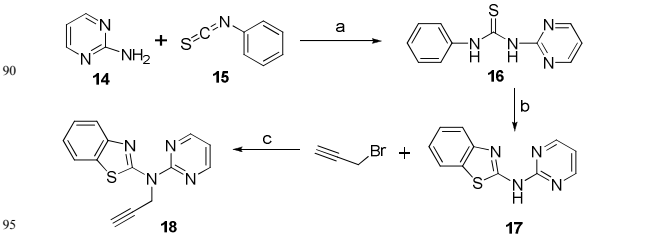


Scheme 1. Reagents and conditions : a) 4-Dimethylaminopyridine (5 mol%), DMF, RT, 4 h; b) [bbim][Br_3], 70 ^{0}C, 40 min.; c) K_2CO_3, DMF, RT, 24 h.

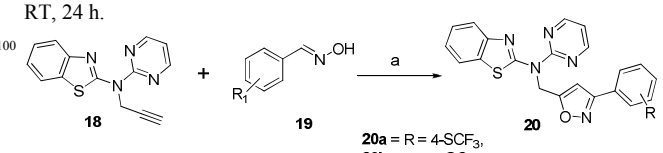


13a = R = 4-SCF_3,
13b = R = 4-OCF_3,
13c = R = 4-OCH_3,
13d = R = 3,4,5-$(OCH_3)_3$,
13e = R = 4-Br,
13f = R = 2-F

Scheme 2. Reagents and conditions: a) aq. NaOCl, Et_3N, 0 ^{0}C → RT, 24 h.



Scheme 3. Reagents and conditions : a) 4-Dimethylaminopyridine (5 mol%), DMF, RT, 3 h; b) [bbim][Br_3], 70 ^{0}C, 40 min.; c) K_2CO_3, DMF, RT, 24 h.



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20d = R = 3,4,5-$(OCH_3)_3$,
20e = R = 4-Br,
20f = R = 2-F

Scheme 4. Reagents and conditions: a) aq. NaOCl, Et_3N, 0 ^{0}C → RT, 24 h.

Biological evaluation

***In vitro* antitumor Evaluation and Structure-Activity Correlations.** The newly synthesised novel Isoxazole derivatives of 6-fluoro-*N*-(6-methoxybenzo[*d*]thiazol-2-yl)benzo[*d*]thiazol-2-amine and *N*-(pyrimidin-2-yl)benzo[*d*]thiazol-2-amine were screened initially for their *in vitro* antitumor activity against the following cancer cell lines: Colon cancer (Colo205), Human leukemic monocyte lymphoma (U937), Breast cancer (MCF7) and Lung cancer (A549) by 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide assay (MTT assay) with etoposide as the standard drug. The data from MTT allowed determination of the inhibitory activities (as IC_{50} in µM) of these compounds as summarized in **Table 1**. Out of the entire series of compounds tested against the above mentioned cell lines, interestingly all the compounds of this series exhibited moderate cytotoxic activity against colon cancer cells i.e., Colo205 with IC_{50} values ranging from 5.04 to 13.39 µM. Among the series, compounds **20b, 20c** and **20e** displayed IC_{50} values of 5.69, 5.04, and 8.82 µM respectively. All the compounds demonstrated least activity against the A549 lung cancer cell line as observed by very high IC_{50} values ranging from 25.43 to 61.93. The cytotoxic response of these effective compounds [**20b, 20c** and **20e]** is Colo-205>U937>MCF-7>A549 **[Table-1]**.

Table 1. *In vitro* cytotoxic effect of compounds **13a-f** and **20a-f** on human colon cancer (Colo205), human leukemic monocyte lymphoma (U937), Breast cancer (MCF7) and lung cancer (A549) cell lines.

Compound	IC50 (µM)			
	Colo205	U937	MCF7	A549
13a	11.08 ± 0.35	42.93 ± 0.25	14.13 ± 0.06	26.54 ± 1.5
13b	9.94 ± 0.22	23.06 ± 0.03	17.40 ± 0.44	37.06 ± 0.75
13c	11.36 ± 0.46	138.23 ± 0.14	32.40 ± 1.82	38.83 ± 0.08
13d	11.78 ± 0.24	44.04 ± 0.42	26.57 ± 0.06	61.93 ± 0.38
13e	7.00 ± 0.51	34.87 ± 0.58	41.15 ± 0.52	32.74 ± 0.62
13f	13.39 ± 0.32	29.45 ± 0.81	28.50 ± 0.84	51.75 ± 0.7
20a	10.10 ± 0.29	19.43 ± 0.09	17.22 ± 0.89	29.80 ± 0.92
20b	5.69 ± 0.06	13.46 ± 0.91	14.76 ± 0.09	31.82 ± 0.23
20c	5.04 ± 0.41	13.9 ± 0.68	30.67 ± 0.64	30.45 ± 1.9
20d	10.21 ± 0.03	26.10 ± 0.74	13.40 ± 0.42	28.38 ± 0.89
20e	8.82 ± 0.31	13.6 ± 0.72	21.12 ± 0.72	31.54 ± 0.74
20f	13.16 ± 0.26	27.56 ± 0.62	19.52 ± 0.66	25.43 ± 0.04
11	30.26 ± 0.78	24.89 ± 1.21	27.76 ± 0.99	24.45 ± 0.67
18	12.04 ± 0.43	15.68 ± 0.45	18.27 ± 0.83	17.35 ± 0.27
Std(Etoposide)	0.47 ± 0.62	10.1 ± 0.75	0.008	4.7 ± 0.85

Cell lines were treated with different concentrations of the compounds as described in Materials and Methods. Cell viability was determined by MTT assay. Compounds were tested in triplicates (n=3). IC_{50} values are indicated as mean +/- SD of three independent experiments. 11 and 18 are the intermediates from which 20a-f are synthesised.

The structural activity relationship studies revealed that the compounds with pyrimidine ring exhibits comparatively substantial anticancer activity than bis benzothiazole derivatives. In particular, compounds **20b, 20c** and **20e** with substituent **4-OCF_3**, **4-OCH_3** and **4-Br** on isoxazole ring show enhanced cytotoxic activity against Colo205 cell line.

20c causes DNA damage *in vitro*

Large-scale analysis of cellular response to anticancer drugs typically focuses on variation in potency (half-maximum inhibitory concentration, (IC_{50})), assuming that it is the most important difference between effective and ineffective drugs or sensitive and resistant cells. But recent studies envisaged the importance of considering the parameters other than potency [IC_{50}] to find out drug response. [31] Etoposide, a known DNA damage causing agent was found to cause strong cytotoxic effect in colon cancer cells and increase the cytotoxic effects of chemotherapeutic agents such as cisplatin in advanced colorectal carcinoma. Studies by Xing et al., 2014 have revealed that the chemosensitivity of colon cancer cells to etoposide was enhanced by FTY720 via the modulation of P-glycoprotein and multidrug resistance protein 1. Also many of the earlier studies of 1990's report use of oral etoposide as second line chemotherapy for colorectal cancer and prostrate cancer.[32-35]

Thus, In order to find out whether **20c** has any possible role in DNA damage *in vitro,* we have conducted alkaline comet assay (single cell gel electrophoresis) [36] in compound treated Colo205 cells. Treatment of cells with either etoposide or **20c** resulted in the formation of comets with significant tail length in more than 90% [i.e 45/50] of treated colon cancer cells when compared to untreated cells where no such tails were found. Bright head and tail formation observed upon treatment with **20c** or etoposide have clearly indicated single strand DNA breaks, whereas negligible or no single strand breaks were observed in control cells as shown in **Figure 2** and **Table 2.**

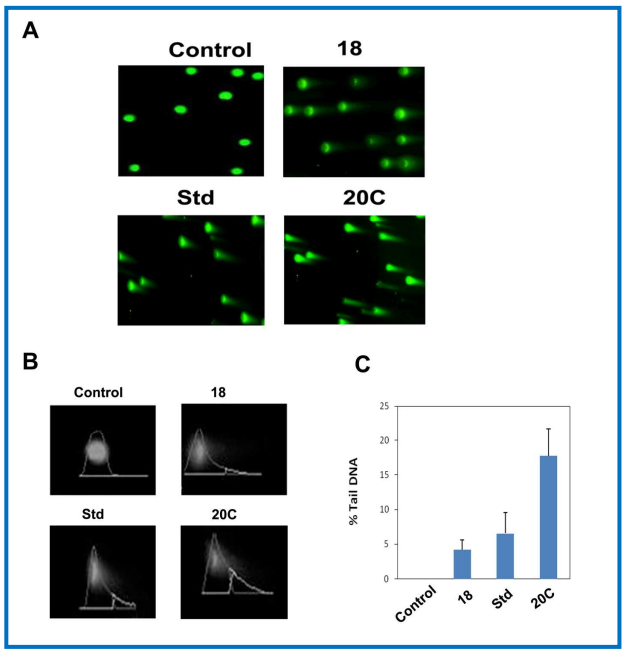

Figure 2. Compound 20c causes DNA damage

(**A**) DNA damage was examined by alkaline comet assay (single cell gel electrophoresis). Control Colo 205 cells as well as cells treated with **18, Std [Etoposide]** and **20c** were subjected to comet assay. **18** is the intermediate from which compounds **20a-f** were synthesized. **Std** is a known positive control for DNA damage. SYBR Green I stained nuclei of cells were analyzed by fluorescence microscopy (Original

magnification, 20X). Here, control cells did not show comet like structures where as treatment with intermediate compound **(18)**, **20c** and **Etoposide** has resulted in comet like structures with tails.(**B**) The extent of DNA damage was determined by Comet Score Software (version 1.5), at least 50 cells/nuclei were analysed for each treatment. (**C**) Semi quantitative analysis of the results presented in (A), expressed as % tail DNA (mean ± S.D.) of three independent experiments, Statistical significance was analyzed by Student's *t*-test (*$P \leq 0.05$)

Table 2. Extent of DNA damage was quantified by measuring comet parameters such as tail length and tail DNA in compound 20c treated Colo205 cells

S. No.	Compound	Tail lenght[a]	%Tail DNA[b]
1	**Control**	0	0
2	**18**	21.4	4.194 ± 1.4
3	**Std**	11.2	6.564 ± 2.9
4	**20c**	27.2	17.72 ± 4.06

[a] refers to the average tail length of cells with COMET. [b] refers to %tail DNA as analysed by the COMET score software. At least 50 cells/ nuclei were analysed for each treatment. The data shown here are representative of three independent experiments

Compound 20c causes G2/M cell cycle arrest

The cell cycle checkpoint is a surveillance mechanism that coordinates with DNA repair by delaying progress of cell cycle following DNA damage. In order to understand the cell cycle regulatory nature of these compounds, flow cytometry was performed. Colo205 cells were treated with respective IC_{50} concentrations of these compounds using Etoposide as positive control for 24h. Compounds caused G2/M cell cycle arrest with apoptotic sub G1 phase cells indicating cell arrest as well as apoptotic nature of these compounds.

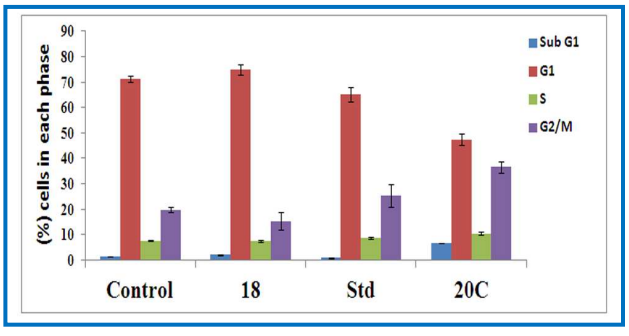

Figure 3. Effect of **20c** on the cell cycle distribution of Colo205 cells. Colo205 cells were treated with **18** (intermediate from which compounds **20a-f** are synthesised), **Std [Etoposide]**, **20c**. The cells were harvested after 24hrs of treatment, subjected to flow cytometry analysis as described in the methods section. Control represents cells treated with DMSO.

Cyclin B1 and Cdk1 form the key Cyclin/Cdk complex that regulate G2/M phase of cell cycle, apart from p53. [37] Therefore, to further strengthen our understanding of mechanism of action of **20c** on Colo205 cells, we also determined the levels of important G2/M phase associated proteins such as Cyclin B1 and Cdk1 by western blot analysis as shown in **Figure 4**. We observed decrease in Cyclin B1 with negligible change in Cdk1. It is obvious that minor changes in levels of key protein(s) can bring large difference(s) in the pattern of cell cycle progression.

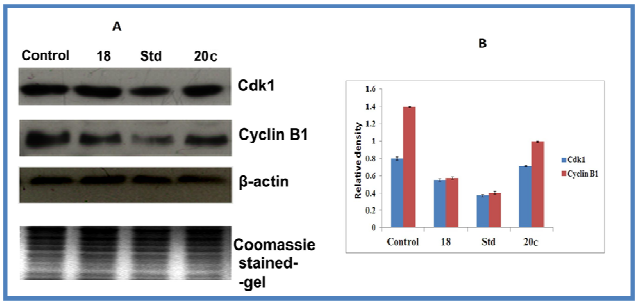

Figure 4. Effect of **20c** on G2/M phase associated proteins. Colo205 cells were treated with **18, Std [Etoposide], 20c** and the whole cell lysates obtained were subjected to western blotting against CyclinB1 and cdk1 proteins (**A**). Representative western blot of three independent experiments is shown. Here β –actin levels and the coomassie stained gel picture show equal loading of samples. (**B**) Represents histogram obtained from densitometric analysis of the bands (relative density against β- actin as arbitrary units) as obtained by the Image J software. Control represents cells treated with DMSO.

Compound 20c induces cell death by p53 dependent apoptotic pathway

p53 is a key modulator of DNA damage and apotosis that involves mitochondria as a key organelle.[38] During DNA damage p53 as a transcriptional activator as well as master regulator of apoptosis gets up-regulated and transactivates a number of genes such as *gadd45*, *p21* and *Bax* that are tightly involved in cell cycle arrest and apoptosis. [39,40] Therefore, we further investigated the plausible effect of compound **20c** in the initiation and activation of the p53 pathway. The levels of p53 and key apoptosis associated proteins (such as Cytochrome c, Apaf1, Caspase 3 and Caspase 9) were determined by western blot analysis in compound **20c** treated Colo205 cells. Interestingly, the western blot results revealed a marked increase in the levels of apoptosis associated mitochondrial targets Apaf1, Caspase 3 [both procaspase 3 and active caspase 3(p17, p21)] and Caspase 9 in **20c** treated Colo205 cells compared to the untreated cells. (**Figure 5**). These results clearly demonstrate that Colo205 cells treated with **20c** cause cell cycle arrest and apoptosis by p53 activation via mitochondrial-dependent pathways.

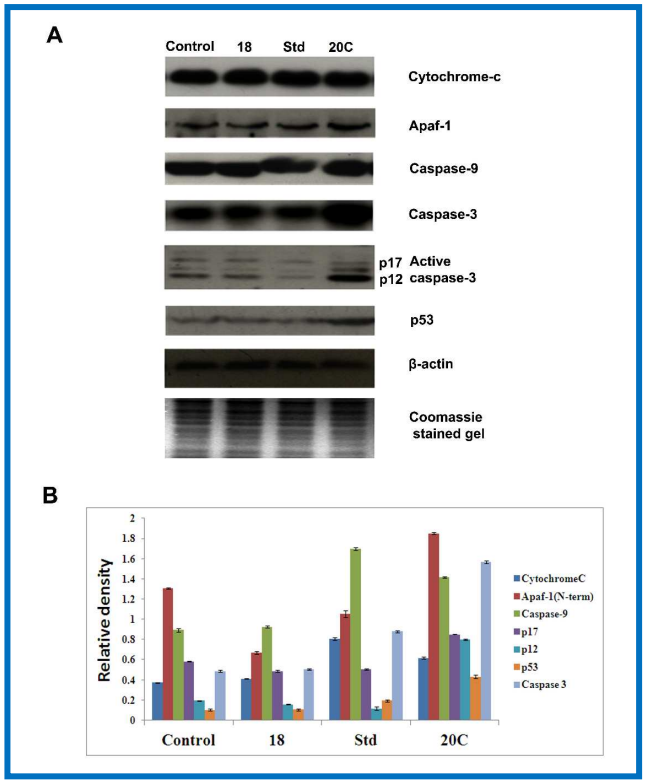

Figure 5. Effect of 20c on apoptosis. Colo205 cells were treated with **18, Std [Etoposide]** and **20c** for 24h and the whole cell lysates were resolved by SDS PAGE followed by western blotting with Cytochrome C, Apaf1, Caspase 9, Caspase 3, p53 antibodies, the major proteins of the p53 dependent apoptotic pathway respectively. Here, a representative western blot of three independent experiments is shown. **(A)** Represents the western blot pictures against their loading control, β –actin. Here coomassie stained gel picture also depicts equal loading of samples. (**B**) Represents histogram obtained from densitometric analysis of the bands (relative density against β- actin as arbitrary units) as obtained by the Image J software. Control represents cells treated with DMSO.

20c inhibits NF-kB via p53 activation

NF-kB is an important transcription factor which contributes to malignant progression and therapeutic resistance of the major forms of human cancer. [41,42] Consecutively, to assess the antitumor effect of **20c** on cell proliferation, we examined the levels of key protein of cell proliferation i.e., NF-kB by western blot analysis.The results demonstrated that compound **20c** inhibited NFkB as indicated by reduction in the levels of this protein compared to that of control (**Figure 6**). Thus, this data clearly shows p53 activation with concomitant reduction in NF-kB in treated cells that might regulate chemosensitivity and apoptosis.

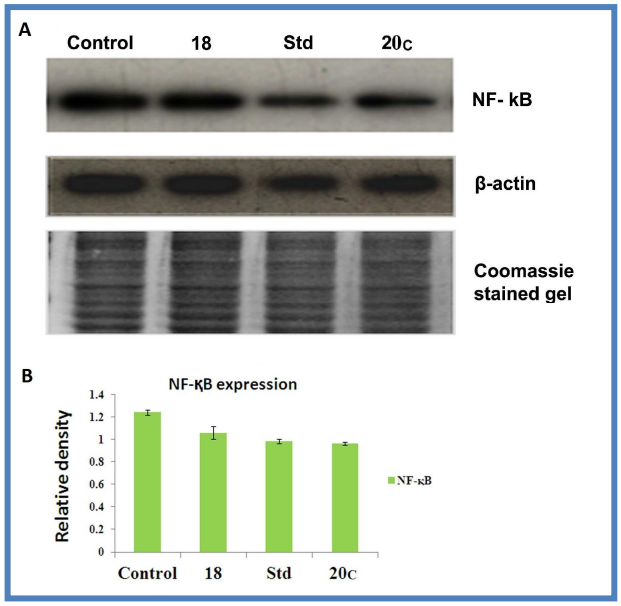

Figure 6. Effect of **20c** on NF-kB. Colo 205 cells were treated with **18, Std [Etoposide]** and **20c** for 24h and the whole cell lysates were resolved by SDS PAGE followed by western blotting with anti- NFkB antibody, A representative western blot of three independent experiments is shown. (**A**) Represents the western blot pictures of the individual protein against their loading control β –actin and the coomassie stained gel picture shows equal loading of samples. (**B**) Represents densitometric analysis of the bands (relative density against β- actin as arbitrary units) as obtained by the Image J software. Control represents cells treated with DMSO.

Effect on expression of transcriptional targets of p53

p53 dependent transcriptional targets such as Bax and Bcl2 present in the mitochondria are the key pro-apoptotic and anti-apoptotic proteins, and their relative ratio determines the vulnerability of cancer cells to apoptosis. [43]

Western blotting analysis revealed decreased level of Bcl2 expression with concomitant increase in the levels of Bax in compound treated cells (**Figure 7**) as compared to that of control. These results indicated that compound **20c** up-regulates the Bax /Bcl2 ratio which in turn activates the caspase 3 cascade, the key protein that regulates apoptotic signaling.

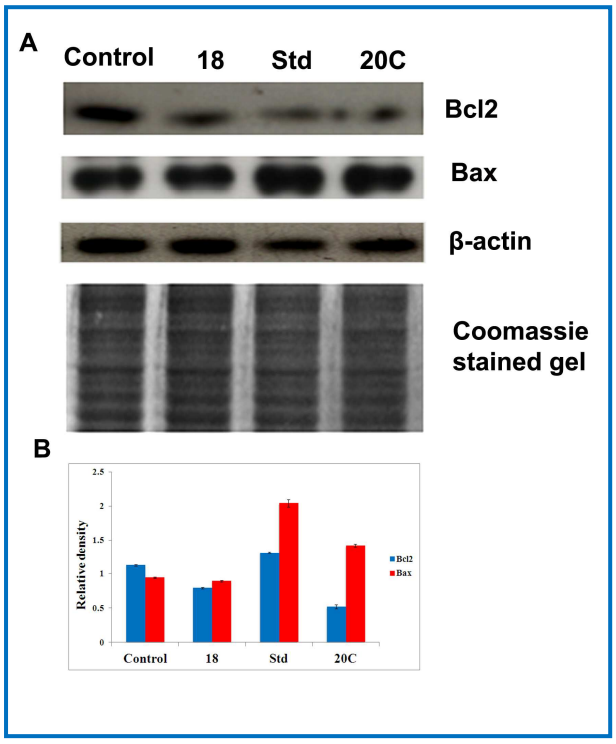

Figure 7. Effect of **20c** on Bcl2 family of Proteins. Colo205 cells were treated with **18, Std [Etoposide]** and **20c** for 24h and the whole cell lysates were subjected to western blotting with Bax and Bcl2 antibodies. (A) Representative western blot of three independent experiments is shown. β –actin and the Coomassie stained gel picture show equal loading of samples. (B) Represents histogram obtained from densitometric analysis of the bands (relative density against β- actin as arbitrary units) as obtained by the Image J software. Control represents cells treated with DMSO.

Conclusion

The synthesis and biological evaluation of a series Isoxazole derivatives of 6-fluoro-*N*-(6-methoxybenzo[*d*]thiazol-2-yl)benzo[*d*]thiazol-2-amine and *N*-(pyrimidin-2-yl)benzo[*d*]thiazol-2-amine has been accomplished. Evaluation against the four different cancer cell lines revealed compounds within this series to have appreciably higher antitumor activity against the colorectal cancer, colo-205 cell line (IC_{50} values in the range of 5.04 to 13.39 μM). Further, among the entire series, compound **20c** displayed significant antitumor activity on Colo205 cells than on other cancer cells. Delineation of compound **20c** mode of action reveals that the observed cytotoxic activity appears to emerge by DNA damage, through a p53 dependent apoptotic pathway. These findings identify compound **20c** as a novel promising candidate in this series which can be taken up for detailed *in vivo* biological studies in colon cancer model systems.

Acknowledgements

We are thankful to Dr. J. S. Yadav, Former Director, IICT for providing facilities, T.D. thank UGC, AN and U. K. thank CSIR, New Delhi for the award of fellowship. We are thankful to Y. Suresh, Chemical biology Department, CSIR-IICT, for technical assistance in providing the FACS data. R.M.K thanks S.E.R.C., Department of Science & Technology, Government of India for financial assistance under the Fast Track Scheme for young scientists (SR/ FT/CS-031/2010).

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