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Synthesis and biological evaluation of novel 1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylic acid derivatives and their precursors as antileukemic agents

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ABSTRACT

We report here the synthesis and preliminary evaluation of novel 1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylic acid derivatives **6**(**a–k**) and their precursors **5**(**a–k**) as potential chemotherapeutic agents. In each case, the structures of the compounds were determined by FTIR, ^{1}H NMR and mass spectroscopy. Among the synthesized molecules, methyl 1-(4-methoxyphenethyl)-2-(4-fluoro-3-nitrophenyl)-1*H*-benzimidazole-5-carboxylate (**5a**) induced maximum cell death in leukemic cells with an IC_{50} value of 3 μM. Using FACS analysis we show that the compound **5a** induces S/G2 cell cycle arrest, which was further supported by the observed down regulation of CDK2, Cyclin B1 and PCNA. The observed downregulation of proapoptotic proteins, upregulation of antiapoptotic proteins, cleavage of PARP and elevated levels of DNA strand breaks indicated the activation of apoptosis by **5a**. These results suggest that **5a** could be a potent anti-leukemic agent.

Among different types of cancers, leukemia is one of the major causes of cancer related deaths.[1,2] Leukemia originates from hematopoietic stem cells or cells at different stages of myeloid or erythroid differentiation which spread throughout the body. Although, the success of clinical trials in identifying new agents and treatment modalities has been significant, current treatments suffer from many limitations such as side effect of the drugs and drug resistance. Hence, the identification of novel, efficient and less toxic anticancer agents remains an important and challenging task in cancer biology.

The benzimidazole nucleus is the key building block for a variety of compounds that play crucial roles in the function of a number of biologically important molecules.[3] Benzimidazole derivatives have shown different therapeutic properties such as antiulcer,[4] antihelminthic,[5] antihypertensive,[6] anticoagulant,[7] antiallergic,[8] analgesic,[9] anti-inflammatory,[10] antimicrobial,[11] antiviral,[12] antiparasitic[13] and antioxidant.[14] It is also reported that, the benzimidazole nucleus is an essential part of many antineoplastic derivatives.[15] TREANDA® (bendamustine hydrochloride) containing alkylating group and benzimidazole component has been used for the treatment of patients with chronic lymphocytic leukemia (CLL) and has been approved by FDA.

Because of their significant medicinal importance, the synthesis of substituted benzimidazoles has become a focus of synthetic organic chemistry. Earlier we showed the antiproliferative effect and mechanism of induction of apoptosis by various bioactive heterocyclic compounds and reported the impact of electron releasing and withdrawing groups on the induction of apoptosis.[16–24] In continuation with our efforts in search of potential anticancer agents, we have synthesized a series of novel 1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylic acid derivatives **6**(**a–k**) and their precursors **5**(**a–k**) by introducing different substituted aryl groups at 2nd position of benzimidazole nucleus.

Here, we report the synthesis and screening of antiproliferative activities of a series of novel 1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylic acid derivatives **6**(**a–k**) and their precursors **5**(**a–k**). Among the compounds, **5a** is identified as a potential therapeutic agent for leukemia. We find that **5a** induces DNA damage leading to cell cycle arrest at S and G2/M phases culminating into apoptosis.

Synthesis of 1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylic acid derivatives was outlined in Scheme 1. 4-Fluoro-3-nitro-benzoic acid **1** was dissolved in dichloromethane (MDC).

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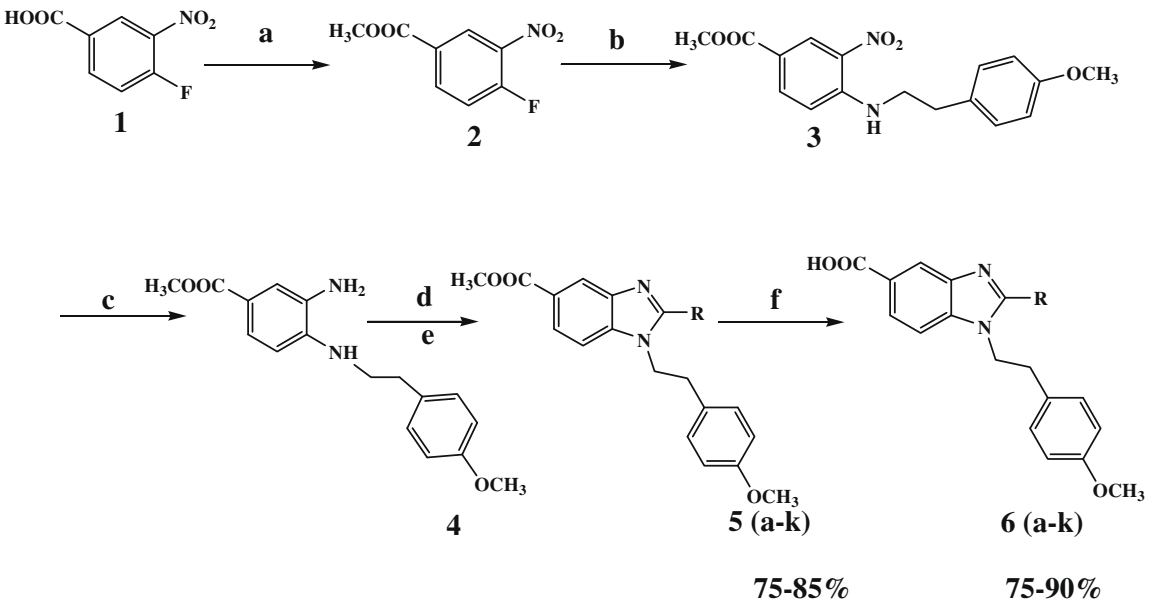


Scheme 1. Synthesis of benzimidazole derivatives **5**(**a–k**) and **6**(**a–k**). Reagents and conditions: (a) Thionyl chloride, Methanol, MDC, reflux condition; (b) 2-(4-methoxyphenyl)ethanamine, DIPEA, DMF, rt, 3 h; (c) Fe powder, NH4Cl, IPA, 70 °C, 4 h; (d) R–COCl, TEA, MDC, rt, 3 h; (e) CH3COOH, 90 °C, 8 h; (f) LiOH, THF/water (2:1), reflux condition.

Methanol and thionyl chloride was added to it, and under reflux condition the compound **2** was formed. Nucleophilic aromatic substitution of fluorine group with 2-(4-methoxyphenyl)ethanamine gave the product **3**. The aryl nitro group was then reduced to amine **4** using NH_4Cl solution and 20% iron (II) powder. The selective acylation of primary aromatic amine followed by cyclization gave the precursors **5**(**a–k**). The hydrolysis of **5**(**a–k**) gave the final compounds **6**(**a–k**). Compounds **5**(**a–k**) and **6**(**a–k**) were obtained in good yield, purified by column chromatography using hexane and ethyl acetate, and recrystallized using diethyl ether and hexane. The resulting compounds were characterized by ^{1}H NMR, FTIR, and LCMS analysis (Fig. 1, Table 1 and Suppl. Fig. S1, S2, S3 and S4).

Trypan blue exclusion assay was the first line of our investigation, where we assessed the effect of benzimidazole derivatives **5**(**a–k**) and **6**(**a–k**) on viability of different leukemic cell lines (K562 and CEM). The leukemic cells were treated with different benzimidazole derivatives at concentrations of 10, 100 and 250 μM. Since the compounds were dissolved in DMSO, the cells with DMSO (equivalent to DMSO used in 250 μM) were used as vehicle control. The cells were counted at intervals of 24 h till they attained stationary phase. Results showed that the addition of benzimidazole derivatives **5**(**a–k**) and **6**(**a–k**) affected the viability of cells (Suppl. Fig. S5 A–D) in a dose- and time-dependent manner. In both cell lines tested, the derivatives, **5a, 5e–g, 5i, 5k, 6–h** and **6j** showed remarkable effect on cell viability at concentrations of 100 and 250 μM, within 48 h itself. However, in the case of other derivatives the effect was limited. The DMSO control, corresponding to the highest concentrations of drugs tested did not show any significant toxic effect. The cytotoxicity induced by benzimidazole derivatives **5**(**a–k**) and **6**(**a–k**) was further verified using MTT assay (Fig. 2 and Suppl. Fig. S6 A–D). Based on the trypan blue and MTT assays, IC_{50} values were calculated for both K562 and CEM cells (Table 1).

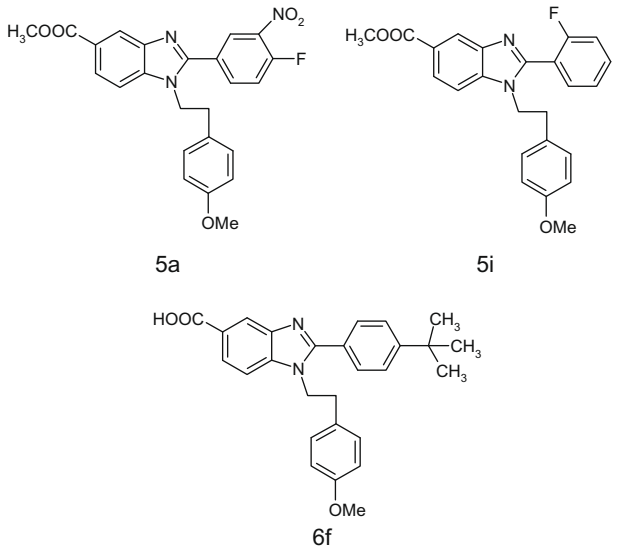


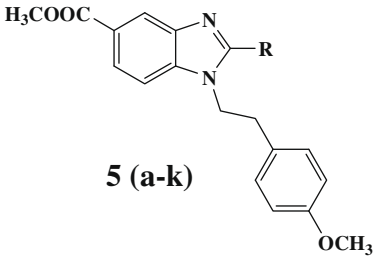
Figure 1. The structure of the benzimidazole derivative **5a**, **5i** and **6f**.

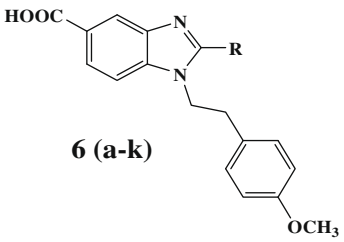
Release of LDH is an indicator of cell injury and hence cell death. To confirm the cytotoxicity exhibited by some of the compounds (**5a**, **5i** and **6f**; Fig. 1) we have carried out an LDH assay. Results showed a dose- and time-dependent increase in the LDH release upon treatment with the compounds, further confirming the above results (Fig. 3).

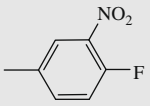
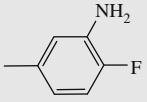
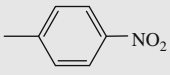
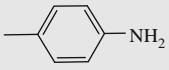
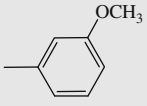
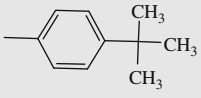
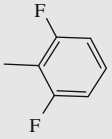
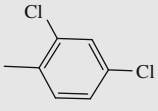
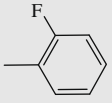
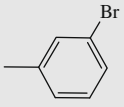
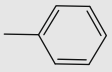
Studies thus far described clearly show that **5a** affects the viability of the cells significantly. Therefore, we were interested in understanding the mechanism by which **5a** induces cell death. First, we tested whether **5a** induces cell death by affecting cell proliferation. Results of tritiated thymidine incorporation assay showed a time- and dose-dependent decrease in incorporation of [^{3}H]-thymidine upon treatment with **5a** in K562 cells (Fig. 4A). These studies indicated that **5a** could be interfering with DNA replication affecting cell division and hence proliferation.

Many chemotherapeutic compounds have been shown to have antiproliferative effects by inhibiting cell cycle at certain checkpoints.[25] Similarly, certain compounds are believed to function via cell cycle-mediated apoptosis.[26,27] In order to study whether **5a** could inhibit cell proliferation by cell cycle arrest, FACS analysis was performed after treatment with **5a**. Results showed that the cell cycle progression was arrested at S and G2 phases (Fig. 4B and Suppl. Fig. S7). Therefore, our studies suggested that growth

Table 1
IC_{50} value of **5**(**a**–**k**) and **6**(**a**–**k**) compounds on K562 and CEM cells





Compound	R	IC_{50} (μM)		Compound	IC_{50} (μM)	
		K562	CEM		K562	CEM
5a		3	4	**6a**	135	132
5b		78	75	**6b**	180	182
5c		120	122	**6c**	250	245
5d		180	175	**6d**	>250	>250
5e		136	138	**6e**	200	195
5f		70	68	**6f**	40	42
5g		84	85	**6g**	80	85
5h		>250	>250	**6h**	110	102
5i		56	55	**6i**	240	244
5j		>250	>250	**6j**	124	120
5k		64	65	**6k**	>250	>250

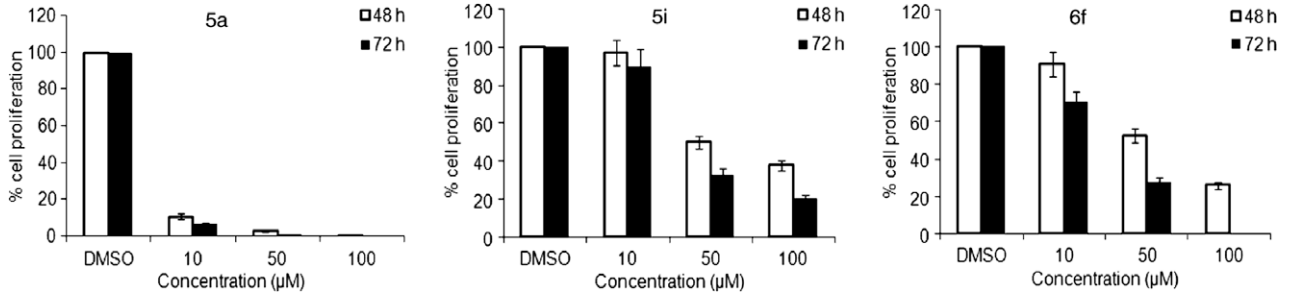


Figure 2. Determination of cell proliferation by MTT assay. K562 cells were cultured with different concentrations of **5a**, **5i** or **6f** for 48 and 72 h. After harvesting the cells, MTT (0.5 mg/ml) was added and processed. The absorbance at 570 nm was measured using ELISA plate reader.

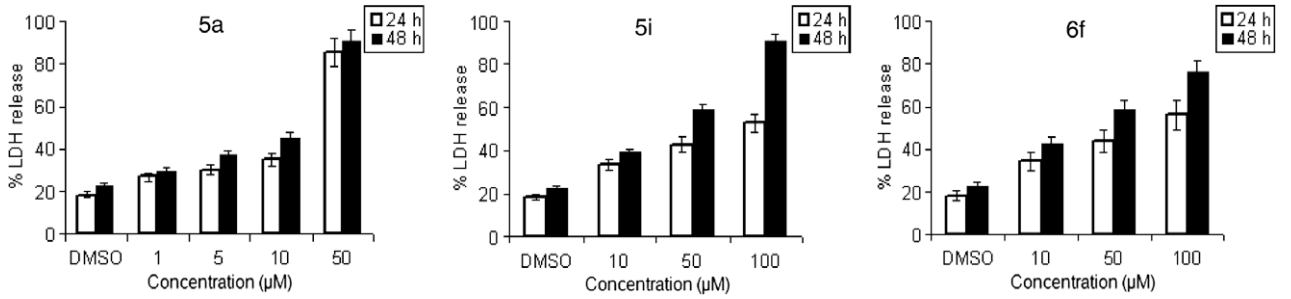


Figure 3. Measurement of LDH release following treatment with **5a**, **5i** and **6f**. After the exposure of K562 cells with different concentrations of **5a**, **5i** and **6f** for 24 and 48 h, the release of LDH was measured at 490 nm. Results are presented as percentage of LDH release.

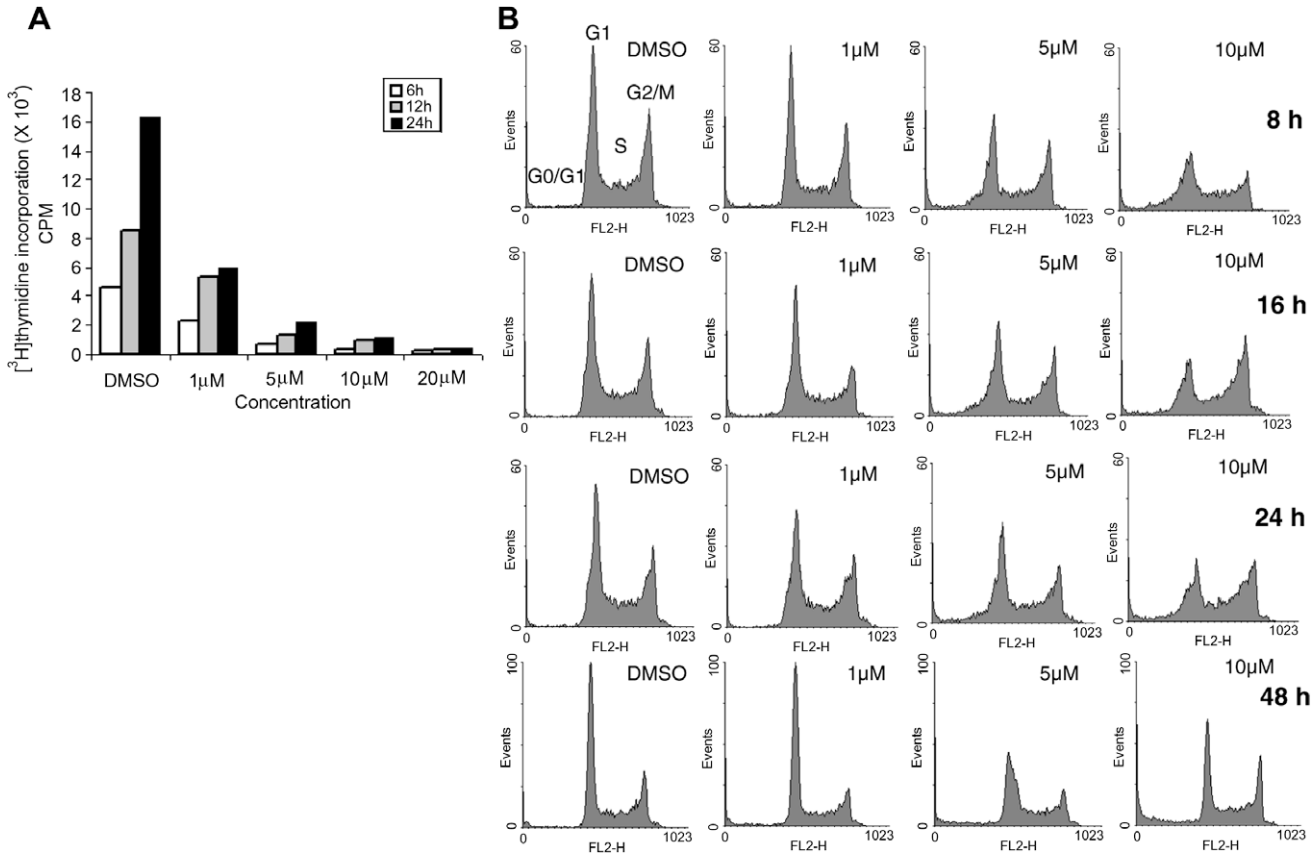


Figure 4. Compound **5a** affects cell division and progression of cell cycle in leukemic cells. (A) Tritiated thymidine assay. Different concentrations of **5a** were treated with K562 cells for indicated time. Histogram shows the dose-dependent incorporation of tritiated thymidine into DNA. (B) FACS analysis of **5a** treated (1, 5, and 10 μM) K562 cells. DMSO treated cells were used as vehicle control. (C) Treatment of **5a** leads to the alteration in the level of replication related proteins. The deregulation of p-Histone 3, PCNA, Cyclin B1, CDK2 and p21 are studied using western blotting. Tubulin was used as the internal control. Histograms in the right panel show the normalized level of proteins studied following **5a** treatment.

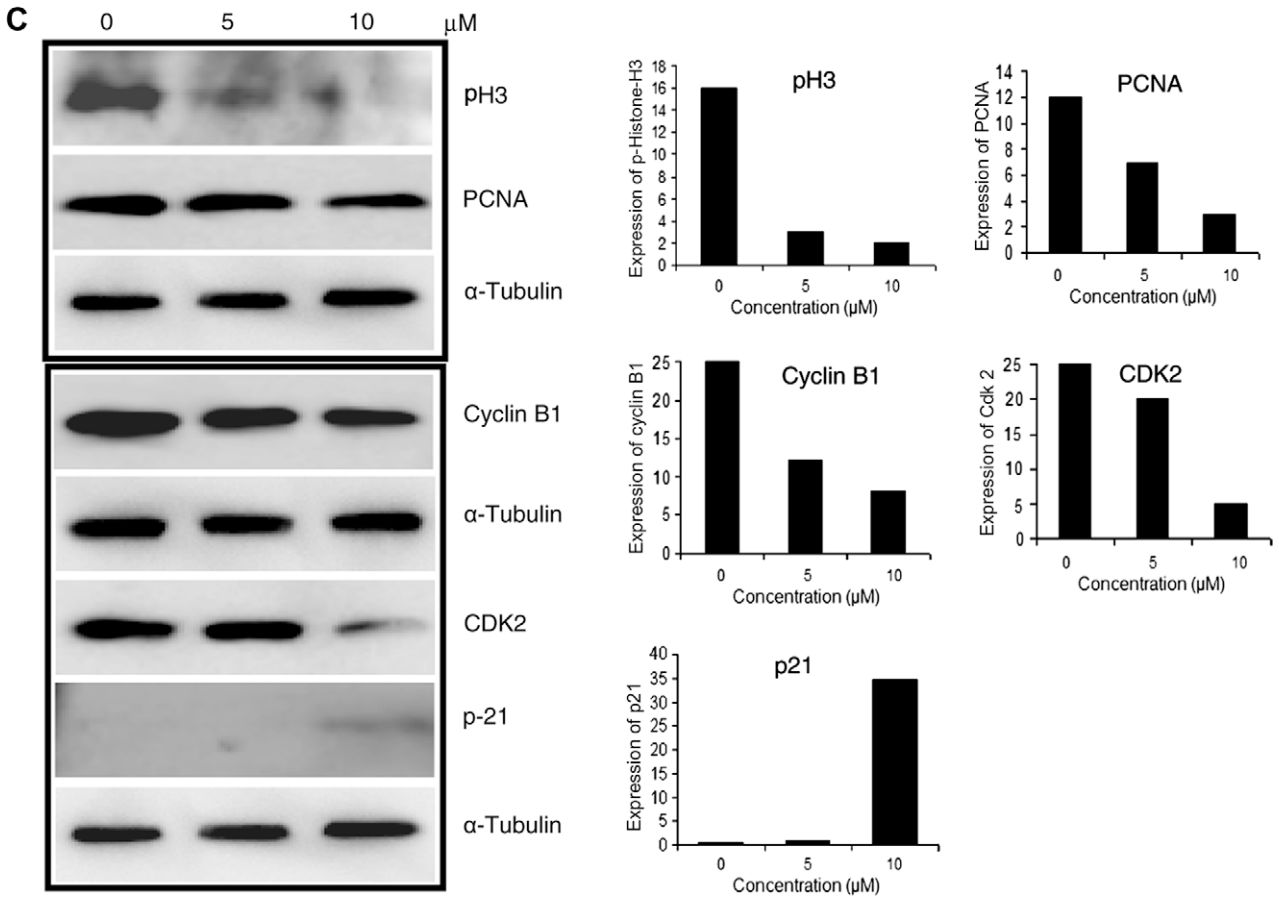


Fig 4. (*continued*)

inhibition observed could be mediated through a DNA replication defect followed by cell cycle arrest leading to apoptosis.

Since a cell cycle arrest was observed, we were interested in testing the expression levels of replication related proteins in K562 cells following treatment with **5a**. Western blot analysis showed that, the levels of p-histone H3 (marker for cell division) reduced upon treatment with **5a** at concentrations of 5 and 10 μM. However, robust expression was seen in the control (Fig. 4C). These results indicated that histone H3, (a histone normally phosphorylated at M phase) was not phosphorylated when cells were treated with **5a**. Moreover, we noted that the levels of expression of PCNA, another protein required for replication was reduced upon treatment with **5a** (Fig. 4C). In addition to this we find that **5a** treatment leads to a concentration dependent down regulation of cyclin B1 explaining the observed cell cycle arrest in G2/M phase. At 10 μM concentration, we also found that **5a** could induce down regulation of CDK2. This is consistent with the observed S phase arrest. Since earlier reports have shown that the presence of p21 could lead to the inhibition of PCNA and CDK2, we were interested to test whether **5a** treatment activates p21. Western blot analysis showed that though the p21 level was very low in the normal cells, treatment with **5a** induced activation of p21 (Fig. 4C), further justifying the downregulation of PCNA and CDK2. Hence, our results suggest that **5a** downregulates expression of critical cell cycle related proteins to induce cell cycle arrest.

The formation of distinct DNA fragments of oligonucleosomal size is a biochemical hallmark of apoptosis in many cells. Hence we were interested to see whether **5a** could induce DNA damage. To test this, K562 cells treated with 10, 50 and 100 μM of **5a, 5i** or **6f** for 72 hr were harvested and used for extraction of chromosomal DNA. The extracted DNA was run on agarose gel. The observed smear could be the result of DNA breakage at multiple positions across the chromosomal DNA (Suppl. Fig. S8). Compound **5i** and **6f** showing maximum strand breaks at 100 μM whereas **5a** showed good amount of DNA strand breakage even at 10 μM. In the case of DMSO control, there were only limited strand breaks. These results indicate that **5a** is able to induce significant amounts of DNA strand breaks including double-strand breaks (DSBs). Although, we did observe DNA strand breaks, we could not see DNA fragments of oligonucleosomal size. This suggests that most likely **5a** might be inducing damage in the DNA, leading to strand breaks. However, such a hypothesis needs to be verified further.

Finally we check the effect of **5a** on expression proteins associated with apoptosis, like BAD, BCL2 and PARP. Results showed that **5a** treatment led to the upregulation of proapoptotic protein, BAD in a time-dependent manner (Fig. 5). In contrast, the expression of antiapoptotic protein BCL2 went down immediately after **5a** treatment (Fig. 5). Poly (ADP-ribosyl) polymerase (PARP) is a single-strand break repair enzyme (116 kDa). It is known that upon activation of apoptotic pathway, caspase cleaves PARP into 85 and 27 kDa polypeptides.[28] By immunoblotting analysis using anti-PARP, we found that the addition of **5a** led to significant PARP cleavage, resulting in the accumulation of the 85 kDa product (Fig. 5). The maximum PARP cleavage was seen after 12 h treatment of **5a**. (Fig. 5). Hence, these results suggest that **5a** treatment affects the ratio of proapoptotic:antiapoptotic proteins normally seen in the cells, and that leads to the activation of apoptosis.

The observed structure–activity relationship indicated that activity of both the series of compound varied at different levels.

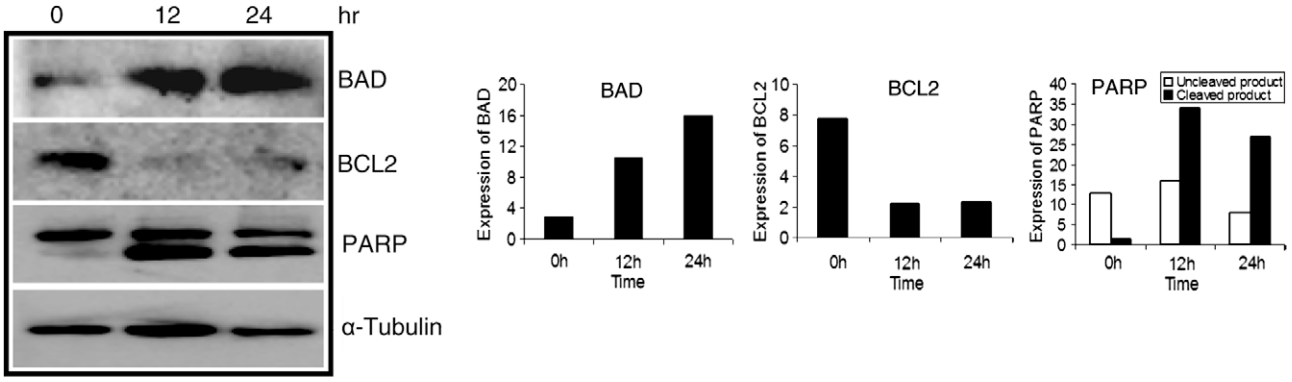


Figure 5. Compound **5a** alters expression of apoptotic proteins in K562 cells. Cell lysate was prepared from K562 cells after treating with 30 μM of **5a** for 12 and 24 h (labelled as '12' and '24', respectively). DMSO treated cells grown for 24 h was used as vehicle control (labelled as '0'). Approximately, 20 μg of protein per sample was resolved on SDS–PAGE and transferred to a PVDF membrane. The membrane was probed for the expression of BAD, BCL2 and PARP using specific primary antibody and appropriate secondary antibody. The α-tubulin was used as an internal loading control. The histogram showing quantification of respective gels are shown on the right.

When we compare **5(a–k)** series (ester) with **6(a–k)** series (carboxylic acid) of compounds, we note that most of the compounds in ester derivatives exhibited better activity than carboxylic acid derivatives. However, we did not observe any significant difference in the activity exhibited by these compounds between the cell lines tested. Among the ester derivatives, it is noteworthy to mention that, compound **5a**, having a fluoro at the para position and a nitro at the meta position exhibited growth inhibitory activity at an IC_{50} value of 3 μM, whereas replacement of the same with only ortho fluoro group (**5i**) and nitro group with amine group (**5b**) decreases the activity by 19 and 25-fold, respectively (Table 1). Furthermore, nitro (**5c**) and amine group (**5d**) at the para position and methoxy group (**5e**) at meta position exhibited low activity with IC_{50} value ranging from 75 to 180 μM in both the cell lines (Table 1). When we introduced tertiary butyl group (**5f**) and fluorine groups (**5g** and **5i**) improved cytotoxicity was observed. A significant loss in the activity was observed when fluorine was replaced with chloro (**5h**) and bromo (**5j**) groups. Although it is difficult to explain the observed variations in the activity with respect to substituents in growth inhibition, it is important to point out that phenyl ring without any substituents in fact exhibited only a moderate activity. In carboxylic acid derivatives **6**(**a–k**), compound **6f** containing tertiary butyl group showed good activity relative to all other compounds of the same series.

In summary, our SAR study shows that both ester and carboxylic acid derivatives exhibited different levels of antiproliferative activity upon treatment on leukemic cells. Further studies are underway to understand the structure-activity relationship of these novel benzimidazole derivatives. The screening of 22 novel compounds has led to the discovery of methyl 2-(4-fluoro-3-nitrophenyl)-1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylate (**5a**), containing electron-withdrawing nitro ($-NO_2$) and fluorine (F) groups, which showed an excellent inhibition on human leukemic cells with an IC_{50} of 3 μM. Tritiated thymidine incorporation assay suggested that **5a** inhibits the DNA replication affecting the cell proliferation. Besides, flow cytometric analysis indicated that such a block on DNA synthesis could lead to cell cycle arrest at S/G2 phase. DNA fragmentation studies in conjunction with immunoblotting and FACS analysis further suggest that following cell cycle arrest, **5a** triggers activation of apoptosis. However, the molecular mechanism through which this molecule elicits its effect in the induction of apoptosis is currently under investigation. Further, the role DNA repair proteins in this context are also being investigated. Hence, the present study provides a new insight of this novel benzimidazole derivative serving as a potential therapeutic agent against leukemia.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.103.

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