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# Novel rhodanine derivatives induce growth inhibition followed by apoptosis

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ABSTRACT

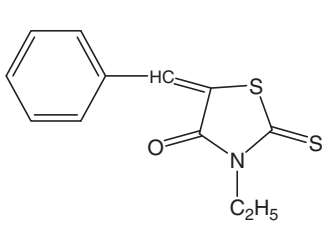
We have designed and synthesized three novel compounds, 5-isopropylidiene derivatives of 3-dimethyl-2-thio-hydantoin (**ITH-1**), 3-ethyl-2-thio-2,4-oxazolidinedione (**ITO-1**), and 5-benzilidene-3-ethyl rhodanine (**BTR-1**), and have tested their chemotherapeutic properties. Our results showed that all three compounds induced cytotoxicity in a time- and concentration-dependent manner on leukemic cell line, CEM. Among the compounds tested, **BTR-1** was 5- to 7-fold more potent than **ITH-1** and **ITO-1** when compared by trypan blue and MTT assays. $IC_{50}$ value of **BTR-1** was estimated to be <10 μM. Both cell cycle analysis and tritiated thymidine assays revealed that **BTR-1** affects DNA replication by inducing a block at S phase. **BTR-1** treatment led to increased level of ROS production and DNA strand breaks suggesting activation of apoptosis for induction of cell death.



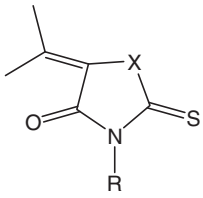
Cancer is one of the difficult diseases to be cured, and very few effective drugs are available. The development of novel, efficient, and less toxic anticancer agents remains an important and challenging goal in medicinal chemistry. Understanding the molecular mechanism involved in cancers will lead to identification of novel anticancer agents. Changes in DNA, RNA, and protein levels due to mutations have been analyzed in many cancers, including leukemia and lymphoma.[1–4] Recently, extensive efforts were made towards characterization of the mechanism of chromosomal translocations involved in many lymphoid cancers.[5,6] The proteins mostly responsible for leukemia and lymphoma in the recent past are Recombination Activating Genes (RAGs, the enzyme responsible for antibody diversity)[1,7–9] and Activation Induced Deaminase (AID, an enzyme responsible for somatic hypermutation and class switch recombination).[2,10–12]

Leukemia is one of the major types of cancers affecting a significant segment of the population. In fact, leukemia is the most frequent childhood cancer, with 26% of all cases, and 30% mortality.[13] Although the incidence rate for this disease remains relatively unchanged, some success has been attained in its treatment. However, the current treatments have many limitations. This includes side effects induced by the drugs and the development of acquired drug resistance.[14] Thus, the need for the development of effective anti-cancer therapeutic agents with well-defined pharmacokinetic properties is of importance.

Rhodanine derivatives have been proven to be attractive compounds due to their outstanding biological activities and have undergone rapid development as anticonvulsant, antibacterial, antiviral, and antidiabetic agents.[15,16] At the same time, these have also been reported as hepatitis C virus (HCV) protease inhibitors[17] and used as inhibitors of uridine diphospho-*N*-acetylmuramate/L-alanine ligase.[18] Recently, substituted rhodanines were investigated for tau aggregation inhibitor properties.[19] Rhodanines are classified as nonmutagenic[20] and a long-term study on the clinical effects of the rhodanine-based Epalrestat as an anti-diabetic, dem-




BTR-1




ITO-1; R = $C_2H_5$, X = O
ITR-1; R = $C_2H_5$, X = S
ITH-1; R = $CH_3$, X = $CH_3$

**Figure 1.** The five membered heterocyclic core; rhodanines (X = S), thiohydantoin (X = $NCH_3$), and thioxooxazolidine (X = O).

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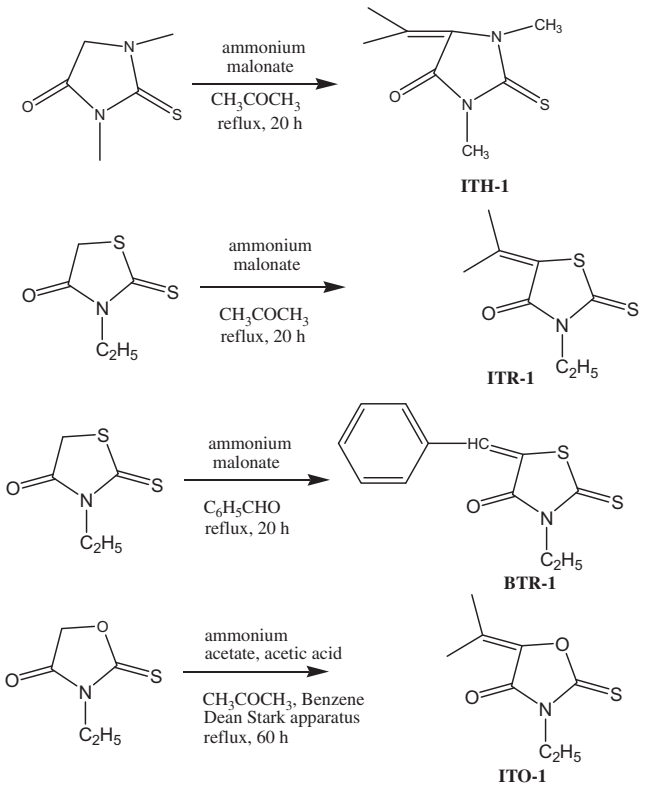
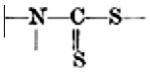
onstrated that it is well tolerated.[21] Additionally, rhodanines have been designed as inhibitors of various enzymes such as bacterial β-lactamase and Mur ligases.[22] Rhodanine derivatives were found to have marked mildew-proofing activity. It is interesting to note that the new mildew-proofing agents contain the structure




**Scheme 1.** Synthesis of the five member heterocyclic cores.



present in many plant fungicides (tetramethylthiuram disulfide and the salts of dithiocarbamic acid), as well as a carbonyl group conjugated with an ethylenic linkage, found in another class of fungicides.[23] Due to various possibilities of chemical derivatization of the rhodanine ring, rhodanine-based compounds will probably remain a privileged scaffold in drug discovery. Therefore, the synthesis of these compounds is of considerable interest. However, the anticancer properties of rhodanine derivatives were never investigated extensively.

Thus, in the present study we report synthesis of three novel rhodanine derivatives, 5-isopropylidiene derivates of 3-methyl-2-thio-hydantoin (**ITH-1**), 3-ethyl-2-thio-2,4-oxazolidinedione (**ITO-1**) and 5-benzilidene-3-ethyl rhodanine (**BTR-1**). The biological activity of the compounds was assayed using various cytotoxic and cellular assays. Results showed that **BTR-1** was many fold more potent than **ITH-1** and **ITO-1** with an $IC_{50}$ value around 10 μM. Further, we show that **BTR-1** affects DNA replication by inducing a block at S phase followed by induction of cell death by apoptosis.

*Chemical synthesis*. In our previous study, **ITR-1** showed encouraging results and was capable of inducing cytotoxic activity and apoptosis in CEM with an $IC_{50}$ of 40 μM,[24] which makes it promising for further synthesis and optimization by QSAR studies. For synthesis, we employed a very simple approach and focused on the heterocycle as well as its substituents. Initially, we focused on the central heterocycle itself, replacing the original rhodanine core of **ITR-1** with other heterocycles and in these experiments, we prepared rhodanines (X = S), thiohydantoin (X = $NCH_3$), and thioxooxazolidine (X = O) (Fig. 1) as per the Scheme 1.

*ITH-1, ITO-1, and BTR-1 induces cytotoxicity in T-cell leukemic cell line in a dose- and time-dependent manner*. In order to evaluate the cytotoxic effect of **ITH-1**, **ITO-1**, and **BTR-1** on T-cell leukemic cell line (CEM), we used trypan blue assay as described in Supplementary Methods. CEM cells were treated with 10, 50, 100, or 250 μM of **ITH-1**, **ITO-1**, and **BTR-1**. Since the compounds were dissolved in

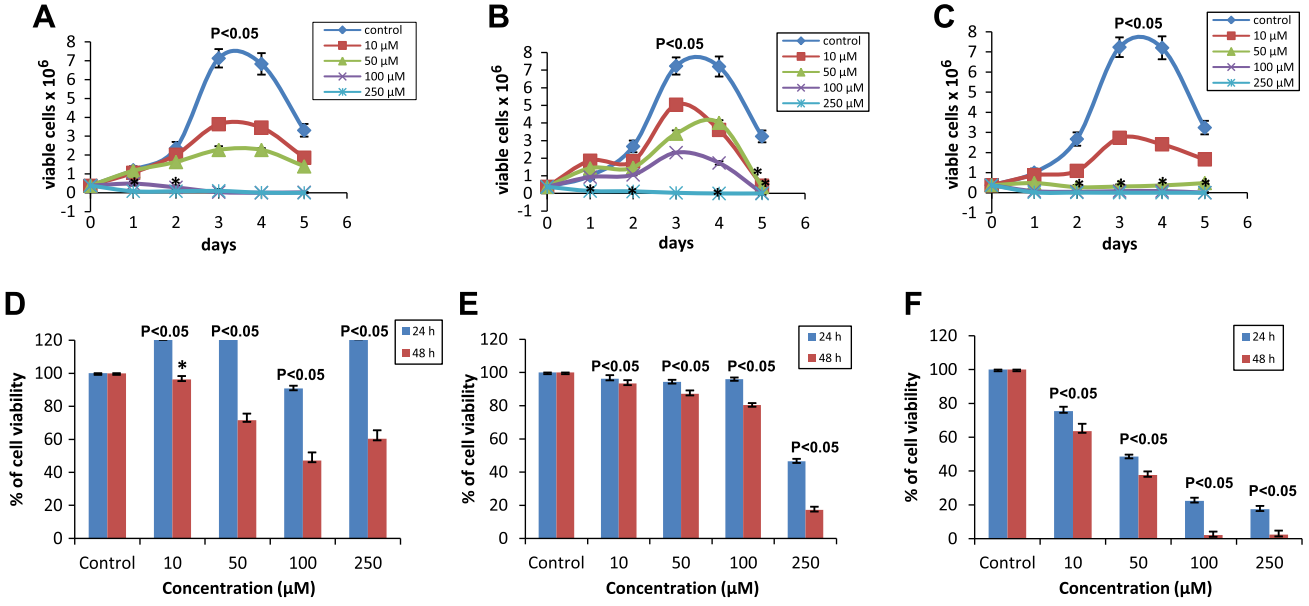



**Figure 2.** Cytotoxicity of different rhodanine derivatives on leukemia cell line. (A–C) Determination of cell viability on T-cell leukemia cell line, CEM following addition of ITH-1 (A), ITO-1(B), and BTR-1 (C) (10, 50, 100, and 250 μM) by trypan blue assay. DMSO was used as vehicle control. Live cells were counted at an interval of 24 h following addition of compounds, till culture reached stationary phase and the data was represented as a graph. Error bars based on independent experiments are indicated. (D–F) Determination of cell proliferation by MTT assay. CEM cells were cultured with ITH-1 (D), ITO-1 (E), and BTR-1 (F) as described in 'panel A', harvested after 24 and 48 h and subjected to MTT assay and data is presented as bar diagram. The percentage of viable cells was calculated considering DMSO treated cells as 100% and plotted. In all the cases, error bars indicate SD (standard deviation) from three independent experiments. *p* values ($p < 0.01$, $p < 0.05$) were calculated comparing control cells with treated cells. $p > 0.05$ are represented by asterisks (*).

DMSO, the cells with DMSO were used as vehicle control. Following addition of the compound, cells were counted at intervals of 24 h until the control cells attained stationary phase. Results showed that cell growth was affected with increase in duration of treatment and concentration of the compound (Fig. 2A–C). The effect was limited in case of **ITH-1**, **ITO-1** even when 100 μM was used, whereas in case of **BTR-1**, there was significant reduction in the cell number even when 10 μM was used for the study. However, concentration of 250 μM resulted in cell death in all three cases (Fig. 2A–C). The $IC_{50}$ of **ITH-1** was approximately 70 and 40 μM and **ITO-1** was 80 and 50 μM, when estimated at 48 and 72 h, respectively. Interestingly for BTR-1, $IC_{50}$ value was 8 and 6 μM at 48 and 72 h, respectively. These results suggest that **ITH-1**, **ITO-1**, and **BTR-1** induce cytotoxicity in human leukemic cells in a dose- and time-dependent manner.

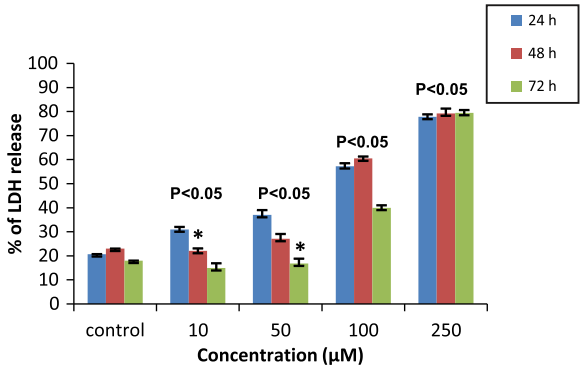



**Figure 3.** Detection of LDH release induced by BTR-1. After exposure of CEM cells with BTR-1 (10, 50, 100, and 250 μM for 24, 48, and 72 h), LDH release was measured at 490 nm. Results are presented as percentage of LDH release. Error bars based on independent experiments are indicated. Error bars indicate SD (standard deviation) from three independent experiments. *p* values ($p < 0.05$) were calculated comparing control cells with treated cells. $p > 0.05$ are represented by asterisks (*).

The effect of **ITH-1**, **ITO-1**, and **BTR-1** on proliferation of T-cell leukemic cells was further verified using MTT assay. CEM cells were treated with 10, 50, 100, and 250 μM of compound and were subjected to MTT assay after 24 and 48 h (Fig. 2D–F). Results showed that cell viability was affected at higher concentration in case of **ITH-1**, **ITO-1** while the effect was more pronounced in the case of **BTR-1**. In case of **BTR-1**, cell viability was affected as low as 10 μM. Interestingly, when normal T-lymphocyte cell line, 8E5, was used for the study, none of the compounds were cytotoxic, except in the case of **BTR-1**, which showed a limited sensitivity at the highest concentration (250 μM) and that could be due to nonspecific interaction (Supplementary Fig. 1). Based on both trypan blue and MTT assays, we find that potency of **BTR-1** ($IC_{50}$ value <10 μM) is 5- and 7-fold higher than **ITH-1** and **ITO-1**, respectively. Thus we have used **BTR-1** for further studies.

LDH release assay was performed to test the cell membrane damage induced by **BTR-1**. For this, CEM cells were cultured with 10, 50, 100, and 250 μM of **BTR-1** and LDH released was measured at 24, 48, and 72 h. Consistent with above results, we observed a dose- and time-dependent increase in LDH release, further confirming the cytotoxic potential of **BTR-1** (Fig. 3).

Compounds **ITH-1**, **ITO-1**, and **BTR-1** were employed for induction of cytotoxic activity and apoptosis in CEM cells. The rhodanine heterocycle **BTR-1** appeared to be most potent followed by thiohydantoin **ITH-1** and oxazolidinedione **ITO-1**. The thioxo group in rhodanines is a carboxylic acid bioisoster by size has low electronegativity and has ability to build hydrogen bonds, which could be attributed for the activity.[25] The activity of **BTR-1** was found to be higher than **ITR-1**[24] which was tested earlier and this may be due to the increase in the delocalization of the electrons. It further indicates that the activity is in the order of electronegativity values of the atom X. As the electronegativity values increase from S to O through N, the activity decreases in the same order. When the electronegativity increases probably it will attract more electrons and disturb the delocalisation of electrons between the double bond and the carbonyl group of the heterocyclic ring.

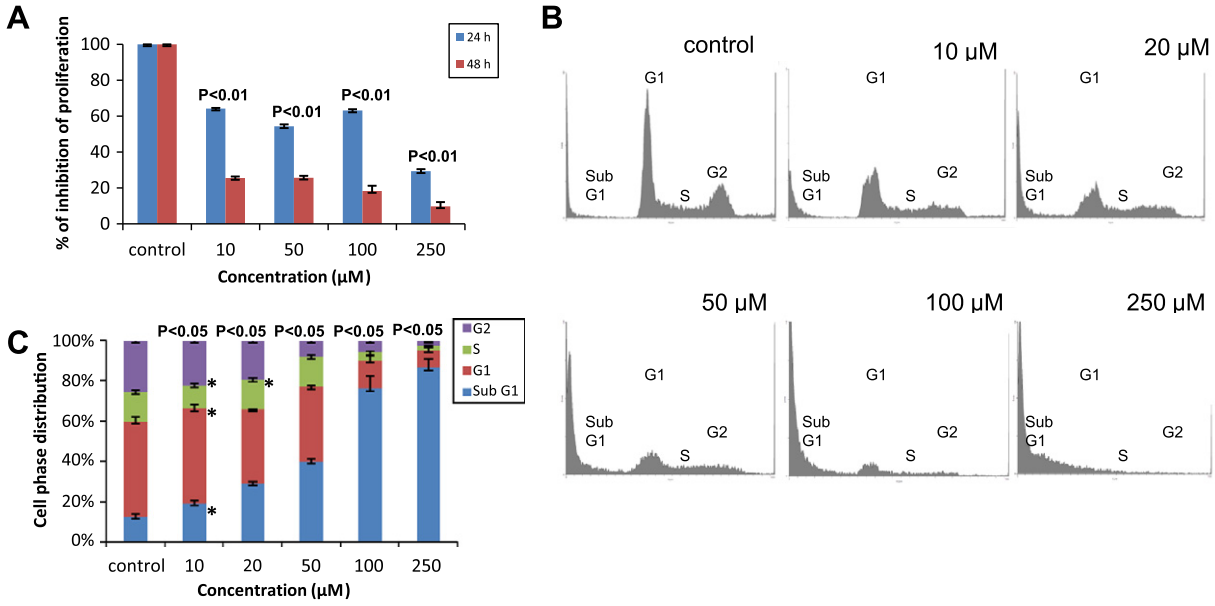



**Figure 4.** Determination of effect of BTR-1 on cell division by [$^3$H]-thymidine incorporation assay and cell cycle analysis. (A) [$^3$H]-thymidine assay to determine effect of BTR-1 treatment on CEM cells. [$^3$H]-thymidine was added to BTR-1 treated cells (10, 50, 100, or 250 μM) and radioactive counts per minute (cpm) were calculated and plotted as a bar diagram. The data presented is derived from independent experiments and error bars are indicated. (B and C) Flowcytometric analysis of CEM cells following BTR-1 treatment. BTR-1 treated cells (10, 20, 50, 100, and 250 μM) or vehicle control were harvested after 48 h. The cells were fixed and stained with propidium iodide and quantified by flow cytometry and presented as histogram (B). For each sample, 10,000 cells were used for sorting. Bar diagram showing quantification of percentage of cells in the $G_0/G_1$, G1, S, and $G_2$/M phase of the cell cycle (C). Error bars indicate SD (standard deviation) from three independent experiments. *p* values ($p < 0.05$) were calculated comparing control cells with treated cells and $p > 0.05$ are represented by asterisks (*).

*BTR-1 affects the proliferation of CEM cells*. The potential of **BTR-1** to inhibit cell division was tested by tritiated thymidine incorporation assay. To test this, cultured CEM cells were incubated in the presence of [$^3$H] thymidine following addition of **BTR-1**. Results showed that **BTR-1**-treatment led to the reduction in incorporation of [$^3$H] thymidine, in all concentrations tested especially after 48 h (Fig. 4A). These results suggest that **BTR-1** affects the cell viability by inhibiting cell division probably by interfering with DNA replication. However, it is also possible that, in addition to its effect on cell division, it could directly induce apoptosis.

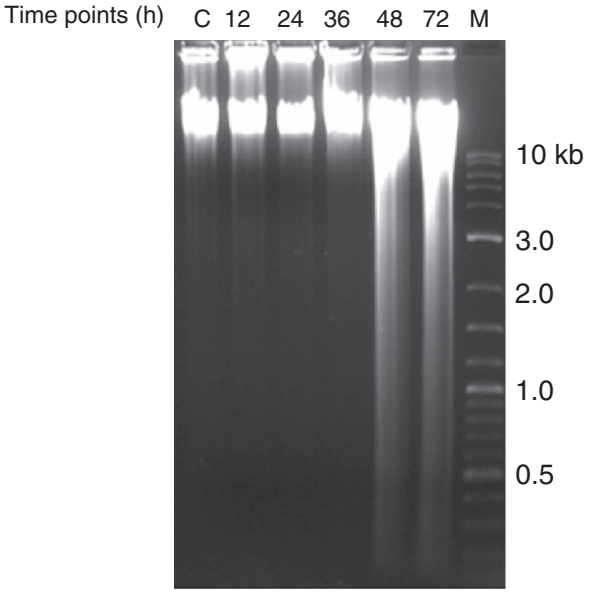



**Figure 5.** Agarose gel profile showing DNA fragmentation. The chromosomal DNA was extracted from CEM cells, following treatment with 20 μM BTR-1. The purified DNA was then resolved on a 1% agarose gel. Lane C, DMSO; lanes 2–6, 12–72 h, respectively. 'M' is molecular weight ladder (2 log DNA ladder).

FACS analysis was performed to determine whether **BTR-1** has any effect on cell cycle progression. CEM cells were stained with propidium iodide after 48 h of treatment (10, 20, 50, 100, and 250 μM) and subjected to FACS as described in Supplementary Methods. Histogram of vehicle (DMSO) treated cells showed a standard cell cycle pattern, which includes G1 and G2 separated by S phase (Fig. 4B and C). The subG1 phase (mostly dead cells) was not prominent. Interestingly, upon addition of **BTR-1**, a concentration dependent change was observed in the cell cycle pattern (Fig. 4B and C) leading to accumulation of cells in subG1 and decline of G2/M and G1. Importantly results showed a transient cell cycle arrest at S phase in low doses of treatment (10, 20, and 50 μM) (Fig. 4C). Hence, these results indicate that **BTR-1** may interfere with cell division by inducing S phase arrest followed by apoptosis. However, more studies are required to understand the mechanism of cell cycle arrest.

*BTR-1 induces DNA fragmentation*. FACS analysis is also indicative of the integrity of genome as propidium iodide staining was performed. The observed accumulation of cells at G0 phase indicated fragmentation of the DNA. In order to test the extent of DNA breaks induced by **BTR-1**, CEM cells treated with 20 μM of **BTR-1** were harvested after 12, 24, 36, 48, and 72 h and used for gel electrophoresis. The results showed fragmentation of the chromosomal DNA leading to a smear in the lanes representative of cells treated with **BTR-1** (Fig. 5). Such breakage was extensive when cells were incubated for 48 and 72 h with **BTR-1**. These results further confirm the activation of cell death pathways upon treatment with **BTR-1**.

*ROS production by BTR-1*. We examined whether **BTR-1** treatment (20 μM) led to ROS production in CEM cells as detailed in Supplementary Methods. Results showed that there was ROS production at 2 and 4 h of treatment with **BTR-1** (Fig. 6C–E). The $H_2O_2$ induced ROS production was used as a positive control (Fig. 6F). However, ROS production was weaker when the incubation period

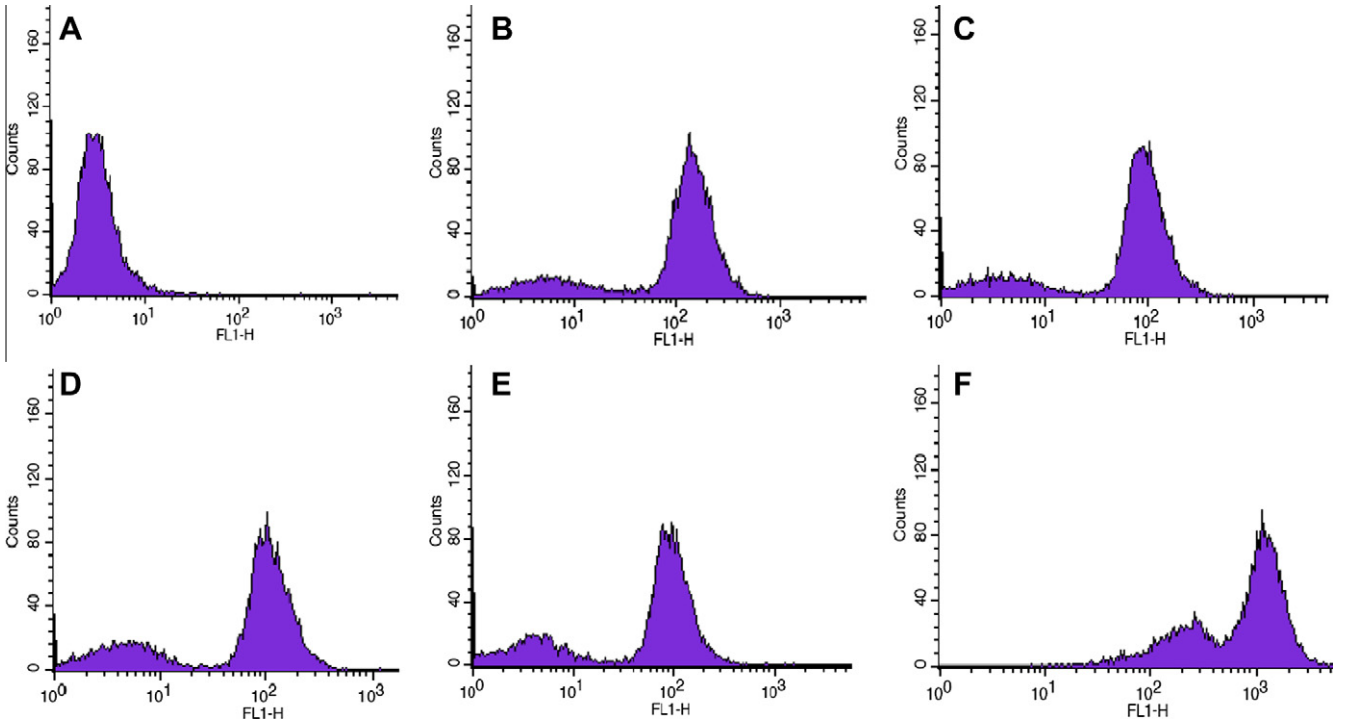



**Figure 6.** Effect of BTR-1 on ROS formation. CEM cells were treated with BTR-1 (20 μM) for 1 (C), 2 (D), and 4 h (E). ROS production was quantified by flow cytometry and presented. Cells treated with (B) and without (A) dye are also shown. $H_2O_2$ was used as a positive control (F).

was more than 4 h (data not shown). Hence, our results suggest that ROS generation could be an intermediate step in **BTR-1** induced cytotoxicity.

Thus, in the present study, we have designed and synthesized three novel derivatives of 5-isopropylidiene, **ITH-1**, **ITO-1**, and **BTR-1**. We find that among the compounds tested, **BTR-1** was many fold potent in inducing cytotoxicity than **ITH-1** and **ITO-1** with an $IC_{50}$ value of <10 μM. It affected cell division by inducing a block at S phase, which finally led to the activation of apoptosis.

*Conflict of interest*. Authors disclose that there is no conflict of interest.

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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.2010.08.084.

## References and notes

1. Nambiar, M.; Kari, V.; Raghavan, S. C. *Biochim. Biophys. Acta* **2008**, *1786*, 139.
2. Nickoloff, J. A.; De Haro, L. P.; Wray, J.; Hromas, R. *Curr. Opin. Hematol.* **2008**, *15*, 338.
3. Aplan, P. D. *Trends Genet.* **2005**, *22*, 46.
4. Rowley, J. D. *Nat. Rev. Cancer* **2001**, *1*, 245.
5. Raghavan, S. C.; Lieber, M. R. *Bioessays* **2006**, *28*, 480.
6. Raghavan, S. C.; Swanson, P. C.; Wu, X.; Hsieh, C. L.; Lieber, M. R. *Nature* **2004**, *428*, 88.
7. Raghavan, S. C.; Lieber, M. R. *Cell Cycle* **2004**, *3*, 762.
8. Raghavan, S. C.; Kirsch, I. R.; Lieber, M. R. *J. Biol. Chem.* **2001**, *276*, 29126.
9. Marculescu, R.; Le, T.; Simon, P.; Jaeger, U.; Nadel, B. *J. Exp. Med.* **2002**, *195*, 85.
10. Dorsett, Y.; McBride, K. M.; Jankovic, M.; Gazumyan, A.; Thai, T. H.; Robbiani, D. F.; Di Virgilio, M.; San-Martin, B. R.; Heidkamp, G.; Schwickert, T. A.; Eisenreich, T.; Rajewsky, K.; Nussenzweig, M. C. *Immunity* **2008**, *28*, 630.
11. Okazaki, I. M.; Kotani, A.; Honjo, T. *Adv. Immunol.* **2007**, *94*, 245.
12. Lieber, M. R.; Yu, K.; Raghavan, S. C. *DNA Repair (Amst.)* **2006**, *5*, 1234.
13. Kavitha, C. V.; Nambiar, M.; Ananda Kumar, C. S.; Choudhary, B.; Muniyappa, K.; Rangappa, K. S.; Raghavan, S. C. *Biochem. Pharmacol.* **2009**, *77*, 348.
14. Robert, J.; Jarry, C. *J. Med. Chem.* **2003**, *46*, 4805.
15. Ohishi, Y.; Mukai, T.; Nagahara, M.; Yajima, M.; Kajikawa, N.; Miyahara, K.; Takano, T. *Chem. Pharm. Bull. (Tokyo)* **1990**, *38*, 1911.
16. Momose, Y.; Meguro, K.; Ikeda, H.; Hatanaka, C.; Oi, S.; Sohda, T. *Chem. Pharm. Bull. (Tokyo)* **1991**, *39*, 1440.
17. Sudo, K.; Matsumoto, Y.; Matsushima, M.; Fujiwara, M.; Konno, K.; Shimotohno, K.; Shigeta, S.; Yokota, T. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 643.
18. Sim, M. M.; Ng, S. B.; Buss, A. D.; Crasta, S. C.; Goh, K. L.; Lee, S. K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 697.
19. Bulic, B.; Pickhardt, M.; Khilstunova, I.; Biernat, J.; Mandelkow, E. M.; Mandelkow, E.; Waldmann, H. *Angew Chem. Int. Ed. Engl.* **2007**, *46*, 9215.
20. Zeiger, E.; Anderson, B.; Haworth, S.; Lawlor, T.; Mortelmans, K.; Speck, W. *Environ. Mutagen.* **1987**, *9*, 1.
21. Hotta, N.; Akanuma, Y.; Kawamori, R.; Matsuoka, K.; Oka, Y.; Shichiri, M.; Toyota, T.; Nakashima, M.; Yoshimura, I.; Sakamoto, N.; Shigeta, Y. *Diabetes Care* **2006**, *29*, 1538.
22. Frlan, R.; Kovac, A.; Blanot, D.; Gobec, S.; Pecar, S.; Obreza, A. *Molecules* **2008**, *13*, 11.
23. Krishman, P. S. *Nature* **1951**, *168*, 171.
24. Ravi, S.; Chiruvella, K. K.; Rajesh, K.; Prabhu, V.; Raghavan, S. C. *Eur. J. Med. Chem.* **2010**, *45*, 2748.
25. Patani, G. A.; LaVoie, E. J. *Chem. Rev.* **1996**, *96*, 3147.