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Benzisothiazolones arrest the cell cycle at the G_2/M phase and induce apoptosis in HeLa cells†

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Anticancer activities of a series of benzisothiazolones having alkyl, aryl and aralkyl substituents on the nitrogen atom and the mechanistic basis of cytotoxicity are presented. Cellular responses like DNA laddering, disruption of mitochondrial membrane potential and caspase-3 activation on incubation of HeLa cells with representative compounds from this group suggested the induction of apoptosis through an intrinsic pathway. Their ability to arrest the cell cycle at the G_2/M phase was confirmed by flow cytometric analysis.

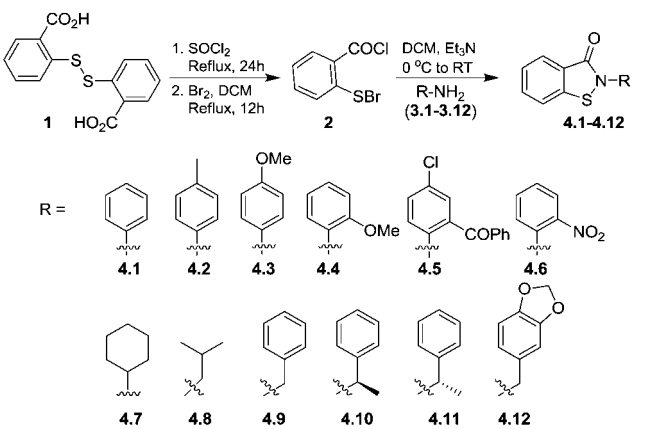
Introduction

Heterocyclic compounds containing sulfur and nitrogen atoms are known to exhibit interesting biological properties.[1] Among these, isothiazolones and benzisothiazolones (BIT) constitute an important class due to the presence of electrophilic sulfur as part of the ring.[2,3] The capability of these compounds to form disulfide bonds with sulfur nucleophiles or to chelate biologically relevant metals such as zinc[4,5] has been correlated with their activities against human leukocyte elastase,[6–9] RNA polymerase, proteases,[10,11] HIV-NcP-7,[12] telomerase,[13,14] blood platelet aggregation,[15] microbes,[16–18] and proliferation.[19,20] Recently, Groutas and co-workers have evaluated the activities of benzisothiazolone derivatives against fungal strains,[21] and viruses causing Dengue and West Nile fever.[22] Experiments involving representative compounds later revealed that they probably act by targeting the respiratory pathway and induce the formation of reactive oxygen species.[23] During our efforts to develop new protease inhibitors based on benzisothiazolones, we came across their anti-proliferative effect on various cancer cell lines. As many anticancer agents are known to cause apoptosis,[24] we examined whether the most cytotoxic benzisothiazolones from the present study are also capable of inducing similar effects on the susceptible HeLa cells. Results from these cytotoxicity evaluation and cell-response studies are discussed below.

Synthesis

The synthesis of benzisothiazolones (BITs) **4.1** to **4.12** commenced from the commercially available dithiodibenzoic acid (**1**).[2] In a typical procedure, **1** was converted to dithiodibenzoyl chloride using thionyl chloride and then reacted with bromine in dichloromethane to get bromosulfenylbenzoyl chloride (**2**). This on treatment with aniline (**3.1**) in the presence of triethylamine afforded **4.1** in 39% yield (Scheme 1). Inclusion of other aniline derivatives with electron donating or withdrawing substituents (**3.2–3.6**) in the scheme gave compounds **4.2–4.6** in 10–66% yields. Reaction of **2** with cyclohexylamine (**3.7**), isobutylamine (**3.8**) and benzylamine (**3.9**) resulted in the formation of compounds **4.7–4.9** respectively.

Compounds **4.1–4.9** were then subjected to cytotoxicity evaluation on HeLa (human cervical adenocarcinoma), SiHa (human cervical squamous cell carcinoma) and SW480 (human colon adenocarcinoma) cells using the MTT assay.[25] Among these, the cyclohexylamine and benzylamine derived benzisothiazolones,



Scheme 1 Synthesis of benzisothiazolone derivatives from dithiodibenzoic acid (**1**).

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Table 1 IC_{50} (in μM) values of BIT derivatives in human cancer cells[a]

Compounds	HeLa	SiHa	SW480
4.7	24.8	48.3	48.8
4.8	31.8	38.1	47.8
4.9	20.6	25.1	23.8
4.10	43.7	32.3	43.1
4.11	30.9	35.9	41.8
4.12	37.3	29.3	46.3

[a] Cells were treated with various concentrations of BIT for 72 h using DMSO as control and the viability was determined by the MTT assay.

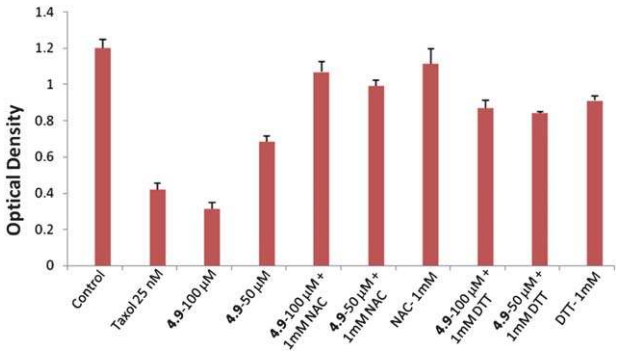

Fig. 1 Bar diagram showing the effect of NAC and DTT on the anticancer activity of **4.9**.

4.7 and **4.9**, showed IC_{50} values of 24.8 and 20.6 μM against HeLa cells (Table 1). They respectively showed IC_{50} values of 48.3 μM and 25.1 μM against SiHa cells, and 48.8 μM and 23.8 μM against SW480 cell lines. The corresponding values for **4.8** were slightly higher and ranged between 31.8 and 47.8 μM. The aniline derivatives **4.1–4.6** although soluble in DMSO started precipitating (visible only under a microscope) in the cell culture medium and hence were not taken forward for the estimation of IC_{50} values. Based on the structure of **4.9**, a second line of derivatives were then prepared and evaluated against all the three cell lines (**4.10–4.12**). Since these compounds did not show significant improvement in their potencies in comparison with **4.7** and **4.9** (Table 1) the latter two were selected for detailed mechanistic investigations in HeLa cells.

As mentioned, benzisothiazolones possess an electrophilic sulfur having a high tendency to form adducts with compounds containing thiol functionalities. In order to see whether such a reaction is responsible for the observed cytotoxicity, we co-administered *N*-acetyl cysteine (NAC) along with the compound **4.9** during the MTT assay. As shown in Fig. 1, this completely abolished the cytotoxicity (compare bars 3 and 5 in Fig. 1) suggesting the possibility of such covalent adduct formation with important cellular targets. A comparable result was obtained on using dithiothreitol (DTT) in place of NAC (compare bars 3 and 8 in Fig. 1).[11]

Mechanistic insight into the cytotoxic effect by 4.7 and 4.9

Small-molecule anticancer agents usually exert a cytotoxic effect by inducing apoptosis, a process characterized by both

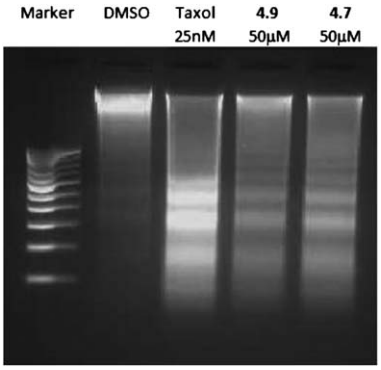

Fig. 2 Gel picture showing DNA fragmentation induced by benzisothiazolones **4.7** and **4.9** in HeLa cells.

morphological and molecular changes such as cell shrinkage, externalization of phosphatidyl serine and DNA fragmentation.[26] Based on the sequence of events involved, apoptosis can be extrinsic or intrinsic.[27] The former usually is initiated by the interaction of extracellular factors such as TNF (Tumor Necrosis Factor) with the death receptors and the subsequent propagation of signals *via* activation of caspase-8. The intrinsic pathway on the other hand occurs after the internalization of cytotoxic agents through the cell membrane. Their interaction with cellular targets then activates specific signals that prepare the cells for programmed cell death. Since the apoptotic response of cancer cells after benzisothiazolone treatment has never been studied systematically, we carried out a series of experiments and the results are outlined below.

The cleavage of nuclear DNA into oligonucleosomal fragments usually occurs in response to apoptotic stimuli. Since these fragments appear like a ladder in electrophoretic gels, it is termed as DNA laddering and is considered a hallmark of apoptosis.[28] DNA fragmentation assay results after incubating compounds **4.7** and **4.9** with HeLa cells for 72 h are presented in Fig. 2. DNA was stained with ethidium bromide after electrophoresis on a 2% agarose and then visualized under UV light. In Fig. 2, lane 1 represents the DNA marker whereas lanes 2 and 3 show the stained DNA in cells treated with DMSO (control) and taxol, respectively. The results after treating HeLa cells with compounds **4.9** (50 μM) and **4.7** (50 μM) shown respectively in lanes 4 and 5 suggest that both **4.9** and **4.7** induce DNA fragmentation.

Apoptosis induction is also associated with the disruption of mitochondrial membrane potential and release of Cytochrome *c* into the cytosol. The state of the membrane can be followed using cationic JC1 dye having a tendency to accumulate in the interior of mitochondria.[29] JC1 in the monomeric form emits in the green region, whereas its aggregates will be orange/red.[30] This dye aggregates in mitochondria with normal membrane potential but remains monomeric when the potential is low. As shown in Fig. 3A, untreated HeLa cells appeared orange but treatment with taxol induced noticeable disruption of the membrane potential as indicated by emission in the green region (Fig. 3B). The response from compounds **4.9** and **4.7** was similar to that of taxol, which suggested significant disruption

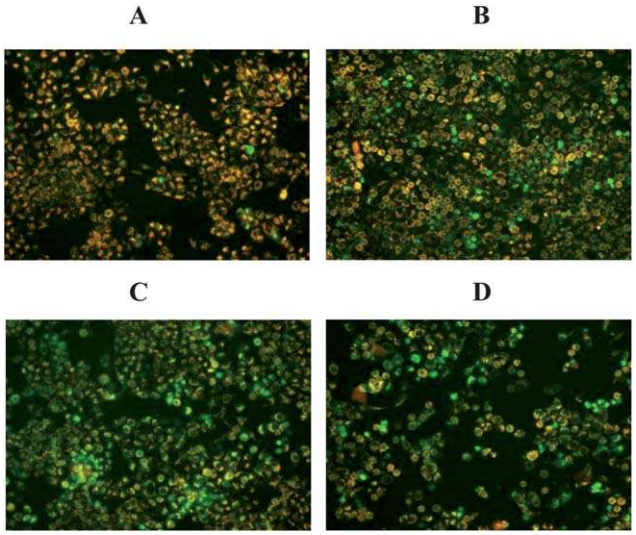

Fig. 3 (A) JC-1 localized in the mitochondria of HeLa cells, (B) HeLa cells after the treatment of 12 nM taxol, (C and D) HeLa cells treated with **4.9** (50 μM), and **4.7** (50 μM), respectively.

of the potential at the concentration tested (Fig. 3C and D). To understand the effect of **4.9** and **4.7** on the cell cycle,[31] they were incubated with HeLa cells for 24 h, fixed, stained using propidium iodide and analyzed by flow-cytometry.[32] The results of

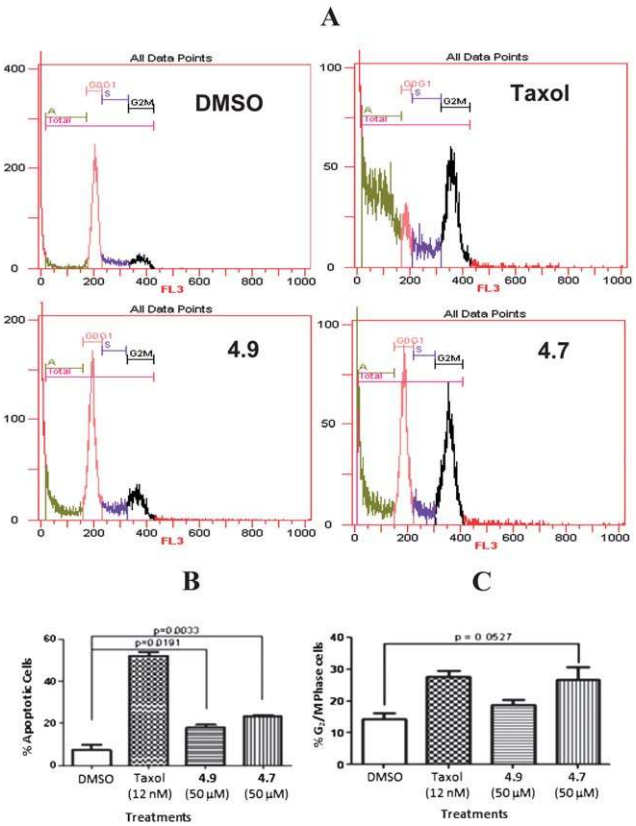

Fig. 4 (A) *X*-axis indicates the DNA content and *Y*-axis indicates the cell count; each graph shows the distribution of the G_0/G_1 phase, S phase, G_2/M phase and apoptotic levels. Data represent the mean ± SEM of three independent experiments. (B) % of apoptotic cells and (C) % of the G_2/M phase cells.

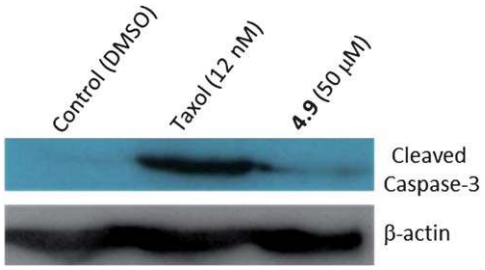

Fig. 5 HeLa cells treated with the **4.9** at the indicated concentration for 48 h were lysed. The lysates were resolved on a 10% SDS-PAGE, transferred on to a nitrocellulose membrane and probed for cleaved caspase-3. The same blot was then re-probed for actin.

these experiments are summarized in Fig. 4A–C which show the accumulation of cells in the G_2/M phase, depletion of cells in the G_0/G_1 phase and an increase in apoptotic fraction in their presence. Exposure of HeLa cells to compounds **4.9** and **4.7** at a 50 μM concentration for 48 h resulted in 18% and 23.4% of apoptotic cells respectively, whereas only 7.3% of apoptosis could be seen in untreated cells (Fig. 4B). Similarly, there were 18.6% and 26.6% of the G_2/M phase cells in these cases compared to 14.3% for untreated cells (Fig. 4C).

Apoptosis signals will finally lead to the processing of pro-caspase-3 to caspase-3,[33] which then assists the degradation of cellular proteins. We have looked for the presence of caspase-3 after treating HeLa cells with **4.9**. The western blot showing the presence of cleaved caspase-3 after incubating HeLa cells with taxol (12 nM) and **4.9** (50 μM) is shown in Fig. 5. The presence of this enzyme in cells treated with **4.9** clearly shows the processing of pro-caspase-3 and induction of apoptosis.

As mentioned, apoptosis induction is characteristic of a number of known anticancer agents.[24] While the effect of taxol is obvious from control experiments presented here, compounds such as mimosine[34] (which interfere with DNA replication and cause oxidative stress) are also known to trigger all the cellular responses typical of apoptosis discussed here. Apart from benzisothiazolones, our laboratory has investigated the anticancer activities of a number of redox-active 1,2-dihydroquinolines. Some compounds from this group, which showed low micromolar IC_{50} values, have also shown comparable effects.[35]

Conclusion

In conclusion, we have synthesized and evaluated the anticancer activities of a number of benzisothiazolones. The importance of electrophilic sulfur for their anticancer activity became evident when they were co-administered with NAC or DTT during the MTT assay. Using representative derivatives, we have shown that they induce apoptosis characterized by cellular responses like DNA fragmentation, disruption of mitochondrial membrane potential and activation of caspase-3. The subsequent flow cytometric analysis involving the same compounds (**4.7** and **4.9**) clearly showed that they are capable of arresting the cell cycle at the G_2/M phase. The results presented here raise

the hope that this class of compounds can be developed into new anticancer leads by proper structure optimization.

Acknowledgements

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