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Hybrid molecules containing benzo[4,5]imidazo-[1,2-*d*][1,2,4]thiadiazole and α-bromoacryloyl moieties as potent apoptosis inducers on human myeloid leukaemia cells

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Abstract—The synthesis and biological activity of a series of hybrids **1**–**5** prepared combining a benzo[4,5]imidazo[1,2-*d*][1,2,4]thiadiazole and different benzoheterocyclic α-bromoacryloyl amides have been described and their structure–activity relationships discussed. All these hetero-bifunctional compounds were highly cytotoxic against the human myeloid leukaemia cell lines HL-60 and U937 (IC_{50} 0.24–1.72 μM), significantly superior to that of both alkylating units alone. In human myeloid leukaemia HL-60 cells we observed that these compounds suppress survival and proliferation by triggering morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death. The apoptosis induced by these compounds is mediated by caspase-3 activation and is also associated to an early release of cytochrome *c* from the mitochondria.

Cancer treatment by chemotherapy kills target cells primarily by inducing apoptosis.[1] As known, apoptosis or programmed cell death is an intrinsic mechanism of self destruction that is inherent in every cell of the body, occurring under a wide range of physiological and pathological conditions.[2] It plays an essential and protective mechanism against neoplastic development in the organism by eliminating genetically damaged excess cells that have been improperly induced to divide by mitotic stimulus.[3] There are two alternative pathways that initiate apoptosis: one is mediated by death receptors on the cell surface, referred to as the 'extrinsic pathway'. The other is mediated by mitochondria, referred to as the 'intrinsic pathway'.[4] In both pathways, intracellular cysteinyl aspartate-specific proteases (caspases) are activated and subsequently cleave proteins that will ultimately lead to the morphological manifestation of apoptosis.[5] Apoptotic cells are characterized by specific morphological and biochemical changes, including DNA condensation and fragmentation, skeletal disruption, decrease in adhesion and intracellular contacts, blebbing of the plasma membrane, cell shrinkage and apoptotic body formation.[6]

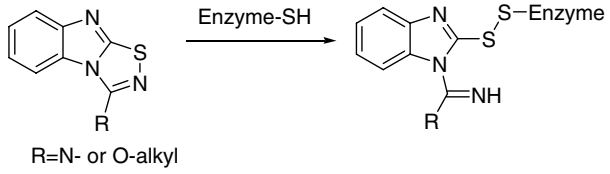

Chart 1.

In recent years, several research groups have disclosed the use of benzo[4,5]imidazo[1,2-*d*][1,2,4]thiadiazoles

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([1,2,4]BTHDs) as inhibitors targeting cysteine residue of biomolecules.[7] As shown in Chart 1, the proposed enzymatic mechanism involves the nucleophilic attack of the active site cysteine thiol at the sulfur atom, leading to the formation of a S–S bond with concomitant N–S ring opening.[8]

It is worthy to note that the [1,2,4]BTHD system displayed a lack of reactivity towards other nucleophiles such as amines and alcohols.[9] An appropriate C-3 substituent (R) in the tricyclic [1,2,4]BTHD can enhance both enzyme affinity and reactivity.[10]

The pyrroloiminoquinone cytotoxic alkaloids Discorhabdin A[11] and Discorhabdin G[12] are characterized by the presence of a α-bromoacryloyl alkylating moiety of low chemical reactivity, unusually for cytotoxic compounds. In fact α-bromoacrylic acid is not per se cytotoxic (IC_{50} on L1210 cells being greater than 120 μM). The reactivity of the α-bromoacryloyl moiety has been hypothesized to be based on a first-step Michael-type nucleophilic attack, followed by a further reaction of the no longer vinylic bromo substituent α to the carbonyl, leading successively to a second nucleophilic substitution or to beta elimination.[13] The same moiety is present in a series of potent anticancer distamycin-like minor groove binders, for example, PNU-166196 (brostallicin), an α-bromoacrylamido derivative of four-pyrrole distamycin homologue ending with a guanidino moiety, which is presently undergoing Phase II clinical trials (Chart 2).[14]

Following the finding that both benzo[4,5]imidazo[1,2-*d*][1,2,4]thiadiazole and α-bromoacryloyl moieties may act as thiol trapping agents, in this article we report the preparation and biological evaluation of a novel series of conjugates **1**–**5** incorporating these two moieties in their structures. One is an α-bromoacrylic derivative of benzoheterocyclic rings (compounds **6**–**9**), such as indole, *N*-methyl indole, benzofuran and benzothiophene, tethered via a flexible propyl/hexyldiamino spacer, to the C-3 position of tricyclic [1,2,4]BTHD system.

The convergent synthetic strategy followed by the synthesis of hybrid compounds **1**–**5** is outlined in Scheme 1, in which the two parts of each conjugate, **6**–**9**[15] and **11**–**12**, were prepared separately before coupling, according to previously described methods. The substituted diamines **11** and **12** were prepared, according to the procedure previously described by

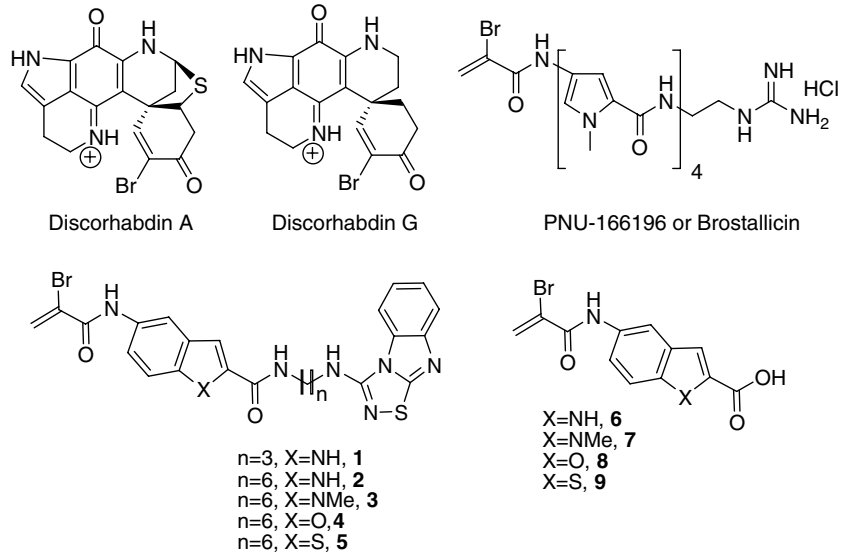
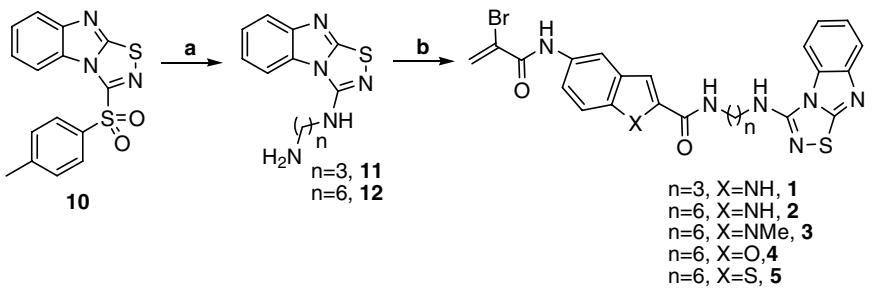


Chart 2.



Scheme 1. Reagents and conditions: (a) 5 equiv of 1,3-diaminopropane or 1,6-diaminohexane, 2 equiv of TEA, 2 h, DMF; (b) 6–9, EDCI, HOBt, DMF, rt, 24 h.

Leung-Toung,[16] submitting to nucleophilic displacement via an S_NAr mechanism the tricyclic 3-*p*-tosylbenzo[4,5]imidazo[1,2-*d*][1,2,4]thiadiazole **10** with 1,3-diaminopropane and 1,6-diaminohexane, respectively. The hybrid compounds **1**–**5** were achieved, in acceptable yields, by the selective condensation of diamines **11** and **12** with the α-bromoacryloyl benzoheterocyclic carboxylic acid derivatives **6**–**9**. This condensation was performed using an excess (2 equivalents) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCl) as coupling agent, in dry DMF as solvent at room temperature and with identical reaction times (24 h).

In Table 1, we have reported the in vitro antiproliferative activity of hybrid compounds **1**–**5** against the human myeloid leukaemia HL-60 and U937 cell lines and human melanoma SK-MEL-1 cells.

α-Bromoacrylamido benzoheterocycles **6**–**9** and benzo [4,5]imidazo[1,2-*d*] [1,2,4]thiadiazol-3-ylalkyldiamine derivatives **11**–**12** resulted inactive against these three cell lines ($IC_{50} > 20$ μM). Both Discorhabdin A ($IC_{50} = 70$ nM in the HCT-116 assay)[11] and brostallicin ($IC_{50} = 1.9$ nM against L1210 cells)[14] appeared more potent than **1**–**5**. These latter hybrids were found to inhibit the growth and cell viability of human HL-60 and U937 cells in a dose-dependent manner as determined by the 3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyl tetrazolium bromide (MTT) dye-reduction assay (Table 1).[17] Similar results were obtained with the human melanoma cell line SK-MEL-1, however the IC_{50} values were higher than on human myeloid cells. The relationship between the heteroatom in the benzoheterocycle and antiproliferative activity revealed that compounds **1** and **2**, with N-unsubstituted indole, were the most active. Comparing these two derivatives the antiproliferative activity decreased on increasing the length of the chain from three (**1**) to six (**2**) methylenic units. In the series of derivatives **2**–**5** characterized by the same alkyl chain ($n = 6$), the greatest potency was exhibited by compound **2**, constituted by **12** and the α-bromoacrylamidoindole derivative **6**, with IC_{50} values of 0.55, 0.40 and 3.02 μM against HL-60, U937 and SK-MEL-1 cells, respectively. The least potent compounds of this series were the conjugates **4** and **5**, which included the benzofuran and benzothiophene residues **8** and **9**, respectively. This hybrid molecule was found to be more than 2-fold less active with respect to **2**. A progressive decrease in antiproliferative activity was observed replacing indole **2** with *N*-methyl indole **3** to end with benzofuran **4** and benzothiophene **5**.

Table 1. Effects of compounds **1**–**5** on the growth of HL-60, U937 and SK-MEL-1 cells cultured

Compound	IC_{50} (μM)		
	HL-60	U937	SK-MEL-1
1	0.29 ± 0.03	0.24 ± 0.03	2.09 ± 0.39
2	0.55 ± 0.18	0.40 ± 0.11	3.02 ± 0.62
3	0.84 ± 0.06	0.89 ± 0.06	4.50 ± 0.49
4	1.72 ± 0.65	0.74 ± 0.12	8.95 ± 0.45
5	1.24 ± 0.12	0.91 ± 0.05	8.04 ± 0.78

The data shown represent means(±S.E.M.) of three independent experiments with three determinations in each.

These data do not allow the identification of the molecular target(s) of these novel antiproliferative compounds. Preliminary DNase I footprinting experiments sustain the concept that these molecules retain low ability to alkylate DNA also at high concentration (10 μM).[18] This is expected, since brostallicin exhibits low DNA alkylating activity,[14] on the other hand, benzo[4,5]imidazo[1,2-*d*] [1,2,4]thiadiazoles are known to exert their main action through chemical reactions with cysteine residues of proteins.

To elucidate the possible mechanism(s) of action mediating cell growth inhibition, we have tested the effects of hybrids **1**–**5** to induce apoptosis, using HL-60 cells as experimental system.[19] As shown in Figure 1 (upper panel), compounds **1**–**5** (3 μM, 4 h) induced morphological changes, as visualized by phase contrast microscopy.

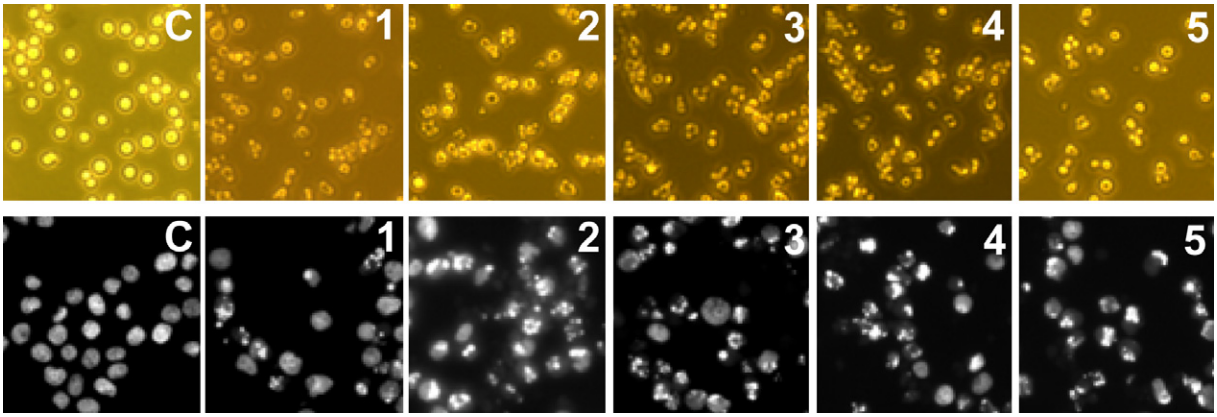


Figure 1. Morphological changes and Apoptosis induction by compounds **1**, **2**, **3**, **4** and **5** on HL-60 cells. Upper panel: HL-60 cells were incubated with medium alone (C, control), or compounds **1**, **2**, **3**, **4** and **5** (3 μM). Images of cells in culture were obtained using an inverted phase contrast microscope. Lower panel: photomicrographs of representative fields of HL-60 cells cultured in absence (C, control) or presence of 3 μM of the indicated compounds for 4 h and then stained with Hoechst 33258.

As shown in lower panel, cells exposed to 3 μM of compounds for 4 h displayed condensation of chromatin[20] and the appearance of apoptotic bodies by fluorescence microscopy after DNA staining with Hoechst 33258, meanwhile control HL-60 cells exhibited normal features, with the nuclei round and homogeneous.

Using QFM (quantitative fluorescence microscopy) the least potent compound was **1**, meanwhile compounds **2**–**5** displayed similar values in the percentage of apoptotic cells (Fig. 2) at times and doses assayed. We also examined whether these compounds induced chromosomal DNA fragmentation, which is considered the end point of the apoptotic pathway and results from activation of caspase-activated endonuclease.[21]

A biochemical hallmark of apoptosis is the fragmentation of genomic DNA into integer multiples of 180-bp units, resulting in a characteristic ladder on agarose gel electrophoresis. As expected, the results demonstrate that exposure to 3 μM of compounds **1**–**5** results in endonucleolytic DNA cleavage, which then led to the formation of DNA ladder in HL-60 and U937 cells (Fig. 3).

During apoptosis, mitochondria suffer specific damage that results in loss of their function. Release of cytochrome *c*, the sole water-soluble component of the electron transfer chain, can potentially halt the electron transfer, leading to failure in maintaining membrane potential. For this reason, we also examined the effect of compounds on cytochrome *c* translocation from the mitochondria into the cytosol.

The representative Western blot analysis showed that the monoclonal antibody for cytochrome *c* detected a single band at the expected size of 15 kDa (Fig. 4a). Cytochrome *c* release was observed in the presence of doses as low as 3 μM of compounds **1**–**5**.

A central role for caspase-3 in cell death is supported by involvement of this executioner caspase in the apoptotic response to diverse stimuli.[22]

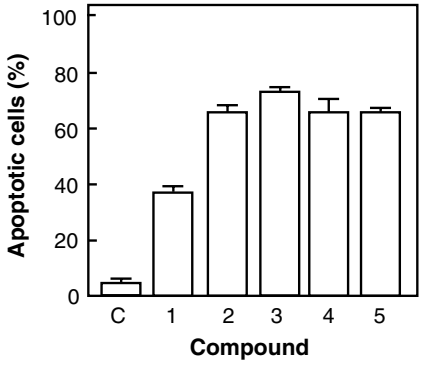


Figure 2. Quantitative analysis of apoptotic cells by fluorescence microscopy. HL-60 cells were treated with no drug (C, control) or 3 μM of the indicated compounds for 4 h. Cells were stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation and quantitated by fluorescence microscopy. The results of a representative experiment are shown, and each point represents the average (±SE) of triplicate determinations.

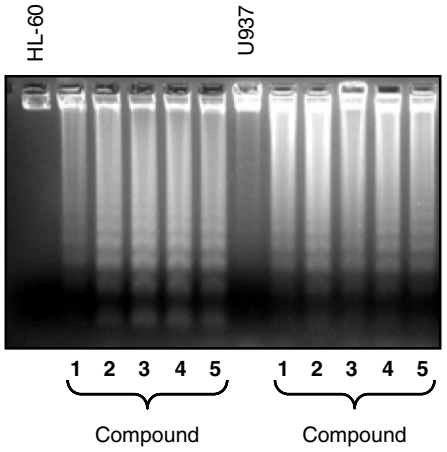


Figure 3. HL-60 and U937 cells were treated with 3 μM of compounds for 4 h and total cellular DNA was isolated and stained with ethidium bromide after electrophoresis on a 2% agarose gel. Internucleosomal DNA fragmentation was visualized under UV light.

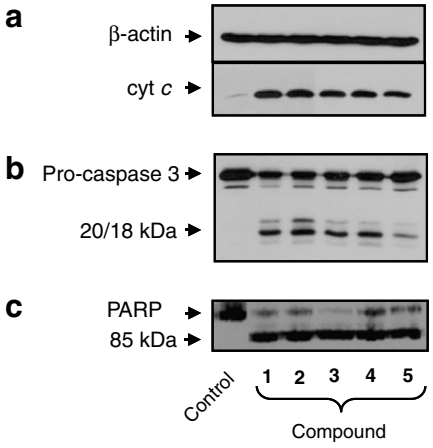


Figure 4. (a) Western blot analysis of cytochrome *c* release. HL-60 cells were treated with 3 μM of compounds and harvested at 4 h. Cytosolic lysates were analyzed by immunoblotting with an anti-cytochrome *c* antibody. β-Actin was used as loading control. (b) Representative Western blot showing the processing of the Mr 32,000 caspase-3 precursor to the p20 and p18 cleavage products after treatment with 3 μM of compounds. (c) Cleavage of poly(ADP-ribose) polymerase (PARP). The cells were treated as above and equal amounts of proteins from cell lysates were loaded in each lane and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by blotting with an anti-PARP monoclonal antibody.

Caspase-3 is responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP). This protein plays an important role in chromatin architecture and DNA metabolism.[23] PARP that is a 116 kDa DNA repair enzyme is cleaved to produce an 85 kDa fragment during apoptosis.

To assess whether these compounds activate caspase-3, lysates were subjected to immunoblotting with anti-caspase-3. Treatment of HL-60 cells with these compounds

resulted in cleavage of caspase-3 (Fig. 4b). Similar results were obtained in U937 cells (results not shown). In addition, these compounds also induced PARP cleavage. As expected, the typical apoptotic 85 kDa fragment was visualized upon treatment of the cells with 3 μM of compounds **1**–**5** (Fig. 4c).

When considered together, the results shown in Figures 1–4 consistently indicate that compounds **1**–**5** are able to induce apoptosis as demonstrated by fulfilling two criteria: (i) identification of nuclear changes associated to apoptosis using fluorescence microscopy, and (ii) DNA laddering on agarose gel electrophoresis. All these derivatives induced extensive PARP hydrolysis, considered to be one of the major hallmarks of apoptosis.

In conclusion, a new class of apoptosis inducer, as hybrid derivatives based on 3-substituted benzo[4,5]imidazo[1,2-*d*] [1,2,4]thiadiazole linked with a polymethylene spacer to α-bromoacryloyl amido benzoheterocycles was described. Relatively highest sensitivity to the compounds here described was found for human myeloid leukaemia HL-60 and U937 cell lines, while the human melanoma SK-MEL-1 cell line was less sensitive.

Further, our results point to the fact that the compounds investigated induce apoptosis in HL-60 cells through activation of caspase-3. We have observed that these hybrid molecules suppress proliferation of HL-60 cells by triggering morphological changes and internucleosomal DNA fragmentation. Further investigations are necessary to determine the detailed pathway of programmed cell death by these compounds.

Based on the structure–activity relationships of this limited series of derivatives, this preliminary study would encourage us to synthesize more advanced lead compounds by chemical modifications (i.e., optimal length of the linker, effects of the presence of both electron-releasing and electron-withdrawing substituents on the [1,2,4]BTHD).

Acknowledgments

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