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Discovery of cytotoxic and pro-apoptotic compounds against leukemia cells: Tert-butyl-4-[(3-nitrophenoxy) methyl]-2,2-dimethyloxazolidine-3-carboxylate

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ABSTRACT

Aims: We evaluated biological activity in leukemia cells lines of *R* and *S* enantiomers of *tert*-butyl 4-[(3-nitrophenoxy)-methyl]-2,2-dimethyloxazolidine-3-carboxylate (BNDC).
Main methods: Cytotoxic activity was assessed by MTT assay. Flow cytometry assays were used to determined DNA fragmentation (Propidium Iodide-PI staining) and phosphatidylserine exposure (Annexin-V and PI staining). DNA condensation was evaluated by fluorescence microscopy using double-staining in leukemia cells (Hoechst and PI). Caspase activities were measured using Z-VAD-FMK, a non-selective caspase inhibitor, by flow cytometry and Z-DEVD-AMC, a selective caspase-3 substrate, by fluorescence spectrometry.
Key findings: Both enantiomers displayed cytotoxic activity against leukemia cell lines (HL60, HL60.Bcl-2, HL60.Bcl-XL and Jurkat) with low toxicity against human peripheral blood mononuclear cell — PBMC based on IC_{50} values. In HL60 cell lines, compounds induce exposure of phosphatidylserine and DNA fragmentation, which could be blocked by pretreatment of cells with Z-VAD-FMK. Confirming this observation, both enantiomers induced caspase-3 activation. Additional analysis revealed an increased percentage of apoptotic cells (defined as those with fragmented nuclei and condensed chromatin) after treatment with compounds.
Significance: Taken together, the results indicate that BNDC compounds exhibited cytotoxic and pro-apoptotic activities and have a potential for developing a new class of anticancer drugs.

Introduction

The investigation of the biological activities of novel synthetic compounds is the first step in the discovery of new drugs (Keseru and Makara, 2006; Braña and Sánchez-Migallón, 2006). The discovery process begins with screening a large number of compounds selected from chemical libraries to identify new hit compounds with biological activity (Langer et al., 2009). New series of compounds structurally related to these *hit* compounds are evaluated for their structure–activity relationship to select a prototype or *lead* compound (Bleicher et al., 2003). In the discovery of drugs for cancer treatment, in vitro cytotoxicity assays are important for identifying new *hit* compounds. Once a new compound is identified, it is necessary to investigate its biological action mechanisms (Keseru and Makara, 2006), which include DNA polymerase or topoisomerase inhibition, and DNA alkylation. Induction of apoptosis is another drug activity indicator in cancer cells (Hickman, 1992; Makin and Hickman, 2000; Li et al., 2006).

Programmed cell death (apoptosis) is a natural process for removing unwanted cells, such as those with potentially harmful mutations, aberrant substratum attachment, or alterations in cell-cycle control (Fesik, 2005). The classical apoptosis are divided in two well described pathways: extrinsic and intrinsic. In the extrinsic pathway, endogenous factors like tumor necrosis factor and TRAIL binding on 'death receptors' such as TRAIL receptors and Tumor Necrosis Factor Receptor 1 and trigger apoptosis by activating the initiator protease caspase-8, which directly activates the effector protease caspase 3 (Hengartner, 2000). Intrinsic pathway is initiated by cellular stress which leads the mitochondria to release cytochrome c, apoptotic protease activating factor-1 and AIF into the citosol (Hengartner, 2000). This mitochondrial release of proteins is controlled by Bcl-2 family, which is composed of anti-apoptotic (e.g. Bcl-2, Bcl-XL, MCL-1) and pro-apoptotic (e.g. Bax, Bid, Bak) proteins, that regulate the membrane potential of mitochondria and endoplasmic reticulum, being crucial to transmit pro-apoptotic signals to cell death (Vander Heiden

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and Thompson, 1999; Janumyan et al., 2003). Once in the cytoplasm, the procaspase-9, cytochrome c and apoptotic protease activating factor-1 form an apoptosome complex that activates caspase-9, which in turn activates the caspase effectors. The active caspase effectors such as caspases 3, 6 and 7 are responsible for most of the morphological changes related to the phenomenon of apoptosis, such externalization of phosphatidylserine (PS), chromatin condensation and DNA fragmentation (Wang et al., 2005; Van Noorden, 2001; Ghavami et al., 2009).

Over the past decade, substantial advances have been made in the field of apoptosis-based therapeutics. Evasion has been identified as one of the main processes associated with the pathophysiology of cancer, which is thus associated with the aberrant regulation of regulators and effectors of apoptotic death (Fleischer et al., 2006). Most of the changes in proteins involved in cell death signaling are often resistant to chemotherapy and are more difficult to treat using chemotherapeutic agents that act primarily by inducing apoptosis (Fesik, 2005). Therefore, the ability to understand and manipulate the machinery of cell death is a clear goal of medical research. Apoptosis-targeting therapies are now advancing from preclinical/clinical trials to actual application. This is a remarkable achievement, considering that apoptotic cell death is a relatively recent discovery (Fischer and Schulze-Osthoff, 2005; Jana and Paliwal, 2007).

Using this background, we performed the screening of a series of compounds from an in-house chemical library against four cancer cell lines to identify new cytotoxic compounds that target the apoptotic machinery. Four chemically related compounds displayed cytotoxicity against Jurkat and HL60 cells. The structures of compounds (**1**–**4**) are shown in Fig. 1. To investigate drug resistance, we used the HL60 cell line ectopically expressing anti-apoptotic proteins that include Bcl-2 and Bcl-XL since these proteins are often over-expressed in various human cancers, thereby conferring drug resistance (Brumatti et al., 2003). The values of IC_{50} indicated that *R* and *S* enantiomers of Tert-butyl-4-[(3-nitrophenoxy)methyl]-2,2-dimethyloxazolidine-3-carboxylate — BNDC (compounds **3** and **4**) were more active in the HL60 cell line, with minor toxicity against normal human peripheral blood mononuclear cells — PBMC . These compounds induced DNA fragmentation, phosphatidylserine exposure, morphological changes and caspase-3 activation, suggesting that apoptosis was the mechanism most likely to be involved. Thus, our data point to BNDC as cytotoxic molecules that could provide a new scaffold for apoptosis inducers useful in the development of a novel class of anti-cancer agents.

Material and methods

Synthetic route

Compounds **1** and **2** were prepared as described elsewhere (Dias et al., 2009). In the same vein, compounds **3** and **4** were prepared as follows. Initially, the commercially available protected serine **5** (*R* or *S* enantiomer) was reacted with $LiBH_4$ in THF/CH_3OH at −10 °C to give the corresponding alcohol **6** (Garner and Park, 1987). Reaction of **6** (*R* or *S* enantiomer) with 3-nitrophenol under Mitsunobu conditions is described below.

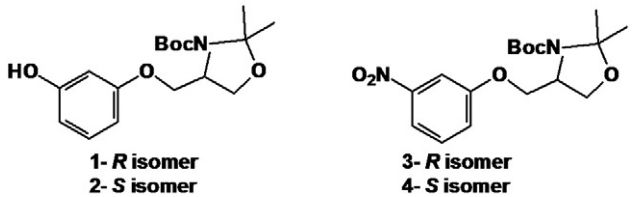


Fig. 1. Structures of compounds **1**–**4**.

*Synthesis of (R)-tert-butyl 4-(3-(nitrophenoxy)methyl)-2,2-dimethyl-oxazolidine-3-carboxylate (**3**)*

Diethyl azodicarboxylate (0.26 mL, 1.65 mmol) was added to a mixture of 3-nitrophenol (0.215 g, 1.54 mmol), alcohol **6** (*R* enantiomer) (0.24 g, 1.04 mmol) and PPh_3 (0.43 g, 1.65 mmol) in toluene (12 mL) at room temperature under an argon atmosphere and the reaction mixture was stirred for 7 h at 80 °C. The solution was cooled to room temperature and washed with 1.0 M NaOH solution (20 mL) and then H_2O (3×20 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel (*n*-hexane/EtOAc 9:1) to give the desired **3** (0.29 g, 81%). Melting point: 59.6–60.1 °C. $[\alpha]D^{20}$ −70.3 (*c* 1.0, $CHCl_3$). HRMS (ESI+) calculated for $C_{17}H_{24}N_2O_6Na$ (M + Na), 375.1532. Found: 375.1540. ^1H NMR (500 MHz, DMSO-d_6, 363 K) δ 1.45 (s, 9H, *t*-butyl); 1.48 (s, 3H, CH_3); 1.55 (s, 3H, CH_3); 3.96–4.24 (m, 5H H-4, H-5, H-6); 7.43–7.45 (d, 1H, J_{12-11} = 8.0 Hz, H-12).; 7.58 (t, 1H, J_{11-12} = J_{11-10} = 8.0 Hz, H-11); 7.74 (bs, 1H, H-8); 7.80 (d, 1H, J_{10-11} = 8.0 Hz, H-10). ^{13}C NMR (125 MHz, DMSO-*d*6, 363 K) δ 23.3 (CH_3), 26.2 (CH_3); 27.5 ((CH_3)$_3$C); 55.1(C-4); 64.4 (C-5); 67.5 (C-6); 79.2 ((CH_3)$_3$C); 92.9 (C-2); 108.9, (C-8); 115.2 (C-10); 121.3 (C-12); 130.1 (C-11); 148.6 (C-9); 150.9 (C═O); 158.6 (C-7).

Synthesis of (S)-tert-butyl 4-[(3-nitrophenoxy)methyl]-2,2-dimethyl-oxazolidine-3-carboxylate (4)

Compound **4** (0.21 g, 73%) was prepared from the alcohol **6** (*S* enantiomer, 0.19 g, 0.82 mmol) under the same conditions and reagent proportions as described above in preparing **3**. Melting point: 59.2–60 °C. $[\alpha]D^{20}$ 73.3 (c 1.0, $CHCl_3$). HRMS (ESI+) calculated for $C_{17}H_{24}N_2O_6Na$ (M + Na), 375.1532. Found: 375.1534. ^1H NMR and ^{13}C NMR spectra of **4** were identical to those of **3**, as expected.

Leukemia cell lines

HL60 cells (human promyelocytic leukemia HL-60 cells), HL60.Bcl-2 and HL60.Bcl-XL (HL60 cells ectopically expressing Bcl-2 and Bcl-XL, respectively) and human immortalized line of T lymphocyte (Jurkat cells) were provided by Gustavo Amarante-Mendes, PhD (São Paulo University, Brazil). All lineages were cultivated to log phase in RPMI 1640 Sigma-Aldrich (St. Louis, MO), supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO BRL, Grand Island, NY), enriched with 2 mM of L-glutamine and 10% fetal bovine serum. All cultures were maintained at 37 °C in a humidified incubator with 5% CO_2.

PBMC

The PBMC were prepared using the protocol previously described by Gazzinelli et al., 1983. Briefly, PBMC samples were obtained through agreement with Minas Gerais Hematology and Hemotherapy Center Foundation — HEMOMINAS (protocol no 105/2004) from healthy adult volunteers of both sexes by centrifugation of heparinized venous blood over Ficoll cushion (Sigma-Aldrich, St. Louis, MO). PBMC were collected from the interphase after Ficoll separation and washed three times in RPMI-1640 before further processing (Gazzinelli et al., 1983). All cultures were carried out in RPMI-1640 medium Sigma-Aldrich (St. Louis, MO), supplemented with 5% (v/v) heat-inactivated, pooled AB (GIBCO/BRL, Grand Island, NY) sera and 2 mM L-glutamine. An antibiotic/antimicotic solution containing 1000 U/mL penicillin, 1000 μg/mL streptomycin and 25 μg/mL fungisone (GIBCO/BRL, Grand Island, NY) was added to control fungal and bacterial contamination.

Analysis of cell viability

The leukemia cell lines and PBMC were cultured in 96 wells plate at densities of 50,000 cells/well and 100,000 cells/well, respectively, in a final volume of 200 μL/well. The plates were pre-incubated in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 24 h to allow adaptation of cells prior to the addition of the test compounds. All substances were dissolved in dimethyl sulfoxide (DMSO) prior to dilution. The half maximal inhibitory concentration (IC_{50}) was determined over a range of concentrations (10 nM–100 μM). All cell cultures were incubated in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 48 h. Cell viability was estimated by measuring the rate of mitochondrial reduction of yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO) to insoluble purple formazan crystals (Mosmann, 1983). After incubation with the test compounds, MTT solution (20 μL; 5 mg/mL) was added to each well and incubated for 4 h. Afterward, the supernatant was removed and 200 μL of 0.04 M HCl in isopropyl alcohol were added to dissolve the formazan crystal. The optical densities (OD) were measured with a spectrophotometer at 590 nm. Results were normalized with DMSO control (0.05%) and expressed as a percentage of cell viability inhibition. Interactions of compounds and media were estimated on the basis of the variations between the drug-containing medium and drug-free medium to control for false-positive or false-negative results. The half maximal inhibitory concentration (IC_{50}) values were obtained graphically from dose–effect curves using Prism 5.0 (GraphPad Software Inc.). The cytotoxicity of cisplatin was evaluated under the same experimental conditions for comparison.

DNA fragmentation assay

Cell cycle status and quantification of DNA fragmentation (hypodiploid DNA-content) were performed by propidium iodide (PI) staining according to Nicoletti et al. (1991). HL60 cells were treated with the four compounds at the final concentration of 50 μM and incubated in a 5% CO_2/95% air-humidified atmosphere at 37 °C for 24 h. Afterward, the cells were centrifuged, resuspended in a hypotonic fluorochrome solution — HFS (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated at 4 °C for 4 h. The PI fluorescence of 20,000 individual nuclei was measured using a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Data were analyzed using FlowJo software 7.5.5 (TreeStar Inc, CA).

Investigation of DNA condensation by Hoechst staining

After incubation with the compounds (50 μM), nuclei were double-stained with Hoechst 33342 (HST) and PI. The cells were examined by fluorescence microscopy (Carl Zeiss, Germany) with two filters (DAPI fluorescent filter, excitation 340–380 nm, barrier filter 430 nm and a rhodamine filter, excitation 530–560 nm, barrier filter 580 nm). For each experiment, three microscopic fields were photographed with an AxioCam MRm ZEISS (Carl Zeiss, Germany) and results were averaged. For each field, one photograph of HST and one of PI were taken for comparison. The images were analyzed and merged using Image J software 1.43r (National Institute of Health, USA). The cell nuclei were counted and classified as normal nuclei, condensed nuclei or fragmented nuclei. Cells double-stained with HST and PI were considered late apoptotic or necrotic, depending on the morphology of the nuclei. Apoptotic cells were defined as HST+/PI-cells with condensed nuclei or fragmented nuclei (Thuret, et al., 2003).

Evaluation of phosphatidylserine externalization

Apoptotic cells were detected using an Annexin-V FITC Apoptosis Kit (Invitrogen Corporation, USA) and differentiated into viable, early, and late apoptotic, as previously described (Martin, et al., 1995). Briefly, the cells were treated with compounds (50 μM) and incubated for 24 h at 37 °C in an atmosphere of 5% CO_2. The cells were washed with PBS and then resuspended in Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM, NaCl, 2.5 mM $CaCl_2$). They were double-stained with an Annexin-V FITC and PI buffer and incubated at room temperature for 15 min in the dark. Following incubation, the cells were analyzed in FACScalibur flow cytometer (BD Biosciences) and the data analyzed by FlowJo 7.5.5 (TreeStar Inc, CA).

Detection of caspase activity

Caspase activation was assessed by pre-treatment with benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), a broad-spectrum caspase inhibitor. In assays of DNA fragmentation inhibition, cells were incubated for 40 min with 40 μM Z-VAD-FMK (Biomol, Research Laboratories, Plymouth Meeting, PA, USA) before being treated with enantiomers at 50 μM. After 24 h of incubation, the DNA content was evaluated as previously described. Control cultures were treated with the appropriate amount of DMSO (0.05%). Caspase-3 activation was determined by EnzChek® Caspase-3 Assay Kit #1 (Molecular Probes, USA). Briefly, the cells were treated with a lysis buffer and incubated on ice for 30 min. The lysed cells were centrifuged at 5000 rpm for 5 min in a microcentrifuge and the supernatant collected. Microplate wells were filled with 50 μL of the supernatant from each sample and 50 μL of substrate working solution with 200 μM of Z-DEVD-AMC. The samples were incubated in the dark at room temperature for 30 min. Afterward, the plate was analyzed in a fluorescence spectrophotometer (Cary Eclipse, Varian, USA) with excitation and emission wavelengths of 342 nm and 482 nm, respectively.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated from HL60 cells using 1 mL of Trizol® reagent according to the protocol provided by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, United States of America — USA). RNA samples were treated with Turbo DNA-free kit (Ambion Inc., Foster, CA, USA). First-strand complementary DNA (cDNA) was synthesized from 2 μg total RNA using the Superscript first-strand synthesis system (Invitrogen Inc., Carlsbad, CA, USA). After denaturing the RNA template and primers (25 pmol of each reverse oligonucleotide primer) at 70 °C for 10 min, 40 U reverse transcriptase was added in the presence of RT buffer

Table 1
Sequences of primers used in quantitative RT-PCR.

Primers	Nucleotide sequence	Size	Fragment size	GenBank access number
BaxForw	5′-TCTACTTTGCCAGCAAACTGGTGC-3′	24 nt	80 bp	NM_138765.3
BaxRev	5′-TGTCCAGCCCATGATGGTTCTGAT-3′	24 nt		
Bcl2Forw	5′-ATTTCCTGCATCTCATGCCAAGGG-3′	24 nt	90 bp	NM_000657
Bcl2Rev	5′-TGTGCTTTGCATTCTTGGACGAGG-3′	24 nt		
BclXlForw	5′-ATGACCAGACACTGACCATCCACT-3′	24 nt	99 bp	NM_001191
BclXlRev	5′-ATGTAGTGGTTCTCCTGGTGGCAA-3′	24 nt		
GapdHForw	5′-TTCCAGGACCAAGATCCCTCCAAA-3′	24 nt	86 bp	XM_001725661
GapdHRev	5′-ATGGTGGTGAAGACACCAGTGAAC-3′	24 nt		

(50 mM KCl, 20 mM Tris–HCl, pH 8.4), 4 μL dNTP mix (250 μM each), 40 U RNase inhibitor and RNase-free water to complete the final volume. The reaction mixture (50 μL) was incubated at 43 °C for 1 h, then stopped at 4 °C and used immediately for PCR or kept at −80 °C until use.

Real-time PCR was carried out in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using the *Power* Sybr® Green Master Mix Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Experiments were performed in two independent experiments, and each sample run in duplicate in each experiment. Samples were run on 96-well optical PCR plates in a final reaction volume of 25 μL. The PCR parameters were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

The primers used for PCR amplification of Bax, Bcl-2, Bcl-XL and Gapdh, are listed in Table 1. Gapdh (Glyceraldehyde-3-Phosphate Dehydrogenase) gene was used as a reference gene to normalize target gene expression. Specific primers were designed using the sequences obtained in GeneBank, through the Blast program (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Subsequently, all sequences were designed and analyzed using the Integrated DNA Technologies website program (http://www.idtdna.com).

The PCR results were analyzed based on the ΔCT, which is the primary source of data variability. Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle.

Statistical analysis

All results were expressed as the mean ± SD of three independent experiments carried out, at least, in triplicate. These data were analyzed using Student's *t*-test for paired comparisons between compound and DMSO control. One-way ANOVA test was used to analyze target gene expression. Results were considered statistically significant when $P<0.05$.

Results

Synthetic route for preparation of BNDC

The compounds **1** and **2**, R and S tert-butyl 4-((3-hydroxyphenoxy) methyl)-2,2-dimethyloxazolidine-3-carboxylate — BHDC, were prepared as described elsewhere (Dias, et al., 2009). Using a similar strategy, compounds **3** and **4** (BNDC) were prepared, according to the synthetic route shown in Scheme 1. Initially, the commercially available protected serine 5 (R or S enantiomer) was reacted with $LiBH_4$ in THF/CH_3OH at −10 °C to give the corresponding alcohol **6** (Garner and Park, 1987; Meffre, et al., 1994). Reaction of **6** (R or S enantiomer) with 3-nitrophenol under Mitsunobu conditions at 80 °C produced **3** or **4**, respectively (Pave, et al., 2003).

Cytotoxic activity of BNDC

To evaluate the antitumor potential of our compounds, we used two wild-type leukemia cell lines, Jurkat cells (human T cell lymphoblast-like) and HL60 cells (human promyelocytic leukemia cells), along with two cell lines HL60.Bcl-2 and HL60.Bcl-XL (HL60 cells with ectopic expression of Bcl-2 and Bcl-XL, respectively) known to be resistant to anti-cancer drugs. Cytotoxic effects on normal cells were evaluated using PBMC from healthy donors. The IC_{50} values for the reduction of cell viability by these compounds using tumors and normal cells are summarized in Table 2.

Table 2
Concentrations of investigated compounds that induced 50% decrease (IC_{50}) in malignant and peripheral blood mononuclear cell survival.

Cell lines	1	2	3	4	Cisplatin
HL60	>100	75.25 ± 3.82	44.82 ± 5.30	12.22 ± 2,13	1.69 ± 0.44
HL60.Bcl-2	>100	>100	>100	25.66 ± 0.52	5.77 ± 3.98
HL60.Bcl-XL	>100	78.21 ± 6.62	>100	12.20 ± 5.55	4.91 ± 3.00
Jurkat	>100	74.44 ± 5.66	25.12 ± 5.03	13.61 ± 2.30	2.24 ± 0.66
PBMC	>100	43.31 ± 7.26	71.61 ± 14.94	60.46 ± 11.24	31.87 ± 14.54

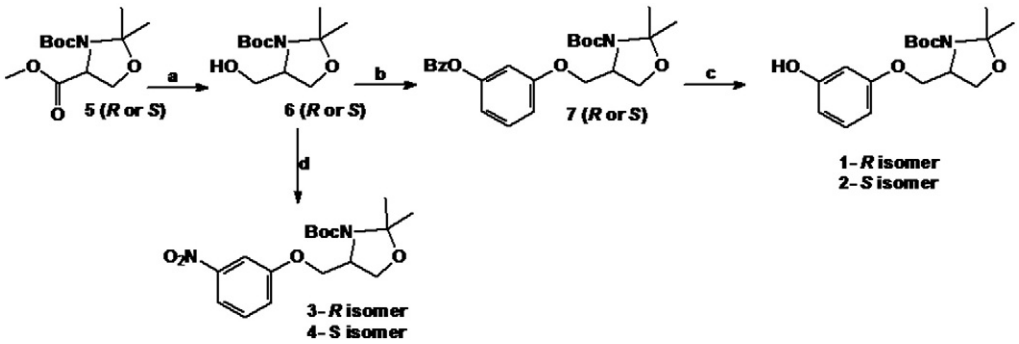
Leukemia cells lines and PBMC were incubated with drugs in concentrations ranging from 0.1 to 100 μM, at 37 °C in a 5% CO_2 atmosphere for 48 h. Viability was determined by MTT assay. Each data represents mean ± SD from three (leukemia cells) or seven (PBMC) independent experiments, performed in triplicate. Cisplatin was used as positive control.

HL60 cells were more susceptible to treatment with compounds than HL60.Bcl-2 and HL60.Bcl-XL, which ectopically express anti-apoptotic proteins, but Jurkat cells were as susceptible as the HL60 cells. Compound **1** was inactive in the tested concentration for all cell lines. Compound **2** displayed cytotoxic effects on at least one resistant lineage, while compound **4**, as the most active compound, was active against all the lineages. The compound **4** presented similar IC_{50} values against HL60, HL60.Bcl-XL and Jurkat cell lines that were significantly smaller than those observed against HL60.Bcl-2 (Student *T*-test, $P<0.05$). Compound **3** displayed cytotoxic effects on Jurkat and HL60, but it was inactive in HL60.Bcl-2 and HL60.Bcl-XL. Similar profile was obtained for cisplatin, IC_{50} value against HL60 cells was three-times lower than those observed against HL60.Bcl-2 and HL60 Bcl-XL cells. Cytotoxic activity differences between compounds **3** and **4** on Jurkat, HL60, HL60.Bcl-2 and HL60.Bcl-XL indicate an isomer-dependent effect (Student's *t*-test, $P<0.05$).

When considering the BNDC compounds, the IC_{50} values for tumor cells were lower than those for normal PBMC. Compound **4** was approximately five times less cytotoxic to PBMC than to HL60 and Jurkat cells, and for compound **3**, the corresponding IC_{50} values were approximately two-times lower. Cisplatin was around fifty-times less cytotoxic to PBMC than to leukemia cells (HL60 and Jurkat).

Apoptotic nuclear changes induced by BNDC

To investigate whether apoptosis was triggered by treatment with the new compounds, DNA fragmentation and chromatin condensation



Scheme 1. Synthetic scheme for preparation of BHDC (compounds **1** and **2**) and BNDC (compounds **3** and **4**). Reagents and conditions: a) $LiBH_4$, THF, MeOH, −10 °C; b) 3-benzoylphenol, DEAD, PPH_3, toluene, 80 °C; c) sodium methoxide/methanol, 0 °C; d) 3-nitrophenol, DEAD, PPH3, toluene, 80 °C.

Table 3
DNA fragmentation induction by compounds **1**–**4** in leukemia cell lines.

Substances	HL60	Jurkat
Control	6.70 ± 3.41	5.37 ± 2.37
Cisplatin	92.22 ± 2.48*	47.64 ± 11.00*
1	16.99 ± 2.37*	5.27 ± 2.69
2	17.90 ± 3.44*	3.30 ± 1.17
3	71.78 ± 17.78*	7.02 ± 4.44
4	65.97 ± 15.72*	11.98 ± 6.18

HL60 and Jurkat cells were incubated with 50 μM of each compound, at 37 °C in a 5% CO_2 atmosphere for 24 h. DNA content was assessed by flow cytometry analysis of cells labeled with propidium iodide. Each data represents mean ± SD from 3 different experiments.
* $P<0.05$, Student *T*-test.

was evaluated after 24 h of incubation with 50 μM of compounds (Nicoletti, et al., 1991; Nagata, 2000). Significant increases in DNA fragmentation were detected after 24 h of treatment with compounds **3** and **4** (50 μM) in HL60 cells ($P<0.05$, Student's *t*-test), but corresponding increases were not observed in Jurkat cells (Table 3). Compounds **1** and **2** were clearly less potent than compounds **3** and **4** in HL60 cells.

Consistent with these results, morphological changes in cell nuclei were observed in HL60 cells after treatment with BNDC compounds, as shown in Fig. 2. The treatment of cells with enantiomers **3** and **4** resulted in a marked increase in secondary necrosis in the apoptotic population containing single or multiple clumps of rounded, bright and condensed chromatin within a pale cytoplasm, different of control cell nuclei that exhibited diffused and homogeneous staining of the chromatin. The results revealed that the morphological features characteristic of apoptosis (chromatin condensation and nuclear fragmentation) was present in HL60 cells following exposure to compounds **3** and **4** or to cisplatin.

Apoptotic membrane changes induced by BNDC

The discrimination between cell death by necrosis and apoptosis is important for understanding the mechanism of action involved in the cytotoxicity of new compounds. For this purpose the exposure of PS was investigated. Under normal conditions, these phospholipids are only present in the inner leaflet of the plasma membrane, but after oxidative disordering it appears also in the outer side of the membrane (Martin, et al., 1995).

Control cells presented 90.84 ± 1.22% of cell without labeling, different than found to compound **3** (70.71 ± 8.52%) and compound **4** (72.16 ± 5.20%) after 24 h of incubation (Student *T*-test, $P<0.05$). Treatment of cells with enantiomers **3** and **4** (50 μM) increased the proportion of cells in late apoptosis (20.4% and 22.1% of Annexin-V/PI double-positive cells, respectively), being similar effects observed with cisplatin treatment under the same conditions, as shown in Fig. 3.

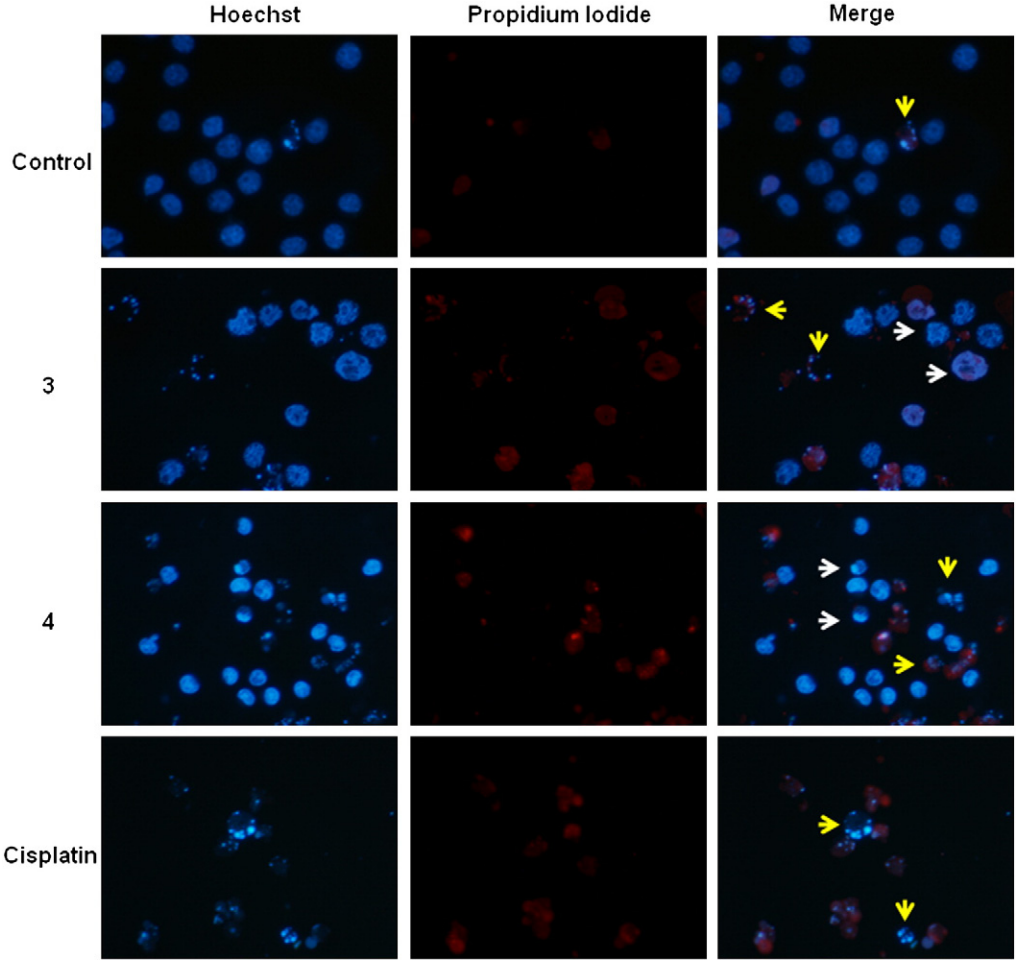


Fig. 2. BNDC compounds induce morphological alterations in HL60 cells. Cells were incubated with compounds **3** and **4** (50 μM) or cisplatin for 24 h. The double staining cell with Hoechst 33342 and propidium iodide distinguishes some typical features of apoptosis such as condensed chromatin (white arrows) and fragmented nuclei (yellow arrows). Overlap of the two images allows the detection of double-labeled cells suggesting late apoptosis at this time.

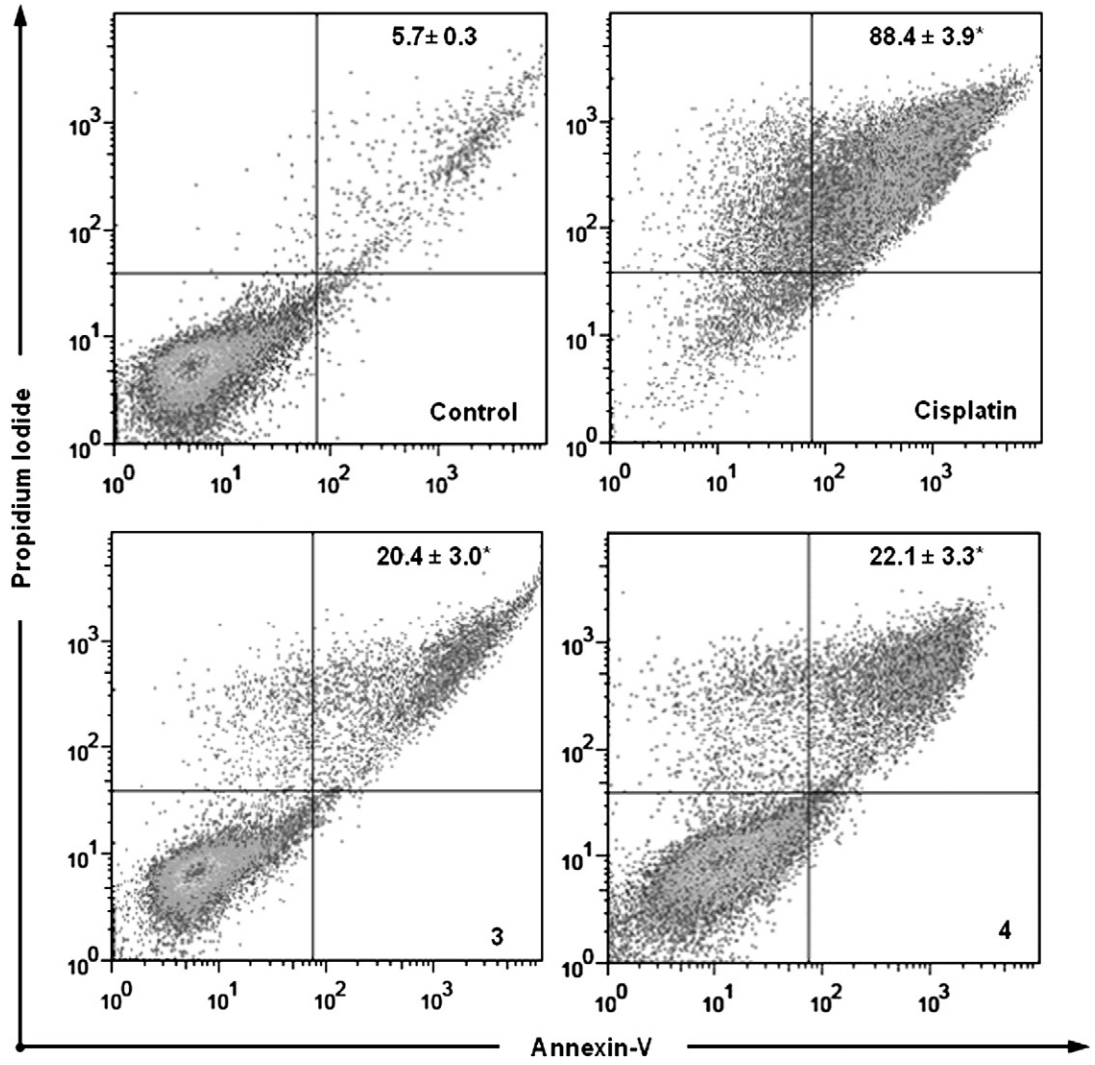


Fig. 3. BNDC compounds induce phosphatidylserine exposure in HL60 cells. The cells were incubated with the enantiomers **3** and **4** at the concentration of 50 μM for 24 h. The cells were stained with annexin V-FITC and propidium iodide. Analyses were performed by flow cytometry. Each data represents mean ± SD from three independent experiments (*P<0.05, *T*-test Student).

Caspase-dependent activity induced by BNDC

Having demonstrated that the cytotoxic effects of enantiomers **3** and **4** on HL60 cells induced DNA fragmentation, chromatin condensation and externalization of phosphatidylserine, we next investigated whether the compounds induce a caspase-dependent apoptotic pathway. HL60 cells were pretreated with Z-VAD-FMK (40 μM) for 40 min and then treated with BNDC compounds (50 μM) and incubated for 24 h, followed by PI staining and flow cytometry analyses. As expected, the treatment with the compounds **3** and **4** significantly increased the subdiploid DNA content of HL60 cells, when compared with the DMSO control (Student *T*-test, P<0.05). However, cells pretreated with Z-VAD-FMK and then treated with compounds **3** and **4** showed reduced levels of subdiploid DNA content (Fig. 4), similar to the levels in the control, suggesting that the compounds induced apoptosis by a caspase-dependent mechanism.

As caspase-3 has been specifically implicated as the caspase responsible for DNA fragmentation and chromatin condensation, this end point was examined in HL60 cells in response to treatment with compounds **3** and **4**. Caspase-3-like activity was measured by monitoring fluorescence levels generated by proteolytic cleavage of the Z-DEVD-AMC substrate. HL60 cells were treated with compounds **3** and **4** (50 μM) for 24 h. Exposure to compounds **3** and **4** increased caspase-3-like activity 4.36- and 2.48-fold, when compared with control, respectively (P<0.05, Student *T*-test). Cisplatin was included as a positive control (Fig. 5). Our results demonstrated that cell death induced by BNDC compounds involved activation of the classical pathway of apoptosis.

Changes in mRNA expression of Bcl-2 family induced by BNDC

BNDC enantiomers displayed cytotoxic activity and pro-apoptotic effects against leukemia cell lines. In order to observe the effect of BNDC in gene expression profile of Bcl-2 family, a Real-time PCR was performed to the specific target genes (Bax, Bcl-2 and Bcl-XL) in HL60 cells lines treated for 24 h with compound **4** in concentrations of 6.25 μM, 12.5 μM, 25 μM and 50 μM, as shown in Fig. 6. Compound **4** induced a concentration-dependent increase in *mRNA* expression of Bax and Bcl-XL in HL60 cells lines. Bcl-2 expression of *mRNA* decreased after treatment with 50 μM of compound **4**. HL60 cells with DMSO vehicle were used as control.

Discussion

The present paper describes the discovery of a new class of compounds with cytotoxic and pro-apoptotic activities. In drug discovery,

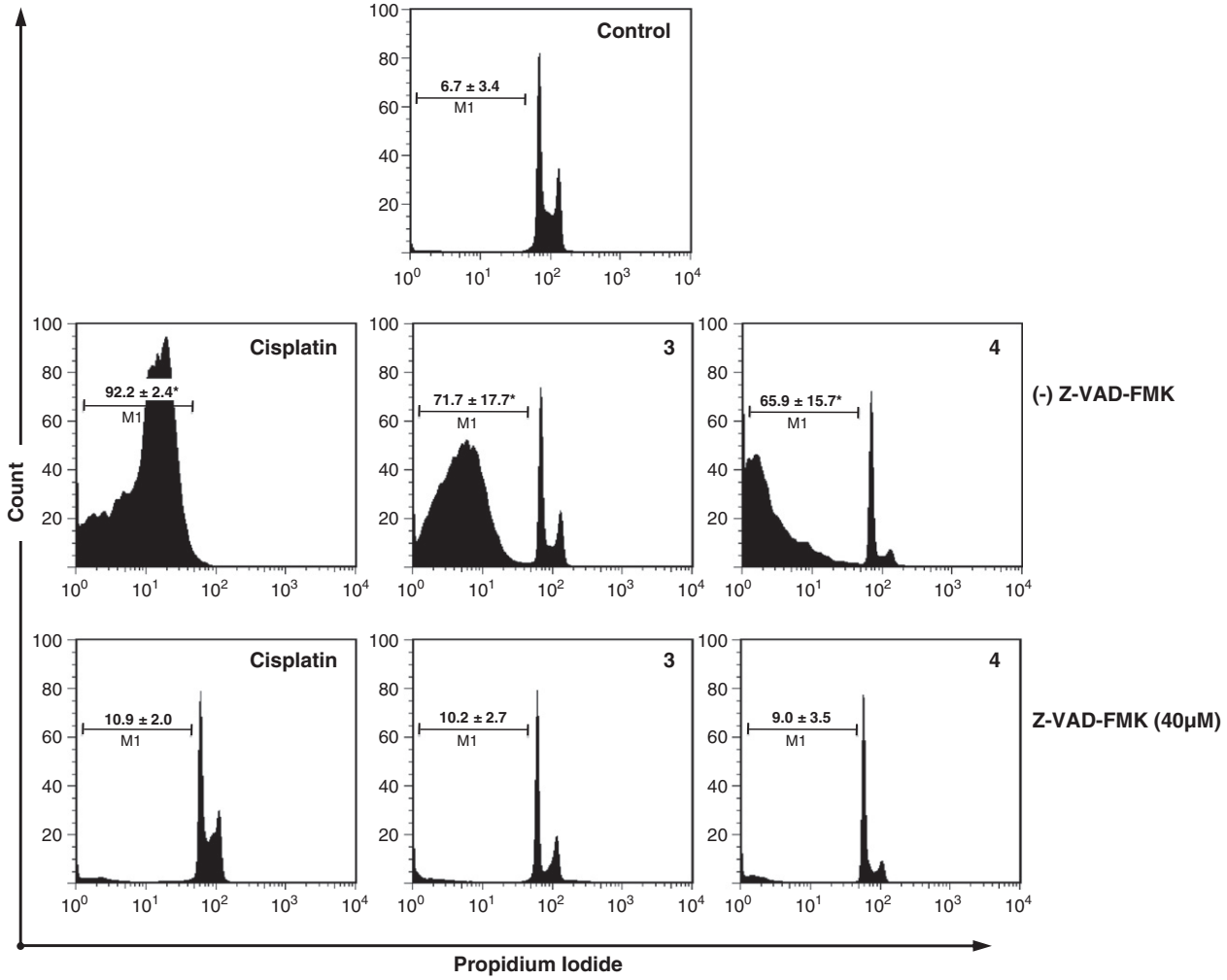


Fig. 4. BNDC compounds induce caspase-dependent apoptosis in HL60 cells. HL60 cells were pretreated with 40 μM of Z-VAD-FMK for 40 min and incubated with compounds **3** and **4** for 24 h. The DNA content was assessed by staining with PI and flow cytometry analysis. Cells containing subdiploid DNA (fragmented DNA) is indicated as M1 in each histograms. Cisplatin, positive control is demonstrated. Each data represents mean ± SD from three independent experiments (*P<0.05, *T*-test Student).

low toxicity and high efficacy are the main criteria in selecting *hit* compounds as candidates for drug development (Keseru and Makara, 2006; Langer et al., 2009). To demonstrate that the new BNDC compounds are useful hit compounds with anticancer potential, we evaluated their cytotoxicity toward cancer and normal cell lineages. The BNDC enantiomers are the first representative compounds of a new class with high potential, since the exchange of a hydroxyl group by the nitro group significantly increases (up to five-fold) the cytotoxicity effectiveness of these compounds. Furthermore an enantiomer-dependent effect was observed in BNDC and BHDC compounds, which suggest site-specific effects of these compounds. In the present study a relevant finding is that cytotoxicity effects of BNDC and BHDC against HL60 cells are greater than for HL60. Bcl-2, as we know the stable expression of these anti-apoptotic proteins in HL60 cells converted this apoptosis-sensitive cell line into resistant cells against anticancer drugs (Brumatti et al., 2003). These results strongly suggest that cytotoxicity effect induced by the BHDC and BNDC compounds are involved with apoptosis. Interestingly, the ectopic expression of Bcl-XL did not alter the cytotoxic activity of compounds **2** and **4**. According to these data, 50 μM of compound **4** induces an increase in mRNA expression of Bcl-XL, but this phenomenon does not seem to influence the cytotoxic effects in HL60 cells. On the other hand, this treatment presents downregulation of Bcl-2 and upregulation of Bax mRNA expression, which suggest a differential activity through Bax/Bcl-2 pathways by BNDC. In normal cells, Bcl-XL preferentially associates with Bak, while Bcl-2 associate with Bax to trigger apoptosis, being a differential action in Bax/Blc-2 pathway a good strategy to develop new anticancer drugs against cancer cells with Bcl-XL overexpression (Willis et al., 2005; Park et al., 2004; Liu et al., 2005).

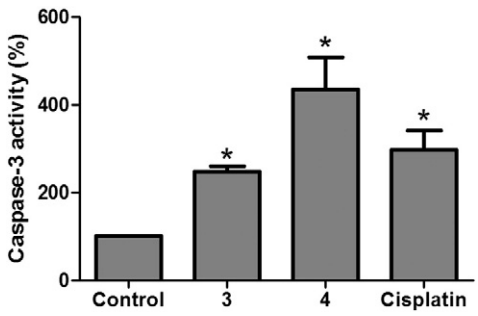


Fig. 5. BNDC compounds induce caspase-3 activation in HL60 cells. The cells were incubated with the enantiomers **3** and **4** in the concentration of 50 μM for 24 h. The fluorescence generated by exposure to substrate Z-DEVD-AMC was quantified in a fluorescence spectrophotometer. Each data represents mean ± SD from 3 different experiments (*P<0.05, *T*-test Student).

Based on these data, it was evaluated more closely the phenomenon of apoptosis triggered by the cytotoxic effect of the compounds against the two susceptible lineages, HL60 and Jurkat. With regard to apoptotic nuclear morphology, the DNA condensation and

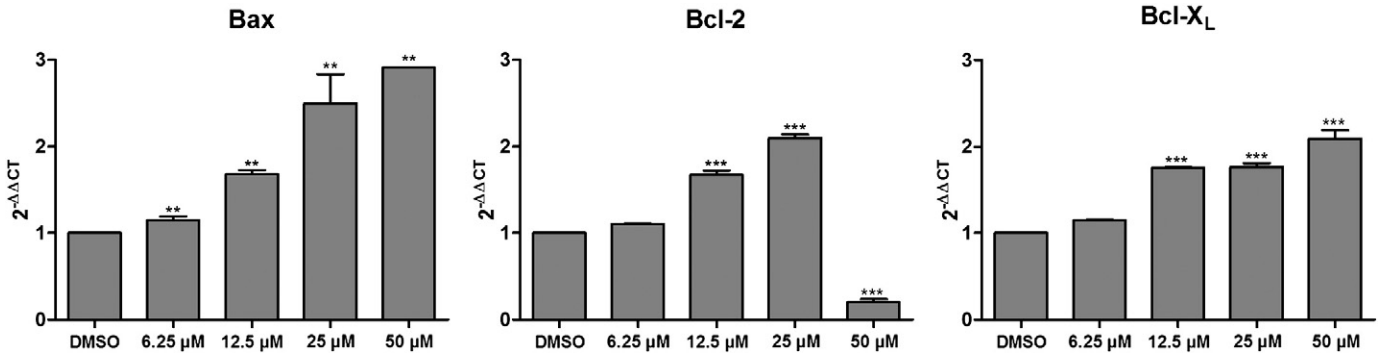


Fig. 6. Compound **4** induces change in mRNA expression of Bax, Bcl-2 and Bcl-XL in HL60 cells. HL60 were treated with different concentrations of compound **4** (50 μM, 25 μM, 12.5 μM and 6.25 μM) and incubated for 24 h. Data are plotted as mean ± SD of $2^{-\Delta\Delta CT}$, which is directly proportional to the relative gene expression **P<0.001, ***P<0.0001 (One-way ANOVA test).

fragmentation, these events occurred in HL60 cells when treated with BNDC and BHDC compounds, being the nitro compounds the most potential apoptotic inductor. The Jurkat cells demonstrated to be resistant to DNA fragmentation after 24 h of incubation with compounds, being the differences between HL60 and Jurkat apoptosis with apoptosis inducers also observed in other works (Reis et al., 2010; Kizawa et al., 2006). Karpinich et al. (2002) demonstrated that Jurkat cells lack expression of two critical pro-apoptotic proteins responsible for induction of apoptosis by intrinsic pathway: Bax and P53. The HL60 cells, also lack p53 protein, but normally express Bax protein (Brumatti et al., 2003; Koeffler et al., 1986; Shimizu and Pommier, 1997). Thus, the pro-apoptotic activity observed for these compounds possible depend on Bax protein to trigger apoptosis, which explains the greater resistance to DNA fragmentation in Jurkat cells. Although no difference in DNA fragmentation was found after treatment with both enantiomers **3** and **4**, a relevant observation was that the peak characteristic of cells in G0/G1 phase was preserved in the treatments with both compounds, which was not observed in the control of cisplatin. Cisplatin causes DNA damage when interacting with DNA forming interstrand cross-link and protein–DNA cross-link, which may lead to expression of p53 protein and G1 phase cell cycle arrest and apoptosis, in the early phase of this phenomenon and is dose-dependent (Gonzalez et al., 2001). In our experiments, cisplatin was used at higher concentration (50 μM) and DNA measurement was performed after 24 h, where total DNA fragmentation is observed. In cells treated with BNDC, it is remarkable that viable cells were the majority after incubation, which corroborates with G0/G1 peak found in the analysis of DNA content measurement. The compounds seem to act in synthesis phase and/or G2/Mitose, which is characteristic of substances that act during cell division or causing DNA damage (Karpinich et al., 2002; Gonzalez et al., 2001).

Furthermore, the DNA fragmentation triggered by compounds **3** and **4** was related to the activation of caspases, once the caspase inhibitor Z-VAD-FMK was able to block this effect, indicating that caspase cascades were involved in the apoptotic cell death induced by the new enantiomers. Consistent with these results, compounds **3** and **4** promoted caspase-3 activation. Caspase-3 is activated specifically during apoptosis (in the intrinsic and extrinsic pathways) and is one of the most important markers of apoptotic activity. These enzymes normally remain in the cytoplasm of cells as pro-enzymes, are particularly activated in the presence of a pro-apoptotic stimulus and are responsible for the major cellular degradation found in the nucleus and in the cytoplasm (Thornberry, 1998; Wang et al., 2005). A pro-apoptotic signal activates the initiator caspases, such as 8, 9 and 10, which in turn, activate effector caspases such as caspases 3, 6 and 7 that bring about most of the morphological changes related to apoptosis (Wang et al., 2005; Van Noorden, 2001). In addition, compounds **3** and **4** showed more activity in induction of caspase-3 compared to cisplatin after 24 h of treatment. These results point to different kinetics of apoptosis induction. At this time, cisplatin cell control is in the end of the apoptosis process and the most part of cells present total DNA fragmentation, which is associated with late-phase of apoptosis. As observed by the caspase activity, there are differences on activation of caspase-3, that is time-course and cell-type dependent (Kolfschoten et al., 2002; Gonzalez et al., 2001). Consistent with these data, analysis of phosphadidilserine exposure to measure necrosis/apoptosis ratio, demonstrated that HL-60 cells treated with cisplatin presents large number of cells in late apoptosis and necrosis differently from BNDC, that can be related to a distinct mechanism of action of cisplatin (Gonzalez et al., 2001). Likewise, caspase-3 activation also induces cleavage of lamin and nuclear mitotic apparatus proteins that maintain the structural integrity of the nucleus (Ziegler and Groscurth, 2004). Added to all of our findings, analysis of nuclear morphology using Hoechst 33258 and PI staining in HL60 treated with BNDC and cisplatin, corroborates with activation of caspase-dependent apoptotic machinery.

Even though the new compounds **3** and **4** demonstrated pro-apoptotic activity in our assays at 50 μM, there are screening programs designed to discover new hit compounds from assays at higher concentrations. Keseru and Makara (2006) described that hit compounds could be detected by high-throughput screening (HTS) techniques, in which, single-point experiments are typically performed with small molecules at concentrations of 1–50 μM with a 30–50% activity cutoff to identify potential hit compounds. In agreement with this report, Severson et al. (2008) screened 84,000 compounds against influenza virus at 114 μM and several high-activity compounds were revealed. The authors performed a structure–activity relationship (SAR) analysis of compounds to identify the most promising to lead compounds for further design and synthesis. The evaluation of targeted analogs of various molecular scaffolds should help to identify compounds exhibiting greatly improved potency and selectivity, which can be subsequently, developed into clinically useful therapeutic agents. In this context, the enantiomers **3** and **4** are interesting compounds that can be further modified by simple chemical manipulation in order to improve activity (Keseru and Makara, 2006; Bleicher et al., 2003; Li et al., 2006). Although the exact mechanism(s) by which these compounds can activate the apoptotic cascade in HL60 cells remains to be elucidated, our results provide a clear correlation between DNA fragmentation, morphological alterations and caspase activation consistent with a pro-apoptotic potential of the new enantiomers.

Conclusion

In conclusion, through this work we have identified new cytotoxic agents with pro-apoptotic activity, preferentially toward tumor cell division. Furthermore, our results suggest enantiomer-specific effects

and that relatively small changes in the structure of these molecules could lead to new compounds with improved cytotoxic and pro-apoptotic activity. Studies in this direction are already under way and will be reported in due course.

Conflict of interest

There are no competing interests.

Acknowledgment

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