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The design, synthesis and anticancer activity of new nitrogen mustard derivatives of natural indole phytoalexin 1-methoxyspirobrassinol

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Nitrogen mustards *cis*-1-methoxy-2-deoxy-2-[*N*,*N*-bis(2′-chloroethyl)amino]spirobrassinol (**4**) and *trans*-1-methoxy-2-deoxy-2-[N,N-bis(2′-chloroethyl)amino]spirobrassinol (**5**) derived from 1-methoxyspirobrassinol, an indole phytoalexin produced by the Japanese radish *Raphanus sativus* var. *hortensis* were designed as prospective dual-action compounds with DNA-alkylating effect and glutathione-depleting effects that may sensitize cancer cells to alkylating agents. Both new compounds demonstrated cytostatic/cytotoxic effects on various leukemia and ovarian cancer cell lines and dsDNA-destabilizing effects *in vitro*. Compound **4**, the more promising of the two compounds, exerts earlier onset of anticancer effects on Jurkat cells via induction of apoptosis compared to the traditional alkylating anticancer agent melphalan. In addition, it demonstrated higher potency on ovarian cancer OVCAR-3 cell line and lower fold resistance between Jurkat and Jurkat-M cells selected for the resistance to melphalan. Therefore, compound **4** may be less affected by certain cancer drug resistance mechanisms than melphalan and it may become a prototype of a new class of anticancer active nitrogen mustards that combine DNA-damaging and DNA-damage-sensitizing properties.

Key words: nitrogen mustard, indole phytoalexin, methoxyspirobrassinol, DNA-damaging compounds, drug resistance

The era of modern cancer chemotherapy started in 1942 when nitrogen mustard-3 (trichlormethine, HN3) was for the first time rationally applied to treat x-ray-resistant advanced lymphoma [1, 2]. Soon thereafter, other nitrogen mustards, including chlormethine (HN2) and N,N,N',N'-tetrakis-(2-chloro-ethyl)-propane-1.3-diamine (SK136) were found to be effective in Hodgkin's disease, lymphomas, and chronic myelocytic (CML) and lymphocytic (CLL) leukemias, but not against acute leukemias [3] and a wide range of solid tumors [4, 5]. Later, other nitrogen mustards were designed and evaluated in order to improve pharmacokinetics, efficacy, and selectivity for particular tissues. Among them were orally active chlorambucil [6], melphalan originally targeted against high phenylalanine-uptake melanomas [7], and cyclophosphamide developed as a prodrug targeting phosphoamidase-overexpressing cancers [8]. Similarly, uracil mustard (uramustine) effective against CLL and lymphomas was designed with the expectation of more efficient delivery to target DNA [9], and hydantoin-containing spirohydantoin mustard (spiromustine) was designed to cross the blood-brain barrier for the treatment of CNS tumors [10]. Rational design of anticancer-active nitrogen mustards directed against specific tissues and molecular targets continues at the present time, as demonstrated, for example, by the development of tallimustine, a distamycin A-derived nitrogen mustard [11], and 4-anilinoquinoline-linked aniline mustards targeting AT-rich regions of the minor groove of DNA [12].

In this paper we report on the design, synthesis and anticancer properties of nitrogen mustards derived from 1-methoxyspirobrassinol (1), an indole phytoalexin produced by the Japanese radish *Raphanus sativus* var. *hortensis*, upon infection by the plant pathogen, *Pseudomonas cichorii* [13]. We have previously reported that 2-amino analogues of this natural compound *cis*-1-methoxy-2-deoxy-2-(N-piperidyl)spirobrassinol (2) and trans-1-methoxy-2-deoxy-2-(N-piperidyl)spirobrassinol (3) (Figure 1) demonstrate anticancer properties against various human solid tumor and leukemia cell lines, due to, at least in part, their glutathione-depleting effect [14]. Considering the role of glutathione in anticancer drug resistance, including resistance to nitrogen mustards [15], we have rationally designed new prospective anticancer agents *cis*-1-methoxy-2-deoxy-2-[*N*,*N*-bis(2′-chloroethyl)amino]spirobrassinol (4) and trans-1-methoxy-2-deoxy-2-[N,N-bis(2′-chloroethyl)a

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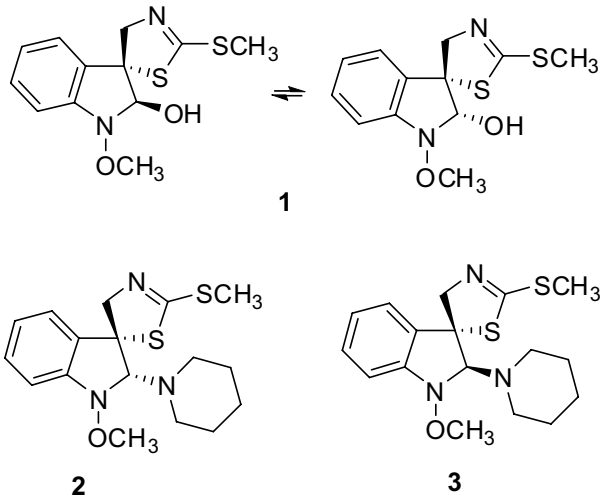


Figure 1. 1-methoxyspirobrassinol (1), *cis*-1-methoxy-2-deoxy-2-(N-piperidyl)spirobrassinol (2) and *trans*-1-methoxy-2-deoxy-2-(N-piperidyl)spirobrassinol (3)

mino]spirobrassinol (5), that were expected to combine the DNA-damaging (alkylating) functionality of nitrogen mustards with the glutathione-depleting, and potentially, the drug resistance-suppressing effect of the 2-amino analogues of 1-methoxyspiroibrassinol (1).

Materials and methods

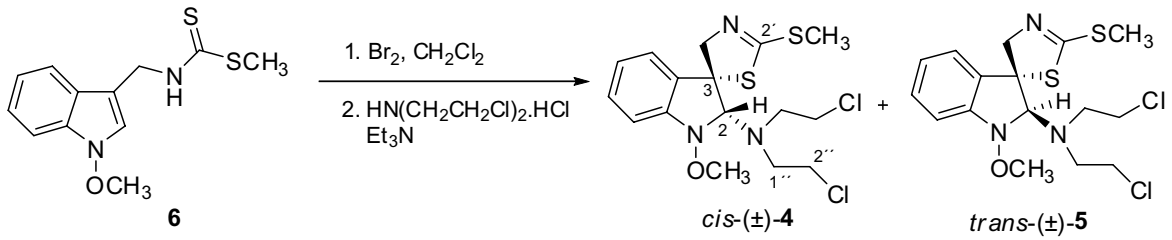
Compounds and synthesis. Synthesis of target compounds 4 and 5 (Figure 2) was performed from 1-methoxybrassinin (**6**) [16] by a spirocyclization reaction previously used for the synthesis of 1-methoxyspirobrassinol (**1**) [17]. A solution of bromine in anhydrous dichloromethane (0.96 mL, 0.413 mmol, stock solution prepared by dissolving 0.04 mL of Br_2 in 1.76 mL of anhydrous dichloromethane) was added to a stirred solution of 1-methoxybrassinin (0.375 mmol) in anhydrous dichloromethane (4 mL). After stirring at room temperature for 5 min a solution of bis-(2-chloroethyl)amine hydrochloride (0.602 g, 3.375 mmol) and triethylamine (0.721 g, 0.993 mL, 7.125 mmol) in anhydrous dichloromethane (10 mL) was added. After being stirred for 5 min, the reaction mixture was diluted by dichloromethane (8 mL), successively washed with 16 mL of water and 2×16 mL of saturated aqueous solution of sodium chloride, and the obtained dichloromethane solution filtered with charcoal. The residue obtained after the evaporation of solvent was subjected to flash chromatography on silica gel (15 g, Merck 230-400 mesh), eluent hexane/ethyl acetate 5:1 affording diastereoisomeric products 4 (0.070g, 45.9%) and 5 (0.015g, 9,8%).

The diastereoisomeric structure of the products *cis*-(±)-4 and *trans*-(±)-5 was determined by NOE differential and 2D NOESY NMR experiments, which was done in a similar way as for compounds 2 and 3 [14]. Compound 2 was synthesized following Mezencev et al [14].

For all biological assays, stock solutions of compounds 2, 4 and 5 were prepared in DMSO at concentrations of 40 mM, and frozen at -80°C prior to their use. Melphalan was dissolved in 70% ethanol acidified with HCl at concentration 10 mM, and cisplatin was dissolved in 0.9% sodium chloride solution at 6.6 mM. Compound 5 is less promising than compound 4 due to its mostly lower cytostatic/cytotoxic potency; therefore, it was only tested in resazurine-based and DNA-melting assays.

Cell cultures. Human ovarian cancer OVCAR-3 (NCI, Bethesda, MD, USA) and human acute T-lymphoblastic leukemia (T-ALL) Jurkat cells subclone A3 (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 medium with L-glutamine, supplemented with 10% FBS and 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B at 37°C in humidified air with 5% CO_2. Human T-ALL Jurkat-M cells were selected for resistance to melphalan by growing Jurkat cells in RPMI-1640 medium with L-glutamine, 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, and gradually increasing the concentration of melphalan from 1 μM to 7.5 μM over 10 weeks. Prior to the glutathione and antiproliferative/cytotocitiy assays, Jurkat and Jurkat-M cells were grown for 1 week in MEM medium supplemented with 5% FBS, glutamine and antibiotics without melphalan. Human acute promyelocytic leukemia HL-60 cells (ATCC, Manassas, VA) were maintained in suspension in IMDM with 4 mM L-glutamine, supplemented with 20% FBS and 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B at 37°C in humidified air with 5% CO_2. Human ovarian cancer HEY-CTPL cell line was prepared by stable transfection of HEY cells (Gordon W. Mills, Department of Molecular Therapeutics, the University of Texas, M. D. Anderson Cancer Center, TX, USA) with IMG-800-6 nega-

Figure 2. Synthesis of *cis*- and *trans*-1-methoxy-2-deoxy-2-[N,N-bis(2′-chloroethyl)amino]spirobrassinols (4 and 5) from 1-methoxybrassinin (6)



tive control plasmid from Gene Suppressor System IMG-803 (Imgenex, San Diego, CA, USA) following manufacturer's protocol and maintained at 37°C in humidified air with 5% CO_2 in RPMI-1640 medium with L-glutamine, 10% FBS, 600 μg/mL G418, 100 IU/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B.

Antiproliferative/cytotoxic effects. The effects of compounds **4**, **5**, and melphalan on the growth and viability of Jurkat, Jurkat-M, HL-60, OVCAR-3, and HEY-CTPL cells were evaluated at an arbitrarily selected time point of 72 hours by resazurin-based assay [18] using the *in vitro* toxicology assay kit TOX-8 (Sigma-Aldrich, St. Louis, MO, USA). Jurkat, Jurkat-M, HL-60 and OVCAR-3 cells were plated in 96-well black-walled plates (100 μL per well; cell density 100,000 cells/mL) in phenol red-free MEM medium with glutamine, supplemented with 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, and grown at 37°C in humidified air with 5% CO_2 for 24h. HEY-CTPL cells were plated in RPMI-1640 medium with glutamine, 10% FBS, 600 μg/mL G418, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Thereafter, 100 μL of full MEM growth medium containing tested compounds at concentrations 0, 0.2, 2, 20 and 200 $\mu mol.L^{-1}$ were added in quadruplicates and cells were incubated for 72 hours. After that 20 μL of the TOX-8 reagent were added to each well and incubated for the next 1-2.5 hours. The increase of fluorescence was measured at a wavelength of 590 nm using an excitation wavelength of 560 nm. The emission of control wells (no drug treatment) after the subtraction of a blank was taken as 100% and the results for treatments were expressed as a percentage of the control. All experiments were performed 3 times. IC_{50} values (concentrations of tested agents that inhibited growth of cell cultures to 50% of the untreated control) were determined by GraphPad Prism for Windows version 5.01 (GraphPad Software, Inc.).

Glutathione Assay. To explore the possible role of reduced glutathione (GSH) in the resistance of Jurkat-M cells to melphalan, cellular GSH was quantified in Jurkat and Jurkat-M cells using the GSH-Glo Glutathione Assay (Promega Corporation, Madison, WI, USA), a luminescence assay that is based on the conversion of luciferin-NT into luciferin in the presence of GSH and glutathione S-transferase. Cells were grown for one week at 37°C in humidified air with 5% CO_2 in MEM medium supplemented with 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Thereafter, about 20,000 Jurkat and Jurkat-M cells in 50 μL of PBS were plated into 96-well white-walled plates in quadruplicates, and GSH was quantified following manufacturer's protocol for mammalian cells in suspension. The amount of GSH was expressed as blank-corrected arbitrary luminescence units per 10^5 cells.

Determination of apoptosis by caspase 3/7 activity. Activities of caspase-3 and -7 were measured upon cleavage of their pro-fluorescent substrate Z-DEVD-R110 using Apo-One Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA). Jurkat cells were plated in MEM medium supplemented with 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B into 96-well black-walled plates at density 100,000 cells/mL and treated for 8 hours with compound **4**, melphalan, and solvent control (all tested agents at final concentration 10 μM). The assay was performed following manufacturer's protocol (each treatment and untreated control in quadruplicates). Activity of caspase-3 and -7 was measured as fluorescence at λex/ λem = 499nm/521 nm and expressed as relative fluorescence units after the subtraction of the blank.

Determination of necrosis/apoptosis by FACS. Jurkat cells were grown in RPMI-1640 medium with phenol red and glutamine, supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (37 °C; humidified air with 5% CO_2). Cells were exposed to compound 4 at 50 μM or DMSO solvent control for 48 hrs. The level of apoptosis and necrosis was determined by FACS analysis of cells stained with annexin V-FITC and propidium iodide (PI) using annexin V-FITC Apoptosis Detection Kit PF032 (Calbiochem, San Diego, CA, USA) following manufacturer's rapid binding protocol. Experiments were performed in 3 biological replicates. Results were interpreted and cytotoxicity was calculated following previously reported procedure [19].

Kinetics of cytotoxicity. Cytotoxicity of compound 4 and melphalan on Jurkat cells was determined with CytoTox-Glo Cytotoxicity Assay (Promega, Madison, WI, USA). In this homogenous, luminescent assay, the number of dead cells with lost membrane integrity was measured via distinct protease activity using luminogenic substrate alanyl-alanyl-phenylalanyl-aminoluciferin. Number of viable cells was determined from increase in the number of dead cells after addition of digitonin to lyse remaining live cells at the end of drug treatment. Cytotoxicity was expressed as blank-corrected arbitrary luminescence units and cell viability as percentage of increase of luminescence after addition of digitonin to drug treated cells compared to untreated control cells. Jurkat cells were plated in MEM medium supplemented with 5% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B into 96-well white-walled plates at density 100,000 cells/mL (50 μL of cell suspension per well). Thereafter, 50 μL of drugs dissolved in full MEM medium were added to appropriate wells and cells were treated for arbitrarily selected time points of 2, 8 and 24 hours (all drugs at final concentration 10 μM). The assay was performed according to cytotoxicity assay and viability (by lysis) assay protocols.

Effect on differentiation of leukemia HL60 cells. The differentiation of promyelocyte HL-60 leukemia cells to granulocyte lineage was evaluated by a respiratory-burst NBT reduction assay as previously described [20]. Prior to the treatment by compound **4**, cells were maintained at a logaritmic growth phase and seeded at density 0.2 x 10^6 cells/mL (passage 5). Compound **4** was added at final concentrations of 10 and 100 μM. The final concentration of DMSO added with tested com-

pounds was 0.25% (V/V). Positive and negative controls were prepared by adding DMSO to the suspension of HL-60 cells at final concentrations of 1.25% and 0.25%, respectively. After 96 hours of continuous incubation, cells were counted by a Vi-CELL XR cell counter (Beckman Coulter) using the trypan blue dye exclusion method, and aliquots of cell suspensions (3 mL) were centrifuged at 400g for 7 minutes. The pellets were re-suspended in 1 mL complete IMDM medium. Thereafter, 1 mL of PBS solution containing 20 ng/mL of PMA (Sigma-Aldrich) and 2 mg/mL of NBT (Sigma-Aldrich) was added to the cell suspensions and incubation was allowed to proceed for 1 h at 37°C. At the end of incubation, 0.4 mL of cold 2M HCl were added and insoluble formazan was obtained by centrifugation at 700g for 10 min, dissolved in 1 mL DMSO, and the OD of the solution (200 µl per well in 96-well microplate) was measured by microplate reader at 590 nm. The experiments were performed in triplicate and the extent of differentiation was expressed as mean OD_{590nm} per 10^6 cells.

DNA melting analysis. Calf thymus (CT) DNA (Sigma-Aldrich, St. Louis, MO, USA) at 500 µg/mL in a buffer solution (10 mM TRIS, 5 mM NaCl, 0.5 mM EDTA; pH 7.5) was treated with compounds **4**, **5**, melphalan (Sigma-Aldrich), and cisplatin (Sigma-Aldrich) at final concentrations of 1, 10 and 100 µM for each agent for 48 hours at 37° C. After the incubation, DNA was extracted by phenol-chloroform-isoamylalcohol (25:24:1), precipitated with 3 M sodium acetate and 100% ethanol at -20°C overnight. DNA pellets were dissolved in 10 mM phosphate buffer (pH 7.5), and the melting curves were recorded by CARY IE UV-Visible Spectrophotometer in 1-cm quartz sample cells by measuring the absorbance at 260 nm and temperature from 25°C to 95°C with gradient 1°C/min using 10 mM phosphate buffer as a reference. The melting temperature (T_m) was determined as the temperature corresponding to a maximum on the first-derivation profile of the melting curves [21]. Each DNA drug treatment was performed in triplicate.

Formation of DNA interstrand crosslinks. Amount of interstrand crosslinks was determined in CT DNA (390 µg/mL) treated with compound **4** or melphalan at 100 µM (as described in DNA melting analysis) and in DNA from HL-60 cells exposed to compound **4** or melphalan. HL-60 cells were plated in full IMDM medium at density 500,000 cells/mL (humidified air with 5% CO_2; 37 °C) and exposed to compound **4** or melphalan for 48 hrs at a concentration of 100 µM. Thereafter, cellular DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following manufacturer's protocol and its purity and concentration were determined by spectrophotometry from double ratio 260 nm/280 nm and 260 nm/230 nm using the NanoDrop ND-1000 spectrophotometer. The amounts of interstrand crosslinks were determined by ethidium bromide fluorescence assay as described previously [22]. The percentage of cross-linked DNA was determined from ethidium bromide fluorescence intensities by means of formula:

$$CRL = \frac{ft - fn}{1 - fn} \times 100\%$$

where CRL = the percentage of interstrand cross-linked DNA in treated cells; *ft*= ratio of fluorescence after thermal denaturation to fluorescence before denaturation of DNA from treated cells, *f*n = ratio of fluorescence after denaturation to fluorescence before denaturation of DNA from untreated cells. The excitation wavelength was 525 nm and the emission wavelength was 580 nm.

Statistical analysis. The statistical significance of differences between mean values was determined using two-tailed, unpaired (two-sample) Student's t-test with Welch's correction for unequal variances. For DNA crosslink experiment, statistical significance between calculated mean percentages of cross-linked DNA and hypothetical value of 0% for untreated controls was determined using two-tailed one-sample Student's t-test. P-values ≤ 0.05 were considered statistically significant Standard deviations of parameters derived from experimental data were calculated using error propagation calculator (http://laffers.net/tools/error-propagation-calculator.php). Error bars in graphs represent standard errors of means (SEM).

Results

Synthesis. *cis*-1-Methoxy-2-deoxy-2-[*N*,*N*-bis(2´-chloroethyl)amino]spirobrassinol (**4**): Colorless crystals, mp 119-120°C (dichlormethane-hexane), R_f (hexane/ethyl acetate, 5:1) 0.38. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.57 (3H, s, SCH_3); 3.19 (2H, ddd, *J* = 5.6 Hz, *J* = 8.6 Hz, *J* = 14.2 Hz, H-1´´); 3.31 (2H, ddd, *J* = 6.3 Hz, *J* = 10.6 Hz, *J* = 14.5 Hz, H-1´´); 3.54 (2H, ddd, *J* = 5.6 Hz, *J* = 10.6 Hz, *J* = 15.1 Hz, H-2´´); 3.62 (2H, ddd, *J* = 6.3 Hz, *J* = 8.8 Hz, *J* = 5.1 Hz, H-2´´); 3.93 (3H, s, OCH_3); 4.26 (1H, s, H-2); 4.30 (1H, d, *J* = 15.1 Hz, H-4´b); 4.49 (1H, d, *J* = 15.1 Hz, H-4´a); 6.95 (1H, d, *J* = 7.8 Hz, H-7); 7.01 (1H, ddd, *J* = 1.0 Hz, *J* = 7.5 Hz, *J* = 7.6Hz, H-5); 7.23 (1H, d, *J* = 7.6 Hz, H-4); 7.26 (1H, ddd, *J* = 1.2 Hz, *J* = 7.5 Hz, *J* = 7.8 Hz, H-6). ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.3 (SCH_3); 43.3 (C-2´´); 55.9 (C-1´´); 63.2 (OCH_3); 70.1 (C-3); 74.9 (C-4`); 94.4 (C-2); 112.9 (C-7); 123.2 (C-4); 124.2 (C-5); 129.5 (C-4a); 130.0 (C-6); 148.8 (C-7a); 165.3 (C-2`). MALDI TOF MS *m/z* (%) 408.0513 $[M+H]^+$ (76), 406.0570 $[M+H]^+$ (100), 301.0649 (45), 299.0691 (64), 270.0429 (63), 268.0467 (73), 265.0443 (81), 233.026 (49), 219.0667 (37). Anal. Calcd. for $C_{16}H_{21}Cl_2N_3OS_2$: C, 47.29; H, 5.21; N, 10.34. Found: C, 47.48; H, 4.95; N, 10.56

trans-1-Methoxy-2-deoxy-2-[*N*,*N*-bis(2´-chloroethyl)amino]spirobrassinol (**5**): Colorless oil, R_f (hexane/ethyl acetate, 5:1) 0.54. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.56 (3H, s, SCH_3); 3.16 (4H, m, H-1´´); 3.55 (4H, m, H-2´´); 3.92 (3H, s, OCH_3); 4.05 (1H, d, *J* = 15.6 Hz, H-4´), 4.58 (1H, s, H-2); 4.62 (1H, d, *J* = 15.6 Hz, H-4´); 6.95 (1H, d, *J* = 7.8 Hz, H-7); 7.01 (1H, ddd, *J* = 1.0 Hz, *J* = 7.5 Hz, *J* = 7.6Hz, H-5); 7.26 (1H, ddd, *J* = 1.2 Hz, *J* = 7.5 Hz, *J* = 7.8 Hz, H-6); 7.29 (1H, dd, *J* = 1.1 Hz, *J* = 7.6 Hz, H-4). ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.3 (SCH_3); 42.7 (C-2´´); 55.8 (C-1´´); 63.2 (OCH_3); 70.1 (C-3); 70.4 (C-4`); 96.5 (C-2); 112.6 (C-7); 123.7 (C-4); 123.8 (C-5); 127.8 (C-4a); 130.0 (C-6); 149.5 (C-7a); 165.1 (C-2`). Anal. Calcd. for $C_{16}H_{21}Cl_2N_3OS_2$: C, 47.29; H, 5.21; N, 10.34. Found: C, 47.53; H, 5.49; N, 10.60

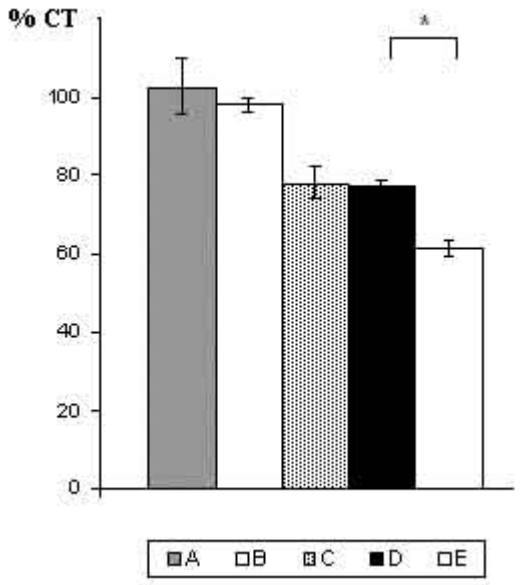


Figure 3. Cytostatic/cytotoxic effects of compound 5 (A), compound 2 (B), melphalan (C), compound 2 + melphalan (D) and compound 4 (E) on OVCAR-3 cells. All compounds individually or in combination were tested at 10 μM/72hrs. %CT: viability percentage of treated vs untreated control cells; *: p = 0.000761

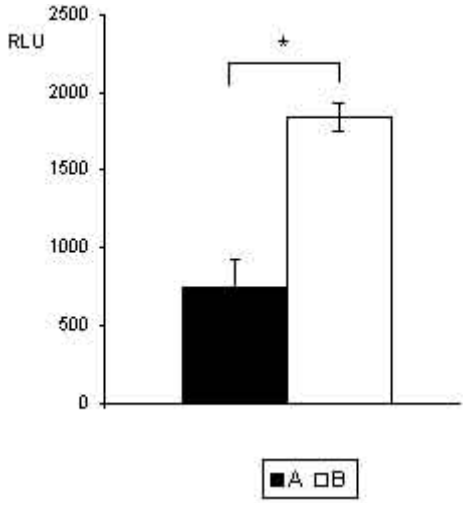


Figure 4. Determination of reduced glutathione in Jurkat (A) and Jurkat-M (B) cells (no drug treatment). RLU = blank-corrected arbitrary luminescence units normalized per 10^5 cells. Error bars: standard errors of means. *: p=0.0175

Antiproliferative/cytotoxic effects. IC_{50} values for compounds **4**, **5**, and melphalan on Jurkat, Jurkat-M (melphalan-resistant), HL-60, HEY-CTPL, and OVCAR-3 cells are presented in Table 1. New nitrogen mustards **4** and **5** displayed lower potency than melphalan on Jurkat, Jurkat-M and HL-60 leukemia cells and on HEY-CTPL ovarian cancer cells; however, compound **4** was significantly more potent than melphalan on ovarian cancer OVCAR-3 cells (p=0.0063). Compound **4** also was more cytostatic/cytotoxic on OVCAR-3 cells than combination of melphalan and compound **2** (all drugs at 10 μM, Figure 3).

Compared to Jurkat cells, Jurkat-M cells were more resistant to all tested nitrogen mustards ($p < 0.05$); nevertheless, compound **4** demonstrated lower fold resistance than melphalan (2.1 vs. 8.6, respectively). The fold resistance for compound **5** was similar to that of melphalan (8.2). Interestingly, compound **4** was less potent on Jurkat cells, but more potent on HL-60, HEY-CTPL and OVCAR-3 cells than diastereomeric compound **5**. Compound **4** appears to be slightly more potent than compound **5** for Jurkat-M cells, but the difference between IC_{50} values is not considered statistically significant by our criteria (p=0.06).

Table 1. IC_{50} values of compounds 4, 5 and melphalan (MEL) on Jurkat, Jurkat-M (melphalan-resistant), HL-60, HEY-CTPL, and OVCAR-3 cells (72 hr; resazurin-based assay, SEM = standard error of mean).

IC_{50}±SEM [μM]	Melphalan	4	5
Jurkat	0.25±0.01	1.87±0.14	0.93±0.08
Jurkat-M	2.16±0.37	3.94±0.40	7.61±1.32
HL-60	0.17±0.09	4.15±0.03	11.31±1.23
HEY-CTPL	4.20±0.31	7.60±0.74	>100
OVCAR-3	24.09±3.00	11.46±0.68	36.47±5.09

GSH-assay. The level of reduced glutathione in melphalan-resistant Jurkat-M cells was found to be significantly higher than that in the parent Jurkat cell line (Figure 4, p=0.0175).

Apoptosis by caspase-3 and -7 activity. Jurkat cells treated with compound **4** at 10 μM for 8 hours display significantly higher caspase-3 and -7 activity than untreated control cells (p=0.0077, Figure 5) and cells treated with melphalan under the same conditions (p=0.0149). There was no statistically significant difference in caspase-3 and -7 activities in cells treated

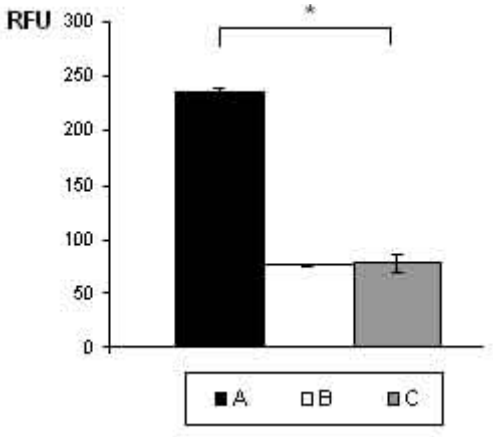


Figure 5. Induction of apoptosis by compound 4 (A), and melphalan (B) in Jurkat T-ALL cells determined by caspase-3 and -7 activity. Cells were treated for 8 hours at drug concentrations of 10 μM. C = untreated Jurkat cells; RFU = blank-corrected relative fluorescence units; *: p=0.0077

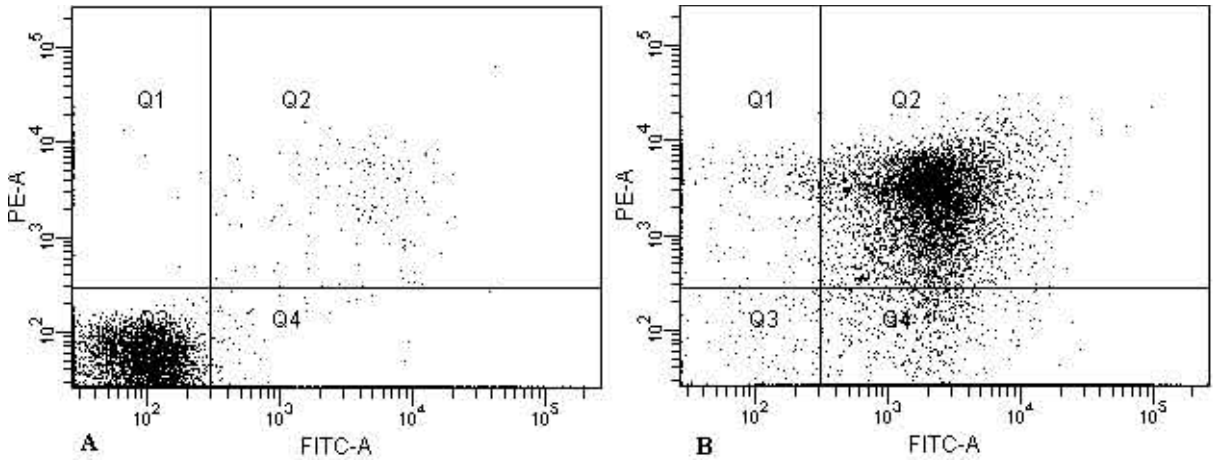


Figure 6. FACS determination of necrosis and apoptosis in Jurkat cells treated by DMSO solvent control (A) and compound 4 (B) for 48 hours at concentrations 0.125% and 50 μM, respectively. Q1 (Annexin V^-/PI^+ cells); Q2 (Annexin V^+/PI^+ cells); Q3 (Annexin V^-/PI^- cells); Q4 (Annexin V^+/PI^- cells)

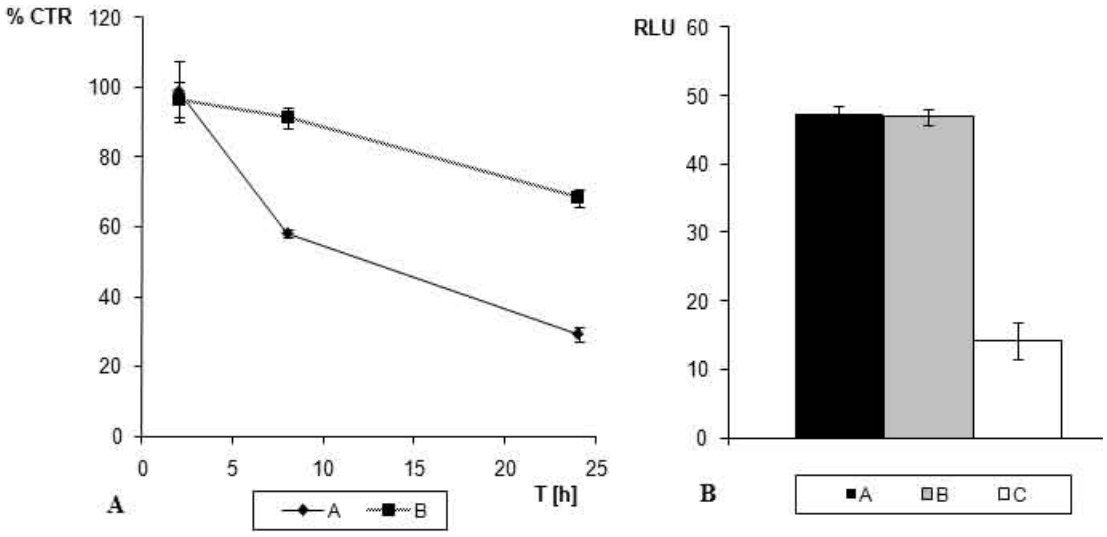


Figure 7. A: Viability (by cell lysis) of Jurkat cells treated with 10 μM of compound 4 (A) and melphalan (B) for 2, 8 and 24 hours.
B: Cytotoxicity associated with membrane disruption in Jurkat cells treated for 24 hours with 10 μM of compound 4 (A) and melphalan (B). C = untreated Jurkat cells. P-values: A/C=3.57x10^{-7}, B/C=4.23x10^{-7}, A/B=0.798.

with melphalan and untreated control cells (p=0.82). Overall, the result indicates that under these experimental conditions compound **4**, but not melphalan, induces early cell death by apoptosis (at ~8 hrs).

Table 2. FACS distributions of Jurkat treated cells (50 μM compound 4 / 48 hrs) and control cells (0.125% DMSO / 48 hrs). Q1 (Annexin V^-/PI^+), Q2 (Annexin V^+/PI^+), Q3 (Annexin V^-/PI^-), Q4 (Annexin V^+/PI^-). SEM=standard error of mean.

Q±SEM	Q1 [%]	Q2 [%]	Q3 [%]	Q4 [%]	Q1+Q2+Q4 [%]
Control cells	0.43±0.09	1.07±0.18	88.90±0.55	9.60±0.29	11.10±0.35
Treated cells	2.57±0.28	55.27±0.88	2.17±0.57	39.97±0.69	97.81±1.15

Apoptosis/necrosis by FACS. Representative scattergrams of PI vs. FITC fluorescence intensities from 3 experimental replicates are presented for treated and untreated control cells (Figure 6).

Quadrants in the scatterplots represent: Q1 – Annexin V^-/PI^+ (cells with permeabilized membranes/cell nuclei/debris); Q2 – Annexin V^+/PI^+ (late apoptosis and/or necrotic cells); Q3 – Annexin V^-/PI^- (viable cells), and Q4 -Annexin V^+/PI^- (early apoptotic cells). After 48 hr, Jurkat cells treated with compound **4** at 50 μM displayed mostly late apoptosis and/or necrosis (Table 2 and Figure 6). Calculated cytotoxicity from this experiment for compound **4** is 86.2% (SEM = 0.69%), which is comparable to that of melphalan (82.3%) under the same experimental conditions (data not shown).

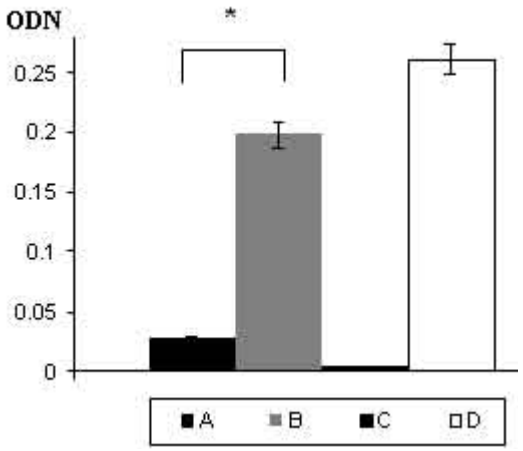


Figure 8. Effect of compound 4 at 10 μM (B) and 100 μM (C) on the differentiation of HL-60 cells into granulocyte lineage. A: negative control (0.25% DMSO); D: positive control (1.25% DMSO); ODN = normalized OD_{590nm} per 10^6 cells; *: p≤0.05.

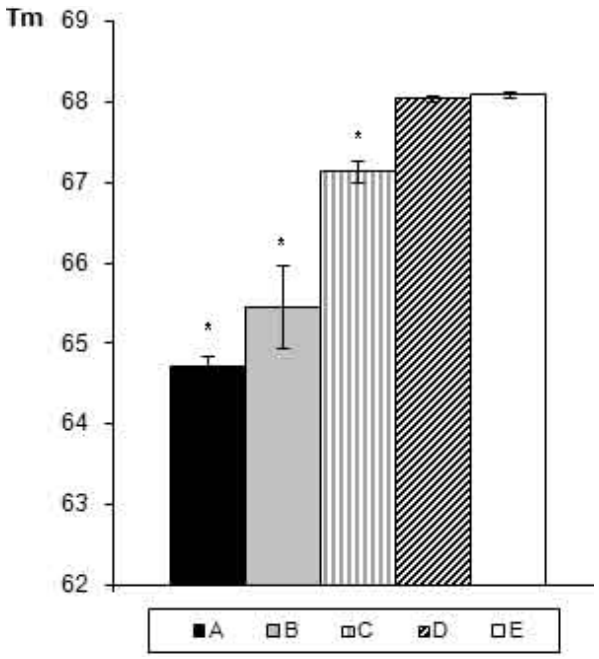


Figure 9. Melting temperature (Tm in °C) of calf thymus DNA treated with 10 μM of: melphalan (A); cisplatin (B); compound 4 (C); compound 5 (D). Untreated control DNA (E); *: p ≤ 0.05 (compared to E).

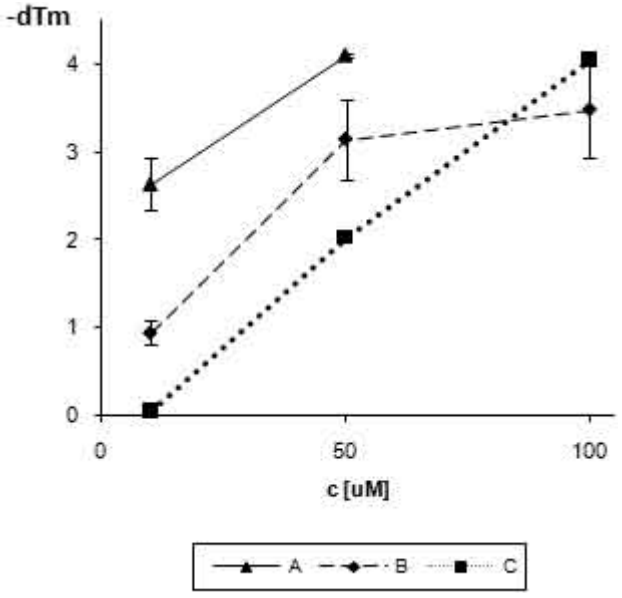


Figure 10. Change of melting temperature (-dTm in °C) of calf thymus DNA treated with different concentrations of cisplatin (A), compound 4 (B) and compound 5 (C).

Kinetics of cytotoxicity. Both compound **4** and melphalan at 10 μM induced significant cytotoxicity associated with cell membrane disruption in Jurkat cells after 24 hours ($p=3.57x10^{-7}$ and $4.23x10^{-7}$; Figure 7B) but not after 2-hour and 8-hour treatments (data not shown). However, compound **4** caused a greater decrease in the proportion of viable Jurkat cells after 8 and 24 hours than melphalan (Figure 7A). These data demonstrate earlier onset of anticancer effects of compound **4** compared to melphalan. In addition, the early effects of compound **4** at time point of 8 hrs are not associated with loss of membrane integrity.

Differentiation. Compound **4** at concentration 10 μM, but not at concentration 100 μM strongly induced differentiation of cultured HL-60 cells into granulocyte lineage (Figure 8).

DNA melting. Compound **4**, but not compound **5**, significantly decreased the melting temperature of calf thymus DNA treated with these compounds at concentrations of 10 μM (p=0.05 and 0.30, respectively) (Figure 9). Compound **5** significantly decreased the melting temperature of DNA only at higher concentrations (50 and 100 μM) with respective p-values of $1x10^{-5}$ and $3x10^{-5}$. The effect of compounds **4** and **5** on the DNA melting temperature is concentration dependent and qualitatively the same as effect of the traditional agent cisplatin (Figure 10). Melting curves of CT DNA modified with cisplatin at 100 μM did not yield reliable Tm values.

Interstrand crosslinks. Compound **4** and melphalan significantly induced formation of interstrand crosslinks in calf thymus DNA (p=0.0095 and 0.0105, respectively) and in cellular DNA of HL-60 cells (p=0.0025 and 0.0098, Figures 11 and 12). In both cases melphalan induced significantly higher amounts of interstrand crosslinks than compound **4** (p=0.0395 and 0.0254). Results of the experiment using HL-60 cells provide evidence that both melphalan and compound **4** penetrate across the cell membrane and translocate to the nucleus where they cause DNA damage via formation of interstrand crosslinks.

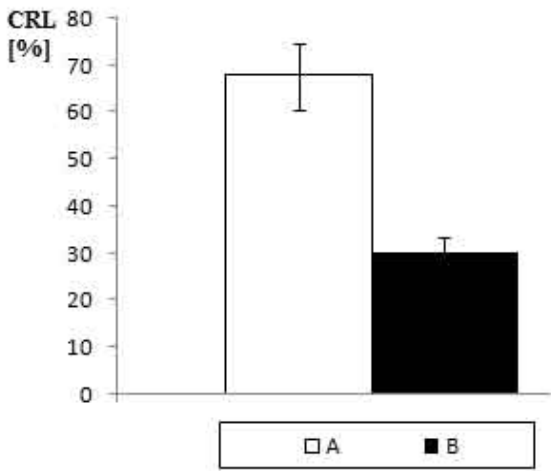


Figure 11. Percentage of interstrand crosslinks (CRL) in calf thymus DNA treated with melphalan (A) and compound 4 (B) at 100 μM for 48 hrs/37 °C. CRL of untreated DNA is by definition 0%. p-values: A/B=0.0394, A/hypothetical mean 0%=0.0105, B/hypothetical mean 0%=0.0095.

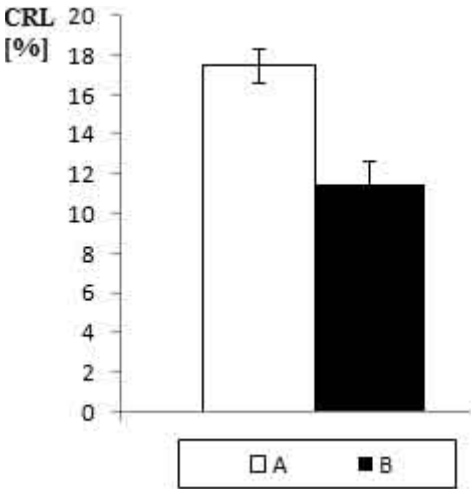


Figure 12. Percentage of interstrand crosslinks (CRL) in HL-60 cellular DNA. Cells at initial density of 500,000 /mL were treated with melphalan (A) or compound 4 (B) at 100 μM for 48 hrs/37 °C. CRL of untreated DNA is by definition 0%. p-values: A/B=0.0254, A/hypothetical mean 0%=0.0025, B/hypothetical mean 0%=0.0098.

Discussion

Indole phytoalexin-derived nitrogen mustards **4** and **5** were designed as dual-action compounds that comprise two elements: a bis(2′-chloroethyl)amine alkylating group and 2-amino substituted 2-deoxy-1-methoxyspirobrassinol moiety with glutathione-depleting activity [14]. As a result, they were expected to possess a higher potency against cancer cells than the traditional alkylating agent melphalan, at least in cells with glutathione-mediated resistance to alkylating agents. In our experiments, melphalan after 72-hour treatment, exhibited higher antiproliferative/cytotoxic potency than both new nitrogen mustards in all leukemia cell lines, including Jurkat-M cells selected for the resistance to melphalan. On the other hand, the fold resistance between Jurkat and Jurkat-M cells was remarkably lower for compound **4** compared to compound **5** and melphalan, suggesting that compound 4 is less affected by resistance mechanism/s developed in Jurkat-M cells than compound **5** and melphalan. Jurkat-M cells have about 2.5-times higher levels of reduced glutathione and 9-times higher IC_{50} for melphalan than their parent Jurkat cell line, which is consistent with other reports on cancer cells with glutathione-mediated melphalan-resistance [23] and suggests that glutathione is, at least in part, responsible for the resistance of Jurkat-M cells to melphalan.

The higher potency of compound **4** against ovarian cancer OVCAR-3 cells may be a promising finding, considering the fact that this cell line was initially established from malignant ascites of a patient with ovarian papillary adenocarcinoma pre-treated with cyclophosphamide, doxorubicin and cisplatin, and displays high resistance *in vitro* to clinically relevant concentrations of these drugs [24]. Compound **4**, combining glutathione-depleting and alkylating moieties in one molecule, was more effective against OVCAR-3 cells than combination of its building blocks and functional units, compound **2** and melphalan. This finding demonstrates that, at least for OVCAR-3 cells and the employed concentration, the design of compound **4** was successful.

The unexpectedly large difference in potencies of diastereomeric nitrogen mustards **4** and **5** on HEY-CTPL cells, not observed in other cell lines, demonstrates the important role of configuration on the anticancer effects of these compounds; however, the underlying mechanism responsible for the difference remains to be elucidated. Due to its less promising cytostatic/cytotoxic potencies, compound 5 was not involved in further tests.

Unlike melphalan, compound **4** strongly induced apoptosis of Jurkat cells after 8-hour treatment, demonstrated by the activation of effector caspase-3 and -7. This finding is further supported by results of cytotoxicity kinetics that demonstrate significantly decreased viability without the loss of membrane integrity of cells treated for 8 hours with compound **4**, but not with melphalan, compared to untreated control (p=0.0245 and 0.2134, respectively). Furthermore, compound **4** induced a significantly greater decrease in viability of Jurkat cells after 24-hour treatment than melphalan (p=0.05), but essentially the same level of cytotoxicity associated with the loss of membrane integrity (RLU = 47.26 and 46.89, respectively, p=0.798). Therefore, it can be concluded that compound **4**, unlike melphalan**,** induces early apoptosis of Jurkat cells *in vitro* and demonstrates earlier overall onset of anticancer effects on Jurkat cells than melphalan, which demonstrates delayed onset of apoptotic or non-apoptotic cell death under the same conditions.

After 72-hour treatment there was no remarkable difference in levels of total cell death (apoptosis and primary or secondary necrosis) induced by compound **4** and melphalan at 50 μM concentrations.

Compound **4**, the more promising of the two new anticancer agents, promoted differentiation of promyelocytic HL-60 leukemia cells into the granulocyte lineage. So far there have been few reports suggesting that nitrogen mustards may promote cell differentiation in addition to their cytotoxic effects [25]. This mechanism also may contribute to the overall anticancer effects of this compound, especially at lower concentrations, while at higher concentrations the cell killing effect likely prevails over stimulation of differentiation.

Several anticancer active nitrogen mustards, including prednimustine, estramustine and bendamustine, were designed as dual-action compounds combining an alkylating bis(2'-chloroethyl)amine group with some other pharmacologically active molecules linked via tether. Prednimustine, a conjugate of chlorambucil and prednisolon, is essentially a prodrug of alkylating agent chlorambucil [26]. However, estramustine [27] and bendamustine [28] do not show alkylating effects and their anticancer mode of action includes stabilization of microtubule dynamics and inhibition of mitotic checkpoints/mitotic catastrophe, respectively. Compounds **4** and **5** were designed and synthesized as dual-action agents with DNA alkylating effects enhanced by a simultaneous depletion of cellular glutathione. Considering the lack of alkylating effects of some anticancer-active nitrogen mustards, the verification of DNA alkylating properties of compounds **4** and **5** was warranted. In our experiments, both new nitrogen mustards, similar to the traditional alkylating agents melphalan and cisplatin, decreased the melting temperature of CT DNA treated with these compounds. This finding provides evidence that compounds **4** and **5** interact with DNA in a way similar to that of melphalan and cisplatin and induce conformational distortions destabilizing dsDNA. The same effect on DNA melting temperature was previously reported for other DNA-interacting anticancer agents [21, 29]. Furthermore, the alkylating effect of compound **4** was confirmed by its capability to induce formation of DNA interstrand crosslinks in CT DNA and HL-60 cellular DNA.

It was previously reported that the cytotoxicity of melphalan on melanoma B16 cells correlates with the percentage of interstrand crosslinks induced in cellular DNA [30]. Therefore, the confirmed interstrand crosslinking capability of compound 4 may be, at least in part, responsible for its anticancer effects. Compound **4** induces a substantially lower amount of interstrand crosslinks than melphalan in CT DNA ($p=0.0394$), which may be interpreted as a result of lower reactivity of compound **4** with DNA. Similarly, compound **4** induces a lower amount of interstrand crosslinks than melphalan in DNA of HL-60 cells ($p=0.0254$) treated with these agents. However, the difference between amounts of crosslinks induced by melphalan and compound **4** is lower in HL-60 cells than in CT DNA (6% vs. 37.2%) which is indicative of some additional effects, such as drug transport, detoxification, or DNA damage repair, that negatively affect the effect of melphalan more than the effect of compound **4**.

Glutathione-mediated resistance of cancer cells affects the efficacy of various anticancer drugs, including alkylating agents. For example, ovarian cancer cells highly resistant to cisplatin were shown to have strikingly increased levels of glutathione [31]. Moreover, possible importance of the pharmacological depletion of glutathione for the treatment of resistant or recurrent glioblastomas and melanomas with melphalan is investigated by ongoing clinical trials (ClinicalTrials.gov identifiers NCT00005835 and NCT00661336, respectively).

Although the evidence for a preserved glutathione-depleting effect of 2-amino-2-deoxy-1-methoxyspirobrassinol moiety in the newly synthesized alkylating agents is not yet available, the higher cytostatic/cytotoxic effect of compound **4** than that of melphalan on OVCAR-3 cells, and lower fold resistance on Jurkat-M cells suggest that this compound may be less affected by certain resistance mechanism/s, such as glutathione-mediated drug inactivation, than the traditional nitrogen mustard agent, melphalan.

In conclusion, new anticancer agents *cis*- and *trans*-1-methoxy-2-deoxy-2-[N,N-bis(2′-chloroethyl)amino]spirobrassinol (**4** and **5**) with DNA-alkylating and glutathione-depleting moieties were designed and synthesized. Both compounds demonstrate cytostatic/cytotoxic effects against various leukemia and ovarian cancer cell lines and dsDNA destabilizing effects *in vitro*. Compared to the traditional DNA alkylating agent melphalan, compound **4** exerts earlier onset of anticancer effects on Jurkat cells via induction of apoptosis. Furthermore, compound **4** demonstrates higher cytostatic/cytotoxic effect on OVCAR-3 cells and lower fold resistance on Jurkat-M cells, which suggests that it may be less affected by certain cancer drug resistance mechanisms than melphalan. Combination of alkylating and glutathione depleting properties may be beneficial for design of new anticancer agents.

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