Antitumoral activity of new polyamine-naphthoquinone conjugates

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Abstract. Polyamine-naphthoquinone conjugates 5a-c were synthesized by nucleophilic displacement of 2-methoxy-lawsone 3a, 2-methoxylapachol 3b and 2-methoxy-*nor*-lapachol 3c with the polyamine *N*1-Boc-*N*5-Bn-spermidine 4. Unprotected derivatives 6a-c were synthesized to evaluate the effect of the protective Boc group on the activity of compounds 5a-c. The colorimetric MTT assay was used to evaluate their cytotoxic activity. All compounds were active against human lines of promyelocytic leukemia (HL-60), lung cancer (GLC4), Burkitt lymphoma (Daudi) and a mouse breast tumor (Ehrlich carcinoma), but only unprotected 6a-c showed activity against the human line of melanoma (MV-3). IC_{50} values were obtained from dose response curves by linear regression. DNA fragmentation was measured by quantification of the subG1 peak of the cell cycle. Apoptosis of HL-60 treated with 5c was dose-dependent. The amount of DNA fragmentation observed by exposure of HL-60 to 25 μM of compounds 5a-c and 6a-c is compatible with the decrease in viability induced by the drugs at this concentration. Production of ROS was measured by H2-CFDA. Kinetics of HL-60 DNA fragmentation and ROS formation by 5c indicated that production of ROS precedes cell death. In conclusion, spermidine-1,4-naphthoquinone conjugates exhibited an increase in activity compared with the natural products and induced apoptosis of tumor cell lines by a mechanism that is mediated, at least in part, by ROS production.

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Introduction

Despite all technological advances the treatment of cancer is still a challenge and cases of cure are rare. Natural products and chemical modification of antitumoral substances are amongst the most important strategies used in the search of new antineoplastic drugs.

Although the concept of conjugation of polyamine to drugs as a strategy to enhance cytotoxic activity was reported several years ago it remains little documented. This study showed that derivatives obtained by conjugation of 1,4-naphthoquinones with polyamine spermidine present an increase in the antitumoral activity as compared with the starting compounds.

Coupling of polyamines to cytotoxic compounds may represent a very attractive anticancer chemotherapeutic strategy. Polyamines such as N1-(3-aminopropyl)-1,4-butanediamine (spermidine, 1) are present in high concentrations in rapidly proliferating cells (1-4). Polyamine analogues that vary slightly in structure from natural polyamines might compete with naturally occurring polyamines for critical cellular binding sites (5-7). Thus, tagging spermidine analogues to a chemotherapeutic agent may enhance their activity either by facilitating uptake (via polyamine uptake systems) or by competition with polyamines for 'critical binding sites' in the cells. Indeed, it was demonstrated that linkage of polyamines to cytotoxic compounds facilitates their entry into tumor cells possessing a polyamine uptake system and increases their selectivity to DNA (8). Thus, the ability of the bifunctional alkylating agent chlorambucil, to form interstrand crosslinks with double stranded DNA, increased 10^4-fold after conjugation with spermidine (9).

1,4-Naphthoquinones are widely distributed in nature and many clinically important antitumor drugs containing a quinone moiety show anticancer activity. Lapachol, 2-hydroxy-3-prenyl-1,4-naphthoquinone, was shown to be active against the Walker-256 carcinoma and Yoshida sarcoma (10). Synthetic derivatives of lapachol, such as 2ß-O-glycoside-tetraacetate (11) and furano-1,4-naphthoquinones (12) showed

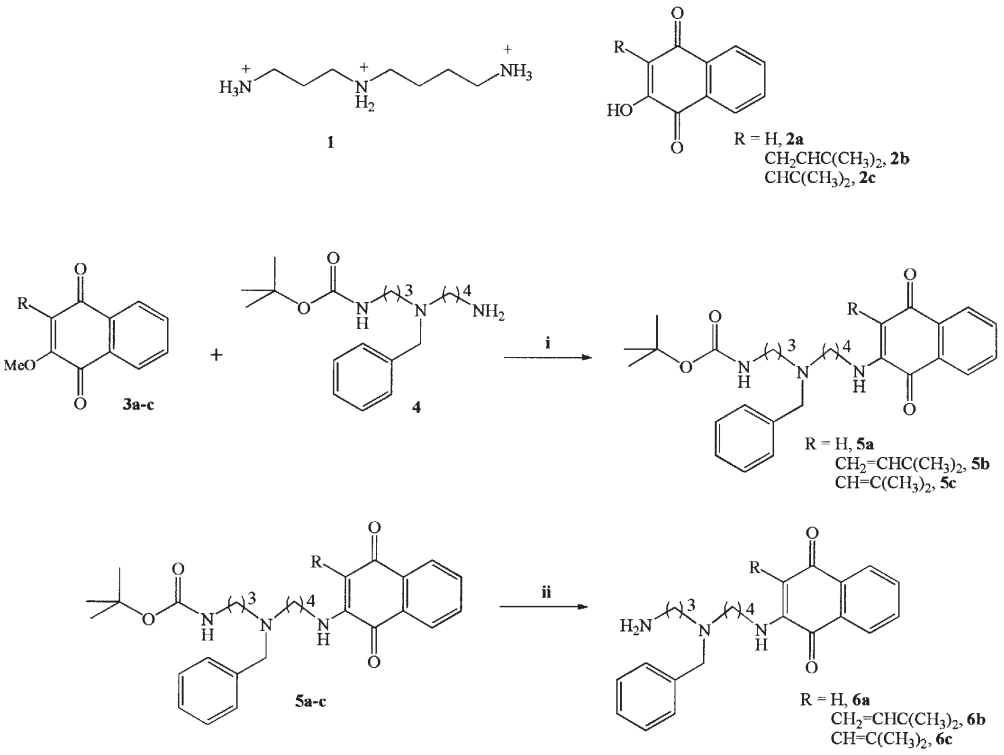

Figure 1. Structures of compounds, reagents and conditions. (i) MeOH, reflux, 24 h; (ii) CF_3COOH/CH_2Cl_2, 0˚C-RT, 4 h.

potent antitumor activity against leukemia and solid tumor cell lines, respectively. Studies of the antitumor properties and mechanisms of action of quinone derivatives have shown that they can act as topoisomerase inhibitors, via DNA intercalation. Their toxicity can also be explained by oxidative stress via generation of reactive oxygen species (ROS) (13).

Recently we synthesized polyamine-naphthoquinone conjugates from lawsone 2a, lapachol 2b and *nor*-lapachol 2c by nucleophilic displacement of the respective methoxy-naphthoquinones 3a-c with the polyamine (PA) *N*1-Boc-*N*5-Bn-spermidine 4 (Fig. 1) and showed that the resulting 1,4-naphthoquinone-polyamine conjugates 5a-c are potent inhibitors of DNA-topoisomerase II-α (14). Herein we describe the preparation of novel unprotected derivatives 6a-c and the evaluation of the cytotoxicity of both protected and unprotected 1,4-naphthoquinone-polyamines 5a-c and 6a-c against human lines of leukemia (HL-60), lung cancer (GLC4), melanoma (MV-3), Burkitt lymphoma (Daudi) and a mouse breast tumor (Ehrlich carcinoma). We also analyzed the ability of the compounds to induce apoptosis in the human promyelocytic leukemia cell line HL-60 and the production of ROS by 5c. In addition to demonstrating an increase in cytotoxicity of the polyamine-naphthoquinone conjugates, the results presented also show that these derivatives induced apoptosis of the HL-60 cells by a mechanism that probably involved ROS production.

Materials and methods

Reagents and apparatus. All reagents were purchased from Merck or Aldrich. Solvents were distilled prior to use. TLC analyses were performed on 0.2 mm plates (Merck), visualized with short-wave length UV light. Column chromatography (CC) was performed on silica gel G_{60} (230-400 mesh, ASTM, Merck). Infra-red spectra (KBr pellets or as film on a NaCl disk) were recorded on a Nicolet-Magna 760 FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on a Bruker DRX-200 e DRX-300 (200 or 300 MHz and 75 or 50 MHz) spectrometers, with TMS as the internal reference, and $CDCl_3$ as the solvent. High-resolution mass spectra were obtained on a QT micromass spectrometer with electron spray ionization.

Syntheses of polyamine-naphthoquinone conjugates 5a-c. Polyamine-1,4-naphthoquinone conjugates 5a-c (14) were synthesized as follows: i) methylation of lapachol 2b and *nor*-lapachol 2c with dimethylsulphate in acetone and potassium carbonate, to yield 3b (77%) (15) and 3c (71%) (16), respectively, and synthesis of methoxylawsone (3a) from the sodium salt of 1,2-naphthoquinone-4-sulfonic acid (17); ii) preparation of the protected derivative of spermidine 4 in a four-step synthesis (18,19); iii) nucleophilic displacement of the methoxyquinones 3a-c with compound 4. To the solution 4 (110.0 mg; 0.40 mmol) in MeOH (2 ml) in a sealed tube, was added a solution of 3b (100.0 mg; 0.30 mmol) in MeOH (5 ml). The reaction was stirred at 90˚C under argon, for 24 h, after which time the solvent was removed under reduced pressure. The product was purified by flash column chromatography (hexane/EtOAc, 9:1) and obtained as reddish brown oil 5b (174.0 mg, 75%). Rf=0.7 (hexane/EtOAc, 1:1). IR (film) ν_{max}/cm^{-1}: 3347, 3063, 3027, 2930, 2863, 1712, 1669, 1602, 1570, 1515, 1271, 721, 698. ^1H NMR (200 MHz, $CDCl_3$): δ 1.43 (s, 9H), 1.60 (s, 4H), 1.68 (brs, 3H), 1.73 (brs, 3H), 2.42 (t, *J*=6.4 Hz, 2H), 2.48 (t, *J*=6.4 Hz, 2H), 3.15 (m, 2H), 3.36-3.45 (m, 4H), 3.52 (s, 2H), 5.06 (m, 1H), 5.25 (m, 1H), 5.67 (m, 1H), 7.56 (dt, *J*=7.5 and 2.0 Hz, 1H), 7.67 (dt,

J=7.5 and 2.0 Hz, 1H), 7.99 (dd, *J*=7.5 and 2.0, 1H) and 8.09 (dd, *J*=7.5 and 2.0 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 18.2, 23.8, 24.3, 25.8, 26.6, 28.6, 29.2, 39.3, 45.0, 52.7, 53.4, 58.9, 77.9, 115.4, 123.2, 126.2, 127.2, 128.7, 130.5, 132.0, 133.6, 134.4, 139.4, 145.7, 156.1, 183.1. MS Found: 560.3488 [5b+1]$^+$; calc. for $[C_{34}H_{46}N_3O_4]^+$: 560.3488.

Similar procedure was followed for the syntheses of compounds 5a and 5c. Product 5c was purified as above using hexane/EtOAc 8:2 as eluent, and obtained as brown-reddish oil. 5c (132 mg, 61%). Rf=0.50 (hexane/EtOAc, 1:1). IR (film) ν_{max}/cm^{-1}: 3354-3347, 3063, 3027, 2972, 2806, 1712, 1678, 1602, 1515, 1252, 1170, 721, 698. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.43 (s, 9H), 1.48 (brs, 3H), 1.56-1.68 (m, 4H), 1.91 (brs, 3H), 2.38 (t, *J*=6.5 Hz, 4H), 2.46 (t, *J*=6.5 Hz, 2H), 3.14 (t, *J*=6.2 Hz, 4H), 3.52 (s, 2H), 5.38 (m, 1H), 5.92 m, 1H), 6.10 (m, 1H), 7.26-7.35 (m, 5H), 7.55 (dt, *J*=7.5 and 1.6 Hz, 1H), 7.63 (dt, *J*=7.5 and 1.6 Hz, 1H), 8.03 (dd, *J*=7.5 and 1.6 Hz, 1H) and 8.06 (dd, *J*=7.5 and 1.6 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 20.3, 24.4, 25.6, 27.0, 28.1, 28.6, 28.7, 44.4, 53.0, 53.5, 59.0, 79.0, 113.5, 118.1, 126.2, 126.5, 127.2, 128.5, 129.0, 130.1, 132.1, 133.8, 134.7, 138.7, 139.5, 144.8, 156.2, 183.1, 183.5. MS Found: 546.3332 [5c+1]$^+$; calcd. for $[C_{33}H_{44}N_3O_4]^+$ 546.3332.

Product 5a was purified as above using hexane/EtOAc 8:2 as eluent and obtained as a brown-reddish oil (307.8 mg, 98%). Rf=0.43 (hexane/EtOAc, 1:1). IR (film) ν_{max}/cm^{-1}: 3353, 3063, 3003, 2973, 2805, 1708, 1680, 1606, 1509, 1254, 1171, 729. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.44 (s, 9H), 1.59 (m, 6H), 2.43 (t, *J*=6.2 Hz, 2H), 2.50 (t, *J*=6.2 Hz, 2H), 3.11 (m, 4H), 3.53 (s, 2H), 5.28 (brs, 1H), 5.68 (s, 1H), 5.99 (s, 1H), 7.30 (m, 5H), 7.61 (brt, *J*=7.5 Hz, 1H), 7.73 (brt, *J*=7.5 Hz, 1H), 8.05 (d, *J*=7.5 Hz, 1H), 8.11 (d, *J*=7.5 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 24.6, 25.9, 26.1, 28.5, 34.0, 42.4, 52.4, 53.1, 58.9, 78.9, 126.2-129.0, 131.9, 134.7, 139.2, 149.2, 157.3, 183.1, 184.3. MS Found: 492.2862 [5a+1]$^+$; calcd. for $[C_{29}H_{38}N_3O_4]^+$ 492.2862.

Removal of the protecting group from 5a-c to give 6a-c. To the solution 5a (62.7 mg; 0.13 mmol) in MeOH (20 ml) was slowly added a solution of TFA (0.20 ml; 2.6 mmol) in CH_2Cl_2 (5 ml) at 0˚C. After 10 min the reaction was taken to room temperature and kept under stirring for 4 h until total consumption of the starting material. The solvent was removed under reduced pressure; addition of 10% $KHCO_3$ (10 ml) was followed by extraction with CH_2Cl_2 (3x20 ml). The organic phase was dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to give the free amine 6a (50.3 mg, 99%), as a red oil that was purified by flash chromatography (EtOAc/MeOH, 6:4). [R_f=0.2 (CH_2Cl_2/MeOH/Et_3N 10:89:1)]. IR (film) ν_{max}/cm^{-1}: 3353, 3063, 3003, 2973, 2805, 1708, 1606, 1509, 721, 698. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.59 (m, 6H), 2.43 (t, *J*=6.2 Hz, 2H), 2.50 (t, *J*=6.2 Hz, 2H), 3.11 (m, 4H), 3.53 (s, 2H), 5.28 (brs, 1H), 5.68 (s, 1H), 5.99 (s, 1H), 7.30 (m, 5H), 7.61 (brt, *J*=7.5, 1H), 7.73 (brt, *J*=7.5, 1H), 8.05 (d, *J*=7.5, 1H) and 8.11 (d, *J*=7.5Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 24.6, 25.9, 26.1, 28.5, 34.0, 42.4, 52.4, 53.1, 58.9, 126.2, 129.0, 131.9, 134.7, 139.2, 149.2, 183.1, 184.3 MS Found: 392.2359 [6a+1]$^+$; calcd. for $[C_{24}H_{29}N_3O_2]^+$ 392.2338.

Similar procedure was followed for the syntheses of compounds 6b and 6c. Product 6b was purified as above using EtOAc/MeOH 6:4 as eluent and obtained as brown-reddish oil (57.9 mg, 97%). [R_f=0.2 (CH_2Cl_2/MeOH/Et_3N 10:89:1)]. IR (film) ν_{max}/cm^{-1}: 3347, 3063, 3027, 2930, 2863, 1712, 1602, 1570, 1515, 721, 698. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.60 (m, 4H), 1.68 (brt, 3H), 2.42 (t, *J*=6.4 Hz, 2H), 2.48 (t, *J*=6.4 Hz, 2H), 3.15 (m, 2H), 3.36-3.45 (m, 4H), 3.52 (s, 2H), 5.06 (m, 1H), 5.25 (m, 1H), 5.67 (m, 1H), 7.56 (brt, *J*=7.5 Hz, 1H), 7.67 (brt, *J*=7.5 Hz, 1H), 7.99 (dd, *J*=7.5 Hz, 1H), 8.09 (dd, *J*=7.5 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 18.2, 23.8, 24.3, 25.8, 26.6, 29.2, 39.3, 45.0, 52.7, 53.4, 58.9, 115.4, 123.2, 126.2, 127.2, 128.7, 130.5, 132.0, 133.6, 134.4, 139.4, 145.7, 183.1. MS Found: 460.2943 [6b+1]$^+$; calcd. for $[C_{29}H_{37}N_3O_2]^+$ 460.2964.

Product 6c was purified as above using EtOAc/MeOH, 6:4 as eluent as red oil (55.5 mg, 96%) [R_f=0.2 (CH_2Cl_2/MeOH/Et_3N 10:89:1)]. IR (film) ν_{max}/cm^{-1}: 3354-3347, 3063, 3027, 2972, 2806, 1712, 1602, 1515, 1170, 721, 698. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.46 (m, 5H); 1.53-1.69 (m, 4H); 1.88-1.96 (s, 3H); 2.34-2.51 (m, 4H, *J*=6.3); 2.61-2.63 (t, 2H, *J*=6.3Hz); 3.04-3.13 (t, 2H, *J*=6.3); 3.50 (s, 2H); 5.34 (br s, H); 5.91 (t, 1H); 6.10 (s, 1H); 7.27-7.36 (m, 5H); 7.54-7.72 (dt, 2H, *J*=7.5); 7.96-8.16 (dd, 2H, *J*=7.5). ^{13}C NMR (50 MHz, $CDCl_3$): δ 20.3, 24.5, 25.6, 26.9, 28.0, 39.9, 44.4, 39.9, 53.5, 59.0, 113.5, 118.0, 126.1, 126.5, 127.3, 128.5, 129.1, 130.6, 132.0, 133.8, 134.7, 135.3, 138.7, 139.5, 144.8, 183.1, 183.5. MS Found: 446.2837 [6c+1]$^+$; calcd. for $[C_{28}H_{35}N_3O_2]^+$ 446.2807.

Cell lines. The human cancer cell lines HL-60 (leukemia), GLC4 (small cell lung cancer), MV-3 (melanoma), Daudi (Burkitt lymphoma) and Ehrlich carcinoma cells (mouse breast) were maintained in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY, USA), 10 U penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO_2. The medium was changed every 3 days. For adherent cells, confluent monolayers were gently washed with phosphate-buffered saline (PBS), pH 7.2, and after trypsinization the cells were suspended in culture medium.

Cytotoxic assay. The drug cytotoxicity was assessed by the MTT assay as described previously (20). Briefly, 180 μl of cell suspension (10^4 per well) was distributed in 96-well plates and pre-incubated for 24 h at 37˚C/5% CO_2 to allow stabilization of the culture. Test compounds were dissolved in DMSO and diluted in medium for use. Cells were exposed to 20 μl of medium, different concentrations of compounds 2a-c, 4, 5a-c, 6a-c or DMSO (at the same concentration carried by the compounds, control) as well as cisplatin or quercetin (positive controls). After 48 h incubation the culture was treated with 20 μl MTT (5 mg/ml) and kept for 4 h at 37˚C before being centrifuged and the supernatant discarded. The formazan produced by reduction of MTT by viable cell was dissolved in DMSO and the optical density was measured in an ELISA reader (BenchMark, Bio-Rad, CA) at 570 nm (reference 630 nm). All determinations were carried out in

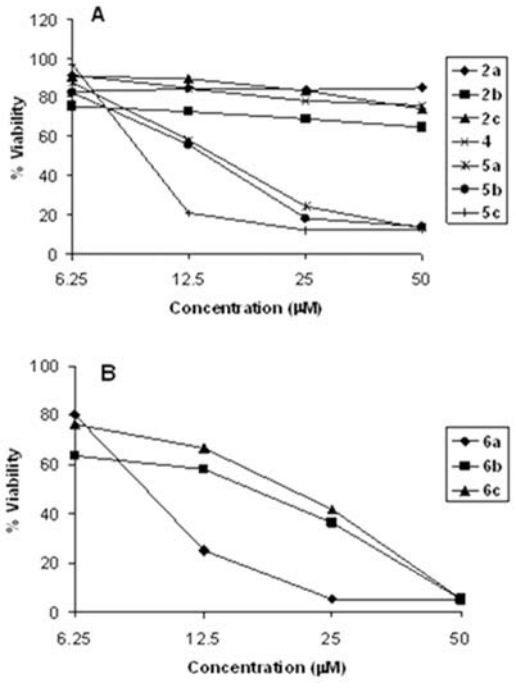

Figure 2. Effects of the compounds on HL-60 viability. Cells (10^4 per well) were distributed in 96-well plates and treated with the indicated concentration of the compounds: (A) 2a-c, 4 and 5a-c; (B), 6a-c for 48 h. Cell viability was assessed by MTT and expressed percentage of cells treated with DMSO at the same concentration carried by the compounds (100%). Each value represents the average of at least three independent experiments performed in triplicate.

triplicate and the average standard error was always <5%. The IC_{50} values were calculated from concentration-response curves by linear regression analysis.

Apoptosis assay. Apoptosis (DNA-fragmentation) was evaluated by cell cycle analysis and subdiploid populations were considered apoptotic (21). HL-60 cells ($2x10^4$/well) were platted as described for MTT assay. After 24 h resting, cells were treated with medium, 25 μM of 5a-c or 6a-c or different concentrations (6.25, 12.5, 25, 50 μM) of 5c, and incubated for another 48 h. After this time, cells were harvested and suspended in 180 μl of HFS Hypotonic Fluorescent Solution (50 μg/ml propidium iodide (PI) and 0.1% Triton X-100 in 0.1% Na citrate buffer). After 1 h incubation in the dark at 4˚C the DNA content was measured by flow cytometry (FL-2) (FACSCalibur, Becton-Dickinson, San Jose, CA). Data acquisition and analysis were controlled by Cellquest software version 3.1f.

Determination of reactive oxygen species (ROS). H_2-CFDA was used to evaluate the formation of ROS as described by Sasaki *et al* (22). Briefly, HL-60 cells were plated and treated with compound 5c under the same conditions described for apoptosis detection. After 48 h cells were harvested and suspended in 160 μl 10 μM H_2-CFDA and ROS generation relative to controls were determined by flow cytometry (FL-1) (FACSCalibur, Becton-Dickinson). Data acquisition and analysis were controlled by Cellquest software version 3.1f.

Results

This work showed that conjugation of polyamines with 1,4-naphthoquinones resulted in an increase in the antitumoral activity of the original compounds.

The *in vitro* cytotoxic activities of both protected (5a-c) and unprotected (6a-c) conjugates was evaluated against cancer human cell lines [leukemia (HL-60), melanoma (MV-3), lung

Table I. Cytotoxic activities of spermidine-naphthoquinone derivatives on tumor cell lines.

	IC_{50} ± SD (μM)				
	Cell lines				
Compounds	Ehrlich	HL-60	MV-3	GLC4	Daudi
Lawsone 2a	>50	>50	>50	>50	>50
Lapachol 2b	>50	>50	>50	>50	>50
Nor-lapachol 2c	>50	>50	>50	>50	>50
4	>50	>50	>50	>50	>50
5a	>50	16.48±0.3	>50	19.66±1.0	24.80±2.0
5b	45.74±4.3	14.01±0.5	>50	17.80±0.6	17.31±1.5
5c	47.37±4.9	12.51±1.3	>50	15.12±1.6	30.02±2.9
6a	30.71±1.8	10.54±0.5	39.11±0.5	14.91±0.8	26.67±1.9
6b	30.73±3.0	16.81±1.3	45.50±1.3	25.95±2.2	29.91±0.3
6c	25.56±2.3	21.91±1.1	42.39±2.5	28.28±1.8	30.23±0.5
Cisplatin	-	-	94.45±1.1	49.75±2.4	49.92±0.1
Quercetin	44.00±0.4	-	-	-	-

Data expressed as means ± SD for three independent experiments performed in triplicates.

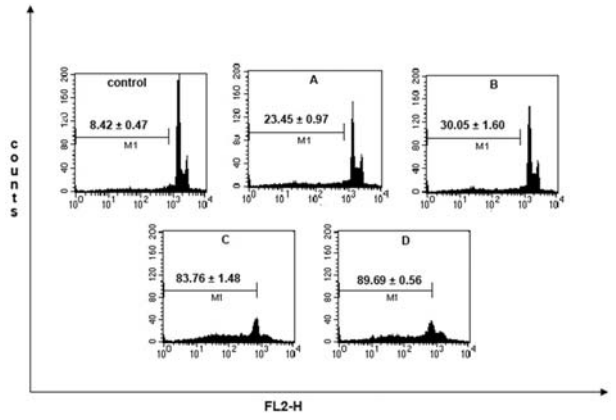

Figure 3. Induction of apoptosis is dose-dependent. HL-60 cells were treated with medium (control), 6.25 (A); 12.5 (B); 25 (C); or 50 μM (D) of 5c for 48 h and DNA-fragmentation (sub-G1 peak of the cell cycle) was measured by flow cytometry. Results are representative of three independent experiments performed in triplicates.

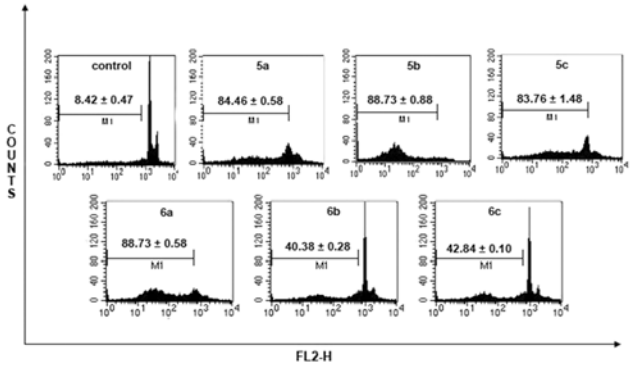

Figure 4. Compounds 5a-c and 6a-c induced apoptosis of HL-60. Cells were treated for 48 h with medium (control) or 25 μM of the 1,4-naphthoquinones 5a-c, 6a-c and DNA fragmentation was measured by flow cytometry. Results are representative of three independent experiments performed in triplicates.

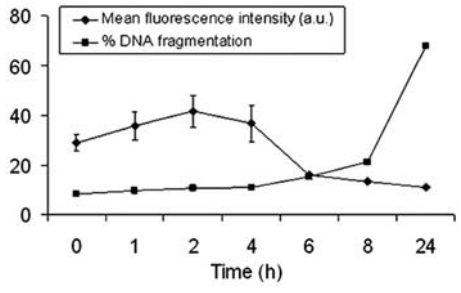

Figure 5. Kinetics of ROS formation and DNA fragmentation. HL-60 cells were treated with 25 μM 5c for the indicated times and generation of ROS (evaluated with H_2-CFDA) and (%) of DNA fragmentation were measured by flow cytometry. Generation of ROS was expressed as mean fluorescence intensity (MIF). Experiment representative of three others performed in triplicate.

cancer (GLC4), Burkitt lymphoma (Daudi) and a mouse breast tumor (Ehrlich carcinoma)]. Cell viability was measured by MTT 48 h after cells were exposed to increasing concentrations of the conjugates (6.25, 12.5, 25 and 50 μM) or the equivalent amounts of DMSO (control). All cell lines showed a dose-dependent decrease in viability when exposed to compounds 5a-c and 6a-c. Results for HL-60 are shown in Fig. 2.

The IC_{50} values were calculated by linear regression analysis from at least three independent experiments performed in triplicates. The cytotoxic activities, expressed as IC_{50} values, are summarized in Table I.

To gain insight into the mechanisms of cytotoxicity of compounds 5a-c and 6a-c we analyzed their ability to induce DNA fragmentation (apoptosis) in HL-60 cells. Measurement of the subG1 peak in the cell cycle, characteristic of apoptosis, showed that treatment of HL-60 cells for 48 h with various concentrations (6.25, 12.5, 25 and 50 μM) of 5c resulted in a dose-dependent DNA fragmentation (Fig. 3). Induction of apoptosis was not a specific property of 5c as all derivatives induced DNA fragmentation at the dose of 25 μM after 48 h of incubation (Fig. 4).

To investigate if the cytotoxic activity of the naphthoquinone derivatives involved changes in the redox status of the cells, we used H2-CFDA to evaluate the production of ROS in HL-60 treated with 25 μM of 5c. Evaluation of DNA fragmentation was performed at the same conditions as used to measure ROS (Fig. 5). Production of ROS by 5c was time-dependent, reaching a maximum after 2 h of exposure to 5c then decreasing. Interestingly, under the same conditions, DNA fragmentation started after 6 h exposure to 5c indicating that generation of ROS precedes apoptosis.

Discussion

The purpose of this study was to investigate the *in vitro* cytotoxic activities of various 2-spermidine-3-R-1, 4-naphthoquinone conjugates against cancer cell lines and to assess the effect of the R-side chain and the amine Boc protective group on the activity of the conjugates.

The results obtained from treatment of cancer cell lines with the spermidine-1, 4-naphthoquinone conjugates demonstrated an increase in activity in comparison with the natural products 2a-c (Fig. 2). However, the IC_{50} obtained for the polyamine-1,4-naphthoquinone conjugates did not show difference with variations of the R-side chain (lawsone, lapachol or *nor*-lapachol).

Observation that a Boc containing derivative of taxol, taxotere, showed better activity than taxol in several assays (23,24) called attention for its possible role on antitumoral activity. In this study, removal of the amine protective group (Boc) in compounds 5a-c did not lead to increased activity of 6a-c towards HL-60, GLC4 and Daudi, however, it resulted in improved activity against Ehrlich carcinoma and especially MV-3 melanoma against which 5a-c were inactive (Table I). It should be noted that unprotected compounds 6a-c showed an improvement in cytotoxicity that was about half of that observed against the other cell lines (IC_{50} around 39-45 μM for MV-3 and 10-30 μM for the other cell lines) and even higher than cisplatin (positive control). Although the general improved activity upon removal of the Boc group could be associated to a decreased lipophilicity of the resulting conjugates, our previous work on the cytotoxic activity of amino derivatives of lapachol 2b has evidenced the opposite trend, i.e. derivatives of the least polar amines were generally

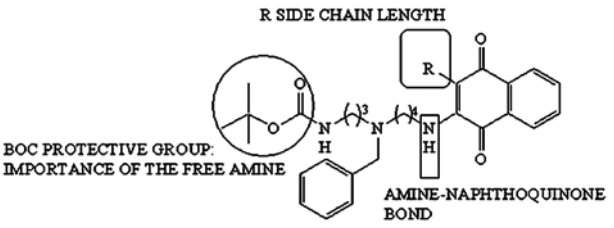

Figure 6. Pharmacophoric groups of spermidine-1,4-naphthoquinone conjugates identified from the cytotoxic activity results against cancer cell lines.

the most active (25). One might therefore speculate that the Boc group is cleaved intracellularly and that the efficacy observed is coming from the de-protected molecules, in which case the MV-3 melanoma cells might lack the metabolic capability to remove the Boc group.

Several chemotherapeutic agents have been reported to exert antitumor effects by inducing apoptosis of cancer cells. This was also demonstrated for the naturally occurring 1,4-naphthoquinone lapachol 2b from which compounds 5b and 6b were synthesized (26). Derivatives 5a-c and 6a induced the highest amounts of DNA fragmentation. Interestingly, whereas a decrease in DNA fragmentation was observed in cells treated with 6b-c compared to 5b-c, removal of the Boc group in 5a did not result in change in the activity of 6a. However, the decreased DNA fragmentation observed with 6b-c is in accordance with the viability data obtained after exposure of HL60 to 25 μM of these compounds. Together these results led to the identification of three pharmacophoric groups in the spermidine-1,4-naphthoquinone conjugates, as illustrated in Fig. 6.

It has been suggested that the cytotoxic effects of naphthoquinones are mainly due to their ability to inhibit DNA topoisomerase-II and to the formation of semiquinone radical that can transfer an electron to oxygen to produce super oxide. Both semiquinone and super oxide of quinones can generate the hydroxyl radical, which causes DNA strand breaks. As 1,4-naphthoquinones contain the quinone group they have the ability to accept one or two electrons to form the corresponding 'reactive oxygen species' (ROS) (13). In most cases, the deleterious effect of ROS is a function of activation of intracellular cell-death circuit and involvement of ROS at different phases of the apoptotic pathway has been clearly established. Indeed, interference of ROS in the association of cytochrome *c* with cardiolipin at the inner mitochondrial membrane facilitates the release of the pro-apoptotic cytochrome c and induction of apoptosis (27). In a previous study (14) we showed that while coupling of spermidine group to naphthoquinones led to an increase in their cytotoxic activity, it did not change the ability of conjugates 5a-c to inhibit DNA topoisomerases. Here we demonstrated that this coupling also did not block the ability of 5c to generate ROS. Together, these data support previous suggestions for a role of ROS as an intermediate in the anti-tumoral activity of quinones (13).

In conclusion, conjugation of 2-spermidine with 1,4-naphthoquinones increased their *in vitro* cytotoxic activity. All conjugates induced DNA fragmentation, a process that may involve ROS generation as suggested by data obtained with 5c. Our results corroborate work of other groups where modification of 1,4-naphthoquinones also induced increase in antitumoral activity reinforcing the importance of this approach for the development of more active drugs (28-30).

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