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PRECLINICAL STUDIES

Comparison of the cytotoxic effect of lapachol, α-lapachone and pentacyclic 1,4-naphthoquinones on human leukemic cells

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Summary The pentacyclic 1,4-naphthoquinones **1a**–**d** were cytotoxic (IC_{50}~2–7 μM) to human leukemic cell lines K562 (oxidative stress-resistant), Lucena-1 (MDR phenotype) and Daudi. Fresh leukemic cells obtained from patients, some with the MDR phenotype, were also sensitive to these compounds. The pentacyclic 1,4-naphthoquinones **1a** and **1c** induced apoptotic cell death in cells from leukemic patients as determined by flow cytometry. Conversely, the cell lines were highly insensitive to lapachol (**2**) and α-lapachone (**3**). Mitomycin-C inhibited cell proliferation at concentrations as low as 0.5 μM. The low toxicity against lymphocytes activated by phytohemagglutinin shows that these compounds are selective for the cancer cells studied. Previous data suggest that these compounds (**1a**–**d**) can be bioactivated in situ by reduction followed by rearrangement leading to enones, which are powerful alkylating agents. In contrast, lapachol (**2**) and β-lapachone (**3**), which cannot be bioactivated by reduction, showed little activity against the same cell lines.

Keywords Naphthoquinones · Lapachol · α-Lapachone · Leukemia · Multidrug resistance · Oxidative stress

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Introduction

Naturally occurring quinones and their analogs are important sources of cytotoxic compounds [1–4]. For example, dactinomycin, anthracycline antibiotics (daunorubicin, doxorubicin, idarubicin and mitoxantrone), bleomycins and mitomycin-C have been clinically used for cancer chemotherapy [5–9]. New natural and synthetic cytotoxic compounds of this group have been described in the recent years [5]. The *para*-quinone moiety present in the structure of most of them may participate in the cell redox cycle, acting as a precursor of reactive oxygen species and leading to oxidative stress [1, 2, 5, 6]. Some quinones also act as intercalating agents in the DNA molecule [5] or as inhibitors of enzymes essential for DNA duplication and nucleotide biosynthesis [10–12], or they can be activated in situ by reduction, leading to conjugated intermediates which are powerful alkylating agents [13–17].

As part of a program directed at the discovery of new anticancer drugs, we synthesized a series of pentacyclic 1,4-naphthoquinones of type **1** (Fig. 1) [18]. These compounds were designed as molecular hybrids of the cytotoxic naphthoquinones lapachol (**2**) and α-lapachone (**3**), isolated from *Tabebuia* spp. [1, 2] and natural pterocarpan (**4**), a cytotoxic isoflavonoid isolated from *Petalostemon purpureus* [19–21]. These pentacyclic 1,4-naphthoquinones were cytotoxic to MCF-7, a breast cancer

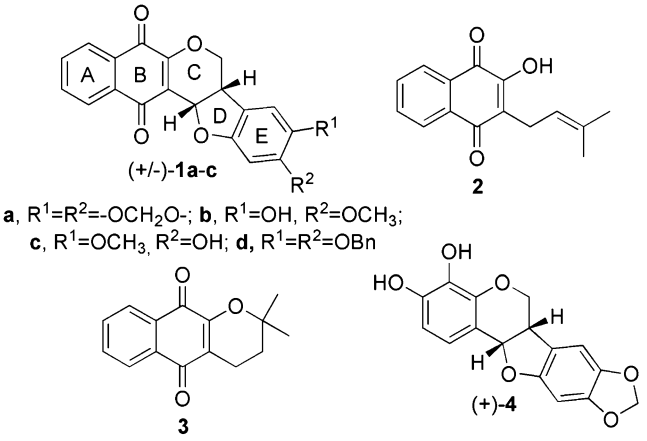

Fig. 1 Pentacyclic naphthoquinones **1a**–**d**, lapachol (**2**), α-lapachone (**3**) and natural pterocarpan (**4**)

cell line (Table 1), whereas α-lapachone **(3)** was inactive on these cells [18, 22].

It has been reported that MCF-7 cells are resistant to oxidative stress [11]. Since they are sensitive to the 1,4-naphthoquinones, it was felt that the cytotoxicity of these compounds should be studied in other oxidative stress-resistant cell lines as well as in tumor cells obtained from patients.

For this study three human leukemic cell lines were chosen. Daudi, a cell line that does not contain elevated levels of Bcl-2 protein [26], is sensitive to oxidative stress and undergoes lipid peroxidation and apoptosis following the removal of serum, required to maintain growth in vitro. K562 has a high content of glutathione [11]. Lucena-1 [23, 24] was originally selected from K562 for resistance to the *Vinca* alkaloid vincristine (multidrug-resistant phenotype). Lucena-1 cells are also very resistant to oxidative stress; they have a high content of catalase and overexpress ABCB1, a transmembrane protein of the ABC superfamily of transporters which plays an important role in the process of MDR [25]. In addition to these cell lines, fresh leukemic cell samples obtained from patients were also analyzed.

Materials and methods

Cell lines

The human leukemic cell lines Daudi, K562 and its MDR variant, Lucena-1, were all maintained in RPMI-1640 medium, supplemented with 50 μM β-mercaptoethanol, 25 mM HEPES, pH adjusted to 7.4 with NaOH, 60 mg/L penicillin, 100 mg/L streptomycin and 10% fetal calf serum (FCS), inactivated at 56°C for 1 h. Daudi cells were supplemented with 20% FCS. Vincristine sulfate (60 nM) was added to Lucena-1 cells in order to maintain the MDR phenotype.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were obtained by fractionating heparinized blood from healthy volunteers or chronic myeloid leukemia patients admitted to the Hematology Service of the Brazilian National Cancer Institute. Blood was heparinized and fractionated on Ficoll-Hypaque (Hystopaque) by density gradient centrifugation. The PBMC fraction was washed twice and resuspended in RPMI-1640, supplemented as described above, and the cell density was adjusted to 10^6 cells/mL. Cells were incubated with 5 μg/mL of the mitogen phytohemagglutinin (PHA), in the presence or absence of the compounds being tested. The study was approved by the Research Ethics Committee of the Brazilian National Cancer Institute and written informed consent was obtained from the participants according to the Declaration of Helsinki.

Cell treatment

Leukemic cell lines were exposed to the compounds in culture for 72 h. Briefly, 2×10^4 cells/mL in 200 μL were seeded in 96-well microtiter plates in drug-free medium or in medium containing different concentrations of each

Table 1 Effect of compounds **1a**–**d**, **2**, **3** and mitomycin-C on the growth of human cell lines

Cell line	1a	1b	1c	1d	2	3	Mit. C
K562	4.63±0.90	2.18±0.28	4.50±0.64	3.46±0.60	16.04±3.21	42.71±5.57	0.47±0.08
Lucena-1	5.47±0.35	2.57±0.07	4.49±1.44	3.64±0.25	20.84±6.48	47.44±10.47	2.75±0.60
Daudi	6.74±0.62	2.85±0.75	6.08±0.82	6.31±0.79	25.55±5.06	69.36±10.06	0.45±0.03
MCF-7	29.0±1.30	5.3±0.70	7.8±0.60	>100	ND	38.0[a]	ND
PBMC	23.25±0.12	ND	23.50±1.28	8.56±0.54	ND	ND	4.03±1.35

Results are reported as IC_{50} values±SD (concentration required to inhibit cell growth by 50%) in micromolar. Data represent the means of three independent experiments, with each concentration tested in triplicate. Assays were performed as described in the "Materials and methods" section

ND not done

[a] Ref. [22]

compound and maintained for 72 h at 37°C in an atmosphere of 5% CO_2, and cell viability was then measured.

Cell viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) colorimetric assay. MTT can be reduced by dehydrogenases present in active mitochondria of living cells. After 72 h of incubation in the presence or absence of the drugs being tested, 20 μL of MTT (5 mg/mL) was added to each well. Plates were then kept at 37°C in 5% CO_2 for 3 h. After centrifugation, 200 μL of DMSO was added to all wells in order to dissolve the dark blue crystals formed by the reduction of MTT. Absorbance was measured with an ELISA reader at 490 nm. Absorbance was directly proportional to the amount of formazan (reduction product) present, indicating the percentage of living cells.

Analysis of apoptosis

Leukemic cells from patients were cultured for 24 h at 37°C in the presence of 5 μM **1a** and **1c**. The percentage of apoptotic cells was assessed using the Annexin V assay. Cells were stained with FITC-labeled Annexin V as described by our group [31].

Detection of MDR phenotype

To measure ABCB1 and ABCC1 expression, leukemic cells were incubated with anti-ABCB1 or anti-ABCC1 monoclonal antibody for 30 min. After incubation, cells were washed and incubated with rabbit anti-mouse antibody for another 30 min.

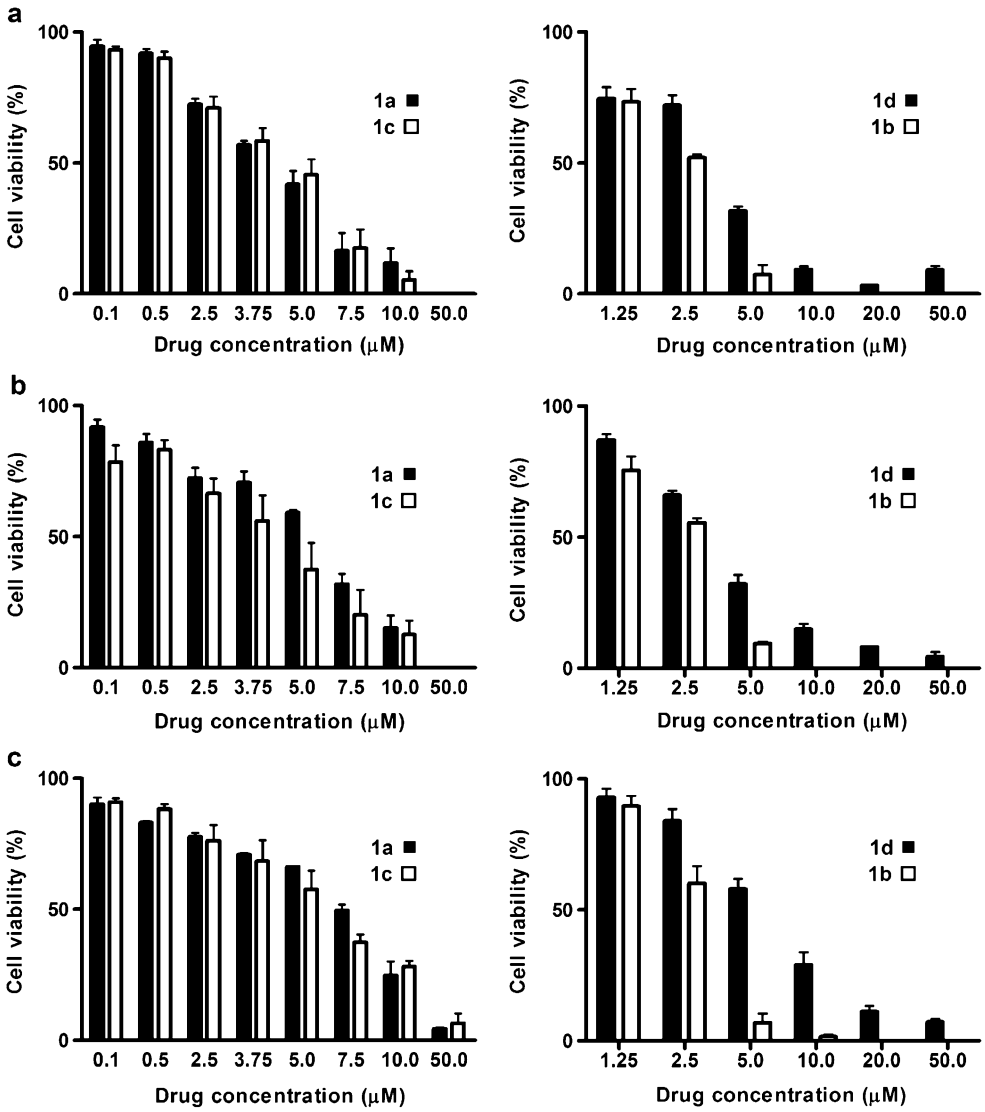

Fig. 2 Effects of compounds **1a–d** on the viability of (**a**) K562, (**b**) Lucena-1 and (**c**) Daudi cell lines. Viability was determined by MTT assay after 72 h incubation with the drugs. Control cells were treated with diluent. Each point of the experiment was performed in triplicate and results are reported as the mean+SD of at least three independent experiments

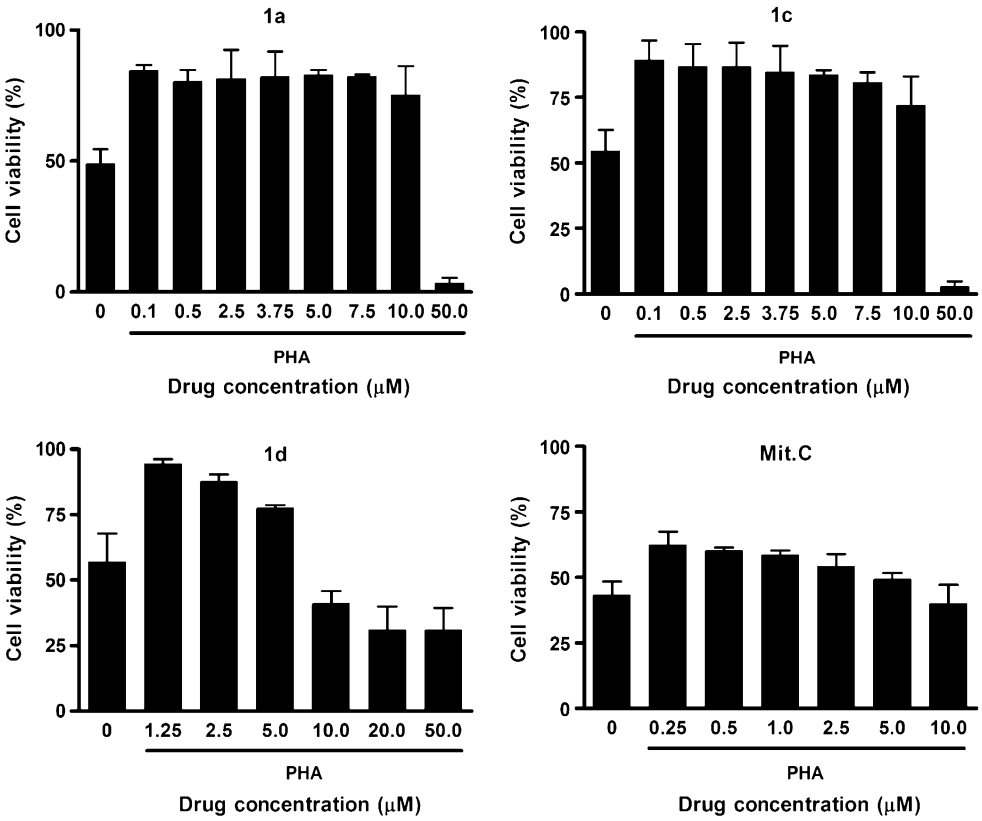

Fig. 3 Effect of compounds **1a**, **1c**, **1d** and mitomycin-C on the viability of normal human peripheral blood mononuclear cells (PBMC). Viability was determined by MTT assay after 72 h incubation with the drugs. PBMC were stimulated with the mitogen PHA (5 μg/mL). Control cells were treated with diluent and no PHA. Each point of the experiment was performed in triplicate and results are reported as the mean+SD of at least three independent experiments

For the determination of MDR activity, leukemic cells were incubated with rhodamine-123 plus cyclosporin A. Both ABCB1 and ABCC1 expression levels and functional activity were determined by flow cytometry [32].

Results

Compounds **1a**–**d** were synthesized as described by our laboratory [18]. Their cytotoxic effects on MCF-7, K562, Lucena-1 and Daudi cell lines are shown in Table 1. As previously reported [18], the potency toward MCF-7 cells was dependent on the of substitutions at the E-ring. Compounds **1b** and **1c**, bearing a phenol group at this ring, were fivefold and fourfold more active, respectively, than **1a**, in which the oxygen atoms are part of the apolar methylenedioxy group. Compound **1d**, bearing two apolar benzyloxy groups, exhibited the lowest activity on the MCF-7 cell line (Table 1).

In contrast, the cytotoxic effect of these quinones on the K562, Lucena-1 and Daudi cell lines (Table 1) was less dependent on the substitutions at the E-ring: compounds **1a**–**d** were almost equipotent. Interestingly, K562 and Lucena-1 were slightly more sensitive than Daudi cells (Table 1 and Fig. 2). The toxicity toward human peripheral blood mononuclear cells (PBMC) activated by PHA was also determined for **1a**, **1c** and **1d**. Compounds **1a** and **1c** had IC_{50} values above 23 μM, whereas for compound **1d** the IC_{50} value was 8.56 μM (Table 1 and Fig. 3).

Lapachol (**2**) and α-lapachone (**3**), lacking D- and E-rings in their structures, required extremely high concentrations to induce cytotoxicity in the same cells. Mitomycin-C, a drug used as a reference that shares the *para*-quinone moiety with **1a**–**d**, inhibited cell growth by nearly 50% when 0.5 μM was used.

The cell lines K562 and Lucena-1 are chronic myeloid leukemias (CML), whereas Daudi derives from a Burkitt's lymphoma. Studies using compounds **1a** and **1c** suggested that **1a** induced apoptosis while **1c** induced both apoptosis and necrosis. Compounds **1a** and **1c** were tested against patients' leukemic cells, with eight samples being from

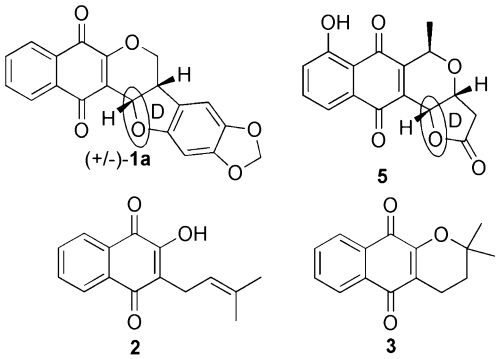

Fig. 4 Structural comparison of naphthoquinones **1a**, **2**, **3** and **5**

patients with CML and one from a patient with acute lymphoid leukemia (ALL). Apoptosis was measured after 24 h incubation with 5 μM of the compounds. When CML samples were tested, the median percentage of apoptosis obtained with both compounds was 12.5% (range=2% to 42% for **1a** and 2% to 60% for **1c**). In the only sample from an ALL patient, 39% of the cells died of apoptosis in the first 24 h with either compound. Other leukemia cells were also tested: two acute myeloid leukemia (AML) and two chronic lymphoid leukemia (CLL) samples were sensitive to compounds **1a** and **1c**. Furthermore, nine of the 13 patients' samples tested expressed ABCB1 and 11 expressed ABCC1, another transmembrane protein of the ABC superfamily of transporters that is also involved in the mechanism underlying multidrug resistance. These results, when taken together with those on cell lines, suggest that compounds **1a** and **1c** are not substrates for these ABC transporters.

Discussion

Xenobiotic quinones can be converted into cytotoxic semiquinone radicals through one-electron reduction by enzymes such as NADH cytochrome *b*5 reductase and NADPH:cytochrome P-450 reductase [1, 27, 28]. Recently, cyclic voltammetric studies performed with **2** in the presence and absence of oxygen clearly indicated the formation of semiquinone anion and HOO• [29]. Alternatively, quinones can be reduced by a two-electron process mediated by NAD(P)H quinone oxidoreductase (NQO1 or DT-diaphorase) [27, 28, 30], leading to the corresponding hydroquinone which can be tranformed into more polar products to be excreted. It has been proposed that **2** inhibits DT-diaphorase [30]. It was also reported that **3** inhibits topoisomerase II [10] and this effect seems to be associated with the redox properties of this compound since it is reversed in the presence of antioxidants [11].

It is reasonable to suppose that our pentacyclic 1,4-naphthoquinones **1a**–**d** can undergo a similar metabolic reaction, acting via oxidative stress mainly on Daudi cells, which are sensitive to oxidative stress [26]. However, for K562 and Lucena-1, cells that are resistant to oxidative stress [11, 23, 24], this mechanism should be less important. In contrast to **2** and **3**, compounds **1a**–**d** present a C–O bound at the D-ring and in principle could be activated by reduction as suggested for kalafungin (**5**), an antibiotic produced by various species of *Streptomyces* [15]. Kalafungin (**5**) acts as an antibiotic [15] through a mechanism similar to those proposed for mitomycin C and analogs [5–7, 16], and for model quinomethanes [13, 14, 17]. After reduction of kalafungin (**5**) by NQO1, the resulting hydroquinone undergoes a rearrangement, leading to a Michael *bis*-acceptor which reacts with essential nucleophiles in the cell, as proposed by Moore and Czeniak [15]. Since compounds **1a**–**d** are structurally correlated with kalafungin (**5**) (Fig. 4), we believe that, like kalafungin (**5**), these compounds can be activated by two-electron reduction, generating an electrophilic species that could act as a pro-alkylating agent, forming covalent bonds with DNA, glutathione and other essential nucleophiles. Work is in progress to investigate this mechanistic possibility.

In contrast to MCF-7 cells, the cytotoxic effect of **1a**–**d** on leukemic cells was less dependent on the pattern of substitution at the E-ring, with compounds **1a**–**d** almost equipotent on these cell lines and compounds **1a** and **1c** having similar effects on patients' samples. Furthermore, these compounds were active against cell lines and patients' samples expressing the multidrug resistance phenotype. This indicates that they are not substrates for ABCB1 or ABCC1, and can reach intracellular concentrations required for the cytotoxic effect.

In conclusion, the fact that compounds **1a**–**d** were active on K562 and Lucena-1 cell lines (oxidative stress-resistant) indicates that the bioreductive activation of pentacyclic naphthoquinones type **1** could be responsible for the cytotoxic effect on these cells. The low toxicity against PBMC activated by PHA indicates that compounds **1a** and **1c** show some degree of selectivity for the cancer cells studied. Furthermore, leukemic cells obtained from patients were also sensitive to compounds **1a** and **1c**. Therefore, our data suggest that the pentacyclic quinones studied herein might be important for the treatment not only of solid tumors, but also of multidrug-resistant leukemias.

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