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Report

Synthetic 1,4-anthracenediones, which block nucleoside transport and induce DNA fragmentation, retain their cytotoxic efficacy in daunorubicin-resistant HL-60 cell lines

Mingfu Wu,[1] Buna Wang,[1] Elisabeth M Perchellet,[1] Bonnie J Sperfslage,[1] Heidi A Stephany,[2] Duy H Hua[2] and Jean-Pierre Perchellet[1]

[1]Anti-cancer Drug laboratory, Division of Biology, Ackert Hall and [2]Department of Chemistry, Kansas State University, Manhattan, KS 66506-4901, USA.

Anthracene-1,4-dione and 6,7-dichloro-1,4-anthracenedione (code names AQ1 and AQ4, respectively) are cytostatic (IC_{50}: 53 and 110 nM, respectively) and cytotoxic (IC_{50}: 100 and 175 nM, respectively) in wild-type drug-sensitive HL-60-S tumor cells at day 4 *in vitro*. Therefore, the antitumor effects of these drugs were assessed and compared to those of daunorubicin (DAU) in HL-60-RV and HL-60-R8 tumor cells, which are, respectively, P-glycoprotein-positive and -negative multidrug-resistant (MDR) sublines. In contrast to DAU, which loses its cytostatic [resistance factors (RFs): 30.3–31.8] and cytotoxic (RFs: 48.8–58.1) activities in MDR sublines, AQ1 inhibits cell proliferation (RFs: 0.9–1.3) and cell viability (RFs: 1.4–1.6) as effectively in HL-60-RV and HL-60-R8 as in HL-60-S cells. Similarly, DAU decreases the rate of DNA synthesis less effectively in MDR sublines (RFs: 8.0–13.3) but AQ1 inhibits the incorporation of [^{3}H]thymidine into DNA to the same degree in HL-60-S as in HL-60-RV and HL-60-R8 cells (RFs: 0.9–1.1). In contrast to DAU, which is ineffective, the advantage of AQ1 is its ability to block the cellular transport of purine and pyrimidine nucleosides in HL-60-S cells, an effect which persists in the MDR sublines (RFs: 1.1). AQ4, which mimics to a lesser degree all the antitumor effects of AQ1, except the inhibition of adenosine transport, also retains its effectiveness in MDR sublines (RFs: 1.1–3.1). The peaks of DNA cleavage caused by DAU and AQ1 in HL-60-S cells shift to lower concentrations with increasing times of drug exposure but DAU loses most of its ability to induce DNA fragmentation in MDR sublines, whereas the levels of AQ1-induced DNA cleavage at 16 and 24 h are nearly equivalent in HL-60-S, HL-60-RV and HL-60-R8 cells. Because they not only mimic the antitumor effects of DAU in the nM range but also block nucleoside transport and remain effective in tumor cells that have developed different mechanisms of MDR, AQ1 and AQ4 analogs might be valuable to develop new means of polychemotherapy. [© 2001 Lippincott Williams & Wilkins.]

Key words: 1,4-Anthracenediones, DNA synthesis and fragmentation, nucleoside transport, tumor cell growth and viability, wild-type and multidrug-resistant HL-60 cells.

Introduction

Several natural and synthetic quinones function as bioreductive alkylating agents and have antitumor activity.[1,2] Their cytotoxicity may be due to two competing mechanisms: soft electrophilic arylation and redox cycling oxidation.[3] While complete two-electron reduction of the quinone ring by DT diaphorase produces a stable hydroquinone, partial one-electron reduction of the quinone ring by NADPH-oxidizing enzymes yields an unstable semiquinone free radical (FR) that can spontaneously autoxidize at the expense of molecular O_2 to generate a cascade of reactive O_2 species and FRs, which can induce DNA damage, lipid peroxidation and cytotoxicity. However, the various quinone antitumor agents used clinically, such as anthracycline antibiotics, mitomycin C and benzoquinone derivatives, have a complex chemical structure with a number of functional groups and the exact contribution of the quinone group to their

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Correspondence to J-P Perchellet, Anti-Cancer Drug Laboratory, Kansas State University, Division of Biology, Ackert Hall, Manhattan, KS 66506-4901, USA.
Tel: (+1) 785 532-7727; Fax: (+1) 785 532-6653;
E-mail: jpperch@ksu.edu

antitumor activity remains uncertain.[4–7] The anthracycline quinone antibiotics doxorubicin (DOX, adriamycin) and daunorubicin (DAU, daunomycin) covalently bind to and intercalate into DNA, inhibit DNA replication and RNA transcription, are DNA topoisomerase (Topo) II poisons, produce oxidative stress and damage biomembranes, induce DNA breakage and chromosomal aberrations, trigger apoptosis, and have a wide spectrum of anticancer activity.[4,5,7–12] Since the clinical effectiveness of DOX and DAU is severely limited by their cumulative cardiotoxicity and ability to induce multidrug resistance (MDR), it is important to develop quinone antitumor drugs with improved bioactivity.[4]

Recently, we discovered that, in contrast to its inactive precursor quinizarin (1,4-dihydroxy-9,10-anthraquinone, code name AQ2), anthracene-1,4-dione (1,4-anthraquinone, code name AQ1) is cytostatic and cytotoxic in the same nM range as DAU in the L1210 leukemic cell system *in vitro*.[13] Like DAU, AQ1 inhibits the rates of DNA, RNA and protein syntheses, and may arrest early stages of cell cycle progression to decrease the mitotic index of L1210 cells. However, AQ1 has the additional advantage of also blocking the cellular transport of both purine and pyrimidine nucleosides, an effect which DAU cannot do.[13] Moreover, AQ1 induces as much DNA cleavage in L1210 cells as camptothecin and DAU, two anticancer drugs producing DNA strand breaks and known to inhibit DNA Topo I and II activities, respectively. The mechanism by which AQ1 induces DNA fragmentation is inhibited by actinomycin D, cycloheximide and aurintricarboxylic acid, suggesting that AQ1 activates endonucleases and triggers apoptosis.[13] The abilities of AQ1 to block nucleoside transport, inhibit DNA synthesis and induce DNA fragmentation are irreversible upon drug removal, suggesting that this compound may rapidly interact with various molecular targets in cell membranes and nuclei to disrupt the functions of nucleoside transporters and nucleic acids, and trigger long-lasting antitumor effects which persist after cessation of drug treatment.[13] Because of its potency and dual effects on nucleoside transport and DNA cleavage, the use of bifunctional AQ1 with antileukemic activity in the nM range *in vitro* might provide a considerable advantage in polychemotherapy to potentiate the action of antimetabolites and sensitize MDR tumor cells.

Antimetabolites inhibit the synthesis of nucleic acid precursors but tumor cells may escape such inhibition by relying on their nucleoside transport system to incorporate precursors already made from the outside. By blocking this rescue pathway, nucleoside transport inhibitors, such as dipyridamole (DPR), may potentiate or prolong the antitumor activities of methotrexate (MTX) and 5-fluorouracil (5-FU).[14–21] Interestingly, nucleoside transport may also play a role in MDR and the classic probes used to experimentally block nucleoside transport have been shown to sensitize MDR cells to the action of conventional anticancer drugs.[22–26] Therefore, the present study was undertaken to determine whether AQ1, a potent DNA-damaging quinone antitumor drug which has the unique ability to block nucleoside transport, would retain its effectiveness in human HL-60 promyelocytic leukemia sublines that have already developed different mechanisms of resistance to DAU.

Reduced drug accumulation and/or altered drug distribution is a dominant feature of tumor cells that develop cross-resistance to several structurally and/or mechanistically unrelated anticancer agents, such as Vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes. MDR is achieved by ATP-dependent extrusion of anticancer drugs from tumor cells overexpressing P-glycoprotein (P-gp) and/or multidrug resistance-associated protein (MRP).[27,28] In contrast to their drug-sensitive wild-type (WT) HL-60-S parental counterparts, the P-gp$^+$ HL-60-RV cells and the P-gp$^-$ HL-60-R8 cells, which overexpress MRP, are two MDR sublines that have, respectively, been isolated for resistance to vincristine and DOX but also display cross-resistance to a number of other cytotoxic agents.[29–35]

Materials and methods

Cell culture and drug treatments

All solutions of AQ2 (from Aldrich, Milwaukee, WI) and synthetic AQ1 and AQ2 analogs were dissolved and diluted in dimethyl sulfoxide (DMSO), whereas DAU (from Sigma, St Louis, MO) solutions were dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl.[13,36–38] Suspension cultures of WT, drug-sensitive, human HL-60-S promyelocytic leukemia cells (from ATCC, Manassas, VA) were maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 8.25% fortified bovine calf serum (FCS; Hyclone, Logan, UT) and penicillin (100 IU/ml)–streptomycin (100 μg/ml), and incubated in the presence or absence of drugs at 37°C in a humidified atmosphere containing 5% CO_2. The P-gp$^+$ HL-60-RV cells and the P-gp$^-$ HL-60-R8 cells shown to overexpress MRP, two MDR sublines provided by Dr Melvin S Center (Kansas State University), were similarly maintained in RPMI 1640 medium in the absence of drugs.[29–35] Every 4 weeks, these HL-60-RV

and HL-60-R8 sublines were exposed to 41 nM DAU for 48 h to stabilize their MDR phenotype. This concentration of DAU, which is not cytotoxic to MDR HL-60 sublines, was removed from the culture medium at least 48 h before experimentation. Since drugs were supplemented to the culture medium in 1-μl aliquots, the concentration of vehicle in the final incubation volume (0.5 ml) did not affect the basal activity levels in control tumor cells incubated in the absence of drugs.[13,36–38] Resistance factors (RFs) were determined by dividing the IC_{50} of the MDR cells by that of the sensitive WT parent cell line.

Cell proliferation and viability assays

For tumor cell growth, WT and MDR HL-60 cells lines were resuspended in fresh FCS-containing RPMI 1640 medium, seeded in triplicate at an initial density of 1.5×10^4 cells/0.5 ml and incubated at 37°C in 48-well Costar cell culture plates (Costar, Cambridge, MA). Tumor cells were grown for 4 days in the presence or absence (control) of drugs and their density was monitored every 24 h using a Z1 dual threshold Coulter counter (Beckman Coulter, Miami, FL).[13,36–38] For tumor cell viability, WT and MDR HL-60 cells suspended in FCS-containing RPMI 1640 medium (initial density 3.75×10^4/0.5 ml) were grown at 37°C in 48-well Costar cell culture plates for up to 4 days in the presence or absence (control) of drugs to evaluate their cytotoxicity. The viability of drug-treated cells was assessed from their ability to bioreduce the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product that absorbs at 490 nm.[39] After 4 days in culture, control and drug-treated cell samples (about 10^6/0.5 ml/well for controls) were further incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS:PMS (2:0.1) reagent and their relative cell viability was estimated by recording the absorbance at 490 nm using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL).[13,36–38] Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were subtracted from the results.

Nucleoside transport and DNA synthesis

WT and MDR HL-60 cells (1.24×10^6 cells/0.5 ml) were preincubated for 15 min at 37°C in the presence or absence (control) of drugs and then exposed to 1 μCi of [2,8-^{3}H]adenosine (30 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO) or [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) for only 30 s to, respectively, assess the cellular uptake of purine and pyrimidine nucleosides over such very short period of time.[13,23,36,38] Reactions were diluted with 2 ml of ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and the unincorporated radiolabel was removed by centrifugation at 200 *g* for 10 min. After washing thrice with 2 ml of ice-cold PBS, intact cell pellets were harvested by centrifugation and incubated for 30 min in 1 ml of hypotonic lysis buffer (HLB) containing 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 0.2% Triton X-100. Cell lysates were mixed with 6 ml of Bio-safe II (Research Products International, Mount Prospect, IL) and counted to estimate the cellular uptake of [^{3}H]adenosine or [^{3}H]thymidine. Drug inhibition was expressed as percentage of [^{3}H]adenosine or [^{3}H]thymidine transported into vehicle-treated control tumor cells over a similar 30 s period.[13,23,36,38] To estimate the rate of DNA synthesis, WT and MDR HL-60 cells were resuspended in fresh FCS-containing RPMI 1640 medium at a density of 8.28×10^5 cells/0.5 ml, incubated at 37°C for 90 min in the presence or absence (control) of drugs and then pulse-labeled for an additional 30 min with 1 μCi of [^{3}H]thymidine. The incubation was terminated by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). After holding on ice for 15 min, the acid-insoluble material was recovered over Whatman GF/A glass microfiber filters and washed thrice with 2 ml of 5% TCA and twice with 2 ml of 100% EtOH. After drying the filters, the radioactivity bound to the acid-precipitable material was determined by liquid scintillation counting (LSC) in 6 ml of BioSafe NA (Research Products International).[13,36–38]

DNA cleavage

Drug-induced DNA cleavage was determined by intact chromatin precipitation, using WT and MDR HL-60 cells which were prelabeled with 1 μCi of [^{3}H]thymidine for 2 h at 37°C, washed with 3×1 ml of ice-cold PBS, collected by centrifugation and resupended in fresh FCS-containing RPMI 1640 medium at a density of 0.5×10^5 cells/0.5 ml.[11,13,36–38,40,41] Such tumor cells containing prelabeled DNA were then incubated at 37°C for 24 h in the presence or absence (control) of drugs. After centrifugation at 200 *g* for 10 min to discard the drugs and wash the cells, the intact cell pellets were lysed for 20 min in 0.5 ml of cold HLB, centrifuged at 12 000 *g* for 10 min to collect the supernatants and resuspended in 0.5 ml of HLB. After another similar centrifugation, the radioactivities in the

pooled supernatants (detergent-soluble low molecular weight DNA fragments) and the pellet (intact chromatin DNA) were determined by LSC: % DNA fragmentation=[c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100.[11,13,36–38,40,41] Before being counted in 6 ml of BioSafe NA, the intact pelleted chromatin was incubated for 2 h at 60°C in the presence of 0.6 ml of NCS tissue solubilizer (Amersham).[40]

Results

Drugs

The chemical structures and code names of the AQ1 and AQ2 analogs synthesized to be tested for their antileukemic activity in WT and MDR HL-60 cell lines *in vitro* are shown in Figure 1 and the correct nomenclatures of these compounds are as follows. AQ3: 6,7-dichloro-1,4-dihydroxy-9,10-anthracenedione; AQ4: 6,7-dichloro-1,4-anthracenedione; AQ5: 6,7-dichloro-2-(methylamino)-1,4-anthracenedione hydrochloride; AQ6: 6,7-dichloro-2-(methylamino)-1,4-anthracenedione; AQ7: 2-(methylamino)-1,4-anthracenedione. AQ2, which is used to prepare AQ1, has no cytotoxicity whatsoever (Figure 2). In contrast, AQ1 and its 6,7-dichloro analog, AQ4, can decrease the viability of HL-60-S cells by 81 and 56%, respectively, at 256 nM, and by 99 and 85%, respectively, at 640 nM (Figure 2). Interestingly similar treatments with AQ3, AQ5, AQ6 and AQ7 are totally ineffective. Hence, none of these 9,10-anthacenediones or methylamino-substituted 1,4-anthracenediones are able to reduce HL-60-S tumor cell viability at day 4 (Figure 2). Since in our studies 1-μl aliquots of drugs are added to 0.5-ml incubation volumes of tumor cells, the final concentration of 640 nM AQ3 could not be tested because a stock solution 500 times more concentrated of this compound was not soluble in DMSO.

Inhibition of WT and MDR HL-60 cell growth and viability

The concentration-dependent inhibitions of HL-60-S, HL-60-RV and HL-60-R8 cell proliferation by DAU at day 4 are characterized by IC_{50} values of 3.3, 105 and 100 nM, respectively (Figure 3A), demonstrating that the effectiveness of DAU as a cytostatic agent is greatly compromised in MDR sublines (RFs: 30.3–31.8). In contrast, the concentration-dependent inhibitions of HL-60-S, HL-60-RV and HL-60-R8 cell proliferation by AQ1 are characterized by similar IC_{50} values of 53, 68 and 45 nM, respectively, and the RFs (0.9–1.3) are negligible, indicating that AQ1 retains its effectiveness as a cytostatic agent in MDR sublines (Figure 3B). Although its antiproliferative activity in HL-60-S cells (IC_{50}: 110 nM) is slightly less potent than that of AQ1, AQ4 also retains its effectiveness as a cytostatic agent in MDR HL-60-RV (IC_{50}: 225 nM) and HL-60-R8 (IC_{50}: 200 nM) cells (RFs: 1.8–2.0) (Table 1). Similarly, RFs of 48.8–58.1 are observed between the abilities of DAU

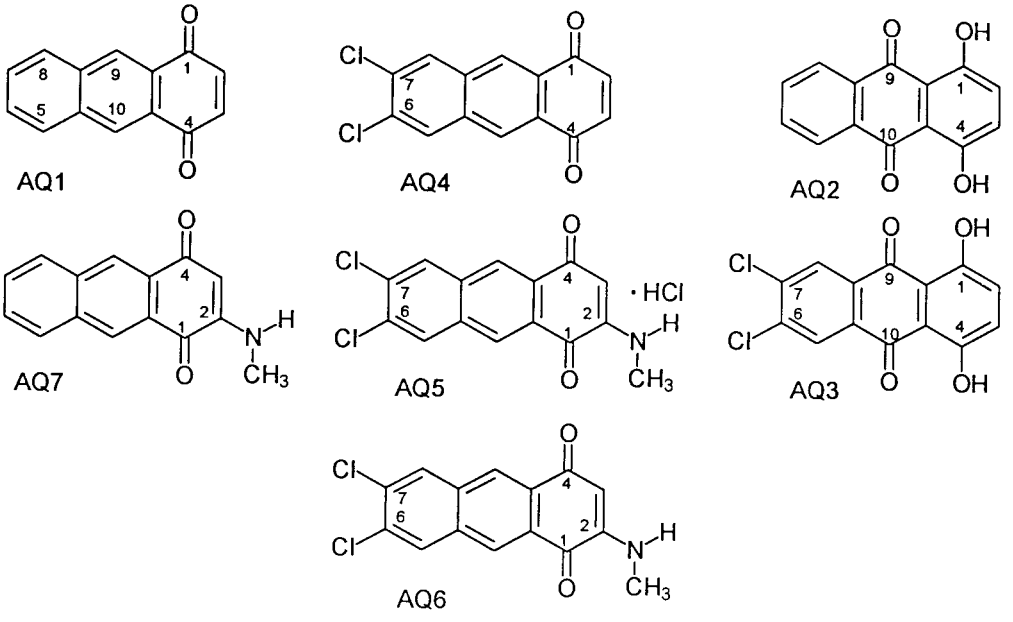

Figure 1. Stuctures and code names of the AQ1 and AQ2 analogs tested for their antileukemic activity in human WT and MDR HL-60 cells *in vitro*.

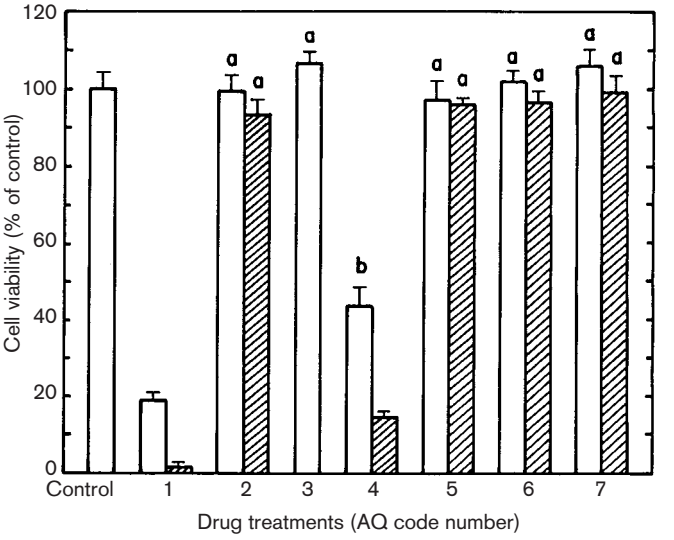

Figure 2. Comparison of the ability of 256 (open) and 640 nM (striped) concentrations of AQ analogs to inhibit HL-60-S cell viability at day 4 *in vitro*. Cell viability results are expressed as percentage of the net absorbance of MTS/formazan after bioredution by vehicle treated control cells ($A_{490\ nm}$=1.584 ± 0.062, 100 ± 4%) after 4 days in culture. The blank value ($A_{490\ nm}$=0.367 at day 4) for cell-free culture medium supplemented with MTS:PMS reagents has been substracted from the results. Bars: mean ± SD (n=3). [a]Not different from control; [b]$p < 0.0005$, smaller than control but $p < 0.005$, greater than 256 nM AQ1.

to inhibit, in a concentration-dependent manner, the viabilities of WT HL-60-S cells (IC_{50}: 4.3 nM) and MDR HL-60-RV (IC_{50}: 250 nM) and HL-60-R8 (IC_{50}: 210 nM) cells at day 4 *in vitro* (Figure 4A). In contrast to DAU, AQ1 reduces tumor cell viability as effectively in HL-60-S (IC_{50}: 100 nM) cells as in HL-60-RV (IC_{50}: 135 nM) and HL-60-R8 (IC_{50}: 155 nM) cells and the RFs (1.4–1.6) between the cytotoxic effects of AQ1 in WT and MDR cells are negligible (Figure 4B). Under similar conditions, AQ4, which is a cytotoxic agent slightly less potent than AQ1 in WT HL-60-S cells (IC_{50}: 175 nM) at day 4, also retains its effectiveness in the MDR HL-60-RV (IC_{50}: 360 nM) and HL-60-R8 (IC_{50}: 550 nM) sublines with only minimal RF values (2.1–3.1) (Table 1).

Inhibition of DNA synthesis and nucleoside transport in WT and MDR tumor cells

The concentration-dependent inhibitions of DNA synthesis by DAU in HL-60-S (IC_{50}: 150 nM), HL-60-RV (IC_{50}: 2000 nM) and HL-60-R8 (IC_{50}: 1200 nM) tumor cells are characterized by RF values of 8.0–13.3 (Figure 5A). In contrast, AQ1 inhibits the incorporation of [^{3}H]thymidine into DNA as effectively in HL-60-S (IC_{50}: 1400 nM) as in MDR HL-60-RV (IC_{50}: 1200 nM) and HL-60-R8 (IC_{50}: 1500 nM) tumor cells, and there are no significant RFs (0.9–1.1) (Figure 5B). Similarly, AQ4 retains its potency and inhibits the rate of DNA synthesis as much in HL-60-S (IC_{50}: 800 nM) as in HL-60-RV (IC_{50}: 1000 nM) and HL-60-R8 (IC_{50}: 1300 nM) cells (RFs: 1.3–1.6) (Table 1). DAU, which fails to inhibit nucleoside transport in murine L1210 cells,[13,36] is also unable to do so in human HL-60-S parental cells (Figure 6) and their MDR sublines (data not shown). However, AQ1 is a nucleoside transport inhibitor as effective in HL-60-S (Figure 6) as in L1210 tumor cells.[13,36] Moreover, the ability of AQ1 to block the cellular transport of [^{3}H]adenosine (Figure 6A) and [^{3}H]thymidine (Figure 6B) in HL-60-S cells (IC_{50}: 1800 and 1400 nM, respectively) persists in HL-60-RV (IC_{50}: 2800 and 1500 nM, respectively) and HL-60-R8 (IC_{50}: 1300 and 1600 nM, respectively). The RFs between the inhibitory effects of AQ1 on purine (0.7–1.6) and pyrimidine (1.1) nucleoside transport in WT and MDR leukemic cells are thus negligible (Figure 6). Like AQ1, AQ4 also inhibits the cellular transport of [^{3}H]thymidine in HL-60-S (IC_{50}: 1000 nM) and remains effective in HL-60-RV (IC_{50}: 1100 nM) and HL-60-R8 (IC_{50}: 2000 nM) cells, as indicated by the negligible RF values (1.1–2.0) observed between the WT and MDR cells lines (Table 1). However, in contrast to AQ1, AQ4 fails to significantly alter the cellular uptake of [^{3}H]adenosine in all three WT and MDR HL-60 cell lines studied (Table 1), suggesting that AQ4 specifically blocks the pyrimidine nucleoside transporters without interfering with the purine nucleoside transport system.

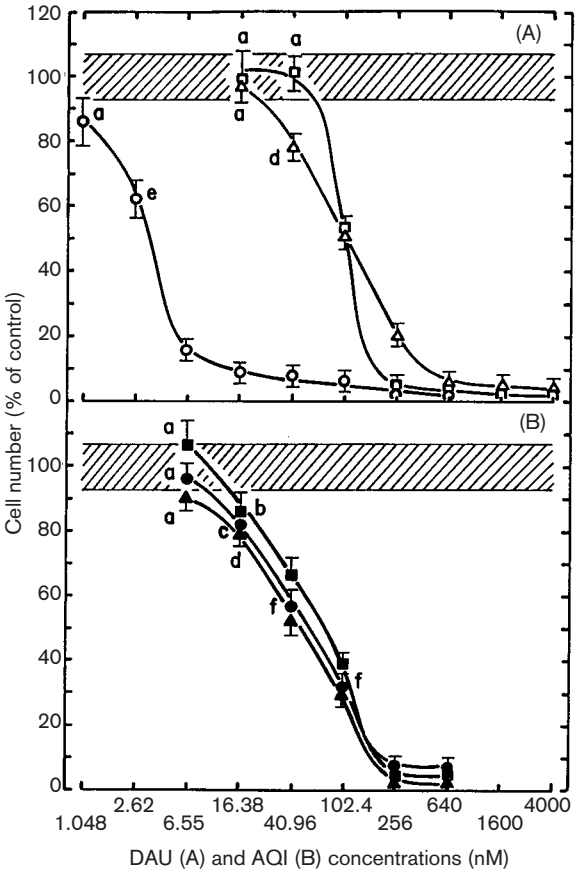

Figure 3. Comparison of the concentration-dependent inhibition of HL-60-S (circles), HL-60-RV (squares) and HL-60-R8 (triangles) cell proliferation by DAU (open symbols in A) and AQ1 (closed symbols in B) at day 4 *in vitro*. Cell growth results are expressed as percentage of the numbers of vehicle-treated control tumor cells (142 627 $\pm$ 9955 cells/ml, 100 $\pm$ 7%, striped areas) after 4 days in culture. Bars: means $\pm$ SD (n=3). [a]Not different from control; [b]$p<0.05$, [c]$p<0.025$, [d]$p<0.01$ and [e]$p<0.005$, smaller than control; [f]not different from effects on HL-60-RV and HL-60-R8 cells.

Induction of DNA cleavage in WT and MDR tumor cells

HL-60-S, HL-60-RV and HL-60-R8 cells containing [^{3}H]thymidine prelabeled DNA were used to quantitatively compare the ability of DAU and AQ1 to induce DNA fragmentation at 16 or 24 h in WT and MDR tumor cell lines. As reported in L1210 cells,[13,36] the concentration-dependent induction of DNA cleavage caused by DAU in HL-60-S cells at 16 h is biphasic, peaking at 81% in response to 1.6 μM but declining back to, or even below, control level (15%) at higher 10–25 μM concentrations of DAU (Figure 7A). In accord with its weaker ability to induce DNA cleavage in L1210 cells,[13] the biphasic concentration-dependent levels of DNA fragmentation caused by AQ1 in HL-60-S cells at 16 h peak at 58% in response to 4 μM before dropping back to, or even below, control level at 10–25 μM (Figure 7C). Interestingly, both peaks of DNA cleavage caused by DAU and AQ1 at 16 h shift to lower concentrations of drugs as the duration of these treatments increases to 24 h. For instance, as compared to their effects at 16 h (Figure 7A), 0.041–0.102 μM concentrations of DAU become effective at 24 h and 0.256–0.64 μM DAU mimic the maximal induction of DNA cleavage (84%) caused by 1.6 μM DAU at 24 h, whereas 4 μM DAU loses its ability to trigger DNA fragmentation at 24 h (Figure 7B). Similarly, and as compared to their effects at 16 h (Figure 7C), 1.6 μM AQ1 gains the ability to maximally induce DNA cleavage at 24 h, whereas 4 μM AQ1 loses most of its effectiveness as a DNA-damaging treatment at 24 h (Figure 7D). The concentrations of DAU producing the peak and plateau of DNA cleavage in WT tumor cells at 16 and 24 h lose most, if not all, of their ability to trigger DNA fragmentation in the MDR tumor sublines. For example, the magnitudes of DNA cleavage caused by

Table 1. Comparison of the antitumor activities of AQ4 in WT and MDR HL-60 cells *in vitro*

Cellular activities inhibited[a]	HL-60-S	HL-60-RV		HL-60-R8	
	IC_{50} (nM)[b]	IC_{50} (nM)[b]	RF[c]	IC_{50} (nM)[b]	RF[c]
Proliferation	110 $\pm$ 6	225 $\pm$ 20	2.04	200 $\pm$ 15	1.82
Viability	175 $\pm$ 7	360 $\pm$ 27	2.06	550 $\pm$ 35	3.14
DNA synthesis	800 $\pm$ 75	1000 $\pm$ 117	1.25	1300 $\pm$ 111	1.63
Nucleoside transport:					
purine	NS[d]	NS[d]		NS[d]	
pyrimidine	1000 $\pm$ 52	1100 $\pm$ 31	1.10	2000 $\pm$ 132	2.00

[a]The conditions of the assays and the determinations of the results were identical to those described for AQ1 in Materials and methods.
[b]IC_{50} values: nM concentrations of AQ4 (means $\pm$ SD, n=3) that inhibit cellular activities by 50%.
[c]RF: IC_{50} in MDR cells/IC_{50} in WT cells.
[d]The cellular uptake of [^{3}H]adenosine is not significantly inhibited by the increasing concentrations of AQ4 tested up to 4 μM.

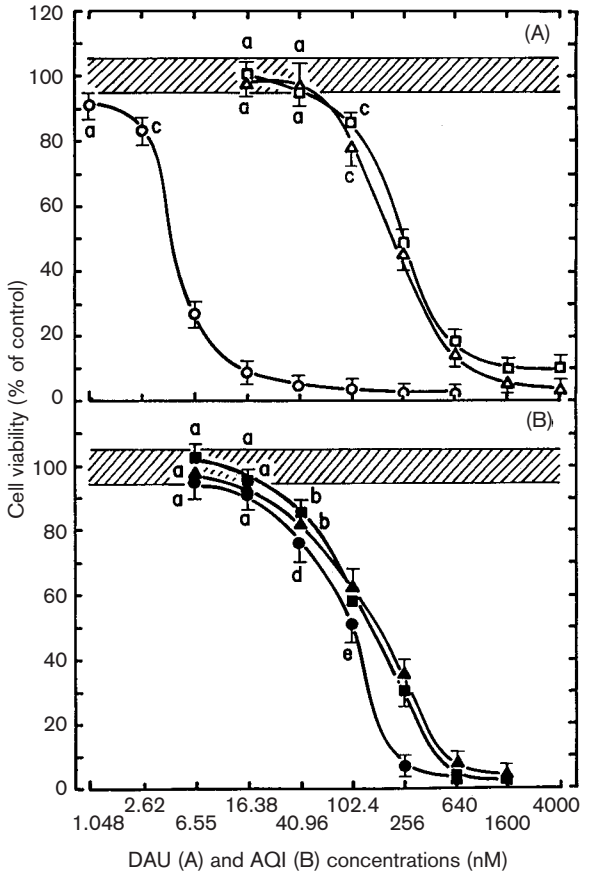

Figure 4. Comparison of the concentration-dependent inhibition of HL-60-S (circles), HL-60-RV (squares) and HL-60-R8 (triangles) cell viability by DAU (open symbols in A) and AQ1 (closed symbols in B) at day 4 *in vitro*. Cell viability results are expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells ($A_{490\ nm}$=1338±0.068, 100±5%, striped areas) after 4 days in culture. The blank value ($A_{490\ nm}$=0.345 at day 4) for cell-free culture medium supplemented with MTS:PMS reagent has been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.025$ and [c]$p<0.01$, smaller than control; [d]$p<0.005$, smaller than control but not different from effects on HL-60-RV and HL-60-R8 cells; [e]not different from effects on HL-60-RV and HL-60-R8 cells.

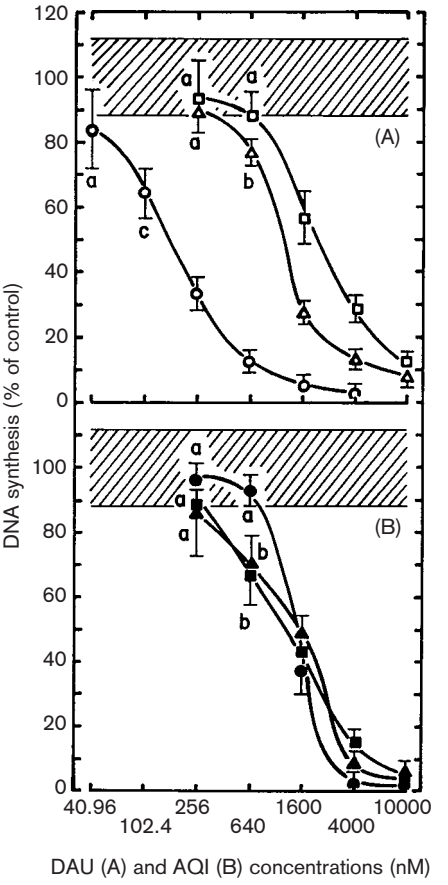

Figure 5. Comparison of the concentration-dependent inhibition of DNA synthesis by DAU (open symbols in A) and AQ1 (closed symbols in B) in HL-60-S (circles), HL-60-RV (squares) and HL-60-R8 (triangles) cells *in vitro*. DNA synthesis in vehicle-treated control cells was 20 916±2468 c.p.m. (100±12%, striped areas). The blank value (559±67 c.p.m.) for control cells incubated and pulse-labeled at 2°C with 1 μCi of [^{3}H]thymidine has been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.025$ and [c]$p<0.01$, smaller than control.

1.6 μM DAU at 16 and 24 h are not only reduced by 58–64% in HL-60-R8 cells, but such smaller responses can only be induced after 24 h by higher concentrations of 4–10 μM DAU in the HL-60-RV subline (Figure 7A and B). In contrast, the time- and concentration-dependent levels of DNA fragmentation induced by AQ1 in HL-60-S cells at 16 and 24 h persist more or less unaltered in the HL-60-RV and HL-60-R8 sublines (Figure 7C and D).

Discussion

AQ1, which has been shown to inhibit L1210 cell growth and viability in the same nM range as DAU,[13] is also cytostatic (IC_{50}: 53 nM) and cytotoxic (IC_{50}: 100 nM) to HL-60-S cells in the present study. The antitumor effects of AQ1 are mimicked, with one exception, by AQ4, suggesting that the 6,7-dichloro substitution prevents this AQ1 analog from interacting with the purine nucleoside transport system (Table 1). The observation that AQ5, AQ6 and AQ7 are not cytostatic and cytotoxic suggests that, in contrast to

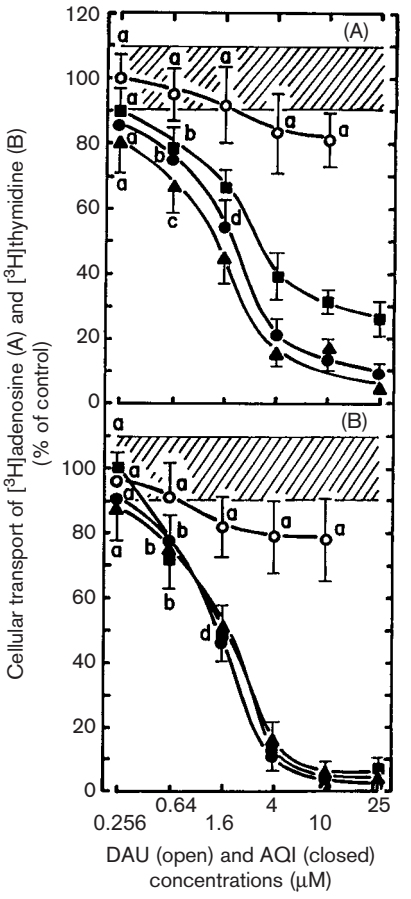

Figure 6. Comparison of the concentration-dependent inhibition of the cellular transport of purine (A) and pyrimidine (B) nucleosides by DAU (open symbols) and AQ1 (closed symbols) in HL-60-S (circles), HL-60-RV (squares) and HL-60-R8 (triangles) cells *in vitro*. Results are expressed as percentage of [^{3}H]adenosine (22 438 $\pm$ 2109 c.p.m., 100 $\pm$ 9%, striped area in A) and [^{3}H]thymidine (14 889 $\pm$ 1439 c.p.m., 100 $\pm$ 10%, striped area in B) transported into vehicle-treated control cells over 30 s. Bars: means $\pm$ SD (n=3). [a]Not different from control; [b]$p < 0.05$ and [c]$p < 0.01$, smaller than control; [d]not different from effects on HL-60-RV and HL-60-R8 cells.

the 6,7-dichloro substitution, the 2-(methylamino) addition abolishes the antitumor activity of the AQ1 skeleton (Figure 1). In accord with the fact that AQ2 is ineffective in the murine L1210 cell system,[13] AQ2 and AQ3 also fail to alter human HL-60-S cell viability, substantiating the finding that, with or without 6,7-dichloro addition, the dihydroxy-substituted anthracene skeleton of AQ2 with its internal quinoid ring is devoid of antitumor activity (Figure 1). Hence, it is reasonable to assume that, with or without 6,7-dichloro substitution, the AQ1 framework with its external *para*-quinone may be responsible for its potent antitumor activity in L1210 and HL-60-S cells.

To the best of our knowledge, we are the first to report that AQ1 and AQ4 are potent quinone antitumor drugs that retain their effectiveness in DAU-resistant leukemic sublines. The cytotoxic activities of a limited number of quinoid structures with one (benzoquinones), two (naphthoquinones) or three rings (anthraquinones, AQs) and various substitutions have been reviewed.[1,13,36,38,42,43] Several groups of natural AQs found in plants, soil, fungi, lichens, insects, marine animals and cultures of bacteria, including some quinone pigments, contain interesting antitumor antibiotics.[1] The potent antitumor activities of AQ1 and AQ4 are all the more remarkable that, among naturally occurring quinones, the number of bioactive 1,2-, 1,4- and 9,10-AQs appears quite limited, and only five of them elicit antitumor effects *in vivo*.[1,42,43] Only four antitumor AQs are active *in vitro*, all of them being 9,10-AQs.[1,42,43] The 1,4-AQ analog viocristin and its isomer isoviocristin are antibiotics that inhibit macromolecule synthesis in microorganisms. The antitumor activities of oncocalyxones A and B, two 1,4-AQs from *Auxemma oncocalyx*, are nearly equipotent in WT and MDR SW1573 lung tumor cells, which overexpress MRP.[44,45] However, the IC_{50} values for the cytotoxic effects of the above natural compounds in a spectrum of tumor systems including HL-60 cells range from 0.76 to 18 μg/ml,[44,45] whereas the 9–175 nM IC_{50} values reported for the cytostatic and cytotoxic effects of AQ1 and AQ4 in L1210 and HL-60-S cells are equivalent to IC_{50} values ranging from 1.8 to 36 ng/ml, suggesting that the synthetic AQ1 and AQ4 compounds used in the present study may be antitumor drugs 21–10 000 times more potent than these phytochemicals.

Among synthetic aminoalkylamino-AQs, mitoxantrone is the only 9,10-AQ equipotent to DOX and DAU.[43,46] Mitoxantrone, a DNA-damaging agent, which inhibits mammary tumor cell growth and viability *in vitro* and *in vivo*, is somewhat effective in MDR sublines, synergistic with MTX and 5-FU, and less cardiotoxic than DOX (reviewed in Perchellet *et al.*[38]). Cytotoxic dihydroxy-9,10-AQ analogs include morindaparvin A, which has a methylenedioxy group and antileukemic activity, altersolanol A, which is phytotoxic towards potato, tomato and pea, and aloe-emodin, which has significant antileukemic activity in mice and may be used as a starting point for the synthesis of the anthracycline antitumor antibiotics.[1] However, the IC_{50} values for the cytotoxicity of aloe-emodin range from 1 to 100 μM in various tumor cell

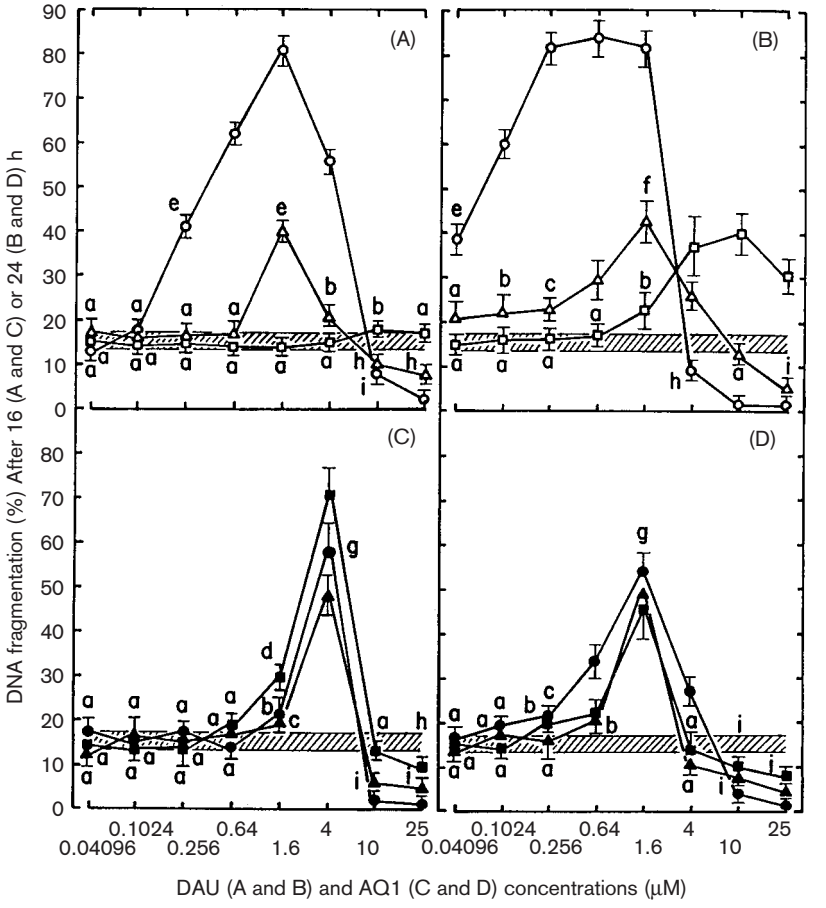

Figure 7. Comparison of the concentration-dependent induction of DNA cleavage by DAU (open symbols in A and B) and AQ1 (closed symbols in C and D) at 16 (left panels) and 24 (right panels) h in HL-60-S (circles), HL-60-RV (squares) and HL-60-R8 (triangles) cells containing ^{3}H-prelabeled DNA *in vitro*. Results are expressed as (c.p.m. in supernatant/c.p.m. in supernatant+pellet) × 100 at 16 or 24 h. For untreated controls (15.3 ± 1.6% DNA fragmentation, striped areas at 16 and 24 h), the supernatant (DNA fragments) is 12 738 ± 1248 c.p.m. and the pellet (intact DNA) is 70 626 ± 8051 c.p.m. Bars: means ± SD (n=3). [a]Not different from control; [b]$p < 0.05$, [c]$p < 0.025$, [d]$p < 0.005$ and [e]$p < 0.0005$, greater than control; [f]$p < 0.01$, smaller than the effect of 0.1024 μM DAU in HL-60-S cells; [g]not different from the effects of similar concentration of AQ1 in HL-60-RV and HL-60-R8 cells; [h]$p < 0.01$ and [i]$p < 0.005$, smaller than control.

lines, suggesting that such hydroxy-AQ present in *Aloe vera* leaves is far less effective than AQ1 and AQ4.[47] Similarly, the concentration-dependent cytotoxic effects of the 9,10-AQs barleriaquinone-I and -II in MCF-7 human breast adenocarcinoma cells *in vitro* are characterized by IC_{50} values of 8–10 μM or greater,[48] suggesting that these natural 9,10-AQs extracted from the roots of *Barleria buxifolia* have much weaker antitumor potential than the synthetic AQ1 analogs tested in our study. The pigment carminic acid, which is a tetrahydroxy-9,10-AQ compound and the coloring principle of cochineal in scale insects, shows antitumor activity in rat and is a potent feeding deterrent to ants, suggesting that its natural function may be to act as a defense against predation.[1] Other miscellaneous polyhydroxy-9,10-AQs showing antitumor antibiotic activity include the kidamycin group of closely related AQ di-*C*-glycosides, hedamycin, which is susceptible to quick photodeactivation, and the indomycin pigments.[1] 1,3,6,8-Tetrahydroxy-9,10-AQ derivatives from the sponge-associated fungus *Microsphaeropsis* species have been shown to inhibit the activities of protein kinase C, cyclin-dependent kinase 4 and the tyrosine kinase domain of the epidermal growth factor receptor in cell-free assays *in vitro* (IC_{50}: 18.5–43.5 μM).[49] The potent antitumor activities of AQ1 and AQ4 detected in our study are quite unexpected since the three-ring AQ is essentially

non-toxic to freshwater algae[50] and is neither mutagenic *in vitro* nor *in vivo*.[51] The antitumor activities of various substituted AQ1 analogs and of different AQs, such as 9,10-AQs, with or without hydroxyl functions remain to be investigated. Interestingly, *ortho*-quinones have been reported to be the most toxic, whereas internal quinones with benzenoid substitutions on both sides of the quinoid ring are not toxic at saturation.[52] Methoxy-, hydroxy- and chloro-substitutions may decrease the toxicity of quinoid compounds, which can no longer efficiently conjugate or arylate protein thiols.[52] In contrast, since the hydrophilicity or hydrophobicity of AQ substituents controls the fraction of chlorophyll accessible to quinone in chloroplasts, hydroxyl substituents enhance the ability of 9,10-AQ to quench the singlet photoexcited state of light-harvesting chlorophyll (LHC), thereby reducing even more the population of LHC and its fluorescence intensity.[53] Since the ability of AQ1 to bind to nucleoside transporters, interact covalently with DNA, inhibit Topo activities and affect the production of FRs is still unknown, it is rather premature to speculate on the antitumor potential of various substitutions and quinone or hydroquinone functionalities of the AQ1 skeleton.

The magnitude at which AQ1 inhibits HL-60-S, HL-60-RV and HL-60-R8 cell proliferation (IC_{50}: 45–68 nM) matches its ability to decrease cell viability (IC_{50}: 100–155 nM) in the same WT and MDR tumor cell lines. The cytostatic and cytotoxic effects of each concentrations of AQ1 increase with the time in culture, suggesting that the effectiveness of AQ1 as an inhibitor of tumor cell proliferation and viability *in vitro* is a combination of drug concentration and duration of drug exposure. Decreased tumor cell viability after AQ1 and AQ4 treatment *in vitro* may be a reliable predictor of anticancer activity *in vivo*.[54] Although the antitumor effects of AQ1 are nearly equipotent to those of DAU in the L1210 tumor cell system,[13] in the HL-60-S leukemic system, AQ1 inhibits tumor cell growth, viability and DNA synthesis, respectively, 16, 23 and 9 times less effectively than DAU (Figures 3–5). Moreover, concentrations of AQ1 at least 2.5 times greater than those of DAU are required to induce more than 50% of DNA cleavage in HL-60-S cells at 16 and 24 h (Figure 7). Nevertheless, the magnitudes of the inhibitory and stimulatory effects triggered by DAU and somewhat higher concentrations of AQ1 are nearly identical in HL-60-S cells. However, the critical finding is that the abilities of AQ1 and AQ4 to inhibit tumor cell growth, viability and DNA synthesis in HL-60-S cells persist unaltered in the HL-60-RV and HL-60-R8 cells lines (RFs: 0.9–3.1), while DAU becomes dramatically less effective against DNA synthesis (RFs: 8–13.3), cell proliferation (RFs: 30.3–31.8) and cell viability (RFs: 48.8–58.1) in these MDR HL-60 sublines than in their parental WT counterparts. Finally, AQ1, which, in contrast to DAU, has the unique ability to rapidly block the cellular transport of both purine and pyrimidine nucleosides in HL-60-S cells, retains such additional advantage in the MDR HL-60 sublines (Figure 6). Even though the 6,7-dichloro-substitution may prevent the AQ1 skeleton from interfering with the cellular transport of purine nucleoside, AQ4 can still inhibit the pyrimidine nucleoside transporters as effectively in WT as in MDR (RFs: 1.1–2.0) HL-60 tumor cells (Table 1). This preliminary study suggests that, in contrast to anthracycline antibiotics, quinone antitumor drugs that can also block nucleoside transport may have a more versatile mechanism of action that can circumvent MDR.

The apparent discrepancy between the nM concentrations of AQ1 sufficient to totally inhibit HL-60-S, HL-60-RV and HL-60-R8 cell growth and viability (Figures 3 and 4), and the higher μM concentrations of AQ1 required to block nucleoside transport (Figure 6), inhibit DNA synthesis (Figure 5) and maximally induce DNA cleavage (Figure 7) may be due in part to different experimental conditions and cellular responses to various periods of drug exposure: the rates of nucleoside transport over 30 s and DNA synthesis over 30 min are inhibited in WT and MDR tumor cells treated for only 15–90 min with AQ1 and the peak levels of DNA cleavage occur 16–24 h after AQ1 treatment, whereas the more spectacular reductions of tumor cell growth and viability are the results of 4-day long AQ1 treatments.

Although the cellular uptake, retention, metabolism and half-life of radiolabeled AQ1 in cell culture remain to be determined, our preliminary L1210 data suggest that AQ1 rapidly triggers long-lasting inhibitory and damaging events that persist upon drug removal.[13] For instance, the irreversibility of the inhibitions of nucleoside transport and DNA synthesis caused by AQ1 suggests that the presence of this drug in the extracullular medium becomes irrelevant after 15–60 min, presumably because it has already bound to membrane nucleoside transporters and, perhaps, also been internalized to interact with nuclear enzymes involved in DNA metabolism. Since both responses are irreversible in L1210 cells and share similar IC_{50} values in L1210[13] and WT or MDR HL-60 cell lines (1.2–1.6 μM), the rapid inhibition of [^{3}H]thymidine incorporation into DNA caused by AQ1 (Figure 5B) is most likely due to its ability to immediately block the cellular transport of nucleosides (Figure 6). In L1210 cells, it takes 24 h to fully reveal the extent of DNA cleavage achieved by 2- to 5-h pretreatments with AQ1

and DAU.[13] The irreversibility of this response suggests that the DNA-damaging events triggered by AQ1 and DAU within the first 2–5 h can proceed uninterrupted for the next 19–22 h in the absence of these drugs in the extracellular medium and are sufficient to induce the same maximal levels of DNA fragmentation as when the drugs are maintained in the culture medium for the entire 24-h period of incubation.[13]

The fact that, within 16–24 h, AQ1 can induce almost as much DNA fragmentation as the known Topo II inhibitor DAU in L1210[13] and WT and MDR HL-60 cells (Figure 7) suggests that the ability of AQ1 to produce DNA strand breaks may play a major role in its mechanism of antitumor activity. Since the shape of the concentration–response curve for the effects of AQ1 on DNA cleavage resembles the biphasic response to DAU, which has already been reported and discussed before,[11,13,36] it is tempting to speculate that the mechanisms by which AQ1 and DAU induce DNA fragmentation share some similarity. The Topo II-associated lesions occurring in drug-treated tumor cells may facilitate subsequent internucleosomal DNA fragmentation by endogenous nucleases and trigger apoptosis.[11] An active apoptotic pathway may be involved in the inductions of DNA cleavage by AQ1 and DAU, which are inhibited by actinomycin D, cycloheximide and aurintricarboxylic acid.[13] In spite of their increasing cytotoxicity, the higher concentrations of 4–25 μM AQ1 tested in HL-60-S cells might inhibit RNA and protein syntheses,[13] reduce the level of Topo targets, inactivate caspases and endonucleases and/or arrest cell cycle traverse to such excessive degree that they might actually block their own ability to sustain the molecular mechanisms required for internucleosomal DNA fragmentation and apoptosis, and produce the paradoxical biphasic curves of DNA cleavage shown in Figure 7.[13,36] This hypothesis is substantiated by the observation that, because DAU loses its overall effectiveness in MDR cells, its ability to both stimulate the active process of apoptotic DNA fragmentation at low concentrations and block the same process at high concentrations appears to be severely impaired in HL-60-RV and HL-60-R8 cells. As a result, the submaximal and maximal levels of DNA cleavage caused by 0.04–1.6 μM DAU are totally lost, drastically reduced and/or shifted toward higher concentrations, which consequently lose some, it not all, of their ability to decrease the level of DNA cleavage below control (Figure 7A and B). Taken together, the facts that the inhibitions of nucleoside transport/DNA synthesis and the biphasic elevation and decline of DNA cleavage (Figure 7C and D) caused by AQ1 are irreversible in L1210 cells[13] and remain unaltered in MDR HL-60 cells, suggest that this new quinone antitumor drug may rapidly and tightly interact with various membrane and intracellular targets to disrupt the structures/functions of nucleoside transporters, nucleic acids and proteins, and to trigger long-lasting antitumor events, which persist after cessation of drug treatment and in DAU-resistant tumor cells.

The multifactorial mechanisms of MDR to anthracycline quinone antibiotics may include altered expressions of P-gp, Topo II and MRP, increased DNA repair and glutathione-dependent detoxifying enzyme activities, and alterations in cell cycle progression, differentiation and apoptotic pathways.[27,28,55–59] MDR is sometimes associated with increases in the number of nucleoside transporters and their rate of transport, resulting in the increased uptake of adenosine[22] and nucleoside salvage pathway limiting the efficacy of MTX and 5-FU.[14–21] Non-toxic concentrations of DPR enhance the cellular uptake of DOX, and potentiate its cytotoxicity *in vitro* and *in vivo*.[24] DPR circumvents DOX resistance,[25,26] and its analog, BIBW 22, is a bifunctional modulator which reverses the MDR phenotype by interfering with both P-gp and nucleoside transport in MDR cells.[23] Incidentally, the nucleoside transport system may contribute to DOX transport in HL-60 cells.[60] It would be of interest to determine which of the mechanisms of MDR AQ1 and AQ4 can overcome, and whether these quinone antitumor drugs can potentiate the action of antimetabolites.

Conclusion

Although less potent, AQ1 and AQ4 mimic the ability of DAU to inhibit DNA synthesis and the proliferation and viability of WT HL-60 cells in the nM range *in vitro*. The advantage of AQ1 and AQ4 lies with the fact that, in contrast to DAU, these quinone antitumor drugs can also block nucleoside transport and retain their effectiveness in HL-60 sublines that have developed different mechanisms of MDR. Further studies are warranted to elucidate in detail the molecular mechanism of action and demonstrate the anticancer potential *in vivo* of AQ1 and AQ4 analogs, which might be valuable to develop new means of polychemotherapy.

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