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Report

1,4-Anthraquinone: an anticancer drug that blocks nucleoside transport, inhibits macromolecule synthesis, induces DNA fragmentation, and decreases the growth and viability of L1210 leukemic cells in the same nanomolar range as daunorubicin *in vitro*

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1,4-Anthraquinone (AQ) was synthesized and shown to prevent L1210 leukemic cells from synthesizing macromolecules and growing *in vitro*. In contrast, its dihydroxy-9,10-anthraquinone precursor, quinizarin, was inactive. The antitumor activity of AQ was compared to that of daunorubicin (DAU), which is structurally different from AQ but also contains a quinone moiety. AQ is equipotent to DAU against L1210 tumor cell proliferation (IC_{50}: 25 nM at day 2 and 9 nM at day 4) and viability (IC_{50}: 100 nM at day 2 and 25 nM at day 4), suggesting that its cytostatic and cytotoxic activities are a combination of drug concentration and duration of drug exposure. Since AQ does not increase but rather decreases the mitotic index of L1210 cells at 24 h, it is not an antitubulin drug but might arrest early stages of cell cycle progression. Like DAU, a 1.5–3 h pretreatment with AQ is sufficient to inhibit the rates of DNA, RNA and protein syntheses (IC_{50}: 2 μM) determined over 30–60 min periods of pulse-labeling in L1210 cells *in vitro*. In contrast to DAU, which is inactive, a 15 min pretreatment with AQ has the advantage of also inhibiting the cellular transport of both purine and pyrimidine nucleosides (IC_{50}: 2.5 μM) over a 30 s period *in vitro*. Hence, AQ may prevent the incorporation of [^{3}H]adenosine and [^{3}H]thymidine into DNA because it rapidly blocks the uptake of these nucleosides by the tumor cells. After 24 h, AQ induces as much DNA cleavage as camptothecin and DAU, two anticancer drugs producing DNA strand breaks and known to, respectively, inhibit topoisomerase I and II activities. However, the concentration-dependent induction of DNA cleavage by AQ, which peaks at 1.6–4 μM and disappears at 10–25 μM, resembles that of DAU. The mechanism by which AQ induces DNA cleavage is inhibited by actinomycin D, cycloheximide and aurintricarboxylic acid, suggesting that AQ activates endonucleases and triggers apoptosis. The abilities of AQ 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. Because of its potency and dual effects on nucleoside transport and DNA cleavage, the use of bifunctional AQ with antileukemic activity in the nM range *in vitro* might provide a considerable advantage in polychemotherapy to potentiate the action of antimetabolites and sensitize multidrug-resistant tumor cells. [© 2000 Lippincott Williams & Wilkins.]

Key words: 1,4-Anthraquinone, DNA cleavage, L1210 cells, macromolecule synthesis, mitotic index, nucleoside transport, tumor cell growth and viability.

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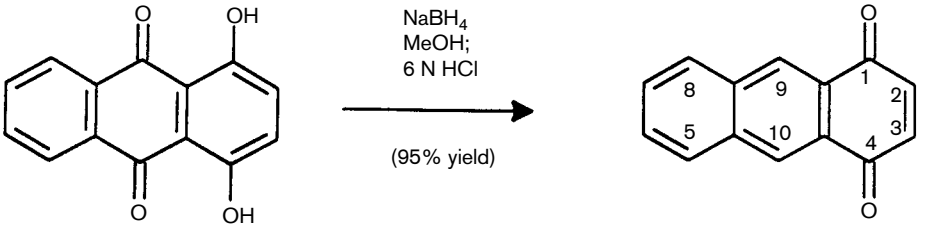
Introduction

As precursors of reactive quinone methides, several natural and synthetic quinones function as bioreductive alkylating agents and have antitumor activity.[1–6] The cytotoxicity of quinones may be due to two

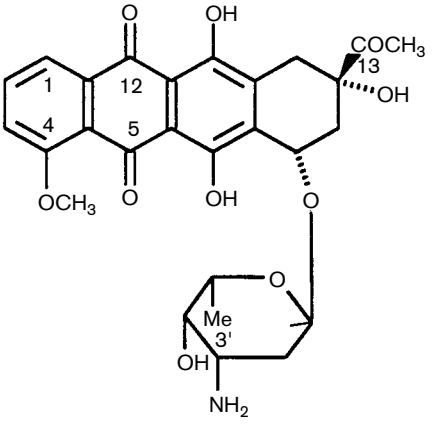
competing mechanisms: soft electrophilic arylation and redox cycling oxidation.[7–9] While complete two-electron reduction of the quinone ring by DT diaphorase produces a stable hydroquinone, partial one-electron reduction of the quinone ring by NADPH-oxidizing enzymes yields an unstable semiquinone free radical (FR) that can spontaneously autoxidize at the expense of molecular O_2 to generate a cascade of reactive O_2 species (ROS) 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 active functional groups and the exact contribution of the quinone group to their antitumor activity remains uncertain.[10–13] The anthracycline quinone antibiotics doxorubicin (ADR, 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.[10,11,13–18]

Many important cancer chemotherapeutic agents contain a quinoid ring in their chemical structure[10,12,13] and there is a need to synthesize anthracyclinone analogs with reduced cardiotoxicity;[1] however, to the best of our knowledge, the antitumor potential of 1,4-anthraquinone (AQ, anthracene-1,4-dione) has never been evaluated before (Figure 1). A limited number of quinoid structures with one (benzoquinones), two (naphthoquinones) or three rings (anthraquinones) and various substitutions may induce cytotoxic effects by a variety of mechanisms, which makes it difficult to assess structure–activity relationships.[1,19–24] Based on short-term photosynthetic inhibition, three-ring compounds are generally non-toxic to freshwater algae but two-ring compounds are more toxic than related one-ring compounds.[20] However, several groups of natural anthraquinones found in plants (seeds, roots, rhizomes, tubers, stems, leaves, flowers, fruits, woods, barks and saps), soil, fungi, lichens, insects, marine animals and cultures of bacteria, including some quinone pigments, contain



Quinizarin

1,4-Anthraquinone (AQ)



Daunorubicin (DAU)

Figure 1. Comparative structures of the quinone compounds tested for their antileukemic activity *in vitro*.

interesting antitumor antibiotics.[1] In addition to synthetic anthracycline antibiotic drugs, a few natural anthracyclinones, which are characterized by a linear fused tetracyclic system, have encouraging antitumor activity.[1]

Quinizarin (1,4-dihydroxy-9,10-anthraquinone), which is used as a precursor of AQ synthesis in Hua's laboratory (Figure 1), has no antileukemic activity whatsoever when tested in Perchellet's laboratory at concentrations ranging from 2.62 nM to 1.6 μM in the L1210 tumor cell system *in vitro* (data not shown). Therefore, the present study was undertaken to determine if, in contrast to its inactive precursor, quinizarin, and as compared to the potent antitumor anthracyclinone glycoside, DAU, AQ has any antitumor activity (Figure 1). Interestingly, we found that AQ is cytostatic and cytotoxic to L1210 tumor cells in the same nM range than DAU *in vitro*. Moreover, our preliminary data indicate that AQ may have an intriguing bifunctional molecular mechanism of action, blocking nucleoside transport and actively inducing DNA fragmentation in order to prevent leukemic cells from synthesizing macromolecules, proliferating and remaining viable *in vitro*.

Materials and methods

Cell culture and drug treatments

All solutions of synthetic AQ and tricyclic pyrone H10, quinizarin (Aldrich, Milwaukee, WI), vincristine (VCR; a gift from Lilly Research Laboratories, Indianapolis, IN), camptothecin (CPT), actinomycin D (Act-D), cycloheximide (CHX) and aurintricarboxylic acid (ATA; all Sigma, St Louis, MO) were dissolved and diluted in dimethyl sulfoxide (DMSO), whereas DAU (from Sigma) solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl.[25–29] Suspension cultures of murine L1210 lymphocytic leukemia cells (ATCC, Rockville, MD) 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. Since drugs were supplemented to the culture medium in 1 μl aliquots, the concentration of DMSO in the final incubation volume (0.5 ml) never exceeded 0.2%, and did not affect the rates of macromolecule syntheses, growth and survival in control L1210 cells incubated with vehicle in the absence of drugs.[25–29] For drug removal, incubates were spun at 200 *g* for 10 min, drug-containing supernatants were discarded, and intact cells were washed thrice with 1 ml of the above RPMI 1640 culture medium and resuspended in 0.5 ml of fresh medium for further incubation.

Cell proliferation assay

For tumor cell growth, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium, seeded in triplicate at an initial density of 1×10^4 cells/0.5 ml and incubated at 37°C in 48-well Costar cell culture plates (Costar, Cambridge, MA). Except when otherwise specified, cells were grown for 4 days in the presence or absence (control) of drugs and their density was monitored every 24 h using a Coulter counter (Coulter Electronics, Luton, UK).[25–29]

Cell viability assay

L1210 cells suspended in FCS-containing RPMI 1640 medium were grown at 37°C in 48-well Costar cell culture plates for up to 4 days in the presence or absence (control) of AQ to evaluate drug cytotoxicity. Decreasing concentrations of cells, such as 9×10^4 and 1×10^4 cells/0.5 ml/well, were initially plated in triplicate at time 0 in order to collect control samples with approximately equal cell densities after 2 and 4 days in culture, respectively. The viability of AQ-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 which absorbs at 490 nm.[30] At the appropriate time after drug treatment, 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).[28,29] Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were substracted from the results.

Macromolecule synthesis

For nucleic acid and protein syntheses, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium at a density of about $1.25–1.6 \times 10^6$ cells/0.5 ml. Except when otherwise specified, the cells

were 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 [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) to estimate the rate of DNA synthesis. In reversibility studies, preincubated cells were spun, washed, resuspended in fresh medium and then pulse-labeled with [^{3}H]thymidine for 30 min in the presence (+) or absence (−) of drugs. For RNA and protein syntheses, cells were incubated at 37°C for 3 h in the presence or absence (control) of drugs and then pulse-labeled for an additional 1 h with 2 μCi of [5,6-^{3}H]uridine (46 Ci/mmol; ICN Biomedicals, Irvine, CA) or 2.5 μCi of L-[3,4,5-^{3}H]leucine (120 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO), respectively. The incubations were 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 microfibre 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 10 ml of Bio-Safe NA (Research Products International, Mount Prospect, IL).[25–29]

Nucleoside transport

L1210 cells (about $1.2–1.7 \times 10^6$ cells/0.5 ml) were preincubated for 15 min at 37°C in the presence or absence (control) of AQ and then exposed to 1 μCi of [2,8-^{3}H]adenosine (30 Ci/mmol; American Radiolabeled Chemicals) or [^{3}H]thymidine for only 30 s to, respectively, assess the cellular uptake of purine and pyrimidine nucleosides over such very short period of time.[28,29,31] In reversibility studies, preincubated cells were spun, washed, resuspended in fresh medium and then exposed to [^{3}H]thymidine for 30 s in the presence (+) or absence (−) of drugs. 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 9 ml of Bio-Safe II (Research Products International) and counted to estimate the cellular uptake of [^{3}H]adenosine or [^{3}H]thymidine. Drug inhibition was expressed as percent of [^{3}H]adenosine or [^{3}H]thymidine transported into vehicle-treated control cells over similar 30 s periods.[28,29,31]

Mitotic index

L1210 cells (about 0.5×10^6/0.5 ml of FCS-containing RPMI 1640 medium) were incubated in triplicate for 24 h at 37°C in the presence or absence of AQ or known antimitotic drugs and collected by centrifugation at 200 *g* for 10 min to determine their mitotic index. For hypotonic treatment, cells were resuspended in 1 ml of 75 mM KCl for 20 min at 4°C. After fixation in 1 ml of MeOH:acetic acid (3:1), the final cell pellets were collected by centrifugation, resuspended in 75 μl of MeOH:acetic acid (3:1), dispensed onto glass slides, air dried and stained by spreading 40 μl of 0.1% crystal violet under a coverslip.[27–29] The percent of cells in mitosis was determined microscopically by counting 500 cells/slides. The mitotic index was calculated as the percent of mitotic cells in drug-treated cultures divided by the percent of mitotic cells in non-treated controls.[27–29]

DNA cleavage

Drug-induced DNA cleavage was determined by intact chromatin precipitation, using L1210 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 resuspended in fresh FCS-containing RPMI 1640 medium at a density of about 1×10^6 cells/0.5 ml.[17,28,29,32] Except when otherwise specified, such cells containing prelabeled DNA were then incubated at 37°C for 24 h in the presence or absence (control) of AQ or drugs known to induce DNA fragmentation. After centrifugation at 200 *g* for 10 min to discard the drugs and wash the cells, the intact cell pellets were lysed for 30 min in 0.5 ml of HLB, centrifuged at 12,000 *g* for 30 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: percent DNA fragmentation=[c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100.[17,28,29,32] In reversibility studies, the drugs were either maintained in the medium for the whole 24 h period of incubation or the cells were spun and washed to remove the drugs after the first 2 or 5 h, and then resuspended in 0.5 ml of fresh medium to complete the 24 h period of incubation in the absence of drugs.

Results

Drugs

Quinizarin, which is not cytotoxic to L1210 cells when tested at 2.62 nM to1.6 μM over a 4 day period *in vitro* (data not shown), is available commercially and the synthesis of AQ, which is a known compound, has already been reported (Figure 1).[33–36] For this study, AQ was prepared (Figure 1) in 95% yield (orange solids) by the reduction of quinizarin with four equivalents of sodium borohydride in MeOH (g/20 ml) at 0°C for 1 h followed by the addition of 6 N HCl (g/12 ml).[36] The antileukemic effects of AQ were assessed and compared to those of the well-known anthracycline antibiotic DAU, a clinically valuable anticancer drug which is structurally very different from AQ but also contains a quinone moiety (Figure 1).[10]

Inhibition of tumor cell growth and viability by AQ

At concentrations of 102.4 nM or above, DAU inhibits almost totally the proliferation and viability of L1210 cells at day 4 but these maximal cytostatic and cytotoxic activities of DAU can be mimicked by similar concentrations of AQ, and the smallest concentrations of AQ and DAU that can induce significant antiproliferative and cytotoxic effects after 4 days are 6.55 and 16.38 nM, respectively (Figures 2 and 3). These relative potencies of AQ and DAU can easily be compared using the full concentration–response curves of Figures 2 and 3, where the striped areas at 100% represent the control levels of L1210 cell growth and viability after 2 and 4 days in culture. The magnitudes of the cytostatic and cytotoxic effects of both AQ and DAU are clearly related to the combination of their increasing concentration and duration of action. For instance, 16.38 nM AQ and DAU are moderately cytostatic (42 and 27% inhibition, respectively) and very weakly cytotoxic (14 and 24% inhibition, respectively) at day 2 but, respectively, decrease L1210 cell proliferation by 62 and 46% and L1210 cell viability by 42 and 46% at day 4 (Figures 2 and 3). As a result, the antiproliferative activities of AQ and DAU are, respectively, characterized by IC_{50}

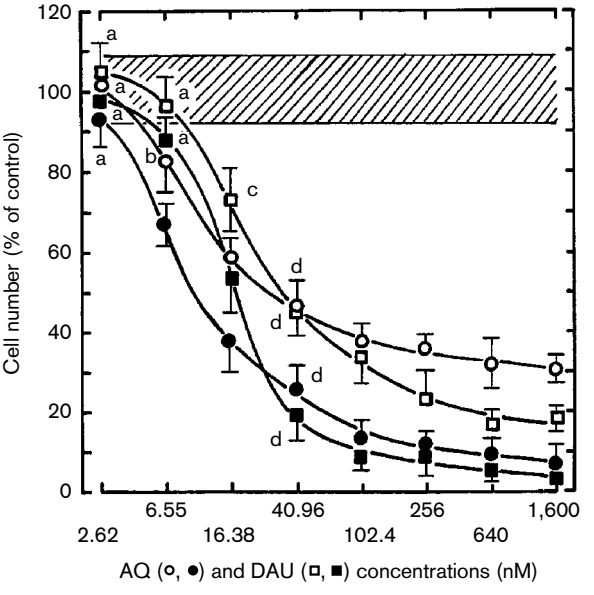


Figure 2. Comparison of the concentration-dependent inhibitions of L1210 cell proliferation by AQ (circles) and DAU (squares) at days 2 (open symbols) and 4 (closed symbols) *in vitro*. Cell growth results are expressed as percent of the numbers of vehicle-treated control cells (100 ± 9%, striped area) after 2 (221 145 ± 20 434 cells/ml) and 4 (1 453 458 ± 125 579 cells/ml) days in culture. Bars: means ± SD (n=3). [a]Not different from control; [b]$P<0.05$ and [c]$P<0.025$, smaller than control; [d]no difference between similar concentrations of drugs on the same day.

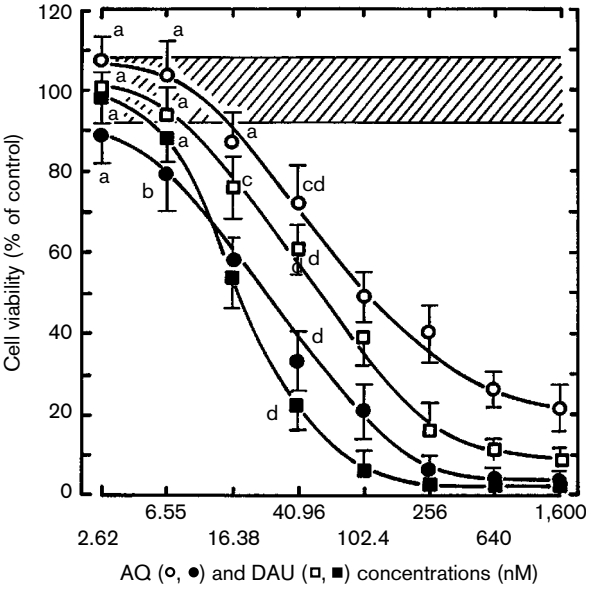


Figure 3. Comparison of the concentration-dependent inhibitions of L1210 cell viability by AQ (circles) and DAU (squares) at days 2 (open symbols) and 4 (closed symbols) *in vitro*. Cell viability results are expressed as % of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells (100 ± 8%, striped area) at days 2 ($A_{490\ nm}$=1.284 ± 0.098) and 4 ($A_{490\ nm}$=1.146 ± 0.095). Blank values ($A_{490\ nm}$=0.356 and 0.348 at days 2 and 4) for cell-free culture medium supplemented with MTS:PMS reagent have been substracted from the results. Bars: means ± SD (n=3). [a]Not different from control; [b]$P<0.05$ and [c]$P<0.025$, smaller than control; [d]no difference between similar concentrations of drugs on the same day.

values of 25 and 32 nM at day 2 but 9 and 17 nM at day 4, suggesting that AQ and DAU are similarly cytostatic in the L1210 tumor system *in vitro* (Figure 2). Moreover, the inhibitions of tumor cell viability by AQ and DAU are, respectively, characterized by IC_{50} values of 100 and 55 nM at day 2 but 25 and 17 nM at day 4, suggesting that AQ and DAU are also similarly cytotoxic in this leukemic system *in vitro* (Figure 3). Indeed, the fact that AQ is an antileukemic agent equipotent to DAU is substantiated by the observation that, at a concentration of 40.96 nM, the antiproliferative and cytotoxic effects of AQ and DAU are not significantly different (Figures 2 and 3).

Inhibition of DNA synthesis and nucleoside transport by AQ

A 2 h treatment with AQ is sufficient to inhibit, in a concentration-dependent manner, the rate of DNA synthesis determined over a 30 min period of pulse-labeling in L1210 cells *in vitro* (Figure 4). DNA synthesis is totally inhibited by 10 and 25 μM AQ but, as compared to DAU which becomes effective against DNA synthesis at 0.64 μM, concentrations of 1.6 μM or above must be used to demonstrate the inhibitory effect of AQ on DNA synthesis. Hence, the concentration-dependent inhibitions of DNA synthesis by AQ (IC_{50}: 2 μM) and DAU (IC_{50}: 1 μM) suggest that, under these experimental conditions, DAU prevents L1210 cells from synthesizing DNA 2 times more effectively than AQ (Figure 4).

A critical finding is that, in contrast to DAU which serves as a negative control in the assays, a 15 min treatment with AQ is sufficient to block, in a concentration-dependent manner, the cellular transport of both [^{3}H]adenosine and [^{3}H]thymidine occurring over only 30 s *in vitro* (Figure 5). The transport of these radiolabeled purine and pyrimidine nucleosides is totally inhibited in L1210 cells treated with 10 and 25 μM AQ. However, concentrations of 4 and 10 μM DAU are totally unable to significantly alter the cellular transport of [^{3}H]adenosine and [^{3}H]thymidine (Figure 5), even though such concentrations of DAU maximally inhibit the incorporation of [^{3}H]thymidine into DNA used to assess the rate of DNA synthesis (Figure 4). Interestingly, the concentration–response curves for the inhibitory effects of AQ on the cellular transport of purine (IC_{50}: 2.5 μM) and pyrimidine (IC_{50}: 2.5 μM) nucleosides (Figure 5) and the rate of DNA synthesis (Figure 4) are nearly identical and share almost similar IC_{50} values, suggesting that the inhibition of [^{3}H]thymidine incorporation into DNA caused by AQ at 2 h (Figure 4) may largely be due to the ability of this three-ring quinone antitumor drug to immediately block the uptake of [^{3}H]thymidine by the cells (Figure 5).

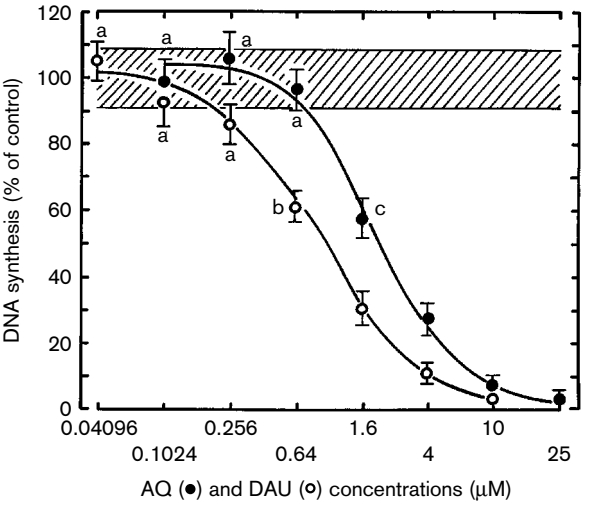


Figure 4. Comparison of the concentration-dependent inhibitions of DNA synthesis by AQ (●) and DAU (○) in L1210 cells *in vitro*. DNA synthesis in vehicle-treated control cells was 22 089±2010 c.p.m. (100±9%, striped area). The blank value (1074±39 c.p.m.) for cells incubated and pulse-labeled at 2°C with 1 μCi of [^{3}H]thymidine has been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$P<0.01$, smaller than control; [c]$P<0.005$, smaller than control and greater than similar concentration of DAU.

Moreover, the abilities of 4 and 10 μM AQ to, respectively, inhibit nucleoside transport by 71 and 92% and DNA synthesis by 84 and 96% are both irreversible upon drug removal (Figure 6), suggesting that, after a 15–60 min pretreatment, the presence of AQ in the culture medium is no longer required to continually block the cellular transport of [^{3}H]thymidine and thereby prevent its incorporation into DNA. For the sake of comparison, the inhibitions of nucleoside transport and DNA synthesis caused by the novel tricyclic pyrone analog H10, which are fully reversible upon drug removal,[26,28] are demonstrated as positive controls under similar experimental conditions in this L1210 tumor assay system (Figure 6).

Inhibition of RNA and protein syntheses by AQ

Besides DNA synthesis, a 3 h treatment with AQ can also inhibit, in a concentration-dependent manner, the rates of RNA and protein syntheses determined over 60 min periods of pulse-labeling in L1210 cells *in vitro*

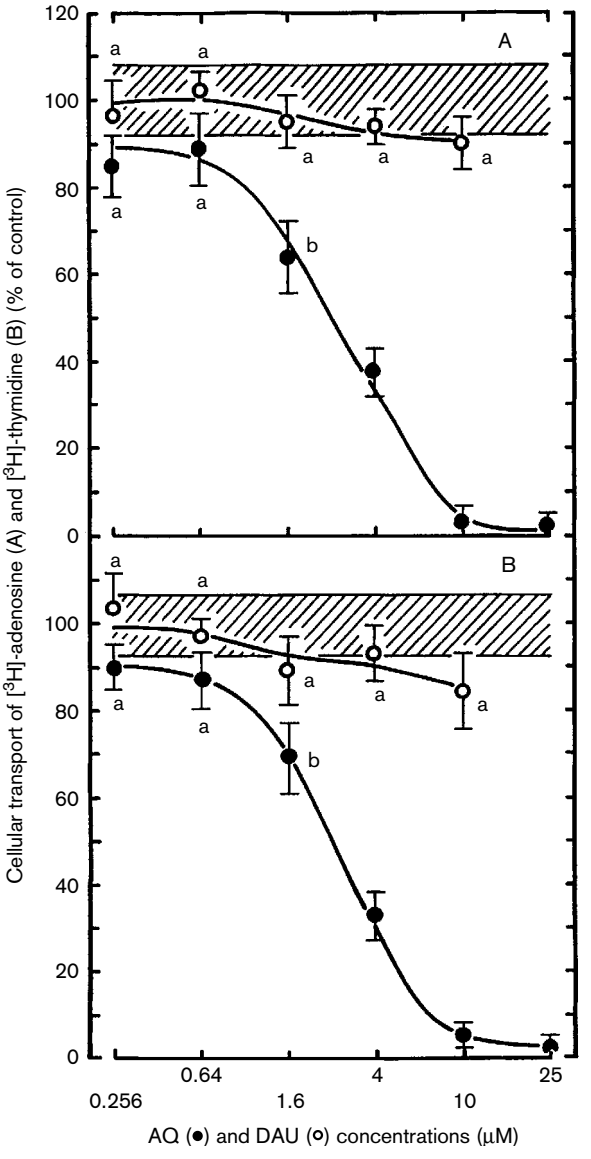


Figure 5. Comparison of the concentration-dependent inhibitions of the cellular transport of purine (A) and pyrimidine (B) nucleosides by AQ (●) and DAU (○) in L1210 cells *in vitro*. Results are expressed as percent of [^{3}H]adenosine (55 311 ± 4497 c.p.m., 100 ± 8%, striped area in A) and [^{3}H]thymidine (13 049 ± 965 c.p.m., 100 ± 7%, striped area in B) transported into vehicle-treated control cells over 30 s. Bars: means ± SD (n=3). [a]Not different from control; [b]$P<0.01$, smaller than control.

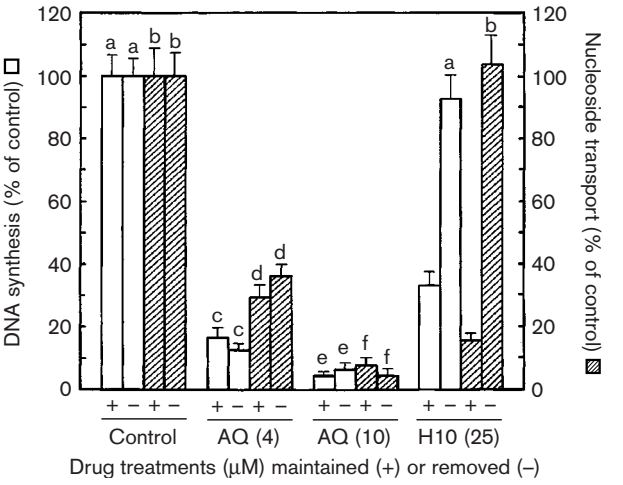


Figure 6. Irreversibility of the inhibitory effects of AQ on DNA synthesis (open) and nucleoside transport (striped) in L1210 cells *in vitro*. The reversible inhibitory effects of the tricyclic pyrone H10 are demonstrated in the same experiments. The cellular uptake of [^{3}H]thymidine over 30 s (striped) and the rate of [^{3}H]thymidine incorporation into DNA over 30 min (open) were determined in cells, respectively, preincubated at 37°C for 15 or 60 min in the presence or absence (control) of 4 and 10 μM AQ or 25 μM H10. After preincubation, either the drugs were maintained in the culture medium (+) or the cells were spun, washed, and resuspended in fresh medium in order to remove the drugs (−). Nucleoside transport results are expressed as the % of [^{3}H]thymidine transported into vehicle-treated control cells over 30 s (12 051 ± 1048 c.p.m., 100 ± 9%, striped control +; 15 483 ± 1053 c.p.m., 100 ± 7%, striped control −). DNA synthesis results are expressed as percent of [^{3}H]thymidine incorporation into DNA of vehicle-treated control cells over 30 min (30 751 ± 1968 c.p.m., 100 ± 6%, open control +; 25 508 ± 1479 c.p.m., 100 ± 6%, open control −). The blank value (1360 ± 82 c.p.m.) for 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). Values with similar superscripts are not significantly different from each other.

(Figure 7). The concentration–response curves for the inhibitions of RNA and protein syntheses (Figure 7) by AQ are nearly identical to that for the inhibition of DNA synthesis (Figure 4): concentrations of AQ equal to (Figure 7A) or greater than 0.64 μM (Figure 7B) must be used to demonstrate effectiveness, total inhibition is achieved at 10–25 μM and the IC_{50} values in all cases are 2 μM (Figures 4 and 7). Under similar conditions, DAU inhibits RNA synthesis (IC_{50}: 0.8 μM) 2.5 times more effectively than AQ but is exactly as potent as AQ against protein synthesis (IC_{50}: 2 μM) (Figure 7).

Effects of AQ on the mitotic index

Control populations of L1210 cells cultured for 24 h in the absence of drugs contain only 1.69% of mitotic cells. In relation with their ability to block tubulin polymerization and cell cycle progression in M phase, 24 h treatments with VCR and the tricyclic pyrone H10 respectively produce 9.9- and 5.7-fold increases in the mitotic index.[26–28] Such known microtubule destabilizing anticancer drugs, therefore, serve as positive controls in this antimitotic assay.[26–29] In contrast, none of the concentrations of AQ tested, even those in

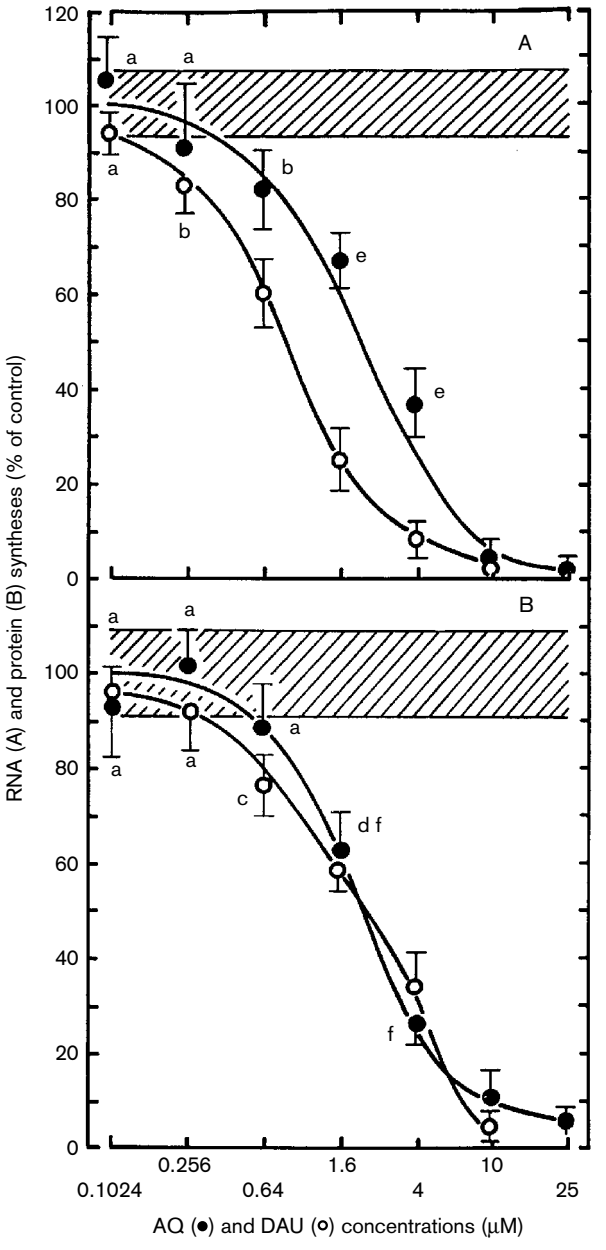


Figure 7. Comparison of the concentration-dependent inhibitions of RNA (A) and protein (B) syntheses by AQ (●) and DAU (○) in L1210 cells *in vitro*. RNA and protein syntheses in vehicle-treated control cells were 66 633±4398 c.p.m. (100±7%, striped area in A) and 14 948±1386 c.p.m. (100±9%, striped area in B), respectively. The blank values (1331±117 and 2073±248 c.p.m.) for cells, respectively, incubated and pulse-labeled at 2°C with 2 μCi of [^{3}H]uridine (A) or 2.5 μCi of [^{3}H]leucine (B) have been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$P<0.05$, [c]$P<0.025$ and [d]$P<0.01$, smaller than control; [e]$P<0.005$, greater than similar concentrations of DAU; [f]not different from similar concentrations of DAU.

the 0.64–4 μM range that are already highly cytostatic and cytotoxic at 24 h, are able to raise the mitotic index of L1210 cells (data not shown), suggesting that AQ is not an antimitotic drug that disrupts microtubule dynamics to trigger its anticancer activity. In relation with its known ability to first accumulate cells in G_2 and then inhibit cell cycle traverse as its concentration increases (reviewed in Perchellet *et al.*[29]), 0.256–1.6 μM concentrations of DAU actually decrease the percent of mitotic cells by 86–95% (data not shown). At 0.256 μM, AQ is ineffective but since the highest concentrations of 0.64–4 μM AQ tested all significantly decrease the percent of mitotic cells by 43–76% (data not shown), AQ might also prevent tumor cell cycle progression to mitosis.

Induction of DNA cleavage by AQ

L1210 cells containing [^{3}H]thymidine-prelabeled DNA were used to quantitatively determine whether AQ could induce DNA fragmentation over a 24 h period *in vitro*. CPT and DAU, two anticancer drugs known to induce DNA strand breaks by, respectively, inhibiting Topo I and II activities (reviewed in Perchellet *et al.*[29]),[14] are used as positive controls in this DNA fragmentation assay (Figure 8). As reported before with anthracycline quinone antibiotics (reviewed in Perchellet *et al.*[29]), the concentration-dependent induction of DNA cleavage caused by 24 h DAU treatments is biphasic, peaking at 46% in response to 1.6 μM DAU but declining back to control level (5%) at higher concentrations of DAU (Figure 8).[17,18] In contrast, the concentration-dependent increase of DNA cleavage produced by 24 h CPT treatments (Figure 8) reaches 58% in response to 1.6 μM CPT but remains at a plateau of maximal stimulation (60–72%) at higher concentrations of CPT (reviewed in Perchellet *et al.*[29]). After 24 h, the maximal levels of DNA cleavage caused by 1.6 (41%) and 4 (68%) μM AQ, respectively, match those induced by 1.6 μM DAU and 1.6–25 μM CPT (Figure 8). However, at concentrations greater than 4 μM, AQ loses its ability to induce DNA fragmentation (Figure 8). Hence, concentrations of AQ 2.5 times higher than those of DAU are required to induce such peak of DNA cleavage at 4 μM but the overall shape of the concentration–response curve for the ability of AQ to break DNA resembles that of DAU rather than that of CPT (Figure 8), suggesting that the DNA-damaging effects of AQ and DAU might share some similarity.

Interestingly, L1210 cells treated for only 2 and 5 h with 1.6 and 4 μM AQ (data not shown) have the same levels of DNA fragmentation at 24 h (33–39 and 67–

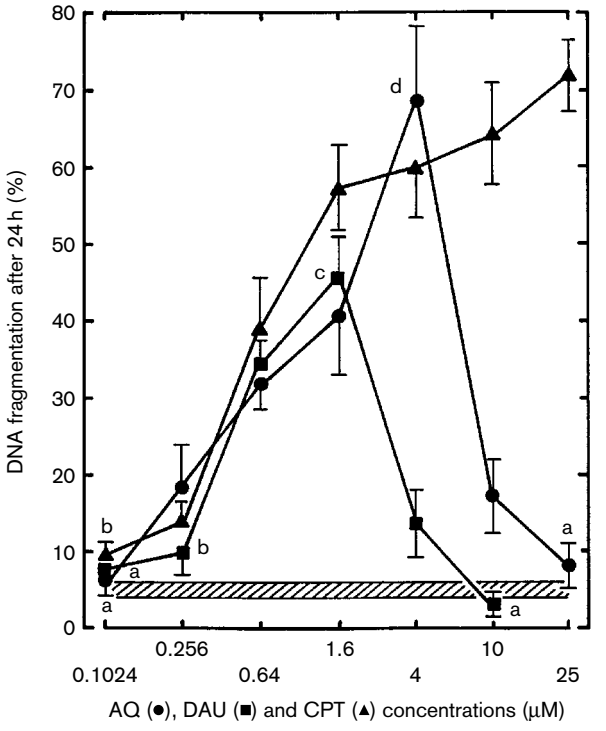


Figure 8. Comparison of the concentration-dependent effects of AQ (●) and the known DNA-damaging anticancer drugs DAU (■) and CPT (▲) on DNA cleavage in L1210 cells containing ^{3}H-prelabeled DNA *in vitro*. Results are expressed as [c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100 at 24 h. For untreated controls (5.2 ± 0.7% DNA fragmentation, striped area), the supernatant (DNA fragments) is 791 ± 109 c.p.m. and the pellet (intact DNA) is 14 538 ± 2108 c.p.m. Bars: means ± SD (n=3). [a]Not different from control; [b]$P<0.05$, greater than control; [c]$P<0.05$, smaller than 1.6 μM CPT but not different from 1.6 μM AQ; [d]$P<0.05$, greater than 1.6 μM DAU but not different from 1.6, 4, 10 and 25 μM CPT.

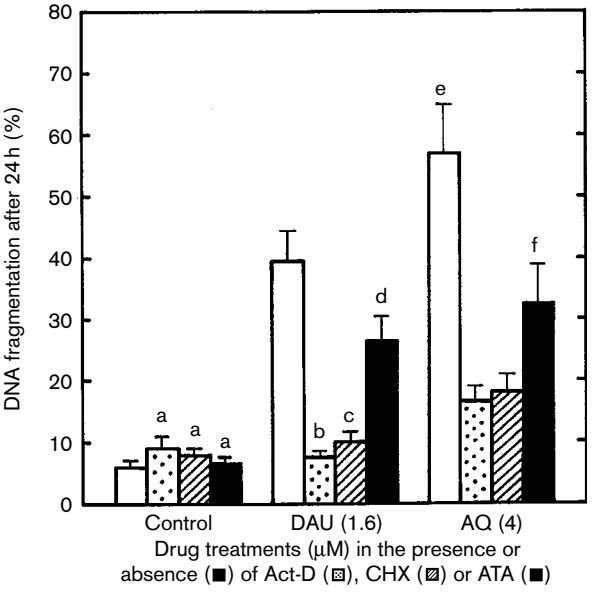


Figure 9. Inhibitory effects of Act-D, CHX and ATA on AQ- and DAU-induced DNA cleavage in L1210 cells *in vitro*. Cells containing prelabeled DNA were preincubated at 37°C for 2 h in the presence or absence (open) of Act-D (2 μg/ml, dotted), CHX (50 μg/ml, striped) or ATA (200 μM, closed) and, after supplementing their culture medium with either vehicle (control), 1.6 μM DAU or 4 μM AQ, these incubations were continued for an additional 24 h to determine the level of DNA cleavage. For drug-untreated controls preincubated and incubated in the presence of vehicle only (5.8 ± 1.0% DNA fragmentation), the supernatant is 967 ± 170 c.p.m. and the pellet is 15 705 ± 2889 c.p.m. Bars: means ± SD (n=3). [a]Not different from vehicle-treated control; [b]not different from control and Act-D alone; [c]not different from CHX alone but $P<0.025$, greater than control; [d]$P<0.025$, smaller than DAU alone; [e]$P<0.05$, greater than DAU alone; [f]$P<0.025$, smaller than AQ alone.

69%, respectively) than if they are exposed for the whole 24 h incubation period to 1.6 and 4 μM AQ (34 and 68%, respectively). Under similar conditions, 1.6 μM DAU and CPT also rapidly trigger molecular events, which are irreversible and produce identical levels of DNA fragmentation at 24 h (32–39 and 54–59%, respectively), whether or not those drugs are maintained in the culture medium after 2 or 5 h (data not shown). It should be noted that no significant elevation of DNA cleavage is detectable in this assay after 2 or 5 h of drug exposure and that the increases of DNA fragmentation caused by AQ, DAU and CPT only appear after 12 h to reach a maximal level at 24 h (data not shown), suggesting that the irreversible events triggered by those drugs during the initial 2–5 h still require a substantial period of time, irrespective of the continual presence or absence of drugs, to fully induce DNA fragmentation. These results suggest that, like anticancer drugs known to induce DNA strand breaks, AQ rapidly interacts with cellular targets to induce long-lasting DNA-damaging effects, which develop and persist after drug removal.

Finally, non-toxic concentrations of Act-D, CHX and ATA, which by themselves do not alter the basal level of DNA cleavage in L1210 cells after a 2 h preincubation period followed by 24 h of treatment (Figure 9), were tested for their ability to affect the molecular mechanisms by which AQ and DAU induce DNA fragmentation. Such treatments with Act-D, CHX and ATA, which are known to, respectively, inhibit RNA and protein syntheses and endonuclease activity,[17] either totally abolished or drastically reduced the abilities of both AQ and DAU to induce DNA fragmentation at 24 h (Figure 9). For instance, considering that the control level of DNA cleavage is 6%, the levels of DNA fragmentation induced by DAU

(39%) and AQ (57%) at 24 h are, respectively, inhibited by 88–94 and 65–78% in the presence of Act-D or CHX, and by 36 and 49% in the presence of ATA (Figure 9). These preliminary data, therefore, suggest that, like DAU and ADR,[17] AQ may use, at least partially, an active process of apoptosis that requires gene expression, the synthesis of new RNA and proteins, and the activation of endogenous nucleases in order to induce DNA fragmentation.

Discussion

To the best of our knowledge, this is the first report demonstrating that AQ is an antitumor agent equipotent to DAU against L1210 cell proliferation and viability *in vitro*. The magnitudes at which 6.55–256 nM concentrations of AQ inhibit tumor cell proliferation (IC_{50}: 9 nM at day 4) closely match their cytotoxic activities (IC_{50}: 25 nM at day 4) (Figures 2 and 3). AQ is also nearly equipotent to DAU against DNA, RNA and protein syntheses (IC_{50}: 2 μM at 2–3 h) in L1210 tumor cells (Figures 4 and 7). Moreover, AQ inhibits DNA synthesis in relation with its effectiveness against the cellular transport of purine and pyrimidine nucleosides (IC_{50}: 2.5 μM at 15 min), suggesting that it prevents DNA assembly because it rapidly blocks, in the same concentration-dependent and irreversible manner, the cellular uptake of DNA precursors (Figures 4–6). In addition, AQ may use an active process of apoptosis to induce DNA fragmentation to the same maximal levels than clinically valuable anticancer drugs (Figures 8 and 9), suggesting that the ability of AQ to break DNA may play a significant role in its molecular mechanism of antitumor activity. Because of its ability to interact with both membrane and nuclear targets to block nucleoside transport, inhibit nucleic acid and protein syntheses, cleave DNA, and reduce tumor cell growth and viability in the same nM range than DAU *in vitro*, AQ may represent a novel class of bifunctional quinone antitumor drugs valuable to develop new means of polychemotherapy.

This preliminary study has focused on AQ but its exact molecular mechanism of action remains to be elucidated, and further studies would be required to establish conclusive relationships between the chemical structures of specific AQ analogs and their antitumor activities. The potent antitumor activity of AQ is all the more remarkable that, among naturally occuring quinones, the number of bioactive 1,2-, 1,4- and 9,10-AQs appears quite limited.[1] The 1,4-AQ analog viocristin and its isomer isoviocristin are antibiotics that inhibit macromolecule synthesis in microorganisms.[1] 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 aloeemodin, which has significant antileukemic activity in mice and may be used as a starting point for the synthesis of the anthracycline antitumor antibiotics.[1] 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] Besides the famous anthracycline antibiotics ADR and DAU and the less cardiotoxic aclacinomycin A, other anthracyclinones with interesting antitumor activity include carminomycinone, which is 4-*O*-demethyldaunomycinone, nogalarol derivatives, such as nogalamycin and menogarol, and the aklavinone, daunomycinone and adriamycinone glycosides.[1]

Other types of biological interactions have been reported for a number of benzoquinones, naphthoquinones and AQs, which are capable of inhibiting purified glutathione S-transferase activities *in vitro*,[19] inducing aquatic toxicity in the *Tetrahymena pyriformis*[24] and *Selenastrum capricornutum*[20] assay systems, blocking viral infectivity,[23] and quenching chlorophyll fluorescence in chloroplasts.[22] The potent antitumor activity of AQ detected in our study is quite unexpected since the three-ring AQ is essentially nontoxic to freshwater algae[20] and is neither mutagenic *in vitro* nor *in vivo*.[21] In contrast the one-ring 1,4-benzoquinone and the two-ring 1,4-naphthoquinone are the most toxic in freshwater algae,[20] and their substituted analogs effectively inhibit influenza virus infectivity by blocking the conformational change in viral hemagglutinin which is a prerequisite for fusion peptide exposure, membrane fusion, viral entry and infection.[23] Since the precursor quinizarin, which has a dihydroxy-substituted anthracene skeleton with an internal quinoid ring, is inactive, it is reasonable to assume that the AQ framework with its external *para*-quinone may be responsible for its potent antitumor activity (Figure 1). The antitumor activities of various substituted AQs and of different anthraquinones, such as 9,10-anthraquinones, 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 substitution on both sides of the quinoid ring are not toxic at saturation.[24] Substitution of the 1,4-benzoqui-

none ring by electron-donating methoxy or hydroxy groups decreases toxicity, 2,5-substitution being less toxic than 2,6-substitution.[24] Hence, fully substituted tetramethoxy- and tetrachloro-1,4-benzoquinone derivatives, which cannot conjugate or arylate protein thiols, are significantly less toxic than 1,4-benzoquinone.[24] In contrast, since the hydrophilicity or hydrophobicity of AQ substituents controls the fraction of chlorophyll accessible to quinone in chloroplasts, hydroxy 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.[22] Since the ability of AQ to bind to nucleoside transporters, interact covalently with DNA, inhibit Topo activities, and affect the production of ROS and FRs is still unknown, it is rather premature to speculate on the antitumor potential of various substitutions and quinone or hydroquinone functionalities of the AQ skeleton.

The cytostatic and cytotoxic effects of each concentrations of AQ increase with the time in culture (Figures 2 and 3), suggesting that the effectiveness of AQ 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 AQ treatment may be a reliable predictor of anticancer activity.[37] Overall, AQ is a very potent antileukemic agent, reducing L1210 cell proliferation and viability in the same nM range as DAU, and inhibiting DNA, RNA and protein syntheses in the same μM range as DAU (Figures 2, 3, 4 and 7). Moreover, 1.6 and 4 μM concentrations of AQ, respectively, produce exactly the same percent of DNA fragmentation as the peak of DNA cleavage caused by 1.6 μM DAU and the plateau of DNA cleavage induced by 1.6–25 μM CPT (Figure 8). However, in addition to mimicking all the antitumor effects of DAU studied, our data demonstrate that AQ remarkably blocks the cellular transport of both purine and pyrimidine nucleosides, which DAU cannot do (Figure 5), suggesting that the less complex AQ structure may have a more versatile mechanism of action and be advantageous in polychemotherapy to potentiate the anticancer effects of antimetabolites and circumvent multidrug resistance (MDR).

For both AQ and DAU, concentrations in the 16.38–102.4 nM range are sufficient to inhibit tumor cell growth and viability (Figures 2 and 3), whereas higher concentrations in the 0.64–4 μM range must be used to inhibit macromolecule synthesis (Figures 4 and 7) and maximally induce DNA fragmentation (Figure 8). However, these apparent discrepancies may be due in part to different experimental conditions and cellular responses to various periods of drug exposure: the rates of nucleic acid and protein syntheses over 30–60 min are inhibited in cells treated for only 2–3 h with AQ or DAU, whereas the level of DNA cleavage and the reduction of tumor cell growth/viability are the results of 1- and 2- or 4-day-long drug treatments, respectively. It should be noted that no significant DNA fragmentation can be detected within the first 2–5 h of AQ or DAU treatments and that concentrations of AQ and DAU of 40.96–102.4 nM or above must be used to induce substantial and consistent antiproliferative and cytotoxic effects after only 24 h of drug exposure (data not shown).

Our preliminary data suggest that AQ may rapidly trigger long-lasting inhibitory and damaging events which persist upon drug removal. For instance, the irreversibility of the inhibitions of nucleoside transport and DNA synthesis caused by AQ suggests that the presence of this drug in the extracellular medium becomes irrelevant after 15–60 min (Figure 6) to maintain membrane nucleoside transporters and DNA synthesis inhibited. Incidentally, the DNA cleavage caused by AQ, DAU and CPT is undetectable at 2 and 5 h, becomes significant at 12 h, and reaches a maximal level at 24 h (data not shown). Hence, it takes 24 h to fully reveal the extent of DNA cleavage achieved by 2–5 h pretreatments with the above drugs. The irreversibility of this response suggests that the DNA-damaging events triggered by AQ, DAU and CPT 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.

In contrast to VCR and the novel tricyclic pyrone H10, which are microtubule de-stabilizing anticancer drugs known to, respectively, interact with the Vinca and colchicine binding sites on tubulin to block microtubule assembly and the progression of tumor cells undergoing mitosis,[26–28] the inability of AQ to raise the mitotic index of L1210 cells after 24 h suggests that this compound is not a mitotic spindle poison and neither interacts with tubulin nor alters the polymerization/depolymerization of microtubules in order to induce its cytostatic/cytotoxic effects. In fact, anticancer quinones have been reported to cause G_2 arrest,[13] and the ability of 0.64, 1.6 and 4 μM concentrations of AQ to increasingly reduce the percent of mitotic cells at 24 h (data not shown) like the increasing concentrations of DAU known to first block tumor cells in G_2 and then prevent cell cycle traverse all together (reviewed in Perchellet *et al.*[29]) suggests that AQ might also block early stages of cell

cycle progression to prevent tumor cells from reaching the M phase.

The fact that, within 24 h, AQ can produce as much internucleosomal DNA fragmentation in L1210 cells as the known Topo I and II inhibitors CPT and DAU (Figure 8) suggests that the ability of AQ 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 AQ on DNA cleavage resembles the biphasic response to DAU,[17] which has already been reported and discussed before (reviewed in Perchellet *et al.*[29]), it is tempting to speculate that the mechanisms by which AQ and DAU induce DNA fragmentation share some similarity and that DNA cleavage is not always necessary for high concentrations of 4–10 μM DAU and 10–25 μM AQ to be cytotoxic (Figure 8). The Topo II-associated DNA lesions occuring in cells exposed to anthracycline quinone antibiotics may facilitate subsequent internucleosomal DNA fragmentation by endogenous nucleases and trigger apoptosis.[17] Since apoptosis is an active and cell cycle phase-specific process, which requires the expression of specific genes, the syntheses of new RNA and proteins and the activation of endonuclease enzymes, inhibitors of such mechanisms can prevent DNA fragmentation in anthracycline-treated cells (reviewed in Ling *et al.*[17]). Hence, the abilities of the inhibitor of RNA synthesis Act-D, the inhibitor of protein synthesis CHX and the inhibitor of endonuclease activity ATA to similarly block or reduce the mechanisms of DNA fragmentation by DAU and AQ (Figure 9) suggest that, just like 0.256–1.6 μM DAU, lower concentrations of 0.256–4 μM AQ (Figure 8) might also activate endonucleases and trigger apoptosis in order to break DNA and induce tumor cell killing. In spite of their increasing cytotoxicity, the highest concentrations of 4–10 μM DAU and 10–25 μM AQ tested in our study might inhibit RNA and protein syntheses (Figure 7), reduce the level of Topo II targets, inactivate endonucleases and/or arrest cell cycle traverse to such degrees that they actually block the active molecular mechanisms required for internucleosomal DNA fragmentation and apoptosis, and produce the paradoxical biphasic curve of DNA cleavage shown in Figure 8.[17] Taken together, the irreversibilities of the inhibitions of nucleoside transport/DNA synthesis (Figure 6) and of the induction of DNA fragmentation (data not shown) upon AQ removal suggest that, because AQ may rapidly and tightly interact with various membrane and intracellular targets, its presence is soon no longer required in the medium 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.

The clinical effectiveness of ADR and DAU is limited by their cumulative cardiotoxicity and ability to induce MDR.[10] Combining drugs, which target different molecules and achieve complementary or synergistic antitumor effects, is an important strategy in cancer chemotherapy. AQ, which inhibits nucleoside transport and induces DNA cleavage, might disrupt a wider spectrum of molecular targets in populations of unsynchronized tumor cells than another drug affecting a single of these events. For nucleotide synthesis, cells use purine and pyrimidine nucleosides generated either through *de novo* synthesis or through the utilization of salvage pathways. MDR is sometimes associated with increases in the number of nucleoside transporters and their rate of transport, resulting in the increased uptake of adenosine.[38] By blocking the rescue effect of exogenous nucleosides, nucleoside transport inhibitors may potentiate or prolong the antitumor activity of antimetabolites which inhibit the *de novo* pathway for nucleoside synthesis.[39–43] Moreover, nucleoside transport inhibitors may also circumvent ADR resistance by interfering with both P-glycoprotein and nucleoside transport in MDR cells.[44,45] As a bifunctional inhibitor of nucleoside transport and inducer of DNA cleavage, AQ might be valuable in polychemotherapy to potentiate the antitumor activities of methotrexate and 5-fluorouracil, and sensitize MDR tumor cells that have become unresponsive to the cytotoxicities of other conventional DNA-damaging anticancer agents.

Conclusion

AQ may be a novel and potent bifunctional anticancer drug, which blocks the cellular transport of purine and pyrimidine nucleosides and induces DNA fragmentation by an active apoptotic process in order to inhibit nucleic acid and protein syntheses and decrease the proliferation and viability of leukemic cells in the same nM range as DAU *in vitro*. Further studies are warranted to elucidate in detail the molecular mechanism of action and demonstrate the anticancer potential *in vivo* of AQ, which might be valuable to develop new means of polychemotherapy.

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