

Novel substituted 1,4-anthracenediones with antitumor activity directly induce permeability transition in isolated mitochondria

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Abstract. Synthetic analogs of 1,4-anthraquinone (AQ code number), which block nucleoside transport, decrease DNA, RNA and protein syntheses, trigger cytochrome *c* release without caspase activation, induce apoptotic DNA fragmentation and inhibit the proliferation of wild-type and multidrug resistant tumor cells in the nM range *in vitro*, rapidly cause the collapse of mitochondrial transmembrane potential in cell and cell-free systems. Because mitochondrial permeability transition (MPT) requires more than depolarization to occur, antitumor AQs were tested for their ability to directly trigger specific markers of MPT in isolated mitochondria. In contrast to a spectrum of conventional anticancer drugs that are inactive, various AQs interact with isolated mitochondria in a concentration- and time-dependent manner to rapidly cause large amplitude swelling and Ca²⁺ release in relation with their effectiveness against L1210, HL-60 and LL/2 tumor cells *in vitro*. Indeed, the lead antitumor AQ8, AQ9 and AQ17 are also the most effective inducers of MPT in isolated mitochondria, whereas all AQ derivatives devoid of anti-proliferative activity also fail to trigger mitochondrial swelling and Ca²⁺ release. Moreover, the ability of 4 μM AQ17 to maximally induce mitochondrial swelling and Ca²⁺ release within 15 min is similar to that of classic MPT-inducing agents, such as 5 μg/ml alamethicin, 200 μM atractyloside, 5 μM phenylarsine oxide, 100 μM arsenic trioxide and a 100 μM Ca²⁺ overload. Interestingly, AQ17 requires a priming concentration of 20 μM Ca²⁺ to trigger mitochondrial swelling and Ca²⁺ release and these 0.1 μM ruthenium red-sensitive MPT events are abolished by 1 μM cyclosporin A, 2 mM ADP and 20 μM bongkreikic acid, which block components

of the permeability transition pore (PTP), and also inhibited by 50-100 μM of various ubiquinones, which interact with the quinone binding site of the PTP and raise the Ca²⁺ load required for PTP opening. Hence, antitumor AQs that target isolated mitochondria and trigger MPT might directly interact with components of the PTP to induce conformational changes that increase its Ca²⁺ sensitivity and transition from the closed to the open state.

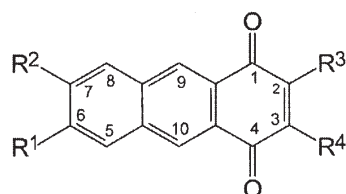
Introduction

In contrast to their inactive precursor quinizarin (AQ2, 1,4-dihydroxy-9,10-anthraquinone), synthetic analogs (AQ code number) of 1,4-anthraquinone (AQ1, anthracene-1,4-dione) inhibit L1210 and wild-type, drug-sensitive, HL-60 tumor cell proliferation within 2-4 days, and DNA, RNA and protein syntheses within 2-3 h (1-4). They reduce the mitotic index within 24 h, suggesting that they arrest early stages of cell cycle progression. New RNA and protein syntheses, and the activation of endonucleases are required to sustain the active mechanism by which low concentrations of AQs induce as much apoptotic DNA fragmentation at 24 h as 20(S)-camptothecin and daunorubicin (DAU, daunomycin) (1,2). One advantage over DAU is that AQs totally block the cellular uptake of both purine and pyrimidine nucleosides within 15 min (1,2), an antitumor effect that might potentiate the action of antimetabolites. Because these antitumor effects persist upon drug removal, AQs may rapidly and irreversibly interact with various molecular targets in cell membranes and nuclei. Moreover, AQs retain their efficacy in multidrug-resistant (MDR) HL-60-RV and HL-60-R8 sublines that have different mechanisms of resistance to DAU. Taken together, these findings suggest that these novel synthetic AQ1 analogs might have a wider spectrum of molecular targets than DAU, and might be valuable in polychemotherapy to circumvent mechanisms of MDR (2).

Based on their ability to decrease by 50% the metabolic activity of L1210 and HL-60 cells at day 4 (IC₅₀ values) in the nM range, using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS):phenazine methosulfate (PMS) (2:0.1) assay reagent to assess mitochondrial metabolism, 6-methyl-1,4-anthracenedione (AQ8), 6-bromomethyl-1,4-anthracenedione (AQ9)

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AQ8, AQ9, AQ14-AQ17

Compounds					IC ₅₀ values (nM) ^a		
Code	Residues				L1210	HL-60	LL/2
No.	R ¹	R ²	R ³	R ⁴	cells	cells	cells
AQ8	CH ₃	H	H	H	29 ± 1	87 ± 4	760 ± 52
AQ9	CH ₂ Br	H	H	H	26 ± 1	79 ± 3	680 ± 87
AQ14	CH ₂ -(1,3,4,6-tetra-O-acetyl-β-D-glucosamino)	H	H	H	196 ± 18	254 ± 23	813 ± 74
AQ15	A hydrogen chloride salt of AQ14	H	H	H	---	1,980 ± 76	---
AQ16	CH ₂ -[(-)-quinic acid ester]	H	H	H	524 ± 34	919 ± 78	---
AQ17	[di(t-butylxy)phosphonyloxy]methyl	H	H	H	23 ± 2 ^b	133 ± 9 ^c	654 ± 48 ^b

Figure 1. Concentrations of AQs required to inhibit by 50% (IC₅₀ values) the metabolic activity of L1210, HL-60 and LL/2 tumor cells, using the MTS:PMS assay at day 4 *in vitro*. ^aMeans ± SD (n=3); ^bnot different from AQ9; ^cP<0.005, greater than AQ9.

and 6-hydroxymethyl-1,4-anthracenedione (AQ10), which have more antiproliferative activity than AQ1, are our current lead antitumor compounds (3,4). Since AQ2 with its internal quinoid B-ring is devoid of antitumor activity, it is reasonable to assume that the external para-quinone is responsible for the potent antitumor activity of the AQ1 framework. Structure-activity relationships indicate that the bioactivity of AQ1 analogs is hindered by the presence of a substituent on their external quinone A-ring, whereas the functionalization of the C6-methyl group on the C-ring of AQ8 may enhance the antitumor activity (5). Compounds AQ1 and AQ8-10 also inhibit irreversibly the proliferation of Lewis lung carcinoma (LL/2) cells *in vitro*, suggesting that the classic LL/2 tumor model would be appropriate to assess the effectiveness of these drugs against solid primary tumor growth and metastasis in mice *in vivo* (5). The potent antitumor effects of AQ8-10 *in vitro* are all the more remarkable that, among synthetic or naturally occurring quinones, the number of bioactive 1,2-, 1,4- and 9,10-AQs appears quite limited, very few elicit antitumor effects *in vivo* and, with the exception of mitoxantrone, none of them seems to match the potency of our lead AQs *in vitro* (1-5). The antiproliferative and cytotoxic activities of AQ9 (NSC 727286) have been confirmed in all 60 human tumor cell lines of the NCI's *in vitro* antitumor screen. Although this drug is somewhat more potent against ascitic than solid tumors, the selectivity pattern indicates that a wide spectrum of human tumor cells are sensitive to AQ9 inhibition (5). As an attempt to improve their antitumor activity, novel C-6 substituted AQ structures have been synthesized and tested in the present study.

Compounds AQ8-10 trigger several early and late markers of apoptosis in wild-type and MDR HL-60 cells, including cytochrome *c* (Cyt *c*) release, caspase-2, -8, -9 and -3 activation and poly(ADP-ribose) polymerase-1 cleavage within 6 h and internucleosomal DNA fragmentation within 24 h (3). AQ8 and

AQ9 induce a caspase-independent release of mitochondrial Cyt *c* and a caspase-2-mediated activation of initiator caspase-8 and -9 and effector caspase-3 in HL-60 cells by a mechanism which does not involve Fas signaling since it is insensitive to antagonistic anti-Fas and anti-FasL monoclonal antibodies (3). Also the caspase-8 inhibitor benzyloxycarbonyl (z)-Ile-Glu-Thr-Asp (IETD)-fluoromethyl ketone (fmk) does not prevent AQ8-induced Cyt *c* release and caspase-2, -9 and -3 activation, but the caspase-2 inhibitor z-Val-Asp-Val-Ala-Asp (VDVAD)-fmk totally blocks AQ8-induced caspase-9, -3 and -8 activities without altering AQ8-induced Cyt *c* release. Antitumor AQ treatments, therefore, can fully release mitochondrial Cyt *c* without caspase activation but AQ-induced caspase-2 activity may be an apical event required for the activation of a downstream cascade of other initiator and effector caspases (3). Fluorescent probes that are specific of mitochondrial transmembrane potential ($\Delta\Psi_m$) were used to demonstrate that antitumor AQ analogs, which can trigger Cyt *c* release without caspase activation and retain their ability to induce apoptosis in DAU-resistant HL-60 cells, might have the advantage of directly targeting mitochondria in cell and cell-free systems to cause the collapse of $\Delta\Psi_m$ ($\downarrow\Delta\Psi_m$) that is linked to permeability transition pore (PTP) opening (6).

Using JC-1 dye, the abilities of various AQs to induce the $\downarrow\Delta\Psi_m$ in wild-type and MDR HL-60 cells are rapid, within 2.5-10 min, irreversible after drug removal, concentration-dependent in the 0.256-10 μM range, and related to their antitumor activities *in vitro* but not prevented by caspase-2 or -8 inhibitor pretreatments, suggesting that activations of these apical caspases upstream of mitochondria are not involved in this process (6). Antitumor AQs also mimic the abilities of the known depolarizing agents, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), alamethicin, gramicidin A and 100 μM CaCl₂, to directly induce, within 15 min, the $\downarrow\Delta\Psi_m$ in isolated mitochondria prepared from mouse liver

Table I. Comparison of the direct MPT-inducing activities of various antiproliferative AQs in isolated mitochondria.

Treatment ^a	Markers of MPT	
	Mitochondrial swelling Absorbance at 540 nm ^b	Mitochondrial Ca ²⁺ release Fluo-5N fluorescence intensity (a.u.) ^b
Control	0.346±0.017	36.6±2.3
100 μM Ca ²⁺	0.202±0.007	605.4±35.1
20 μM Ca ²⁺	0.334±0.021 ^c	41.3±3.3 ^c
+ AQ1	0.230±0.009	137.3±8.8
+ AQ2	0.324±0.013 ^d	-
+ AQ3	0.328±0.012 ^d	-
+ AQ4	0.242±0.016	117.1±6.9
+ AQ5	0.330±0.017 ^d	-
+ AQ6	0.326±0.014 ^d	-
+ AQ7	0.331±0.015 ^d	-
+ AQ8	0.221±0.008	150.1±8.1
+ AQ9	0.212±0.008	152.6±11.0
+ AQ10	0.224±0.007	144.9±10.1
+ AQ11	0.265±0.016 ^e	87.1±7.6 ^f
+ AQ12	0.232±0.012	113.5±7.3
+ AQ13	0.326±0.012 ^d	-
+ AQ14	0.242±0.014	111.6±8.9
+ AQ16	0.255±0.013	93.3±6.4
+ AQ17	0.194±0.008 ^g	176.5±9.9 ^g

^aIsolated mitochondria were incubated for 15 min with 4-μM concentrations of AQs in the presence of a 20 μM Ca²⁺ primer; ^bmeans ± SD (n=3); ^cnot different from control; ^dnot different from 20 μM Ca²⁺; ^eP<0.025 and ^fP<0.005, respectively smaller and greater than 20 μM Ca²⁺; ^gP<0.05, smaller or greater than respective AQ9 values. a.u., arbitrary units.

and loaded with rhodamine 123 (Rh 123) dye (6). The fact that 20 μM Ca²⁺, which is insufficient to trigger depolarization on its own, is required to prime the depolarizing effect of AQ9 in isolated mitochondria suggests that antitumor AQs might interact with the PTP to alter its conformation and increase its Ca²⁺ sensitivity. Indeed, such Ca²⁺-dependent ↓ΔΨ_m in isolated mitochondria treated with 1.6 μM AQ9 or 100 μM Ca²⁺ is blocked by ruthenium red (RR), which prevents the Ca²⁺ uniporter from accumulating the exogenously added divalent cation into the mitochondrial matrix (6). DAU is unable to mimic the rapid ↓ΔΨ_m caused by antitumor AQs within 2.5-40 min of treatment in HL-60 cells or isolated mitochondria. Moreover, the ↓ΔΨ_m caused by 1.6 μM AQ9 or 100 μM Ca²⁺ in isolated mitochondria is similarly blocked by cyclosporin A (CsA), bongkreic acid (BA) and decyl-ubiquinone (d-Ub), which prevent PTP opening, suggesting that, in contrast to DAU, antitumor AQs that directly target mitochondria to trigger the Ca²⁺-dependent and CsA-sensitive ↓ΔΨ_m might induce PTP opening and the mitochondrial pathway of apoptosis even in the absence of nuclear signals (6). However, depolarization alone is insufficient to support the hypothesis that AQs might represent a new class of apoptosis-targeted drugs that directly interact with components

of the PTP to induce mitochondrial permeability transition (MPT). Critical MPT events result from the sudden and non-specific permeabilization of the inner membrane (IM) to ions and low molecular weight (MW) solutes and the hyperosmolarity of the matrix, including the entry of water from the cytosol, the large amplitude swelling, the rupture of the outer membrane (OM), and the release of sequestered Ca²⁺. Therefore, as MPT requires more than depolarization to occur, antitumor AQs were tested for their ability to interact with isolated organelles and directly trigger specific markers of MPT, such as mitochondrial swelling and intramitochondrial Ca²⁺ release.

Materials and methods

Drug treatments. The methods to synthesize in relatively few steps and high yield the structures of the AQ1 analogs illustrated in Fig. 1 and listed in Table I have already been reported (4,5). These novel antitumor AQs were synthesized and selective functionalization at the C ring was accomplished by a benzylic bromination reaction. The resulting bromo-derivatives were converted into water-soluble and phosphate ester drugs (4,5). All solutions of AQ2 (Aldrich, Milwaukee, WI), synthetic AQ1 analogs, atractyloside, phenylarsine oxide, d-Ub (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone), 2,3-dimethoxy-5-methyl-p-benzoquinone (Ub₀, ubiquinone-0 or coenzyme Q₀), 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (Ub₅, ubiquinone-5 or coenzyme Q₁), 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Ub₁₀, ubiquinone-10 or coenzyme Q₂) (all from Sigma, St. Louis, MO) and CsA (Calbiochem, La Jolla, CA) were dissolved and diluted in dimethyl sulfoxide (DMSO). Fluo-5N, pentapotassium salt (Molecular Probes, Eugene, OR), ADP (Sigma), and RR (Fluka, Buchs SG, Switzerland) solutions were prepared in double-distilled water (DDW). Alamethicin (Sigma) was formulated in 50% EtOH, arsenic(III) oxide (arsenic trioxide; Sigma) was solubilized in 1 N NaOH before dilution in DDW, and BA (Sigma) was provided in 10 mM Tris-HCl buffer, pH 7.5. All reference anticancer drugs were purchased from Sigma. DAU, doxorubicin (DOX, adriamycin), staurosporine, mitoxantrone, vincristine, vinblastine, etoposide (VP-16), taxol (paclitaxel), nocodazole, tubulazole C, 5-fluorouracil, cis-diammineplatinum (II) dichloride (cisplatin) and camptothecin were all dissolved in DMSO, whereas cytosine β-D-arabinofuranoside (Ara-C) and cyclophosphamide were prepared in DDW and amethopterin (methotrexate; Sigma) was solubilized in 0.1 N NaOH before dilution in DDW. The concentrations of these vehicles in the final incubation volumes never exceeded 0.2% and did not interfere with the data.

Cell cultures and proliferation assays. Suspension cultures of mouse L1210 lymphocytic leukemia, human HL-60 promyelocytic leukemia and mouse LL/2 Lewis lung carcinoma cells (all from ATCC, Manassas, VA) were maintained in continuous exponential growth by twice-a-week passage in RPMI-1640 medium supplemented with 10% fetal bovine calf serum (Atlanta Biologicals, Norcross, GA) and penicillin (100 IU/ml)-streptomycin (100 μg/ml), and incubated in the presence or absence (control) of drugs at 37°C in a humidified

atmosphere containing 5% CO₂ (1-3,6). The proliferation of drug-treated L1210 (initial density 4.5x10³/0.5 ml), HL-60 (initial density 3.75x10⁴/0.5 ml) and LL/2 (initial density 1.75x10³/0.5 ml) tumor cells was assessed from their mitochondrial ability to bioreduce the MTS reagent (Promega, Madison, WI) in the presence of PMS (Sigma) into a water-soluble formazan product that absorbs at 490 nm. After 4 days at 37°C in 48-well Costar cell culture plates, control and drug-treated cell samples (~10⁶/0.5 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 metabolic activity was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL). Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were subtracted from the results (1-3,6).

Isolation of mitochondria. To collect enough organelles to study AQ-induced mitochondrial swelling and Ca²⁺ release, they were isolated by differential centrifugation at 4°C from one female CF-1 (Charles River, Wilmington, MA) mouse liver after an overnight period of fasting to deplete its levels of glycogen and fatty acids (6,7-10). The liver was rinsed, minced with scissors in 10 ml of 10 mM HEPES buffer, pH 7.2, containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 0.1% BSA, and homogenized in 40 ml of this mitochondrial extraction buffer with two slow up/down strokes of a motorized tight-fitting Teflon pestle rotating at ~500 r.p.m. in a glass Potter-Elvehjem tissue grinder. Disrupted cells were centrifuged (1,000 g x 10 min) in a 50-ml conical polypropylene tube to precipitate unlysed cells, nuclei and large membrane fragments. The supernatant was decanted and then recentrifuged (10,000 g x 10 min) in 15-ml Corex borosilicate glass tubes to collect the mitochondrial pellets, which were washed, pooled and resuspended at a final concentration of 50 mg protein/ml of mitochondrial storage buffer, containing 10 mM HEPES, pH 7.2, 225 mM mannitol and 75 mM sucrose (6,7-10). The protein concentrations of the mitochondrial samples were determined using the BCA protein assay kit (Pierce, Rockford, IL).

Mitochondrial swelling. Conventional rod-shaped mitochondria are packed with small and dense cristae, whereas large and swollen mitochondria have diluted matrices with cristae in various stages of disintegration. Matrix configuration is determined by light scattering: the more condensed matrices diffract more light (11-13). Since severely swollen mitochondria with clear matrices and disrupted cristae scatter and absorb much less light than the dense and granular light-absorbing structure of their normal compact counterparts, a decrease in light scattering reflects the degree of mitochondrial swelling. Hence, the large amplitude swelling of mitochondria undergoing AQ-induced MPT was determined spectrophotometrically by monitoring the decrease in apparent absorbance (light-scattering) at 540 nm (9,12,13). To assay mitochondrial swelling, 24-well Costar clear transparent polystyrene cell culture plates were sequentially supplemented with 1 ml of mitochondrial reaction buffer (MRB), containing 10 mM HEPES, pH 7.2, 100 mM sucrose, 65 mM KCl, 5 mM

glutamate, 2.5 mM malate, 1 mM KH₂PO₄ and 20 μM EGTA, and 30 μl of isolated mitochondria (1.5 mg of protein). After incubation of these control or AQ-treated reaction mixtures for 5 min at room temperature, the decrease of absorbance (light scattering) linked to large amplitude mitochondrial swelling was monitored for 2.5-30 min at 540 nm, using a Cambridge model 750 automatic microplate reader.

Mitochondrial Ca²⁺ release. The ability of AQs to directly induce isolated mitochondria to release Ca²⁺ was determined using the pentapotassium salt of Fluo-5N, a cell-impermeant Ca²⁺ indicator, which is not fluorescent in the absence of divalent cation but exhibits strong fluorescence upon binding to extramitochondrial Ca²⁺. Hence, the development of green Fluo-5N fluorescence was linked to the increase of free extramitochondrial Ca²⁺ in the reaction medium, resulting from the release of sequestered Ca²⁺ by mitochondria undergoing AQ- or 100 μM Ca²⁺ overload-induced MPT. In controls, the baseline of green Fluo-5N fluorescence is low since the high ΔΨ_m prevents the release of mitochondrial Ca²⁺. Likewise, when 100 μM Ca²⁺ overload is used to trigger MPT, the RR-sensitive Ca²⁺ uniporter accumulates the cation so rapidly into the matrix that Fluo-5N fluorescence returns to baseline before measurements can be made in the microplate reader, but any subsequent increase of Fluo-5N fluorescence demonstrates the release of intramitochondrial Ca²⁺ caused by AQ- or 100 μM Ca²⁺ overload-induced MPT (9,14). Mitochondrial Ca²⁺ released was assayed in 1.5-ml Eppendorf tubes, which were sequentially supplemented with 1 ml of MRB, 1 μl of dye to obtain a final concentration of 1 μM Fluo-5N, and 20 μl of isolated mitochondria (1 mg of protein). After incubation of these control or AQ-treated reaction mixtures for 5 min at room temperature, 0.2-ml aliquots were transferred to a 96-well Costar white opaque polystyrene assay plate and the increased fluorescence of Fluo-5N, which indicates that the membrane impermeable dye is bound to Ca²⁺ released from mitochondria undergoing MPT, was monitored for 2.5-30 min at 495 nm excitation/518 nm emission, using a Cary Eclipse Fluorescence Spectrophotometer equipped with microplate reader accessory (Varian, Walnut Creek, CA) (9,14).

Results

Inhibition of tumor cell proliferation. The antiproliferative activities of 4 new C6-substituted AQ analogs were compared to those of the current lead antitumor compounds AQ8 and AQ9 (Fig. 1). The nomenclatures of the newly synthesized compounds are: AQ14, 6-glucosaminomethyl-1,4-anthracenedione; AQ15, a hydrogen chloride salt of AQ14; AQ16, 6-quinoylmethyl-1,4-anthracenedione; and AQ17, di-*t*-butyl-[(5,8-dioxo-2-anthracenyl)methyl]phosphate. AQ17, which can be used to prepare water-soluble phosphate ester prodrugs, is nearly equipotent to AQ8 and AQ9 against L1210, HL-60 and LL/2 tumor cell proliferation (Fig. 1). AQ14 and, especially, AQ16 are clearly less effective. Hydrophilic AQ15, which is the least effective against HL-60 cell proliferation, was not further tested in the other tumor cell lines. Because of their potent antitumor effects *in vitro*, AQ8, AQ9 and AQ17 were selected to study the markers of MPT in isolated mitochondria.

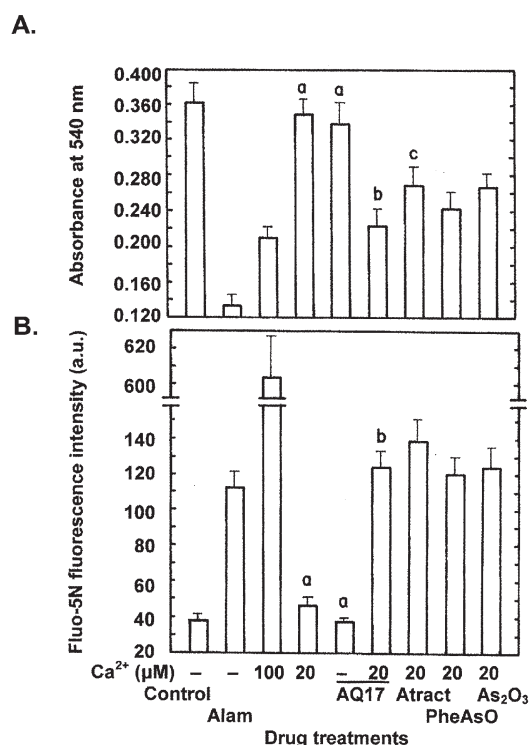


Figure 2. Inducers of MPT in a cell-free system. A, comparison of the abilities of 5 $\mu\text{g}/\text{ml}$ alamethicin (Alam), 20 or 100 μM CaCl_2 , and 4 μM AQ17, 200 μM atractyloside (Atract), 5 μM phenylarsine oxide (PheAsO) or 100 μM arsenic trioxide (As_2O_3), in the presence of a 20 μM Ca^{2+} primer, to directly induce the large amplitude swelling of isolated mitochondria. The decrease in absorbance (light scattering) at 540 nm occurs after 15 min in severely swollen organelles with diluted matrices and disrupted cristae. Control: absorbance at 540 nm of non-treated suspensions of isolated mitochondria (0.362 ± 0.022). Bars: means \pm SD ($n=3$). ^aNot different from control; ^bnot different from 100 μM Ca^{2+} or PheAsO but $P < 0.05$, smaller than Atract or As_2O_3 ; ^c $P < 0.01$, smaller than 20 μM Ca^{2+} . B, comparison of the abilities of the above treatments to directly induce the release of Ca^{2+} sequestered in isolated mitochondria. The development of green Fluo-5N fluorescence (arbitrary units: a.u.) occurs when this membrane impermeable dye, which is not fluorescent in the absence of divalent cation, becomes bound to the extramitochondrial Ca^{2+} released from these drug-treated organelles at 15 min. Control: baseline intensity of Fluo-5N fluorescence in non-treated suspensions of isolated mitochondria (38.2 ± 3.4). ^aNot different from control; ^b $P < 0.0005$, greater than 20 μM Ca^{2+} but not different from Alam, Atract, PheAsO or As_2O_3 .

Induction of MPT. Freshly isolated mitochondria were used to determine whether antitumor AQs would directly target these organelles to induce the large amplitude swelling and release of sequestered Ca^{2+} that are indicative of MPT. The transmembrane channel-forming peptide alamethicin, which functions as an uncoupler of mitochondrial oxidative phosphorylation by dissipating electrochemical gradients across the IM, is used as a positive control to demonstrate the rapid induction of mitochondrial swelling and Ca^{2+} release within 15 min (Fig. 2). Since the open-closed transition of the PTP is Ca^{2+} sensitive and mitochondrial Ca^{2+} overload triggers PTP opening, IM permeabilization, depolarization, swelling and apoptosis, raising the exogenous concentration of Ca^{2+} from a low of 20 μM , which has no effect on its own, to a high of 100 μM immediately induces isolated mitochondria to swell and release their sequestered Ca^{2+} (Fig. 2). Interestingly, 4 μM AQ17, which is inactive alone, requires the presence of a

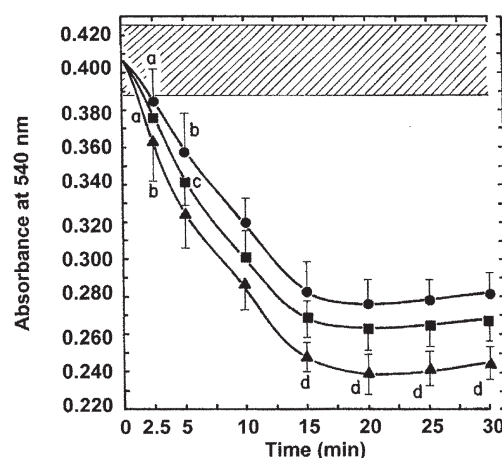


Figure 3. Comparison of the time-dependent inductions of the large amplitude swelling of isolated mitochondria by 4 μM concentrations of AQ8 (●), AQ9 (■) or AQ17 (▲), in the presence of a 20 μM Ca^{2+} primer. Control: average absorbance at 540 nm of non-treated suspensions of isolated mitochondria at all time points studied (0.407 ± 0.019 ; striped area). Alone, 20 μM Ca^{2+} is unable to induce mitochondrial swelling over 30 min (0.390 ± 0.015 , not different from control), whereas a 100 μM Ca^{2+} overload does within 5-10 min and is used as a reference MPT-inducing treatment (0.237 ± 0.009). Bars: means \pm SD ($n=3$). ^aNot different from control; ^b $P < 0.05$ and ^c $P < 0.025$, smaller than control; ^d $P < 0.05$, smaller than AQ9.

20 μM Ca^{2+} primer in order to induce the same mitochondrial swelling and Ca^{2+} release at 15 min as those caused by 100 μM Ca^{2+} overload or other known MPT inducers, including 200 μM atractyloside, 5 μM phenylarsine oxide and 100 μM arsenic trioxide (Fig. 2). Antitumor AQs, therefore, might interact with the PTP to induce conformational changes that increase its sensitivity to the priming concentration of 20 μM Ca^{2+} and trigger its transition to the open state.

Characterization of AQ-induced MPT. The ability of AQ8, AQ9 and AQ17 to induce Ca^{2+} -dependent MPT in isolated mitochondria is a function of time (Fig. 3) and concentration (Fig. 4). At a concentration of 4 μM , AQ8, AQ9 and AQ17 need about 15 min to fully induce mitochondrial swelling (Fig. 3). The Ca^{2+} -dependent mitochondrial swelling induced by AQ17 is slightly greater than those caused by AQ8 and AQ9, starting earlier at 2.5 min and reaching a larger amplitude at 15 min (Fig. 3). When compared at 15 min, the Ca^{2+} -dependent inductions of mitochondrial swelling and Ca^{2+} release by AQ8, AQ9 and AQ17 are similarly concentration dependent, starting at ~256-640 nM and peaking at 4 μM before declining thereafter (Fig. 4). Again, AQ17 appears to be a slightly more potent MPT inducer than AQ8 and/or AQ9 (Fig. 4).

Relevance of AQ-induced MPT. In general, synthetic AQ analogs seem to induce markers of MPT in isolated mitochondria in relation with their antiproliferative and proapoptotic effects in tumor cells *in vitro* (1-6). AQ2 and 6,7-dichloro-1,4-dihydroxy-9,10-anthracenedione (AQ3), which have an internal quinoid ring, and 6,7-dichloro-2-(methylamino)-1,4-anthracenedione hydrochloride (AQ5), 6,7-dichloro-2-(methylamino)-1,4-anthracenedione (AQ6) and 2-(methylamino)-1,4-anthracenedione (AQ7), which

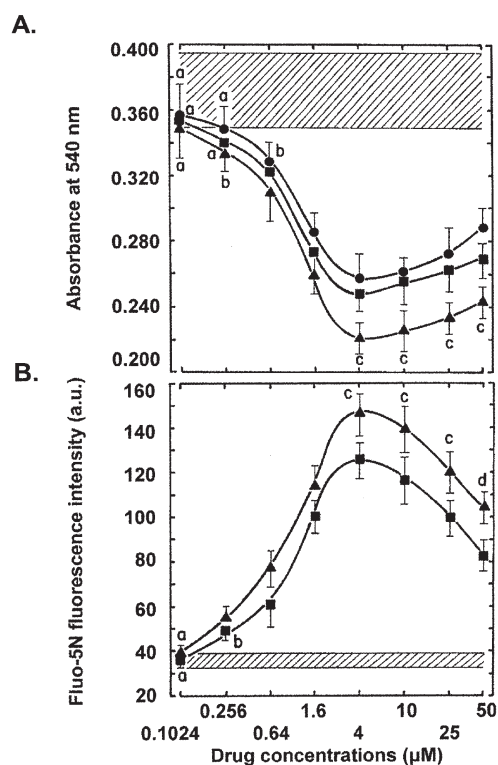


Figure 4. Concentration-dependent induction of MPT by AQs in a cell-free system. A, comparison of the abilities of serial concentrations (plotted on a logarithmic scale) of AQ8 (●), AQ9 (■) or AQ17 (▲), to directly induce, in the presence of a 20 μM Ca^{2+} primer, the large amplitude swelling of isolated mitochondria after 15 min. Control: absorbance at 540 nm of non-treated suspensions of isolated mitochondria (0.372 ± 0.023 , striped area). Alone, 20 μM Ca^{2+} is unable to induce mitochondrial swelling (0.357 ± 0.021 , not different from control), whereas a 100 μM Ca^{2+} overload does and is used as a reference MPT-inducing treatment (0.232 ± 0.009). Bars: means \pm SD ($n=3$). ^aNot different from control; ^b $P < 0.05$, smaller than control; ^c $P < 0.05$, smaller than AQ9. B, comparison of the abilities of serial concentrations of AQ9 (■) or AQ17 (▲), to directly induce, in the presence of a 20 μM Ca^{2+} primer, the release of Ca^{2+} from isolated mitochondria after 15 min. Control: baseline intensity of Fluo-5N fluorescence in non-treated suspensions of isolated mitochondria (35.9 ± 2.9 , striped area). Alone, 20 μM Ca^{2+} does not trigger mitochondrial Ca^{2+} release (43.1 ± 3.8 , not different from control), whereas a 100 μM Ca^{2+} overload does and is used as a reference MPT-inducing treatment (581.0 ± 32.5). ^aNot different from control; ^b $P < 0.025$, greater than control; ^c $P < 0.05$ and ^d $P < 0.025$, greater than AQ9.

have a substituent on their external quinone ring, and 5,8-dioxo-5,8-dihydro-2-anthracenecarboxylic acid (AQ13), which has a carboxylic acid function, have no antitumor effects and all fail to induce mitochondrial swelling in presence of a 20 μM Ca^{2+} primer (Table I). In contrast, 4 μM concentrations of the potent antitumor compounds AQ1, AQ8, AQ9, AQ10 and AQ17 all mimic the MPT-inducing effect of a 100 μM Ca^{2+} overload in isolated mitochondria and induce the most Ca^{2+} -dependent mitochondrial swelling and Ca^{2+} release at 15 min (Table I). Other compounds with weak or moderate antitumor activities, such as 6,7-dichloro-1,4-anthracenedione (AQ4), 6-formyl-1,4-anthracenedione (AQ11), a mixture of 2-bromo-6-methyl- and 2-bromo-7-methyl-1,4-anthracenedione regioisomers (AQ12), AQ14 and AQ16, only partially induce Ca^{2+} -dependent swelling and intramitochondrial Ca^{2+} release in a population of isolated mitochondria (Table I). Antitumor AQs may have a unique ability to directly interact with isolated mitochondria and trigger MPT since various other anticancer

Table II. Comparison of the direct MPT-inducing activities of known antiproliferative and anticancer drugs in isolated mitochondria.

Treatment ^a	Markers of MPT in isolated mitochondria	
	Swelling Absorbance at 540 nm ^b	Ca^{2+} release Fluo-5N fluorescence intensity (a.u.) ^b
Control	0.305 ± 0.015	36.8 ± 2.1
100 μM Ca^{2+}	0.185 ± 0.008	557.2 ± 31.7
20 μM Ca^{2+}	0.292 ± 0.016^c	41.1 ± 3.3^e
+ Daunorubicin	0.329 ± 0.022^d	29.1 ± 2.3^e
+ Doxorubicin	0.321 ± 0.019^d	33.5 ± 2.3^f
+ Staurosporine	0.283 ± 0.012^d	42.2 ± 2.8^d
+ Mitoxantrone	0.313 ± 0.016^d	35.5 ± 2.5^d
+ Vincristine	0.290 ± 0.017^d	40.7 ± 2.2^d
+ Vinblastine	0.291 ± 0.011^d	41.1 ± 2.4^d
+ Etoposide (VP-16)	0.288 ± 0.015^d	46.7 ± 3.4^d
+ Taxol	0.291 ± 0.017^d	41.5 ± 2.7^d
+ Nocodazole	0.287 ± 0.011^d	40.4 ± 2.3^d
+ Tubulazole C	0.303 ± 0.011^d	60.7 ± 5.3^g
+ Methotrexate	0.290 ± 0.013^d	39.3 ± 2.6^d
+ Ara-C	0.289 ± 0.012^d	41.8 ± 3.0^d
+ 5-Fluorouracil	0.286 ± 0.015^d	40.4 ± 2.5^d
+ Cyclophosphamide	0.304 ± 0.011^d	39.6 ± 2.2^d
+ Cisplatin	0.302 ± 0.012^d	40.1 ± 2.2^d
+ Camptothecin	0.288 ± 0.014^d	40.8 ± 2.4^d

^aIsolated mitochondria were incubated for 15 min with 4- μM concentrations of drugs in the presence of a 20 μM Ca^{2+} primer; ^bmeans \pm SD ($n=3$); ^cnot different from control; ^dnot different from 20 μM Ca^{2+} ; ^e $P < 0.01$ and ^f $P < 0.05$, smaller than 20 μM Ca^{2+} ; ^g $P < 0.01$, greater than 20 μM Ca^{2+} .

drugs tested at 4 μM under similar conditions all fail to induce Ca^{2+} -dependent mitochondrial swelling and Ca^{2+} release at 15 min (Table II). This wide spectrum of known DNA-alkylating and -damaging agents, DNA topoisomerase inhibitors, microtubule-interacting compounds and anti-metabolites tested over the 0.256-25 μM range is also unable to trigger markers of MPT in isolated mitochondria within 1 h (data not shown).

Inhibition of AQ-induced MPT. The hypothesis that antitumor AQs might directly interact with components of the PTP to increase its Ca^{2+} sensitivity and trigger its opening is substantiated by the fact that a low and normally ineffective 20 μM Ca^{2+} concentration of exogenous Ca^{2+} now becomes sufficient to prime AQ-induced mitochondrial swelling and Ca^{2+} release (Fig. 5). As expected, this Ca^{2+} -dependent induction of mitochondrial swelling and Ca^{2+} release by 4 μM AQ17 is abolished by 0.1 μM RR, which blocks the Ca^{2+} uniporter (Fig. 5). The ability of 4 μM AQ17 to directly trigger mitochondrial swelling and Ca^{2+} release in the presence of a 20 μM Ca^{2+} primer is also abolished or inhibited by several MPT blockers known to interact with various components of the PTP complex to prevent its opening. These inhibitors

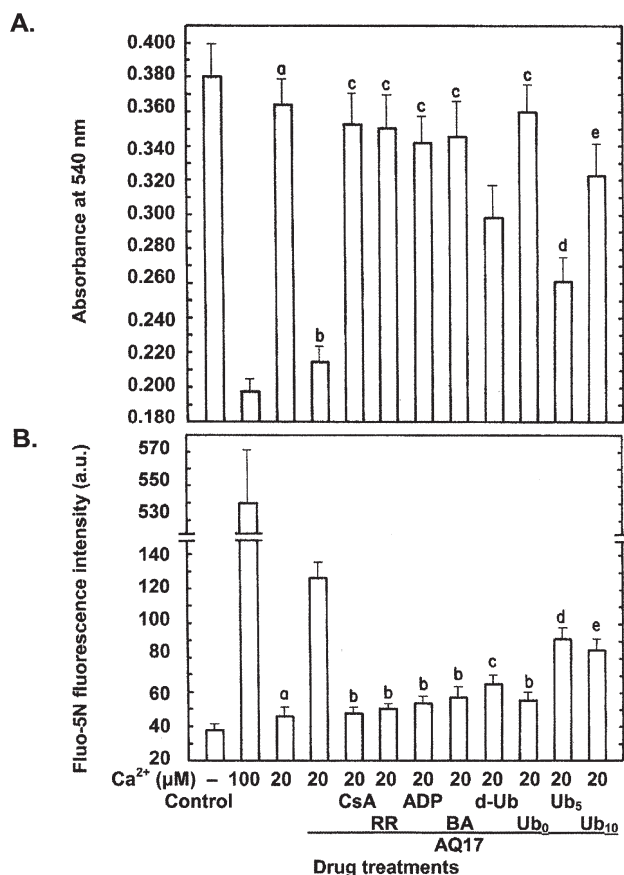


Figure 5. Inhibition of AQ-induced MPT in a cell-free system. A, comparison of the abilities of 1 μM CsA, 0.1 μM RR, 2 mM ADP, 20 μM BA, 100 μM d-Ub, 50 μM Ub₀, 100 μM Ub₅ or 100 μM Ub₁₀ pretreatments to inhibit the large amplitude swelling of isolated mitochondria directly induced after 15 min by 4 μM AQ17 in the presence of a 20 μM Ca²⁺ primer. Control: absorbance at 540 nm of non-treated suspensions of isolated mitochondria (0.380 \pm 0.019). Alone, 20 μM Ca²⁺ is unable to induce mitochondrial swelling, whereas a 100 μM Ca²⁺ overload does and is used as a reference MPT-inducing treatment. Bars: means \pm SD (n=3). ^aNot different from control; ^bnot different from 100 μM Ca²⁺; ^cnot different from 20 μM Ca²⁺; ^dP<0.01, greater than 20 μM Ca²⁺ + 4 μM AQ17; ^eP<0.05, smaller than 20 μM Ca²⁺. B, comparison of the abilities of the above pretreatments to inhibit the release of Ca²⁺ from isolated mitochondria directly induced after 15 min by 4 μM AQ17 in the presence of a 20 μM Ca²⁺ primer. Control: baseline intensity of Fluo-5N fluorescence in non-treated suspensions of isolated mitochondria (37.9 \pm 3.1). Alone, 20 μM Ca²⁺ does not trigger mitochondrial Ca²⁺ release, whereas a 100 μM Ca²⁺ overload does and is used as a reference MPT-inducing treatment. ^aNot different from control; ^bnot different from 20 μM Ca²⁺; ^cP<0.01, greater than 20 μM Ca²⁺; ^dP<0.01 and ^eP<0.005, smaller than 20 μM Ca²⁺ + 4 μM AQ17.

of AQ-induced MPT include 1 μM CsA, which binds to cyclophilin D, 2 mM ADP and 20 μM BA, the natural and synthetic ligands that stabilize the adenine nucleotide translocator (ANT) in an inactive conformation, and 100 μM d-Ub, 50 μM Ub₀, 100 μM Ub₅ and 100 μM Ub₁₀, which interact with the ubiquinone (Ub)/quinone-binding site of the PTP to induce conformational changes that decrease its Ca²⁺ sensitivity (Fig. 5).

Discussion

The PTP functions as a CsA-sensitive and Ca²⁺-, voltage-, pH- and redox-gated channel with several levels of conductance

(15). The intrinsically low permeability of the IM to ions and solutes allows energy conservation in the form of a proton-driven $\Delta\Psi\text{m}$. The primary event in MPT is a sudden and nonspecific permeabilization of the IM to K⁺, Mg²⁺ and Ca²⁺, as well as to solutes of MW <1,500 Da (15,16). Once PTP is open, the electrochemical H⁺ gradient essential for oxidative phosphorylation is dissipated, the $\downarrow\Delta\Psi\text{m}$ and uncoupling of respiration deplete ATP levels, and the hyperosmolarity of the matrix induces H₂O to enter from the cytosol and cause rapid mitochondrial swelling, which may rupture the OM and trigger the release of intramitochondrial Ca²⁺ and proapoptotic proteins (17-18).

PTP opening may be regulated by the $\downarrow\Delta\Psi\text{m}$ and the pH of the matrix but the relationships between depolarization, MPT and apoptosis remain unclear. Even though antitumor AQs trigger mitochondrial depolarization in cells and cell-free systems (6), the $\downarrow\Delta\Psi\text{m}$ may be critical but not sufficient for, or a universal step in, apoptosis (19). MPT is always followed by $\downarrow\Delta\Psi\text{m}$ but $\downarrow\Delta\Psi\text{m}$ is not always followed by MPT (15). Direct $\downarrow\Delta\Psi\text{m}$ caused by CCCP does not induce MPT per se. As MPT requires more than $\downarrow\Delta\Psi\text{m}$ to occur (17), the present findings that antitumor AQs directly induce isolated mitochondria to undergo large amplitude swelling and release their sequestered Ca²⁺ demonstrate that these depolarizing agents are truly capable of rapidly triggering specific markers of MPT.

PTP opening may induce hyperosmolarity of the matrix, which expands. Swelling resulting from solute and H₂O flux from intermembrane space to matrix occurs when there is an osmotic imbalance, but not for very short periods of PTP opening or for lower conductance states of MPT. Low concentrations of pore-impermeant solutes may prevent MPT-dependent swelling *in vitro*. Whether swelling and OM rupture occur after AQ-induced MPT *in vivo* is hard to predict because of the high level of macromolecules in the cytosol (15). An RR-sensitive uniporter driven by the $\Delta\Psi\text{m}$ of the IM is responsible for Ca²⁺ uptake (15). Although it is also stored in the endoplasmic reticulum, Ca²⁺ is mostly sequestered in mitochondria and MPT-induced Ca²⁺ release is a signal of apoptosis (14,16). Ca²⁺ may be released from mitochondria under stress by drugs or Ca²⁺ overloads that open the CsA-sensitive PTP and cause IM permeabilization, $\downarrow\Delta\Psi\text{m}$ and swelling (16,20,21). The IM contains separate routes for Ca²⁺ uptake and release. MPT exhibits a prominent Ca²⁺ dependence. When Ca²⁺ concentration gradients exist between matrix and cytosol or external medium, onset of MPT serves as a mitochondrial Ca²⁺ release channel (15). The facts that all the MPT effects of AQs on isolated organelles require a 20 μM Ca²⁺ primer and an active Ca²⁺ uniporter suggest that these antitumor drugs are likely to lower the threshold of exogenous Ca²⁺ triggering mitochondrial PTP opening, $\downarrow\Delta\Psi\text{m}$, swelling and Ca²⁺ release. Presumably, the intracellular concentration of Ca²⁺ in HL-60 cells might be sufficient to mediate the rapid $\downarrow\Delta\Psi\text{m}$ caused by AQs in mitochondria *in situ* (6).

The ability of antitumor AQs to target mitochondria and rapidly induce the $\downarrow\Delta\Psi\text{m}$ has been demonstrated in both cultured HL-60 cells and suspensions of freshly isolated mitochondria and is not a fluorescence artifact (6). Indeed, when emission wavelengths are scanned over a 513-713 nm

range at the 503-nm excitation wavelength of the Rh 123 assay, antitumor AQs alone have no autofluorescence and do not displace the sharp peak of Rh 123 fluorescence at 520-530 nm either in the presence or absence of isolated mitochondria. But, obviously, these AQs increase the fluorescence intensity of the unquenched Rh 123 released solely in the presence of organelles undergoing PTP opening, thereby confirming the validity of our $\downarrow\Delta\Psi_m$ data and ruling out any non-physiological drug-dye interference in the cell-free system (6). Similarly, when emission wavelengths are scanned at the 495 excitation wavelength of the Fluo-5N assay, antitumor AQs do not emit their own fluorescence or interfere with the basal level of Fluo-5N fluorescence when mitochondria are incubated in the absence of a 20 μM Ca^{2+} primer. However, AQs clearly boost the peak of Fluo-5N fluorescence emitted at 518 nm when the membrane-impermeant dye binds the Ca^{2+} suddenly released from mitochondria undergoing Ca^{2+} primer-dependent PTP opening. Moreover, the most convincing evidence that antitumor AQs directly interact with isolated mitochondria to cause PTP opening and MPT is that, under the same experimental conditions where they trigger $\downarrow\Delta\Psi_m$ and Ca^{2+} release, they rapidly induce large amplitude swelling within 15 min in a simple spectrophotometric assay where no fluorescent dye is used and no fluorescence artifact is possible.

Since synthetic AQs directly interact with isolated mitochondria to rapidly induce MPT events involved in apoptosis, these new quinone antitumor drugs might have a wider spectrum of molecular targets than DAU, bypass upstream mechanisms of MDR and lead to the development of apoptosis-targeted therapies for cancer (22-28). Direct MPT might release proapoptotic molecules independently from the Bcl-2 alterations involved in MDR. Lonidamine triggers $\downarrow\Delta\Psi_m$, Cyt *c* release and apoptosis by a mechanism which directly targets mitochondria and bypasses MDR in DOX-resistant cells. Betulinic acid triggers apoptosis by a direct effect on mitochondria, which is caspase-independent, bypasses the requirement for upstream signaling and persists in tumor cells that have defective apoptotic pathways (7). In contrast to DAU, DOX, cisplatin and VP-16, which do not rapidly target isolated mitochondria (7,29), AQs irreversibly induce mitochondria to lose their $\Delta\Psi_m$ in a caspase-independent manner within 5-15 min in cell and cell-free systems, suggesting that, no matter what other cellular and nuclear interactions or signaling pathways they generate, AQs also have the potential to directly target this key organelle to initiate MPT and apoptosis (6). The reports that mitochondria isolated from cells previously treated for 12-48 h with μM concentrations of DOX or other DNA-damaging drugs are severely impaired are totally different and suggest that, in contrast to AQs, indirect and delayed damage to mitochondria *in situ* is the consequence rather than the cause of DOX-induced cytotoxicity (7,30,31). For instance, the $\downarrow\Delta\Psi_m$ is only observed 2-6 h after 75-100 μM echinocystic acid, 3-6 h after 25 μM parthenolide, 6-48 h after 50 μM cisplatin, 9 h after 10 μM of the carbazole compound LCY-2-CHO, 12 h after 80 μM myricetin, and 24 h after 2-5 μM taxol or 20 μM lovastatin (32-38). Moreover, 5 μM camptothecin and 100 μM *tert*-butyl hydroperoxide cause mitochondrial hyperpolarization at 1 h and need 2.5-5 h or 4-6 h, respectively, to induce the $\downarrow\Delta\Psi_m$ (39,40). Similarly, 500 μM VP-16 must be used to detect rapid MPT events in

isolated mitochondria, whereas lower concentrations of VP-16 require 24-48 h to promote cellular apoptosis (10). The fact that AQ-induced $\downarrow\Delta\Psi_m$, swelling and Ca^{2+} release in isolated mitochondria are blocked by CsA, ADP, BA and various Ubs suggests that antitumor AQs directly interact with components of the PTP complex to cause MPT. The direct action of AQs resembles that of hyperforin, which can induce a rapid and caspase-independent $\downarrow\Delta\Psi_m$ within 30 min, whereas the delayed $\downarrow\Delta\Psi_m$ observed 24-48 h after paclitaxel is merely the consequence of earlier cytotoxic drug interactions (41). Since the supernatant from drug-treated isolated mitochondria is sufficient to induce apoptotic chromatin condensation in isolated nuclei, rapid execution of apoptosis is nucleus independent but involves mitochondria and CsA-sensitive MPT (42). As PTP-targeting drugs, AQs might be useful cancer cell death inducers when classic drugs requiring nuclear action lose their therapeutic efficacy. Because various topoisomerase inhibitors, microtubule-disrupting compounds, antimetabolites and alkylating agents fail to mimic the rapid $\downarrow\Delta\Psi_m$ (6), swelling and Ca^{2+} release caused by AQs in our cell and cell-free systems, most of the current anticancer drugs do not have the option of directly targeting mitochondria like AQs but must first damage other molecular targets in order to generate the signals that trigger the mitochondrial pathway of apoptosis.

Since tumor cell mitochondria play a central role in apoptosis, have elevated resting $\Delta\Psi_m$ as their metabolism shifts from phosphorylating to non-phosphorylating conditions, and may be highly susceptible to depolarizing lipophilic cations that partition across the IM, they might be preferentially targeted by antitumor AQs that cause $\downarrow\Delta\Psi_m$, swelling and Ca^{2+} release (25). However, the role and importance of mitochondrial interaction for the antitumor action of AQs remain to be determined. In general, the most antiproliferative AQs induce the highest MPT responses but structure-activity relationships are difficult to study when mitochondrial $\downarrow\Delta\Psi_m$, swelling and Ca^{2+} release are likely to be all-or-nothing events, based on the sensitivity threshold of individual mitochondria. Hence, different AQs with increasing antitumor activities are probably targeting an increasing fraction of organelles in the population of isolated mitochondria rather than increasing the MPT responses of individual organelles.

Since drug effectiveness is generally a combination of concentration and duration of action, the apparent discrepancy between the μM concentrations of AQs triggering markers of MPT at 15 min and the nM concentrations of AQs inhibiting tumor cell proliferation at 4 days is not unusual and does not necessarily imply that rapid mitochondrial dysfunction is irrelevant to the molecular mechanism of antitumor activity. The 0.64-4 μM concentrations of AQs required to trigger mitochondrial $\downarrow\Delta\Psi_m$, swelling and Ca^{2+} at 15 min are not different from the ones required to block nucleoside transport at 15 min, inhibit DNA, RNA and protein syntheses at 2-3 h, detect caspase activation, Cyt *c* release and PARP-1 cleavage at 6 h, and cause internucleosomal DNA fragmentation at 24 h, whereas low 23-140 nM concentrations of AQs added to cell cultures at time 0 are sufficient to decrease tumor cell proliferation after 4 days (1-6). DAU and other established anticancer drugs also require μM concentrations to rapidly induce DNA damage, macromolecule synthesis inhibition and proapoptotic effects, whereas nM concentrations of these

drugs, which need several days to reveal their antiproliferative potency, are unable to induce detectable alterations of such early molecular targets within minutes or hours (1-6).

The main components of the PTP complex are the voltage-dependent anion channel (VDAC) and ANT which, at the contact sites between the mitochondrial OM and IM, form dynamic megachannels that link the matrix to the cytosol (23,26,43,44). In the OM, the gating of the VDAC may be regulated by interactions with the peripheral benzodiazepine receptor, cytosolic hexokinase II, and Bcl-2 family members that control apoptosis (45-47). Apart from Cyt *c*, the mitochondrial intermembrane space (IMS) contains creatine kinase and other proapoptotic molecules, such as apoptosis-inducing factor (AIF), endonuclease G, Smac/Diablo and htrA2/Omi. The IM contains ANT, which may be regulated by interactions with cyclophilin D, Bcl-2 and Bax, high amounts of cardiolipin, which ensure membrane fluidity and Cyt *c* oxidase function, and the electron-transporting complexes (I-V) of the mitochondrial respiratory chain, which pump protons out of the matrix to the IMS to generate the $\Delta\Psi_m$ that drives the conversion of ADP to ATP (48). The vital exchange of matrix ATP for cytosolic ADP is dependent on the activity of the ANT-VDAC complex linking the IM to the OM (43). Normally, antiapoptotic Bcl-2 and Bcl-x_L might maintain the VDAC in a physiological open state, which allows ATP-ADP exchanges and energy production but is not permeable to Cyt *c* (43). Conversely, proapoptotic Bax and Bak might disrupt VDAC conformation to induce a nonphysiological open state, which permits the release of Cyt *c* and other soluble proteins from the IMS (43). Opening of mitochondrial PTP and channels controlled by Bcl-2 family members combined with osmotic swelling of the matrix and rupture of the OM may cause a 2-step release of apoptogenic proteins from the IMS to the cytosol and nucleus (15,45). But the mechanisms of Cyt *c* release are complex and unclear (46,49). Cyt *c* release is a universal event in apoptosis but this can occur before, independently, or in the absence of $\downarrow\Delta\Psi_m$ and without concomitant PTP opening, mitochondrial swelling and OM rupture (19,43,50-56). CsA inhibits Cyt *c* release in isolated mitochondria but not in tumor cells (29), suggesting that some drugs might directly target mitochondria to release Cyt *c* in a CsA-dependent manner but also induce cellular events that indirectly trigger Cyt *c* release in a CsA-independent manner. The consensus is that AIF release requires MPT and $\downarrow\Delta\Psi_m$, whereas Cyt *c* release is not always dependent on those events. Drug-induced $\downarrow\Delta\Psi_m$ could play a role before to initiate and/or after to magnify the release of Cyt *c*. The $\downarrow\Delta\Psi_m$ causing matrix remodeling might unfold the cristae and redistribute most of the Cyt *c* from the closed cristae, where it is sequestered and resistant to release by agents that disturb the OM, to the IMS where it is more susceptible to be released (11). Following $\downarrow\Delta\Psi_m$, matrix remodeling might not induce but simply facilitate the complete recruitment and release of Cyt *c* (11). Bax activation may be a more plausible candidate for Cyt *c* release than $\downarrow\Delta\Psi_m$ (17). Following activation of the BH3-only molecules, a bifurcated pathway might ensure the complete release of Cyt *c* (57). One arm would activate Bax and Bak to initiate the release of some Cyt *c* across the permeabilized OM in a CsA-insensitive and Ca²⁺- and MPT-independent manner,

whereas another arm would involve Bax/Bak-independent but CsA-sensitive and Ca²⁺- and MPT-dependent events, such as $\downarrow\Delta\Psi_m$ and IM remodeling, required to mobilize and fully release the cristae stores of Cyt *c* (57,58). As drugs that target isolated mitochondria induce the release of AIF (7), it would be of interest to determine whether PTP-interacting AQs would directly trigger a CsA-inhibitable, Ca²⁺-sensitive and MPT-dependent release of AIF and a caspase-independent process of nuclear apoptosis (24).

The open-closed transition of the PTP is regulated by matrix and membrane factors (15). The hypothesis that AQs interact with components of the PTP is substantiated by the finding that the ability of these antitumor compounds to mimic the mitochondrial $\downarrow\Delta\Psi_m$, swelling and Ca²⁺ release caused by known inducers of PTP opening is inhibited by selected PTP blockers. ANT, which regulates the exchange of adenine nucleotides across the IM, is a carrier protein specific for ADP and ATP with two conformational c- and m-states, depending on whether its hydrophilic ligand-binding loop faces the cytoplasm or matrix sides. Proapoptotic drugs and Bcl-2 members which target ANT might induce conformational changes that fix its c-state, block its ability to transport ADP, and induce a CsA-insensitive release of Cyt *c* by an MPT-independent mechanism in the absence of mitochondrial swelling (59). Whether AQs can inhibit the ANT-mediated transport of ADP in isolated mitochondria remains to be studied. Pretreatments with BA and the natural ANT ligand ADP, which fix ANT conformation in the m-state, all suppress the ability of ANT-interacting drugs to induce Cyt *c* release by fixing ANT in its c-state (59). Since BA and ADP inhibit AQ-induced mitochondrial $\downarrow\Delta\Psi_m$ (6), swelling and Ca²⁺ release, such pretreatments might prevent AQs from interacting with ANT to induce conformational changes that trigger MPT and Cyt *c* and AIF releases. ANT-1 and cyclophilin D overexpression promote MPT and induce apoptosis (60). Normally, ANT-bound cyclophilin D may facilitate a Ca²⁺-triggered conformational change that promotes ANT opening (61). Since the mitochondrial effects of AQs are CsA-sensitive, the interaction of CsA with cyclophilin D might form a complex that prevents cyclophilin D from binding to the ANT and strongly inhibits MPT (61,62).

Quinones may interact with the quinone-binding site of the PTP to induce conformational changes that alter its Ca²⁺-binding affinity, making it either less or more sensitive to Ca²⁺, and define the minimal Ca²⁺ load required to induce PTP opening (63-66). (OH)d-Ub stimulates, whereas Ub₀ and d-Ub inhibit, pore opening because they respectively lower or raise the Ca²⁺ load required to initiate MPT (65). Ub₀ may be a stronger PTP blocker than CsA (66). Inactive Ub₅ can block both the stimulatory or inhibitory effects of other PTP-interacting quinones, suggesting that all quinones compete for a common binding site (65). The PTP of isolated mitochondria opens in the presence of 100 μ M Ca²⁺ but does not in the presence of 20 μ M Ca²⁺ or AQs alone. However, AQs induce mitochondrial $\downarrow\Delta\Psi_m$, swelling and Ca²⁺ release in the presence of 20 μ M Ca²⁺, an effect which is inhibited by d-Ub, Ub₀, Ub₅ and Ub₁₀, suggesting that AQs might interact with the Ub-quinone-binding site of the PTP to induce conformational changes that make it possible for priming concentrations of Ca²⁺ to access the Ca²⁺-binding site regulating its transition

from the closed to the open state. AQs might interfere with endogenous Ub, which normally binds to PTP and stabilizes it in the closed conformation. In this context, Ca²⁺ binding and PTP opening can be achieved either by increasing the Ca²⁺ load or by displacing the inhibitory Ub (65). The closed PTP can exist in 2 quinone-liganded states which confer different conformations resulting in different accessibility to Ca²⁺. In one liganded state, a small Ca²⁺ load is sufficient to open the PTP, whereas in the other state, a much higher Ca²⁺ load is required to access the Ca²⁺-binding site that opens the PTP (67). Since AQs lower from 100 to 20 μ M the Ca²⁺ concentration required to induce mitochondrial $\downarrow\Delta\Psi_m$ (6), swelling and Ca²⁺ release, the AQ-liganded state might confer a conformation to the pore that enhances its Ca²⁺ sensitivity.

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