Antitumor Triptycene Analogs Directly Interact with Isolated Mitochondria to Rapidly Trigger Markers of Permeability Transition

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Abstract. *Background: Substituted triptycenes (TT code number), which block nucleoside transport, macromolecule syntheses and DNA topoisomerase activities, induce cytochrome c release and apoptotic DNA fragmentation, inhibit the proliferation of drug-sensitive and -resistant tumor cells in the nM range in vitro and rapidly trigger the collapse of mitochondrial transmembrane potential in cell and cell-free systems. Because mitochondrial permeability transition (MPT) requires more than depolarization, antitumor TTs were tested for their ability to directly trigger specific markers of MPT in isolated mitochondria. Materials and Methods: Large amplitude swelling and Ca^{2+} release were assayed in isolated mitochondria to demonstrate TT-induced MPT. Results: Antitumor TTs interact with isolated mitochondria in a concentration- and time-dependent manner to rapidly cause large amplitude swelling and Ca^{2+} release in relation with their antiproliferative activities in L1210, HL-60 and LL/2 tumor cells in vitro. The ability of 4-10 μM **TT15**, **TT16** and **TT24** to maximally induce mitochondrial swelling and Ca^{2+} release within 20 min is similar to that of classic MPT inducers, such as 5 μg/ml alamethicin, 200 μM atractyloside, 5 μM phenylarsine oxide, 100 μM arsenic trioxide and a 100 μM Ca^{2+} overload. **TT15** requires a priming concentration of 20 μM Ca^{2+} to trigger mitochondrial swelling and Ca^{2+} release and these 0.1 μM ruthenium red-sensitive MPT events are abolished by 1 μM cyclosporin A, 2 mM ADP and 20 μM bongkrekic acid, which block components of the permeability transition pore (PTP), and by 50-100 μM of various ubiquinones, which interact with the quinone binding site of the PTP and raise the Ca^{2+} load required for PTP opening. Conclusion: Antitumor TTs that trigger MPT in isolated mitochondria might interact with components of the PTP to boost its Ca^{2+}-sensitive transition from the closed to the open state and might be valuable to develop mitochondriotoxic drugs that directly activate early components of apoptosis.*

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Unlike their inactive parent triptycene (**TT0**), synthetic analogs (TT code number) with different quinone functionality inhibit DNA, RNA and protein syntheses within 2-3 h, decrease the mitotic index and induce apoptotic DNA fragmentation within 24 h, and reduce L1210 and wild-type, drug-sensitive, HL-60 leukemia cell proliferation within 2-4 days like daunorubicin (DAU). Based on their ability to decrease by 50% the metabolic activity of L1210 and HL-60 cells at day 4 (IC_{50} 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, bisquinones **TT2**, **TT13**, **TT16**, **TT19**, **TT24** and **TT26** are our current lead antitumor compounds (1-9). Bisquinone **TT21** is weaker. The less potent **TT5**, **TT7** and **TT9** are monoquinones with good antitumor activities. The dihydroquinone **TT3** and the diketone **TT8** lack quinone functionality but still elicit apoptotic and antitumor effects *in vitro*. TT bisquinones also inhibit the proliferation of Lewis lung carcinoma (LL/2) cells, an effect which persists after drug removal *in vitro* (9). The cytostatic and cytotoxic activities of **TT13** (NSC 727282) and **TT24** (NSC 727284) have been confirmed in the 60 human tumor cell lines of the NCI's *in vitro* antitumor screen (9).

New RNA and protein syntheses, and the activation of caspases, endonucleases and non-caspase proteases are required to sustain the active mechanism by which TT bisquinones induce as much apoptotic DNA fragmentation at 24 h as 20 (S)-camptothecin (CPT) and DAU. One advantage over DAU is that TT bisquinones totally block

the cellular uptake of both purine and pyrimidine nucleosides within 15 min, an antitumor effect that might potentiate the action of antimetabolites (1, 4). Moreover, TT bisquinones are dual inhibitors of DNA topoisomerase (Topo) I and II activities, which match the Topo I inhibitory effects of CPT and surpass the Topo II inhibitory effects of *m*-amsacrine under conditions where CPT is ineffective in the Topo II assay and etoposide (VP-16) is ineffective in the Topo I assay (6). Because their antitumor effects persist after drug removal, TTs may rapidly and irreversibly interact with various molecular targets in cell membrane and nuclei. In addition, TT bisquinones retain their efficacy in multidrug-resistant (MDR) HL-60-RV and HL-60-R8 sublines that have different mechanisms of resistance to DAU (2). Studies by AnorMED, Inc., (Langley, British Columbia, Canada) confirm that bisquinones **TT15** and **TT24** inhibit the proliferation of human CH1 ovarian carcinoma and RH-30 rhabdomyosarcoma cells, including sublines that resist doxorubicin (DOX), taxol and vincristine (VCR) (9). Taken together, these findings suggest that synthetic TT bisquinones might have a wider spectrum of molecular targets than DAU, and might be valuable in polychemotherapy to circumvent mechanisms of MDR. The anticancer potential of **TT15** was evaluated by AnorMED against xenografts of human CH1 ovarian carcinoma in NCR nude mice *in vivo*. However, this compound is not water soluble and fine suspensions of **TT15** injected *i.p.* 14 days after *s.c.* tumor transplantation slow down but cannot fully block the growth of such pre-existing solid tumors (9). As an attempt to improve their antitumor activity, novel substituted TT structures have been synthesized and tested in the present study.

Antitumor bisquinones **TT2**, **TT13**, **TT16**, **TT19**, **TT24** and **TT26** 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 (PARP-1) cleavage within 6 h and internucleosomal DNA fragmentation within 24 h (5, 7). **TT13** induces a caspase-independent release of mitochondrial Cyt *c* and a caspase-2-mediated activation of initiator caspase-8 and -9 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 (7). And the caspase-8 inhibitor benzyloxycarbonyl (z)-Ile-Glu-Thr-Asp (IETD)-fluoromethyl ketone (fmk) does not prevent **TT13**-induced Cyt *c* release and caspase-2 and -9 activation. But the caspase-2 inhibitor-z-Val-Asp-Val-Ala-Asp (VDVAD)-fmk totally blocks **TT13**-induced caspase-8 and -9 activities without altering **TT13**-induced Cyt *c* release. Antitumor TTs, therefore, can fully release mitochondrial Cyt *c* without caspase activation but TT-induced caspase-2 activity may be an apical event required for the activation of a downstream cascade of other initiator and effector caspases (7). Recently, fluorescent probes that are specific of mitochondrial transmembrane potential ($\Delta\Psi$m) were used to demonstrate that, in contrast to DAU, antitumor TT bisquinones 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 (8).

Using JC-1 dye, the abilities of various TTs to induce the $\downarrow\Delta\Psi$m in wild-type and MDR HL-60 cells are rapid, within 5-20 min, irreversible after drug removal, concentration-dependent in the 0.64-25 μM range, and generally related to their antitumor activities *in vitro* but not prevented by z-VDVAD-fmk or z-IETD-fmk pretreatments, suggesting that activations of apical caspase-2 or -8 upstream of mitochondria are not involved in this process (8). Antitumor TTs also mimic the abilities of the known depolarizing agents, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), alamethicin, gramicidin A and 100 μM $CaCl_2$, to directly induce within 20 min the $\downarrow\Delta\Psi$m in isolated mitochondria prepared from mouse liver and loaded with rhodamine 123 (Rh 123) dye (8). The fact that 20 μM $CaCl_2$, which is insufficient to trigger depolarization on its own, is required to prime the depolarizing effect of **TT2** in isolated mitochondria suggests that antitumor TTs might interact with the PTP to alter its conformation and increase its Ca^{2+} sensitivity. Indeed, such Ca^{2+}-dependent $\downarrow\Delta\Psi$m in isolated organelles treated with 25 μM **TT2** or 100 μM $CaCl_2$ are blocked by ruthenium red (RR), which prevents the Ca^{2+} uniporter from accumulating the exogenously added divalent cation into the mitochondrial matrix (8). DAU is unable to mimic the rapid $\downarrow\Delta\Psi$m caused by antitumor TTs within 5-40 min of treatment in HL-60 cells or isolated mitochondria. Moreover, the $\downarrow\Delta\Psi$m caused by 25 μM **TT2** or 100 μM $CaCl_2$ in isolated mitochondria are similarly blocked by cyclosporin A (CsA), bongkrekic acid (BA) and decylubiquinone (d-Ub), which prevent PTP opening, suggesting that, in contrast to DAU, antitumor TT bisquinones that directly target mitochondria to trigger the Ca^{2+}-dependent and CsA-sensitive $\downarrow\Delta\Psi$m might induce PTP opening and the mitochondrial pathway of apoptosis even in the absence of nuclear signals (8).

However, the $\downarrow\Delta\Psi$m alone is insufficient to support the hypothesis that TTs 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 Ca^{2+}. Hence, as MPT requires more than depolarization to occur, antitumor TTs

were tested for their ability to interact with isolated organelles and directly trigger specific markers of MPT, such as mitochondrial swelling and Ca^{2+} release.

Materials and Methods

Drug treatments. The new method to synthesize the structures of the TT analogs mentioned in this study has already been reported (3, 9). The basic skeleton of TT bisquinones is readily synthesized by a two-step sequence leading to **TT2**, which is then converted to various antitumor analogs. The functionalization of the TT nucleus is achieved by selective bromination followed by addition of alkylamines (3, 9). All solutions of parent **TT0** (Aldrich, Milwaukee, WI, USA), synthetic **TT2** analogs, atractyloside, phenylarsine oxide, d-Ub (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone), 2,3-dimethoxy-5-methyl-*p*-benzoquinone (Ub_0, ubiquinone-0 or coenzyme Q_0), 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone (Ub_5, ubiquinone-5 or coenzyme Q_1), 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Ub_{10}, ubiquinone-10 or coenzyme Q_2) (all from Sigma, St. Louis, MO, USA) and CsA (Calbiochem, La Jolla, CA, USA) were dissolved and diluted in dimethyl sulfoxide (DMSO). Fluo-5N, pentapotassium salt (Molecular Probes, Eugene, OR, USA), 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. 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, USA) 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, USA) 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% CO_2 (1, 2, 4, 5, 7-9). The proliferation of drug-treated L1210 (initial density $4.5x10^3$/0.5 ml), HL-60 (initial density $3.75x10^4$/0.5 ml) and LL/2 (initial density $1.75x10^3$/0.5 ml) tumor cells was assessed from their mitochondrial ability to bioreduce the MTS reagent (Promega, Madison, WI, USA) 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 (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 metabolic activity was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL, USA). Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were substracted from the results (1, 2, 4, 5, 7-9).

Isolation of mitochondria. To collect enough organelles to study TT-induced mitochondrial swelling and Ca^{2+} release, they were isolated by differential centrifugation at 4˚C from one female CF-1 (Charles River, Wilmington, MA, USA) mouse liver after an overnight period of fasting to deplete its levels of glycogen and fatty acids (8, 10-12). 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 about 500 r.p.m. in a glass Potter-Elvejhem 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 (8, 10-12). The protein concentrations of the mitochondrial samples were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

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 (13, 14). 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 TT-induced MPT was determined spectrophotometrically by monitoring the decrease in apparent absorbance (light-scattering) at 540 nm (11, 14). 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 KH_2PO_4 and 20 μM EGTA, and 30 μl of isolated mitochondria (1.5 mg of protein). After incubation of these control or TT-treated reaction mixtures for 5 min at room temperature, the decrease of absorbance (light scattering) linked to large amplitude mitochondrial swelling was monitored for 2.5-40 min at 540 nm, using a Cambridge model 750 automatic microplate reader.

Mitochondrial Ca^{2+} release. The ability of TTs to directly induce isolated mitochondria to release Ca^{2+} was determined using the pentapotassium salt of Fluo-5N, a cell-impermeant Ca^{2+} indicator, which is not fluorescent in the absence of divalent cation but exhibits strong fluorescence upon binding to extramitochondrial Ca^{2+}. Hence, the development of green Fluo-5N fluorescence was linked to the increase of free extramitochondrial Ca^{2+} in the reaction medium, resulting from the release of sequestered Ca^{2+} by mitochondria undergoing TT- or 100 μM Ca^{2+} overload-induced MPT. In controls, the baseline of green Fluo-5N fluorescence is low since the high $\Delta\psi m$ prevents the release of mitochondrial Ca^{2+}. Likewise, when 100 μM Ca^{2+} overload is used to trigger MPT, the RR-sensitive Ca^{2+} 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 Ca^{2+} caused by TT- or 100 μM Ca^{2+} overload-induced MPT (11, 15). Mitochondrial Ca^{2+} 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 TT-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 Ca^{2+} released from mitochondria undergoing MPT, was monitored for 2.5-40 min at 495 nm excitation/ 518 nm emission, using a Cary Eclipse Fluorescence Spectrophotometer equipped with microplate reader accessory (Varian, Walnut Creek, CA, USA) (11, 15). Data were analyzed using the Student's *t*-test with a level of significance set at $p<0.05$.

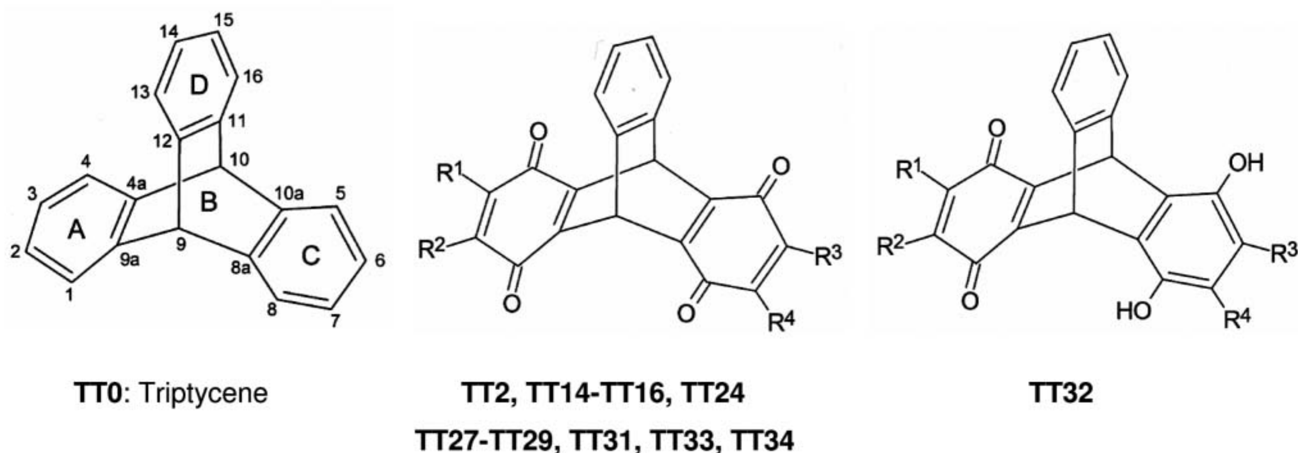
Results

Inhibition of tumor cell proliferation. Because *in vitro* studies suggest that the addition of alkylamino function at C6 and C7 alters TT bioactivity (1-9), the antiproliferative activities of newly synthesized **TT27**- **TT29** and **TT31-34** were compared to those of the current lead antitumor compounds **TT2**, **TT16** and **TT24** (Figure 1). The nomenclatures of these novel substituted TTs, which are mixtures of 2 regioisomers inseparable by silica gel column chromatography, are: **TT27**, 6-[*N*-(2-*t*-butyldimethylsilyloxyethyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4,5,8-triptycenetetraone; **TT28**, 6-[*N*-(4-hydroxybutyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4,5,8-triptycenetetraone; **TT29**, 6-[*N*-[2-*bis*(di-*t*-butyloxyphosphonyloxyethyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4,5,8-triptycenetetraone; **TT31**, 6-[*N*-(2-hydroxyethyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4,5,8-triptycenetetraone; **TT32**, 6-[*N*-[3-(*N*-methyl-*N*-*t*-butyloxycarbonylamino)propyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4-dioxo-5,8-triptycenediol; **TT33**, 6-[*N*-[3-(*N*-methyl-*N*-*t*-butyloxycarbonylamino)propyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4,5,8-triptycenetetraone; and **TT34**, 6-[*N*-[2-*bis*(di-*t*-butyloxyphosphonyloxypropyl)-*N*-methylamino]-2-bromo-3-methoxy-1,4,5,8-triptycenetetraone. Substituted TT analogs are more potent against L1210 than HL-60 cells and their efficacy decreases in relation with the rate of cell proliferation from rapidly growing leukemia to slowly growing LL/2 solid lung tumors (Figure 1). Since the IC_{50} values of previously synthesized **TT14** and **TT15**, which had not been characterized before (1-9), are somewhat higher than those of **TT2**, **TT16** and **TT24** in L1210 and HL-60 cells, these compounds were not evaluated against LL/2 cell proliferation. Among the new compounds, bisquinones **TT28**, **TT29** and **TT33** are the most potent, with antiproliferative activities nearly matching or even surpassing those of the lead antitumor TT compounds in the L1210, HL-60 and LL/2 systems (Figure 1). As bisquinones **TT27** and **TT31** are only moderately effective against fast-growing L1210 and HL-60 leukemic cells, they were not evaluated against slow-growing LL/2 lung carcinoma cells. And new bisquinone **TT34**, which is by far the least effective against LL/2 cell proliferation, was not tested any further. Interestingly, the antiproliferative activity of monoquinone **TT32** is almost comparable to that of some of the lead antitumor TT bisquinones in most cell lines.

Induction of MPT. Freshly isolated mitochondria were used to determine whether antitumor TTs 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 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 20 min (Figure 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+} (Figure 2). Interestingly, 4-10 μM **TT15**, which is inactive alone, requires the presence of a 20 μM Ca^{2+} primer in order to induce the same mitochondrial swelling and Ca^{2+} release at 20 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 (Figure 2). Antitumor TTs, 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 TT-induced MPT. The ability of **TT15**, **TT16** and **TT24** to induce Ca^{2+}-dependent MPT in isolated mitochondria is a function of time (Figure 3) and concentration (Figure 4). At a concentration of 10 μM, **TT15**, **TT16** and **TT24** need about 20 min to fully induce mitochondrial swelling (Figure 3). The Ca^{2+}-dependent mitochondrial swelling induced by **TT15** is slightly greater than that caused by **TT16**, starting earlier at 2.5 min and reaching a larger amplitude at 20 min (Figure 3). When compared at 20 min, the Ca^{2+}-dependent inductions of mitochondrial swelling and Ca^{2+} release by **TT15**, **TT16** and **TT24** are similarly concentration dependent, starting at about 640 nM and peaking at 10 μM before declining thereafter (Figure 4). Again, **TT15**, which is effective around 256 nM, appears to be a slightly more potent MPT inducer than **TT16** (Figure 4).

Relevance of TT-induced MPT. In general, synthetic TT analogs seem to induce markers of MPT in isolated



Compounds		IC_{50} values (nM)[a]		
Code	Residues	L1210 cells	HL-60 cells	LL/2 cells
TT2	R^1 = OMe, R^2 = R^3 = R^4 = H	125 ± 12	275 ± 23	962 ± 68
TT14	R^1 = NHMe•HCl, R^2 = Br, R^3 = R^4 = H	270 ± 15	349 ± 42	---
TT15	R^1 = Br, R^2 = NHMe, R^3 = R^4 = H	192 ± 23	522 ± 71	---
TT16	R^1 = OMe, R^2 = Br, R^3 = NMe_2, R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe_2	116 ± 10	338 ± 35	967 ± 94
TT24	R^1 = OMe, R^2 = Br, R^3 = NHMe, R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NHMe	48 ± 3	286 ± 13	1,029 ± 76
TT27	R^1 = OMe, R^2 = Br, R^3 = NMe(CH_2CH_2OSi-*t*-$BuMe_2$), R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe(CH_2CH_2OSi-*t*-$BuMe_2$)	352 ± 39	474 ± 38	---
TT28	R^1 = OMe, R^2 = Br, R^3 = NMe($CH_2CH_2CH_2CH_2OH$), R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe($CH_2CH_2CH_2CH_2OH$)	69 ± 10	154 ± 18	741 ± 74
TT29	R^1 = OMe, R^2 = Br, R^3 = NMe[CH_2CH_2OPO(O-*t*-Bu)$_2$], R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe[CH_2CH_2OPO(O-*t*-Bu)$_2$]	100 ± 8	258 ± 24	784 ± 94
TT31	R^1 = OMe, R^2 = Br, R^3 = NMe(CH_2CH_2OH), R^4 = H R^1 = OMe, R^2 = Br, R^4 = H, R^3 = NMe(CH_2CH_2OH)	203 ± 17	740 ± 56	---
TT32	R^1 = OMe, R^2 = Br, R^3 = NMe($CH_2CH_2CH_2NMeBoc$), R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe($CH_2CH_2CH_2NMeBoc$)	188 ± 18	234 ± 15	867 ± 83
TT33	R^1 = OMe, R^2 = Br, R^3 = NMe($CH_2CH_2CH_2NMeBoc$), R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe($CH_2CH_2CH_2NMeBoc$)	97 ± 14	256 ± 19	503 ± 48
TT34	R^1 = OMe, R^2 = Br, R^3 = NMe[$CH_2CH_2CH_2OPO$(O-*t*-Bu)$_2$], R^4 = H R^1 = OMe, R^2 = Br, R^3 = H, R^4 = NMe[$CH_2CH_2CH_2OPO$(O-*t*-Bu)$_2$]	---	---	3,391 ± 249

Figure 1. *Concentrations of TTs required to inhibit the metabolic activity of L1210, HL-60 and LL/2 tumor cells by 50% (IC_{50} values), using the MTS:PMS assay at day 4 in vitro.* [a]*Means±SD (n=3).*

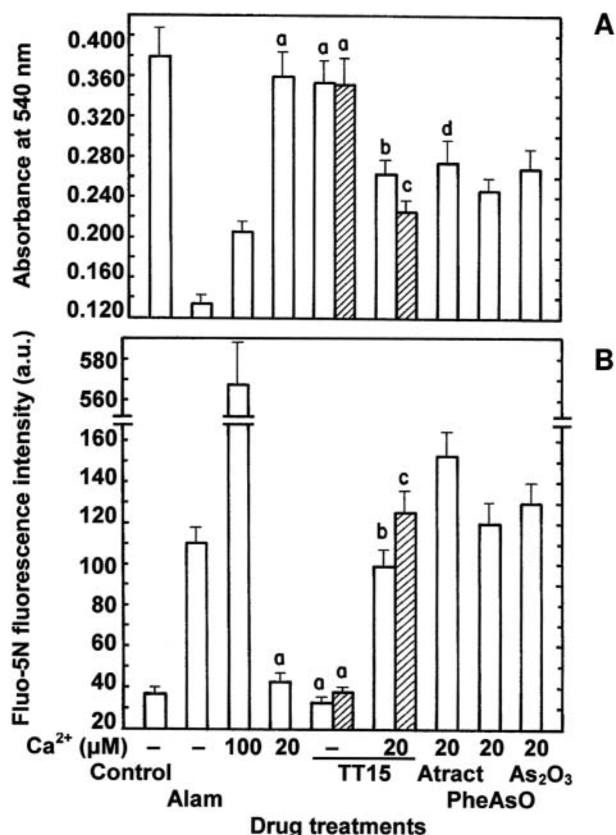


Figure 2. *Inducers of MPT in a cell-free system. A) Comparison of the abilities of 5 μg/ml alamethicin (Alam), 20 or 100 μM $CaCl_2$, and 4 (open) or 10 μM (striped) concentrations of* ***TT15****, 200 μM atractyloside (Atract), 5 μM phenylarsine oxide (PheAsO) or 100 μM arsenic trioxide (As_2O_3), 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 20 min in severely swollen organelles with diluted matrices and disrupted cristae. Control: absorbance at 540 nm of non-treated suspensions of isolated mitochondria (0.380±0.028). Bars: means±SD (n=3). [a]Not different from control; [b]not different from Atract, PheAsO or As_2O_3; [c]$p<0.025$, smaller than 20 μM Ca^{2+} + 4 μM* ***TT15*** *but not different from 100 μM Ca^{2+}; [d]$p<0.01$, 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 20 min. Control: baseline intensity of Fluo-5N fluorescence in non-treated suspensions of isolated mitochondria (37.2±2.7). [a]Not different from control; [b]$p<0.0005$, greater than 20 μM Ca^{2+} but not different from Alam; [c]$p<0.025$, greater than 20 μM Ca^{2+} + 4 μM* ***TT15****, $p<0.05$, smaller than Atract but not different from Alam, PheAsO or As_2O_3.*

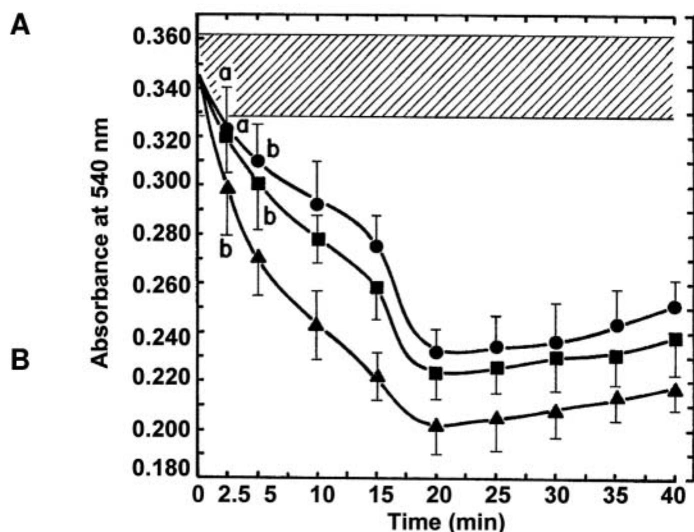


Figure 3. *Comparison of the time-dependent inductions of the large amplitude swelling of isolated mitochondria by 10 μM concentrations of* ***TT15*** *(▲),* ***TT16*** *(●) or* ***TT24*** *(■), in the presence of a 20 μM Ca^{2+} primer. Control: average absorbance at 540 nm of non-treated suspensions of isolated mitochondria at all time points studied (0.345±0.017; striped area). Alone, 20 μM Ca^{2+} is unable to induce mitochondrial swelling over 40 min (0.326±0.016, 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 (0.192±0.007). Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$, smaller than control.*

mitochondria in relation with their antiproliferative and proapoptotic effects in tumor cells *in vitro* (1-9). For simplification, only those antitumor TTs inducing the most mitochondrial swelling were also tested for their ability to trigger the release of mitochondrial Ca^{2+} (Table I). The commercially available parent compound **TT0**, which has no antitumor activity, fails to induce mitochondrial swelling and Ca^{2+} release in the presence of a 20 μM Ca^{2+} primer. Even though they elicit some weak apoptotic and antitumor effects *in vitro*, the dihydroquinone **TT3** and the diketone **TT8** lack quinone functionality and are also unable to trigger these MPT markers. But **TT5**, **TT7**, **TT9** and the new **TT32**, which are example of TT monoquinones with weak to moderate antitumor activities, significantly induce mitochondrial swelling in the presence of a 20 μM Ca^{2+} primer. All the other antitumor TT bisquinones with greater antiproliferative activity generally also induce to a greater degree mitochondrial swelling and Ca^{2+} release, the most potent being the lead antitumor **TT2**, **TT16** and **TT24**, with **TT13**, **TT14**, **TT19**, **TT21**, **TT28** and **TT29** not far behind. Some exceptions are bisquinone **TT15**, which is not a lead antiproliferative compound but is consistently the most potent inducer of MPT markers in this study, and bisquinone **TT33**, which is one of the top antiproliferative agent in the series but has only weak MPT-inducing activity in isolated mitochondria (Table I).

Antitumor TTs may have a unique ability to directly interact with isolated mitochondria and trigger MPT since

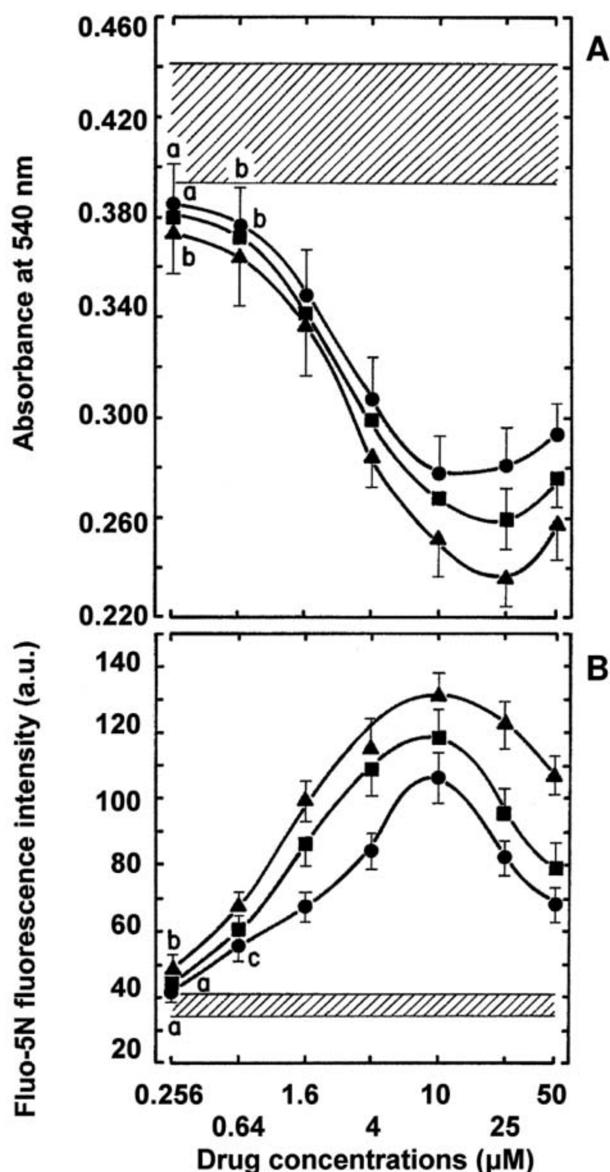


Figure 4. *Concentration-dependent induction of MPT by TTs in a cell-free system. A) Comparison of the abilities of serial concentrations (plotted on a logarithmic scale) of **TT15** (▲), **TT16** (●) or **TT24** (■) to directly induce, in the presence of a 20 μM Ca^{2+} primer, the large amplitude swelling of isolated mitochondria after 20 min. Control: absorbance at 540 nm of non-treated suspensions of isolated mitochondria (0.418±0.024, striped area). Alone, 20 μM Ca^{2+} is unable to induce mitochondrial swelling (0.386±0.022, not different from control), whereas a 100 μM Ca^{2+} overload does and is used as a reference MPT-inducing treatment (0.215±0.009). Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$, smaller than control. B) Comparison of the abilities of serial concentrations of **TT15** (▲), **TT16** (●) or **TT24** (■) to directly induce, in the presence of a 20 μM Ca^{2+} primer, the release of Ca^{2+} from isolated mitochondria after 20 min. Control: baseline intensity of Fluo-5N fluorescence in non-treated suspensions of isolated mitochondria (37.9±3.1, striped area). Alone, 20 μM Ca^{2+} does not trigger mitochondrial Ca^{2+} release (44.2±3.9, not different from control), whereas a 100 μM Ca^{2+} overload does and is used as a reference MPT-inducing treatment (562.2±28.7). [a]Not different from control; [b]$p<0.05$ and [c]$p<0.005$, smaller than control.*

Table I. *Comparison of the direct MPT-inducing activities of various antiproliferative TTs 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	0.383±0.019	38.6±2.9
100 μM Ca^{2+}	0.202±0.008	596.1±34.6
20 μM Ca^{2+}	0.358±0.016[c]	45.3±3.4[c]
+ **TT0**	0.351±0.015[d]	47.2±4.1[d]
+ **TT2**	0.249±0.011	105.4±6.7
+ **TT3**	0.333±0.014[d]	---
+ **TT5**	0.318±0.019[e]	---
+ **TT7**	0.311±0.018[e]	---
+ **TT8**	0.339±0.020[d]	---
+ **TT9**	0.322±0.017[e]	---
+ **TT13**	0.271±0.010	95.7±5.6
+ **TT14**	0.257±0.011	94.2±6.6
+ **TT15**	0.218±0.011	126.2±7.1
+ **TT16**	0.246±0.013	107.3±8.4
+ **TT19**	0.255±0.012	97.7±7.2
+ **TT21**	0.259±0.009	84.9±6.4
+ **TT24**	0.236±0.011	117.0±7.7
+ **TT25**	0.297±0.019[f]	---
+ **TT26**	0.274±0.016[g]	76.0±6.1
+ **TT27**	0.327±0.012[e]	---
+ **TT28**	0.251±0.013	95.0±6.3
+ **TT29**	0.253±0.014	88.4±7.5
+ **TT31**	0.307±0.017[f]	---
+ **TT32**	0.302±0.018[f]	---
+ **TT33**	0.294±0.019[f]	67.2±5.9[h]

[a]Isolated mitochondria were incubated for 20 min with 10 μM concentrations of TTs in the presence of a 20 μM Ca^{2+} primer; [b]means±SD (n=3); [c]not different from control; [d]not different from 20 μM Ca^{2+}; [e]$p<0.05$, [f]$p<0.025$ and [g]$p<0.005$, smaller than 20 μM Ca^{2+}; [h]$p<0.01$, greater than 20 μM Ca^{2+}.

various other anticancer and antiproliferative drugs tested at 10 μM under similar conditions, including DAU, DOX, staurosporine, mitoxantrone, VCR, vinblastine, VP-16, taxol, nocodazole, tubulozole c, methotrexate, cytosine β-D-arabinofuranoside, 5-fluorouracil, cyclophosphamide, cisplatin and CPT, all fail to induce Ca^{2+}-dependent mitochondrial swelling and Ca^{2+} release at 20 min (data not shown). This wide spectrum of known DNA-alkylating and -damaging agents, Topo inhibitors, microtubule-interacting compounds and antimetabolites 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 TT-induced MPT. The hypothesis that antitumor TTs 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 concentration of exogenous Ca^{2+} now becomes sufficient to prime TT-induced mitochondrial swelling and Ca^{2+} release (Figure 5). As expected, this Ca^{2+}-dependent induction of mitochondrial swelling and Ca^{2+} release by 10 μM **TT15** is abolished by 0.1 μM RR, which blocks the Ca^{2+} uniporter (Figure 5). The ability of 10 μM **TT15** 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 of TT-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 Ub_0, 100 μMUb_5 and 100 μM Ub_{10}, which interact with the ubiquinone (Ub)/quinone-binding site of the PTP to induce conformational changes that decrease its Ca^{2+} sensitivity (Figure 5).

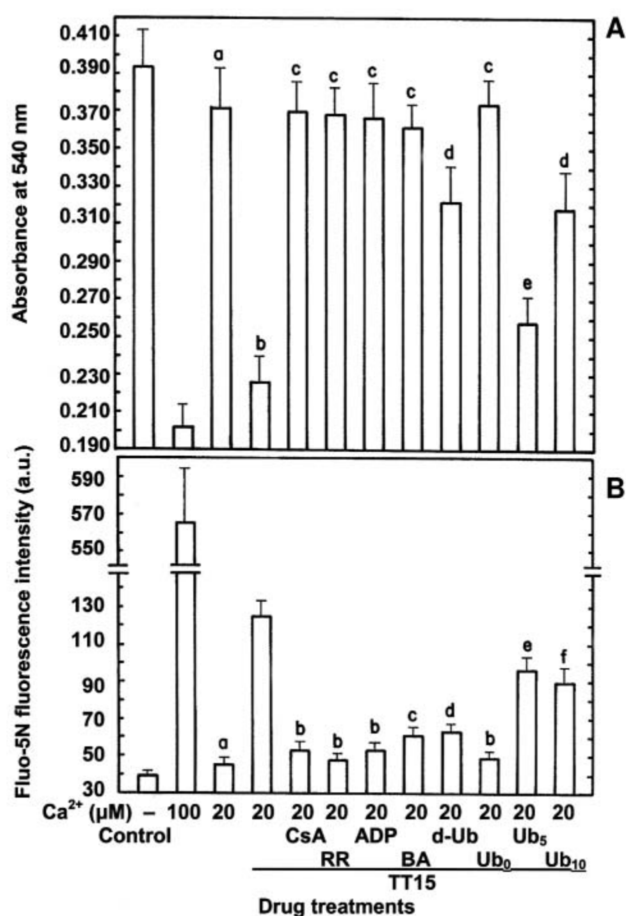


Figure 5. *Inhibition of TT-induced MPT in a cell-free system. A) Comparison of the abilities of 1 μM CsA, 0.1 μM RR, 2 mM ADP, 20 μM BA, 100 μM d-Ub, 50 μM Ub_0, 100 μM Ub_5 or 100 μM Ub_{10} pretreatments to inhibit the large amplitude swelling of isolated mitochondria directly induced after 20 min by 10 μM **TT15** in the presence of a 20 μM Ca^{2+} primer. Control: absorbance at 540 nm of non-treated suspensions of isolated mitochondria (0.394±0.019). Alone, 20 μM Ca^{2+} is unable to induce mitochondrial swelling, whereas a 100 μM Ca^{2+} overload does and is used as a reference MPT-inducing treatment. Bars: means±SD (n=3). [a]Not different from control; [b]not different from 100 μM Ca^{2+}; [c]not different from 20 μM Ca^{2+}; [d]$p<0.05$, smaller than 20 μM Ca^{2+}; [e]$p<0.05$, greater than 20 μM Ca^{2+} + **TT15**. B) Comparison of the abilities of the above pretreatments to inhibit the release of Ca^{2+} from isolated mitochondria directly induced after 20 min by 10 μM **TT15** in the presence of a 20 μM Ca^{2+} primer. Control: baseline intensity of Fluo-5N fluorescence in non-treated suspensions of isolated mitochondria (39.3±2.4). Alone, 20 μM Ca^{2+} does not trigger mitochondrial Ca^{2+} release, whereas a 100 μM Ca^{2+} overload does and is used as a reference MPT-inducing treatment. [a]Not different from control; [b]not different from 20 μM Ca^{2+}; [c]$p<0.025$ and [d]$p<0.005$, greater than 20 μM Ca^{2+}; [e]$p<0.025$ and [f]$p<0.01$, smaller than 20 μM Ca^{2+} + **TT15**.*

Discussion

The PTP functions as a CsA-sensitive and Ca^{2+}-, voltage-, pH- and redox-gated channel with several levels of conductance (16). The intrinsically low permeability of the IM to ions and solutes allows energy conservation in the form of a proton-driven Δψm. The primary event in MPT is a sudden and nonspecific permeabilization of the IM to K^+, Mg^{2+} and Ca^{2+}, as well as to solutes of MW <1,500 Da (16, 17). Once PTP is open, the electrochemical H^+ gradient essential for oxidative phosphorylation is dissipated, the ↓ΔΨm and uncoupling of respiration deplete ATP levels, and the hyperosmolarity of the matrix induces H_2O to enter from the cytosol and cause rapid mitochondrial swelling, which may rupture the OM and trigger the release of intramitochondrial Ca^{2+} and proapoptotic proteins (18).

PTP opening may be regulated by the ↓ΔΨm and the pH of the matrix but the relationships between depolarization, MPT and apoptosis remain unclear. Even though antitumor TTs trigger mitochondrial depolarization in cells and cell-free systems (8), the ↓ΔΨm may be critical but not sufficient for, or a universal step in, apoptotis. MPT is always followed by ↓Δψm but ↓Δψm is not always followed by MPT (16). Direct ↓Δψm caused by CCCP does not induce MPT *per se*. As MPT requires more than ↓Δψm to occur (18), the present findings that antitumor TTs directly induce isolated mitochondria to undergo large amplitude swelling and release their sequestered Ca^{2+} 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. Mitochondrial swelling resulting from solute and H_2O flux from intermembrane space (IMS) 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 TT-induced MPT *in vivo* is hard to predict because of the high level of macromolecules in the cytosol (16). A RR-sensitive uniporter driven by the $\Delta\psi$m of the IM is responsible for Ca^{2+} uptake (16). Although it is also stored in the endoplasmic reticulum, Ca^{2+} is mostly sequestered in mitochondria and MPT-induced Ca^{2+} release is a signal of apoptosis (16, 17). Ca^{2+} may be released from mitochondria under stress by drugs or Ca^{2+} overloads that open the CsA-sensitive PTP and cause IM permeabilization, $\downarrow\Delta\psi$m and swelling (17, 19). The IM contains separate routes for Ca^{2+} uptake and release. MPT exhibits a prominent Ca^{2+} dependence. When Ca^{2+} concentration gradients exist between matrix and cytosol or external medium, onset of MPT serves as a mitochondrial Ca^{2+} release channel (16). The facts that all the MPT effects of TTs on isolated organelles require a 20 μM Ca^{2+} primer and an active Ca^{2+} uniporter suggest that these antitumor drugs are likely to lower the threshold of exogenous Ca^{2+} triggering mitochondrial PTP opening, $\downarrow\Delta\psi$m, swelling and Ca^{2+} release. Presumably, the intracellular concentration of Ca^{2+} in HL-60 cells might be sufficient to mediate the rapid $\downarrow\Delta\psi$m caused by TTs in mitochondria *in situ* (8).

The ability of antitumor TTs to target mitochondria and rapidly induce the $\downarrow\Delta\psi$m has been demonstrated in both cultured HL-60 cells and suspensions of freshly isolated mitochondria and is not a fluorescence artifact (8). Indeed, when emission wavelengths are scanned over a 513-713 nm range at the 503 nm excitation wavelength of the Rh 123 assay, antitumor TTs 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 TTs 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 (8). Similarly, when emission wavelengths are scanned at the 495 excitation wavelength of the Fluo-5N assay, antitumor TTs 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, TTs 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 TTs 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 20 min in a simple spectrophotometric assay where no fluorescent dye is used and no fluorescence artifact is possible.

Since synthetic TTs 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 (20-23). 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 (10). In contrast to DAU, DOX, cisplatin and VP-16, which do not rapidly target isolated mitochondria (10, 24), TTs irreversibly induce mitochondria to lose their $\Delta\Psi$m in a caspase-independent manner within 5-20 min in cell and cell-free systems, suggesting that, no matter what other cellular and nuclear interactions or signaling pathways they generate, TTs also have the potential to directly target this key organelle to initiate MPT and apoptosis (8). 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 TTs, indirect and delayed damage to mitochondria *in situ* is the consequence rather than the cause of DOX-induced cytotoxicity (10, 25). 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 (26-32). Moreover, 5 μM CPT 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 (33, 34). 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 (12). The fact that TT-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 TTs directly interact with components of the PTP complex to cause MPT. The direct action of TTs 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 (35). 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 (36). As PTP-targeting drugs, TTs might be useful cancer cell death inducers when classic drugs requiring nuclear action lose their therapeutic efficacy. Because various Topo inhibitors, microtubule-disrupting compounds, antimetabolites and alkylating agents fail to mimic the rapid $\downarrow\Delta\Psi m$ (8), swelling and Ca^{2+} release caused by TTs in our cell and cell-free systems, most of the current anticancer drugs do not have the option of directly targeting mitochondria like TTs 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 TTs that cause $\downarrow\Delta\Psi m$, swelling and Ca^{2+} release. However, the role and importance of mitochondrial interaction for the antitumor action of TTs remain to be determined. In general, the most antiproliferative TTs 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 TTs with increasing antitumor activities are probably targeting 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 TTs triggering markers of MPT at 20 min and the nM concentrations of TTs 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 1.6-10 μM concentrations of TTs required to trigger mitochondrial $\downarrow\Delta\Psi m$, swelling and Ca^{2+} at 20 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 lower 48-1,029 nM concentrations of TTs added to cell cultures at time 0 are sufficient to decrease tumor cell proliferation after 4 days (1-9). 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 (1-9).

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 (20, 22, 37). 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 (38, 39). Apart from Cyt c, the mitochondrial 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. 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 (37). 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* (37). 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 (37). 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 (16, 38). But the mechanisms of Cyt *c* release are complex and unclear (39). 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 (37, 40-43). CsA inhibits Cyt *c* release in isolated mitochondria but not in tumor cells (24), 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 (13). Following $\downarrow\Delta\Psi m$, matrix remodeling might not induce but simply facilitate the complete recruitment

and release of Cyt *c* (13). Bax activation may be a more plausible candidate for Cyt *c* release than ↓ΔΨm (18). Following activation of the BH3-only molecules, a bifurcated pathway might ensure the complete release of Cyt *c* (44). One arm would activate Bax and Bak to initiate the release of some Cyt *c* across the permeabilized OM in a CsA-insensitive and Ca^{2+}- and MPT-independent manner, whereas another arm would involve Bax/Bak-independent but CsA-sensitive and Ca^{2+}- and MPT-dependent events, such as ↓ΔΨm and IM remodeling, required to mobilize and fully release the cristae stores of Cyt *c* (44). As drugs that target isolated mitochondria induce the release of AIF (10), it would be of interest to determine whether PTP-interacting TTs would directly trigger a CsA-inhibitable, Ca^{2+}-sensitive and MPT-dependent release of AIF and a caspase-independent process of nuclear apoptosis (21).

The open-closed transition of the PTP is regulated by matrix and membrane factors (16).The hypothesis that TTs interact with components of the PTP is substantiated by the finding that the ability of these antitumor compounds to mimic the mitochondrial ↓ΔΨm, swelling and Ca^{2+} 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 2 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 a MPT-independent mechanism in the absence of mitochondrial swelling (45). Whether TTs 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 (45). Since BA and ADP inhibit TT-induced mitochondrial ↓Δψm (8), swelling and Ca^{2+} release, such pretreatments might prevent TTs from interacting with ANT to induce conformational changes that trigger MPT and Cyt *c* and AIF releases. ANT-1 and cyclophilin D overexpressions promote MPT and induce apoptosis (46). Normally, ANT-bound cyclophilin D may facilitate a Ca^{2+}-triggered conformational change that promotes ANT opening. Since the mitochondrial effects of TTs 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 (47).

Quinones may interact with the quinone-binding site of the PTP to induce conformational changes that alter its Ca^{2+}-binding affinity, making it either less or more sensitive to Ca^{2+}, and define the minimal Ca^{2+} load required to induce PTP opening (48-51). (OH)d-Ub stimulates, whereas Ub_0 and d-Ub inhibit, pore opening because they respectively lower or raise the Ca^{2+} load required to initiate MPT (50). Ub_0 may be a stronger PTP blocker than CsA (51). Inactive Ub_5 can block both the stimulatory or inhibitory effects of other PTP-interacting quinones, suggesting that all quinones compete for a common binding site (50). The PTP of isolated mitochondria opens in the presence of 100 μM Ca^{2+} but neither in the presence of 20 μM Ca^{2+} nor TTs alone. But TTs induce mitochondrial ↓Δψm, swelling and Ca^{2+} release in the presence of 20 μM Ca^{2+}, an effect which is inhibited by d-Ub, Ub_0, Ub_5 and Ub_{10}, suggesting that TTs might interact with the Ub/quinone-binding site of the PTP to induce conformational changes that make it possible for priming concentrations of Ca^{2+} to access the Ca^{2+}-binding site regulating its transition from the closed to the open state. TTs might interfere with endogenous Ub, which normally binds to PTP and stabilizes it in the closed conformation. In this context, Ca^{2+} binding and PTP opening can be achieved either by increasing the Ca^{2+} load or by displacing the inhibitory Ub (50). The closed PTP can exist in 2 quinone-liganted states which confer different conformations resulting in different accessibility to Ca^{2+}. In one liganted state, a small Ca^{2+} load is sufficient to open the PTP, whereas in the other state, a much higher Ca^{2+} load is required to access the Ca^{2+}-binding site that opens the PTP (52). Since TTs lower from 100 to 20 μM the Ca^{2+} concentration required to induce mitochondrial ↓Δψm (8), swelling and Ca^{2+} release, the TT-liganted state might confer a conformation to the pore that enhances its Ca^{2+} sensitivity.

Acknowledgements

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