Antitumor triptycene analogs induce a rapid collapse of mitochondrial transmembrane potential in HL-60 cells and isolated mitochondria

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Abstract. Since synthetic analogs of triptycene (TT code number), such as bisquinones TT2 and TT13, can trigger cytochrome *c* release without caspase activation and retain their ability to induce apoptosis in multidrug-resistant (MDR) tumor cells, fluorescent probes of transmembrane potential have been used to determine whether these antitumor compounds might directly target mitochondria in cell and cell-free systems to cause the collapse of mitochondrial membrane potential ($\downarrow\Delta\psi m$) that is linked to permeability transition pore (PTP) opening. Using JC-1 dye, the abilities of various TT analogs 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*. The $\downarrow\Delta\psi m$ caused by TT2 and TT13, which are more potent than mitoxantrone, staurosporine and the reference depolarizing agent, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), in HL-60 cells, are not prevented by caspase-2 or -8 inhibitors, suggesting that activation of these apical caspases upstream of mitochondria is not involved in this process. Antitumor TT analogs (0.64-25 μM) also mimic the abilities of the known depolarizing agents, 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 dye. The fact that 20 μM Ca^{2+}, which is insufficient to trigger depolarization on its own, is required to reveal the depolarizing effect of TT2 in isolated mitochondria suggests that antitumor TT analogs might interact with the PTP to alter its conformation and increase its Ca^{2+} sensitivity. Indeed, such Ca^{2+}-dependent $\downarrow\Delta\psi m$ of isolated mitochondria treated with 25 μM TT2 or 100 μM Ca^{2+} are blocked by ruthenium red. Daunorubicin (DAU) is unable to mimic the rapid $\downarrow\Delta\psi m$ caused by antitumor TT bisquinones 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 Ca^{2+} in isolated mitochondria are similarly blocked by cyclosporin A (CsA), bongkrekic acid and decylubiquinone, which prevent PTP opening, suggesting that, in contrast to DAU, antitumor TT analogs 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.

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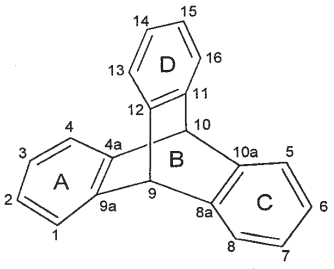
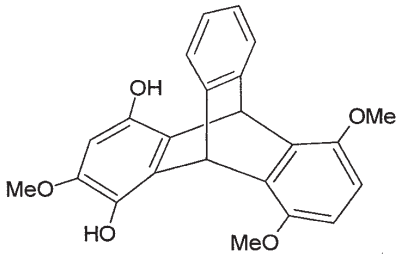
Introduction

Low concentrations of anticancer drugs activate apoptosis while defective apoptotic pathways may contribute to the development of multidrug resistance (MDR) (1). As the anti-cancer activity of doxorubicin (DOX, adriamycin) and daunorubicin (DAU, daumomycin) is limited by their cardiotoxicity and ability to induce MDR, it is important to develop novel quinone antitumor drugs that might overcome mechanisms of apoptosis resistance. Hence, triptycene analogs (TT code number) were synthesized and their antitumor and apoptotic effects were assessed and compared to those of DAU, a valuable anticancer drug that is structurally very different but also contains a para-quinone moiety (2-8). A selection of representative antitumor TT analogs, which may have a wider spectrum of molecular targets than DAU and remain effective in DAU-resistant tumor cells, is shown in Fig. 1. The method of synthesis, correct nomenclatures and bioactivities of these rigid tetracyclic skeletons, which possess either 0, 1 or 2 external quinones, have been reported (2-8).

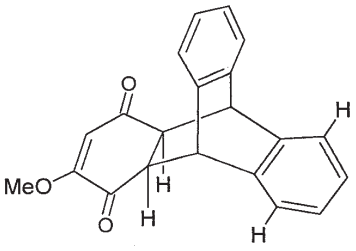
In contrast to their inactive parent compound triptycene (TT0), several synthetic TT analogs with or without quinone functionality may represent a novel class of antitumor drug, which inhibits DNA, RNA and protein syntheses within 2-3 h, decreases the mitotic index and induces DNA fragmentation within 24 h, and reduces the proliferation and viability of murine L1210 lymphocytic and wild-type (WT), drug-



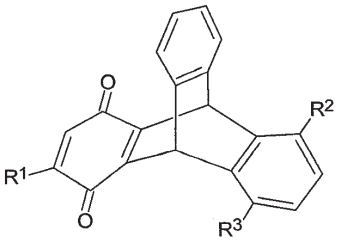
TT0: Triptycene



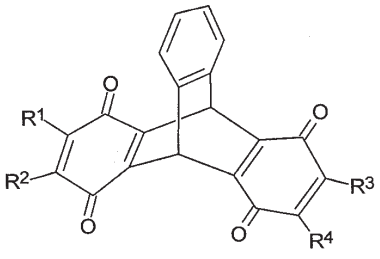
TT3



TT8



TT5: $R^1 = R^2 = R^3 = OMe$
TT7: $R^1 = R^2 = R^3 = H$
TT9: $R^1 = OMe$, $R^2 = R^3 = H$



TT2: $R^1 = OMe$, $R^2 = R^3 = R^4 = H$
TT13: $R^1 = OMe$, $R^2 = Br$, $R^3 = R^4 = H$
TT16: $R^1 = OMe$, $R^2 = Br$, $R^3 = NMe_2$, $R^4 = H$
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = NMe_2$
TT19: $R^1 = NHCH_2CH_2CO_2Et$, $R^2 = Br$, $R^3 = R^4 = H$
TT21: $R^1 = NHCH_2CH_2CO_2$-*t*-Bu, $R^2 = Br$, $R^3 = R^4 = H$
TT24: $R^1 = OMe$, $R^2 = Br$, $R^3 = NHMe$, $R^4 = H$
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = NHMe$
TT26: $R^1 = OMe$, $R^2 = Br$, $R^3 = N(Me)CH_2$-$CH{=}CH_2$, $R^4 = H$
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = N(Me)CH_2$-$CH{=}CH_2$

Figure 1. Chemical structures and code names of the synthetic TT analogs that have been shown to inhibit the viability of L1210 and HL-60 tumor cells in the nM range *in vitro*.

sensitive, human HL-60 promyelocytic leukemia cells within 2-4 days like DAU (2,5). Based on their ability to decrease L1210 and HL-60 cell viability by 50% at day 4 (IC_{50} values), using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium:phenazine methosulfate assay reagent to assess mitochondrial metabolism, bisquinones TT2, TT13, TT16, TT19, TT24 and TT26 are our current lead antitumor compounds (Fig. 1) (7,8). These substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetrones are consistently more potent against L1210 (IC_{50}: 48-135 nM) than against HL-60 cells (IC_{50}: 275-506 nM) (7,8). Bisquinone TT21 is somewhat weaker (IC_{50}: 176 and 567 nM in L1210 and HL-60 cells, respectively). The cytostatic/cytotoxic activities of TT13 (NSC 727282) and TT24 (NSC 727284) have been confirmed in all 60 human tumor cell lines of the NCI's *in vitro* antitumor screen, in which a sulforhodamine B protein assay is used to estimate cell growth and viability after 2 days of continuous drug exposure (8). The less potent TT5, TT7 and TT9 are representative examples of TT monoquinones with good antitumor activities (IC_{50}: 262-374 nM in L1210 cells and 649-941 nM in HL-60 cells). The dihydroquinone TT3 and the diketone TT8 lack quinone functionality but still elicit interesting apoptotic and antitumor effects *in vitro* (IC_{50}: 399-490 nM in L1210 cells and 1,175-1,523 nM in HL-60 cells) (7,8).

New RNA and protein syntheses and the activation of caspase, endonuclease and non-caspase protease enzymes are required to sustain the active mechanism by which low

concentrations of TT bisquinones induce as much apoptotic DNA fragmentation at 24 h as 20(*S*)-camptothecin (CPT) and DAU (2,5). In contrast to DAU, which is ineffective, TT bisquinones have the advantage of totally blocking the cellular transport of both purine and pyrimidine nucleosides within 15 min (2,5). The abilities of TT bisquinones to block nucleoside transport, inhibit DNA synthesis and induce DNA cleavage persist upon drug removal, suggesting that the interactions of these compounds with various molecular targets in cell membrane and nuclei are rapid and irreversible. Moreover, antitumor TT bisquinones are dual inhibitors of DNA topoisomerase (Topo) I and II activities, which match the Topo I inhibitory effect of CPT and surpass the Topo II inhibitory effect 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 (7). In addition to their potency and unusual mechanism of action, TT bisquinones retain their ability to induce caspase-3 activity, poly(ADP-ribose) polymerase-1 (PARP) cleavage and internucleosomal DNA fragmentation, to block nucleoside transport, to inhibit DNA synthesis, and to reduce tumor cell growth and viability in two MDR HL-60-RV and HL-60-R8 sublines that have different mechanisms of resistance to DAU, suggesting that these new quinone antitumor drugs might have other molecular targets than those of DAU and might be valuable in polychemotherapy to potentiate the action of antimetabolites and circumvent MDR (3,6,8).

Recently, antitumor TT bisquinones, TT2, TT13, TT16, TT19, TT24 and TT26, were shown to trigger several early and late markers of apoptosis in WT and MDR HL-60 cells, including cytochrome *c* (Cyt *c*) release, caspase-2, -8, -9 and -3 activation and PARP cleavage within 6 h and internucleosomal DNA fragmentation within 24 h (6,8). Interestingly, 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 (8). Indeed, the antagonistic anti-Fas and anti-FasL monoclonal antibodies do not alter TT13-induced Cyt *c* release and caspase-2, -8 and -9 activation. Also, 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. However, the caspase-2 inhibitor, z-Val-Asp-Val-Ala-Asp (VDVAD)-fmk, totally blocks TT13-induced caspase-8 and -9 activities but not TT13-induced Cyt *c* release, suggesting that induction of apical caspase-2 activity may be required for the activation of a downstream cascade of other initiator and effector caspases during antitumor TT treatments, which can also fully release mitochondrial Cyt *c* without caspase activation (8). Therefore, using fluorescent probes that localize to mitochondria and are specific of mitochondrial transmembrane potential ($\Delta\psi m$), the present study was undertaken to determine whether antitumor TT analogs, which can trigger Cyt *c* release without caspase activation and retain their ability to induce apoptosis in MDR HL-60 cells that are less sensitive to the DNA-damaging effects of DAU, might directly target mitochondria in cell and cell-free systems to cause the collapse of $\Delta\psi m$ ($\downarrow\Delta\psi m$) that is linked to the opening of the permeability transition pore (PTP) and the release of apoptogenic molecules from the mitochondrial intermembrane space (IMS) into the cytosol.

Materials and methods

Drug treatments. A new, short and easy method to synthesize the structures of the 12 TT analogs illustrated in Fig. 1 has been developed (4). The basic skeleton of TT bisquinone is readily synthesized by a two-step sequence leading to TT2, which is then converted to various antitumor analogs, such as TT13-TT26 (4). All solutions of parent TT0 (Aldrich, Milwaukee, WI), synthetic TT analogs, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (d-Ub, decylubiquinone) (both from Sigma, St. Louis, MO), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1 dye; Molecular Probes, Eugene, OR), staurosporine (STS), z-VDVAD-fmk, z-IETD-fmk, cyclosporin A (CsA) and gramicidin A (all from Calbiochem, La Jolla, CA) were dissolved and diluted in dimethyl sulfoxide, whereas mitoxantrone (MITOX), DAU (both from Sigma) and ruthenium red (RR; Fluka, Buchs SG, Switzerland) solutions were prepared in double-distilled water, 2-(6-amino-3-imino-3*H*-xanthe-9-yl)benzoic acid methyl ester (Rh 123 dye, rhodamine 123) and alamethicin (both from Sigma) were formulated in 50% EtOH, and bongkrekic acid (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. Suspension cultures of WT, drug-sensitive, human HL-60 promyelocytic leukemia cells (ATCC, Manassas, VA) were maintained in continuous exponential growth by twice-a-week passage in RPMI-1640 medium supplemented with 8.25% 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% CO_2 (3,6,8). The MDR HL-60-RV cells shown to overexpress P-glycoprotein (P-gp) were similarly maintained in RPMI-1640 medium in the absence of drugs but were exposed every 4 weeks to 41 nM DAU for 48 h, a non-cytotoxic concentration of DAU which stabilized their MDR phenotype and was removed from the culture medium at least 48 h before experimentation (3,6,8). For 10-min pulse-treatment, drug-containing supernatants were discarded after centrifugation (200 g x 5 min) and tumor cells were washed with and resuspended in fresh RPMI-1640 medium for further incubation.

Isolation of mitochondria. Mouse liver was used to isolate enough mitochondria to study TT-induced $\downarrow\Delta\psi m$ (9,10). Mitochondria 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. 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% bovine serum albumin, 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 rpm 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 (9,10). The protein concentrations of the mitochondrial samples were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

Mitochondrial depolarization assay in HL-60 cells. JC-1 is a lipophilic cationic dye used to detect the $\downarrow\Delta\psi m$ that occurs in cells undergoing the early stages of apoptosis because its potential-dependent accumulation and fluorescence emission shift are more specific for mitochondrial than plasma membrane potential (11,12). At high $\Delta\psi m$, JC-1 is internalized and concentrated at the mitochondrial inner membrane (IM) to form micelle-like J-aggregates, which dissociate at lower potential differences to become free JC-1 monomers. Since J-aggregates emit a longer wavelength than JC-1 monomers, the sudden decrease in the JC-1 fluorescence emission ratio of red aggregates/green monomers was detected at 595/535 nm to demonstrate the $\downarrow\Delta\psi m$ in TT-treated HL-60 cells (11,12). Tumor cells were suspended in 1.5-ml Eppendorf tubes at a density of $0.5x10^6$ cells/ml of complete RPMI-1640 medium and preincubated for various periods of time at 37˚C in the presence or absence (control) of TT analogs before being loaded for 30 min with 1 μl of fluorescent dye to obtain a final concentration of 0.3 μM JC-1 for WT HL-60 cells and 0.9 μM JC-1 for MDR HL-60-RV cells. After centrifugation (200 g x 5 min) and washing at room temperature to discard the drugs and unincorporated free dye, the fluorescence readings of the resuspended cell pellets were stabilized over a 20-min period at 37˚C. After transferring 0.2-ml aliquots of these incubates to a 96-well Costar white opaque polystyrene assay plate, the decreased JC-1 fluorescence ratio of red aggregates/green monomers linked to the $\downarrow\Delta\psi m$ was immediately detected at 490 nm excitation and 595/535 nm emission, using a Cary Eclipse Fluorescence Spectrophotometer equipped with microplate reader accessory (Varian, Walnut Creek, CA) (11,12).

Depolarization assay in isolated mitochondria. Because of its charge, solubility and $\Delta\psi m$-driven uptake, the lipophilic cationic dye, Rh 123, is a sensitive and specific probe of $\Delta\psi m$ in isolated mitochondria (13). After Rh 123 has localized to mitochondria, its retention or release is dependent on the maintenance or loss of $\Delta\psi m$ across the IM. The red shift in the excitation and emission spectra and the energy-dependent quenching of Rh 123 fluorescence intensity, which occur upon electrophoretic dye uptake, binding into the matrix space, and redistribution across the polarized IM of coupled mitochondria, are then rapidly and fully reversed when this fluorescent probe is released by uncoupled mitochondria following deenergization and $\downarrow\Delta\psi m$ (13). To assay the $\downarrow\Delta\psi m$ in isolated mitochondria, 1.5-ml Eppendorf tubes were sequentially supplemented with 1 ml of mitochondrial reaction buffer, 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, 4 μl of fluorescent dye to obtain a final concentration of 1 μM Rh 123, and 10 μl of isolated mitochondria (0.5 mg of protein). After incubation for 5 min at room temperature in the presence or absence (control) of various concentrations of TT analogs, 0.2-ml aliquots of these reaction mixtures were transferred to a 96-well Costar white opaque polystyrene assay plate and the unquenched fluorescence of the Rh 123 dye, which is released from mitochondria and indicative of the $\downarrow\Delta\psi m$, was monitored over a 5- to 30-min period at 503 nm excitation/525 nm emission, using a Cary Eclipse fluorescence microplate reader (13). Data were analyzed using the Student's t-test with a level of significance set at $P<0.05$.

Results

Mitochondrial depolarization in WT tumor cells. The $\downarrow\Delta\psi m$ caused by 25 μM CCCP is limited for up to 20 min but severe when HL-60 cells are incubated for 40 min or more in the presence of this reference depolarizing agent, suggesting that CCCP is mostly effective when added before, rather than after, the last 30-min period of JC-1 loading (Fig. 2A). In contrast, increasing 1.6-10 μM concentrations of bisquinone TT2 cause a rapid and steady $\downarrow\Delta\psi m$, which begins as early as 2.5 min and reaches a maximal level at 10-20 min, indicating that this new mitochondrial depolarizing drug is equally effective when added before or after the fluorescent dye (Fig. 2A). Since a 2.5-min treatment with 1.6 μM TT2 and a 40-min treatment with 10 μM TT2 are sufficient to match the $\downarrow\Delta\psi m$ caused by 25 μM CCCP in HL-60 cells over similar incubation periods, antitumor TT2 is a mitochondrial depolarizing agent at least 2.5 times more potent than CCCP in this cell system. No alteration of $\Delta\psi m$ is detected in HL-60 cells incubated with 10 μM DAU, even after 3 h (Fig. 2A).

Because CCCP was used as a positive control in other experiments, HL-60 cells were always incubated for 10 min in the presence of TT drugs prior to the 30-min period of loading with JC-1 in order to compare their effects to the maximal $\downarrow\Delta\psi m$ caused by this reference agent at 40 min (Fig. 2A). Under these conditions, all antitumor TT bisquinones tested at 4 and 10 μM are able to mimic the $\downarrow\Delta\psi m$ caused by 25 μM CCCP in HL-60 cells, 10 μM TT2 being again equipotent to 25 μM CCCP (Fig. 2B). When extremes are compared, TT2, which is clearly more effective than TT21 at reducing the viability of HL-60 cells (8), is also more potent than this compound at inducing the $\downarrow\Delta\psi m$ in this tumor cell system (Fig. 2B). TT2 and TT13, which are, apparently, some of the most effective mitochondrial depolarizing TT analogs, were retained for further studies in HL-60 cells. TT0, which has no cytotoxicity (8), also fails to alter $\Delta\psi m$ when tested up to 10 μM in HL-60 cells (Fig. 3A). But the other antitumor TT analogs with 0 or 1 quinoid ring, which are less cytotoxic than bisquinone TT2 (8), also produce $\downarrow\Delta\psi m$ effects that are weaker than those of TT2 (Fig. 3A). It should be noted that the monoquinones, TT5, TT7 and TT9, are not better inducers of $\downarrow\Delta\psi m$ than the dihydroquinone, TT3, and the diketone, TT8, which have no quinone functionality (Fig. 3A).

TT2 and TT13 cause the $\downarrow\Delta\psi m$ in HL-60 cells at 40 min in the same concentration-dependent manner and are both more potent than the reference agent, CCCP (Fig. 3B). The

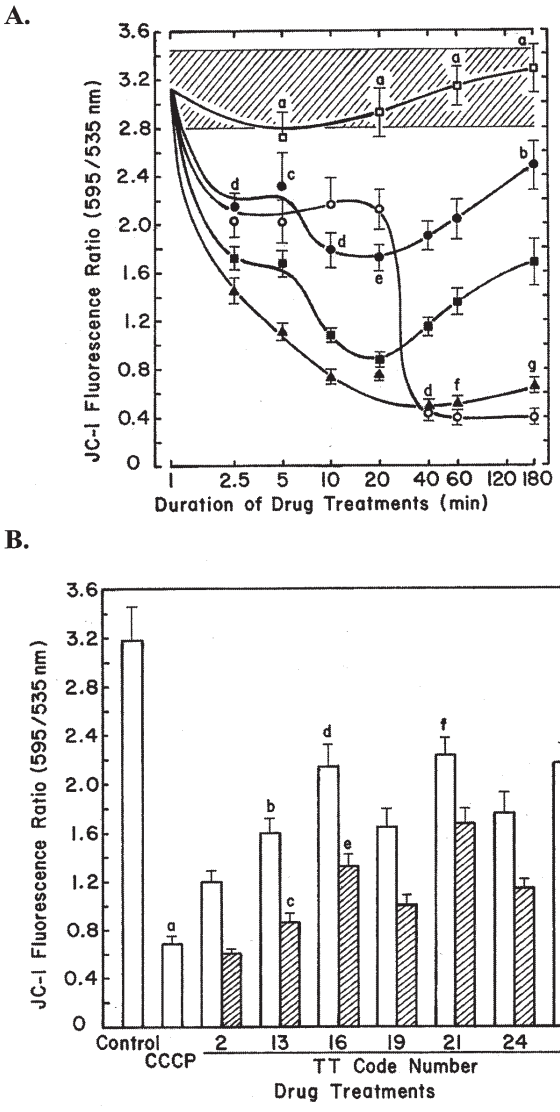


Figure 2. TT analogs induce mitochondrial depolarization in WT tumor cells. A, time-dependent decrease of the red:green fluorescence ratio of JC-1 aggregates:monomers indicating ↓Δψm in HL-60 cells treated with 25 μM CCCP (○), 10 μM DAU (□), or 1.6 (●), 4 (■) and 10 μM (▲) TT2. JC-1 was loaded for the last 30 min of incubation. Tumor cells were incubated for the indicated periods of time (plotted on a logarithmic scale) in the presence or absence (control) of the above concentrations of drugs, which were consequently added either before (180-40 min) or after (20-2.5 min) the fluorescent dye. Control, JC-1 fluorescence ratio in non-treated cells (3.12±0.32, striped area). Bars, means ± SD (n=3). [a]Not different to control; [b]P<0.05, smaller than control; [c]P<0.05, smaller than control but not different to CCCP or DAU; [d]not different to CCCP; [e]P<0.05, smaller than CCCP; [f]P<0.025 and [g]P<0.005, greater than CCCP. B, comparison of the abilities of 4- (open columns) and 10-μM (striped columns) concentrations of lead antitumor TT bisquinones to induce ↓Δψm after 40 min in HL-60 cells. A 25 μM CCCP treatment is used as a reference depolarizing agent. Control, 3.19±0.27. [a]Not different to 10 μM TT2; [b]P<0.01, greater than TT2 but not different to TT19 and TT24; [c]P<0.01, greater than TT2 and smaller than TT24 but not different to TT19; [d]P<0.05, greater than TT24 but not different to TT21 and TT26; [e]P<0.025, smaller than TT21 but not different to TT24 and TT26; [f]P<0.01, smaller than control; [g]P<0.01, greater than TT24 but not different to TT21.

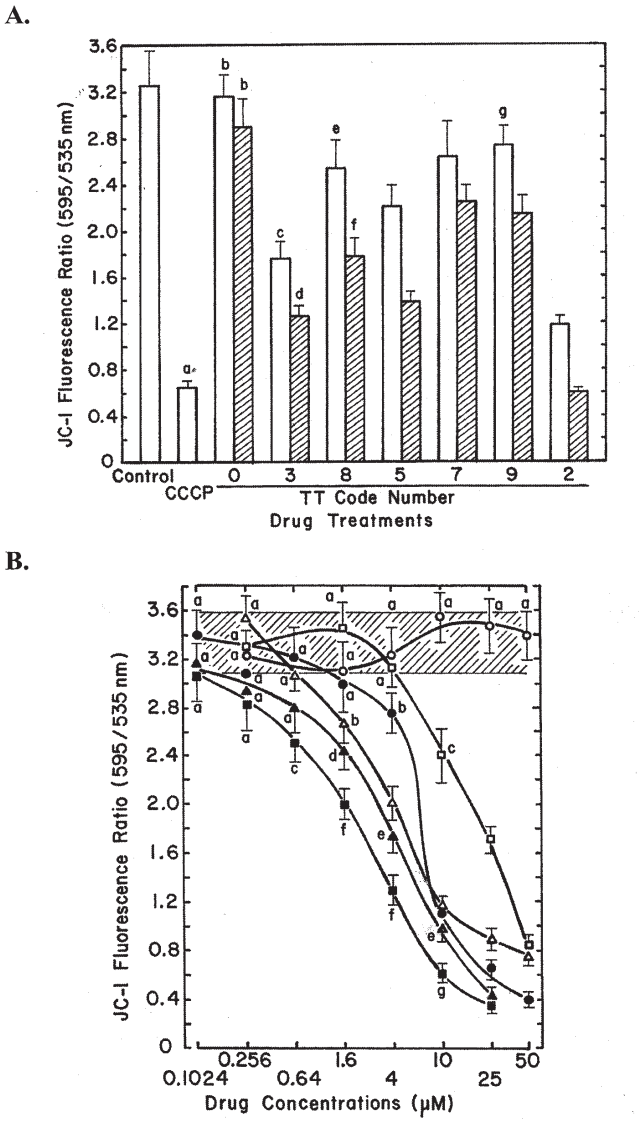


Figure 3. TT analogs induce mitochondrial depolarization in WT tumor cells. A, comparison of the abilities of 4- (open columns) and 10-μM (striped columns) concentrations of parental compound and antitumor TT analogs with different numbers of quinoid rings to induce ↓Δψm after 40 min in HL-60 cells. A 25 μM CCCP treatment is used as a reference depolarizing agent. JC-1 was loaded for the last 30 min of incubation. Control, red:green fluorescence ratio of JC-1 aggregates:monomers in non-treated cells (3.26±0.30). Bars, means ± SD (n=3). [a]Not different to 10 μM TT2; [b]not different to control; [c]P<0.005, greater than TT2 and P<0.05, smaller than TT5; [d]P<0.0005, greater than TT2 but not different to TT5; [e]not different to TT5, TT7 and TT9; [f]P<0.025, greater than TT5 and smaller than TT7, and P<0.05, smaller than TT9; [g]P<0.05, smaller than control. B, comparison of the abilities of serial concentrations (plotted on a logarithmic scale) of TT2 (■), TT13 (▲), MITOX (△), STS (□), DAU (○) and CCCP (●) to induce ↓Δψm after 40 min in HL-60 cells. Control, 3.34±0.25, striped area. [a]Not different to control; [b]P<0.025 and [c]P<0.01, smaller than control; [d]P<0.01, smaller than control but not different to MITOX; [e]not different to MITOX; [f]P<0.025 and [g]P<0.005, smaller than TT13.

concentration-dependent induction of ↓Δψm by TT2, which is slightly more potent than TT13, starts at 0.64 μM and appears to be maximal at 25 μM. Under similar conditions, DAU is unable to cause mitochondrial depolarization in HL-60 cells, even at 25- to 50-μM concentrations (Fig. 3B). MITOX, which is nearly as potent as TT13, and STS, which is somewhat less effective than CCCP, are the only other drugs tested that are capable of causing the concentration-dependent ↓Δψm in HL-60 cells at 40 min (Fig. 3B). Various other anticancer drugs tested over the 1.6-50 μM range, including vincristine, vinblastine, VP-16, taxol, nocodazole, tubulozole C, metho-

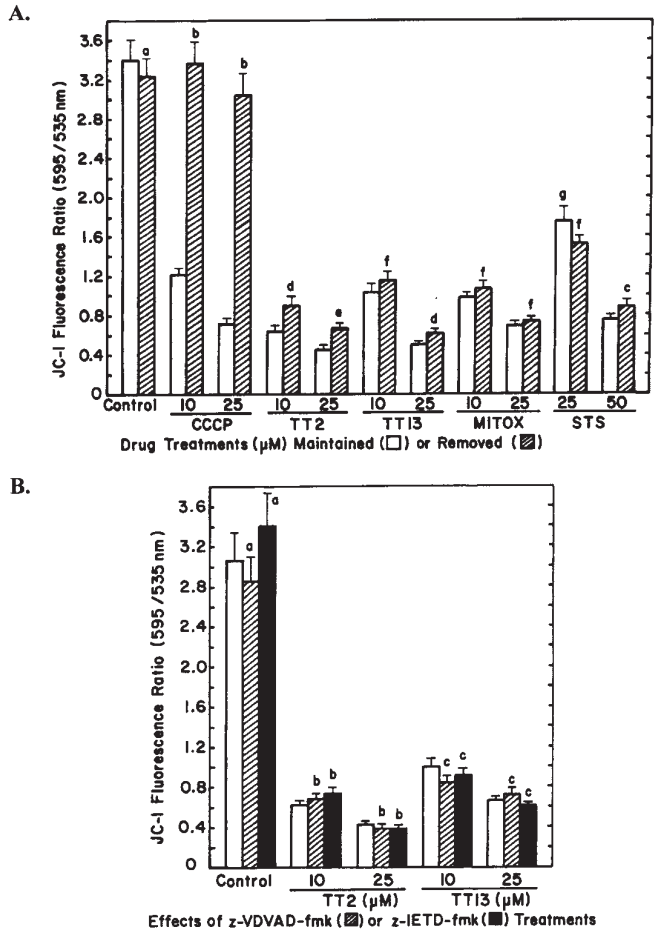


Figure 4. TT analogs induce mitochondrial depolarization in WT tumor cells. A, in contrast to 10-25 μM CCCP, the $\downarrow\Delta\psi$m caused by a 10-min pulse-treatment with 10-25 μM TT2, TT13 and MITOX or 25-50 μM STS is irreversible 30 min after drug removal in HL-60 cells. The drugs were either maintained in the medium for the whole 40-min period of incubation (open columns) or removed after the first 10 min (striped columns) in order to complete the remaining 30 min of incubation in the absence of drugs. JC-1 was loaded for the last 30 min of incubation. Control, JC-1 fluorescence ratio in non-treated cells similarly incubated for 40 min (3.40±0.21, open) or spun, washed and resuspended in fresh medium after the first 10 min of the 40-min period of incubation (3.24±0.17, striped). Bars, means ± SD (n=3). [a]Not different to the other control; [b]not different to both controls; [c]P<0.05, [d]P<0.025 and [e]P<0.01, greater than similar concentrations of drugs maintained for 40 min in the incubation medium; [f]not different to similar concentrations of drugs maintained for 40 min; [g]P<0.0005, smaller than control. B, pretreatments with specific inhibitors of initiator caspase-2 and -8 activities do not prevent antitumor TT bisquinones from inducing $\downarrow\Delta\psi$m after 40 min in HL-60 cells. Tumor cells were preincubated for 30 min in the presence of dimethyl sulfoxide vehicle (open columns) or 25-μM concentrations of z-VDVAD-fmk (striped columns) or z-IETD-fmk (solid columns) and, after supplementing their culture medium with either vehicle (control) or 10- and 25-μM concentrations of TT2 or TT13, these incubations were continued for an additional 40 min to determine the $\downarrow\Delta\psi$m. JC-1 was loaded for the last 30 min of incubation. Control, JC-1 fluorescence ratio in non-treated cells similarly incubated in the absence of caspase inhibitor (3.06±0.28, open). [a]Not different to control in the absence of caspase inhibitor; [b]not different to similar concentrations of TT2 in the absence of caspase inhibitor; [c]not different to similar concentrations of TT13 in the absence of caspase inhibitor.

trexate, cytosine ß-D-arabinofuranoside, 5-fluorouracil, cyclophosphamide, *cis*-dichlorodiammine platinum (*cis*-DDP) and CPT, all fail to alter $\Delta\psi$m in HL-60 cells at 40 min (data not shown).

To determine whether or not these mitochondrial depolarizing effects are reversible, HL-60 cells were exposed to 10-25 μM CCCP, TT2, TT13 or MITOX and 25-50 μM STS for only 10 min before these drugs were removed, and the magnitudes of drug-induced $\downarrow\Delta\psi$m were assayed as usual at 40 min (Fig. 4A). Because the $\downarrow\Delta\psi$m caused by CCCP in HL-60 cells at 40 min disappears when this agent is removed at 10 min, the mitochondrial depolarizing effect of CCCP, which is rapidly and fully reversible, may require the continual presence of the compound in the incubation medium in order to persist up to 40 min (Fig. 4A). In contrast, 10-min pulse-treatments with TT2, TT13, MITOX and STS are sufficient to trigger as much $\downarrow\Delta\psi$m at 40 min as when these drugs are maintained in the culture medium for the whole 40-min period of incubation. Hence, the mechanisms by which antitumor TT analogs, MITOX and STS rapidly induce the $\downarrow\Delta\psi$m in HL-60 cells are irreversible and persist after drug removal (Fig. 4A).

Since apical caspase-2 and -8 may be involved upstream of mitochondria in the apoptotic pathway, the same 25 μM z-VDVAD-fmk and z-IETD-fmk pretreatments shown to totally abolish TT13-induced caspase-2 and -8 activities at 6 and 8 h, respectively, in HL-60 cells (8) were used to determine whether they would also block the very early and rapid mitochondrial-depolarizing effects of antitumor TT analogs in this tumor cell system. However, this was not the case (Fig. 4B). These specific caspase-2 and -8 inhibitors were both unable to prevent 10-25 μM TT2 and TT13 from inducing their usual $\downarrow\Delta\psi$m in HL-60 cells at 40 min. Such initiator caspases, therefore, are unlikely to play a role in the molecular mechanism by which antitumor TT analogs interact with mitochondria to trigger their depolarization (Fig. 4B).

Mitochondrial depolarization in MDR tumor cells. Since antitumor TT bisquinones retain their ability to induce caspase-3, -8 and -9 activities and PARP cleavage at 6 h, internucleosomal DNA fragmentation at 24 h and cytostatic/cytotoxic effects at 2-4 days in MDR HL-60 sublines, the mitochondrial-depolarizing abilities of TT2, TT13, DAU and MITOX were assessed and compared in the P-gp-positive HL-60-RV cells, which, in earlier studies, proved to be the most resistant to the action of DAU (3,6,8). Indeed, TT2, TT13 and MITOX all retain their ability to induce after 40 min the same $\downarrow\Delta\psi$m in MDR HL-60-RV cells (Fig. 5) as that previously observed in the WT parental HL-60 cell line (Figs. 3B and 4A). These lead antitumor TT bisquinones and MITOX at 4-25 μM are even more effective mitochondrial-depolarizing agents than CCCP at 25-50 μM in this MDR tumor subline (Fig. 5). Not surprisingly, 10-25 μM DAU, which is unable to cause $\downarrow\Delta\psi$m in WT cells (Figs. 2A and 3B), does not do it in the MDR subline either (Fig. 5).

Depolarization in isolated mitochondria. Freshly isolated mitochondria were used to determine whether antitumor TT analogs would directly target these organelles to induce the $\downarrow\Delta\psi$m that is one of the markers of mitochondrial permeability transition (MPT). Positive controls, such as CCCP and the transmembrane channel-forming peptides, alamethicin and gramicidin A, which function as uncouplers of mitochondrial oxidative phosphorylation by dissipating electrochemical

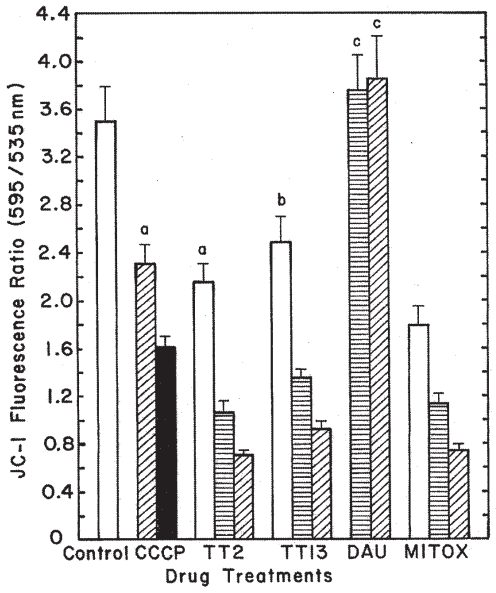


Figure 5. TT analogs induce mitochondrial depolarization in MDR tumor cells. Comparison of the abilities of 4 (open columns), 10 (horizontal stripes), 25- (diagonal stripes) or 50-μM (solid columns) concentrations of the lead antitumor TT bisquinones, TT2 and TT13, DAU, MITOX and the reference depolarizing agent, CCCP, to induce $\downarrow\Delta\psi$m after 40 min in MDR HL-60-RV cells. JC-1 was loaded for the last 30 min of incubation. Control, JC-1 fluorescence ratio in non-treated cells (3.49±0.30). Bars, means ± SD (n=3). [a]P<0.005 and [b]P<0.01, smaller than control; [c]not different to control.

gradients across the IM, directly cause rapid and massive $\downarrow\Delta\psi$m in isolated mitochondria within 20 min (Fig. 6). Since the open-closed transition of the PTP is Ca^{2+}-sensitive (14,15) and mitochondrial Ca^{2+} overload triggers PTP opening, IM permeabilization, $\downarrow\Delta\psi$m, swelling and apoptosis (16), 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 the $\downarrow\Delta\psi$m in our suspension of isolated mitochondria (Fig. 6). As expected, this Ca^{2+} overload-induced $\downarrow\Delta\psi$m is totally blocked by RR, which prevents the Ca^{2+} uniporter from accumulating the exogenously added divalent cation into the mitochondrial matrix (Fig. 6). The $\downarrow\Delta\psi$m caused by 100 μM Ca^{2+} in isolated mitochondria is also abolished or inhibited by several MPT blockers known to interact with various components of the PTP complex to prevent its opening, including CsA, which binds to cyclophilin D; BA, a ligand that stabilizes the adenine nucleotide translocator (ANT) in an inactive conformation; and d-Ub, which interacts with the ubiquinone (Ub)/quinone-binding site of the PTP to induce conformational changes that decrease its Ca^{2+} sensitivity (Fig. 6) (14,15).

Interestingly, 25 μM TT2, which is inactive alone, requires the presence of 20 μM Ca^{2+} in order to induce the same $\downarrow\Delta\psi$m in isolated mitochondria at 20 min as that caused by 100 μM Ca^{2+} (Fig. 6). Antitumor TT bisquinones, therefore, might interact with the PTP to induce conformational changes that increase its Ca^{2+} sensitivity, so that a low and normally ineffective 20 μM concentration of exogenous Ca^{2+} now becomes sufficient to prime PTP opening during TT analog treatment (Fig. 6). This hypothesis is substantiated by the fact that TT2-induced $\downarrow\Delta\psi$m in isolated mitochondria is abolished in the presence of RR (Fig. 6). Since the Ca^{2+}-dependent depolarizing effect of TT2 in isolated mitochondria is also blocked by CsA, BA and d-Ub, direct interactions with the Ub/quinone-binding site or other components of the PTP complex might be involved in the mechanism by which antitumor TT analogs cause rapid $\downarrow\Delta\psi$m in cell and cell-free systems (Fig. 6).

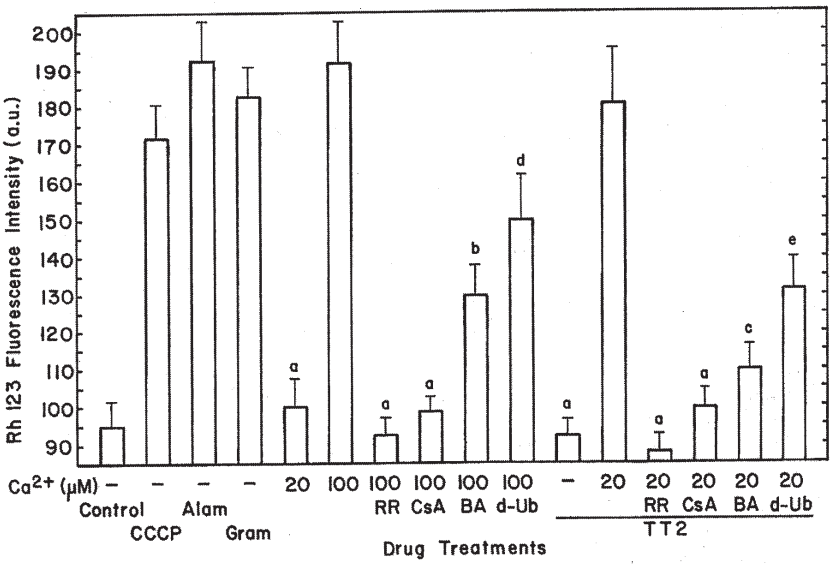


Figure 6. TT analogs induce mitochondrial depolarization in a cell-free system. Comparison of the abilities of 4 μM CCCP, 40 μg/ml alamethicin, 3 μM gramicidin A, 20-100 μM $CaCl_2$ and 25 μM TT2 to directly induce $\downarrow\Delta\psi$m in isolated mitochondria, based on the unquenched fluorescence (arbitrary units) of Rh 123 released from these drug-treated organelles at 20 min. The depolarizations of isolated mitochondria directly caused by a 100 μM Ca^{2+} overload or by 25 μM TT2, which requires the presence of a 20 μM Ca^{2+} primer, are both blocked or inhibited by 0.1 μM RR, 1 μM CsA, 50 μM BA and 100 μM d-Ub pretreatments. Control, Rh 123 fluorescence intensity in non-treated suspensions of isolated mitochondria (95.0±6.7). Bars: means ± SD (n=3). [a]Not different to control; [b]P<0.005 and [c]P<0.05, greater than control; [d]P<0.025, smaller than 100 μM Ca^{2+}; [e]P<0.01, smaller than 25 μM TT2 in the presence of 20 μM Ca^{2+}.

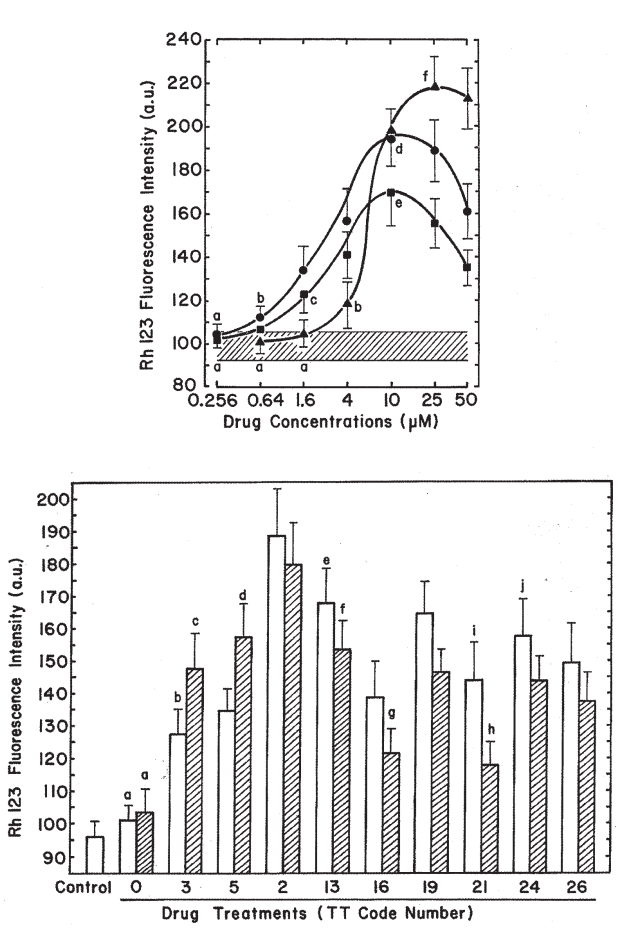


Figure 7. TT analogs induce mitochondrial depolarization in a cell-free system. A, comparison of the abilities of serial concentrations (plotted on a logarithmic scale) of TT2 (•) TT13 (■) and STS (▲) to directly induce, in the presence of a 20 μM Ca^{2+} primer, $\downarrow\Delta\psi$m in isolated mitochondria, based on the unquenched fluorescence (arbitrary units) of Rh 123 released from these drug-treated organelles at 20 min. Control, Rh 123 fluorescence intensity in non-treated suspensions of isolated mitochondria (98.9±6.5, striped area). Alone, 20 μM Ca^{2+} is unable to depolarize isolated mitochondria (102.4±5.9; not different to control), whereas a 100 μM Ca^{2+} overload does and is used as a reference depolarizing treatment (189.8±9.9). Bars, means ± SD (n=3). [a]Not different to control; [b]P<0.05 and [c]P<0.025, greater than control; [d]not different to 25 μM TT2 but P<0.05 and P<0.025, greater than 50 and 4 μM TT2, respectively; [e]not different to 25 μM TT13 but P<0.05 and P<0.025, greater than 4 and 50 μM TT13, respectively; [f]not different to 10 and 50 μM STS. B, comparison of the abilities of 10- (open columns) and 25-μM (striped columns) concentrations of parental compound (TT0) and antitumor TT analogs with 0 (TT3), 1 (TT5) or 2 (TT2, TT13, TT16, TT19, TT21, TT24 and TT26) quinoid rings to directly induce, in the presence of a 20 μM Ca^{2+} primer, $\downarrow\Delta\psi$m in isolated mitochondria at 20 min. Control: 96.0±4.8. 20 μM Ca^{2+} alone: 101.1±6.0, not different to control. 100 μM Ca^{2+} overload: 187.6±13.1. [a]Not different to control; [b]not different to 10 μM TT5, TT16 and TT21 but P<0.05, smaller than 10 μM TT26; [c]not different to 25 μM TT5, TT13, TT19, TT24 and TT26 but P<0.025, greater than 25 μM TT16; [d]not different to 25 μM TT2; [e]not different to 10 μM TT2, TT19, TT24 and TT26; [f]P<0.05, smaller than 25 μM TT2; [g]P<0.01 and [h]P<0.025, greater than control; [i]P<0.05, smaller than 10 μM TT13; [j]P<0.05, smaller than 10 μM TT2.

The Ca^{2+}-dependent inductions of $\downarrow\Delta\psi$m by bisquinones T2 and TT13 in isolated mitochondria at 20 min start at 0.64-1.6 μM and peak at 10 μM before declining thereafter (Fig. 7A). Under similar conditions, the Ca^{2+}-dependent induction of $\downarrow\Delta\psi$m by STS requires higher concentrations, starting at 4 μM and peaking at 25 μM (Fig. 7A). None of the various other anticancer agents tested over the 1.6-50 μM range, even including MITOX, is able to induce a significant $\downarrow\Delta\psi$m in isolated mitochondria incubated for 20 min in the presence of 20 μM Ca^{2+} (data not shown). Finally, 10-25 μM TT0, which has no antitumor activity (8) and does not induce mitochondrial depolarization in HL-60 cells, also fails to do so in the cell-free system (Fig. 7B). But all other antitumor TT analogs compared at 10-25 μM are capable of triggering the Ca^{2+}-dependent $\downarrow\Delta\psi$m in isolated mitochondria at 20 min (Fig. 7B). Even though the depolarizing effects of the dihydroquinone, TT3, and the monoquinone, TT5, at 10 μM are smaller than those of most but not all of the antitumor TT bisquinones, it seems that, despite their role and importance, the presence or number of quinone functions might not be absolutely required for antitumor TT analogs to directly interact with and increase the Ca^{2+} sensitivity of the PTP in order to trigger the depolarization of isolated mitochondria (Fig. 7B).

Discussion

The ability of antitumor TT analogs to cause a very rapid $\downarrow\Delta\psi$m in WT and MDR tumor cells and isolated mitochondria substantiates the hypothesis that, with or without quinone functionality, these new synthetic compounds might have a wider spectrum of molecular targets than DAU. The interest of TT analogs lies in the fact that, in addition to mimicking the antitumor effects of DAU tested so far *in vitro*, they have the advantage of directly targeting mitochondria to cause $\downarrow\Delta\psi$m, rapidly blocking the cellular transport of purine and pyrimidine nucleosides, inhibiting both Topo I and II activities, and retaining their efficacy in MDR cells, which DAU is unable to do under similar experimental conditions (2-8). The finding that our lead antitumor TT bisquinones cause a very rapid, Ca^{2+}-dependent and CsA-sensitive $\downarrow\Delta\psi$m in isolated mitochondria suggests that, irrespective of the initial and massive DNA damage these dual Topo I and II inhibitors (7) are likely to induce in tumor cells to indirectly trigger the intrinsic mitochondrial pathway of apoptosis like other conventional DNA-interacting anticancer drugs (8), TT2 and TT13 might represent a new class of apoptosis-targeted drugs that could directly target mitochondria *in situ* and interact with components of the PTP complex to induce MPT, release apoptogenic molecules in the absence of nuclear signal and, possibly, bypass mechanisms of apoptosis resistance. Such direct (through PTP interaction) and indirect (through sequential DNA damage, PARP activation and cytosolic and mitochondrial NAD^+ depletion) (17,18) means of inducing MPT and apoptosis might explain why multitargeted TT bisquinones remain effective in MDR tumor cells that have become less sensitive to the DNA-damaging effects of DAU (3,6,8), an anticancer drug which, according to our data, does not have the option of directly targeting mitochondria to rapidly cause their $\downarrow\Delta\psi$m.

The development of novel anticancer drugs that target mitochondria and directly activate early components of the apoptotic pathway, such as MPT, might bypass upstream mechanisms of MDR, including P-gp-mediated drug efflux, drug metabolism abnormalities, mutations of Topo enzymes, enhanced DNA repair and defective cell-cycle checkpoints (19-22). Directly permeabilizing mitochondrial membranes

might trigger the release of proapoptotic molecules independently from the Bcl-2 alterations involved in MDR. Lonidamide triggers ↓Δψm, 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 involving p53 or CD95 (9).

In contrast to conventional drugs such as DAU, DOX, *cis*-DDP and VP-16, which do not rapidly target isolated mitochondria in this and other studies (9,23-25), TT2 and TT13 irreversibly induce mitochondria to lose their Δψm in a caspase-independent manner within 15-20 min or less in cell and cell-free systems, suggesting that, irrespective of the other cellular and nuclear interactions or signaling pathways that they generate, TT drugs have also the potential to directly target this key organelle to initiate MPT and proapototic events. The reports that mitochondria isolated from cells or tissue previously treated for 12-48 h with high μM concentrations of DOX or other DNA-damaging drugs are severely impaired are totally different and suggest that, in contrast to TT analogs, indirect and delayed damage to mitochondria *in situ* is the consequence rather than the cause of DOX-induced cytotoxicity (9,26,27). Similarly, huge 500-μM concentrations of 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 TT2-induced ↓Δψm in isolated mitochondria is blocked or inhibited by CsA, BA and d-Ub, suggesting that antitumor TT bisquinones directly interact with components of the PTP complex to trigger this marker of MPT. The direct action of TT analogs might resemble that of hyperforin, which can induce a rapid and caspase-independent ↓Δψm within 30 min, whereas the delayed ↓Δψm observed 24-48 h after paclitaxel indicates that apoptosis is merely the consequence of earlier cytotoxic drug interactions (28). 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 (29). As PTP-targeting drugs causing ↓Δψm, synthetic TT analogs might be useful cancer cell death inducers when classic drugs requiring nuclear action lose their therapeutic efficacy.

Besides DAU, the failure of a wide spectrum of other Topo inhibitors, microtubule-disrupting compounds, antimetabolites and alkylating agents to mimic the rapid ↓Δψm induced by TT2 and TT13 in both cell and cell-free systems (data not shown) suggests that most of the current anticancer drugs do not have the option of directly targeting mitochondria like synthetic TT analogs but must first damage or disrupt other molecular targets in order to generate the signals that ultimately trigger the mitochondrial pathway of apoptosis. TT analogs and STS are the only compounds which consistently induce rapid ↓Δψm in both HL-60 cells and isolated mitochondria. The fact that MITOX, which is a mitochondrial-depolarizing agent as potent as TT13 in WT and MDR HL-60 cells, becomes totally inactive in isolated mitochondria (data not shown) possibly suggests that drug metabolism or other cellular targets absent from the cell-free system might be required to mediate the rapid ↓Δψm caused by this anticancer drug. It should be noted that, when emission wavelengths are scanned over a 513-713 nm range at the 503 nm excitation wavelength of the Rh 123 assay, antitumor TT analogs alone have no autofluorescence, do not displace the sharp peak of Rh 123 fluorescence at 525-530 nm either in the presence or absence of isolated mitochondria, but, obviously, 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 ↓Δψm data and ruling out any non-physiological drug-dye interference in the cell-free system.

The voltage-dependent anion channel (VDAC or porin) and ANT are the main components of the PTP complex which, at the contact sites between the mitochondrial outer membrane (OM) and IM, cooperate to form dynamic mega-channels that link the mitochondrial matrix to the cytosol (20,22,30). 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 (31-33). Besides 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 convert H^+ and O_2 to H_2O, and pump protons out of the matrix to the IMS to generate the Δψm 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 (30). Normally, antiapoptotic Bcl-2 and Bcl-x_L might maintain the VDAC in a physiological state, which facilitates ATP-ADP exchanges to sustain energy production but is not permeable to Cyt *c* (30). Conversely, proapoptotic Bax and Bak might disrupt VDAC conformation and integrity to induce a non-physiological open state, which permits the release of Cyt *c* and other soluble proteins from the IMS (30). The components of the megachannel responsible for MPT that may be targeted or regulated by TT drugs that act upstream of mitochondria to induce apoptosis remain to be identified (19,22).

The PTP functions as a CsA-sensitive and Ca^{2+}-, voltage-, pH- and redox-gated channel with several levels of conductance (34). 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 non-specific permeabilization of the IM to K^+, Mg^{2+} and Ca^{2+}, as well as to solutes of molecular weight <1,500 Da (34). Once such 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 causes the entry of water from the cytosol and a rapid mitochondrial swelling, which may rupture the OM and lead to the release of intra-mitochondrial Ca^{2+} and proapoptotic molecules from the IMS (16,31,34,35). PTP opening may be regulated by both ↓Δψm and the pH of the matrix but the relationships between ↓Δψm, MPT and apoptosis remain unclear. Changes in Δψm may

be critical but not sufficient for, or a universal step in, the apoptotic process (36). MPT is always followed by $\downarrow\Delta\psi m$ but $\downarrow\Delta\psi m$ is not always followed by MPT (34). For instance, direct $\downarrow\Delta\psi m$ caused by CCCP does not induce MPT *per se*. Moreover, 0.5 μM VP-16 causes mitochondrial hyperpolarization, whereas VP-16 concentrations <1-5 μM must be used to cause $\downarrow\Delta\psi m$ after 24 h, a late occurrence in HL-60 cells undergoing VP-16-induced apoptosis (37). In contrast, the ability of TT analogs to directly and rapidly cause $\downarrow\Delta\psi m$ might be advantageous for their apoptotic and antitumor activities since fast-growing cancer cells exhibit higher $\Delta\psi m$ and bioenergetic states than senescent, quiescent or normal dividing cells (38). Because MPT requires more than $\downarrow\Delta\psi m$ to occur, the ability of antitumor TT analogs to interact with mitochondria and trigger other MPT markers, such as IM permeabilization, swelling and Ca^{2+} release (35), should be established.

In addition, it would be of interest to determine whether the $\downarrow\Delta\psi m$ caused by TT2 and TT13 might be required for AIF rather than Cyt *c* release (31,35). Opening of PTP and channels controlled by Bcl-2 family members may cause a 2-step release of apoptogenic proteins from the IMS to the cytosol and nucleus (33,34). As a result of MPT being linked to $\downarrow\Delta\psi m$ and equilibration of matrix and cytosolic ion and metabolite concentrations, osmotic swelling of the matrix may rupture the OM and achieve the further release of proapoptotic factors from the IMS (39). Drugs that directly target isolated mitochondria trigger the release of AIF, which translocates to the nucleus and may be required upstream of caspase-8 cleavage to induce apoptotic DNA degradation by caspase-independent activation of nuclease (9,39-41). STS, which mimics the ability of TT2 and TT13 to rapidly trigger $\downarrow\Delta\psi m$, induces an MPT-dependent release of AIF responsible for a caspase-independent process of nuclear apoptosis, whereas VP-16, which fails to induce such rapid $\downarrow\Delta\psi m$ in our study, is unable to mimic the AIF effect of STS (21). The mechanisms of Cyt *c* release are complex and still unclear (32,42). Channels may be formed by the translocation and oligomerization of the proapoptotic Bax and Bak to the OM, a process induced by certain BH3-only members. Pores may result from the interactions between Bax subfamily members and the VDAC (33). And the OM may rupture from the swelling of mitochondria undergoing MPT when the surface of the IM with its unfolded cristae becomes much larger than that of the surrounding OM (32,42). Cyt *c* release is a universal event in apoptosis but can occur before, independently, or in the absence of $\downarrow\Delta\psi m$ and without concomitant PTP opening, mitochondrial swelling and OM rupture (36,40,41,43-46). Since the mitochondrial respiratory chain uses Cyt *c* to regulate the $\Delta\psi m$ and maintain ATP generation, Cyt *c* release might cause $\downarrow\Delta\psi m$ and death by ATP depletion (36). But the consensus is that AIF release requires MPT and $\downarrow\Delta\psi m$, whereas Cyt c release is not always dependent on those events. 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 usually sequestered and resistant to release by agents that disrupt the OM, to the IMS where it is more susceptible to release (47). Following $\downarrow\Delta\psi m$ and uncoupling, therefore, matrix remodeling might not induce but simply facilitate the complete recruitment and release of Cyt *c* (47). Bax activation may be a more plausible candidate for Cyt *c* release than $\downarrow\Delta\psi m$ (35). Following activation of the BH3-only molecules, Bid, Bad and Bim, a bifurcated pathway might ensure the complete release of Cyt *c* (48). 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 $\downarrow\Delta\psi m$ and IM remodeling, required to mobilize and fully release the cristae stores of Cyt *c* (48). The regulatory mechanisms by which mitochondrial-depolarizing TT analogs trigger the release of AIF, Cyt *c* and other apoptogenic molecules from the IMS remain to be studied.

ANT, a PTP component that 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 nucleotide ligand-binding loop faces the cytoplasm or matrix sides. Proapoptotic drugs and Bcl-2 members which directly 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 (49,50). Pretreatments with BA and the natural ANT ligands, ADP and ATP, 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 (49). Since BA interferes with TT2-induced $\downarrow\Delta\psi m$, it would be of interest to determine whether TT analogs interact with ANT to induce conformational changes that trigger Cyt *c* release and block the ANT-mediated transport of ADP in isolated mitochondria.

CsA and Ca^{2+} are critical regulators of MPT. ANT-1 and cyclophilin D overexpression promotes MPT and induces apoptosis (51). Normally, ANT-bound cyclophilin D may facilitate a Ca^{2+}-triggered conformational change that promotes ANT opening (52). The interaction of CsA with cyclophilin D, therefore, might form a complex that prevents cyclophilin D binding to the ANT and strongly inhibits MPT (52,53). The ability of CsA to block TT2-induced $\downarrow\Delta\psi m$ in isolated mitochondria suggests that TT analogs might interact with the ANT to induce PTP opening. However, CsA pretreatments fail to prevent the rapid $\downarrow\Delta\psi m$ caused by TT analogs in HL-60 cells (data not shown), suggesting that several mechanisms might be involved in the mitochondrial depolarizing activity of these drugs in intact cells.

Since TT2 requires a priming concentration of Ca^{2+} to trigger RR-sensitive $\downarrow\Delta\psi m$ in isolated mitochondria, it is assumed that the endogenous concentration of Ca^{2+} in HL-60 cell cultures is sufficient to mediate such depolarizing action of TT analogs in mitochondria *in situ*. Ca^{2+} loading triggers Ca^{2+}-induced $\downarrow\Delta\psi m$ and Cyt *c* release, which are prevented by CsA. Bcl-2 and Bcl-x_L might prevent PTP opening by maintaining a physiological uptake of mitochondrial Ca^{2+}, whereas Bax and Bak relocation to the endoplasmic reticulum (ER) might release sarcoplasmic ER ATPase and induce the redistribution of Ca^{2+} from ER stores to mitochondria, thereby triggering MPT and apoptosis (54,55).

Ca^{2+}-dependent and CsA-sensitive MPT is an exclusive characteristic of quinones which only redox cycle. Ca^{2+}-

independent and CsA-insensitive $\downarrow\Delta\psi m$ and MPT can be induced by electrophilic quinones which are potent arylators but do not redox cycle. However, not all antitumor TT analogs inducing $\downarrow\Delta\psi m$ have quinone functionality and, if their MPT effects are both CsA-sensitive and -insensitive, TT analogs might have mixed chemical reactivities and could elicit both oxidative and electrophilic responses in mitochondria. Since CsA blocks TT2-induced $\downarrow\Delta\psi m$ in isolated mitochondria (Fig. 6) but not in HL-60 cells (data not shown), CsA might be effective against the oxidative component of quinone-induced toxicity but might not protect cells from the toxic effects resulting from arylation of critical nucleophiles by electrophilic quinone drugs (56). Differential sensitivity to CsA suggests that both the redox cycling and arylating activities of TT2 and TT13 might be involved in MPT induction. Redox cycling quinones might induce MPT by altering the regulation of CsA-sensitive PTP, whereas arylating quinones might directly depolarize mitochondrial membranes, an effect which, depending on the availability of matrix Ca^{2+}, might be expressed as CsA-insensitive MPT (56).

Regulatory quinones that interact with the Ub/quinone-binding site of the PTP may induce conformational changes that alter its Ca^{2+}-binding affinity, making it either less or more sensitive to Ca^{2+}, define the minimal Ca^{2+} load required to induce PTP opening, and modulate its open-closed transition (14,15). (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 (15). Inactive Ub_5 can block both the stimulatory or inhibitory effects of other PTP-interacting quinones, suggesting that all quinones may compete for a common binding site (15). 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 TT analogs alone. However, antitumor TT analogs with 0, 1 or 2 quinoid rings can increasingly induce $\downarrow\Delta\psi m$ in the presence of 20 μM Ca^{2+}, an effect which is inhibited by d-Ub, suggesting that these hydrophobic drugs might increasingly 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.

Synthetic TT analogs might interfere with the action of 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 (15). 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 (57). Since TT analogs lower from 100 to 20 μM the Ca^{2+} concentration required to induce $\downarrow\Delta\psi m$, the TT drug-liganted state might confer a conformation to the pore that enhances its Ca^{2+} sensitivity.

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