Antitumor triptycene bisquinones: a novel synthetic class of dual inhibitors of DNA topoisomerase I and II activities

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Synthetic triptycene analogs (TT code number) mimic the antitumor effects of daunorubicin in the nanomolar range *in vitro*, but have the advantage of blocking nucleoside transport and retaining their efficacy in multidrug-resistant (MDR) tumor cells. Since TT bisquinones induce poly(ADP-ribose) polymerase-1 cleavage at 6 h and internucleosomal DNA fragmentation at 24 h, which are, respectively, early and late markers of apoptosis, these lead antitumor drugs were tested for their ability to trigger the DNA topoisomerase (Topo) inhibitions responsible for the initial and massive high-molecular-weight cleavage of DNA required for tumor cells to commit apoptosis. Interestingly, antitumor TTs have the unusual ability to inhibit, in a concentration-dependent manner, the relaxation of supercoiled plasmid DNA catalyzed by both purified human Topo I and II enzymes. However, if there is a relationship between the ability of TT analogs to inhibit Topo activities and their quinone functionality and cytotoxicity, it is far from perfect, suggesting that other molecular targets may be involved in the mechanism of action of these antitumor drugs. Moreover, one of the most cytotoxic TT bisquinone, 6-bromo-7-methoxy- or 7-bromo-6-methoxy-2-*N*-methylamino-1 *H*,4 *H*,5 *H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4,5,8-tetraone (TT24), inhibits Topo II activity more effectively than amsacrine (*m*-AMSA) and matches the Topo I inhibitory effect of camptothecin (CPT). The dual inhibitory activity of TT24 is substantiated by the findings that TT24 mimics the action of *m*-AMSA in the Topo II assay, where the Topo I inhibitor CPT is ineffective, and also mimics the action of CPT in the Topo I assay, where the Topo II inhibitor etoposide is ineffective. Because of their ability to target nucleoside transport and topoisomerase activities, synthetic TT bisquinones might represent a novel class of bifunctional drugs valuable to develop new means of polychemotherapy and circumvent MDR. *Anti-Cancer Drugs* 14:503–514 © 2003 Lippincott Williams & Wilkins.

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Introduction

Although the exact bioactivity of their quinoid ring remains unclear, the few quinone antitumor drugs used clinically may be cytotoxic because of their involvement in soft electrophilic arylation and redox cycling oxidation [1]. The anthracycline quinone antibiotics doxorubicin (DOX, adriamycin) and daunorubicin (DAU, daunomycin) covalently bind to and intercalate into DNA, inhibit DNA replication and RNA transcription, are DNA topoisomerase (Topo) II poisons, produce oxidative stress and damage biomembranes, induce DNA breakage and chromosomal aberrations, trigger apoptosis, and have a wide spectrum of anticancer activity [2–7]. Since the clinical effectiveness of DOX and DAU is severely limited by their cumulative cardiotoxicity and ability to induce multidrug resistance (MDR), it is important to develop new quinone antitumor drugs with improved bioactivity [2].

Recently, we found that, in contrast to their inactive parent compound triptycene (code name TT0), several synthetic analogs (TT code number) with or without quinone functionality may represent a novel class of bifunctional antitumor drugs, which inhibit DNA, RNA and protein syntheses, induce DNA cleavage, and decrease the mitotic index, proliferation and viability of murine L1210 lymphocytic and human HL-60 promyelocytic leukemia cells like DAU [8–10]. Interestingly, the six lead antitumor compounds identified so far, TT2, TT13, TT16, TT19, TT21 and TT24, are all TT

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bisquinones, the latter compound being cytotoxic in the nanomolar range of DAU *in vitro* [9]. The critical finding is that, in contrast to DAU, lead antitumor TT bisquinones have the additional advantage of also blocking the cellular transport of purine and pyrimidine nucleosides and retaining their effectiveness in MDR HL-60 sublines that have already developed different mechanisms of resistance to DAU [8–10]. These preliminary studies suggest that antitumor TT analogs might have other molecular targets than those of DAU, and might be valuable in polychemotherapy to potentiate the action of antimetabolites and circumvent MDR [8–10].

In contrast to the early cleavage of DNA into large 50- to 300-kbp fragments, an initial signaling event that may induce tumor cells treated with relatively low concentrations of DNA-damaging anticancer drugs to commit apoptosis, the secondary endonucleolytic cleavage of DNA at internucleosomal linker sites to produce small 180- to 200-bp mono- and oligonucleosomal fragments at 24 h is a late molecular marker concurrent with morphological evidence of apoptosis [11]. The abilities of antitumor TT bisquinones to induce internucleosomal DNA fragmentation at 24 h have been demonstrated by two different techniques, using tumor cells containing [^{3}H]thymidine-prelabeled DNA to detect low-molecular-weight DNA fragments after intact chromatin precipitation or agarose gel electrophoresis to visualize the typical pattern of DNA laddering indicative of apoptosis. TT2 induces as much DNA fragmentation at 24 h as 20(*S*)-camptothecin (CPT) and DAU, two anticancer drugs producing DNA strand breaks and known to inhibit Topo I and II activities, respectively [8]. Moreover, increasing concentrations of TT2, TT24 and DAU produce the same biphasic increase and decrease of internucleosomal DNA fragmentation at 24 h, suggesting that the mechanisms by which these drugs induce DNA damage might share some similarities. Even though they are increasingly cytotoxic, higher than optimal concentrations of DAU and TT analogs might inhibit RNA and protein syntheses, inactivate enzymes and/or arrest cell cycle traverse to such excessive degrees that they actually block their own ability to sustain the molecular events required for the active process of apoptotic DNA fragmentation [8–10]. This hypothesis is substantiated by the finding that the abilities of TT24 and DAU to maximally induce internucleosomal DNA fragmentation at 24 h are inhibited by actinomycin D, cycloheximide, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone (z-IETD-fmk), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and $ZnSO_4$, suggesting that RNA and protein syntheses and cysteine and serine protease and nuclease activities might be required for these drugs to trigger DNA fragmentation through an active apoptotic pathway that involves caspase, non-caspase protease and endonuclease activations [9].

Cleavage and inactivation of poly(ADP-ribose) polymerase-1 (PARP-1) is an early event required for cells committed to apoptosis because it prevents the detection and repair of DNA damage, it blocks the depletion of NAD^+ and ATP causing necrotic cell death, and it enhances nuclease activity [12–15]. Since PARP-1 cleavage at 6 h is one of the first detectable protein-degradation events occurring after early high-molecular-weight cleavage of chromatin DNA, but before the late and low-molecular-weight internucleosomal fragmentation of DNA at 24 h, detection of the specific 85-kDa fragment of PARP-1 cleavage can serve as an early and sensitive marker of apoptosis in tumor cells treated with DNA-damaging anticancer drugs [12–15]. In contrast to TT0, all lead antitumor TT bisquinones share the ability to fully induce PARP-1 cleavage in wild-type (WT), drug-sensitive, HL-60-S cells [16]. Treatments with 1.6 μM concentrations of TT24 and DAU similarly induce maximal PARP-1 cleavage at 6 h in WT HL-60-S cells. However, in MDR HL-60-RV cells, PARP-1 cleavage is still induced by 4 μM TT24, but not by 4–10 μM DAU [16]. Since the abilities of TT24 and DAU to induce PARP-1 cleavage are inhibited by the cysteine protease inhibitor z-VAD-fmk, but not by the serine protease inhibitor TPCK, caspase activation is likely responsible for PARP-1 cleavage after DAU and TT24 treatments [16]. The fact that both z-VAD-fmk and TPCK can inhibit the internucleosomal fragmentation of DNA caused by DAU and TT24 at 24 h indicates that, in addition to executioner caspases, other non-caspase serine proteases, which are unable to target PARP-1 at 6 h, can also play a role in the mechanism by which these quinone antitumor drugs induce apoptotic DNA fragmentation at 24 h. Interestingly, a 1-h pulse treatment with TT24 or DAU is sufficient to induce the same level of PARP-1 cleavage at 6 h as when the drugs are maintained in the medium for the whole 6-h period of incubation [16]. Taken together, the facts that the cleavage of PARP-1, the inhibition of nucleoside transport/DNA synthesis and the biphasic elevation and decline of DNA fragmentation caused by TT2 and TT24 are irreversible in L1210 and HL-60-S cells, and remain unaltered in MDR HL-60 sublines suggest that substituted TT bisquinones may rapidly and tightly interact with various membrane and intracellular targets to disrupt the structures/functions of nucleoside transporters, nucleic acids and proteins, and to trigger long-lasting apoptotic and antitumor events, which persist after cessation of drug treatment and in DAU-resistant tumor cells [8–10,16]. Hence, the next goal is to explore further back in time to characterize which initial and massive molecular damaging events antitumor TT analogs are triggering in the first place in order to irreversibly

commit tumor cells to make an apoptotic decision within 1 h, resulting in early PARP-1 cleavage at 6 h and late internucleosomal DNA fragmentation at 24 h.

Some anticancer drugs may cause DNA single-strand breaks (SSBs), double-strand breaks (DSBs) and DNA–protein cross-links (DPCs), either directly by interacting with DNA or indirectly by targeting Topo I and II activities [2,4,17–19]. Such DNA-damaging events are then likely to induce apoptosis in tumor cells by triggering nuclear signals upstream of mitochondria [3,6,7,20–22]. Intercalation models have been postulated for the actions of CPT and DAU, which inhibit Topo I and II activities, respectively, by stabilizing the normally transient and reversible DNA–Topo I or II cleavable complexes [4,17–19,22]. Such stabilized ternary complexes of DNA–drug–Topo I or II may subsequently need to be processed by cellular machineries, such as collisions or interactions with DNA replication forks, transcription elongation complexes, DNA helicases and the ubiquitin/26S proteasome pathway, in order to be converted to long-lived and highly cytotoxic DNA DSBs, induce apoptosis and exert their lethality [4,17–19,22–25]. DNA-interacting antitumor agents may inhibit Topo I and II activities by two mechanisms: some drugs poison Topos by trapping these enzymes on DNA to form stabilized ternary complexes, while other catalytic inhibitors prevent these enzymes from binding to DNA and suppress the formation of cleavable complexes [4,17–19,22]. Because a few agents, such as intoplicine, saintopin, aclarubicin, actinomycin D, fagaronine derivatives, the indenoquinorine derivative TAS-103, the acridine derivative XR5000, the phenazine XR11576, the pentafluorinated epipodophylloid F11782, the pyrazolo[1,5-α]indole derivative GS-5, disulfiram, ellagic acid and the natural alkylphenol compound elenic acid, have recently been reported to inhibit both Topo I and II activities [26–39], the present study was undertaken to determine whether our lead antitumor TT bisquinones could target both of these nuclear enzymes. Indeed, TT24 is a novel dual inhibitor, which inhibits Topo II activity more effectively than amsacrine [*m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide] and matches the Topo I inhibitory effect of the pentacyclic alkaloid CPT, suggesting that these important molecular targets may also play a role in its mechanism of antitumor activity.

Materials and methods

Drugs and biochemicals

A new, short and easy method to synthesize the structures of the 10 TT analogs (code names TT2, TT3, TT5, TT7, TT8, TT9, TT13, TT16, TT19 and TT21) illustrated in Figure 1 has been developed [40]. The syntheses of TT24 and TT26 (Fig. 1) will be reported elsewhere. All stock solutions of 10 mM parent TT0 (Aldrich, Milwaukee, WI), synthetic TT analogs, *m*-AMSA, etoposide (VP-16, 4′-demethylepipodophyllotoxin–9-[4,6-*O*-ethylidene-β-D-glucopyranoside]) and CPT (all purchased from Sigma, St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO), stored frozen at −80°C and freshly diluted with double-distilled water before the Topo assays. Purified human Topo II (p170 form, IIα) and I enzymes, supercoiled (SC) plasmid pRYG DNA, and standards of linear (L) and relaxed (R) pRYG DNA markers were obtained from TopoGEN (Columbus, OH).

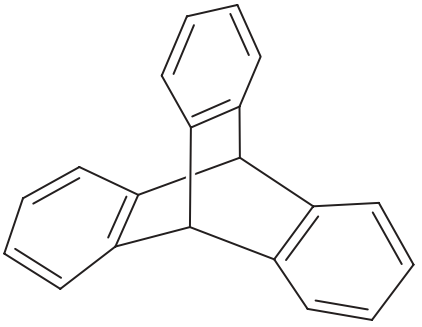
Cell viability assay

Suspension cultures of murine L1210 lymphocytic 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 (FCS; Premium Select, hybridoma qualified, triple 0.1 μm filtered, lot no. F0060; 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. Since TT analogs or DAU (Sigma) were supplemented to the culture medium in 1-μl aliquots, the concentration of vehicle (0.2% DMSO) in the final incubation volume (0.5 ml) did not affect the proliferation, viability, DNA synthesis, nucleoside transport, and basal levels of DNA fragmentation and PARP-1 cleavage in control tumor cells incubated in the absence of drugs [8–10]. For tumor cell viability, L1210 cells suspended in FCS-containing RPMI 1640 medium (initial density 4700 cells/0.5 ml) were grown in triplicate at 37°C in 48-well Costar cell culture plates for up to 4 days in the presence or absence (control) of increasing concentrations of drugs to evaluate their cytotoxicity [8,9]. The viability of drug-treated cells was assessed from their ability to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) reagent (Promega, Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product which absorbs at 490 nm [41]. After 4 days in culture, control and drug-treated cell samples (about 10^6/0.5 ml/well for controls) were further incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS : PMS (2 : 0.1) reagent and their relative cell viability was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL). Blank values for culture medium supplemented with MTS : PMS reagent in the absence of cells were subtracted from the results [8–10].

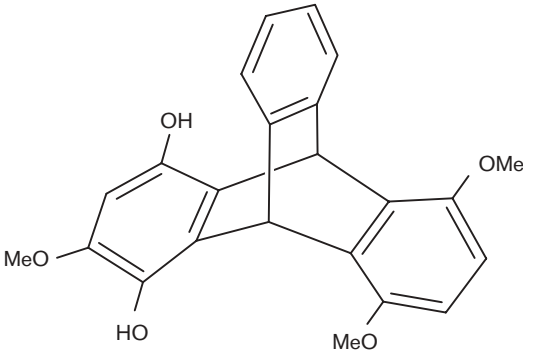
Topo I and II inhibition assays

The abilities of antitumor TT analogs to prevent the relaxation of SC pRYG plasmid DNA catalyzed by purified human Topo I or II enzymes were assessed and

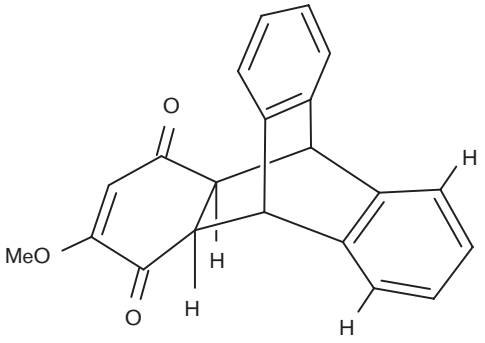
Fig. 1



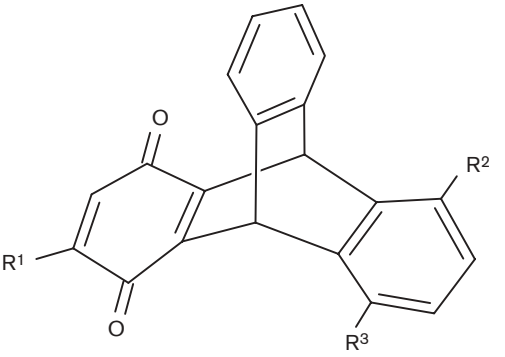
TT0: Triptycene (IC_{50} = NS)[a]



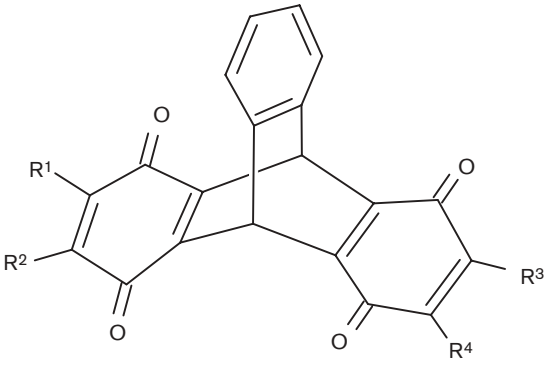
TT3: (IC_{50} = 399 ± 37 nM)[b]



TT8: (IC_{50} = 490 ± 38 nM)



TT5: $R^1 = R^2 = R^3 = OMe$ (IC_{50} = 374 ± 19 nM)
TT7: $R^1 = R^2 = R^3 = H$ (IC_{50} = 287 ± 25 nM)[c]
TT9: $R^1 = OMe$, $R^2 = R^3 = H$ (IC_{50} = 262 ± 17 nM)



TT2: $R^1 = OMe$, $R^2 = R^3 = R^4 = H$ (IC_{50} = 125 ± 12 nM)
TT13: $R^1 = OMe$, $R^2 = Br$, $R^3 = R^4 = H$ (IC_{50} = 135 ± 9 nM)[d]
TT16: $R^1 = OMe$, $R^2 = Br$, $R^3 = NMe_2$, $R^4 = H$ (IC_{50} = 116 ± 10 nM)[d]
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = NMe_2$
TT19: $R^1 = NHCH_2CH_2CO_2Et$, $R^2 = Br$, $R^3 = R^4 = H$ (IC_{50} = 110 ± 8 nM)[d]
TT21: $R^1 = NHCH_2CH_2CO_2$-*t*-Bu, $R^2 = Br$, $R^3 = R^4 = H$ (IC_{50} = 176 ± 14 nM)[e]
TT24: $R^1 = OMe$, $R^2 = Br$, $R^3 = NHMe$, $R^4 = H$ (IC_{50} = 48 ± 3 nM)[f]
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, $R^4 = H$ (IC_{50} = 116 ± 9 nM)[d]
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = N(Me)CH_2$-CH=CH_2

compared to those of known Topo I (CPT) and Topo II (*m*-AMSA, VP-16) inhibitors, using the drug screening kits from TopoGEN. For Topo II activity, the reaction mixture was 16 µl of 50 mM Tris–HCl buffer, pH 8.0, containing 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, and 0.25 µg of SC pRYG DNA as substrate. For Topo I activity, the reaction mixture was 17 µl of 10 mM Tris–HCl buffer, pH 7.9, containing 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol and 0.25 µg of SC pRYG DNA as substrate. Appropriate concentrations of antitumor drugs or DMSO vehicle only were supplemented to these mixtures in 2-µl aliquots and the reactions were started by the addition of either 2 µl of human Topo II (4 U) or 1 µl of human Topo I (1 U), respectively. After incubation for 30 min at 37°C, the Topo II and I reactions were terminated by the addition of 2 µl of 10% SDS and digested for 15 or 30 min, respectively, at 37°C in the presence of proteinase K (50 µg/ml). After addition of 1/10 volume of agarose gel loading buffer (0.25% bromophenol blue, 50% glycerol), samples were extracted with an equal volume of chloroform : isoamyl alcohol (24 : 1) by centrifugation at 14 000 *g* for 10 s and analyzed by agarose gel electrophoresis. DNA samples (15 µl of the blue-colored upper layer from the chloroform : isoamyl alcohol extraction) were loaded onto a 1% agarose gel, separated by electrophoresis for 3–4 h at 57 V in 1 × TAE buffer (50 × stock solution is 242 g of Tris base, 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA), stained with ethidium bromide (EB; 0.5 µg/ml) for 1 h and rinsed in distilled water for 30 min. The DNA bands were visualized and photographed with Polaroid 667 film under UV light at 312 nm, using a FisherBiotech model 88A variable-intensity UV transilluminator. Appropriate standards of L (0.05 µg/µl) and R (0.25 µg/µl) pRYG DNA markers were simultaneously electrophoresed in each Topo II assay, whereas standards of R plasmid DNA (0.05 µg/µl) were simultaneously run in each Topo I assay.

Results

Inhibition of tumor cell viability

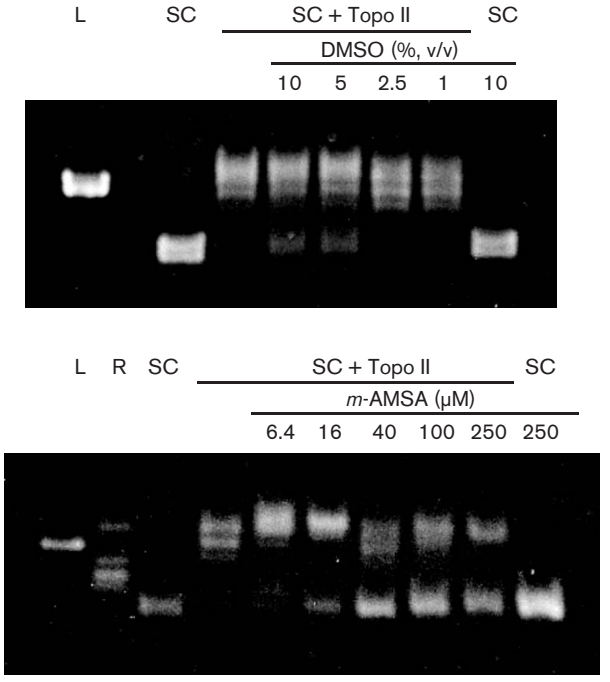
The chemical structures and code names of the antitumor TT analogs (serial numbers indicate the order of synthesis) selected to be tested for their ability to inhibit the catalytic activities of Topo I and II *in vitro* are depicted in Figure 1. The known parent compound TT0 is commercially available, but all other TT analogs under study were synthesized in Hua's laboratory by *in situ* oxidation of substituted dihydroxybenzene followed by [4 + 2] cycloaddition with 1,4-dimethoxyanthracene and then oxidation [8–10,16]. This new, short and easy method to synthesize substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetrones has been reported [40]. The correct nomenclatures and bioactivities of most of these rigid tetracyclic skeletons, which possess either 0, 1 or 2 external quinones, have already been described up to TT24 in the L1210 and HL-60 tumor cell systems [8–10,16,40]. The nomenclature of the new bisquinone TT26 is 2-(*N*-allyl-*N*-methylamino)-6-bromo-7-methoxy-9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetrone. The antileukemic effects of these TT analogs were assessed and compared to those of DAU, a clinically valuable anticancer drug that is structurally very different but also contains a *para*-quinone moiety [8–10,16,40]. In contrast to their parent compound TT0, which is inactive up to 4 µM, all other TT analogs selected for this Topo study have potent antitumor activities, based on the comparative list of nanomolar concentrations required to inhibit by 50% the viability of L1210 cells after 4 days in culture *in vitro* (Fig. 1). Because of its excellent cytotoxicity, the new TT26 increases our current pool of lead antitumor TT bisquinones, in which TT2, TT13, TT16, TT19, TT21 and TT24 are also included. TT21 is slightly less potent than the others but the most effective among these TT bisquinones is TT24, which inhibits L1210 tumor cell viability with an IC_{50} value of 48 nM and is the focus of the present Topo study (Fig. 1). However, it should be noted that DAU, the well-known anthracyclinone characterized by a fused linear tetracyclic system with an internal quinone and a glycosidic moiety, has an IC_{50} value of 25–30 nM under similar conditions [8,9,40]. Although somewhat less potent, TT5, TT7 and TT9 are representative examples of TT monoquinones closely related to the above TT bisquinones, and with good antitumor activities in the L1210 system (Fig. 1). The dihydroquinone TT3 and the diketone TT8 have also been selected for this Topo study because they demonstrate the fact that synthetic TT analogs lacking quinone functionality can still elicit interesting antitumor effects in L1210 cells (Fig. 1).

Inhibition of Topo II catalytic activity

Antitumor TT analogs were tested for their ability to inhibit the catalytic activity of purified human Topo II *in vitro* by measuring the relaxation of SC plasmid pRYG

Chemical structures and code names of the synthetic TT analogs tested for their abilities to inhibit the relaxation activities of Topo I and II *in vitro*. The concentrations of TT analogs required to inhibit by 50% the viability of murine L1210 tumor cells at day 4 *in vitro* were assessed and compared as indicated in Materials and methods. Cell viability results (means ± SD, $n = 3$) were expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control tumor cells ($A_{490\,nm} = 1.533 \pm 0.104$, 100 ± 7%) after 4 days in culture. The blank value ($A_{490\,nm} = 0.386$) for cell-free culture medium supplemented at day 4 with MTS : PMS reagent was subtracted from the results. IC_{50} values were calculated from linear regression of the slopes of the log-transformed concentration–survival curves. [a]Not significantly different from control when tested up to 4 µM; [b]$p < 0.05$, smaller than TT8, but not different from TT5; [c]$p < 0.01$, smaller than TT5, but not different from TT9; [d]not different from TT2; [e]$p < 0.01$, greater than TT2 but $p < 0.005$, smaller than TT9; [f]$p < 0.0005$, smaller than TT2.

Fig. 2

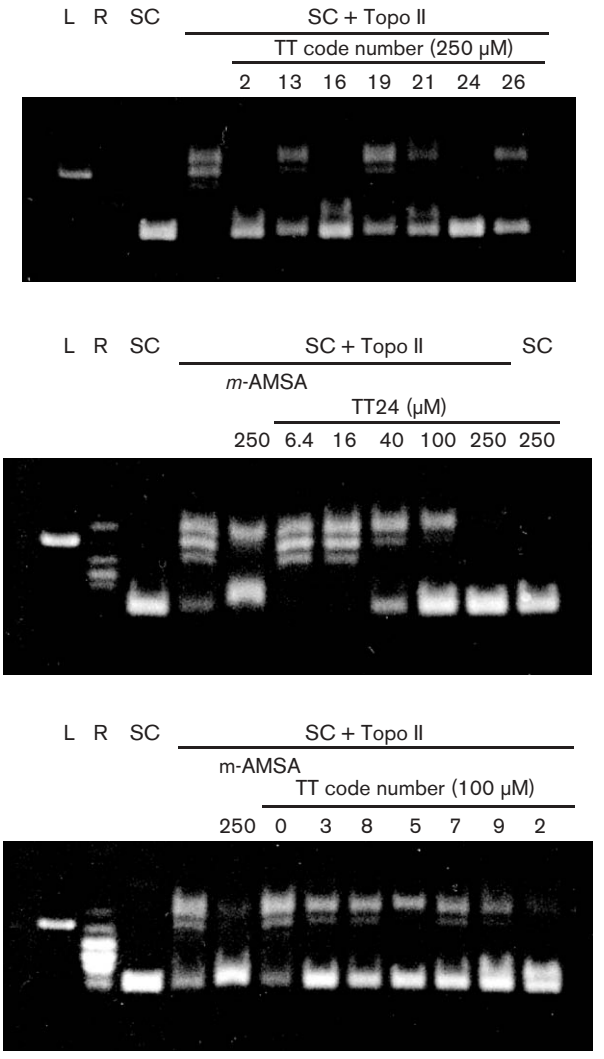


Characterization of the effects of DMSO vehicle and the known Topo II inhibitor *m*-AMSA in the Topo II reaction system. (Top) DMSO was tested at final concentrations of 1, 2.5, 5 and 10% (v/v) in the Topo II relaxation assay mixture. (Bottom) Concentration-dependent inhibition of Topo II catalytic activity by 6.4–250 μM *m*-AMSA. L: linear DNA marker; R: relaxed pRYG DNA marker; SC: supercoiled pRYG plasmid DNA substrate.

DNA, which is a pUC19 derivative in which the 206-bp *Sph*I–*Nar*I fragment of pUC19 has been replaced with a 245-bp *Sph*I–*Acc*I fragment upstream of the human adult β-globin gene promoter. Hence, pRYG DNA is ideal for Topo II assay because such 245-bp fragment contains a 54 bp alternating repeat of purine/pyrimidine sequence that constitutes a single, high-affinity Topo II cleavage and recognition site [42]. First, various concentrations of DMSO vehicle were tested in the absence of drugs to verify that they did not interfere with the relaxation of SC pRYG DNA measured in the Topo assays. The effects of DMSO alone are shown in the presence or absence of Topo II (Fig. 2, top), but similar data were also observed in the Topo I assay (data not shown). At its highest 10% concentration tested, DMSO alone does not alter SC plasmid DNA in the absence of enzyme, and the lower concentrations of 1 and 2.5% DMSO do not alter the ability of purified human Topo II to fully induce the same relaxation of SC DNA as when the enzyme is tested in the absence of DMSO. However, higher 5 and 10% concentrations of DMSO seem to interfere with the relaxation assay since they decrease significantly the catalytic activity of Topo II (Fig. 2, top). Hence, the final concentrations of DMSO vehicle used to test the inhibitory effects of *m*-AMSA, VP-16, CPT and the antitumor TT analogs in the Topo II and I relaxation assay mixtures never exceeded 2.5% (v/v), the levels demonstrated not to interfere with the catalytic activities of these enzymes. The known Topo II inhibitor *m*-AMSA was used as a positive control to standardize the conditions of the Topo II relaxation assay (Fig. 2, bottom). Under conditions where SC pRYG DNA is fully relaxed by 4 U of Topo II, the inhibitory effect of *m*-AMSA is clearly concentration dependent over a 6.4–250 μM range (Fig. 2, bottom). However, even the highest 250 μM concentration of *m*-AMSA tested can only partially inhibit the relaxation of SC plasmid DNA catalyzed by this amount of Topo II enzyme. It should be noted that the negative control of 250 μM *m*-AMSA alone does not alter SC DNA in the absence of Topo II (Fig. 2, bottom).

All lead antitumor TT bisquinones were able to inhibit Topo II activity, based on the substantial amount of residual SC plasmid DNA observed in the presence of 250 μM TT2, TT13, TT16, TT19, TT21, TT24 and TT26 (Fig. 3, top). As suggested by the presence or absence of bands of R DNA and by the intensity of the residual bands of SC DNA, TT2, TT16 and TT24 appear to inhibit Topo II relaxation activity more effectively than TT13, TT19, TT21 and TT26 (Fig. 3, top), a result which is somewhat inconsistent with the fact that, except the most potent TT24 and the weaker TT21, all these lead antitumor TT bisquinones are equally cytotoxic in the tumor cell viability assays (Fig. 1). Nevertheless, since 250 μM TT24 is clearly the most cytotoxic in Fig. 1 and totally prevents the relaxation of SC plasmid DNA by Topo II in Figure 3 (top), the concentration-dependent inhibitory effect of this lead antitumor TT bisquinone was determined in Figure 3 (middle) to identify the lowest concentration of TT24 able to match the magnitude of Topo II inhibition caused by the positive control of 250 μM *m*-AMSA. Low concentrations of 6.4 and 16 μM TT24 are ineffective but, starting at 40 μM, the Topo II inhibitory effect of TT24 is clearly concentration dependent and, based on the intensity of the residual bands of SC DNA, 100 μM TT24 seems to match or even surpass the Topo II inhibitory effect of 250 μM *m*-AMSA (Fig. 3, middle). Moreover, in contrast to the partial inhibitions of Topo II activity achieved by 250 μM *m*-AMSA in Figures 2 (bottom) and 3 (middle), the 250 μM concentrations of TT2, TT16 (Fig. 3, top) and TT24 (Fig. 3, top and middle) can fully inhibit the relaxation of SC plasmid DNA catalyzed by 4 U of Topo II, substantiating the hypothesis that these new lead antitumor compounds might be about 2.5 times more potent than *m*-AMSA against Topo II activity. It should be noted that the negative control of 250 μM TT24 alone does not alter SC plasmid DNA in the absence of Topo II enzyme (Fig. 3, middle). Antitumor TT analogs

Fig. 3



Inhibition of Topo II catalytic activity by antitumor TT analogs. (Top) Comparison of the abilities of 250 μM concentrations of the lead antitumor TT bisquinones TT2, TT13, TT16, TT19, TT21, TT24 and TT26 to inhibit Topo II activity. (Middle) Concentration-dependent inhibition of Topo II activity by 6.4–250 μM TT24. (Bottom) Comparison of the abilities of 100 μM concentrations of TT0 and antitumor TT analogs with 0 (TT3, TT8), 1 (TT5, TT7, TT9) or 2 (TT2) quinoid rings to inhibit Topo II activity. A positive control of 250 μM *m*-AMSA has been included (middle, bottom) for the sake of comparison. The conditions of the assay were identical to those of Fig. 2.

containing different numbers of quinoid rings (Fig. 1) were used to determine whether there is a relationship between their quinone functionality and ability to inhibit Topo II catalytic activity (Fig. 3, bottom). The inactive parent compound TT0, which is not cytotoxic (Fig. 1), does not alter Topo II activity either, whereas all the other antitumor TT analogs tested in Figure 3 (bottom) are capable of inhibiting Topo II activity to various degrees whether or not they possess quinone functionalities. However, in contrast to the bisquinone TT24, which, at 100 μM almost totally inhibits the relaxation of SC DNA by Topo II and matches the magnitude of Topo II inhibition caused by 250 μM *m*-AMSA, the other antitumor TT analogs with 0 or 1 quinoid rings only partially inhibit Topo II activity when tested at 100 μM (Fig. 3, bottom). Based on their abilities to decrease the upper bands of newly R plasmid DNA and increase the bottom bands of residual SC DNA during this enzymatic reaction, the dihydroquinone TT3 and the diketone TT8, which have no quinoid ring, appear to inhibit Topo II activity slightly less effectively than the monoquinones TT5, TT7 and TT9, but the difference is not obvious (Fig. 3, bottom). Similar data (not shown) were obtained in the Topo I assay.

Inhibition of Topo I catalytic activity

Lead antitumor TT bisquinones were also tested for their ability to inhibit the catalytic activity of purified human Topo I *in vitro* by measuring the relaxation of SC plasmid DNA substrate in a reaction buffer specifically formulated for use with Topo I (eukaryotic Topo II activities require $MgCl_2$ and ATP). The known Topo I inhibitor CPT was used as a positive control to standardize the conditions of the Topo I relaxation assay (Fig. 4, top). Under conditions where SC plasmid DNA is nearly fully relaxed by 1 U of Topo I, the inhibitory effect of CPT is clearly concentration dependent. CPT is ineffective at 2 μM, but increasing 10–250 μM concentrations of CPT produce decreases in the upper bands of R DNA that are correlated with concomitant increases in the lower bands of residual SC DNA (Fig. 4, top). However, even the highest 250 μM concentration of CPT tested can only partially inhibit by about 50% the relaxation of SC plasmid DNA catalyzed by this amount of Topo I enzyme. It should be noted that the negative control of 250 μM CPT alone does not alter SC plasmid DNA in the absence of Topo I (Fig. 4, top). Under experimental conditions where SC plasmid DNA is fully relaxed by Topo I, all lead antitumor TT bisquinones were able to partially inhibit Topo I activity more or less effectively than the positive control of 250 μM CPT, based on the substantial amount of residual SC plasmid DNA observed in the presence of 250 μM TT2, TT13, TT16, TT19, TT21, TT24 and TT26 (Fig. 4, middle). Taken together, the decreased bands of R DNA and the increased amounts of residual SC DNA observed in reactions performed in the presence of lead antitumor TT bisquinones indicate that TT2, TT13, TT16 and TT24 inhibit Topo I activity more effectively than TT19, TT21 and TT26 (Fig. 4, middle). Except for the fact that TT21 is consistently one of the weakest and TT24 consistently one of the best, these comparative Topo I inhibitory effects (Fig. 4, middle) do not exactly match the respective inhibitory abilities of these lead antitumor TT bisquinones in the tumor cell viability (Fig. 1) and Topo II relaxation assays (Fig. 3, top). Interestingly, TT2 and TT16 appear to match,

whereas TT24 and, especially, TT13 may even slightly surpass the Topo I inhibitory effect of CPT, suggesting that these latter two antitumor TT bisquinones are at least equipotent to, or might even be slightly more potent than, this reference Topo I-inhibiting anticancer drug (Fig. 4, middle). Moreover, TT24 inhibits Topo I activity in the same concentration-dependent manner (Fig. 4, bottom) as CPT (Fig. 4, top). The lowest concentration of TT24 that can significantly inhibit Topo I activity is 10 μM (Fig. 4, bottom), an observation which might be noteworthy since this compound fails to significantly inhibit Topo II activity at 16 μM (Fig. 3, middle). As observed with CPT in Figure 4 (top), the highest 250 μM concentration of TT24 tested can also inhibit Topo I activity by about 50% without altering by itself the integrity of SC plasmid DNA substrate in the absence of Topo I enzyme (Fig. 4, bottom).

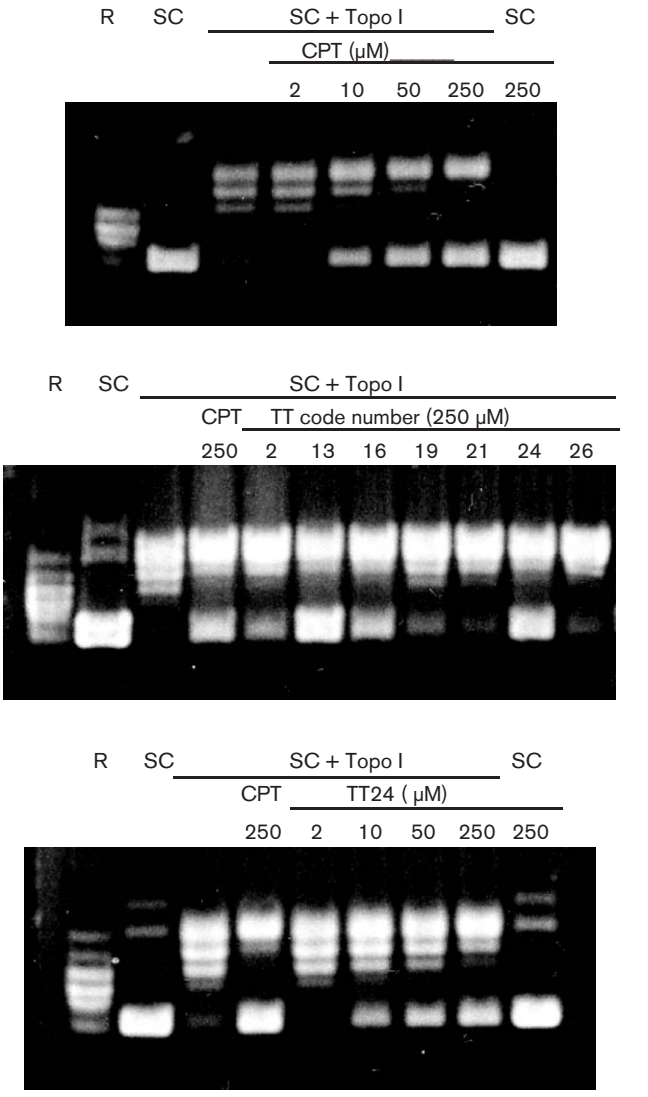
Demonstration of dual Topo I and II inhibition

Finally, the dual inhibitory activity of TT24 is substantiated by the fact that, in contrast to the Topo I inhibitor CPT, which is ineffective in the Topo II inhibition assay, the new Topo I inhibitor TT24 mimics the ability of the specific Topo II inhibitor *m*-AMSA to block the relaxation of SC plasmid DNA catalyzed by Topo II (Fig. 5, top). Similarly, in contrast to the Topo II inhibitor VP-16, which is ineffective in the Topo I inhibition assay, the new Topo II inhibitor TT24 mimics the ability of the specific Topo I inhibitor CPT to block the relaxation of SC plasmid DNA catalyzed by Topo I (Fig. 5, bottom).

Discussion

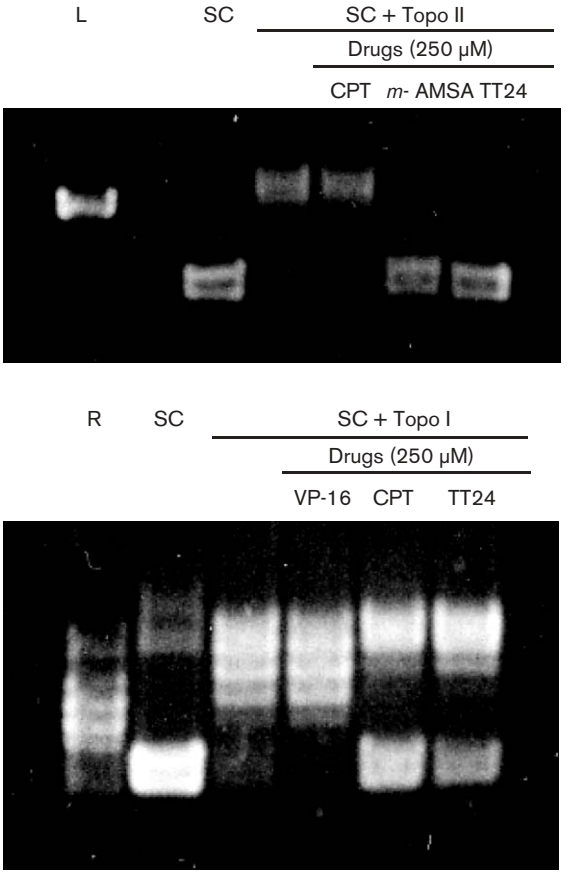
With its simple structure easy to synthesize and mass produce [40], TT24 inhibits Topo I activity like CPT, a natural plant product with a complex structure more difficult to synthesize. Even though IC_{50} values for the cytotoxicity of TT bisquinones are higher in HL-60 than in L1210 cells [10], the inhibition of L1210 tumor cell viability by the new Topo II inhibitor TT24 compares well with that of DAU, a proven anticancer drug already used clinically. Since the parent TT0 structure alters neither Topo activity nor tumor cell viability, the ability of synthetic TT analogs to target Topo I and II may be linked to their antitumor activity. However, structure–activity relationships are difficult to establish and the quinone functionality of various TT analogs is not clearly correlated to their Topo inhibitory activities. Because the quinone functionality of certain anticancer drugs may be linked to their ability to alkylate DNA and produce toxic reactive O_2 species (ROS) and free radicals (FRs) [1,43], it is tempting to speculate that such events may play a role in TT24-induced DNA damage, apoptosis and cytotoxicity. Indeed, selected antitumor TT analogs with 2, 1 or 0 quinoid rings generally produce decreasing levels of Topo II inhibition, PARP-1 cleavage and internucleosomal DNA fragmentation, but this is not a general rule [8,9,16]. Not all TT bisquinones qualify as lead antitumor compounds and the overall antitumor effects of various TT structures are not consistently correlated to their quinone functionalities, suggesting that other mechanisms besides Topo enzymes and nucleoside transporters might be involved in their cytostatic/cytotoxic actions [8–10,16]. For instance, other TT bisquinones not selected among the current seven lead antitumor compounds are less effective than the mono-

Fig. 4



Comparison of the effects of antitumor TT analogs and the known Topo I inhibitor CPT in the Topo I relaxation assay. (Top) Concentration-dependent inhibition of Topo I catalytic activity by 2–250 μM CPT. (Middle) Comparison of the abilities of 250 μM concentrations of the lead antitumor TT bisquinones TT2, TT13, TT16, TT19, TT21, TT24 and TT26 to inhibit Topo I activity. (Bottom) Concentration-dependent inhibition of Topo I activity by 2–250 μM TT24. A positive control of 250 μM CPT has been included (middle, bottom) for the sake of comparison. R: relaxed pRYG DNA marker; SC: supercoiled pRYG plasmid DNA substrate.

Fig. 5



Dual inhibition of Topo I and II activities by the lead antitumor TT bisquinone TT24. (Top) the effects of 250 μM concentrations of the Topo I inhibitors CPT and TT24 are tested together in the Topo II inhibition assay and compared to a positive control of 250 μM *m*-AMSA. (Bottom) The effects of 250 μM concentrations of the Topo II inhibitors VP-16 and TT24 are tested together in the Topo I inhibition assay, and compared to a positive control of 250 μM CPT. The conditions of the Topo II (top) and I (bottom) assays were identical to those of Figures 3 and 4, respectively.

quinones TT5, TT7 and TT9 [8,9], and TT3, which has no quinone functionality, is often equipotent to its structural monoquinone equivalent TT5 [8,9,16].

Topos are ubiquitous nuclear enzymes that alter the helical configuration or topology of DNA [44,45]. The role of these enzymes, which is to introduce or remove DNA superhelical tensions, tie or untie DNA knots and catenate or decatenate circular DNA molecules, is required to achieve key cellular processes, such as replication, transcription, recombination, repair and chromatin assembly [44,45]. Two main families of Topos, types I and II, have been isolated from prokaryotes and eukaryotes, and six enzymes belonging to the three subfamilies IA (DNA Topos IIIα and IIIβ), IB (DNA Topo I and mitochondrial DNA Topo I) and IIA (DNA Topos IIα and IIβ) have been identified in human cells [45,46]. Both Topo I and II carry out similar enzymatic reactions by respectively introducing transient DNA SSBs and DSBs, which allow the alternations of DNA topology required for various nucleic acid functions [44–49]. The enzymatic cycles of Topo I and II can be divided into four steps, which include binding of the enzyme to DNA, DNA cleavage, strand passage and DNA-religation [44–49]. A DNA strand is being cleaved while the Topo enzyme binds to the nucleic acid. A covalent enzyme–DNA intermediate (cleavable complex) linked by a phosphotyrosine bond is produced when the phenolic OH of a tyrosine residue in the catalytic pocket attacks the phosphodiester backbone of DNA [44–49]. Topo I, which is typically monomeric, reacts with a single strand of DNA to induce SSBs, whereas Topo II, which is dimeric, interacts simultaneously with both strands of DNA to produce DSBs with a 4-bp stagger because each subunit contributes a tyrosine that cuts one strand of DNA [44–49].

Several potent anticancer drugs selectively target Topo I or II, including CPT, which specifically inhibits Topo I activity, and DAU, DOX, *m*-AMSA, VP- 16 and teniposide, which preferentially inhibit Topo II activity [4,17–25,28,47–51]. Moreover, Topo inhibitors can be further divided into two broad classes according to their different mechanisms of action [47–51]. In one class of drugs, 'Topo poisons' stabilize the normally transient Topo–DNA cleavable complexes by forming permanent ternary complexes in which drugs, Topos and nicked DNA are covalently linked during the catalytic cycle of the enzymes. In another class of drugs, 'Topo inhibitors' interfere with the catalytic function of the enzymes without trapping the transient Topo–DNA cleavable complexes. Interestingly, most of the Topo-targeting anticancer drugs currently used clinically belong to the class of 'Topo poisons', but the exact mechanism by which lead antitumor TT bisquinones inhibit Topo I and II activities remains to be investigated [47–51].

Some 'Topo poisons' may even have different mechanisms of action. For instance, VP-16 and CPT are Topo poisons that do not affect steps prior to DNA cleavage by the enzyme, but block the enzyme at some point after DNA cleavage by directly preventing religation [4,17–19,22,23,47,48,50,51]. Other Topo poisons like ellipticine and azatoxin do not directly block DNA religation, but may increase the enzymatic cleavage of DNA and the subsequent level of Topo II covalently bound to DNA [52,53]. In any case, both of these inhibitory mechanisms might lead to the same outcome as increased levels of Topo enzymes covalently trapped to DNA would eventually block the religation of the normally transient DNA nicks. However, the detailed mechanisms by which Topo poisons cause these enzymes to stimulate the cleavage and block the religation of DNA remain unclear

and the interactions of antitumor TT analogs with DNA would have to be characterized in order to understand their mechanism of Topo I and II inhibition. Indeed, Topo II poisons may either alkylate DNA, intercalate into DNA, or have no or only weak DNA interactions, suggesting that multiple targets may be involved in their molecular mechanisms of action [51].

'Topo poisons' may be cytotoxic to tumor cells because DNA replication forks collide with such stabilized Topo–drug–DNA covalent ternary complexes to produce long-lived and highly cytotoxic DNA DSBs, although collisions with other enzyme complexes that track along DNA, such as RNA polymerases and DNA helicases, may also be involved [4,20–25,51]. Since the generation of such irreversible DNA damage may induce cells to commit to an apoptotic decision, apoptosis may be an important mechanism of tumor cell killing by Topo-targeting drugs [4,6,7,16–22,47–51]. Whether early and massive high-molecular-weight cleavage of DNA subsequent to Topo I and II inhibition is the nuclear signal by which antitumor TTs trigger apoptosis upstream of mitochondria remains to be elucidated.

'Topo inhibitors', which do not significantly increase the level of trapped Topo–DNA complexes, have also different ways of inhibiting the catalytic activities of these enzymes. For example, Topo II is an ATP-dependent enzyme and derivatives of coumarin antibiotics, such as coumermycins and novobiocin, inhibit Topo II activity by acting as competitive inhibitors of ATP [54,55]. Other 'Topo inhibitors' may kill cells by disrupting essential cellular functions that rely on Topo catalytic activity rather than by generating irreversible DNA damage. For instance, Topo II activity is required during mitosis and its inhibition kills cells undergoing mitosis [51,56], but this mechanism is unlikely to be involved in the action of antitumor TTs since they decrease the mitotic index, suggesting that they block cell cycle progression and prevent tumor cells from reaching and accumulating in M phase [8]. As several cellular processes are involved in Topo activities and because drugs targeting Topos may interact differently with DNA, the present TT analog study adds to the list of chemically diverse Topo inhibitors that are likely to have different mechanisms of antitumor activity.

The protocols of the enzymatic assays used in the present study are designed to screen bioactive compounds for their abilities to inhibit the catalytic activities of Topo I and II responsible for the relaxation of SC plasmid DNA substrate [57,58], and demonstrate that antitumor TTs directly inhibit purified human Topo I and II activities in a cell-free system *in vitro*. Since the catalytic activities of both Topo enzymes are monitored by following the transformation of SC covalently closed circular (CCC) plasmid DNA substrate (disappearance of the bottom bands in Figs 2–5) into R CCC plasmid DNA (appearance of the top bands in Figs 2–5), non-EB gels have been used because they are more effective than EB-containing gels for easily separating the SC CCC DNA from the R CCC DNA. Under these experimental conditions, different electrophoretic patterns may be observed for the top bands because they contain different forms of nicked or open circular DNA and R CCC DNA, which migrate close together but cannot be easily resolved by non-EB gels. The present study demonstrates that antitumor TT analogs have the unusual ability to inhibit, in a concentration-dependent manner, the DNA relaxation activities of both Topo I and II enzymes. Interestingly, the most cytotoxic TT bisquinone, TT24, inhibits Topo II activity 2.5 times more effectively than *m*-AMSA and matches the Topo I inhibitory effect of CPT, although it may be effective against Topo I at lower concentrations than against Topo II. Some Topo II inhibitors have been suggested to induce a non-specific inhibition of Topo I relaxation activity that does not involve the trapping of covalent DNA–Topo I cleavable complexes when high concentrations of such DNA-interacting drugs may saturate the DNA and prevent the enzyme from binding to it [59]. It is not known whether the antitumor TTs target more specifically one or the other enzyme and the Topo I and II inhibitory effects of TT24 would have to be confirmed in WT and MDR tumor cell systems before we can conclude that lead antitumor TT bisquinones are novel Topo I and II inhibitors at least as potent as those anticancer drugs currently used clinically.

The known DNA intercalator DAU poisons Topo II activity by stabilizing the normally transient covalent Topo II–DNA intermediate and creating irreversible ternary complexes, which are then converted to the DNA DSBs responsible for DAU-induced apoptosis [2,4,17–19,22,48,50,51]. Since TT analogs appear to mimic all the effects of DAU tested so far, it is reasonable to assume that these different types of quinone antitumor drugs might share some common molecular targets and mechanisms of action [8–10,16]. However, the ability of TT24 to covalently bind to or intercalate into DNA remains to be established and further studies would be required to determine whether antitumor TT analogs stabilize DNA–Topo cleavable complexes, inhibit Topo I and II activities by acting as 'Topo poisons' or 'Topo inhibitors', and are able to generate the DNA DSBs, SSBs and DPCs that would be required to trigger apoptosis within a few hours in tumor cells. Moreover, many cellular processes may be involved in the commitment to apoptosis and it is possible that TTs may also alter the structure/function of other molecular targets besides DNA in order to induce apoptosis. For instance, quinone antitumor drugs, which are metabolized into

unstable semiquinone FRs, might also trigger apoptosis by increasing the generation of ROS and FRs [1,43]. Furthermore, some DNA-damaging anticancer drugs might also directly target mitochondria to trigger permeability transition pore opening, cytochrome *c* release and apoptosis through a mitochondrial pathway independent of the nucleus [60]. Incidentally, the dual localization of Topos to mitochondria and nucleus has been reported [45,46].

A major finding is that antitumor TTs rapidly block the cellular transport of purine and pyrimidine nucleosides, an effect which DAU cannot do [8–10]. In addition, lead antitumor TT bisquinones retain their ability to block nucleoside transport, trigger PARP-1 cleavage, induce internucleosomal DNA fragmentation and reduce cell viability in HL-60 sublines that have already developed different mechanisms of MDR to DAU, suggesting that these new drugs are not removed by the P-glycoprotein and MDR-associated protein pumps [10,16]. Finally, the present study demonstrates that, under conditions where a known Topo II inhibitor is inactive in the Topo I inhibition assay, the new Topo II inhibitor TT24 has the advantage of also inhibiting Topo I activity. Taken together, these preliminary studies suggest that the mechanism of action of antitumor TT analogs may involve other additional molecular targets besides those shared with DAU.

So far, very few antitumor drugs have been reported to inhibit both Topo I and II activities, and none of them has been shown to target nucleoside transporters [26–39]. For instance, actinomycin D is a poison for both Topo I and II, but is not clinically useful [26,30,61]. Moreover, the anthracycline aclarubicin is a potent catalytic inhibitor of Topo II, but is a Topo I poison much weaker than CPT [62]. In addition, some epipodophylloids exhibit dual inhibitory effects without stabilizing the cleavable complexes induced by either Topo I or II, but not all of them are toxic against tumor cells [34]. Hence, it is significant to demonstrate that, in addition to their cytotoxic potency and bifunctional mechanism of action, lead antitumor TT bisquinones are equipotent to CPT against Topo I and perhaps even more potent than *m*-AMSA against Topo II. Dual inhibitors of Topo I and II might have significant therapeutic advantage over agents targeting a single type of Topo because of the differing roles and expressions of these enzymes within the cell cycle [33,45]. Moreover, dual inhibitors of Topo I and II might be able to circumvent mechanisms of MDR attributable to mutation or down-regulation of a single type of target enzyme [32,33].

Conclusion

The antitumor TT bisquinone TT24 may be a novel dual inhibitor of Topo I and II activities, which matches the Topo I inhibitory effect of CPT and surpasses the Topo II inhibitory effect of *m*-AMSA under conditions where CPT is ineffective in the Topo II assay and VP-16 is ineffective in the Topo I assay. In addition to blocking nucleoside transport, the ability of lead antitumor TT analogs to target Topo I and II may play a role in the mechanisms by which these drugs trigger apoptotic DNA fragmentation and reduce the viability of WT and MDR tumor cells.

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