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Preclinical study

Triptycenes: a novel synthetic class of bifunctional anticancer drugs that inhibit nucleoside transport, induce DNA cleavage and decrease the viability of leukemic cells in the nanomolar range *in vitro*

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In contrast to their inactive parent compound triptycene (code name TT0), several triptycene (TT) analogs (code names TT1 to TT13), most of them new compounds, were synthesized and shown to prevent L1210 leukemic cells from synthesizing macromolecules and growing *in vitro*. The most potent rigid tetracyclic quinones synthesized so far are TT2 and its C2-brominated derivative, TT13. The antitumor activity of TT2 has been compared to that of daunomycin (DAU), a clinically valuable anthracycline antibiotic which is structurally different from TT2 but also contains a quinone moiety. TT2 inhibits the proliferation (IC_{50}: 300 nM at day 2 and 150 nM at day 4) and viability (IC_{50}: 250 nM at day 2 and 100 nM at day 4) of L1210 cells to the same maximal degree as DAU, suggesting that the cytostatic and cytotoxic activities of TT2 are a combination of drug concentration and duration of drug exposure. Since TT2 does not increase the mitotic index of L1210 cells at 24 h like vincristine, it is unlikely to be an antimitotic drug that disrupts microtubule dynamics. Like DAU, a 1.5–3 h pretreatment with TT2 is sufficient to inhibit the rates of DNA, RNA and protein syntheses determined over 30–60 min periods of pulse-labeling in L1210 cells *in vitro* (IC_{50}: 6 μM). In contrast to DAU, which is inactive, a 15 min pretreatment with TT2 has the advantage of also inhibiting the cellular transport of nucleosides occuring over a 30 s period *in vitro* (IC_{50}: 6 μM), suggesting that TT2 prevents the incorporation of [^{3}H]thymidine into DNA because it rapidly blocks the uptake of [^{3}H]thymidine by the tumor cells. After 24 h, TT2 induces as much DNA cleavage as camptothecin and DAU, two anticancer drugs producing DNA strand breaks and known to respectively inhibit DNA topoisomerase I and II activities. Interestingly, the abilities of TT2 to block nucleoside transport, inhibit DNA synthesis and induce DNA fragmentation are irreversible upon drug removal, suggesting that this compound may rapidly interact with various molecular targets in cell membranes and nuclei to disrupt the functions of nucleoside transporters and nucleic acids, and trigger long-lasting antitumor effects which persist after cessation of drug treatment. Because inhibition of nucleoside transport is highly unusual among DNA-damaging drugs, the use of bifunctional TTs with antileukemic activity in the nM range *in vitro* might provide a considerable advantage in polychemotherapy to potentiate the action of antimetabolites and sensitize multidrug-resistant tumor cells. [© 1999 Lippincott Williams & Wilkins.]

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

Introduction

As precursors of reactive quinone methides, many natural and synthetic quinones function as bioreductive alkylating agents and have antitumor activity.[1-6] The cytotoxicity of quinones may be due to two competing mechanisms: soft electrophilic arylation and redox cycling oxidation.[7-9] While complete two-electron reduction of the quinone ring by DT diaphorase produces a stable hydroquinone, partial one-electron reduction of the quinone ring by NADPH-oxidizing enzymes yields an unstable semiquinone free radical (FR) that can spontaneously autoxidize at the expense of molecular O_2 to generate a cascade of

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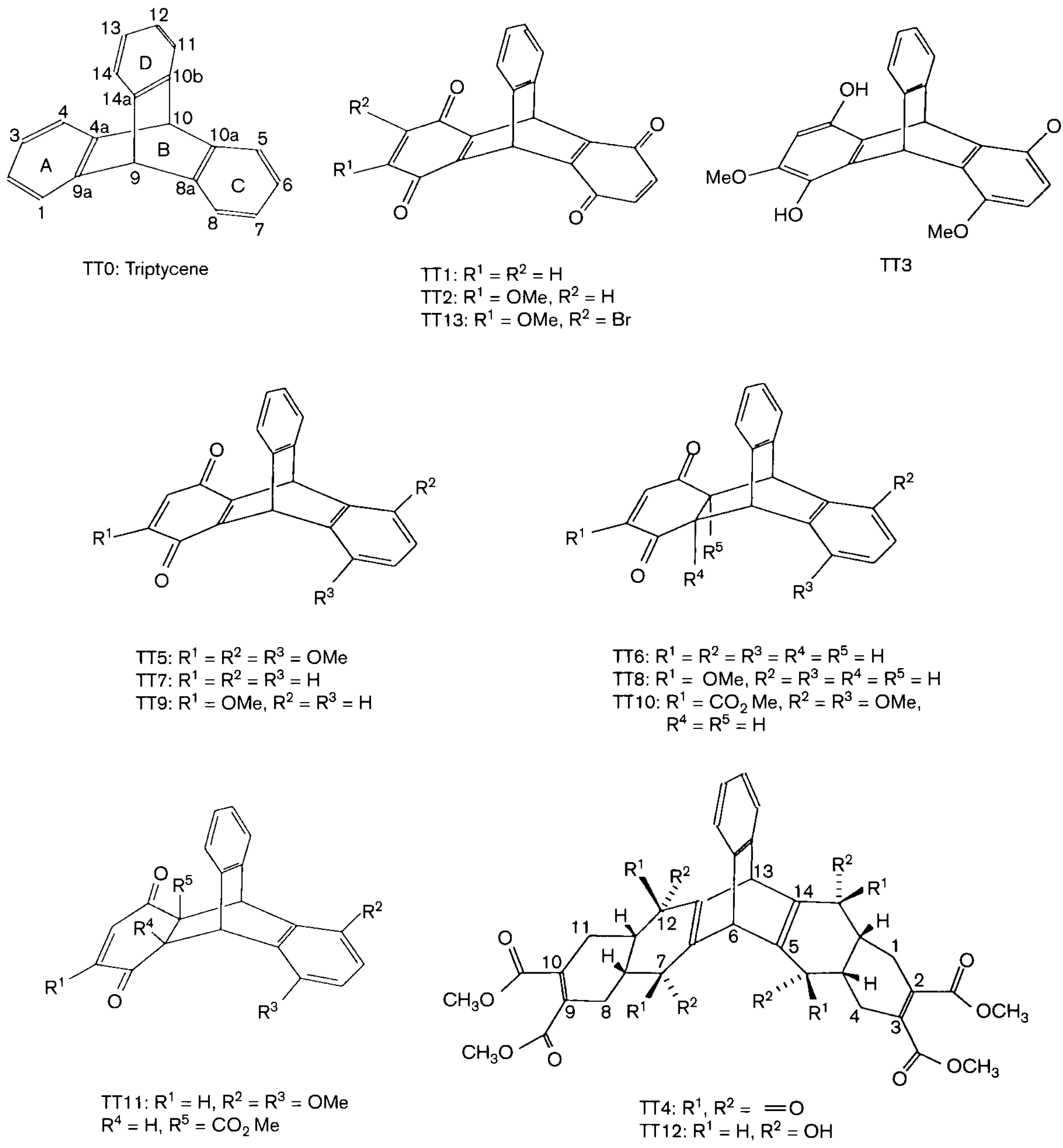
reactive O_2 species (ROS) and FRs, which can induce DNA damage, lipid peroxidation and cytotoxicity. However, most quinone antitumor agents used clinically, such as anthracycline antibiotics, mitomycin C and benzoquinone derivatives, have a complex chemical structure with a number of active functional groups and the exact contribution of the quinone group to their antitumor activity remains uncertain.[10-13] The anthracycline quinone antibiotics adriamycin (ADR) and daunomycin (DAU) covalently bind to and intercalate into DNA, inhibit DNA replication and RNA transcription, are DNA topoisomerase (Topo) II poisons, produce oxidative stress and damage biomembranes, induce DNA breakage and chromosomal aberrations, trigger apoptosis, and have a wide spectrum of anticancer activity.[10,11,13-18]

When tested in Perchellet's laboratory at concentrations ranging from 0.25 to 4 μM, the tetracyclic structure of triptycene (TT), a known and commercially available compound (code name TTO in Figure 1),[19-21] has no antileukemic activity whatsoever in the L1210 tumor cell system *in vitro* (data not shown). Very few



TTO: Triptycene

TT1: $R^1 = R^2 = H$
TT2: $R^1 = OMe$, $R^2 = H$
TT13: $R^1 = OMe$, $R^2 = Br$

TT3

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

TT6: $R^1 = R^2 = R^3 = R^4 = R^5 = H$
TT8: $R^1 = OMe$, $R^2 = R^3 = R^4 = R^5 = H$
TT10: $R^1 = CO_2Me$, $R^2 = R^3 = OMe$, $R^4 = R^5 = H$

TT11: $R^1 = H$, $R^2 = R^3 = OMe$
$R^4 = H$, $R^5 = CO_2Me$

TT4: $R^1, R^2 = {=}O$
TT12: $R^1 = H$, $R^2 = OH$

Figure 1. Chemical structures and code names of the TT analogs tested for their antileukemic activity *in vitro*.

TT derivatives, such as TT1 and TT7 (Figure 1), have been reported in the literature and, to the best of our knowledge, their antitumor activity has never been tested before.[22-27] Since many important cancer chemotherapeutic agents contain a quinone ring in their chemical structure,[10,12,13] the present study was undertaken to determine if, in contrast to their inactive parent compound TT0, various TT analogs with or without monoquinone or bisquinone functionality have any antitumor activity. A shorter and easier method was developed in Hua's laboratory to synthesize the structures of the 13 TT analogs (code names TT1 to TT13) illustrated in Figure 1. Interestingly, we found that several new compounds, such as the TT bisquinones TT2 and TT13 (Figure 1), are cytostatic and cytotoxic to L1210 tumor cells in the nM range *in vitro*. Moreover, our preliminary data indicate that these TT analogs may have an intriguing bifunctional molecular mechanism of action, blocking nucleoside transport and inducing DNA cleavage in order to prevent leukemic cells from synthesizing macromolecules, proliferating and remaining viable *in vitro*.

Materials and methods

Cell culture and drug treatments

TT0 was purchased from Aldrich (Milwaukee, WI). All solutions of synthetic TTs and tricyclic pyrone H10, vincristine (VCR; a gift from Lilly Research Laboratories, Indianapolis, IN) and camptothecin (CPT; from Sigma, St Louis, MO) were dissolved and diluted in dimethyl sulfoxide (DMSO), whereas DAU (from Sigma) solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl.[28-31] Suspension cultures of murine L1210 lymphocytic leukemia cells (ATCC, Rockville, MD) were maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 7.5% fortified bovine calf serum (FCS; HyClone, Logan, UT) and penicillin (100 IU/ml)-streptomycin (100 μg/ml) and incubated in the presence or absence of drugs at 37°C in a humidified atmosphere containing 5% CO_2. Since drugs were supplemented to the culture medium in 1 μl aliquots, the concentration of DMSO in the final incubation volume (0.5 ml) never exceeded 0.2%, and did not affect the rates of macromolecule syntheses and growth in L1210 cells.[28-31] Control cells incubated in the absence of drugs were similarly treated with vehicle only and, in every experiment, all incubates received the same volume of solvent. For drug removal, incubates were spun at 200 *g* for 10 min, drug-containing supernatants were discarded, and intact cells were washed thrice with 1 ml of the above RPMI 1640 culture medium and resuspended in 0.5 ml of fresh medium for further incubation.

Cell proliferation assay

For tumor cell growth, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium, plated at an initial density of 1×10^4 cells/0.5 ml and incubated in 48-well Costar cell culture plates (Costar, Cambridge, MA). Except when otherwise specified, cells were grown for 4 days in the presence or absence of drugs and their density was monitored every 24 h using a Coulter counter (Coulter Electronics, Luton, UK).[28-31]

Cell viability assay

L1210 cells suspended in FCS-containing RPMI 1640 medium were grown in 48-well Costar cell culture plates for up to 4 days in the presence or absence of TTs to evaluate drug cytotoxicity. Decreasing concentrations of cells, such as 1×10^5 and 1.11×10^4 cells/0.5 ml/well, were initially plated at time 0 in order to collect control samples with approximately equal cell densities after 2 and 4 days in culture, respectively. The viability of TT-treated cells was assessed from their ability to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product which absorbs at 490 nm.[32] At the appropriate time after drug treatment, cell samples (about 10^6/0.5 ml/well for controls) were further incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS:PMS (2:0.1) reagent and their relative cell viability was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL).[31] Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were substracted from the results.

Macromolecule synthesis

For nucleic acid and protein syntheses, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium at a density of about $1.1\text{-}1.6 \times 10^6$ cells/0.5 ml. Except when otherwise specified, the cells were incubated at 37°C for 90 min in the presence or absence of drugs and then pulse-labeled for an additional 30 min with 1 μCi of [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) to estimate the rate of DNA synthesis. For RNA and protein syntheses, cells were incubated at 37°C for 3 h in the presence or absence of drugs and then pulse-labeled for an additional 1 h with 2 μCi of [5,6-^{3}H]uridine (46 Ci/mmol; ICN Biomedicals, Irvine, CA) or 2.5 μCi of [4,5-^{3}H]L-leucine (100 Ci/mmol; Moravek Biochemicals, Brea, CA), respectively. The incubations were terminated by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). After holding on ice for 15 min, the acid-insoluble material was recovered over Whatman GF/A glass microfiber filters and washed thrice with 2 ml of 5% TCA and twice with 2 ml of 100% EtOH. After drying the filters, the radioactivity bound to the acid-precipitable material was determined by liquid scintillation counting (LSC) in 10 ml of Bio-Safe NA (Research Products International, Mount Prospect, IL).[28-31]

Nucleoside transport

L1210 cells (about 1.5 × 10^6 cells/0.5 ml) were preincubated for 15 min at 37°C in the presence or absence of TTs and then exposed to 1 μCi of [^{3}H]thymidine for only 30 s to assess the cellular uptake of nucleoside over such a very short period of time.[31,33] Reactions were diluted with 2 ml of ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and the unincorporated radiolabel was removed by centrifugation at 200 *g* for 10 min. After washing thrice with 2 ml of ice-cold PBS, intact cell pellets were harvested by centrifugation and incubated for 30 min in 1 ml of hypotonic lysis buffer (HLB) containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.2% Triton X-100. Cell lysates were mixed with 9 ml of Bio-Safe II (Research Products International) and counted to estimate the cellular uptake of [^{3}H]thymidine. Drug inhibition was expressed as per cent of [^{3}H]thymidine transported into vehicle-treated control cells over a similar 30 s period.[31,33]

Mitotic index

L1210 cells (about 1 × 10^6/0.5 ml of FCS-containing RPMI 1640 medium) were incubated in triplicate for 24 h at 37°C in the presence or absence of TTs or known antimitotic drugs and collected by centrifugation at 200 *g* for 10 min to determine their mitotic index.[34,35] For hypotonic treatment, cells were resuspended in 1 ml of 75 mM KCl for 20 min at 4°C. After fixation in 1 ml of MeOH:acetic acid (3:1), the final cell pellets were collected by centrifugation, resuspended in 75 μl of MeOH:acetic acid (3:1), dispensed onto glass slides, air dried and stained by spreading 40 μl of 0.1% crystal violet under a coverslip.[30,31] The percentage of cells in mitosis was determined microscopically by counting 500 cells/slides. The mitotic index was calculated as the percentage of mitotic cells in drug-treated cultures divided by the percentage of mitotic cells in non-treated controls.[30,31,34,35]

DNA cleavage

Drug-induced DNA cleavage was determined by intact chromatin precipitation, using L1210 cells which were prelabeled with 1 μCi of [^{3}H]thymidine for 2 h at 37°C, washed with 3 × 1 ml of ice-cold PBS, collected by centrifugation and resuspended in fresh FCS-containing RPMI 1640 medium at a density of ~1 × 10^6 cells/0.5 ml.[17,31,36] Except when otherwise specified, such cells containing prelabeled DNA were then exposed for 24 h to TTs and drugs known to induce DNA fragmentation. After centrifugation at 200 *g* for 10 min to discard the drugs and wash the cells, the intact cell pellets were lysed for 30 min in 0.5 ml of HLB, centrifuged at 12 000 *g* for 30 min to collect the supernatants and resuspended in 0.5 ml of HLB. After another similar centrifugation, the radioactivities in the pooled supernatants (detergent-soluble low molecular weight DNA fragments) and the pellet (intact chromatin DNA) were determined by LSC: % DNA fragmentation=[c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100.[17,31,36]

Results

Drugs

The nomenclature of the parent compound TT0, which is not cytotoxic to L1210 cells when tested at 0.25–4 μM over a 4-day period *in vitro* (data not shown), is 9,10-dihydro-9,10-*o*-benzenoanthracene (Figure 1). The TT quinones under study were synthesized in Hua's laboratory by an *in situ* oxidation of substituted dihydroxybenzenes followed by [4+2] cycloaddition with 1,4-dimethoxyanthracene and then oxidation. This method of synthesis will be reported in detail elsewhere. The chemical structures of the TTs tested for their antileukemic activity *in vitro* are shown in Figure 1 (serial numbers indicate the order of synthesis) and their correct nomenclatures are as follows. TT1: 9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4,5,8-tetraone. TT2: 2-methoxy-9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4,5,8-tetraone. TT13: 2-bromo-3-methoxy-9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4,5,8-tetraone. TT3: 1,4-dihydroxy-2,5,8-trimethoxy-9,10-dihydro-9,10[1′,2′]benzenoanthracene. TT5: 2,5,8-trimethoxy-9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4-dione. TT7: 9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4-dione. TT9: 2-methoxy-9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4-dione. TT6: (4aS*,9aR*)-4a,9,9a,10-tetrahydro-9,10[1′,2′]benzenoanthracene-1,4-dione. TT8: (4aS*,9aR*)-2-methoxy-4a,9,9a,10-tetrahydro-9,10 [1′,2′] benzenoanthracene-1,4-dione. TT10: (4aS*,9aR*)-5,8-dimethoxy-2-methoxycarbonyl-4a,9,9a,10-tetrahydro-9,10[1′,2′]benzenoanthracene-1,4-dione. TT11: (4aS*,9aR*)-5,8-dimethoxy-4a-methoxycarbonyl-9,9a,10-trihydro-9,10[1′,2′]benzenoanthracene-1,4-dione. TT4: (4aS*,7aR*,11aS*,14aR*)-tetramethyl-1,4,4a,5,6,7,7a,8,11,11a,12,13,14,14a-tetradecahydro-5,7,12,14-tetraoxo-6,13[1′,2′]benzenopentacene-2,3,9,10-tetracarboxylate. TT12: (4aS*,5S*,7R*,12S*,14R*,7aR*,11aS*,14aR*)-tetramethyl-1,4,4a,5,6,7,7a,8,11,11a,12,13,14,14a-tetradecahydro-5,7,12,14-tetrahydroxy-6,13[1′,2′]benzenopentacene-2,3,9,10-tetracarboxylate.

Inhibition of tumor cell growth and viability by TTs

When tested at 256 nM over a 4 day period, several TTs inhibit the rate of tumor cell growth in relation to their ability to decrease tumor cell viability (Figure 2). Although most of these drugs are much more active at higher concentrations, at this lower concentration of 256 nM, TT4 and TT12 are inactive, and TT11 and TT10 have only marginal and weak inhibitory effects. In contrast, 256 nM TT3, TT5, TT6 and TT8 have moderate cytostatic (32–45% inhibition) and cytotoxic (41–54% inhibition) activities. Under similar conditions, TT1, TT7 and TT9 are more effective, and can reduce the number and viability of L1210 cells at 4 days by 55–57 and 61–66%, respectively. However, the two most potent cytostatic and cytotoxic TT quinones synthesized so far are clearly TT2 and its C2-brominated derivative TT13, which, at 256 nM, can inhibit leukemic cell proliferation and viability by 67–68 and 73–75%, respectively (Figure 2). Therefore, the antileukemic effects of the most bioactive TT2 were further assessed and compared to those of known anticancer drugs already used clinically.

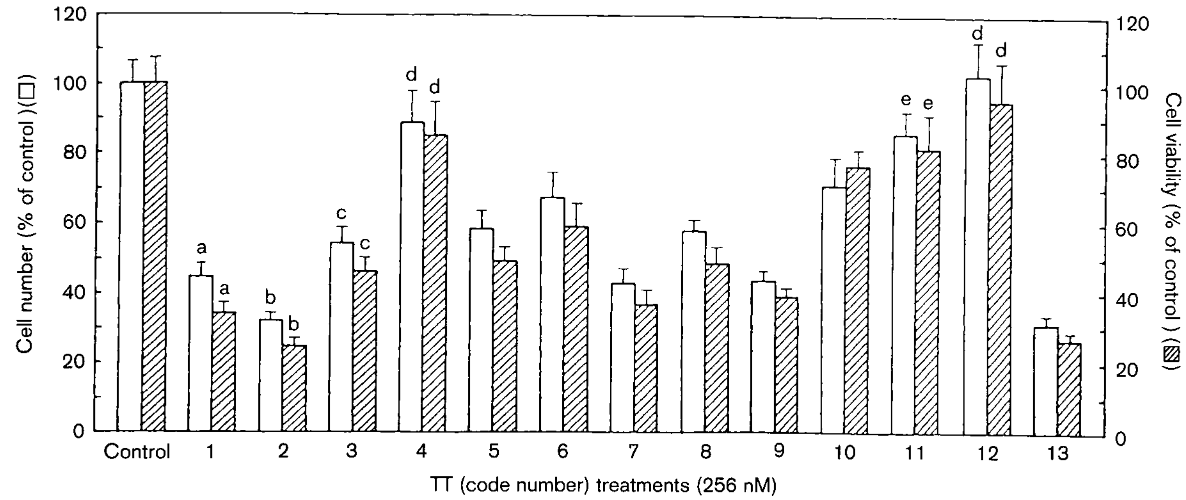


Figure 2. Comparison of the abilities of novel TTs to inhibit the growth (open) and viability (striped) of L1210 cells after 4 days *in vitro*. For tumor cell growth and viability, L1210 cells were, respectively, seeded in triplicate at initial densities of 1×10^4 and 1.11×10^4 cells/0.5 ml/well in RPMI 1640 medium containing 7.5% FCS and penicillin (100 IU/ml)–streptomycin (100 μg/ml) and grown for 4 days in the presence or absence (control) of 256 nM concentrations of the indicated compounds in a humidified incubator at 37°C with 5% CO_2 in air. Cell density was monitored using a Coulter counter. Cell growth results are expressed as percentage of the number of vehicle-treated control cells after 4 days in culture ($1\,465\,110 \pm 92\,595$; $100 \pm 6\%$; open control). The ability of viable cells/0.5 ml to bioreduce 0.1 ml of MTS:PMS (20:1) reagent over a 3 h incubation period at 37°C was assessed by measuring the absorbance of the water-soluble formazan products at $A_{490\ \mathrm{nm}}$. Cell viability results are expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells ($A_{490\ \mathrm{nm}}=1.164 \pm 0.089$; $100 \pm 8\%$; striped control) after 4 days in culture. The blank value ($A_{490\ \mathrm{nm}}=0.202$) for cell-free medium supplemented with MTS:PMS reagent has been substracted from the results. Bars: means $\pm$ SD ($n=3$). [a]Not different from TT7 and TT9; [b]$p<0.025$, smaller than TT1, TT7 and TT9 but not different from TT13; [c]$p<0.05$, greater than TT1, TT7 and TT9 but not different from TT5, TT6 and TT8; [d]not different from control; [e]$p<0.05$, smaller than control.

DAU is a clinically valuable anthracycline antibiotic, which is structurally very different from the TT quinones but also contains a quinone moiety.[10] At concentrations of 640 nM or above, TT2 inhibits almost totally the proliferation and viability of L1210 cells at day 4 but these maximal cytostatic and cytotoxic activities of TT2 can be mimicked by concentrations of DAU as low as 102 nM, and the smallest concentrations of TT2 and DAU that can induce significant antiproliferative and cytotoxic effects after 4 days are 41 and 16 nM, respectively (Figures 3 and 4). These relative potencies of TT2 and DAU can easily be compared using the full concentration-response curves of Figures 3 and 4, where the striped areas at 100% represent the control levels of L1210 cell growth and viability after 2 and 4 days in culture. The magnitudes of the cytostatic and cytotoxic effects of both TT2 and DAU are clearly related to the combination of their increasing concentration and duration of action. For instance, 16 nM DAU and 41 nM TT2 are ineffective at day 2 but their antiproliferative (Figure 3) and cytotoxic (Figure 4) activities become apparent at day 4. Moreover, 41 nM DAU and 256 nM TT2 are moderately cytostatic (42 and 44% inhibition, respectively) and cytotoxic (32 and 49% inhibition, respectively) at day 2 but, respectively, decrease L1210 cell proliferation by 77 and 68%, and L1210 cell viability by 65 and 79% at day 4 (Figures 3 and 4). As a result, the antiproliferative activities of DAU and TT2 are, respectively, characterized by IC_{50} values of 50 and 300 nM at day 2, but 25 and 150 nM at day 4, suggesting that DAU is about 6 times more cytostatic than TT2 in the L1210 tumor system *in vitro* (Figure 3). Similarly, the inhibitions of tumor cell viability by DAU and TT2 are respectively characterized by IC_{50} values of 70 and 250 nM at day 2, but 30 and 100 nM at day 4, suggesting that DAU is about 3.5 times more cytotoxic than TT2 in this leukemic system *in vitro* (Figure 4).

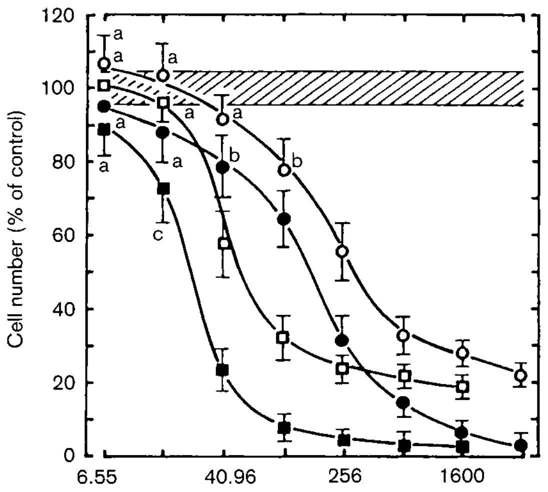


Figure 3. Comparison of the concentration-dependent inhibitions of L1210 cell growth by the TT analog TT2 (circles) and DAU (squares) at days 2 (open symbols) and 4 (closed symbols) *in vitro*. The protocol of the experiment was identical to that of Figure 2. The results are expressed as percentage of the numbers of vehicle-treated control cells (100±4%; striped area) after 2 (213 798±8316 cells/ml) and 4 (1 311 274±55 991 cells/ml) days in culture. Drug concentrations are plotted on a logarithmic scale. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.025$ and [c]$p<0.01$, smaller than control.

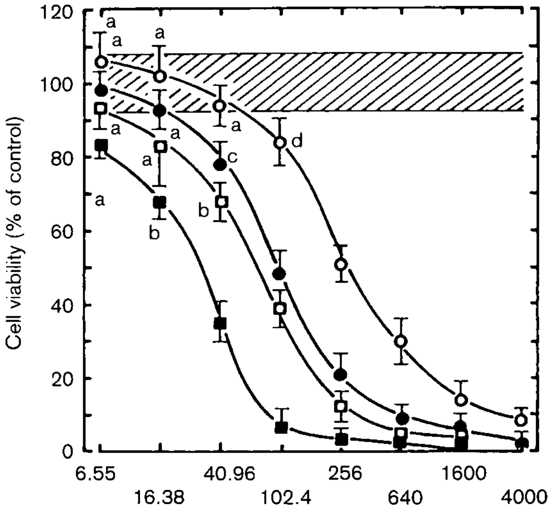


Figure 4. Comparison of the concentration-dependent inhibitions of L1210 cell viability by the TT analog TT2 (circles) and DAU (squares) at days 2 (open symbols) and 4 (closed symbols) *in vitro*. Cells were seeded in triplicate at initial densities of 100 000 or 11 100 cells/0.5 ml/well and, respectively, incubated for 2 or 4 days in the presence or absence (control) of the indicated concentrations of drugs, which are plotted on a logarithmic scale. The ability of viable cells/0.5 ml to bioreduce 0.1 ml of MTS:PMS (20:1) reagent over a 3 h incubation period was assessed as described in Figure 2. Cell viability results are expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells (100±8%; striped area) at days 2 ($A_{490\ nm}$=1.206±0.093) and 4 ($A_{490\ nm}$=1.096±0.086). Blank values ($A_{490\ nm}$=0.185 and 0.189 at days 2 and 4) for culture medium supplemented with MTS:PMS reagent have been substracted from the results. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.005$, [c]$p<0.025$ and [d]$p<0.05$, smaller than control.

Inhibition of DNA synthesis and nucleoside transport by TTs

A 2 h treatment with TT2 is sufficient to inhibit, in a concentration-dependent manner, the rate of DNA synthesis determined over a 30 min period of pulse labeling in L1210 cells *in vitro* (Figure 5). DNA synthesis is totally inhibited by 25 μM TT2 but, as compared to DAU which becomes effective against DNA synthesis at 0.256 μM, concentrations greater than 1.6 μM must be used to demonstrate the inhibitory effect of TT2 on DNA synthesis. Hence, the concentration-dependent inhibitions of DNA synthesis by DAU (IC_{50}: 1 μM) and TT2 (IC_{50}: 6 μM) suggest that, under these experimental conditions, DAU prevents L1210 cells from synthesizing DNA about 6 times more effectively than TT2 (Figure 5).

A critical finding is that, in contrast to DAU which serves as a negative control in the assay, a 15 min treatment with TT2 is sufficient to block, in a concentration-dependent manner, the cellular transport of [^{3}H]thymidine occuring over only 30 s *in vitro* (Figure 6). Nucleoside transport is totally inhibited in L1210 cells treated with 25 μM TT2. However 10 μM DAU is totally unable to alter the cellular transport of [^{3}H]thymidine (Figure 6), even though such concentration of DAU inhibits maximally the incorporation of [^{3}H]thymidine into DNA (Figure 5). Interestingly, the concentration-response curves for the inhibitory effects of TT2 on nucleoside transport (Figure 6) and DNA synthesis (Figure 5) are nearly identical and share similar IC_{50} values of 6 μM, suggesting that the inhibition of [^{3}H]thymidine incorporation into DNA caused by TT2 at 2 h (Figure 5) may largely be due to the ability of this TT quinone to immediately block the uptake of [^{3}H]thymidine by the cells (Figure 6).

Moreover, when compared on an equal 7 μM concentration basis, the different magnitudes at which various TTs inhibit DNA synthesis at 2 h correlate with the different abilities of these compounds to block the cellular transport of nucleosides after 15 min (Figure 7). Indeed, among all TTs tested at this concentration, TT4 and TT12 alter neither DNA synthesis nor nucleoside transport,

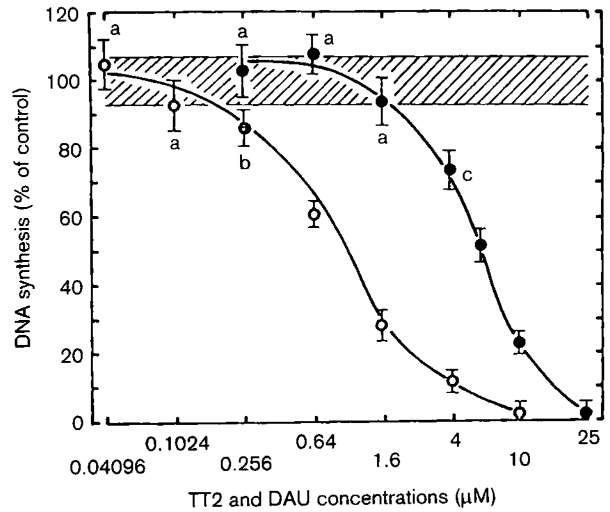


Figure 5. Comparison of the concentration-dependent inhibitions of DNA synthesis by the TT analog TT2 (●) and DAU (○) in L1210 cells *in vitro*. Cells (1.14 × 10^6/0.5 ml of RPMI 1640 medium) were incubated at 37°C for 90 min in the presence or absence (control) of the indicated concentrations of drugs, which are plotted on a logarithmic scale, and then pulse labeled for an additional 30 min to determine the rate of [^{3}H]thymidine incorporation into DNA. DNA synthesis in vehicle-treated control cells was 27 860 ± 1931 c.p.m. (100 ± 7%; striped area). The blank value (916 ± 52 c.p.m.) for cells incubated and pulse labeled at 2°C with 1 μCi of [^{3}H]thymidine has been substracted from the results. Bars: means ± SD (n=3). ^{a}Not different from control; $^b p < 0.05$ and $^c p < 0.01$, smaller than control.

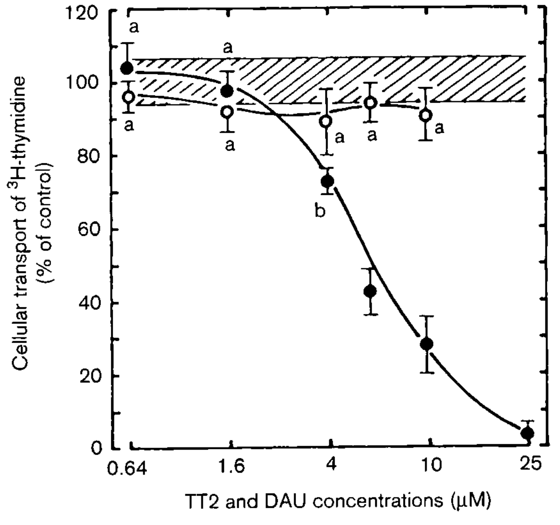


Figure 6. Concentration-dependent inhibition of nucleoside transport by the TT analog TT2 in L1210 cells *in vitro*. Cells (1.48 × 10^6/0.5 ml of RPMI 1640 medium) were preincubated for 15 min at 37°C in the presence or absence (control) of the indicated concentrations of TT2 (●) and DAU (○), which are plotted on a logarithmic scale, before being exposed to 1 μCi of [^{3}H]thymidine for 30 s at 37°C. After washing thrice with PBS, intact cell pellets were harvested by centrifugation, incubated for 30 min in 1 ml of HLB containing 0.2% Triton X-100, and these lysates were mixed with scintillation cocktail and counted to estimate the cellular uptake of [^{3}H]thymidine. Results are expressed as percentage of [^{3}H]thymidine transported into vehicle-treated control cells over 30 s (15 031 ± 872 c.p.m.; 100 ± 6%; striped area). Bars: means ± SD (n=3). ^{a}Not different from control; $^b p < 0.005$, smaller than control.

whereas TT2 and TT13, which block the most the cellular transport of [^{3}H]thymidine (72-75% inhibition), are also the most potent against the incorporation of [^{3}H]thymidine into DNA (69-71% inhibition) (Figure 7). Under similar conditions, TT1, TT3, TT7, TT9 and TT11 inhibit nucleoside transport by 44-57% and DNA synthesis by 41-58%, whereas TT5, TT6, TT8 and TT10 are less effective, inhibiting both responses in the 20-35% range only (Figure 7).

Finally, the abilities of 10 and 25 μM TT2 to, respectively, inhibit nucleoside transport by 67 and 93% and DNA synthesis by 69 and 97% are both irreversible upon drug removal (Figure 8), suggesting that, after a 15-60 min pretreatment, the presence of TT quinone in the culture medium is no longer required to continually block the cellular transport of [^{3}H]thymidine and thereby prevent its incorporation into DNA. For the sake of comparison, the inhibitions of nucleoside transport and DNA synthesis caused by the novel tricyclic pyrone analog H10, which are fully reversible upon drug removal,[29,31] are demonstrated as a positive control under similar experimental conditions in the L1210 tumor assay system (Figure 8).

Inhibition of RNA and protein syntheses by TTs

Besides DNA synthesis, a 3 h treatment with TT2 can also inhibit, in a concentration-dependent manner, the rates of RNA (Figure 9) and protein (Figure 10) syntheses determined over 60 min periods of pulse labeling in L1210 cells *in vitro*. The concentration-response curves for the inhibitions of RNA (Figure 9) and protein (Figure 10) syntheses by TT2 are nearly identical to that for the inhibition of DNA synthesis (Figure 5): concentrations of TT2 greater than 1.6 μM must be used to demonstrate effectiveness, total inhibition is achieved at 25 μM and the IC_{50} value in all cases is about 6 μM. Under similar conditions, DAU inhibits the respective syntheses of RNA (IC_{50}: 0.8 μM) and protein (IC_{50}: 2 μM) about 7.5 and 3 times more effectively than TT2 (Figures 9 and 10).

Effects of TTs on the mitotic index

Control populations of L1210 cells cultured for 24 h in the absence of drugs contain only 1.86% of mitotic

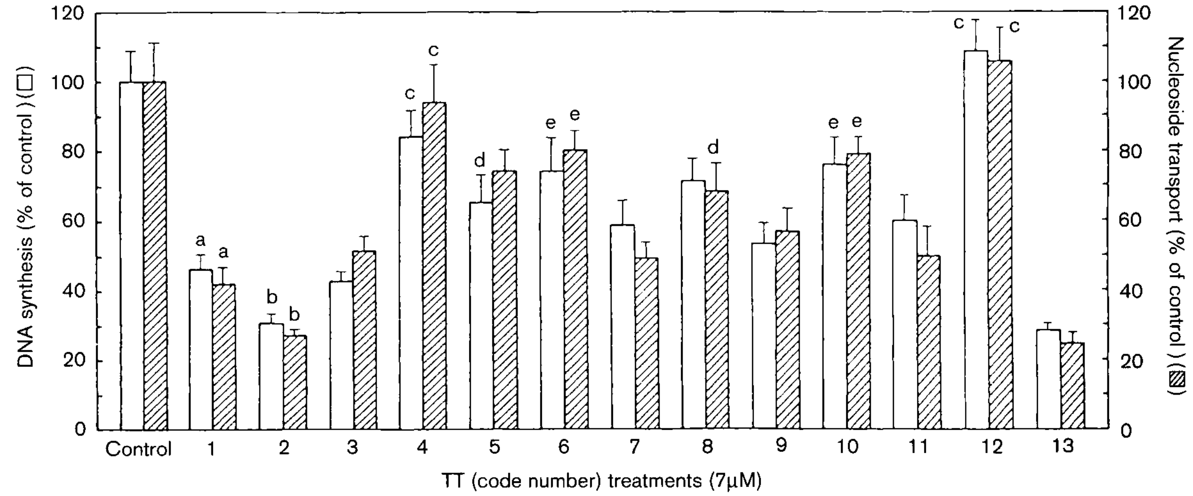


Figure 7. Comparison of the inhibitory effects of novel TT analogs on DNA synthesis (open) and nucleoside transport (striped) in L1210 cells *in vitro*. The cellular uptake of [^{3}H]thymidine (striped) and the rate of [^{3}H]thymidine incorporation into DNA (open) were determined in cells (1.38 × 10^6/0.5 ml of RPMI 1640 medium) respectively preincubated at 37°C for 15 or 90 min in the presence or absence (control) of 7 μM concentrations of the indicated compounds. For the cellular transport of nucleosides, preincubated cells were then exposed to 1 μCi of [^{3}H]thymidine for 30 s at 37°C. Results are expressed as percentage of [^{3}H]thymidine transported into vehicle-treated control cells over 30 s (15 539 ± 1767 c.p.m.; 100 ± 11%; striped control). For DNA synthesis, preincubated cells were then pulse-labeled with 1 μCi of [^{3}H]thymidine for an additional 30 min at 37°C. Results are expressed as percentage of [^{3}H]thymidine incorporation into DNA in vehicle-treated control cells over 30 min (28 505 ± 2594 c.p.m.; 100 ± 9%; open control). The blank value (1075 ± 171 c.p.m.) for cells incubated and pulse-labeled at 2°C with 1 μCi of [^{3}H]thymidine has been substracted from the results. Bars: means ± SD (n=3). ^{a}Not different from TT3, TT7, TT9 and TT11 ^{b}$p<0.01$, smaller than TT1 but not different from TT13; ^{c}not different from control; ^{d}$p<0.025$, greater than TT1; ^{e}$p<0.05$, smaller than control.

cells (Figure 11). In relation with their ability to block tubulin polymerization and cell cycle progression in M phase,[29-31] 24 h treatments with VCR and the tricyclic pyrone H10, respectively, produce 16- and 7-fold increases in the mitotic index (Figure 11). Such known microtubule de-stabilizing anticancer drugs, therefore, serve as positive controls in this antimitotic assay.[29-31] In contrast, none of the concentrations of TT2 tested, even those in the μM range that are highly cytostatic and cytotoxic at 24 h, are able to raise the mitotic index of L1210 cells (Figure 11), suggesting that TT quinones are unlikely to be antimitotic drugs that disrupt microtubule dynamics to trigger their anticancer activity. In relation with its known ability to first accumulate cells in G_2 and then inhibit cell cycle traverse as its concentration increases,[37-44] DAU actually decreases the percentage of mitotic cells by 74-91% (Figure 11). Since the highest concentrations of TT2 tested significantly decrease the percentage of mitotic cells by 38-48% (Figure 11), TTs might also prevent tumor cell cycle progression to mitosis.

Induction of DNA cleavage by TTs

L1210 cells containing $[^3H]$thymidine-prelabeled DNA were used to quantitatively determine whether TTs could induce DNA fragmentation over a 24 h period *in vitro*. CPT and DAU, two anticancer drugs known to induce DNA strand breaks by respectively inhibiting Topo I and II activities,[14,45-48] are used as positive controls in this DNA fragmentation assay (Figure 12). As reported before with anthracycline quinone antibiotics, the concentration-dependent induction of DNA cleavage caused by 24 h DAU treatments is biphasic, peaking at 45% in response to

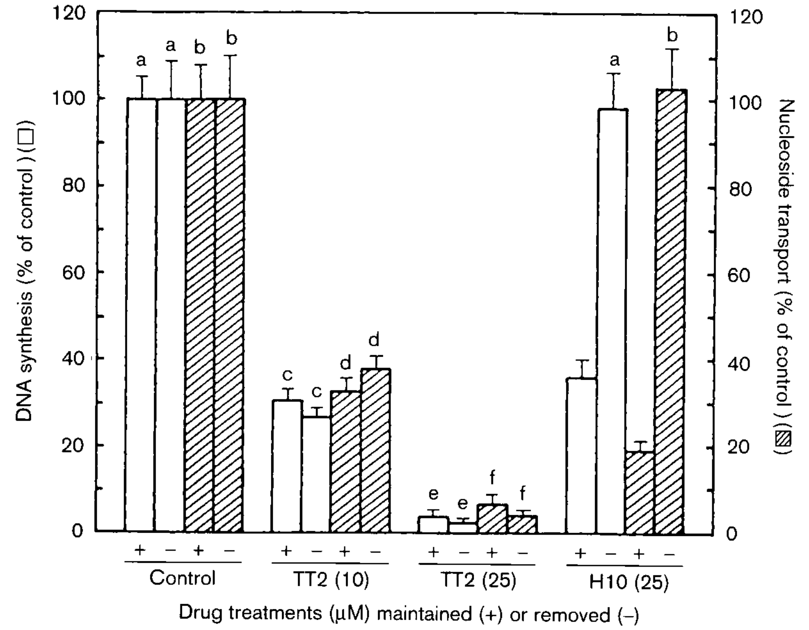


Figure 8. Irreversibility of the inhibitory effects of the novel TT analog TT2 on DNA synthesis (open) and nucleoside transport (striped) in L1210 cells *in vitro*. The reversible inhibitory effects of the tricyclic pyrone H10 are demonstrated in the same experiment. The cellular uptake of $[^3H]$thymidine (striped) and the rate of $[^3H]$thymidine incorporation into DNA (open) were determined in cells (1.64×10^6/0.5 ml of RPMI 1640 medium) respectively preincubated at 37°C for 15 or 60 min in the presence or absence (control) of 10 and 25 μM TT2 or 25 μM H10. The protocol of the experiments and the determination of the results were identical to those of Figures 5 and 6, except that, after preincubation, either the drugs were maintained in the culture medium (+) or the cells were spun, washed and resuspended in fresh medium in order to remove the drugs (−). After preincubation, vehicle-treated controls were similarly spun and washed. For the cellular transport of nucleosides, preincubated cells were then exposed, in the presence (+) or absence (−) of TT2 or H10, to 1 μCi of $[^3H]$thymidine for 30 s at 37°C. Results are expressed as percentage of $[^3H]$thymidine transported into vehicle-treated control cells over 30 s (11 054 $\pm$ 754 c.p.m.; 100 $\pm$ 7%; striped control +; 14 486 $\pm$ 1260 c.p.m.; 100 $\pm$ 9%; striped control −). For DNA synthesis, preincubated cells were then pulse-labeled with 1 μCi of $[^3H]$thymidine for an additional 30 min at 37°C in the presence (+) or absence (−) of TT2 or H10. Results are expressed as percentage of $[^3H]$thymidine incorporation into DNA in vehicle-treated control cells over 30 min (34 052 $\pm$ 1566 c.p.m.; 100 $\pm$ 5%; open control +; 28 811 $\pm$ 2506 c.p.m.; 100 $\pm$ 9%; open control −). The blank value (1328 $\pm$ 86 c.p.m.) for cells incubated and pulse-labeled at 2°C with 1 μCi of $[^3H]$thymidine has been substracted from the results. Bars: means $\pm$ SD (n=3). Values with similar superscripts are not significantly different from each other.

1.6 μM DAU but declining back to control level (5%) at higher concentrations of DAU (Figure 12).[17,18,49-51] In contrast, the concentration-dependent increase of DNA cleavage produced by 24 h CPT treatments reaches 57% in response to 1.6 μM CPT but remains at a plateau of maximal stimulation (60-70%) at higher concentrations of CPT (Figure 12).[52-59] After 24 h, the maximal levels of DNA cleavage caused by 10 μM TT1 (45%) and TT2 (61%), respectively, match those induced by 1.6 μM DAU and CPT (Figure 12). Although concentrations of TTs higher than those of DAU are required to induce such peak of DNA cleavage, the shape of the concentration-response curves for the ability of TT1 and TT2 to break DNA resembles more that of DAU than that of CPT (Figure 12), suggesting that the DNA-damaging effects of TT quinones and DAU might share some similarity.

Overall, the 13 different TTs compared at 7 μM induce various levels of DNA cleavage at 24 h (Figure 13) in relation with their respective cytostatic and cytotoxic activities when tested at 256 nM for 4 days (Figure 2). Indeed, TT2 and TT13, which are the most potent against L1210 cell proliferation and viability (Figure 2), again induce the most DNA fragmentation (54-57%) after 24 h (Figure 13), whereas TT4 and TT12, which have no antileukemic activity in the growth and viability assays (Figure 2), also fail to significantly raise the level of DNA cleavage (4-6%) over control (5%) (Figure 13). TT1, TT7 and TT9, which have good antileukemic activities (Figure 2), also produce substantial levels of DNA fragmentation (31-40%) (Figure 13). Moreover, the extent of DNA cleavage is only 14-18% in L1210 cells treated with TT3, TT5, TT6, TT8 or TT10 and the ability of TT11 to break DNA is minimal (7%) (Figure 13), in relation to the weak and marginal antiproliferative and cytotoxic effects of these compounds in the L1210 system *in vitro* (Figure 2).

Finally, L1210 cells treated for only 3 and 8 h with 10 μM TT1 or TT2 have the same level of DNA fragmentation at 24 h (36-45 or 58-67%, respectively) than if they are exposed for the whole 24 h

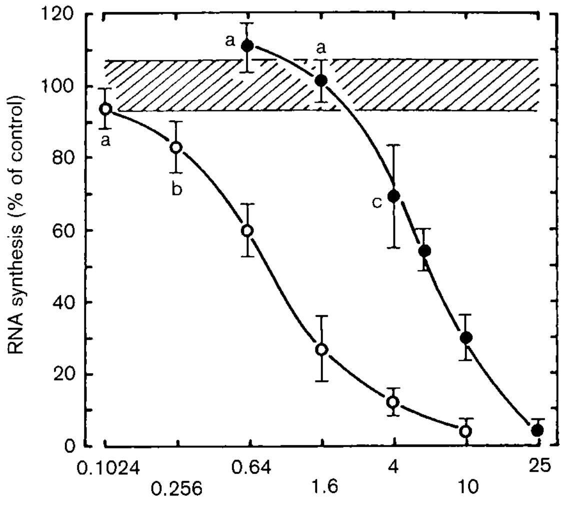


Figure 9. Comparison of the concentration-dependent inhibitions of RNA synthesis by the TT analog TT2 (●) and DAU (○) in L1210 cells *in vitro*. Cells (1.2×10^6/0.5 ml of RPMI 1640 medium) were incubated at 37°C for 3 h in the presence or absence (control) of the indicated concentrations of drugs, which are plotted on a logarithmic scale, and then pulse-labeled for an additional 1 h to determine the rate of [^{3}H]uridine incorporation into RNA. RNA synthesis in vehicle-treated control cells was 47117 ± 3204 c.p.m. ($100 \pm 7\%$; striped area). The blank value (1746 ± 155 c.p.m.) for cells incubated and pulse-labeled at 2°C with 2 μCi of [^{3}H]uridine has been substracted from the results. Bars: means ± SD (n=3). ^{a}Not different from control; $^b p < 0.05$ and $^c p < 0.025$, smaller than control.

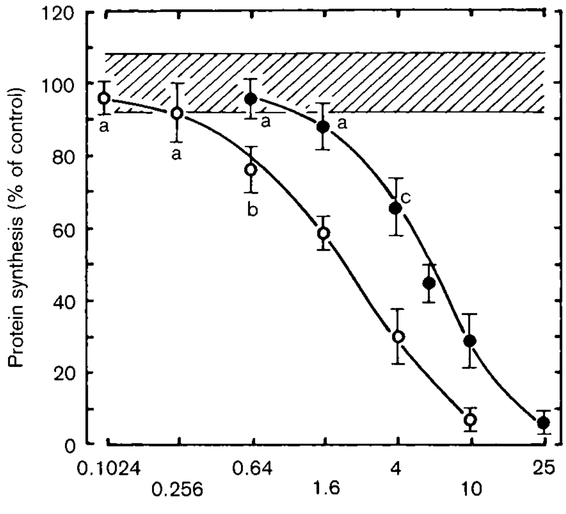


Figure 10. Comparison of the concentration-dependent inhibitions of protein synthesis by the TT analog TT2 (●) and DAU (○) in L1210 cells *in vitro*. Cells (1.51×10^6/0.5 ml of RPMI 1640 medium) were incubated at 37°C for 3 h in the presence or absence (control) of the indicated concentrations of drugs, which are plotted on a logarithmic scale, and then pulse-labeled for an additional 1 h to determine the rate of [^{3}H]leucine incorporation into protein. Protein synthesis in vehicle-treated control cells was 13812 ± 1070 c.p.m. ($100 \pm 8\%$; striped area). The blank value (2436 ± 298 c.p.m.) for cells incubated and pulse-labeled at 2°C with 2.5 μCi of [^{3}H]leucine has been substracted from the results. Bars: means ± SD (n=3). ^{a}Not different from control; $^b p < 0.025$ and $^c p < 0.01$, smaller than control.

incubation period to TT1 or TT2 (40 or 63%, respectively) (Figure 14). Under similar conditions, 1.6 μM DAU and CPT also rapidly trigger molecular events, which are irreversible and produce identical levels of DNA fragmentation at 24 h (33–41 and 51–58%, respectively), whether or not those drugs are maintained in the culture medium after 3 or 8 h (Figure 14). It should be noted that no significant elevation of DNA cleavage is detectable in this assay after 3 and 8 h of drug exposure and that the increases of DNA fragmentation caused by TTs, DAU and CPT only appear after 12 h to reach a maximal level at 24 h (data not shown), suggesting that the irreversible events triggered by those drugs during the initial 3 h still require a substantial period of time, irrespective of the continual presence or absence of drugs, to fully induce DNA fragmentation. These results suggest that, like anticancer drugs known to induce DNA strand breaks, TT quinones interact rapidly with cellular targets to induce long-lasting DNA-damaging effects, which develop and persist after drug removal.

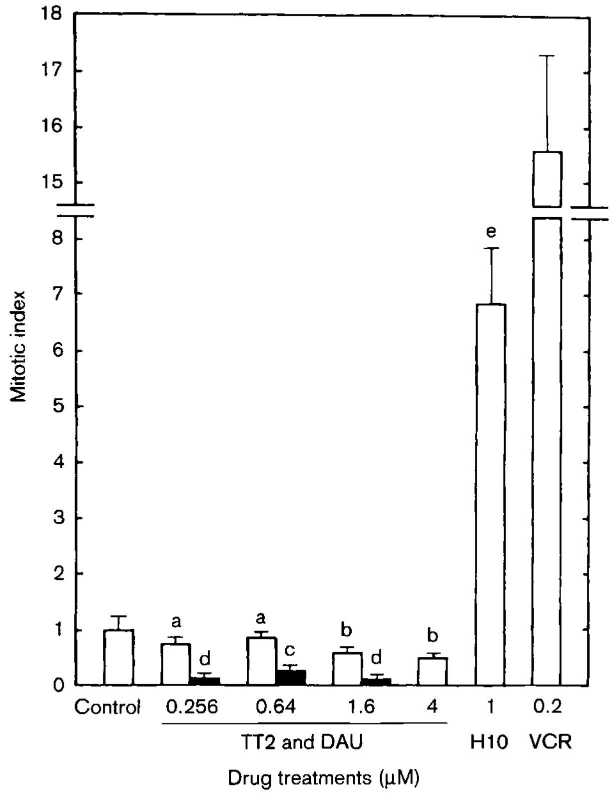


Figure 11. Effects of the novel TT analog TT2 (□) and DAU (■) on the mitotic index of L1210 cells *in vitro*. The antimitotic activities of VCR and the tricyclic pyrone H10 are demonstrated in the same experiment. Cells (10^6/0.5 ml of RPMI 1640 medium) were incubated in triplicate for 24 h at 37°C in the presence or absence (control) of 0.256, 0.64, 1.6 and 4 μM TT2, 0.256, 0.64 and 1.6 μM DAU, 0.2 μM VCR or 1 μM H10. After fixation with MeOH:acetic acid (3:1) and staining with 0.1% crystal violet, about 500 cells/slide were scored for mitotic figures and the mitotic index was expressed as the percentage of mitotic cells in drug-treated cultures divided by the percentage of mitotic cells in non-treated controls. The mean percentage of mitotic cells in control at 24 h was 1.86±0.43%. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$, [c]$p<0.01$ and [d]$p<0.005$, smaller than control; [e]$p<0.005$, greater than control but smaller than VCR.

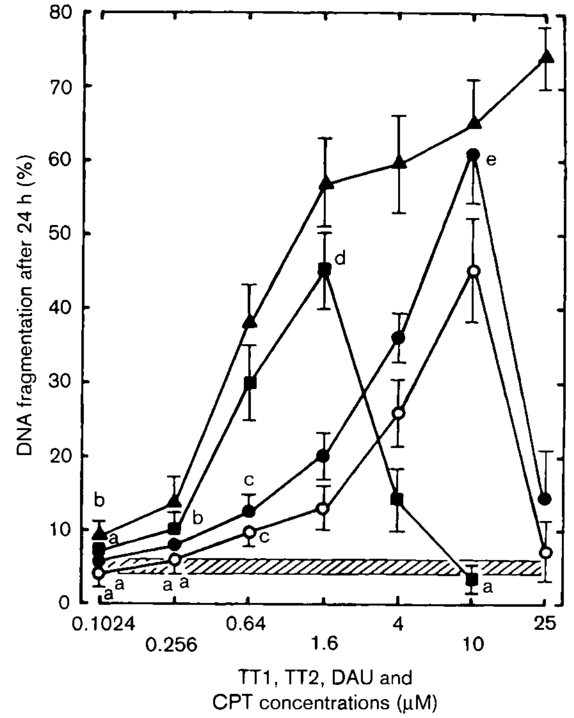


Figure 12. Comparison of the concentration-dependent effects of novel TT analogs and known DNA-damaging anticancer drugs on DNA cleavage in L1210 cells *in vitro*. Cells (10^6/0.5 ml of RPMI 1640 medium) were prelabeled with 1 μCi of [^{3}H]thymidine for 2 h, washed and resuspended in fresh FCS-containing medium, and incubated at 37°C for 24 h in the presence or absence (control) of the indicated concentrations of TT1 (○), TT2 (●), DAU (■) or CPT (▲), which are plotted on a logarithmic scale. After lysing the cells in HLB containing 0.2% Triton X-100, the detergent-soluble DNA fragments present in the supernatants and the intact chromatin DNA remaining in the pellets were separated by centrifugation and their radioactivity estimated by LSC. Results are expressed as [c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100 at 24 h. For untreated controls (5.3±0.6% DNA fragmentation; striped area), the supernatant is 741±79 c.p.m. and the pellet is 13 293±1446 c.p.m. Bars: means±SD (n=3). [a]Not different from control; [b]$p<0.05$ and [c]$p<0.01$, greater than control; [d]$p<0.05$, smaller than CPT but not different from 10 μM TT1; [e]$p<0.05$, greater than TT1 but not different from CPT.

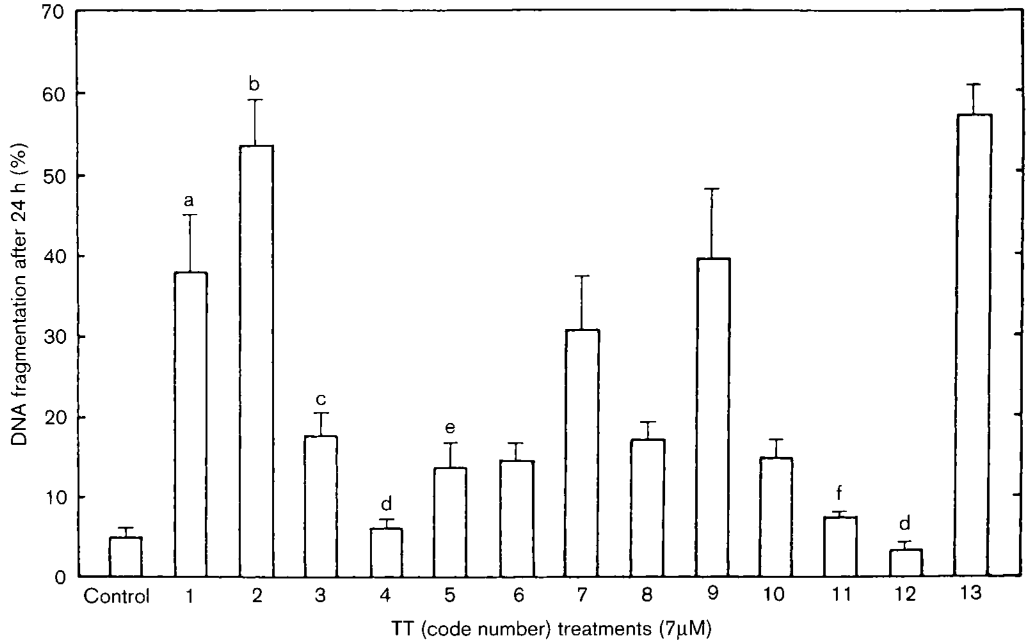


Figure 13. Comparison of the abilities of novel TT analogs to induce DNA cleavage in L1210 cells *in vitro*. Cells containing prelabeled DNA were incubated at 37°C for 24 h in the presence or absence (control) of 7 μM concentrations of the indicated compounds. The protocol of the experiment and the determination of the results were identical to those of Figure 12. Bars: means ± SD (n=3). [a]Not different from TT7 and TT9; [b]$p < 0.05$, greater than TT9 but not different from TT13; [c]$p < 0.05$, smaller than TT7; [d]not different from control; [e]$p < 0.01$, greater than control, $p < 0.025$, greater than TT11 but not different from TT3, TT6, TT8 and TT10; [f]$p < 0.01$, greater than control.

Discussion

To the best of our knowledge, this is the first report demonstrating that several TT analogs are potent antitumor agents *in vitro*. Except TT4 and TT12, which are inactive at the concentrations tested in Figures 2, 7 and 13, our comparison studies show that the different magnitudes at which equimolar concentrations of various TT analogs inhibit tumor cell proliferation match very well the different cytotoxic activities of these compounds (Figure 2). Moreover, the different levels of DNA fragmentation induced by the various TT analogs (Figure 13) match exactly the ranking of these compounds for their cytostatic/cytotoxic effects (Figure 2), suggesting that the ability of TT analogs to break DNA plays a significant role in their molecular mechanism of antitumor activity. In addition, the various TT analogs inhibit DNA synthesis in relation to their effectiveness against nucleoside transport (Figure 7), suggesting that they prevent DNA assembly because they block the cellular uptake of DNA precursors. Because of their ability to interact with both membrane and nuclear targets to block nucleoside transport, inhibit nucleic acid and protein syntheses, cleave DNA, and reduce tumor cell growth and viability in the nM range *in vitro*, these TT analogs may represent a novel synthetic class of bifunctional anticancer drugs valuable to develop new means of polychemotherapy.

This preliminary study has been focused on TT2, the most potent antitumor TT quinone synthesized so far, but its exact molecular mechanism of action remains to be elucidated, and further studies would be required to establish conclusive relationships between the chemical structures of specific TT analogs and their antitumor activities. The fact that the C2-brominated derivative of TT2, TT13, has the same potency than TT2 in all bioassays studied confirms the superior antitumor activity of substituted 2-methoxy-9,10-dihydro-9,10[1′,2′]benzenoanthracene-1,4,5,8-tetraone molecules. All three TT bisquinones, TT1, TT2 and TT13, have excellent antitumor activities, but the two new compounds, TT2 and TT13, respectively, contain a methoxy function at C2 or C3 of the TT1 skeleton, and are significantly more potent than TT1 as inducers of DNA cleavage and inhibitors of nucleoside transport/DNA synthesis and tumor cell growth/viability. The greater antitumor effects of TT2 and TT13 might be due to the greater ability of these TT bisquinones to bind with acidic hydrogen or cationic metal, utilizing C1 and C2 oxygens in TT2 and C3 and C4 oxygens in TT13. Interestingly, the antitumor

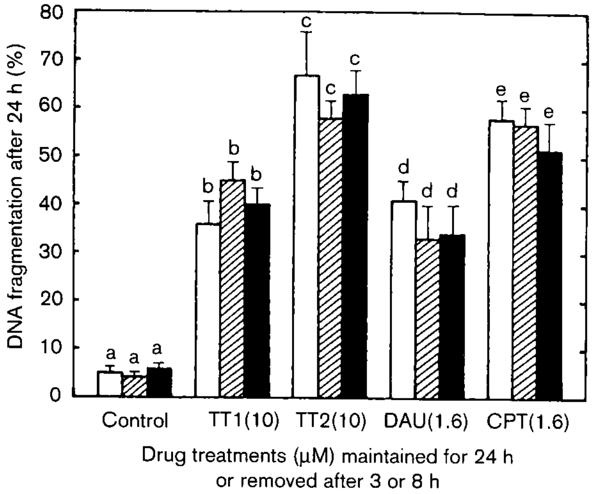


Figure 14. Irreversibility of the effects of novel TT analogs and known DNA-damaging anticancer drugs on DNA cleavage in L1210 cells *in vitro*. Cells containing prelabeled DNA were incubated at 37°C for various periods of time in the presence or absence (control) of 10 μM TT1 or TT2 and 1.6 μM DAU or CPT. The protocol of the experiment and the determination of the results at 24 h were identical to those of Figure 12, except that the drugs were either maintained in the medium for the whole 24 h period of incubation (open) or removed after the first 3 (striped) and 8 (closed) h. After spinning and washing the appropriate samples to remove the drugs, cells were resuspended in 0.5 ml of fresh medium to complete the 24 h period of incubation in the absence of drugs. Vehicle-treated controls were similarly spun and washed at 3 and 8 h. Bars: means ± SD (n=3). Values with similar superscripts are not significantly different from each others. [b]Not different from DAU; [c]$p<0.025$, greater than TT1 but not different from CPT; [d]$p<0.05$, smaller than CPT.

activity of the bisquinone TT1 is pretty much similar to those of TT7 and TT9, which have only one quinone moiety. In contrast to the difference observed between the potencies of TT1 and TT2, the methoxy function of TT9 does not significantly increase its antitumor activity over that of TT7.

Since the parent TT0 is inactive, it is reasonable to assume that the monoquinone, bisquinone and/or methoxy functions of the TT framework may be responsible for the antitumor activity of the most effective TT analogs, although the relative contribution of these individual or combined substituents to the bioactivity of the TT analogs is still unclear. For instance, the pentasubstituted TT dihydroquinone TT3 has good antileukemic activity, especially against DNA synthesis/nucleoside transport (Figure 7), despite its lack of quinone functionality. Surprisingly, the antitumor activity of the monoquinone analog of TT3, TT5, is even smaller than that of TT3. All the R^4,R^5-substituted or dihydro-analogs TT6, TT8, TT10 and TT11 are not quinones but diketones, and their weak antitumor effects might be due to the conformational change in the A ring and the loss of the trigonal molecular shape. TT4 and TT12 are totally inactive and lack any quinone or dihydroquinone moiety, but their sheer size might also prevent such bulky molecules from interacting with nucleoside transporters and reaching intracellular and intranuclear targets to induce antitumor effects. It would be interesting to evaluate the anticancer activity of the bisquinone and bisdihydroquinone derivatives of this class of compounds to determine whether extra six-membered rings attached at the A and C rings increase or decrease such bioactivity. Since the ability of TT analogs to bind to nucleoside transporters, interact covalently with DNA, inhibit Topo activities, and affect the production of ROS and FRs is still unknown, it is rather premature to speculate on the antitumor potential of various substitutions and quinone or hydroquinone functionalities of the TT skeleton.

The cytostatic and cytotoxic effects of each concentrations of TT2 increase with the time in culture (Figures 3 and 4), suggesting that the effectiveness of TT analogs as inhibitors of tumor cell proliferation and viability *in vitro* is a combination of drug concentration and duration of drug exposure. As a result, TT2 inhibits L1210 tumor cell growth at 2 and 4 days with IC_{50} values of 300 and 150 nM, respectively, in relation with its ability to reduce L1210 tumor cell viability with IC_{50} values of 250 nM at day 2 and 100 nM at day 4. The IC_{50} required to reduce tumor cell viability at day 4 (100 nM) may be lower and perhaps more accurate than that observed for tumor cell growth inhibition at day 4 (150 nM) because the Coulter counter data include all viable and non-viable tumor cells that have previously accumulated and remain in the medium at day 4, irrespective of their present metabolic status and reproductive ability. Moreover, decreased tumor cell viability after several days of drug treatment may be a better predictor of anticancer activity than antiproliferation since growth delay may allow survivors to resume dividing and expand clonally once the drug is catabolized or eliminated and its effect is waning.[60]

Overall, the new TT bisquinone TT2 is a potent antitumor agent, which is active in the nM range and is only 3.5-6 times less cytostatic/cytotoxic after 4 days than equimolar concentrations of the clinically proven anthracycline quinone antibiotic DAU, a very potent anticancer drug used as an arbitrary reference in our L1210 tumor cell studies *in vitro* (Figures 3 and 4). When IC_{50} values are compared, TT2 also inhibits the syntheses of DNA, RNA and protein in L1210 cells after 2-3 h about 3-6 times less effectively than DAU

(Figures 5, 9 and 10), and concentrations of TT2 about 6 times higher than those of DAU are also required to induce the same peak of DNA cleavage in L1210 cells after 24 h (Figure 12). However, in addition to mimicking all the antitumor effects of DAU studied, our data demonstrate that TT2 remarkably blocks the cellular transport of nucleosides, which DAU cannot do (Figure 6), suggesting that these novel antitumor TT bisquinones may have a more versatile mechanism of action and be advantageous in polychemotherapy to potentiate the anticancer effects of antimetabolites and circumvent multidrug resistance (MDR).

Although DAU is consistently about 3-6 times more potent than TT2 in almost all bioassays studied, it seems that, for both drugs, concentrations in the 25-150 nM range are sufficient to inhibit tumor cell growth/viability (Figures 3 and 4), whereas higher concentrations in the 0.8-10 μM range must be used to inhibit macromolecule syntheses (Figures 5, 9 and 10) and maximally induce DNA fragmentation (Figure 12). However, these apparent discrepancies may be due in part to different experimental conditions and cellular responses to various periods of drug exposure: the rates of nucleic acid and protein syntheses over 30-60 min are inhibited in cells treated for only 2-3 h with TT2 or DAU, whereas the level of DNA cleavage and the reduction of tumor cell growth/viability are the results of 1 and 4 day drug treatments, respectively. It should be noted that no significant DNA fragmentation can be detected within the first 8 h of TT2 or DAU treatments, and that concentrations of DAU and TT2 respectively greater than 0.25 and 1.6 μM must be used to induce substantial antiproliferative and cytotoxic effects after only 24 h of drug exposure (data not shown). Although the different magnitudes of the antitumor effects of the 13 TT analogs are pretty much consistent throughout the bioassays studied, the greater and perhaps more reliable responses caused by the longer periods of drug exposure might also explain why the ranking of some TT analogs, like TT3 and TT11, for their antiproliferative and cytotoxic potencies at day 4 (Figure 2) matches more closely the ranking of these compounds for their ability to break DNA after 1 day (Figure 13) than the ranking of these compounds for their efficacy against nucleoside transport/DNA synthesis after only 15 min to 2 h (Figure 7).

Although the cellular uptake, retention, metabolism and half-life of radiolabeled TT2 in cell culture remains to be determined, our preliminary data suggest that TT2 rapidly triggers long-lasting inhibitory and damaging events which persist upon drug removal. For instance, the irreversibility of the inhibitions of nucleoside transport/DNA synthesis caused by TT2 suggests that the presence of this drug in the extracellular medium becomes irrelevant after 15-60 min (Figure 8), presumably because it has already bound to membrane nucleoside transporters and, perhaps, also been internalized to interact with nuclear enzymes involved in DNA metabolism. Since both responses are irreversible (Figure 8), share similar IC_{50} values (Figures 5 and 6) and are modulated in parallel by various TT analogs (Figure 7), the rapid inhibition of thymidine incorporation into DNA caused by TT2 is most likely due to its ability to immediately block the cellular transport of this nucleoside. Incidentally, the DNA cleavage caused by CPT, DAU and the TT quinones, TT1 and TT2, is undetectable at 2, 4 and 8 h, becomes significant at 12 h, and reaches a maximum level at 24 h (data not shown). Hence, it takes 24 h to fully reveal the extent of DNA cleavage achieved by 3-8 h pretreatments with the above drugs. The irreversibility of this response suggests that the DNA-damaging events triggered by TT analogs, DAU and CPT within the first 3-8 h can proceed uninterrupted for the next 16-21 h in the absence of these drugs in the extracellular medium and are sufficient to induce the same maximal levels of DNA fragmentation than when the drugs are maintained in the culture medium for the entire 24 h period of incubation (Figure 14).

In contrast to VCR and the novel tricyclic pyrone analog H10, which are microtubule de-stabilizing anticancer drugs known to respectively interact with the vinca and colchicine binding sites on tubulin to block microtubule assembly and the progression of tumor cells undergoing mitosis,[29-31] the inability of TT2 to raise the mitotic index of L1210 cells after 24 h (Figure 11) suggests that TT analogs are not mitotic spindle poisons and neither interact with tubulin nor alter the polymerization/depolymerization of microtubules in order to induce their cytostatic/cytotoxic effects. In fact, anticancer quinones have been reported to cause G_2 arrest,[13] and the ability of increasing concentrations of TT2 to increasingly reduce the percentage of mitotic cells at 24 h (Figure 11) like the increasing concentrations of DAU known to first block tumor cells in G_2 and then prevent cell cycle traverse all together[37-44] suggests that TT quinones might also block early stages of cell cycle progression to prevent tumor cells from reaching the M phase.

The fact that, within 24 h, TT1 and TT2 can produce as much internucleosomal DNA fragmentation in L1210 cells than the known Topo I and II inhibitors CPT and DAU (Figure 12) suggests that the ability of TT quinones to produce DNA strand breaks may play a major role in their mechanism of antitumor activity. Since the shapes of the concentration-response curves for the effects of TT1 and TT2 on DNA cleavage resemble the biphasic response to DAU, which has already been reported and discussed before,[17,49-51] it is tempting to speculate that the mechanisms by which TT quinones and DAU induce DNA fragmentation share some similarity and that DNA cleavage is not always necessary for high concentrations of TT quinones and DAU to be cytotoxic. The Topo II-associated DNA lesions occuring in cells exposed to anthracycline quinone antibiotics may facilitate subsequent internucleosomal DNA fragmentation by endogenous nucleases and trigger apoptosis.[17] Since apoptosis is an active and cell cycle phase-specific process, which requires the expression of specific genes, the syntheses of new RNA and proteins and the activation of endonuclease enzymes, inhibitors of such mechanisms can prevent DNA fragmentation in anthracycline-treated cells.[17] In spite of their increasing cytotoxicity, the highest concentrations of TT1, TT2 and DAU tested in our study might inhibit RNA and protein syntheses, reduce the level of Topo II targets, inactivate endonucleases and/or arrest cell cycle traverse to such degrees that they actually block the molecular mechanisms required for internucleosomal DNA fragmentation and apoptosis, and produce the paradoxical biphasic curve of DNA cleavage shown in Figure 12.[17] Taken together, the irreversibilities of the inhibitions of nucleoside transport/DNA synthesis and of the induction of DNA fragmentation upon TT2 removal suggest that, because TT analogs may rapidly and tightly interact with various membrane and intracellular targets, their presence is soon no longer required in the medium to disrupt the structures/functions of nucleoside transporters, nucleic acids and proteins, and to trigger long-lasting antitumor events, which persist after cessation of drug treatment.

Combining drugs, which target different molecules and achieve complementary or synergistic antitumor effects, is an important strategy in cancer chemotherapy. A TT quinone inhibiting nucleoside transport and inducing DNA cleavage might disrupt a wider spectrum of molecular targets in populations of unsynchronized tumor cells than another drug affecting a single one of these events. Nucleoside transporters in mammalian plasma membranes function by equilibrative (facilitated diffusion) or Na^+-dependent (concentrative) mechanisms. All nucleosides are substrates for the Na^+-independent transporters but Na^+-dependent transporters generally accept purine rather than pyrimidine nucleosides as substrates.[61] Dipyridamole (DPR), 6-(4-nitrobenzylmercapto)purine ribonucleoside (NBMPR) and dilazep are standard potent inhibitors of equilibrative nucleoside transport, whereas phloridzin specifically inhibits Na^+-dependent nucleoside transport.[62,63] Like TT2, NBMPR and DPR also reduce [^3H]thymidine incorporation into DNA.[64] Nucleoside transport inhibitors block equally well the influx and efflux of nucleosides.[65] L1210 cells possess three distinct nucleoside transporters: two equilibrative transporters sensitive (es) or insensitive (ei) to NBMPR, and one Na^+-dependent transporter (cif) of low sensitivity to NBMPR and DPR.[61,63,66,67] An important objective would be to determine if TT2 affects preferentially the bidirectional fluxes of purine and/or pyrimidine nucleosides, mimics or modulates the cellular action of the above nucleoside transporter probes, binds to specific plasma membrane nucleoside transporters and uses such pathway for its cellular uptake.

For nucleotide synthesis, cells use purine and pyrimidine nucleosides generated either through *de novo* synthesis or through the utilization of salvage pathways. MDR is sometimes associated with increases in the number of nucleoside transporters and their rate of transport, resulting in the increased uptake of adenosine.[68] By blocking the rescue effect of exogenous nucleosides, NBMPR, DPR and dilazep may potentiate or prolong the antitumor activity of antimetabolites which inhibit the *de novo* pathway for nucleoside synthesis.[69-73] The clinical effectiveness of ADR and DAU is limited by their cumulative cardiotoxicity and ability to induce MDR.[10] The multifactorial mechanisms of MDR to anthracycline quinone antibiotics may include altered expressions of P-glycoprotein (P-gp), Topo II and multidrug resistance-associated protein, increased DNA repair and glutathione-dependent detoxifying enzyme activities, and alterations in cell cycle progression and apoptotic pathways.[74-80] DPR circumvents ADR resistance[81] and its analog, BIBW 22, is a bifunctional modulator which reverses the MDR phenotype by interfering with both P-gp and nucleoside transport in MDR cells.[33] As bifunctional inhibitors of nucleoside transport and inducers of DNA cleavage, TT quinones might be valuable in polychemotherapy to potentiate the antitumor activity of methotrexate and 5-fluorouracil, and sensitize MDR tumor cells that have become unresponsive to the cytotoxicity of other conventional DNA-damaging anticancer agents.

Conclusion

TT analogs may represent a novel synthetic class of bifunctional anticancer drugs, which block nucleoside transport and induce DNA fragmentation in order to inhibit nucleic acid and protein syntheses, and decrease the proliferation and viability of leukemic cells in the nanomolar range *in vitro*. Further structure-activity studies are warranted to elucidate the molecular mechanism of action and demonstrate the anticancer potential *in vivo* of these TT analogs, which might be valuable to develop new means of polychemotherapy.

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