Anti-Cancer Drugs 2002, **13,** pp. 567–581

Report

Among substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetraones, the lead antitumor triptycene bisquinone TT24 blocks nucleoside transport, induces apoptotic DNA fragmentation and decreases the viability of L1210 leukemic cells in the nanomolar range of daunorubicin *in vitro*

Elisabeth M Perchellet,[1] Bonnie J Sperfslage,[1] Yang Wang,[1] Xiaodong Huang,[2] Masafumi Tamura,[2] Duy H Hua[2] and Jean-Pierre Perchellet[1]

[1]Anti-Cancer Drug Laboratory, Division of Biology, Ackert Hall, and [2]Department of Chemistry, Kansas State University, Manhattan, KS 66506-4901, USA

In contrast to their inactive parent compound triptycene (code name TT0), several new synthetic analogs (TT code number) have antileukemic activities and remain effective in daunorubicin (DAU)-resistant tumor sublines *in vitro*. Among variously substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetraones, a total of six lead antitumor compounds have been identified, and their code names are TT2, TT13, TT16, TT19, TT21 and TT24. These active antitumor triptych structures have bisquinone functionality, and various bromo, methoxy, methylamino and/or dimethylamino substitutions with or without longer alkyl chains on the amino function. Like the anthracycline quinone antibiotic DAU, these triptycene (TT) bisquinones also inhibit DNA synthesis and induce DNA cleavage in relation with their cytotoxic activities, but have the additional advantage of blocking the cellular transport of purine and pyrimidine nucleosides, an effect which DAU cannot do. As demonstrated by intact chromatin precipitation and agarose gel electrophoresis, the ability of TT bisquinones and DAU to induce DNA fragmentation is biphasic with a peak that shifts to lower concentrations with increasing times of drug exposure. The most effective lead antitumor compound, TT24, induces DNA cleavage in the same concentration-dependent manner as DAU at 24 h (similar peak in response to 1.6 μM) and is nearly equipotent to DAU against L1210 tumor cell viability at day 4 (IC_{50} values of TT24 and DAU: 48 and 25 nM, respectively). The mechanism by which TT24 induces DNA fragmentation is inhibited by actinomycin D, cycloheximide, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone, *N*-tosyl-L-phenylalanine chloromethyl ketone and $ZnSO_4$, suggesting that TT bisquinones trigger apoptosis by caspase and endonuclease activation. Since TT24 is cytotoxic in the nanomolar range of DAU, but might have a more versatile mechanism of action than DAU in wild-type and multidrug-resistant tumor cells, this new class of DNA-damaging quinone antitumor drugs inhibiting nucleoside transport might be valuable to develop new means of polychemotherapy. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, DNA synthesis and fragmentation, L1210 cells, nucleoside transport, triptycene bisquinones, tumor cell viability.

This study was supported by grants from the National Institutes of Health (National Cancer Institute 1R01 CA86842-01 and Center of Biomedical Research Excellence 1P20 RR15563-01 with matching funds from the State of Kansas), the National Science Foundation (CHE-0078921), the American Heart Association, Heartland Affiliate (0051658Z and 9951177Z), Great Plains Diabetes Research, Inc., BioServe Space Technologies (NASA NAGW-1197), the Howard Hughes Medical Institute (Biological Sciences Education Grant) and Kansas State University (Center for Basic Cancer Research).

Correspondence to J-P Perchellet, Anti-Cancer Drug Laboratory, Kansas State University, Division of Biology, Ackert Hall, Manhattan, KS 66506-4901, USA.
Tel: (+1) 785 532-7727; Fax: (+1) 785 532-6653;
E-mail: jpperch@ksu.edu

Introduction

Several natural and synthetic quinones function as bioreductive alkylating agents and have antitumor activity.[1,2] Their cytotoxicity may be due to two competing mechanisms: soft electrophilic arylation and redox cycling oxidation.[3] Complete two-electron reduction of the quinone ring by DT diaphorase produces a stable hydroquinone, whereas 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 reactive O_2 species and FRs, which can induce DNA damage, lipid peroxidation and cytotoxicity. However, the few quinone antitumor drugs used clinically have complex chemical structures with a number of active functional groups and the exact contribution of the quinone group to their antitumor activity remains uncertain.[4–7] 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.[4,5,7–12] 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 quinone antitumor drugs with improved bioactivity.[4]

Recently, we found that, with or without quinone functionality, synthetic triptycene (TT) analogs may represent a novel class of bifunctional antitumor drugs that block nucleoside transport, induce DNA cleavage and decrease the viability of murine L1210 leukemic cells in the nanomolar range *in vitro*.[13] Among 13 TT analogs synthesized so far, the TT bisquinone TT2 and its C2-brominated derivative, TT13, are the current lead antitumor compounds.[13] Although less potent, TT2 inhibits DNA, RNA and protein syntheses, induces DNA cleavage, and decreases the mitotic index, proliferation and viability of L1210 lymphocytic leukemia cells like DAU.[13] The critical finding is that, in contrast to DAU, TT2 has the additional advantage of also blocking the cellular transport of nucleosides,[13] an effect which might be very valuable in polychemotherpy to potentiate the action of antimetabolites and circumvent MDR. For instance, by blocking the salvage pathway for the utilization of extracellular nucleosides, inhibitors of nucleoside transport, such as dipyridamole, may potentiate or prolong the antitumor activity of antimetabolites, such as methotrexate (MTX) and 5-fluorouracil (5-FU), which inhibit the *de novo* pathway for nucleoside synthesis.[14–21] Moreover, nucleoside transport may be one of the mechanisms involved in MDR and the classic probes used to experimentally block various nucleoside transporters have been shown to sensitize MDR tumor cells to the action of conventional anticancer drugs, such as DOX.[22–26] Indeed, our preliminary studies indicate that TT2, a potent DNA-damaging quinone antitumor drug which has the unique ability to block nucleoside transport, retains its effectiveness in human HL-60 promyelocytic leukemia sublines that have already developed different mechanisms of resistance to DAU.[27]

In order to explore structure–activity relationships (SARs) and identify more effective lead compounds, 11 new TT analogs (TT14–24) were synthesized and screened in the present study (Figure 1). Their potency and mechanism of action were assessed and compared to those of DAU, TT2 and TT13, the reference quinone antitumor drugs that have already been characterized together in the same L1210 tumor cell system *in vitro*.[13] Interestingly, we discovered four new lead antitumor TT bisquinones, the most potent of them being TT24, a nucleoside transport inhibitor that induces DNA fragmentation by an active process of apoptosis and is, at nanomolar concentrations, nearly as cytotoxic as DAU.

Materials and methods

Cell culture and drug treatments

A new, short and easy method to synthesize the structures of the 12 TT analogs (code names TT2, TT13 and TT14–23) illustrated in Figure 1 has been developed.[28] The synthesis of TT24 (Figure 1) will be reported elsewhere. All solutions of triptycene (code name TT0; Aldrich, Milwaukee, WI), synthetic TT analogs, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma, St Louis, MO), benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) and benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone (z-IETD-fmk) (both from Calbiochem, La Jolla, CA) were dissolved and diluted in dimethylsulfoxide (DMSO), whereas DAU (from Sigma) solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl.[13,27,29–31] $ZnSO_4$ (JT Baker, Phillipsburg, NJ) and actinomycin D–mannitol (Act-D; Sigma) solutions were prepared in H_2O and cycloheximide (CHX; Sigma) was dissolved in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS).[30,31] 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% 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

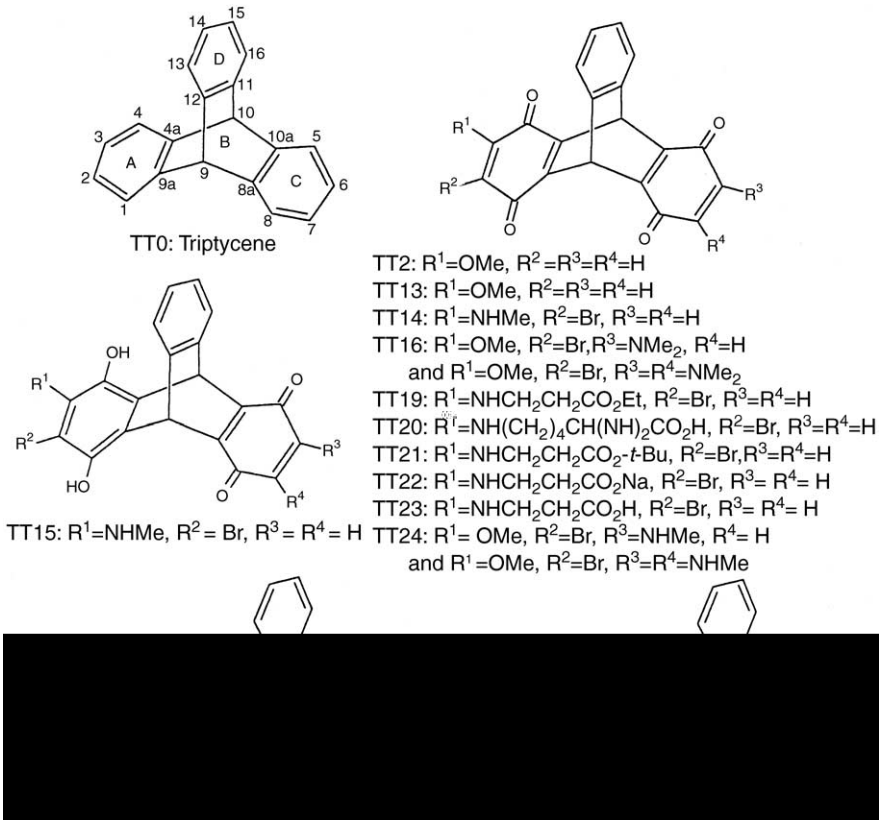

Figure 1. Chemical structures and code names of the TT analogs tested for their antitumor effects *in vitro*.

aliquots, the concentration of vehicle in the final incubation volume (0.5 ml) did not affect the basal activity levels in control L1210 cells incubated in the absence of drugs.[13,27,29–31]

Cell viability assay

For tumor cell viability, L1210 cells suspended in FCS-containing RPMI 1640 medium were grown at 37°C in 48-well Costar cell culture plates for up to 4 days in the presence or absence (control) of drugs to evaluate their cytotoxicity. Decreasing concentrations of cells, such as 75 000 and 4700 cells/0.5 ml/well, were initially plated in triplicate 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 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.[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). Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were subtracted from the results.[13,27,29–31]

Nucleoside transport and DNA synthesis

L1210 cells (10^6/0.5 ml) were preincubated for 15 min at 37°C in the presence or absence (control) of drugs and then exposed to 1 μCi of [2,8-^{3}H]adenosine (30 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO) or [methyl-^{3}H]thymidine (50 Ci/mmol; Amersham, Arlington Heights, IL) for only 30 s to, respectively, assess the cellular uptake of purine and pyrimidine nucleosides over such very short

periods of time.[13,22,27,29–31] Reactions were diluted with 2 ml of ice-cold 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 6 ml of Bio-Safe II (Research Products International, Mount Prospect, IL) and counted to estimate the cellular uptake of [^{3}H]adenosine or [^{3}H]thymidine. Drug inhibition was expressed as percentage of [^{3}H]adenosine or [^{3}H]thymidine transported into vehicle-treated control tumor cells over a similar 30-s period.[13,22,27,29–31] To estimate the rate of DNA synthesis, L1210 cells were resuspended in fresh FCS-containing RPMI 1640 medium at a density of 10^6 cells/0.5 ml, incubated at 37°C for 90 min in the presence or absence (control) of drugs and then pulse-labeled for an additional 30 min with 1 μCi of [^{3}H]thymidine. The incubation was 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 6 ml of Bio-Safe NA (Research Products International, Mt Prospect, IL).[13,27,29–31]

DNA cleavage and apoptosis

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 5×10^5 cells/0.5 ml.[11,13,27,29–31,33,34] Such cells containing prelabeled DNA were then incubated at 37°C for 24 h in the presence or absence (control) of drugs. After centrifugation at 200 *g* for 10 min to discard the drugs and wash the cells, the intact cell pellets were lysed for 20 min in 0.5 ml of ice-cold HLB and centrifuged at 12 000 *g* for 15 min to collect the supernatants. The radioactivities in the supernatants (detergent-soluble low molecular weight DNA fragments) and the pellets (intact chromatin DNA) were determined by LSC: percent DNA fragmentation=[c.p.m. in supernatant/c.p.m. in supernatant+pellet] × 100.[11,13,27,29–31,33,34] Before being counted in 6 ml of Bio-Safe NA, the intact pelleted chromatin was incubated for 2 h at 60°C in the presence of 0.6 ml of NCS tissue solubilizer (Amersham).[33] For apoptosis, L1210 cells were incubated for various periods of time in the presence or absence (control) of drugs and DNA was extracted from samples with equal cell densities (10^6 cells/0.5 ml), using a salting-out procedure.[30,31,35,36] After centrifugation at 200 *g* for 10 min, the cell pellets were washed twice with PBS and lysed overnight at 37°C in 0.34 ml of 10 mM Tris–HCl, pH 8.0, containing 2 mM EDTA, 400 mM NaCl, 1% SDS and proteinase K (0.5 mg/ml). Cell lysates were vortexed for 15 s with 0.1 ml of 6 M NaCl and centrifuged (3000 r.p.m. × 30 min). The supernatants (0.44 ml) were mixed with 0.88 ml of ice-cold 100% EtOH and kept at 4°C for 15 min for DNA precipitation. After centrifugation (13 000 r.p.m. × 15 min) at 4°C, the air-dried DNA pellets were dissolved in 0.34 ml of 10 mM Tris–HCl, pH 8.0, with 1 mM EDTA (TE buffer) and incubated for 2 h at 37°C in the presence of RNase (0.1 mg/ml). After another round of EtOH precipitation and centrifugation, the final air-dried pellets were dissolved in 50 μl of TE buffer and their DNA concentrations determined spectrophotometrically at 260 nm. Equal amounts of DNA samples (6 μg/7.5 μl of TE buffer) were mixed with 1.5 μl of 10 mM Tris–HCl, pH 7.5, containing 50 mM EDTA, 10% Ficoll 400 and 0.4% orange G, and loaded on each lane. About 0.5 μg of 100 bp DNA ladder and 0.75 μg of λ DNA/*Eco*RI+*Hind*III (both from Promega) were similarly applied to each gel to provide size markers in the range 100–1500 and 125–21 226 bp, respectively. Horizontal electrophoresis of DNA samples was performed for 3.7 h at 60 V in 1.5% agarose gels containing ethidium bromide (1 μg/ml) with 90 mM Tris–HCl, pH 8.0, containing 90 mM boric acid and 2 mM EDTA as a running buffer. DNA fragments were visualized and photographed with Polaroid 667 film under UV transillumination.[30,31,36]

Results

Drugs

The chemical structures and code names of the TT analogs (serial numbers indicate the order of synthesis) tested for their antileukemic activity in the L1210 tumor cell system *in vitro* are depicted in Figure 1. The nomenclature of the parent TT0 compound, which is known and commercially

available, is 9,10-dihydro-9,10-[1′,2′]benzenoanthracene. 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.[13,27] This new, short and easy method to synthesize substituted TT quinones has been reported.[28] The correct nomenclature of the TT2 skeleton, which possesses 2 external quinones, is 2-methoxy-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4,5,8-tetraone. TT13 is the C2-brominated derivative of TT2 and its nomenclature is 2-bromo-3-methoxy-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4,5,8-tetraone. Since TT2 and TT13 are the most potent of the rigid tetracyclic quinones we synthesized so far,[13,27] they have been selected as references to assess the effectiveness of the new antitumor compounds in the series. The correct nomenclatures of the new synthetic TT analogs tested in the present study are as follows. TT14: 3-bromo-2-*N*-methylamino-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4,5,8-tetraone. TT15: 3-bromo-5,8-dihydroxy-2-*N*-methylamino-1*H*,4*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4-dione. TT16: 6-bromo-7-methoxy-2-*N*-dimethylamino-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4,5,8-tetraone and 7-bromo-6-methoxy-2-*N*-dimethylamino-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracene-1,4,5,8-tetraone. TT17: dimethyl 6,11-dihydroxy-1,4-dioxo-1*H*,4*H*-5,12-dihydro-5,12-[1′,2′]benzenonaphthacene-8,9-dicarboxylate. TT18: dimethyl 6,11-dihydroxy-1,4-dioxo-5,7,10,12-tetrahydro-1*H*,4*H*-5,12-[1′,2′]benzenonaphthacene-8,9-dicarboxylate. TT19: ethyl 3-[*N*-(3-bromo-1,4,5,8-tetraoxo-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracen-2-yl)]aminopropanoate. TT20: 6-[*N*-(3-bromo-1,4,5,8-tetraoxo-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracen-2-yl)]amino-2-aminohexanoic acid. TT21: *tert*-butyl 3-[*N*-(3-bromo-1,4,5,8-tetraoxo-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracen-2-yl)]aminopropanoate. TT22: sodium 3-[*N*-(3-bromo-1,4,5,8-tetraoxo-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracen-2-yl)]aminopropanoate. TT23: 3-[*N*-(3-bromo-1,4,5,8-tetraoxo-1*H*,4*H*,5*H*,8*H*-9,10-dihydro-9,10-[1′,2′]benzenoanthracen-2-yl)]aminopropanoic acid. TT24: 6-bromo-7-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 and 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 (Figure 1). The antileukemic effects of these TT monoquinones and bisquinones were assessed and compared to those of DAU, a clinically valuable anticancer drug that is structurally very different from TT2 but also contains a *para*-quinone moiety.[13,27] This well-known anthracyclinone is characterized by a linear fused tetracyclic system with an internal quinone and a glycosidic moiety.

Inhibition of tumor cell viability

Treatment with 40.96 nM of the known quinone antitumor drug DAU decreases tumor cell viability at day 4 by 73% and serves as a positive control in the cytotoxicity assay (Figure 2). In contrast to the tetracyclic structure of their parent compound TT0, which is neither cytotoxic at 256 nM (Figure 2) nor in the higher micromolar range (data not shown), 256 nM concentrations of our previous lead antitumor TT bisquinones TT2 and TT13 decrease L1210 tumor cell viability at day 4 by 83–84% (Figure 2). Although most of these substituted TT bisquinones are much more potent at higher concentrations, at this lower concentration of 256 nM, the new hydrophilic molecules TT20, TT22 and TT23 have either no or only weak cytotoxic activities (inhibition by 22–28%). Under similar conditions, the cytotoxic activities of the substituted TT monoquinones TT17 and TT18 are moderate (inhibition by 38–41%). Interestingly, the substituted TT monoquinone TT15 and its bisquinone equivalent TT14 have equally good cytotoxic activities (inhibition by 55–59%) in spite of their different quinone functionalities. However, four new substituted TT bisquinones have excellent cytotoxicities and increase our current pool of lead antitumor compounds in the series (Figure 2). At 256 nM, TT21 inhibits tumor cell viability by 69%, and the new TT16 and TT19 clearly match the cytotoxic effects of the previous TT2 and TT13 (inhibition by 83–88%). However, the critical finding is that 256 nM TT24, which is significantly more potent than TT2, TT13, TT16 and TT19, and inhibits tumor cell viability by 96% (Figure 2), is the most effective of the lead antitumor TT bisquinones synthesized so far and its cytotoxicity compares well with that of a proven anticancer drug already used clinically (Figure 3).

The smallest concentrations of TT24 and DAU that can induce significant cytotoxic effects after 2 days are 102.4 and 40.96 nM, respectively, whereas 256 nM TT24 inhibits almost totally the viability of L1210 cells at day 4 and must be used to mimic the maximal cytotoxic activity of 102.4 nM DAU on the same day (Figure 3). These nearly equivalent potencies of TT24 and DAU can easily be compared

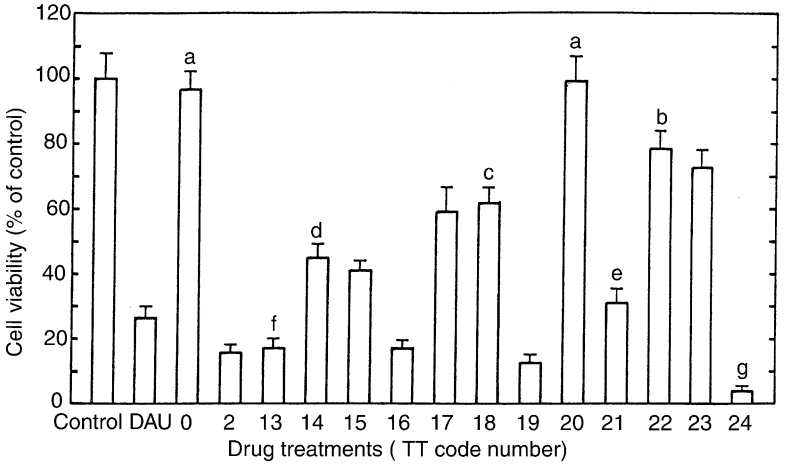

Figure 2. Comparison of the abilities of 256 nM concentrations of TT analogs to inhibit L1210 cell viability at day 4 *in vitro*. The cytotoxic activity of 40.96 nM DAU is demonstrated in the same experiments. Cell viability results are expressed as percent of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control tumor cells ($A_{490\,nm}=1.318\pm0.096$, $100\pm7\%$) after 4 days in culture. The blank value ($A_{490\,nm}=0.294$ at day 4) for cell-free culture medium supplemented with MTS:PMS reagent has been subtracted from the results. Bars: means $\pm$ SD ($n=3$). [a]Not different from control; [b]$p<0.025$, smaller than control, but not different from TT23; [c]$p<0.05$, smaller than TT23, but not different from TT17; [d]$p<0.05$, smaller than TT17, but not different from TT15; [e]$p<0.05$, smaller than TT15; [f]$p<0.01$, smaller than TT21, but not different from TT2, TT16 and TT19; [g]$p<0.005$, smaller than TT19.

using the full concentration–response curves of Figure 3 where the striped area at 100% represents the control levels of L1210 cell viability after 2 and 4 days in culture. The magnitudes of the cytotoxic effects of both TT24 and DAU are clearly related to the combination of their increasing concentration and duration of action. For instance, 16.38 nM DAU and 40.96 nM TT24 are ineffective at day 2 but their cytotoxic activities become obvious at day 4 (inhibitions by 35 and 44%, respectively). Moreover, 40.96 nM DAU and 102.4 nM TT24 are weakly cytotoxic at day 2 (inhibitions by 24 and 28%, respectively) but dramatically inhibit L1210 cell viability by 71 and 74%, respectively, at day 4. As a result, the inhibitions of tumor cell viability by DAU and TT24 are respectively characterized by IC_{50} values of 80 and 154 nM at day 2, but 25 and 48 nM at day 4, suggesting that DAU is only about 1.9 times more cytotoxic than TT24 in this leukemic cell system *in vitro* (Figure 3).

Inhibition of DNA synthesis and nucleoside transport

The concentration-dependent inhibition of DNA synthesis by DAU (IC_{50}: 1 μM) and TT2 (IC_{50}: 6 μM) suggest that, under our experimental conditions, DAU prevents L1210 cells from synthesizing DNA about 6 times more effectively than TT2.[13] As reported previously,[13,27] a 15-min treatment with 7 μM DAU is unable to alter the cellular transports of [^{3}H]adenosine and [^{3}H]thymidine occurring over only 30 s, even though a 2-h treatment with such concentration of DAU inhibits maximally the incorporation of [^{3}H]thymidine into DNA used to assess the rate of DNA synthesis over a 30-min period of pulse-labeling in L1210 cells *in vitro* (Figure 4). Hence, DAU serves as a positive control in the DNA synthesis assay, but as a negative control in the nucleoside transport assay. In contrast to DAU, our previous lead antitumor compounds TT2 and TT13 have been shown to inhibit DNA synthesis in relation with their ability to irreversibly block the cellular transports of purine and pyrimidine nucleosides. Both of these inhibitory effects on DNA synthesis and nucleoside transport share the same IC_{50} value of 6 μM, suggesting that these TT bisquinones decrease the incorporation of radiolabeled nucleosides into DNA at 2 h because they rapidly prevent the nucleoside transport system from incorporating these radiolabeled nucleosides into the cells within 15 min.[13,27] This hypothesis is substantiated by the results of Figure 4, which show that all the new TT analogs synthesized inhibit the rate of DNA synthesis in relation with their ability to block nucleoside transport, and that the magnitudes of their inhibitions of DNA synthesis and nucleoside transport

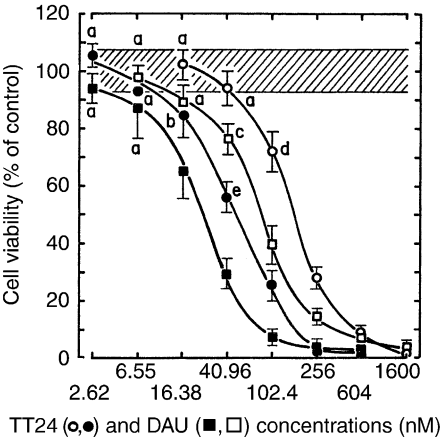

Figure 3. Comparison of the concentration-dependent inhibitions of L1210 cell viability by TT24 (circles) and DAU (squares) at days 2 (open symbols) and 4 (closed symbols) *in vitro*. Cell viability results are expressed as percent of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control tumor cells ($100 \pm 7\%$, striped area) at days 2 ($A_{490\,nm} = 1.294 \pm 0.087$) and 4 ($A_{490\,nm} = 1.275 \pm 0.092$). Blank values ($A_{490\,nm} = 0.265$ and 0.278 at days 2 and 4) for cell-free culture medium supplemented with MTS:PMS reagent have been subtracted from the results. Bars: means $\pm$ SD ($n = 3$). [a]Not different from control; [b]$p < 0.05$, smaller than control but greater than 16.38 nM DAU at day 4; [c]$p < 0.01$, smaller than control and $p < 0.025$, smaller than 40.96 nM TT24 at day 2; [d]$p < 0.01$, smaller than control but $p < 0.005$, greater than 102.4 nM DAU at day 2; [e]$p < 0.005$, greater than 40.96 nM DAU at day 4.

within 15 min to 2 h are linked to their overall cytotoxicity over 4 days in Figure 2. Indeed, the parent compound TT0, which is not cytotoxic (Figure 2), also fails to inhibit nucleoside transport and DNA synthesis (Figure 4). The inactive or marginally cytotoxic molecules TT20, TT22 and TT23 (Figure 2) similarly do not alter or only minimally inhibit DNA synthesis and nucleoside transport (Figure 4). However, TT17 and TT18 inhibit nucleoside transport and DNA synthesis by 24–56%, and TT14 and TT15 inhibit the same responses by 39–71% (Figure 4) in relation with the moderate and good abilities of these groups of compounds to reduce tumor cell viability (Figure 2). Moreover, the four new lead antitumor TT bisquinones all drastically inhibit nucleoside transport and DNA synthesis (Figure 4) in relation with their excellent cytotoxicities (Figure 2). For instance, TT21 decreases nucleoside transport and DNA synthesis by 53–78%, whereas the new TT16 and TT19 mimic the abilities of TT2 and TT13 to inhibit nucleoside transport and DNA synthesis by 62–90% (Figure 4). Interestingly, the most cytotoxic TT bisquinone, TT24 (Figure 2), matches the 97–98% inhibition of DNA synthesis caused by DAU but has the additional advantage of also blocking the cellular transports of purine and pyrimidine nucleosides by 77–83% (Figure 4).

Inductions of DNA cleavage and apoptosis

L1210 cells containing [^{3}H]thymidine-prelabeled DNA were used to quantitatively determine by intact chromatin precipitation whether our lead antitumor TT analogs would induce DNA fragmentation over a 24-h period *in vitro* (Figures 5 and 6) and the results were confirmed by agarose gel analysis (Figure 7). DAU, the quinone antitumor drug known to induce DNA-strand breaks by inhibiting Topo II activity, is used as a positive control in these DNA fragmentation assays and, at 1.6 μM, produces a 17.7-fold increase in the percent of DNA cleavage over control (Figure 5). Our best lead antitumor compound, TT24, which is nearly as cytotoxic as DAU (Figures 2 and 3), also produces a 14.7-fold increase in the level of DNA cleavage when tested at the same concentration of 1.6 μM as DAU (Figure 5). However, all other TT analogs must be compared at 4 μM in order to demonstrate that the various levels of DNA cleavage that they induce at 24 h (Figure 5) are related to their respective cytotoxic activities when tested at 256 nM for 4 days (Figure 2). Indeed, the parent TT0 and its analog TT20, the only two compounds devoid of cytotoxicity (Figure 2), do not damage DNA (Figure 5), whereas the marginally cytotoxic TT22 and TT23 (Figure 2) produce only minimal 1.8- to 2.6-fold increases in the percent of DNA cleavage (Figure 5). However, the levels of DNA cleavage are increased 4.6- to 5.6-fold by the moderately cytotoxic TT17 and TT18, and 9.0- to 10.6-fold by TT14 and TT15, which have good cytotoxicity (Figures 2 and 5). Finally, among the lead antitumor TT bisquinones, the new compounds TT16 and TT19 match the 15.2- to 16.8-fold increases in the percent of DNA cleavage caused by the previous compounds TT2 and TT13, whereas the slightly less cytotoxic TT21 produces a smaller 13.0-fold increase in the percent of DNA cleavage (Figures 2 and 5).

As reported before, the concentration-dependent induction of DNA cleavage caused by DAU is biphasic,[13,27,29] starting at 102.4 nM, peaking 32–39% above control level (2.5% DNA fragmentation) in L1210 cells treated for 24 h with 0.64–1.6 μM DAU,

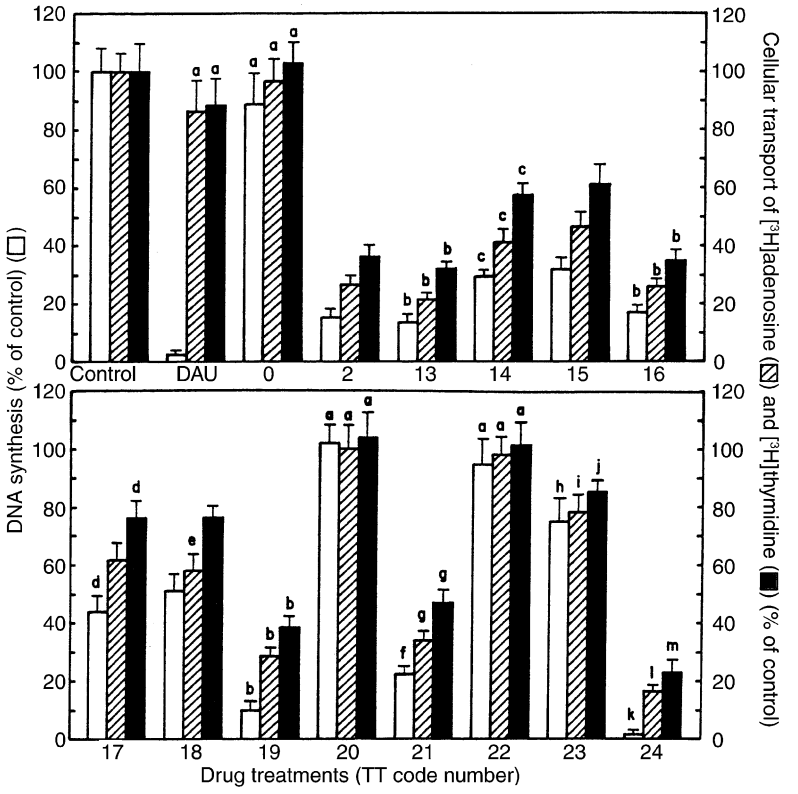

Figure 4. Comparison of the abilities of 7 μM concentrations of TT analogs to inhibit DNA synthesis (open) and the cellular transports of purine (striped) and pyrimidine (closed) nucleosides in L1210 cells *in vitro*. The effects of 7 μM DAU on DNA synthesis and nucleoside transport are demonstrated in the same experiments. DNA synthesis results are expressed as percent of [^{3}H]thymidine incorporation into DNA of vehicle-treated control tumor cells over 30 min (19 914 $\pm$ 1553 c.p.m., 100 $\pm$ 8%, open control). The blank value (680 $\pm$ 87 c.p.m.) for cells incubated and pulse-labeled at 2°C with 1 μCi of [^{3}H]thymidine has been subtracted from the results. Nucleoside transport results are expressed as percent of [^{3}H]adenosine (17 711 $\pm$ 1009 c.p.m., 100 $\pm$ 6%, striped control) or [^{3}H]thymidine (7589 $\pm$ 653 c.p.m., 100 $\pm$ 9%, closed control) transported into vehicle-treated control tumor cells over 30 s. Bars: means $\pm$ SD (n=3). [a]Not different from respective controls; [b]not different from the respective effects of TT2; [c]$p < 0.05$, greater than the respective effects of TT21, but not different from those of TT15; [d]$p < 0.05$, greater than the respective effects of TT15, but not different from those of TT18; [e]$p < 0.05$, greater than the effect of TT15, but not different from that of TT17; [f]$p < 0.05$, greater than the effects of TT2 and TT16; [g]$p < 0.05$, greater than the respective effects of TT2 and TT19; [h]$p < 0.025$, greater than TT18 but $p < 0.05$, smaller than TT22; [i]$p < 0.05$, greater than TT17 but $p < 0.025$, smaller than TT22; [j]$p < 0.05$, greater than TT18 but $p < 0.05$, smaller than control; [k]$p < 0.005$, smaller than TT2 and TT19, but not different from DAU; [l]$p < 0.01$ and $p < 0.05$, smaller than TT2 and TT13, respectively; [m]$p < 0.025$ and $p < 0.05$, smaller than TT2 and TT13, respectively.

but declining back to (7% above control at 4 μM), or even below (1.9 and 2.4% below control at 10 and 25 μM, respectively), control level at higher concentrations of DAU (Figure 6). Although our best lead antitumor compound TT24 is slightly less potent and does not fully match the magnitude of DNA cleavage produced by DAU, the shape of the concentration-dependent induction of DNA cleavage caused by this substituted TT bisquinone in L1210 cells is similarly biphasic and resembles that of DAU, with a peak at 1.6 μM and higher concentrations increasingly losing their ability to induce DNA fragmentation, suggesting that the mechanisms by which TT analogs and DAU induce DNA damage might share some similarity (Figure 6). For instance, DNA breakage is initiated at 256 nM TT24, peaks 30% above the control percent of DNA fragmentation at 1.6 μM, but rapidly declines toward or even below (1.5 and 2.3% below control at 4 and 10 μM, respectively) the control percent of DNA fragmentation at higher concentrations of TT24 (Figure 6).

Time- and concentration-dependent analyses of drug-induced internucleosomal DNA cleavage by agarose gel electrophoresis further demonstrate that

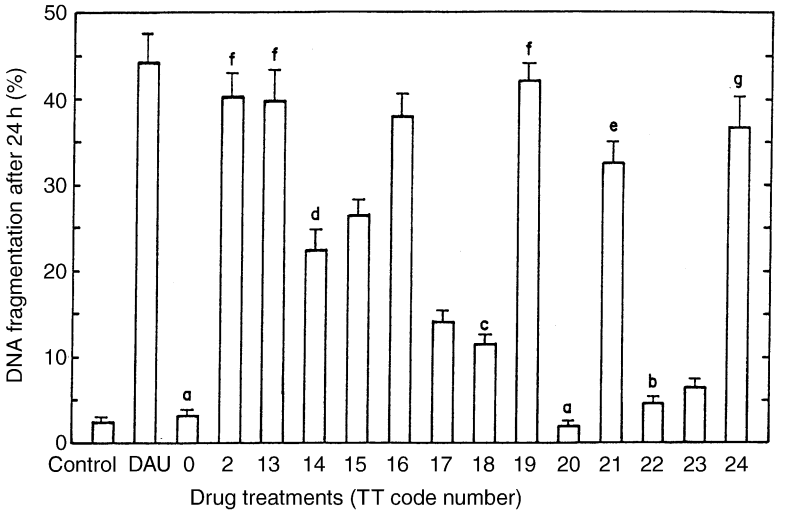

Figure 5. Comparison of the abilities of 4 μM concentrations of TT analogs to induce DNA cleavage at 24 h in L1210 cells containing ^{3}H-prelabeled DNA *in vitro*. The DNA-damaging effects of TT24 and DAU are compared at 1.6 μM in the same experiments. Results are expressed as [c.p.m. in supernatant/c.p.m. in supernatant+pellet] $\times$ 100 at 24 h. For untreated control tumor cells ($2.5 \pm 0.2\%$ DNA fragmentation), the supernatant (DNA fragments) is 1992 ± 167 c.p.m. and the pellet (intact DNA) is $78\,236 \pm 6884$ c.p.m. Bars: means $\pm$ SD ($n=3$). [a]Not different from control; [b]$p < 0.025$, greater than control, but not different from TT23; [c]$p < 0.005$, greater than TT23, but not different from TT17; [d]$p < 0.01$, greater than TT17, but not different from TT15; [e]$p < 0.025$, greater than TT15 but $p < 0.05$, smaller than TT16; [f]not different from TT16; [g]$p < 0.05$, smaller than DAU.

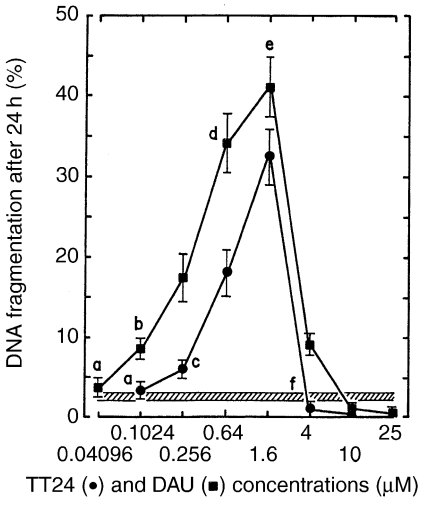

Figure 6. Comparison of the concentration-dependent inductions of DNA cleavage by TT24 (circles) and DAU (squares) at 24 h in L1210 cells containing ^{3}H-prelabeled DNA *in vitro*. Results are expressed as [c.p.m. in supernatant/c.p.m. in supernatant+pellet] $\times$ 100 at 24 h. For untreated control tumor cells ($2.5 \pm 0.3\%$ DNA fragmentation, striped area), the supernatant (DNA fragments) is 2235 ± 232 c.p.m. and the pellet (intact DNA) is $85\,900 \pm 9277$ c.p.m. Bars: means $\pm$ SD ($n=3$). [a]Not different from control; [b]$p < 0.005$ and $p < 0.0005$, greater than TT24 and control, respectively; [c]$p < 0.005$, greater than control but smaller than DAU; [d]$p < 0.005$ and [e]$p < 0.05$, greater than respective concentrations of TT24; [f]$p < 0.005$, smaller than control.

our lead TT bisquinones are truly DNA-damaging anticancer agents which, although they are somewhat less potent than DAU, can similarly induce a biphasic increase and decrease of DNA fragmentation characterized by a peak that shifts toward lower concentrations with increasing times of drug exposure (Figure 7). Indeed, no or very little DNA cleavage is visualized in controls, whereas DNA cleavage bands with a characteristic pattern of internucleosomal ladder suggestive of apoptosis become increasingly apparent over a 3-day period in L1210 cells treated with 0.64 and 1.6 μM DAU or 4 and 10 μM TT2 (Figure 7A). For instance, the faint bands of low molecular weight DNA fragments observed at 24 h in response to both drugs become clearly more pronounced at 48 h and their intensities plateau afterward (Figure 7A). Interestingly, 1.6 μM DAU and 10 μM TT2, respectively, induce more DNA fragmentation than 0.64 μM DAU and 4 μM TT2 at 24 h, but the opposite is true at 72 h when 0.64 μM DAU and 4 μM TT2 become, respectively, more effective than 1.6 μM DAU and 10 μM TT2, an observation suggesting that the molecular events triggered by lower concentrations of quinone antitumor drugs require substantially longer periods of time to fully induce DNA fragmentation (Figure 7A). When the full concentration-dependent inductions

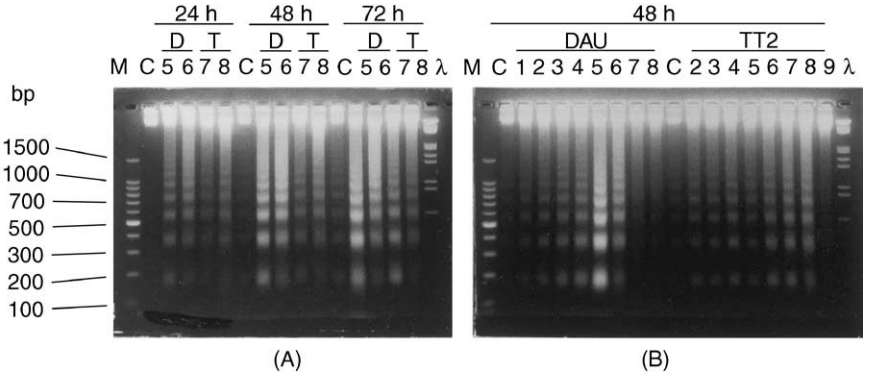

Figure 7. Agarose gel analysis of drug-induced internucleosomal DNA cleavage in L1210 cells *in vitro*. (A) Time courses of DNA fragmentation in cells incubated at 37°C for 1, 2 and 3 days in the presence or absence (control: lanes C) of 0.64 (lanes D5) and 1.6 μM (lanes D6) concentrations of DAU or 4 (lanes T7) and 10 μM (lanes T8) concentrations of TT2. (B) Concentration-dependent levels of DNA fragmentation in cells incubated at 37°C for 2 days in the presence or absence (control: lanes C) of 0.016, 0.041, 0.102, 0.256, 0.64, 1.6, 4 and 10 μM DAU (lanes DAU 1–8) or 0.041, 0.102, 0.256, 0.64, 1.6, 4, 10 and 25 μM TT2 (lanes TT2 2–9). Cellular DNA extracts (6 μg/well) were loaded onto a 1.5% agarose gel containing ethidium bromide (1 μg/ml), separated by electrophoresis for 3.7 h at 60 V and photographed under UV light. A typical ladder pattern indicating the presence of DNA equivalent to the size of single and oligonucleosomes is characteristic of apoptosis. Size markers are shown in lanes M (0.5 μg of 100-bp standard DNA ladder) and λ (0.75 μg of λ DNA/*Eco*RI+*Hind*III markers).

of DNA fragmentation by 16 nM to 10 μM DAU and 41 nM to 25 μM TT2 are compared at 48 h, characteristic ladders of DNA fragments indicative of apoptosis become increasingly visible in response to increasing concentrations of these drugs up to their peaks at 0.64–1.6 μM DAU and 4–10 μM TT2, after which such typical pattern of DNA laddering disappears in response to 10 μM DAU and 25 μM TT2, presumably because the higher concentrations of these cytotoxic drugs block the molecular events required to sustain the active process of apoptotic DNA fragmentation (Figure 7B).

To substantiate this hypothesis, non-toxic concentrations of Act-D, CHX, z-VAD-fmk, z-IETD-fmk, TPCK and $ZnSO_4$, which by themselves do not alter the basal level of DNA cleavage in L1210 cells after a 1-h preincubation period followed by 24 h of treatment,[29–31] were tested for their ability to affect the molecular mechanism by which DAU and TT24 induce DNA fragmentation and apoptosis (Figure 8). Such treatments with Act-D, CHX, z-VAD-fmk, z-IETD-fmk, TPCK and $ZnSO_4$, which are known to, respectively, inhibit RNA and protein syntheses and caspase and endonuclease activities, all reduced more or less the abilities of 1.6 μM concentrations of DAU and TT24 to maximally induce DNA cleavage at 24 h (Figure 8). For instance, considering that the background of DNA cleavage is 2.6% in control tumor cells, the levels of DNA fragmentation induced by DAU (43%) and TT24 (35%) at 24 h are both inhibited by 74–75, 64–65, 56–57, 43, 88–90 and 61% in the presence of Act-D, CHX, z-VAD-fmk, z-IETD-fmk, TPCK and $ZnSO_4$, respectively (Figure 8). These preliminary data suggest that, like DAU, TT24 may use, at least partially, an active process of apoptosis that requires gene expression, new RNA and protein syntheses, and caspase and endonuclease activations in order to trigger DNA fragmentation.

Discussion

Among 24 analogs of TT0 synthesized so far, six excellent lead antitumor compounds have been discovered (Figure 2), all of them substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetraones. TT2 (IC_{50}: 125 nM), TT13 (IC_{50}: 135 nM), TT16 (IC_{50}: 116 nM) and TT19 (IC_{50}: 110 nM) similarly inhibit L1210 tumor cell viability *in vitro*,[28] whereas TT21 (IC_{50}: 176 nM) is slightly less effective[28] and TT24 is clearly the most cytotoxic with an IC_{50} value (48 nM) nearly equipotent to that of DAU (IC_{50}: 25 nM) (Figure 3). The bisquinone TT2 has a methoxy substitution, TT13 is the brominated analog of TT2, TT16 is the dimethylamino derivative of TT13, TT24 is like TT16 but has a methylamino instead of a dimethylamino substitution, and TT19 and TT21 are brominated TT bisquinones with various alkyl chains on their amino function (Figure 1). Our preliminary L1210[13] and HL-60 studies[27] have been focused on TT2, but other substituted TT

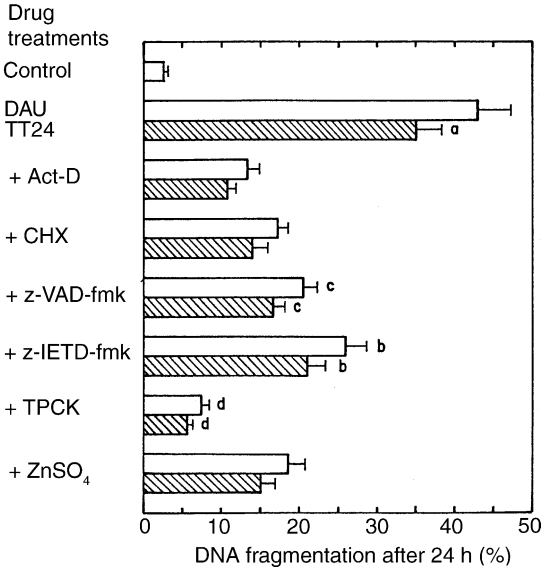

Figure 8. Inhibition of DAU- and TT24-induced DNA cleavage by inhibitors of RNA and protein syntheses, and caspase, non-caspase protease and endonuclease activities in L1210 cells *in vitro*. Tumor cells containing ^{3}H-prelabeled DNA were preincubated at 37°C for 1 h in the presence of vehicle, Act-D (1 μg/ml), CHX (25 μg/ml), 100 μM z-VAD-fmk, 100 μM z-IETD-fmk, 50 μM TPCK or 100 μM $ZnSO_4$ and, after supplementing their culture medium with either vehicle (control), 1.6 μM DAU (open) or 1.6 μM TT24 (striped), these incubations were continued for an additional 24 h to determine the level of DNA cleavage as indicated in Figures 5 and 6. For drug-untreated control tumor cells preincubated and incubated in the presence of vehicle only (2.6 ± 0.2% DNA fragmentation), the supernatant is 2037 ± 177 c.p.m. and the pellet is $76\,932 \pm 7154$ c.p.m. Bars: means $\pm$ SD ($n=3$). $^{a}p < 0.05$, smaller than DAU; $^{b}p < 0.005$, smaller than DAU or TT24 alone, respectively; $^{c}p < 0.05$, smaller than DAU+z-IETD-fmk or TT24+z-IETD-fmk, respectively; $^{d}p < 0.005$, greater than control.

bisquinones share similar bioactivities in the present study (Figures 2, 4 and 5) and their exact molecular mechanism of action would have to be elucidated in order to assess SARs among various TT analogs. Since the parent TT0 framework is inactive, it is reasonable to assume that the bisquinone function and/or bromo, methoxy, methylamino and dimethylamino substitutions of our lead compounds may be responsible for their potent antitumor activities. However, other monoquinone and hydroquinone TT analogs with or without such bromo, methoxy, methylamino and dimethylamino functions also have interesting antitumor activities.[13] For example, TT1 with two quinones is not better than its analog TT7 with only one quinone,[13] and, in the present study, the bisquinone TT14 is equipotent to its monoquinone equivalent TT15 in the cell viability, DNA synthesis, nucleoside transport and DNA cleavage assays. Moreover, the dihydroquinone TT3, which lacks quinone functionality, is as good or even better than its monoquinone equivalent TT5.[13] Hence, it is rather premature to speculate on the antitumor potential of various substitutions and quinone or hydroquinone functionalities of the TT skeleton when the abilities of various TT analogs to bind to nucleoside transporters, interact covalently with DNA, inhibit Topo activities, block cell cycle progression and affect the production of FRs are still unknown. However, it would be of interest to determine whether antitumor TT analogs lacking quinone functionality are less cardiotoxic than DAU or their monoquinone and bisquinone equivalents.

In general, the electrophilic nature of the bisquinone functionalities along with the triptych structure might explain the potent anticancer activities of our lead TT bisquinones (Figure 1). The quinone moiety could undergo addition reactions with amine and thiol nucleophiles, and the triptych structure could intercalate into single- or double-stranded nucleic acid molecules. The rigid α-methoxyketo functionality of compounds TT2 and TT13 could serve as an acceptor of hydrogen bonds. The C2-bromine (electronegative atom) of TT13 might enhance the electrophilicity or reactivity of the adjacent quinone. The monoquinone TT15, which is the dihydro-derivative of TT14, might be oxidized under the conditions of our bioassays to provide the bisquinone TT14 and elicit its antitumor activity. In addition to their disubstituted bromo- and methoxyquinone, TT16 and TT24, respectively, possess α-dimethylaminoquinone or α-methylaminoquinone functions, which could enhance their antitumor activity. Particularly, the α-methylaminoquinone of our best lead antitumor compound, TT24, might provide hydrogen donor–acceptor arrangements that maximize hydrogen bonding. TT19 and TT21 are analogs of TT14 with longer alkyl chains on the C2-amino function, which could increase their hydrophobicity, membrane permeability and cytotoxicity. Increasing the hydrophilicity of TT20, TT22 and TT23 by installing water-solubilized functionalities, such as a carboxylic acid or amino acid or sodium salt derivatives of a carboxylic acid might, in fact, decrease the ability of these compounds to cross cell membranes and explain their poor antitumor activities. Apparently, the extra six-membered ring attached to the A-rings of TT17 and TT18 does not seem to improve the bioactivity of these TT monoquinones (Figure 1).

All TT analogs tested inhibit DNA synthesis and nucleoside transport and induce DNA cleavage in relation with their ability to decrease tumor cell viability (Figures 2, 4 and 5), suggesting that such molecular targets play an important role in the antitumor effects of our lead TT bisquinones, which share the same bifunctional mechanism of action, i.e. blocking nucleoside transport and damaging DNA. Our original lead compound, TT2, inhibits the proliferation and viability of murine L1210 and human wild-type HL-60 leukemic cells in the nanomolar range *in vitro*,[13,27] and its cytostatic, cytotoxic[13] and DNA-damaging effects (Figure 7) increase with the number of days in culture, suggesting that TT bisquinones may be cytostatic and cytotoxic in various tumor cell systems and that their effectiveness as anticancer drugs is a combination of concentration and duration of action. Decreased tumor cell viability after TT24 treatment *in vitro* may be a reliable predictor of anticancer activity *in vivo*,[37] especially since this lead TT bisquinone is almost equipotent to DAU (Figures 3 and 6), a quinone antitumor drug already used clinically. Combining drugs, which target different molecules and achieve complementary or synergistic antitumor effects, is an important strategy in cancer chemotherapy. Although slightly less potent, our antitumor TT bisquinones mimic the ability of DAU to inhibit nucleic acid and protein syntheses, induce DNA fragmentation, and prevent cell cycle progression to M phase.[13,27] However, the critical advantage of TT2, TT13, TT16, TT19, TT21 and TT24 lies with the fact that, in contrast to DAU, they can also block the cellular transports of both purine and pyrimidine nucleosides (Figure 5), suggesting that these bifunctional antitumor TT bisquinones might have a more versatile mechanism of action and disrupt a wider spectrum of molecular targets in populations of unsynchronized tumor cells than DAU. Another attractive feature of TT2 is that it retains its effectiveness in two MDR HL-60-RV and HL-60-R8 sublines that have developed different mechanisms of resistance to DAU, suggesting that antitumor TT bisquinones might not be initially recognized and targeted for ATP-dependent extrusion by tumor cells overexpressing P-glycoprotein and/or multidrug resistance-associated protein and displaying cross-resistance to a number of other cytotoxic agents.[27] Indeed, TT2 inhibits tumor cell growth, viability and DNA synthesis as effectively in the MDR HL-60-RV and HL-60-R8 cell lines as in the wild-type HL-60 cells and the resistance factors (RFs) are negligible (0.9–1.5), whereas DAU becomes dramatically less effective against DNA synthesis (RF: 8.1–11.9), cell proliferation (RF: 22.9–35.7) and cell viability (RF: 23.8–31.3) in these MDR HL-60 sublines than in their parental wild-type counterparts.[27] Moreover, TT2, which, in contrast to DAU, has the unique ability to rapidly block the cellular transports of purine and pyrimidine nucleosides in L1210 and wild-type HL-60 cells, retains such additional advantage in the MDR HL-60 sublines.[27] These findings substantiate the hypothesis that, because of their unusual bifunctional mechanism of action, our lead antitumor TT bisquinones might be more versatile than DAU and able to circumvent MDR.

In addition to synthetic anthracycline antibiotics and a few natural anthracyclinones, a limited number of quinoid structures with one (benzoquinone), two (naphthoquinone, NQ) or three rings (anthraquinone, AQ) and various substitutions have cytotoxic activities;[1,13,27,29,30,38,39] however, with some rare exceptions, none of them are as potent as our lead antitumor TT bisquinones in wild-type and MDR cell lines. At 1 μM, 2-methyl-1,4-NQ (vitamin K_3) is marginally cytotoxic, whereas the NQ isomers of the WS-5995 antibiotics (IC_{50}: 0.65–3.5 μM) and the pyrano-NQ derivatives of lapachol (IC_{50}: 6–80 μM) are several-fold less cytotoxic than TT2.[27,30,40,41] The potent antitumor activity of 1,4-AQ (IC_{50}: 25 and 100 nM in L1210 and wild-type HL-60 cells, respectively) we discovered recently[29,42] is all the more remarkable that the number of bioactive 1,2-, 1,4- and 9,10-AQs appears quite limited and almost all of those with antitumor activity *in vitro* and *in vivo* are 9,10-AQs.[1,38,39] Based on IC_{50} values reported previously, oncocalyxones A and B, two 1,4-AQs from *Auxemma oncocalyx*, appear to be cytotoxic agents 10–529 times less potent than TT2.[13,27,43,44] Among synthetic aminoalkylamino AQs, mitoxantrone is the only 9,10-AQ equipotent to DOX and DAU.[39,45] Mitoxantrone, a DNA-damaging agent which inhibits tumor cell growth and viability *in vitro* and *in vivo*, is somewhat effective in MDR sublines, synergistic with MTX and 5-FU, and less cardiotoxic than DOX (reviewed in Perchellet *et al.*[30]). However, the higher IC_{50} values reported for the cytotoxic effects of aloe-emodin (1–100 μM) and the barleriaquinones (8–10 μM) in various tumor cell lines[46,47] suggest that these natural 9,10-AQ analogs extracted from *Aloe vera* leaves and *Barleria buxifolia* roots have much weaker antitumor potential than our synthetic TT bisquinones.

The apparent discrepancies between the nM concentrations of TT24 sufficient to inhibit tumor cell viability (Figure 3) and the higher micromolar concentrations of TT24 required to block nucleoside transport and DNA synthesis (Figure 4), and maxi-

mally induce DNA cleavage (Figure 6), may be due to the fact that the rates of nucleoside transport over 30 s and DNA synthesis over 30 min are inhibited in L1210 cells treated for only 15–90 min with TT24, and the peak of DNA cleavage occurs 24 h after TT24 treatment, whereas the more spectacular reduction of L1210 cell viability is the result of a 4-day long TT24 treatment. Although the cellular uptake, retention, metabolism and half-life of radiolabeled TT bisquinones remain to be determined, TT2 rapidly triggers long-lasting inhibitory and damaging events that persist upon drug removal.[13] Hence, the irreversibility of the inhibitions of nucleoside transport and DNA synthesis caused by TT2 suggests that this drug is no longer required in the medium after 15–90 min, presumably because it has already bound to membrane nucleoside transporters and, perhaps, also been internalized to interact with nuclear enzymes involved in DNA metabolism.[13] Since both responses are irreversible and share similar IC_{50} values,[13,27] the rapid inhibition of [^{3}H]thymidine incorporation into DNA caused by TT analogs is most likely due to their ability to immediately block the cellular transport of nucleosides (Figure 4). In contrast, DAU blocks the incorporation of precursors into DNA during replication without interfering with the cellular uptake of these DNA precursors (Figure 4). In L1210 cells, it takes 24 h to fully reveal the extent of DNA cleavage induced by 3-h pretreatments with TT2 or DAU.[13] The irreversibility of this response suggests that the DNA-damaging events triggered by TT2 and DAU within the first 3 h can proceed uninterrupted for the next 21 h in the absence of these drugs in the extracellular medium and are sufficient to induce the same maximal levels of DNA fragmentation as when the cells are incubated for the entire 24-h period with the drugs.[13]

Since, within 24 h, the magnitudes and shapes of the concentration–response curves for the abilities of TT2[13,27] and TT24 (Figure 6) to induce DNA fragmentation resemble the biphasic response to the known Topo II inhibitor DAU, which has already been reported and discussed before,[11,13,27,29,42] our lead TT bisquinones might induce DNA-strand breaks by a mechanism which is similar to that of DAU and plays a major role in their antitumor activity. The Topo II-associated lesions occurring in drug-treated tumor cells may facilitate subsequent internucleosomal DNA fragmentation by endogenous nucleases and trigger apoptosis.[11] Since apoptosis is an active and cell cycle phase-specific process, which requires the expression of specific genes, the synthesis of new mRNA and proteins and the activation of caspase and endonuclease enzymes, inhibitors of such mechanism can prevent DNA fragmentation in anthracycline-treated cells (reviewed in Ling *et al.*[11]). Hence, the abilities of the inhibitors of RNA (Act-D) and protein (CHX) syntheses, and caspase (z-VAD-fmk and z-IETD-fmk), non-caspase protease (TPCK) and endonuclease ($ZnSO_4$) activities to similarly reduce the mechanisms of DNA fragmentation by DAU and TT24 (Figure 8) suggest that, just like other clinical and experimental quinone antitumor drugs previously tested,[29–31] our lead TT bisquinones might also activate various sequences of caspases, non-caspase proteases and nucleases in order to trigger apoptosis, break DNA and induce tumor cell killing. Whether death receptor-dependent or -independent, different chemotherapeutic drug-induced apoptotic pathways may converge on the activation of a caspase proteolytic cascade that is amplified by a positive feedback loop involving the release of cytochrome *c* from mitochondria.[48–50] Since TT24-induced DNA cleavage is inhibited by the general caspase inhibitor z-VAD-fmk, which prevents the processing of effector caspase-3 to its active form, and the inhibitor of initiator caspase-8 z-IETD-fmk (Figure 8), both mitochondrial cytochrome *c* release and caspase activation are likely to be involved in the apoptotic pathways by which antitumor TT bisquinones trigger DNA fragmentation in L1210 cells.

In spite of their increasing cytotoxicity, micromolar concentrations of DAU, TT2 and TT24 higher than those inducing the peaks of DNA fragmentation at 24–48 h (Figures 6 and 7) might inhibit RNA and protein syntheses,[13] reduce the level of Topo targets, inactivate caspases and endonucleases, and/or arrest cell cycle traverse to such excessive degrees that they might actually block their own ability to sustain the active molecular mechanisms required for internucleosomal DNA fragmentation and apoptosis, and produce the paradoxical biphasic curves of DNA cleavage shown and discussed before in our L1210 and HL-60 studies.[13,27,29,42] Interestingly, the ability of DAU to both stimulate the active process of apoptotic DNA fragmentation at low concentrations and block the same process at higher concentrations is severely impaired in MDR HL-60 cells, whereas that of TT2 is not.[27,42] Taken together, the facts that the inhibition of nucleoside transport/DNA synthesis and the biphasic elevation and decline of DNA cleavage caused by TT2 are irreversible in L1210 cells[13] and remain unaltered in MDR HL-60 cells[27] 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 antitumor events, which persist

after cessation of drug treatment and in DAU-resistant tumor cells. Further studies are warranted to determine which of the mechanisms of MDR TT24 can overcome and whether this new quinone antitumor drug can potentiate the action of anti-metabolites.

Conclusion

TT24 may be a novel and potent bifunctional anticancer drug, which blocks nucleoside transport and induces apoptotic DNA fragmentation in order to decrease the viability of leukemic cells in the same nanomolar range as DAU *in vitro*. The molecular mechanism of action and anticancer potential of TT24 must be characterized *in vitro* and *in vivo* in order to determine whether this new synthetic class of antitumor TT bisquinones might be valuable to develop improved means of polychemotherapy.

References

1. Thomson RH. *Naturally occurring quinones III: recent advances*. New York: Chapman & Hall 1987.
2. Moore HW. Bioactivation as a model for drug design bioreductive alkylation. *Science* 1977; **197**: 527–32.
3. Monks TJ, Hanzlik RP, Cohen GM, Ross D, Graham DG. Quinone chemistry and toxicity. *Toxicol Appl Pharmacol* 1992; **112**: 2–16.
4. Myers CE, Chabner BA. Anthracyclines. In: Chabner BA, Collins JM, eds. *Cancer chemotherapy: principles and practice*. Philadelphia, PA: Lippincott 1990: 356–81.
5. Rossi L, Moore GA, Orrenius S, O'Brien PJ. Quinone toxicity in hepatocytes without oxidative stress. *Arch Biochem Biophys* 1986; **251**: 25–35.
6. Begleiter A, Leith MK, McClarty G, Beenken S, Goldenberg GJ, Wright JA. Characterization of L5178Y murine lymphoblasts resistant to quinone antitumor agents. *Cancer Res* 1988; **48**: 1727–35.
7. Qiu XB, Schönthal AH, Cadenas E. Anticancer quinones induce pRb-preventable G_2/M cell cycle arrest and apoptosis. *Free Rad Biol Med* 1998; **24**: 848–54.
8. Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989; **58**: 351–75.
9. Mimnaugh EG, Kennedy KA, Trush MA, Sinha BK. Adriamycin-enhanced membrane lipid peroxidation in isolated rat nuclei. *Cancer Res* 1985; **45**: 3296–304.
10. Ganapathi R, Grabowski D, Hoeltge G, Neelon R. Modulation of doxorubicin-induced chromosomal damage by calmodulin inhibitors and its relationship to cytotoxicity in progressively doxorubicin-resistant tumor cells. *Biochem Pharmacol* 1990; **40**: 1657–62.
11. Ling Y-H, Priebe W, Perez-Soler R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. *Cancer Res* 1993; **53**: 1845–52.
12. Ramachandran C, You W, Krishan A. *Bcl-2* and *mdr-1* gene expression during doxorubicin-induced apoptosis in murine leukemic P388 and P388/R84 cells. *Anticancer Res* 1997; **17**: 3369–76.
13. Perchellet EM, Magill MJ, Huang X, Brantis CE, Hua DH, Perchellet JP. 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*. *Anti-Cancer Drugs* 1999; **10**: 749–66.
14. Marina NM, Belt JA. Effects of nucleoside transport inhibitors on thymidine salvage and the toxicity of nucleoside analogs in mouse bone marrow granulocyte-macrophage progenitor cells. *Cancer Commun* 1991; **3**: 367–72.
15. Cao SS, Zhen YS. Potentiation of antimetabolite antitumor activity *in vivo* by dipyridamole and amphotericin B. *Cancer Chemother Pharmacol* 1989; **24**: 181–6.
16. Hughes JM, Tattersall MHN. Potentiation of methotrexate lymphocytotoxicity *in vitro* by inhibitors of nucleoside transport. *Br J Cancer* 1989; **59**: 381–4.
17. Muggia FM, Slowiaczek P, Tattersall MHN. Characterization of conditions in which dipyridamole enhances methotrexate toxicity in L1210 cells. *Anticancer Res* 1987; **7**: 161–6.
18. Grem JL, Fischer PH. Augmentation of 5-fluorouracil cytotoxicity in human colon cancer cells by dipyridamole. *Cancer Res* 1985; **45**: 2967–72.
19. Curtin NJ, Bowman KJ, Turner RN, *et al.* Potentiation of the cytotoxicity of thymidylate synthase (TS) inhibitors by dipyridamole analogues with reduced α_1-acid glycoprotein binding. *Br J Cancer* 1999; **80**: 1738–46.
20. Warlick CA, Sweeney CL, McIvor RS. Maintenance of differential methotrexate toxicity between cells expressing drug-resistant and wild-type dihydrofolate reductase activities in the presence of nucleosides through nucleoside transport inhibition. *Biochem Pharmacol* 2000; **59**: 141–51.
21. Smith PG, Marshman E, Newell DR, Curtin NJ. Dipyridamole potentiates the *in vitro* activity of MTA (LY231514) by inhibition of thymidine transport. *Br J Cancer* 2000; **82**: 924–30.
22. Chen H-X, Bamberger U, Heckel A, Guo X, Cheng Y-C. BIBW 22, a dipyridamole analogue, acts as a bifunctional modulator on tumor cells by influencing both P-glycoprotein and nucleoside transport. *Cancer Res* 1993; **53**: 1974–7.
23. Morgan PF, Fine RL, Montgomery P, Marangos PJ. Multidrug resistance in MCF-7 human breast cancer cells is associated with increased expression of nucleoside transporters and altered uptake of adenosine. *Cancer Chemother Pharmacol* 1991; **29**: 127–32.
24. Kusumoto H, Maehara Y, Anai H, Kusumoto T, Sugimachi K. Potentiation of adriamycin cytotoxicity by dipyridamole against HeLa cells *in vitro* and sarcoma 180 cells *in vivo*. *Cancer Res* 1988; **48**: 1208–12.

25. Ramu N, Ramu A. Circumvention of adriamycin resistance by dipyridamole analogues: a structure–activity relationship study. *Int J Cancer* 1989; **43**: 487–91.
26. Curtin NJ, Turner DP. Dipyridamole-mediated reversal of multidrug resistance in MRP over-expressing human lung carcinoma cells *in vitro*. *Eur J Cancer* 1999; **35**: 1020–6.
27. Wang B, Wu M, Perchellet EM, *et al.* A synthetic triptycene bisquinone, which blocks nucleoside transport and induces DNA fragmentation, retains its cytotoxic efficacy in daunorubicin-resistant HL-60 cell lines. *Int J Oncol* 2001; **19**: 1169–78.
28. Hua DH, Tamura M, Huang X, *et al.* Syntheses and bioactivities of substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetraones. Unusual reactivities with amines. *J Org Chem* 2002; **67**: 2407–12.
29. Perchellet EM, Magill MJ, Huang X, Dalke DM, Hua DH, Perchellet JP. 1,4-Anthraquinone: an anticancer drug that blocks nucleoside transport, inhibits macromolecule synthesis, induces DNA fragmentation, and decreases the growth and viability of L1210 leukemic cells in the same nanomolar range as daunorubicin *in vitro*. *Anti-Cancer Drugs* 2000; **11**: 339–52.
30. Perchellet EM, Sperfslage BJ, Qabaja G, Jones GB, Perchellet JP. Quinone isomers of the WS-5995 antibiotics: synthetic antitumor agents that inhibit macromolecule synthesis, block nucleoside transport, induce DNA fragmentation, and decrease the growth and viability of L1210 leukemic cells more effectively than ellagic acid and genistein *in vitro*. *Anti-Cancer Drugs* 2001; **12**: 401–17.
31. Perchellet EM, Sperfslage BJ, McIlvain CJ, *et al.* Antileukemic activity of synthetic daunomycinone derivatives bearing modifications in the glycosidic moiety. *Anticancer Res* 2002; **21**: 3957–68.
32. Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991; **3**: 207–12.
33. Shi Z, Azuma A, Sampath D, Li Y-X, Huang P, Plunkett W. S-phase arrest by nucleoside analogues and abrogation of survival without cell cycle progression by 7-hydroxystaurosporine. *Cancer Res* 2001; **61**: 1065–72.
34. Kolber MA, Broschat KO, Landa-Gonzalez B. Cytochalasin B induces cellular DNA fragmentation. *FASEB J* 1990; **4**: 3021–7.
35. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215.
36. Lai Y-L, Chen Y-J, Wu T-Y, *et al.* Induction of apoptosis in human leukemic U937 cells by tetandrine. *Anti-Cancer Drugs* 1998; **9**: 77–81.
37. Brown JM. NCI's anticancer drug screening program may not be selecting for clinically active compounds. *Oncol Res* 1997; **9**: 213–5.
38. Driscoll JS, Hazard GF, Wood HB, Goldin A. Structure-antitumor activity relationships among quinone derivatives. *Cancer Chemother Rep* 1974; **4**: 1–362.
39. Zee-Cheng RKY, Cheng CC. Anthraquinone anticancer agents. *Drugs Fut* 1983; **8**: 229–49.
40. Parekh HK, Mansuri-Torshizi H, Srivastava TS, Chitnis MP. Circumvention of adriamycin resistance: effect of 2-methyl-1,4-naphthoquinone (vitamin K3) on drug cytotoxicity in sensitive and MDR P388 leukemia cells. *Cancer Lett* 1992; **61**: 147–56.
41. Krishnan P, Bastow KF. Novel mechanism of cellular DNA topoisomerase II inhibition by the pyranonaphthoquinone derivatives α-lapachone and β-lapachone. *Cancer Chemother Pharmacol* 2001; **47**: 187–98.
42. Wu M, Wang B, Perchellet EM, *et al.* Synthetic 1,4-anthracenediones, which block nucleoside transport and induce DNA fragmentation, retain their cytotoxic efficacy in daunorubicin-resistant HL-60 cell lines. *Anti-Cancer Drugs* 2001; **12**: 807–19.
43. Leyva A, Pessoa C, Boogaerdt F, *et al.* Oncocalyxones A and C, 1,4-anthracenediones from *Auxemma oncocalyx*: comparison with anticancer 1,9-anthracenediones. *Anticancer Res* 2000; **20**: 1029–31.
44. Pessoa C, Silveira ER, Lemos TLG, Wetmore LA, Moraes MO, Leyva A. Antiproliferative effects of compounds derived from plants of northeast Brazil. *Phytother Res* 2000; **14**: 187–91.
45. Zee-Cheng RKY, Cheng CC. Antineoplastic agents. Structure–activity relationship study of bis (substituted aminoalkylamino)anthraquinones. *J Med Chem* 1978; **21**: 291–4.
46. Pecere T, Gazzola MV, Mucignat C, *et al.* Aloe-emodin is a new type of anticancer agent with selective activity against neuroectodermal tumors. *Cancer Res* 2000; **60**: 2800–4.
47. Johnson Inbaraj J, Krishna MC, Gandhidasan R, Murugesan R. Cytotoxicity, redox cycling and photodynamic action of two naturally occurring quinones. *Biochem Biophys Acta* 1999; **1472**: 462–70.
48. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999; **15**: 269–90.
49. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 2000; **256**: 42–9.
50. Nagata S. Apoptotic DNA fragmentation. *Exp Cell Res* 2000; **256**: 12–8.

(Received 12 March 2002; accepted 2 April 2002)