Antitumor triptycene bisquinones induce a caspase-independent release of mitochondrial cytochrome *c* and a caspase-2-mediated activation of initiator caspase-8 and -9 in HL-60 cells by a mechanism which does not involve Fas signaling

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Synthetic triptycene analogs (TT code number) mimic the antitumor effects of daunorubicin (DAU) *in vitro*, but have the advantage of blocking nucleoside transport, inhibiting both DNA topoisomerase I and II activities, and retaining their efficacy in multidrug-resistant (MDR) tumor cells. Since TT bisquinones induce poly(ADP-ribose) polymerase-1 (PARP-1) cleavage at 6 h and internucleosomal DNA fragmentation at 24 h, which are, respectively, early and late markers of apoptosis, these antitumor drugs were tested for their ability to trigger the release of mitochondrial cytochrome *c* (Cyt *c*) and the caspase activation cascade in the HL-60 cell system. Based on their ability to reduce the viability of wild-type, drug-sensitive HL-60-S cells in the nanomolar range, six lead antitumor TT bisquinones have been identified so far: TT2, TT13, TT16, TT19, TT24 and TT26. In accord with the fact that effector caspase-3 is responsible for PARP-1 cleavage, 4 μM concentrations of DAU and these TT bisquinones all maximally induce caspase-3 activity at 6 h in HL-60-S cells, an effect which persists when the drugs are removed after a 1-h pulse treatment. Since caspase-3 may be activated by initiator caspase-9 and -8, it is significant to show that such caspase activation cascade is induced by 4 μM DAU and TT bisquinones at 6 h in HL-60-S cells. Although the relationship is not perfect, the ability of TT analogs to induce caspase-3, -8 and -9 activities may be linked to their quinone functionality and cytotoxicity. Interestingly, 4 μM concentrations of TT bisquinones retain their ability to induce caspase-3, -8 and -9 activities at 6 h in the MDR HL-60-RV cell line where 4 μM DAU becomes totally ineffective. The release of mitochondrial Cyt *c* is also detected within 6 h in HL-60-S cells treated with 4 μM DAU or TT bisquinones, a finding consistent with the fact that Cyt *c* is the apoptotic trigger that activates caspase-9. Caspase-2 and -8 may both act upstream of mitochondria to promote Cyt *c* release, but caspase-2 is already maximally activated 6 h after 4 μM DAU or TT13 treatments, whereas DAU- or TT-induced caspase-8 and -9 activities peak at 9 h. Pre-treatments with 15 μM of the caspase-2 inhibitor benzyloxycarbonyl (z)-Val-Asp-Val-Ala-Asp (VDVAD)-fluoromethyl ketone (fmk) totally block DAU- and TT13-induced caspase-2, -8 and -9 activities, whereas pre-treatments with 15 μM of the caspase-8 inhibitor z-Ile-Glu-Thr-Asp (IETD)-fmk prevent DAU and TT13 from inducing caspase-8 activities without affecting their caspase-2- and -9-inducing activities, suggesting that the induction of apical caspase-2 activity by these drugs may be a critical upstream event required for the activation of other downstream caspases, including caspase-9 and the mitochondrial amplification loop through caspase-8. However, the mechanisms by which DAU and TT13 induce the release of mitochondrial Cyt *c* appear to be caspase-independent since they are both insensitive to similar pre-treatments with 100 μM of these specific caspase-2 and -8 inhibitors. Moreover, pre-treatments with 10 μg/ml of the antagonistic anti-Fas DX2 and ZB4 monoclonal antibodies (mAbs), and the neutralizing anti-Fas ligand (FasL) NOK-1 mAb are all unable to prevent DAU and TT13 from inducing Cyt *c* release and caspase-2, -8 and -9 activities, suggesting that the Fas–FasL signaling pathway is not involved in the mechanism by which these quinone antitumor drugs trigger apoptosis in HL-60 cells. *Anti-Cancer Drugs* 15:929–946 © 2004 Lippincott Williams & Wilkins.

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Introduction

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 reactive O_2 species (ROS) and damage biomembranes, induce DNA breakage and chromosomal aberrations, and have a wide spectrum of anticancer activity [1–8]. Many anticancer drugs, such as DOX and DAU, may activate apoptosis at low concentrations to kill susceptible tumor cells while defective apoptotic signaling pathways may contribute to the development of multidrug resistance (MDR) [3,7–16]. Since the clinical effectiveness of DOX and DAU is severely limited by their cumulative cardiotoxicity and ability to induce MDR, it is important to develop new quinone antitumor drugs with improved bioactivity [1] and determine whether apoptosis plays a decisive role in their molecular mechanism of action.

In contrast to their inactive parent compound triptycene (code name TT0), several synthetic analogs (TT code number) with or without quinone functionality may represent a novel class of bifunctional antitumor drugs, which inhibit DNA, RNA and protein syntheses within 2–3 h, decrease the mitotic index and induce DNA fragmentation within 24 h, and reduce the proliferation and viability of murine L1210 lymphocytic and wild-type (WT), drug-sensitive, human HL-60-S promyelocytic leukemia cells within 2–4 days like DAU [17–20]. Interestingly, the lead antitumor compounds identified so far are all TT bisquinones and, based on their ability to inhibit L1210 leukemic cell growth and mitochondrial metabolism, are cytostatic and cytotoxic in a nanomolar range close to that of DAU *in vitro* [17,19–21]. A critical finding is that TT bisquinones have the additional advantage of blocking the cellular transport of purine and pyrimidine nucleosides within 15 min, an effect which DAU cannot do [17,18,20]. In addition to their potency and unusual mechanism of action, TT bisquinones retain their efficacy in two MDR HL-60-RV and HL-60-R8 sublines that have already developed different P-glycoprotein (P-gp)- and MDR-associated protein-mediated mechanisms of resistance to DAU, suggesting that these new quinone antitumor drugs might have other molecular targets than those of DAU and might be valuable in polychemotherapy to potentiate the action of antimetabolites and circumvent MDR [18].

Some anticancer drugs may cause DNA single-strand breaks (SSBs), double-strand breaks (DSBs) and DNA–protein cross-links (DPCs) either directly by interacting with DNA or indirectly by targeting Topo I and II activities [1,4,22–24]. Such DNA-damaging events are then likely to induce apoptosis in tumor cells by triggering nuclear signals upstream of mitochondria [3,7,8,25]. Intercalation models have been postulated for the actions of 20(*S*)-camptothecin (CPT) and DAU, which, respectively, inhibit Topo I and II activities by stabilizing the normally transient and reversible DNA–Topo I or II cleavable complexes [4,22–25]. Such stabilized ternary complexes of DNA–drug–Topo I or II may subsequently need to collide or interact with DNA replication forks or several other enzyme complexes that track along DNA in order to be converted to long-lived and highly cytotoxic DNA DSBs, induce apoptosis and exert their lethality [4,22–25]. Our recent study demonstrates that antitumor TT bisquinones have the unusual ability to inhibit, in a concentration-dependent manner, the relaxation of supercoiled plasmid DNA catalyzed by both purified human Topo I and II enzymes [21]. TT24, one of the most cytotoxic of these compounds, may be a novel dual inhibitor of Topo I and II activities, which matches the Topo I inhibitory effect of CPT and surpasses the Topo II inhibitory effect of *m*-amsacrine under conditions where CPT is ineffective in the Topo II assay and etoposide (VP-16, 4′-demethylepipodophyllotoxin-9-[4,6-*O*-ethylidene-β-D-glucopyranoside]) is ineffective in the Topo I assay [21]. Since the initial, massive and irreversible high-molecular-weight (MW) cleavage of DNA subsequent to dual Topo I and II inhibition might induce tumor cells to commit to an apoptotic decision, apoptosis might be an important mechanism of tumor cell killing by TT bisquinones that can target both of these nuclear enzymes [4,7,8,21–29].

In contrast to the early cleavage of DNA into large 50- to 300-kbp fragments, an initial signaling event that may induce tumor cells treated with relatively low concentrations of DNA-damaging anticancer drugs to commit apoptosis, the secondary endonucleolytic cleavage of DNA at internucleosomal linker sites to produce small 180- to 200-bp mono- and oligonucleosomal fragments at 24 h is a late molecular marker concurrent with morphological evidence of apoptosis [30,31]. The nuclear poly(ADP-ribose) polymerase 1 (PARP-1) enzyme, which recognizes and is activated by binding to DNA strand breaks, may facilitate DNA repair by dissociating poly(ADP-ribosyl)ated and negatively charged nuclear proteins, principally itself, from nicked DNA, decondensing the chromatin structure and permitting access of the DNA repair machinery to the lesions [32,33]. Hence, cleavage and inactivation of PARP-1 is an early event required for cells committed to apoptosis because it prevents the detection and repair of drug-induced DNA damage, it blocks the depletion of NAD^+ and ATP causing necrotic cell death, and it enhances the activity of Ca^{2+}/Mg^{2+}-dependent endonucleases [32–36]. Since PARP-1 cleavage at 6 h is one of the first detectable protein-degradation events occurring after early high-MW cleavage of chromatin DNA, but before the late and low-MW internucleosomal fragmentation of DNA at 24 h, detection of the specific 85-kDa fragment of PARP-1 cleavage can serve as an early and sensitive marker of

apoptosis in tumor cells treated with DNA-damaging anticancer drugs [32–37]. In contrast to TT0, all lead antitumor TT bisquinones share the ability to fully induce PARP-1 cleavage in WT HL-60-S cells [38]. Treatments with 1.6 μM concentrations of TT24 and DAU similarly induce maximal PARP-1 cleavage at 6 h in WT HL-60-S cells. However, in MDR HL-60-RV cells, PARP-1 cleavage is still induced by 4 μM TT24, but not by 4-10 μM DAU [38]. Since the abilities of TT24 and DAU to induce PARP-1 cleavage are inhibited by the cysteine protease inhibitor benzyloxycarbonyl (z)-Val-Ala-Asp (VAD)-fluoromethyl ketone (fmk), but not by the serine protease inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), caspase activation is likely responsible for PARP-1 cleavage after DAU and TT24 treatments [38].

The abilities of antitumor TT bisquinones to induce internucleosomal DNA fragmentation at 24 h have been demonstrated by two different techniques, using tumor cells containing [^{3}H]thymidine-prelabeled DNA to detect low-MW DNA fragments after intact chromatin precipitation or agarose gel electrophoresis to visualize the typical pattern of DNA laddering indicative of apoptosis [17,18,20]. Since apoptosis is an active ATP-driven and cell cycle phase-specific process that requires the expression of specific genes, the synthesis of new RNA and proteins and the activation of caspases, non-caspase proteases and nucleases, inhibitors of such mechanisms can prevent DNA fragmentation in anthracycline-treated cells [7]. Interestingly, the concentration-dependent inductions of DNA cleavage caused by TT2, TT24 and DAU in L1210 and HL-60-S cells at 24 h are similarly biphasic, suggesting that, even though they are increasingly cytotoxic, higher than optimal concentrations of TT bisquinones and DAU might inhibit RNA and protein syntheses, inactivate enzymes and/or arrest cell cycle traverse to such excessive degrees that they actually abolish their own ability to sustain the active process of apoptotic DNA fragmentation induced by low concentrations of these compounds [17,18,20]. Indeed, the optimal concentrations of 1.6 μM TT24 and DAU, which normally induce peak levels of DNA fragmentation in L1210 cells at 24 h, similarly lose their ability to do so in the presence of actinomycin D, cycloheximide, z-VAD-fmk, z-Ile-Glu-Thr-Asp (IETD)-fmk, TPCK and $ZnSO_4$, suggesting that new RNA and protein syntheses and cysteine and serine protease and nuclease activities might be required for these quinone drugs to trigger DNA fragmentation through an active apoptotic pathway that involves the activation of initiator and executioner caspases, endonucleases and even other non-caspase proteases, which are unable to target PARP-1 at 6 h, but play a role in the mechanism by which TT bisquinones and DAU induce apoptotic DNA fragmentation at 24 h [20,38]. Taken together, the facts that the cleavage of PARP-1, the inhibition of nucleoside transport/DNA synthesis and the biphasic elevation and decline of DNA fragmentation caused by TT2 and TT24 are irreversible in L1210 and HL-60-S cells, and remain unaltered in MDR HL-60 sublines suggest that substituted TT bisquinones might rapidly and tightly interact with various membrane and intracellular targets to disrupt the structures/functions of nucleoside transporters, nucleic acids and proteins, and to trigger long-lasting apoptotic and antitumor events, which persist upon drug removal and in DAU-resistant tumor cells [17,18,20,38].

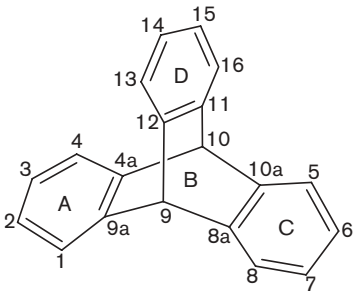
Because neoplastic cells undergoing apoptosis may be phagocytosed without inflammation, lead antitumor TT bisquinones that are the most effective compounds in the series and can induce apoptosis might be valuable to develop new means of chemotherapy. Drug-damaged tumor cells irreversibly committed to the apoptotic pathway exhibit characteristic biochemical and morphological alterations, including mitochondrial dysfunction, cytoplasmic and nuclear condensation, plasma membrane blebbing, DNA degradation into oligonucleosomal fragments, and packaging of cellular components into discrete membrane-bound apoptotic bodies that are rapidly phagocytosed by surrounding cells and macrophages [39]. Whether death receptor (DR)-dependent or -independent, the different apoptosis-signaling pathways induced by various anticancer drugs with distinct primary subcellular targets and mechanisms of action may converge on mitochondria to cause permeability transition (MPT), release apoptogenic factors, and activate a similar caspase proteolytic cascade that is amplified by a positive feedback loop involving the release of cytochrome *c* (Cyt *c*) from mitochondria [16,30,40]. Therefore, the present study was undertaken to further assess and compare the pro-apoptotic effects of synthetic TT analogs and DAU in WT and MDR HL-60 cells. Our findings indicate that antitumor TT bisquinones induce Cyt *c* release and caspase-9, -3 and -8 activities by a Fas-independent signaling pathway which is partially mediated by caspase-2 activation.

Materials and methods

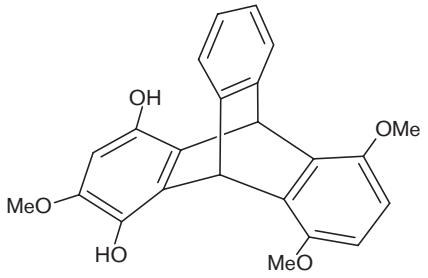
Cell cultures and drug treatments

A new, short and easy method to synthesize the structures of the 10 TT analogs (code names TT2, TT3, TT5, TT7, TT8, TT9, TT13, TT16, TT19 and TT21) illustrated in Figure 1 has been developed [19]. The syntheses of TT24–TT26 (Fig. 1) will be reported elsewhere. All solutions of parent TT0 (Aldrich, Milwaukee, WI), synthetic TT analogs, z-Val-Asp-Val-Ala-Asp (VDVAD)-fmk and z-IETD-fmk (both from Calbiochem, La Jolla, CA) were dissolved and diluted in dimethylsulfoxide (DMSO), whereas DAU (Sigma, St Louis, MO) solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl [17,18,20,21,38,41]. Solutions of antagonistic anti-Fas DX2, anti-Fas ligand

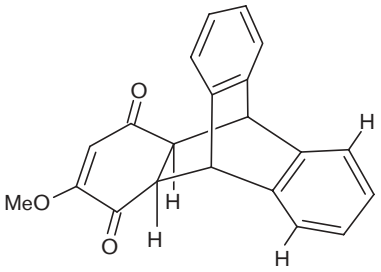
Fig. 1



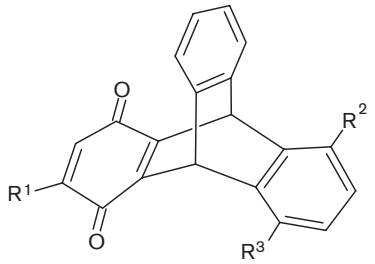
TT0: Triptycene (IC_{50} = NS)[a]



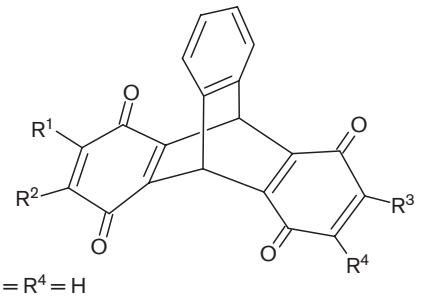
TT3: (IC_{50} = 1175 ± 109 nM)[b]



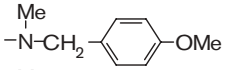
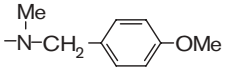
TT8: (IC_{50} = 1523 ± 116 nM)



TT5: $R^1 = R^2 = R^3 = OMe$ (IC_{50} = 649 ± 33 nM)[c]
TT7: $R^1 = R^2 = R^3 = H$ (IC_{50} = 941± 84 nM)
TT9: $R^1 = OMe$, $R^2 = R^3 = H$ (IC_{50} = 785± 52 nM)



TT2: $R^1 = OMe$, $R^2 = R^3 = R^4 = H$ (IC_{50} = 275 ± 23 nM)
TT13: $R^1 = OMe$, $R^2 = Br$, $R^3 = R^4 = H$ (IC_{50} = 316 ± 19 nM)[d]
TT16: $R^1 = OMe$, $R^2 = Br$, $R^3 = NMe_2$, $R^4 = H$ (IC_{50} = 338 ± 35 nM)[d]
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = NMe_2$
TT19: $R^1 = NHCH_2CH_2CO_2Et$, $R^2 = Br$, $R^3 = R^4 = H$ (IC_{50} = 506 ± 34 nM)[e]
TT21: $R^1 = NHCH_2CH_2CO_2$-*t*-Bu, $R^2 = Br$, $R^3 = R^4 = H$ (IC_{50} = 567 ± 40 nM)
TT24: $R^1 = OMe$, $R^2 = Br$, $R^3 = NHMe$, $R^4 = H$ (IC_{50} = 286 ± 13 nM)[d]
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = NHMe$

TT25: $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 =$  (IC_{50} = 1308 ± 107 nM)
and $R^1 = OMe$, $R^2 = Br$, $R^4 = H$, $R^3 =$ 

TT26: $R^1 = OMe$, $R^2 = Br$, $R^3 = N(Me)CH_2–CH=CH_2$, $R^4 = H$ (IC_{50} = 401 ± 49 nM)[f]
and $R^1 = OMe$, $R^2 = Br$, $R^3 = H$, $R^4 = N(Me)CH_2–CH=CH_2$

Chemical structures, code names and concentrations of synthetic TT analogs required to inhibit by 50% the metabolic activity of human HL-60-S cells at day 4 *in vitro*. Cell viability results (means ± SD, $n = 3$) were expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control tumor cells ($A_{490\,nm} = 1.490 \pm 0.083$, 100 ± 6%) after 4 days in culture. The blank value ($A_{490\,nm} = 0.368$) for cell-free culture medium supplemented at day 4 with MTS:PMS reagent was subtracted from the results. IC_{50} values were calculated from linear regression of the slopes of the log-transformed concentration–survival curves. [a]Not significantly different from control when tested up to 4 μM; [b]$p < 0.05$, greater than TT7; [c]$p < 0.05$, greater than TT21; [d]not different from TT2; [e]$p < 0.05$, greater than TT26, but not different from TT21; [f]$p < 0.025$ and $p < 0.05$, respectively greater than TT2 and TT13, but not different from TT16.

(FasL) NOK-1 (both from BD PharMingen, San Diego, CA) and anti-Fas ZB4 (Upstate Biotechnology, Lake Placid, NY) monoclonal antibodies (mAbs) were formulated in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) [41]. Suspension cultures of WT, drug-sensitive, human HL-60-S promyelocytic leukemia cells (ATCC, Manassas, VA) were maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 8.25% fetal bovine calf serum (FCS; Atlanta Biologicals, Norcross, GA) and penicillin (100 IU/ml)/streptomycin (100 µg/ml), and incubated in the presence or absence (control) of drugs at 37°C in a humidified atmosphere containing 5% CO_2 [18,38,41]. The MDR HL-60-RV cells shown to overexpress P-gp were similarly maintained in RPMI 1640 medium in the absence of drugs but were exposed, every 4 weeks, to 41 nM DAU for 48 h to stabilize their MDR phenotype [18,38,41,42]. This concentration of DAU, which is not cytotoxic to MDR HL-60-RV cells, was removed from the culture medium at least 48 h before experimentation. Since drugs were supplemented to the culture medium in 1- or 10-µl aliquots, the concentration of vehicle (0.2% DMSO) in the final incubation volumes (0.5 or 5 ml, respectively) did not interfere with the data [17–21,38,41]. For 1-h pulse treatment in the caspase experiments, drug-containing supernatants were discarded after centrifugation at 200 *g* for 10 min and tumor cells were washed with, and resuspended in, fresh RPMI 1640 medium for further incubation [17,38,41].

Cell viability assay

For tumor cell viability, WT HL-60-S cells suspended in FCS-containing RPMI 1640 medium (initial density: 3.75×10^4 cells/0.5 ml) were grown in triplicate at 37°C in 48-well Costar cell culture plates for up to 4 days in the presence or absence (control) of serial concentrations of drugs to evaluate their cytotoxicity. The viability of drug-treated cells was assessed from their mitochondrial ability to bioreduce the MTS reagent (Promega, Madison, WI) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product which absorbs at 490 nm [43]. After 4 days in culture, control and drug-treated cell samples (about 10^6/0.5 ml/well for controls) were further incubated at 37°C for 3 h in the dark in the presence of 0.1 ml of MTS:PMS (2:0.1) reagent and their relative metabolic activity 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 [17,18,20,21,41].

Fluorogenic assay of caspase activities

Control and drug-treated HL-60-S or HL-60-RV cells (0.4×10^6/0.5 ml of FCS-containing RPMI 1640 medium) were incubated in triplicate for various periods of time at 37°C, collected by centrifugation at 200 *g* for 10 min and washed with 1 ml of ice-cold PBS. Cell pellets were resuspended in chilled 10 mM HEPES buffer, pH 7.4, containing 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM EGTA, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 100 µM digitonin (175 µl for caspase-3 assay, and 120 µl for caspase-2, -8 and -9 assays), and lysed for 10 min on ice [41]. Cell lysates were centrifuged at 14 000 *g* for 20 min at 4°C to precipitate cellular debris and the supernatants were stored frozen at −70°C overnight [41]. The caspase-2-, -3-, -8- and -9-like activities of the lysates were determined in reaction mixtures that contained 50 µl of lysis buffer (blank) or supernatant (control or drug-treated samples) and 50 µl of reaction buffer (100 mM HEPES, pH 7.5, containing 1 mM EDTA, 10 mM EGTA, 10% sucrose and 10 mM of freshly prepared DTT) and that were initiated by the addition of 5-µl aliquots of the respective 5 mM z-VDVAD-7-amino-4-trifluoromethylcoumarin (AFC), 1 mM z-Asp-Glu-Val-Asp (DEVD)-AFC, 1 mM z-IETD-AFC or 5 mM acetyl-Leu-Glu-His-Asp (Ac-LEHD)-AFC stocks of AFC-substrate conjugates (all from Calbiochem) [41]. After incubation for 1 h at 37°C in 96-well Costar white opaque polystyrene assay plates, the fluorescence of the free AFC released upon proteolytic cleavage of the substrate by the appropriate caspase was detected at 400 nm excitation and 505 nm emission, using a Cary Eclipse Fluorescence Spectrophotometer equipped with microplate reader accessory (Varian, Walnut Creek, CA). Arbitrary fluorescence units were quantified with reference to calibration curves ranging from 0.01 to 6 nmol of AFC (from Sigma), the protein concentrations of the supernatants were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), and the VDVAD-, DEVD-, IETD- and LEHD-specific cleavage activities of the samples were expressed as nmol of AFC released/mg of protein [41]. Data of all biochemical experiments were analyzed using Student's *t*-test with the level of significance set at $p < 0.05$.

Western blot analysis of Cyt *c* release

For Cyt *c* analysis, suspensions of control and drug-treated HL-60-S cells were seeded at an initial density of 1.3×10^6 cells/ml and incubated for various periods of time at 37°C in 15 × 60-mm Petri dishes containing final volumes of 5 ml [41]. To determine the release of mitochondrial Cyt *c* into the cytosol, pellets of 6.5×10^6 tumor cells were collected by centrifugation at 200 *g* for 10 min, washed with 1 ml of PBS, and lysed for 30 min on ice with 80 µl of 10 mM HEPES buffer, pH 7.4, containing 100 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM EGTA, 1 mM DTT, 10% sucrose, 1 mM PMSF, leupeptin (0.2 mg/ml), aprotinin (0.2 mg/ml) and 100 µM digitonin [41]. Digitonin permeabilization is required to avoid artifacts due to the mechanical breakage of the outer mitochondrial membrane by dounce homogenization or ultrasonic disruption. Cell lysates were

centrifuged at 14 000 *g* for 20 min and the protein concentrations of the cytosolic supernatants were determined with the BCA Protein Assay Kit. Aliquots of supernatants containing equal 100-μg amounts of proteins were boiled for 5 min in SDS sample loading buffer, resolved by electrophoresis for 75 min at 110 V in a 15% SDS–polyacrylamide gel, and transferred for 1 h to a PVDF sequencing membrane (Immobilon-P^{SQ}; Millipore, Bedford, MA), using a Panther model HEP1 Semidry Electroblotter (Owl Separation Systems, Portsmouth, NH) [38,41]. The blots were blocked with 5% non-fat dry milk in 20 mM Tris–HCl buffer, pH 7.4, with 0.9% NaCl (TBS), containing 0.05% of Tween-20 (TBST) for 2 h at room temperature. Immunodetection of Cyt *c* was conducted at room temperature overnight in TBST containing 2% non-fat dry milk, using 1 μg/ml of mouse anti-Cyt *c* (7H8.2C12) primary mAb (BD PharMingen), which recognizes the 15-kDa denatured form of Cyt *c* [44]. After the blots were rinsed 3 times with TBST, incubated for 1 h at room temperature in TBST containing 2% non-fat dry milk and goat anti-mouse secondary mAb conjugated with horseradish peroxidase (1:50 000 dilution; Oncogene, Boston, MA), and rinsed again 3 times with TBST, Kodak BioMax light film (Eastman Kodak, Rochester, NY) was used to develop images of the immunoreactive bands revealed by enhanced chemiluminescence (CL) staining, using the SuperSignal West Pico CL Substrate (Pierce). The integrated density values of Western blots were compared by digital image analysis (Chemi Imager 5500 with AlphaEaseFC software), using a MultiImage Light Cabinet (Alpha Inotech, San Leandro, CA).

Results

Inhibition of tumor cell viability

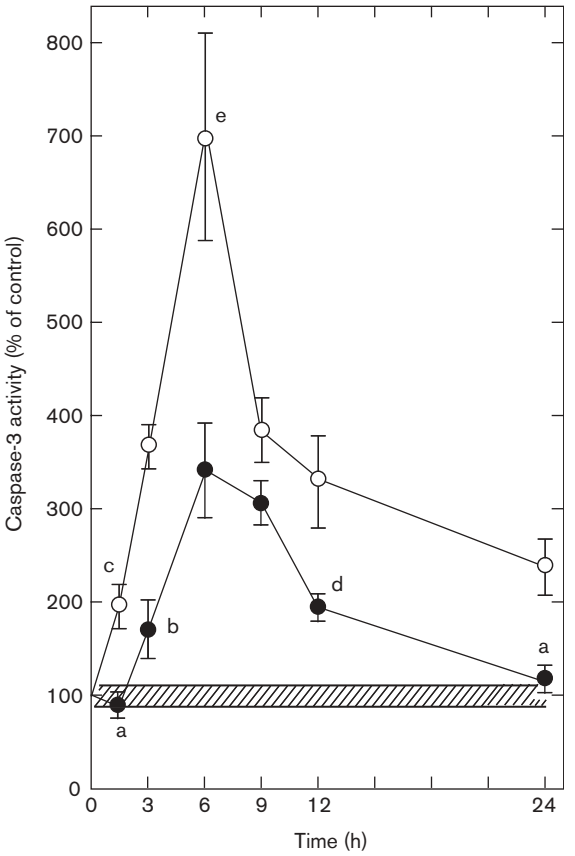
The chemical structures and code names of the antitumor TT analogs (serial numbers indicate the order of synthesis) selected to be tested for their ability to induce markers of apoptosis in the HL-60 tumor cell system *in vitro* are depicted in Figure 1. The known parent compound TT0 is commercially available, but all other TT analogs under study were synthesized by *in situ* oxidation of substituted dihydroxybenzene followed by [4 + 2] cycloaddition with 1,4-dimethoxyanthracene and then oxidation [17–21,38]. This method for synthesizing such substituted 9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetrones has been reported [19]. Except for TT25, the correct nomenclatures and bioactivities of these rigid tetracyclic skeletons, which possess either none, one or two external quinones, have already been described in the L1210 tumor cell system [17,19–21]. The nomenclature of TT25, which is a synthetic precursor of TT24, is 6-bromo-7-methoxy-2-[*N*-(4-methoxybenzyl)-*N*-methylamino]-9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetrone. TT16 and TT24–TT26 are mixtures of regioisomers that are inseparable by silica gel column chromatography. The apoptotic effects of these TT analogs were assessed and compared to those of DAU, a clinically valuable anticancer drug that is structurally very different but also contains a *para*-quinone moiety [17–21,38].

In contrast to their parent compound TT0, which is inactive up to 4 μM, all other TT analogs selected for this apoptosis study have antitumor activities, based on the comparative list of nanomolar concentrations required to inhibit by 50% (IC_{50}) the viability of HL-60-S cells after 4 days in culture *in vitro* (Fig. 1). The 7 TT bisquinones, which inhibit the most the mitochondrial ability of HL-60-S cells to metabolize the MTS:PMS reagent at day 4, are TT2, TT13, TT16, TT19, TT21, TT24 and TT26 (Fig. 1). However, TT21, which is consistently the weakest of them in our biochemical assays, has been dropped from our final list of six lead antitumor compounds. Bisquinone TT25, containing a *N*-methoxybenzyl-*N*-methylamino substitution, is not a lead antitumor compound and was not retained for further study. Similar data have been observed when ranking the cytostatic/cytotoxic potential of these drugs in the L1210 cell system [17,19–21]. However, the same six lead antitumor TT bisquinones are consistently more potent against L1210 cells (IC_{50} values ranging from 48 to 135 nM) [21] than against HL-60-S cells (IC_{50} values ranging from 275 to 506 nM) (Fig. 1). Moreover, it should be noted that, under similar conditions, the cytotoxicity of DAU is characterized by IC_{50} values around 25–30 nM in L1210 cells and 4–18 nM in HL-60 cells [17–21,45]. Although somewhat less potent, TT5, TT7 and TT9 are representative examples of TT monoquinones closely related to the above TT bisquinones, and with good antitumor activities in the L1210 [17,19,21] and HL-60-S [38] cell systems (Fig. 1). The dihydroquinone TT3 and the diketone TT8 have also been selected for this apoptosis study because they demonstrate the fact that synthetic TT analogs lacking quinone functionality can still elicit interesting antitumor effects in L1210 [17,19,21] and HL-60-S [38] cells (Fig. 1).

The antitumor activities of TT13 and TT24 have been confirmed in the 60 human tumor cell line panel of the National Cancer Institute's (NCI) *in vitro* antitumor screen, in which a sulforhodamine B protein assay is used to estimate cell viability and growth after 2 days of continuous drug exposure. With this well-established methodology, the cytostatic/cytotoxic activities of TT13 and TT24 can be demonstrated in all 60 human tumor cell lines, and, although these drugs are slightly more potent against ascitic than solid tumors, the selectivity pattern indicates that a wide spectrum of tumor cells are sensitive to TT bisquinone inhibition (data not shown). For instance, the concentrations of TT24 required to inhibit tumor cell growth by 50% (GI_{50}) at 48 h are 1.12 μM in MOLT-4 and 1.35 μM in K-562 leukemia. For solid tumors, TT24 is the most effective against HOP-92

(GI_{50}: 1.24 μM) and NCI-H522 (GI_{50}: 1.30 μM) non-small lung cancers, HCT-116 and KM12 (GI_{50}: 1.87 μM) colon cancers, SF-539 (GI_{50}: 1.23 μM) and U251 (GI_{50}: 1.41 μM) CNS cancers, LOX IMVI (GI_{50}: 952 nM) and UACC-62 (GI_{50}: 1.47 μM) melanoma, IGROV1 (GI_{50}: 1.35 μM) and OVCAR-3 (GI_{50}: 1.50 μM) ovarian cancers, SN12C (GI_{50}: 1.05 μM) and 786-0 (GI: 1.38 μM) renal cancers, PC-3 (GI_{50}: 1.08 μM) prostate cancer, and MDA-MB-231/ATCC (GI_{50}: 1.41 μM), T-47D (GI_{50}: 1.44 μM) and NCI/ADR-RES (GI_{50}: 1.55 μM) breast cancers. Overall, TT13 is slightly less effective than TT24 in the NCI's antitumor screen, except against HCC-2998 (GI_{50}: 565 nM) colon cancer. Bisquinone TT14, which is 3-bromo-2-*N*-methylamino-9,10-dihydro-9,10-[1,2]benzenoanthracene-1,4,5,8-tetrone (not shown in Fig. 1), is also somewhat less potent than TT24 in the NCI's antitumor screen and has not been selected for this apoptosis study because it is clearly less effective than our current lead antitumor TT bisquinones in our previous biochemical assays [20]. However, MOLT-4 (GI_{50}: 412 nM) and CCRF-CEM (GI_{50}: 472 nM) leukemia, M14 (GI_{50}: 858 nM) melanoma, PC-3 (GI_{50}: 610 nM) prostate cancer and MCF-7 (GI_{50}: 1.30 μM) breast cancer respond quite well to TT14 inhibition (data not shown). Naturally, the concentrations of TT bisquinones required for total growth inhibition (TGI) and to induce a net loss of 50% of the initial cell concentration (LC_{50}) are somewhat higher than the respective GI_{50} values in this NCI's *in vitro* cell line screen.

Fig. 2

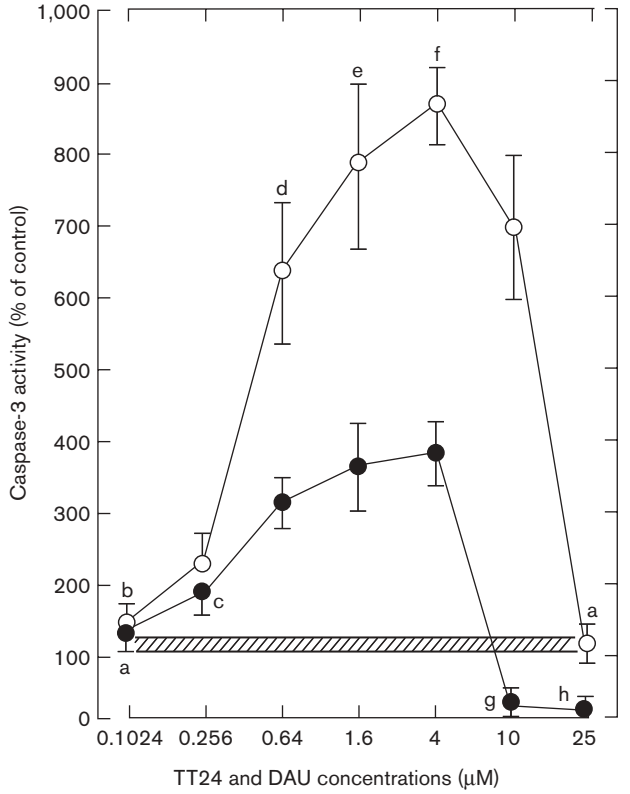


Comparison of the time-dependent inductions of caspase-3-like protease activity by 1.6 μM TT24 (●) and DAU (○) in HL-60-S cells *in vitro*. Results are expressed as percentage of DEVD cleavage activity in vehicle-treated control tumor cells (12.3 ± 1.0 nmol AFC released/mg protein, 100 ± 8%, striped area) at each time point tested. Bars: means ± SD ($n=3$). [a]Not different from control; [b]$p<0.025$, [c]$p<0.005$ and [d]$p<0.0005$, greater than control; [e]$p<0.025$, greater than TT24 at 6 h.

Activation of caspase-3

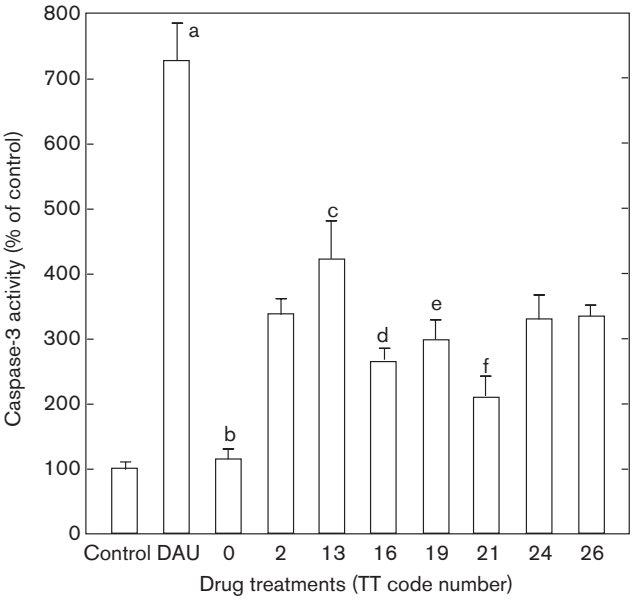
Because the degradation of PARP-1 may be catalyzed by effector caspase-3, the TT bisquinones shown to induce PARP-1 cleavage [38] were tested for their ability to activate caspase-3 in HL-60-S cells. The hypothesis that caspase-3 is involved in DAU- and TT24-induced PARP-1 cleavage at 6 h [38] is substantiated by the fact that DAU and TT24 similarly induce time- (Fig. 2) and concentration-dependent (Fig. 3) elevations of caspase-3 activities, which peak 6 h after treatment of HL-60-S cells with 1.6–4 μM concentrations of these drugs. But the magnitude of DAU-induced caspase-3 activation, which reaches at least 700% of the control, is about 2 times greater than that caused by TT24, which reaches about 350% of the control, and extends over somewhat wider ranges of times and concentrations (Figs 2 and 3), suggesting that DAU may be more effective than TT24 at triggering apoptosis in HL-60 cells. Interestingly, the concentration-dependent abilities of DAU and TT24 to maximally induce caspase-3 activity in HL-60-S cells at 6 h are similarly biphasic and resemble the biphasic increases and decreases of internucleosomal DNA fragmentation caused by these drugs at 24 h [17,18,20], with the activations of caspase-3 increasing up to peaks at 1.6–4 μM followed by declines toward or even below control level for higher 10–25 μM concentrations of DAU and TT bisquinone, which presumably block the active process of apoptosis (Fig. 3). In contrast to their inactive parent compound TT0, which fails to induce caspase-3 activity at 4 μM, the lead antitumor TT bisquinones previously shown to induce PARP-1 cleavage, internucleosomal DNA degradation and antileukemic effects [17–21,38] are all capable of mimicking, albeit to a lesser degree, the activation of caspase-3 caused by 4 μM DAU in HL-60-S cells at 6 h, suggesting that their ability to trigger apoptosis may play an important role in their molecular mechanism of antitumor activity (Fig. 4). TT13 and TT24, two of the most effective inducers of caspase-3 activity, were selected for further study. Finally, HL-60-S cells were exposed to 1.6–4 μM DAU, TT13 and TT24 for only 1 h before these drugs were removed, and caspase-3 activities were assayed as usual at 6 h (Fig. 5). In contrast to the stimulating effects of DAU and TT24, which seem to plateau at 1.6–4 μM, the caspase-3-inducing activity of TT13, which is smaller than that of

Fig. 3



Comparison of the concentration-dependent inductions of caspase-3-like protease activity by TT24 (●) and DAU (○) at 6 h in HL-60-S cells *in vitro*. Results are expressed as percentage of DEVD cleavage activity in vehicle-treated control tumor cells (11.2 ± 1.0 nmol AFC released/mg protein, 100 ± 9%, striped area) at 6 h. Bars: means ± SD ($n = 3$). [a]Not different from control; [b]$p < 0.05$, greater than control, but not different from 0.1024 μM TT24; [c]$p < 0.025$, greater than control, but not different from 0.256 μM DAU; [d]$p < 0.01$, [e]$p < 0.005$ and [f]$p < 0.0005$, respectively, greater than 0.64, 1.6 and 4 μM TT24; [g]$p < 0.005$, greater and [h]$p < 0.0005$, smaller than control.

Fig. 4



Comparison of the abilities of 4 μM concentrations of DAU, TT0 and lead antitumor TT bisquinones to induce caspase-3-like protease activity after 6 h in HL-60-S cells *in vitro*. Results are expressed as percentage of DEVD cleavage activity in vehicle-treated control tumor cells (12.1 ± 1.1 nmol AFC released/mg protein, 100 ± 9%) at 6 h. Bars: means ± SD ($n = 3$). [a]$p < 0.005$, greater than TT13; [b]not different from control; [c]$p < 0.05$, greater than TT2; [d]not different from TT19, but $p < 0.05$, greater than TT21 and smaller than TT24; [e]not different from TT2, TT24 and TT26; [f]$p < 0.005$, greater than control.

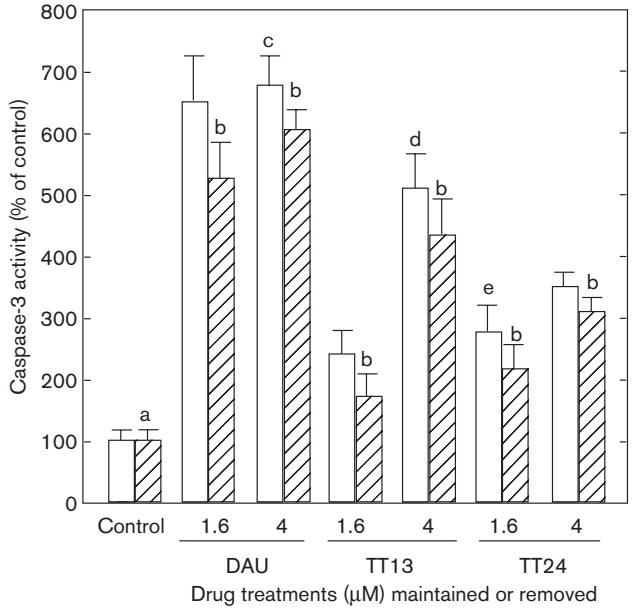
DAU but greater than that of TT24, definitively peaks at 4 μM rather than 1.6 μM (Fig. 5). However, these 1-h pulse treatments with 1.6–4 μM DAU, TT13 and TT24 are sufficient to trigger as much induction of caspase-3 activity at 6 h as when the anticancer drugs are maintained in the culture medium for the whole 6-h period of incubation, suggesting that the mechanisms by which DAU and TT bisquinones rapidly stimulate the apoptotic pathway are irreversible and persist after drug removal (Fig. 5).

Activation of caspase-8 and -9

Because caspase-3 may be proteolytically activated by caspase-9 and, directly or indirectly, by caspase-8, DAU and TT bisquinones were tested for their ability to activate this cascade of key initiator and effector caspases in WT and MDR HL-60 cells at 6 h. Indeed, 1.6–4 μM DAU, TT13 and TT24 all induce initiator caspase-8 and -9 activities in relation with their abilities to activate caspase-3 in HL-60-S cells at 6 h (Fig. 6). As observed for caspase-3 (Figs 4 and 5), TT13 induces caspase-8 and -9 activities to a lesser degree than DAU, but to a greater degree than TT24, especially at 4 μM, a concentration at which TT13 is consistently a more potent caspase activator than at 1.6 μM (Fig. 6). When compared at 4 μM, caspase-3, -8 and -9 activities are maximally induced (between 307 and 796% of the controls) by the lead antitumor bisquinone TT2, but are only partially induced by the other weaker antitumor TT analogs that have no or only one quinoid ring (Fig. 7). Based on the overall magnitudes of these caspase activations, or lack thereof, the diketone TT8 and the dihydroquinone TT3 (102–230% of the controls) appear to induce caspase-3, -8 and -9 activities less effectively than the monoquinones TT5, TT7 and TT9 (128–358% of the controls). And it is noteworthy that the least cytotoxic TT3 and TT8 (Fig. 1), which have no quinone functionality, are also the only compounds that fail to activate caspase-8, whereas TT5, which is the most cytotoxic of the monoquinones in the HL-60 system (Fig. 1), also induces caspase-3, -8 and -9 activities more effectively than TT7 and TT9 (Fig. 7).

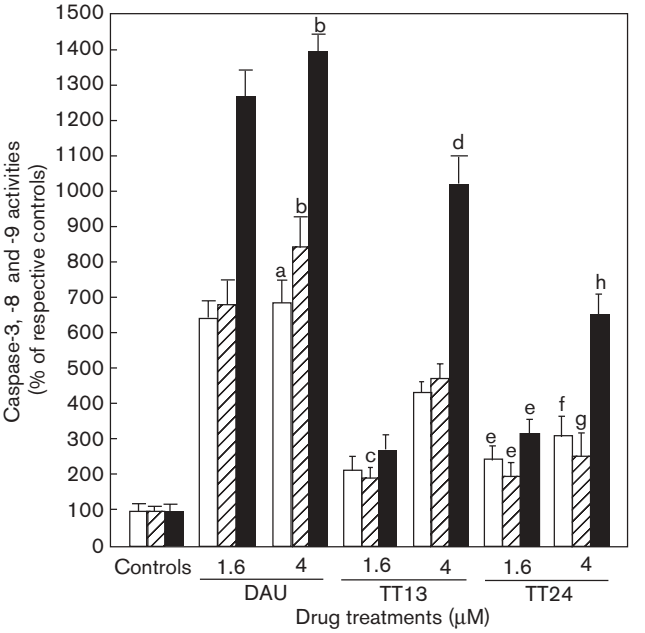
Since antitumor TT bisquinones retain their ability to induce PARP-1 cleavage at 6 h, internucleosomal DNA

Fig. 5



Irreversibility of the abilities of 1.6 and 4 μM concentrations of DAU, TT13 and TT24 to induce caspase-3-like protease activity after 6 h in HL-60-S cells *in vitro*. The drugs were either maintained in the medium for the whole 6-h period of incubation (□) or removed after the first hour (▨) in order to complete the remaining 5 h of incubation in the absence of drugs. Results are, respectively, expressed as percentage of DEVD cleavage activities in vehicle-treated control tumor cells similarly incubated for 6 h (10.8 ± 0.8 nmol AFC released/mg protein, 100 ± 7%, open control) or spun, washed and resuspended in fresh medium after the first 6 h of incubation (12.6 ± 0.8 nmol AFC released/mg protein, 100 ± 6%, striped control). Bars: means ± SD ($n = 3$). [a] $p < 0.005$, smaller than a 1-h treatment with 1.6 μM TT13; [b] not different from the effects of similar concentrations of drugs maintained for 6 h in the incubation medium; [c] not different from a 6-h treatment with 1.6 μM DAU; [d] $p < 0.025$, smaller than a 6-h treatment with 4 μM DAU but $p < 0.01$, greater than a 6-h treatment with 4 μM TT24; [e] not different from a 6-h treatment with 1.6 μM TT13.

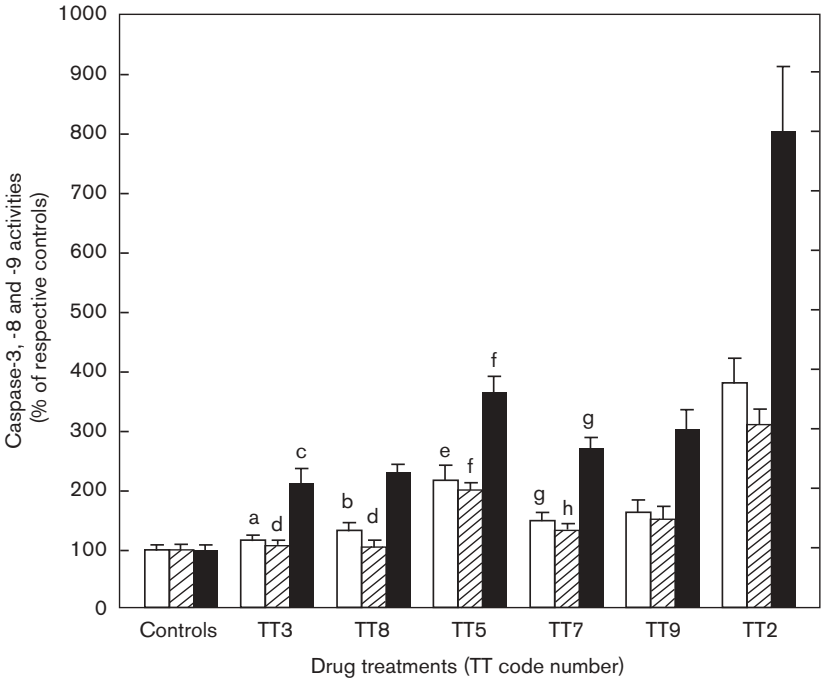
Fig. 6



Comparison of the abilities of 1.6 and 4 μM concentrations of DAU, TT13 and TT24 to induce caspase-3- (□), -8- (▨) and -9-like (■) protease activities after 6 h in HL-60-S cells *in vitro*. Results are respectively expressed as percentage of DEVD (15.2 ± 1.1 nmol AFC released/mg protein, 100 ± 7%, open control), IETD (1.03 ± 0.06 nmol AFC released/mg protein, 100 ± 6%, striped control) or LEHD (2.7 ± 0.3 nmol AFC released/mg protein, 100 ± 11%, closed control) cleavage activities in vehicle-treated control tumor cells at 6 h. Bars: means ± SD ($n = 3$). [a] Not different from 1.6 μM DAU; [b] $p < 0.05$, greater than respective effects of 1.6 μM DAU; [c] $p < 0.005$, greater than control; [d] $p < 0.025$, smaller than 1.6 μM DAU; [e] not different from respective effects of 1.6 μM TT13; [f] not different from 1.6 μM TT24 but $p < 0.01$, smaller than 4 μM TT13; [g] $p < 0.05$, greater than 1.6 μM TT24 but $p < 0.005$, smaller than 4 μM TT13; [h] $p < 0.005$, greater than 1.6 μM TT24, but $p < 0.01$, smaller than 4 μM TT13.

fragmentation at 24 h and cytostatic/cytotoxic effects at 2–4 days in MDR HL-60 sublines, the abilities of DAU, TT13 and TT24 to activate caspase-3, -8 and -9 at 6 h were assessed and compared in the HL-60-RV cells, which, in earlier studies, proved to be the most resistant to the action of DAU [18,38,45]. Interestingly, the ability of DAU to trigger biphasic increases and decreases of caspase-3, -8 and -9 activities in WT HL-60-S cells is totally lost, drastically reduced and/or shifted toward much higher concentrations in the MDR HL-60-RV subline (Fig. 8). For instance, the increasing concentrations of 0.64–4 μM DAU producing submaximal and maximal activations of caspase-3, -8 and -9 (between 380 and 1160% of the controls) in HL-60-S cells become almost totally unable to do so in HL-60-RV cells (between 80 and 160% of the controls). In contrast, the higher 10–25 μM concentrations of DAU, which normally become too toxic to sustain active apoptosis in WT cells, finally begin to raise caspase-3, -8 and -9 activities (between 220 and 570% of the controls) in MDR cells. As a result, a 25 μM concentration of DAU in HL-60-RV cells is barely able to match the magnitudes of caspase-3, -8 and -9 activations caused by 0.64 μM DAU in HL-60-S cells, suggesting that DAU becomes about 39 times less effective at inducing the caspase activation cascade required for apoptosis in this MDR cell system (Fig. 8). In contrast, the concentration-dependent abilities of TT13 and TT24 to induce biphasic increases and decreases of caspase-3, -8 and -9 activities in WT HL-60-S cells remain pretty much unaltered in MDR HL-60-RV cells (Fig. 8). The only exceptions are for the activations of caspase-8 by TT24 and caspase-9 by TT13 and TT24, where, as compared to those in HL-60-S cells, the peaks in HL-60-RV cells may be lower and slightly shifted from 4 to 10 μM, suggesting that, in contrast to DAU, TT13 and TT24 do not lose or lose only 2.5-fold of their effectiveness as activators of caspase-3, -8 and -9 in HL-60-RV cells. The bottom line is that, at 1.6–4 μM, DAU loses most, if not all, of its ability to

Fig. 7



Comparison of the abilities of 4 μM concentrations of antitumor TT analogs with different numbers of quinoid rings to induce caspase-3- (□), -8- (▨) and -9-like (■) protease activities after 6 h in HL-60-S cells *in vitro*. Results are respectively expressed as percentage of DEVD (14.3 ± 1.2 nmol AFC released/mg protein, 100 ± 8%, open control), IETD (1.8 ± 0.2 nmol AFC released/mg protein, 100 ± 9%, striped control) or LEHD (2.6 ± 0.2 nmol AFC released/mg protein, 100 ± 7%, closed control) cleavage activities in vehicle-treated control tumor cells at 6 h. Bars: means ± SD ($n=3$). [a]$p<0.05$, [b]$p<0.025$ and [c]$p<0.005$, greater than respective controls; [d]not different from control; [e]$p<0.025$, greater than TT9, but $p<0.005$, smaller than TT2; [f]$p<0.05$, greater than TT9, but $p<0.005$, smaller than TT2; [g]$p<0.05$, greater than TT8, but not different from TT9; [h]$p<0.05$, greater than TT3, but not different from TT9.

activate initiator and effector caspases in MDR tumor cells, whereas lead antitumor TT bisquinones retain most, if not all, of their ability to do so (Fig. 8).

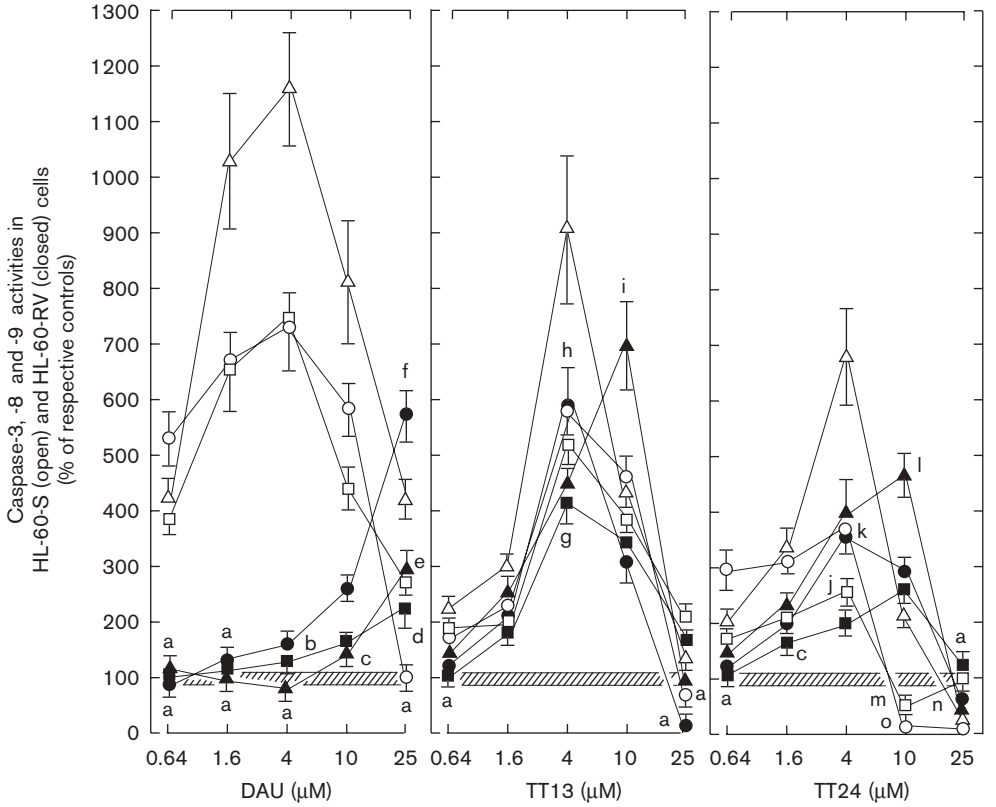
Induction of Cyt *c* release

Because the release of Cyt *c* from the mitochondrial intermembrane space (IMS) into the cytosol may be a limiting factor in caspase-9 activation and represents a central coordinating step in apoptosis [44,46], DAU and TT13, the TT bisquinone shown to activate the most caspase-9 and -3 (Figs 4–6 and 8), were tested for their ability to trigger Cyt *c* release in HL-60-S cells. The abilities of 4 μM DAU and TT13 to induce Cyt *c* release in HL-60-S cells are similarly time dependent (Fig. 9, top). As compared to untreated controls, the 15-kDa bands of cytosolic Cyt *c* become clearly visible at 3 h and fully developed at 6 h, a time which was then selected to determine the concentration dependency of these effects in Figure 9 (middle). Under this condition, 0.64–1.6 μM DAU and 1.6 μM TT13 become increasingly effective at triggering the release of Cyt *c*, which is maximally stimulated in response to 4-10 μM concentrations of these compounds (Fig. 9, middle). When compared at 4 μM, all lead antitumor TT bisquinones produce significant 15-kDa bands of Cyt *c* extracted from the cytosol of HL-60-S cells at 6 h (Fig. 9, bottom). When relative density values are compared, the magnitudes of their Cyt *c* responses appear to match rather well their caspase-inducing activities. For instance, TT13 is slightly more effective than TT24 on both Cyt *c* release (Fig. 9, bottom) and caspase-9 activation (Figs 5, 6 and 8). Moreover, TT13 and TT21 are consistently the most and least potent, respectively, among the other lead antitumor TT bisquinones, TT2, TT16, TT19, TT24 and TT26 sharing more or less similar apoptosis-inducing activities on both Cyt *c* release (Fig. 9, bottom) and caspase-3 activation (Fig. 4).

Roles of initiator caspase-2 and -8 in DAU- and TT13-induced apoptosis

Since caspase-2 and -8 activities might act upstream of mitochondria to promote Cyt *c* release and the activation of the post-mitochondrial initiator caspase-9, it is of interest to show that treatments with 4 μM DAU and TT13 can induce all three initiator caspase-2, -8 and -9 activities tested in a similar time-dependent manner in HL-60-S cells (Fig. 10). However, caspase-2 activity already peaks at 6 h and remains maximally stimulated up

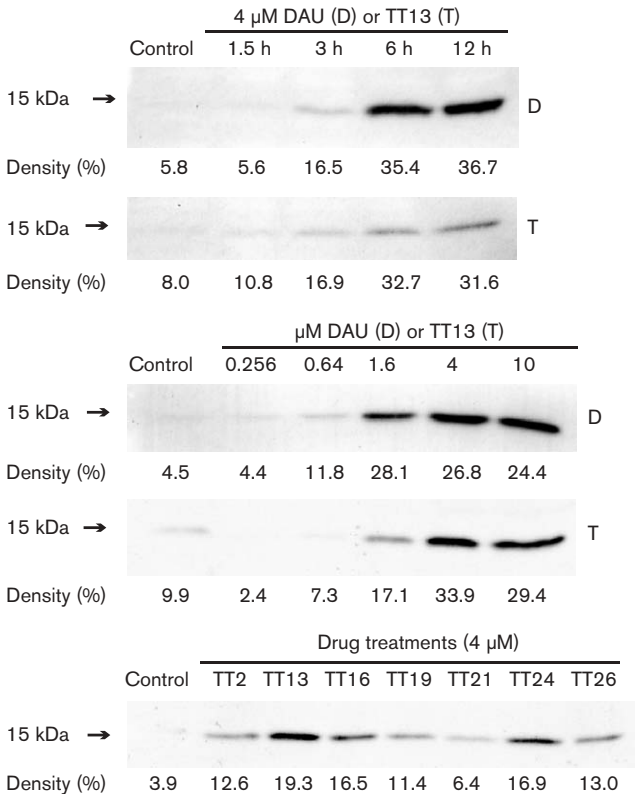
Fig. 8



Comparison of the concentration-dependent inductions of caspase-3- (circles), -8- (squares) and -9-like (triangles) protease activities by DAU, TT13 and TT24 at 6 h in HL-60-S (open symbols) and HL-60-RV (solid symbols) cells *in vitro*. Results are, respectively, expressed as percentage of DEVD (13.45 ± 1.04 nmol AFC released/mg protein), IETD (1.42 ± 0.11 nmol AFC released/mg protein) or LEHD (3.17 ± 0.26 nmol AFC released/mg protein) cleavage activities in vehicle-treated control HL-60-S cells and percentage of DEVD (3.13 ± 0.26 nmol AFC released/mg protein), IETD (0.15 ± 0.01 nmol AFC released/mg protein) or LEHD (1.38 ± 0.11 nmol AFC released/mg protein) cleavage activities in vehicle-treated control HL-60-RV cells at 6 h (100 ± 8%, striped areas). Bars: means ± SD ($n=3$). [a]Not different from respective controls; [b]$p<0.025$ and [c]$p<0.005$, greater than respective controls; [d]not different from the effect of 25 μM DAU in HL-60-S cells, but $p<0.005$, smaller than the effect of 0.64 μM DAU in HL-60-S cells; [e]$p<0.01$, smaller than the effects of 0.64 and 25 μM DAU in HL-60-S cells; [f]not different from the effects of 0.64, 1.6 and 10 μM DAU in HL-60-S cells, but $p<0.05$, smaller than the effect of 4 μM DAU in HL-60-S cells; [g]$p<0.025$, smaller than the effect of 4 μM TT13 in HL-60-S cells; [h]not different from the effect of 4 μM TT13 in HL-60-S cells; [i]not different from the effect of 4 μM TT13 in HL-60-S cells, but $p<0.01$, greater than the effect of 10 μM TT13 in HL-60-S cells; [j]$p<0.025$, greater than the effect of 4 μM TT24 in HL-60-RV cells, but not different from the effect of 10 μM TT24 in HL-60-RV cells; [k]not different from the effect of 4 μM TT24 in HL-60-S cells; [l]$p<0.025$, smaller than the effect of 4 μM TT24 in HL-60-S cells, but $p<0.0005$, greater than the effect of 10 μM TT24 in HL-60-S cells; [m]$p<0.05$, [n]$p<0.01$ and [o]$p<0.0005$, smaller than respective controls.

to 12 h, whereas caspase-8 and -9 activities, which are only partially stimulated at 6 h, need 9 h to be fully induced by 4 μM DAU and TT13 before substantially declining thereafter (Fig. 10). Such different rates of initiator caspase activations might suggest that DAU and antitumor TT bisquinones sequentially induce caspase-2 before caspase-9 and -8 (Fig. 10). Indeed, pre-incubations for 1 h with the specific caspase-2 inhibitor z-VDVAD-fmk (15 μM) totally abolish the abilities of 4 μM DAU and TT13 to maximally induce caspase-2 activity at 6 h and caspase-8 and -9 activities at 8 h in HL-60-S cells (Fig. 11, bottom). In contrast, pre-incubations for 1 h with the caspase-8 inhibitor z-IETD-fmk (15 μM), which totally block DAU- and TT13-induced caspase-8 activities at 8 h, do not prevent 4 μM DAU and TT13 from inducing nearly maximal caspase-2 and -9 activities at 6 and 8 h, respectively (Fig. 11, bottom). However, similar pre-treatments with these caspase-2 and -8 inhibitors tested at 15, 25, 50 (data not shown) and even 100 μM (Fig. 11, top) are all unable to prevent 4 μM DAU and TT13 from triggering maximal or near maximal releases of Cyt *c* from mitochondria in HL-60-S cells at 6 h, indicating that caspase-2 and -8 activations are not absolutely required for Cyt *c* release during DAU and TT bisquinone treatments. Taken together, the results of Figure 11 suggest that initial activation of apical caspase-2 may be critical to mediate the apoptotic pathways by which cytotoxic DAU and TT bisquinones activate the post-mitochondrial caspase-9 and the mitochondrial amplification loop involving caspase-8. However, micromolar concentrations of DAU and synthetic TT bisquinones might also target or disrupt mitochondria to

Fig. 9



Induction of Cyt *c* release by DAU and lead antitumor TT bisquinones in HL-60-S cells *in vitro*. Tumor cells were incubated for the indicated periods of time in the presence or absence (control) of various concentrations of drugs and bands (arrows) of cytosolic Cyt *c* ($M_r \sim 15\,000$) released from the mitochondrial IMS were detected by Western blot analysis. Top: comparison of the time-dependent inductions of Cyt *c* release by 4 μM DAU and TT13. Middle: comparison of the concentration-dependent inductions of Cyt *c* release by 0.256–10 μM DAU and TT13 after 6 h. Bottom: comparison of the abilities of 4 μM concentrations of lead antitumor TT bisquinones to induce Cyt *c* release after 6 h. Relative band densities in equal areas are expressed as the percentage that each box contributes to the total density measured after automatic background correction.

trigger the release of Cyt *c* by other direct or indirect mechanisms which appear to be independent from the pre-mitochondrial action of apical caspases.

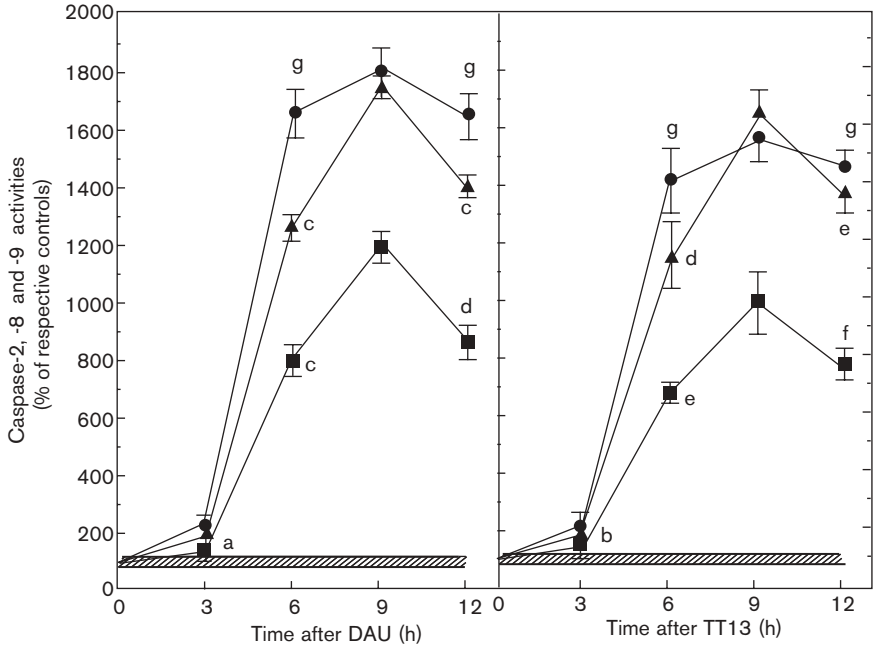
Role of Fas–FasL signaling in DAU- and TT13-induced apoptosis

Since DAU and TT13 induce caspase-8 activity, antagonistic anti-Fas and anti-FasL mAbs have been used to determine whether Fas–FasL signaling is involved in the mechanisms by which these quinone antitumor drugs trigger apoptosis in HL-60-S cells (Fig. 12). The antagonistic anti-FasL NOK-1 mAb is known to neutralize the CD95 ligand, whereas, in earlier studies, the antagonistic anti-Fas DX2 and ZB4 mAbs, which block the CD95 receptor, have been shown to abolish or diminish the abilities of the agonistic anti-Fas CH11 mAb to induce Cyt *c* release and caspase-2, -8 and -9 activities in HL-60-S cells [41]. The same 1-h pre-treatments with blocking or neutralizing anti-Fas and anti-FasL clones (10 μg/ml), however, are totally unable to alter the abilities of 4 μM DAU and TT13 to maximally induce Cyt *c* release (Fig. 12, top) and caspase-2 activity (Fig. 12, bottom) at 6 h and caspase-8 and -9 activities at 8 h (Fig. 12, bottom) in HL-60-S cells, suggesting that the Fas–FasL pathway is neither involved in the mechanisms of DAU- and TT13-induced apoptosis nor even required for the induction of caspase-8 activity by these compounds.

Discussion

Discarding the weaker TT21, six lead antitumor TT bisquinones, including TT2, TT13, TT16, TT19, TT24 and TT26, have been shown to inhibit the proliferation and viability of a wide spectrum of tumor cell lines *in vitro*. Although their potency in the nanomolar range is not as good as that of DAU, they mimic the ability of this proven anticancer drug to trigger Cyt *c* release, caspase-2, -3, -8 and -9 activations, PARP-1 cleavage and internucleosomal DNA degradation [17–20,38]. Since the parent TT0 structure is unable to induce such apoptogenic effects and is not cytostatic/cytotoxic in the micromolar range, the ability of synthetic TT analogs to interact with cellular targets, such as nucleoside transporters and Topo I and II enzymes, and trigger apoptosis may play a significant role in their molecular mechanism of action [17,18,20,21,38]. However, it is unclear whether apoptosis is the cause or merely the consequence of their antitumor activity. Moreover, structure–activity relationships (SARs) are difficult to establish and the quinone functionality of various TT analogs, although important, may not be consistently correlated to their antitumor activities. Because the quinone functionality of certain anticancer drugs may be linked to their ability to alkylate DNA (arylating activity) and produce toxic ROS and free radicals (redox cycling activity) [47,48], it is tempting to speculate that such events may play a role in TT bisquinone-induced DNA damage, apoptosis and cytotoxicity. Indeed, selected antitumor TT analogs with two, one or none quinoid rings generally produce decreasing levels of Topo I and II inhibitions, caspase-3, -8 and -9 activations, PARP-1 cleavage, and internucleosomal DNA fragmentation, but this is not a general rule and there are too many exceptions [17,20,21,38]. Probably because of their different substitutions, not all TT bisquinones qualify as lead antitumor compounds and the overall antitumor effects of various TT structures are not consistently correlated to their quinone functionalities, suggesting that other mechanisms besides nucleoside transporters and Topo and caspase enzymes might be involved in their cytostatic/cytotoxic actions [17, 20,21,38]. For instance, other TT bisquinones not selected among the current six lead antitumor compounds are less effective than the monoquinones TT5,

Fig. 10



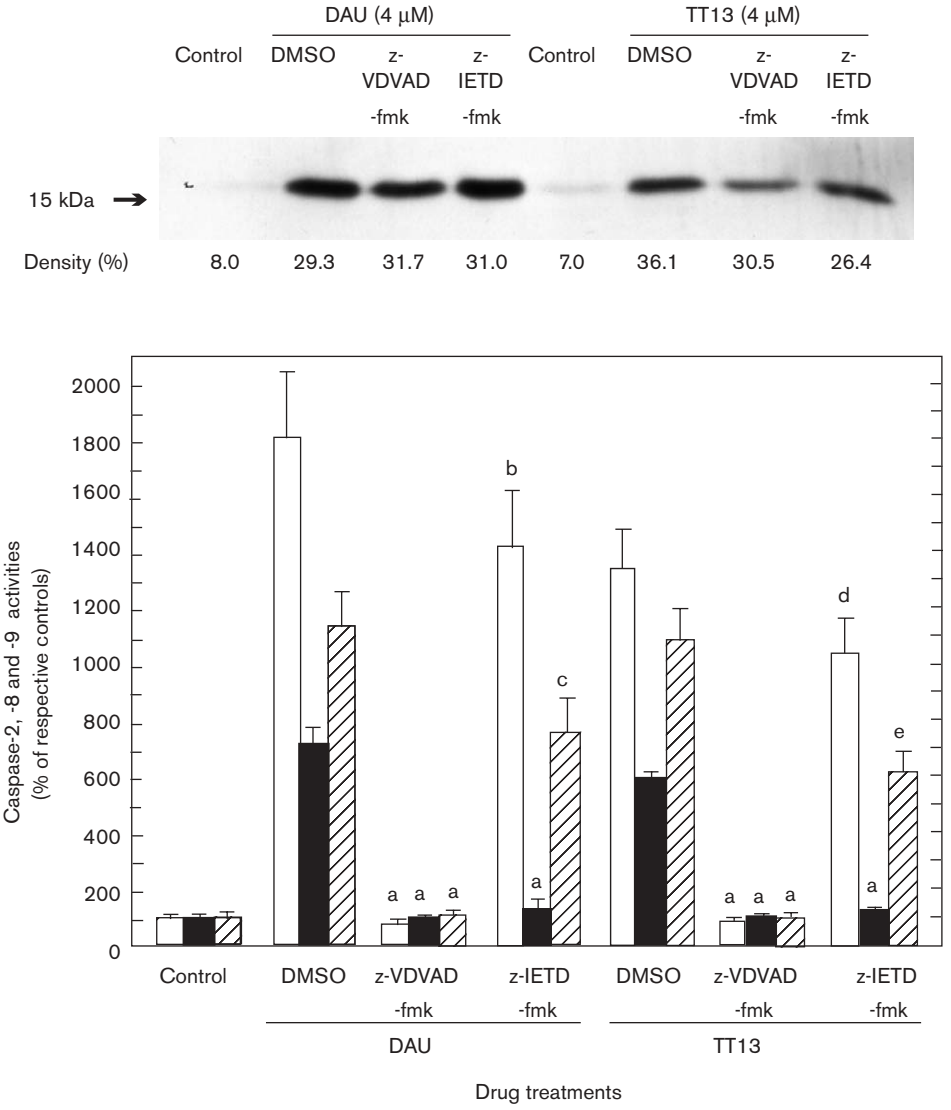
Comparison of the time-dependent inductions of caspase-2- (●), -8- (■) and -9-like (△) protease activities by 4 μM DAU and TT13 in HL-60-S cells *in vitro*. Results are respectively expressed as percentage of VDVAD (2.87 ± 0.27 nmol AFC released/mg protein), IETD (1.65 ± 0.15 nmol AFC released/mg protein), or LEHD (3.57 ± 0.33 nmol AFC released/mg protein) cleavage activities in vehicle-treated control tumor cells (100 ± 9%, striped areas) at each time point tested. Bars: means ± SD ($n=3$). [a]$p<0.01$, greater than control but smaller than caspase-9 and -2 activities; [b]$p<0.005$, greater than control but $p<0.025$, smaller than caspase-9 and -2 activities; [c]$p<0.0005$, [d]$p<0.005$, [e]$p<0.025$ and [f]$p<0.05$, smaller than respective caspase activities at 9 h; [g]not different from respective caspase-2 activities at 9 h.

TT7 and TT9 [17,20] and TT3, which has no quinone functionality, is sometimes equipotent to its structural monoquinone equivalent TT5 [17,20,21,38].

Since the abilities of antitumor TT bisquinones to interact with quinone-metabolizing enzymes, bind to nucleoside transporters, interact covalently with DNA, cause high-MW DNA DSBs, SSBs and DPCs, activate PARP-1 and deplete NAD^+ within 15 min, interact with the quinone-binding site regulating the Ca^{2+} sensitivity of the permeability transition pore (PTP) and induce MPT, block specific phases of cell cycle progression and affect the production of ROS remain to be determined, it is rather premature to elaborate SARs, discuss potential primary molecular targets, and speculate on the nature of the initial and massive damaging events that, within 1 h, induce TT analog-treated tumor cells to commit apoptosis. TT analogs might trigger the different but sequentially related DNA-damaging events suggested to occur during VP-16-induced apoptosis. Rapid, massive and unrepairable DNA damage secondary to Topo inhibition may be the trigger initiating nuclear signals that induce VP-16-treated tumor cells to release their mitochondrial Cyt *c* and undergo apoptosis, thereby activating their post-mitochondrial caspase cascade responsible for PARP-1 cleavage, endonuclease activation and, ultimately, the internucleosomal fragmentation of their DNA, which is one of the late manifestations of the apoptotic process [31,49]. The basis for this hypothesis is that over-expression of anti-apoptotic Bcl-2 or Bcl-x_L, which stabilize mitochondrial pores, abrogate Cyt *c* release and block the post-mitochondrial caspase cascade, inhibit the later apoptotic events culminating in internucleosomal DNA fragmentation without affecting the early formation and repair of DNA DSBs, SSBs and DPCs caused by VP-16 [49].

In the two major pathways of apoptosis, initiator caspase-8 is activated by DR signaling, whereas initiator caspase-9 is activated by cytotoxic drugs and genotoxic events [40,50]. Cytokines trigger the extrinsic pathway in which Fas-mediated signals induce apical caspase-8 activation. However, the involvement of the Fas–FasL signaling pathway in drug-induced apoptosis is controversial [10,12,51–54]. Upon Fas cross-linking by the natural FasL or agonistic anti-Fas CH11 mAb, the receptor multimerization sequentially recruits the adaptor molecule Fas-associated death domain and procaspase-8, which bind to the receptor to form the death-inducing signaling complex where proteolytic activation of caspase-8 takes place. A large amount of activated caspase-8 may directly activate effector caspase-3 and

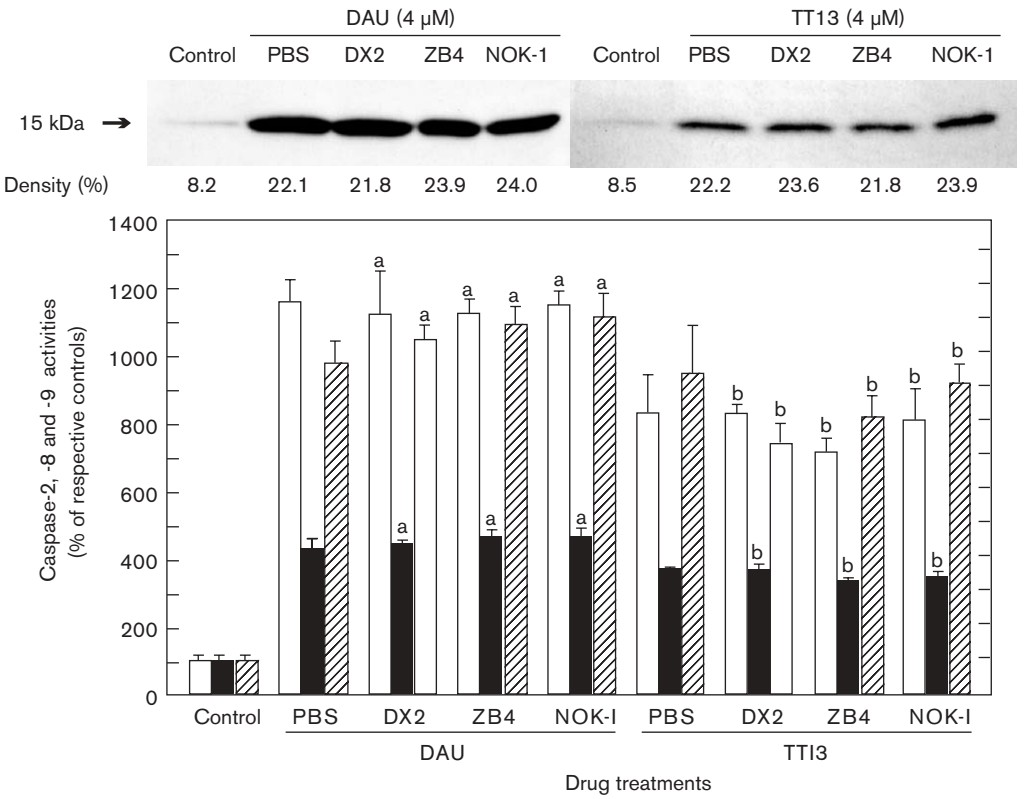
Fig. 11



Comparison of the abilities of caspase-2 and -8 inhibitors to prevent DAU and TT13 from inducing Cyt *c* release (top) and initiator caspase activities (bottom) in HL-60-S cells *in vitro*. Tumor cells were pre-incubated for 1 h in the presence of vehicle, 15 μM (bottom) or 100 μM (top) concentrations of z-VDVAD-fmk or z-IETD-fmk and, after supplementing their culture medium with either vehicle (control) or 4 μM concentrations of DAU or TT13, these incubations were continued for an additional 6 h to determine Cyt *c* release and caspase-2-like activity or for an additional 8 h to determine caspase-8- and -9-like activities. Top: bands (arrow) of cytosolic Cyt *c* ($M_r \sim 15\,000$) released from the mitochondrial IMS were detected by Western blot analysis. Relative band densities in equal areas are expressed as the percentage that each box contributes to the total density measured after automatic background correction. Bottom: caspase-2- (□), -8- (■) and -9-like (▨) activities are respectively expressed as percentage of VDVAD (1.79 ± 0.16 nmol AFC released/mg protein, 100 ± 9%), IETD (1.13 ± 0.08 nmol AFC released/mg protein, 100 ± 7%) or LEHD (2.12 ± 0.16 nmol AFC released/mg protein, 100 ± 7%) cleavage activities in vehicle-treated control tumor cells. Bars: means ± SD ($n = 3$). [a]Not different from respective controls; [b]not different from DAU-induced caspase-2 activity; [c]$p < 0.025$, smaller than DAU-induced caspase-9 activity; [d]$p < 0.05$, smaller than TT13-induced caspase-2 activity; [e]$p < 0.005$, smaller than TT13-induced caspase-9 activity.

the subsequent mitochondrial amplification loop involving the caspase-8-mediated cleavage of Bid [46]. Truncated Bid (tBid) facilitates the insertion, activation and oligomerization of Bax and Bak into the mitochondrial outer membrane where these pro-apoptotic Bcl-2 family members trigger Cyt *c* release, which is the key limiting factor in initiating the post-mitochondrial apoptotic protease cascade [46,55,56]. But a small amount of activated caspase-8 is sufficient to trigger Bid cleavage and Cyt *c* release and might be more efficient to amplify a signal otherwise too weak to directly induce caspase-3 activity and apoptosis [55–57]. DNA-damaging anticancer drugs trigger the mitochondrial-dependent intrinsic pathway in which nuclear-mediated signals likely to involve various p53-responsive genes directly induce the Bax- and Bak-mediated release

Fig. 12



Comparison of the abilities of antagonistic Fas and FasL mAbs to prevent DAU and TT13 from inducing Cyt *c* release (top) and initiator caspase activities (bottom) in HL-60-S cells *in vitro*. Tumor cells were pre-incubated for 1 h in the presence of PBS, antagonistic anti-Fas DX2 and ZB4 mAbs (10 μg/ml) or antagonistic anti-FasL NOK-1 mAb (10 μg/ml) and, after supplementing their culture medium with either vehicle (control) or 4 μM concentrations of DAU or TT13, these incubations were continued for an additional 6 h to determine Cyt *c* release and caspase-2-like activity or for an additional 8 h to determine caspase-8- and -9-like activities. Top: bands (arrow) of cytosolic Cyt *c* ($M_r \sim 15\,000$) released from the mitochondrial IMS were detected by Western blot analysis. Relative band densities in equal areas are expressed as the percentage that each box contributes to the total density measured after automatic background correction. Bottom: caspase-2- (□), -8- (■) and -9-like (▨) activities are respectively expressed as percentage of VDVAD (2.34 ± 0.13 nmol AFC released/mg protein, 100 ± 6%), IETD (1.69 ± 0.08 nmol AFC released/mg protein, 100 ± 5%) or LEHD (2.10 ± 0.11 nmol AFC released/mg protein, 100 ± 6%) cleavage activities in vehicle-treated control tumor cells. Bars: means ± SD ($n = 3$). [a]Not different from respective DAU-induced caspase-2, -8 or -9 activities; [b]not different from respective TT13-induced caspase-2, -8 or -9 activities.

of Cyt *c* [46]. After its release from the mitochondrial IMS into the cytosol, Cyt *c* binds to apoptotic protease-activating factor-1 (Apaf-1), which through its caspase recruitment domain interacts with procaspase-9, resulting in the formation of the apoptosome complex that activates this initiator caspase in the presence of dATP [44,46]. In any case, all apoptotic pathways eventually converge on mitochondria and an activation of caspase-8 that does not require the DR, but is mitochondrial-dependent, may also play a role in drug-induced apoptosis. Hence, caspase-8 is involved in Fas-mediated apoptosis but may also be activated by drugs independently of Fas signaling and downstream of mitochondrial Cyt *c* release to mediate a secondary amplification loop [50]. A drug-inducible apoptotic pathway in which activation of caspase-8, and not caspase-9, forms the apical and mitochondrial-dependent step that subsequently activates the downstream caspases has also been suggested [50]. Among the apoptogenic factors released from mitochondrial IMS into the cytosol that can induce caspase-3 activity, Cyt *c* does not activate caspase-8 but apoptosis-inducing factor (AIF) does, suggesting that cleavage of procaspase-8 downstream of mitochondria requires AIF activity [58].

Even though nuclear and mitochondrial targets have not been studied yet, the present data suggest that antitumor TT bisquinones may trigger apoptosis in HL-60 cells by an intrinsic signaling pathway that does not involve the Fas–FasL system, but is partially caspase-2-dependent. The cellular machinery required for the Fas signaling pathway is present, functional and responsive in HL-60-S cells since the ability of the agonistic anti-Fas CH11 mAb to induce Cyt *c* release and caspase-2, -8 and -9 activities can be prevented in the presence of antagonistic anti-Fas mAbs in this tumor cell system [41]. However, neither the antagonistic anti-Fas DX2 and ZB4 mAbs nor the antagonistic anti-FasL NOK-1 mAb are able to prevent

DAU and TT13 from fully triggering the release of mitochondrial Cyt *c* and the activation of the initiator caspases. Interestingly, the very same neutralizing and blocking mAbs that antagonize Fas-induced apoptosis also fail to inhibit DOX-, DAU- and 1,4-anthraquinone (AQ) analog-induced apoptosis in other studies and/or tumor cell lines, suggesting that the signaling pathways by which these quinone antitumor drugs induce apoptosis are similarly Fas–FasL-independent [10,12,51,59]. Moreover, our studies indicate that both the Fas-mediated effects of CH11 and the Fas-independent effects of DAU, TT bisquinones and AQ analogs on Cyt *c* release and initiator caspase activations in HL-60 cells are definitively p53-independent since p53 is not expressed in an active form in these p53-null HL-60 tumor cells [41,60,61]. Incidentally, bleomycin induces Cyt *c* release and caspase-3 and -8 activities in p53-null HL-60 cells [62], and cisplatin induces Cyt *c* release and caspase-3, -8 and -9 activities in human hepatoma Hep3B cells, which are Fas- and p53-negative [63], suggesting that other anticancer drugs may also trigger apoptosis by a mitochondrial pathway that involves initiator caspase-8.

Caspase-2, which may be the first apical protease activated by DAU, VP-16, AQ analogs and TT bisquinones and required for DNA damage-induced apoptosis, may promote the translocation of pro-apoptotic Bax or tBid to mitochondria that results in the release of Cyt *c*, Smac and AIF [41,64–66]. Based on their rates of activation, caspase-2 is maximally induced at 6 h, whereas caspase-9 and -8 activities peak at 9 h, which may or may not indicate that caspase-2 comes first since submaximal activities of caspase-9 and -8 and maximal activity of caspase-3 are also detected at 6 h. A more convincing argument to suggest that caspase-2 may be activated upstream of the other initiator and effector caspases and control their subsequent activation is that neither caspase-9, -8 nor -3 activations can occur in DAU-, TT13- or AQ analog-treated HL-60 cells when caspase-2 is inhibited by z-VDVAD-fmk [41]. Moreover, the activation of caspase-8 by DAU, TT13 or AQ analog without Fas signaling is likely to occur in the post-mitochondrial sequence since it is blocked in the absence of caspase-9 and -3 activations following caspase-2 inhibition [41]. Because caspase-8 inhibition by z-IETD-fmk does not prevent DAU, TT13 and AQ analog from activating caspase-2, -9 and -3, the mechanism of apoptosis induction by these drugs can proceed in the absence of caspase-8 activation, which may only play a secondary role as part of the mitochondrial amplification loop stimulated by the post-mitochondrial caspase cascade [41].

Our DAU, AQ analog and TT bisquinone studies support the concept that, in drug-induced apoptosis, Cyt *c* release is caspase-independent [41]. But caspase-9 is not the first apical caspase activated [67] since caspase-2 inhibition does prevent caspase-9 activation [41,64–66]. Nuclear caspase-2 may be a primary target of pro-apoptotic nuclear signals and play a role upstream of Cyt *c* release and caspase-9 activation in VP-16 and cisplatin-treated tumor cells [64–66]. VP-16 may induce nuclear damage that signals the caspase-2-mediated release of Cyt *c* at 10 μM, but directly disturb mitochondria to trigger PTP opening and Cyt *c* release at 50 μM [65,66]. The ability of TT bisquinones to directly target mitochondria, induce MPT events and alter the ratio of Bcl-2 family members controlling mitochondrial channels for the release of pro-apoptotic molecules from the IMS is under investigation. Because TT bisquinones fully induce Cyt *c* release, caspase activations, PARP-1 cleavage and internucleosomal DNA fragmentation in HL-60 cells at 4 μM, these drugs may be more potent inducers of apoptosis than VP-16. The fact that z-VDVAD-fmk totally blocks DAU-, AQ analog- and TT13-induced caspase-2, -8 and -9 activations without preventing these drugs from releasing Cyt *c* suggests that, whether or not caspase-2 acts upstream of mitochondria to induce some Cyt *c* release before activating the post-mitochondrial cascade of caspase-9, -3 and -8, the activation of this apical caspase-2 is not absolutely required for DAU-, AQ analog- and TT bisquinone-induced Cyt *c* releases, which can proceed in its absence [41]. The non-essential role of apical caspases in mitochondrial Cyt *c* release is substantiated by the inability of the caspase-8 inhibitor z-IETD-fmk to alter Cyt *c* release by DAU, AQ analog and TT13 [41]. The conclusion is that caspase-2 may play an important role in the mechanisms of DAU-, AQ analog- and TT bisquinone-induced apoptosis but the critical release of mitochondrial Cyt *c* can occur independently from apical caspase activation.

Although they are somewhat less potent than DAU, the interest of synthetic TT bisquinones lies with the fact that, in contrast to DAU, they have the advantage of blocking nucleoside transporters, targeting both Topo I and II enzymes, and retaining their efficacy in HL-60 sublines that have developed different mechanisms of MDR [17,18,20,21,38]. Here, TT13 and TT24 induce caspase-9, -3 and -8 activities almost as effectively in MDR HL-60-RV as in WT HL-60-S cells, a finding which is consistent with the reports that TT bisquinones retain their ability to induce PARP-1 cleavage and internucleosomal DNA fragmentation in these DAU-resistant tumor cells [18,38]. Blocking purine and pyrimidine nucleoside transport and inhibiting both Topo I and II activities might be some of the factors contributing to this circumvention of MDR by TT bisquinones [18,21,38]. Moreover, apoptosis deficiency is strongly associated with the MDR phenotype. DOX induces Apaf-1 expression in WT p53 but not in p53 mutant cell lines. A lack of caspase-3-mediated PARP-1 cleavage is observed in p53 mutant resistant to apoptosis and MDR HL-60 cells may lose surface Fas expression [51]. However, TT

bisquinones can induce PARP-1 cleavage in p53-null WT HL-60-S cells and MDR HL-60-RV cells [38] and, because the Fas pathway is not involved in TT bisquinone-mediated apoptosis in HL-60 cells, p53 deletion and a deficiency in the activation of the Fas system suggested to play a role in MDR appear to be irrelevant to the apoptotic action of our antitumor TT bisquinones. Recently, anticancer drugs including DOX have been shown to down-regulate the mRNA levels of various inhibitor of apoptosis (IAP) proteins in WT but not in MDR HL-60 cells, suggesting that IAPs, which bind to and inhibit the activities of caspase-9, -3, -6 and -7, may be involved in MDR [51]. Whether synthetic TT bisquinones can decrease the levels of anti-apoptotic IAPs to activate caspases as effectively in MDR as in WT HL-60 cells remains to be seen.

Conclusion

Without Fas signaling, TT bisquinones trigger a caspase-independent release of Cyt *c* and a caspase-2-mediated activation of caspase-9 and -8, and retain their ability to induce apoptosis in DAU-resistant HL-60 cells, suggesting that different molecular targets may be involved in the actions of these quinone antitumor drugs.

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References

1 Myers CE, Chabner BA. Anthracyclines. In: Chabner BA, Collins JM (editors): *Cancer Chemotherapy: Principles and Practice*. Philadelphia, PA: Lippincott; 1990, pp. 356–381.
2 Rossi L, Moore GA, Orrenius S, O'Brien PJ. Quinone toxicity in hepatocytes without oxidative stress. *Arch Biochem Biophys* 1986; **251**:25–35.
3 Qiu XB, Schönthal AH, Cadenas E. Anticancer quinone induce pRb-preventable G_2/M cell cycle arrest and apoptosis. *Free Rad Biol Med* 1998; **24**:848–854.
4 Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989; **58**:351–375.
5 Mimnaugh EG, Kennedy KA, Trush MA, Sinha BK. Adriamycin-enhanced membrane lipid peroxidation in isolated rat nuclei. *Cancer Res* 1985; **45**:3296–3304.
6 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**:1657–1662.
7 Ling YH, Priebe W, Perez-Soler R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. *Cancer Res* 1993; **53**:1845–1852.
8 Ramachandran C, You W, Krishan A. *Bcl-2* and *mdr-1* gene expression during doxorubicin-induced apoptosis in murine P388 and p388/R84 cells. *Anticancer Res* 1997; **17**:3369–3376.
9 Skladanowski A, Konopa J. Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumour cells. *Biochem Pharmacol* 1993; **46**: 375–382.
10 Gamen S, Anel A, Lasierra P, Alava MA, Martinez-Lorenzo MJ, Piñeiro A, *et al.* Doxorubicin-induced apoptosis in human T-cell leukaemia is mediated by caspase-3 activation in a Fas-independent way. *FEBS Lett* 1997; **417**:360–364.
11 Côme MG, Skladanowski A, Larsen AK, Laurent G. Dual mechanism of daunorubicin-induced cell death in sensitive and MDR-resistant HL-60 cells. *Br J Cancer* 1999; **79**:1090–1097.
12 Ferraro C, Quemeneur L, Prigent AF, Taverne C, Pevillard JP, Bonnefoy-Berard N. Anthracyclines trigger apoptosis of both G_0–G_1 and cycling peripheral blood lymphocytes and induce massive deletion of mature T and B cells. *Cancer Res* 2000; **60**:1901–1907.
13 Aszalos A, Ladányi A, Bocsi J, Szende B. Induction of apoptosis in MDR1 expressing cells by daunorubicin with combinations of suboptimal concentrations of P-glycoprotein modulators. *Cancer Lett* 2001; **167**: 157–162.
14 Messner PW, Budihardjo II, Kaufman SH. Chemotherapy-induced apoptosis. *Adv Parmacol* 1997; **41**:461–499.
15 Debatin KM. Activation of apoptosis pathways by anticancer treatment. *Toxicol Lett* 2000; **112–113**:41–48.
16 Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 2000; **256**:42–49.
17 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*. *Anticancer Drugs* 1999; **10**:749–766.
18 Wang B, Wu M, Perchellet EM, McIlvain CJ, Sperfslage BJ, Huang X, *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**:1169–1178.
19 Hua DH, Tamura M, Huang X, Stephany HA, Helfrich BA, Perchellet EM, *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**:2907–2912.
20 Perchellet EM, Sperfslage BJ, Wang Y, Huang X, Tamura M, Hua DH, *et al.* 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*. *Anticancer Drugs* 2002; **13**:567–582.
21 Wang B, Perchellet EM, Wang Y, Tamura M, Hua DH, Perchellet JP. Antitumor triptycene bisquinones: a novel synthetic class of dual inhibitors of DNA topoisomerase I and II activities. *Anticancer Drugs* 2003; **14**: 503–514.
22 Froelich-Ammon SJ, Osheroff N. Topoisomerase poisons: harnessing the dark side of enzyme mechanism. *J Biol Chem* 1995; **270**:21429–21432.
23 Pommier Y. DNA topoisomerases and their inhibition by anthracyclines. In: Priebe W (editor): *Anthracycline Antibiotics. New Analogues, Methods of Delivery, and Mechanisms of Action*. Washington, DC: American Chemical Society. *ACS Symp Ser* 1995; **574**:183–203.
24 Kohn KW. Beyond DNA cross-linking: history and prospect of DNA-targeted cancer treatment. Fifteenth Bruce F. Cain Memorial Award lecture. *Cancer Res* 1996; **56**:5533–5546.
25 Li T-K, Liu LF. Tumor cell death induced by topoisomerase-targeting drugs. *Annu Rev Pharmacol Toxicol* 2001; **41**:53–77.
26 Pommier Y, Pourquier P, Fan Y, Strumberg D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta* 1998; **1400**:83–105.
27 Palumbo M, Gatto B, Moro S, Sissi C, Zagotto G. Sequence-specific interactions of drugs interfering with the topoisomerase–DNA cleavage complex. *Biochim Biophys Acta* 2002; **1587**:145–154.
28 Topcu Z. DNA topoisomerases as targets for anticancer drugs. *J Clin Pharm Ther* 2001; **26**:405–416.
29 Walker JV, Nitiss JL. DNA topoisomerase II as a target for cancer chemotherapy. *Cancer Invest* 2002; **20**:570–589.
30 Nagata S. Apoptotic DNA fragmentation. *Exp Cell Res* 2000; **256**:12–18.
31 Compton MM. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev* 1992; **11**:105–112.
32 Duriez PJ, Shah GM. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem Cell Biol* 1997; **75**:337–349.
33 Herceg Z, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res* 2001; **477**: 97–110.
34 Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* 1993; **53**: 3976–3985.

35 Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, *et al.* Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 1995; **81**:801–809.

36 Lazebnik YA, Kaufman SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994; **371**:346–347.

37 Kaufmann SH. Proteolytic cleavage during chemotherapy-induced apoptosis. *Mol Med Today* 1996; **2**:298–303.

38 Wang Y, Perchellet EM, Tamura M, Hua DH, Perchellet JP. Induction of poly(ADP-ribose) polymerase-1 cleavage by antitumor triptycene bisquinones in wild-type and daunorubicin-resistant HL-60 cell lines. *Cancer Lett* 2002; **188**:73–83.

39 Hickman JA, Dive C. *Apoptosis and Cancer Chemotherapy*. Totowa, NJ: Humana Press; 1999.

40 Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999; **15**:269–290.

41 Perchellet EM, Wang Y, Weber RL, Sperfslage BJ, Lou K, Crossland J, *et al.* Synthetic 1,4-anthracenedione analogs induce cytochrome *c* release, caspase-9, -3, and -8 activities, poly(ADP-ribose) polymerase-1 cleavage and internucleosomal DNA fragmentation in HL-60 cells by a mechanism which involves caspase-2 activation but not Fas signaling. *Biochem Pharmacol* 2004; **67**:523–537.

42 Marquardt D, McCrone S, Center MS. Mechanisms of multidrug resistance in HL-60 cells: detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. *Cancer Res* 1990; **50**:1426–1430.

43 Cory AH, Owen JC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991; **3**:207–212.

44 Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* 1996; **86**:147–157.

45 Wu M, Wang B, Perchellet EM, Sperfslage BJ, Stephany HA, Hua DH, *et al.* Synthetic 1,4-anthracenediones, which block nucleoside transport and induce DNA fragmentation, retain their cytotoxicity in daunorubicin-resistant HL-60 cell lines. *Anticancer Drugs* 2001; **12**:807–819.

46 Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, *et al.* Cytochrome *c* and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**:479–489.

47 Powis G. Free radical formation by antitumor quinones. *Free Rad Biol Med* 1989; **6**:63–101.

48 Monks TJ, Hanzlik RP, Cohen GM, Ross D, Graham DG. Quinone chemistry and toxicity. *Toxicol Appl Pharmacol* 1992; **112**:2–16.

49 Kamesaki S, Kamesaki H, Jorgensen TJ, Tanizawa A, Pommier Y, Cossman J. Bcl-2 protein inhibits etopside-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. *Cancer Res* 1993; **53**:4251–4256.

50 Ferreira CG, Span SW, Peters GJ, Kruyt FA, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. *Cancer Res* 2000; **60**:7133–7141.

51 Notarbartolo M, Cervello M, Dusonchet L, Cusimano A, D'Alessandro N. Resistance to diverse apoptotic triggers in multidrug resistant HL60 cells and its possible relationship to the expression of P-glycoprotein, Fas and of the novel anti-apoptosis factor IAP (inhibitory of apoptosis proteins). *Cancer Lett* 2002; **180**:91–101.

52 Villunger A, Egle A, Kos M, Hartmann BL, Geley S, Kofler R, *et al.* Drug-induced apoptosis is associated with enhanced Fas (APO-1/CD95) ligand expression but occurs independently of Fas (APO-1/CD95) signaling in human T-acute lymphatic leukaemia cells. *Cancer Res* 1997; **57**: 3331–3334.

53 McGahon AJ, Costa PA, Daly L, Cotter TG. Chemotherapeutic drug-induced apoptosis in human leukemic cells is independent of the Fas (APO-1/CD95) receptor/ligand system. *Br J Haematol* 1998; **101**:539–547.

54 Tolomeo M, Dusonchet I, Meli M, Grimaudo S, D'Alessandro N, Papoff G, *et al.* The CD95/CD95 ligand system is not the major effector in anticancer drug-mediated apoptosis. *Cell Death Differ* 1998; **5**:735–742.

55 Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94**: 491–501.

56 Scaffidi CA, Fulda S, Srinivasan A, Friesen C, Li F, Feng L, *et al.* Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; **17**:1675–1687.

57 Kuwana T, Smith JJ, Muzio M, Dixit V, Newmeyer DD, Kornbluth S. Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome *c*. *J Biol Chem* 1998; **273**:16589–16594.

58 Fulda S, Scaffidi C, Susin SA, Krammer PH, Kroemer G, Peter ME, *et al.* Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. *J Biol Chem* 1998; **273**:33942–33948.

59 Lorenzo E, Ruiz-Ruiz C, Quesada AJ, Hernández G, Rodríguez A, López-Rivas A, *et al.* Doxorubicin induces apoptosis and CD95 gene expression in human primary endothelial cells through a p53-dependent mechanism. *J Biol Chem* 2002; **277**:10883–10892.

60 Kim SG, Ravi G, Hoffmann C, Jung YP, Kim M, Chen A, *et al.* p53-Independent induction of Fas and apoptosis in leukemic cells by an adenosine derivative, C1-IB-MECA. *Biochem Pharmacol* 2002; **63**: 871–880.

61 Wolf D, Rotter V. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. *Proc Natl Acad Sci USA* 1985; **82**:790–794.

62 Gimonet D, Landais E, Bobichon H, Coninx P, Liautaud-Roger F. Induction of apoptosis by bleomycin in p53-null HL-60 leukemia cells. *Int J Oncol* 2004; **24**:313–319.

63 Kim JS, Lee JM, Chwae Y-J, Kim YH, Lee JH, Kim K, *et al.* Cisplatin-induced apoptosis in Hep3B cells: mitochondria-dependent and -independent pathways. *Biochem Pharmacol* 2004; **67**:1459–1468.

64 Lassus P, Opitz-Araya X, Lazebnik Y. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 2002; **297**:1352–1354.

65 Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Distinct pathways for stimulation of cytochrome *c* release. *J Biol Chem* 2000; **275**: 32438–32443.

66 Robertson JD, Enoksson M, Suomela M, Zhivotovsky B, Orrenius S. Capase-2 acts upstream of mitochondria to promote cytochrome *c* release during etoposide-induced apoptosis. *J Biol Chem* 2002; **277**: 29803–29809.

67 Sien XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J Biol Chem* 1999; **274**:5053–5060.