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ORIGINAL ARTICLE

The cardio-protecting agent and topoisomerase II catalytic inhibitor sobuzoxane enhances doxorubicin-DNA adduct mediated cytotoxicity

Lonnie P. Swift · Suzanne M. Cutts · Abraham Nudelman · Inessa Levovich · Ada Rephaeli · Don R. Phillips

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Abstract
Purpose The importance of understanding the mechanism of action of anticancer agents is sometimes overlooked in the pursuit of new and therapeutically advantageous compounds. Doxorubicin has long been identified as an inhibitor of the DNA-decatenating enzyme topoisomerase II, this being believed to be the major mechanism of action of this drug. However, the complex nature of cytotoxicity induced by doxorubicin suggests that more than one mechanism of action is responsible for cell kill. Investigation into various other cellular effects has shown that doxorubicin can, in the presence of formaldehyde, form doxorubicin-DNA adducts, resulting in enhanced cell death.
Methods We have used six catalytic inhibitors of topoisomerase II (aclarubicin, merbarone, suramin, staurosporine, maleimide and sobuzoxane) to investigate the role of topoisomerase II mediated cell effects in doxorubicin-DNA adduct inducing treatments. Adduct levels were determined by scintillation counting of [^{14}C]doxorubicin-DNA lesions and DNA damage responses by Comet analysis and flow cytometry (apoptosis).
Results Here we show that sobuzoxane inhibits topoisomerase II but in the presence of doxorubicin also enhances the production of doxorubicin-DNA adducts resulting in an enhanced cytotoxic response. We show that the formation of doxorubicin-DNA adducts is mediated by formaldehyde released from sobuzoxane when it is metabolised.
Conclusions Sobuzoxane has also been shown to decrease the normally dose limiting cardiotoxicity commonly exhibited with clinical use of doxorubicin. The potential combination of doxorubicin and sobuzoxane in cancer chemotherapy has two advantages. First, the mechanism of doxorubicin toxicity is shifted away from topoisomerase II inhibition and towards drug-DNA adduct formation which may allow for a lower drug dose to be used and circumvent some drug resistance problems. Second, the addition of a cardioprotecting agent will counteract the commonly dose limiting side effect of cardiac damage resulting from doxorubicin treatment. The importance of the potentiation of cell kill of doxorubicin and sobuzoxane provides a rationalisation of a mechanistic-based combination of anticancer drugs for an improved clinical outcome.

Keywords Sobuzoxane · Doxorubicin · Formaldehyde · Topoisomerase II · Drug-DNA adducts

L. P. Swift · S. M. Cutts · D. R. Phillips (✉)
Department of Biochemistry, La Trobe University,
Bundoora, VIC 3086, Australia
e-mail: d.phillips@latrobe.edu.au

A. Nudelman
Chemistry Department, Bar Ilan University,
Ramat Gan 52900, Israel

I. Levovich · A. Rephaeli
Felsenstein Medical Research Center,
Sackler School of Medicine, Tel Aviv University,
Beilinson Campus, Petach Tikva 49100, Israel

Introduction

Doxorubicin (Adriamycin) is a broad spectrum chemotherapeutic which exhibits activity against breast, lung, thyroid and ovary carcinomas, leukemias, as well as Hodgkin's and non-Hodgkin's lymphomas [1]. Although an efficient anticancer agent, doxorubicin-based treatments are complicated by dose-limiting cardiotoxicity and the development of multi-drug resistance [1]. Cardiotoxicity is due to the

production of reactive oxygen species (ROS) which result when doxorubicin undergoes metabolic reduction [2, 3]. ROS are particularly damaging to cardiac tissues that lack enzymes to detoxify radical species [1]. Multi-drug resistance (MDR) occurs as a result of up-regulation of the membrane associated P-glycoprotein efflux pump [4], resulting in the efflux of doxorubicin (and other drugs) from cells and hence a diminished response to these agents.

Several mechanisms of action have been investigated and collectively explain the broad spectrum of anticancer activity of doxorubicin [5]. Two specific mechanisms of action of doxorubicin are of interest to the current study, formation of formaldehyde-mediated doxorubicin-DNA adducts and the topoisomerase II poisoning capability. Both areas of research have been extensively reviewed [6-10] and are briefly described here.

The formation of doxorubicin-DNA adducts is mediated by formaldehyde. It has been shown that formaldehyde supplies a methylene group which links the 3′-amino group of doxorubicin to the 2-amino group of deoxyguanosine residues of DNA via Schiff base chemistry [11] and that the formaldehyde conjugated complex is the active form of the drug [12]. These adducts are attached covalently to one strand of DNA (and are hence mono-adducts) but are stabilised to the local region of DNA sufficiently that they are resistant to thermal denaturation [12, 13]. Doxorubicin-DNA adducts have been quantitated in MCF-7 breast adenocarcinoma and HL-60 promyelocytic leukemic cells in culture [14, 15]. It was found that doxorubicin-DNA adducts occur in both nuclear and mitochondrial DNA at similar rates [14]. Several formaldehyde releasing prodrugs have been synthesised and characterised, such as AN-9, AN-7 and hexamethylenetetramine [16, 17]. The doxorubicin-DNA adduct forming combination of doxorubicin and formaldehyde-releasing prodrugs are cytotoxic in several cell lines (as reviewed in [6]). This combination treatment has also been shown to shift the mechanism of action of doxorubicin from a topoisomerase II mediated DNA damaging agent to a formaldehyde mediated doxorubicin-DNA adduct forming agent. Doxorubicin-DNA adducts have been shown to be more cytotoxic lesions than topoisomerase II-mediated DNA double strand breaks (dsb), indicating that the clinical cytotoxicity of doxorubicin can be enhanced with an appropriate prodrug combination [15].

Doxorubicin has also been described as a poison of the DNA decatenating enzyme topoisomerase II whereby the drug stabilises the topoisomerase II-DNA cleavable complexes. Topoisomerase II catalyses DNA decatenation, a process essential for replication and transcription of DNA. Topoisomerase II acts to cleave one strand of duplex DNA, allowing a second duplex strand to pass through. Once through, the first strand of duplex DNA is re-sealed [18]. The intermediary complex is referred to as a cleavable complex. Each reaction changes the DNA linking number by two and hydrolyses two ATP molecules [9]. A model for the interaction of topoisomerase II and doxorubicin has been described whereby the drug inserts into the minor groove of duplex DNA alongside the topoisomerase protein during step 3 (Fig. 1), resulting in stabilisation of the cleavable complex through a protein–drug interaction [19]. Stabilisation of the cleavable complex results in the stalling of topoisomerase enzymatic activity and the induction of a DNA dsb [20]. This break is then thought to become a cytotoxic lesion, resulting in apoptosis. Other topoisomerase II poisons include daunorubicin, epirubicin, mitoxantrone and etoposide.

A second category of drugs affecting topoisomerase II are the catalytic inhibitors, including aclarubicin, maleimide, merbarone, staurosporine, suramin and the dioxopiperazines [e.g. ICRF-187, ICRF-193 and sobuzoxane] (see Fig. 1 for structures). Catalytic inhibitors of topoisomerase II are compounds that act on one or more steps of the enzymatic cycle (Fig. 1) [21]. These drugs often possess more than one mechanism of cell toxicity.

The catalytic inhibitor aclarubicin intercalates into DNA modifying the topology of the DNA duplex so that topoisomerase II is prevented from forming the initial non-covalent complex with DNA [22, 23]. Aclarubicin reduces the formation of cleavable complexes observed in the presence of such topoisomerase II poisons as etoposide [22, 24].

The dioxopiperazines ICRF-187, ICRF-193 and ICRF-154 are non-intercalative catalytic inhibitors, these drugs bind directly to topoisomerase II and lock the enzyme in the closed clamp conformation after the DNA religation step (Fig. 1) [25]. Sobuzoxane is a masked version of ICRF-154 originally designed in an attempt to increase the bio-availability of ICRF-154 [26]. Sobuzoxane is currently used as a clinical anticancer agent in Japan and has been shown to be effective for the treatment of lymphomas and adult T-cell leukemias [26, 27]. The ICRF derivatives are known to chelate metal cations and to reverse the free radical generation due to metal-anthracycline complexes which results in one of the major dose limiting side effect of anthracycline toxicity, cardiomyopathy [27–29]. ICRF-193 is known to block the closed clamp form of a topoisomerase II-DNA complex (step 5 of Fig. 1 [25]). To date, the mechanism by which sobuzoxane inhibits topoisomerase II has not been elucidated. Sobuzoxane is degraded to release the active metabolite ICRF-154. It is assumed that due to the similarity of ICRF-154 and ICRF-193 they act on the same step in the topoisomerase II mechanism of action. ICRF-187 (dexrazoxane) is currently used as a clinical agent to decrease cardiac tissue damage resulting from doxorubicin induced free radicals, and has also been shown to decrease ulceration induced by subcutaneous anthracycline injections in mice [30].

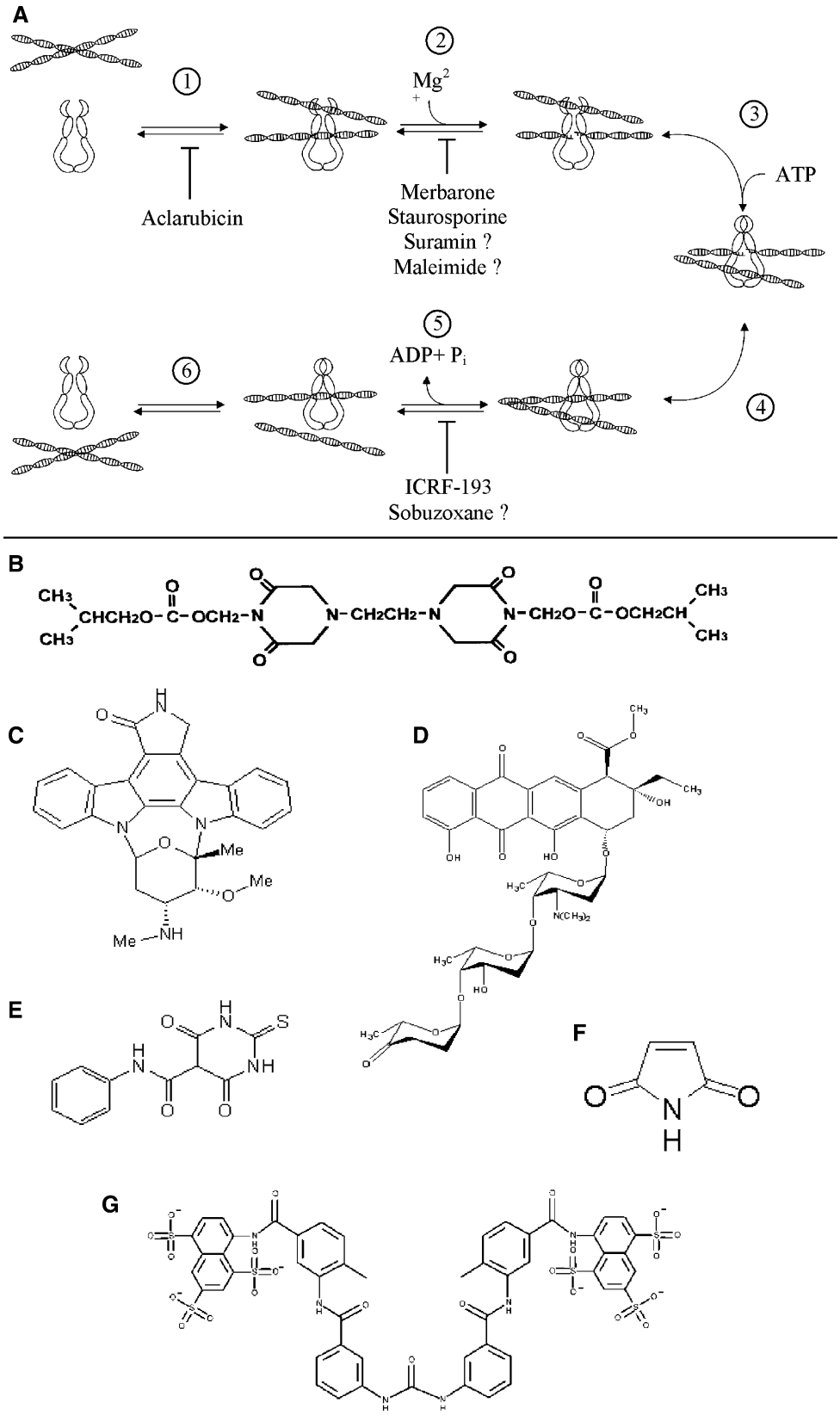

Fig. 1 Mechanism of topoisomerase II decatenation of DNA and sites of catalytic inhibition. The mechanism of DNA decatenation by topoisomerase II involves binding of the enzyme to DNA (**a**) (step 1), followed by a transient DNA-dsb in strand 1 (step 2). A conformational change in the enzyme then allows the unbroken DNA strand (strand 2) to pass through the broken strand of DNA (step 3) termed the "cleavable complex". Step 3 is the point in the topoisomerase II cycle where doxorubicin interferes. The broken strand of DNA will then be rejoined (step 4) and released from the protein clamp (step 5). Finally, strand 2 (the unbroken strand) will be released from the enzyme (step 6) which results in a change in DNA linkage of 2. The steps where topoisomerase II catalytic inhibitors sobuzoxane (**b**), staurosporine (**c**), aclarubicin (**d**), merbarone (**e**), maleimide (**f**) and suramin (**g**) act are indicated [31, 36, 40]

Suramin inhibits the catalytic activity of purified topoisomerase II in vitro as assayed by the reduction of the levels of topoisomerase II relaxed supercoiled DNA, and the decreased decatenation of kinetoplast DNA [31]. Suramin does not bind to DNA and does not induce DNA cleavage in vitro but does block the ability of topoisomerase II

poisons from inducing cleavable complex induced DNA damage [31]. The exact step in the topoisomerase II catalytic cycle that is affected by suramin is not known, but is assumed that it blocks the cleavage of DNA in step 2 (Fig. 1). In the clinic suramin is active against a variety of tumours including adenocarcinomas, lymphomas, renal and prostate cancers [32]. Suramin also affects the activity of several kinases, including phosphatidylinositol kinase, diacylglycerol kinase and protein kinase C [33, 34]. Staurosporine, also a protein kinase inhibitor, interferes with the transfer of phosphodiester bonds from DNA to topoisomerase and hence inhibits strand scission of the duplex DNA (step 2 Fig. 1) [35].

Merbarone has been shown to inhibit DNA relaxation catalysed by topoisomerase II, but did not inhibit topoisomerase II binding to DNA. Merbarone has been found to reduce etoposide induced topoisomerase II-DNA cleavage and has been suggested to act at the DNA scission step of topoisomerase II action (step 2 Fig. 1) which is independent of ATP [36]. Possessing significant activity against several murine tumour models and curative activity against L1210 and P388 leukemia models [37, 38] merbarone has undergone Phase-II clinical trials [39].

Topoisomerase II catalytic activity has been shown to be inhibited by maleimide in vitro, as exemplified by the inhibition of etoposide-induced cell kill [40]. However, the mechanism of this inhibitory activity is not known, but is thought to be due to covalent modification of topoisomerase cysteine residues, thereby reducing the amount of catalytically active enzyme [40].

This study aimed to investigate the effect of topoisomerase II catalytic inhibitors on doxorubicin-induced topoisomerase II-mediated dsb and on formaldehyde-mediated doxorubicin-DNA adducts. Inhibitors were used to reduce the cellular effect of doxorubicin on topoisomerase II-induced dsb and the comparative effect on doxorubicin-DNA adducts were assessed. We discovered that sobuzoxane, a bio-available drug containing the cardioprotective agent ICRF-154, released formaldehyde when degraded intracellularly, and this shifted the mechanism of doxorubicin cytotoxicity to that of formation of doxorubicin-DNA adducts. The adducts enhanced the apoptotic response to doxorubicin/sobuzoxane treatments compared to doxorubicin or sobuzoxane single agent treatments in HL-60 cells.

Materials and methods

Materials

Doxorubicin (a gift from Pfizer, Milan, Italy) was dissolved in Milli-Q water to a stock concentration of 1 mM and stored at −20°C. The topoisomerase II inhibitors aclarubicin, suramin and sobuzoxane (MST-16) were purchased through Sigma-Aldrich, Castle Hill, NSW, Australia, merbarone and maleimide were from Merck Biosciences, Melbourne, VIC, Australia, and staurosporine from Roche Diagnostics, Castle Hill, NSW, Australia. Suramin (25 mM), maleimide (1 mM) and aclarubicin (10 μM) were dissolved in Milli-Q water while sobuzoxane (30 mM), merbarone (50 mM) and staurosporine (10 μM) were all dissolved in DMSO. Tris-saturated ultrapure phenol was purchased from Invitrogen, San Diego, CA, USA, and formaldehyde from Crown Scientific, Minto, NSW, Australia. [14-^{14}C]doxorubicin hydrochloride (54 mCi/mmol) was obtained from GE Healthcare, Bucks, UK, and the QIAamp blood kit was from Qiagen, Hilden, Germany. Glycogen was from Roche Molecular Biosciences, Lawrence, KS, USA, Pivaloyloxymethyl butyrate (AN-9) was synthesised as described previously [41] and was diluted into DMSO using glass vials and glass syringes.

Cell lines

The promyelocytic leukemic cell line HL-60 was obtained from the American Type Culture Collection (Catalogue number CCL-240). HL-60 cell lines were maintained in RPMI 1640 media (Sigma, Munich, Germany) supplemented with 10% foetal calf serum (Trace Scientific, Melbourne, VIC, Australia) at 37°C, 5% CO_2 in the absence of antibiotics.

Drug treatments

Cells (1×10^6) were seeded in 10 cm Petri dishes in 10 ml of complete RPMI-1640 or at the same concentration in 2 ml volumes in six-well plates. Once treated, cells were harvested by centrifugation at 1,000 rpm for 5 min and washed once in PBS. Topoisomerase II inhibiting drugs were used to prevent or stabilise the formation of topoisomerase II-mediated DNA dsb. Cells were pre-treated with topoisomerase inhibitors for a specified time (typically 30 min) to evaluate the potential of each inhibitor to block topoisomerase II-mediated damage compared to controls. Inhibitor concentrations used were optimised, based on literature values, to provide optimal inhibition of topoisomerase II mediated dsb without inducing apoptosis as a single agent (where possible). The final concentration of DMSO in cell media did not exceed 0.5%.

Detection of [^{14}C]-doxorubicin labelled adducts

HL-60 cells were seeded in six well plates and incubated with varying concentrations of topoisomerase II inhibitors and/or [^{14}C]doxorubicin/AN-9 for desired times, then harvested [42]. The genomic DNA was extracted using the

QIAamp blood kit with the single modification that lysis incubation temperature was decreased to 50°C and extended to 30 min to reduce the thermal denaturation of doxorubicin-DNA adducts. Extracted DNA was subjected to two phenol extractions and one chloroform extraction before being precipitated in ammonium acetate and ethanol. Pellets were resuspended in 100 µl TE buffer and DNA concentration calculated at 260 nm. A 90 µl aliquot was added to 1 ml of Optiphase Hisafe (PerkinElmer, Melbourne, VIC, Australia) scintillation mixture and the incorporation of [^{14}C]doxorubicin into DNA was quantitated in a Wallac 1410 Liquid Scintillation Counter and calculated as doxorubicin adducts per 10 kb.

Comet assay (single cell gel electrophoresis)

The Comet assay was based on methods developed by Hartley et al. [43] and Salti et al. [44] as previously described [15]. Briefly, 0.3 ml of cell sample was mixed with 1 ml of molten low gelling temperature agarose and added to a pre-coated slide being set under a 40 × 22 mm^2 coverslip. Set samples were lysed in ice-cold Lysis Buffer for 1 h [43] (100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris–HCl, pH 10.5 plus 1% TritonX-100) followed by 4 × 15 min washes in ice cold Milli-Q H_2O (both lysis and wash step performed on ice). Sample slides were incubated in Alkali Electrophoresis Buffer [44] (300 mM NaOH and 1 mM EDTA) for 1 h followed by electrophoresis for 30 min at 30 V (1 V/cm) at 4°C.

After electrophoresis, slides were flooded with 1 ml neutralisation buffer (0.5 M Tris–HCl and pH 7.5) washed twice with PBS before being stained with 2 × 1 ml of 2.5 µg/ml propidium iodide for 5 min. Stain was rinsed off with Milli-Q water and comet tails were analysed utilising a fluorescence microscope and the Komet software, Kinetic Imaging, UK. Comets (50) were counted and the average Olive Tail Moment (OTM) obtained [45]. All Comet results are representative of the average OTM of three or more individual experiments with the error being the standard error.

Analysis of apoptosis by flow cytometry

Apoptosis was analysed as previously described [15]. Cells (1×10^6 in 10 ml or 2×10^5 in 2 ml) were pelleted and fixed in 70% ethanol for 30 min at room temperature. Fixed cells were washed once in PBS and then resuspended in DNA staining solution (25 µg/ml propidium iodide and 100 µg/ml RNase A in PBS) and incubated at 37°C for 30 min in the dark. Samples were analysed on a FACSCalibur flow cytometer employing CellQuest software (BD Biosciences, San Jose, CA, USA). The FL2-H filter was used to measure the stained nucleic acids of each cell (event) to assess the percentage of sub-G1 events for each sample as a measure of cells undergoing apoptosis as described previously [46]. Doublet discrimination was performed. Experiments were repeated and the data is representative of the average plus or minus the standard error. This method for measuring apoptosis was compared to results obtained by other methods (DNA fragmentation, trypan blue exclusion and cell growth inhibition [15] as well as caspase-3 and morphology analysis [data not shown]) and found to be reproducible and representative of the cellular responses to these agents.

Quenching of doxorubicin fluorescence by topo II inhibitors

The fluorescence of doxorubicin (1 µM) was measured at excitation and emission wavelengths of 480 ± 5 and 595 ± 5 nm, respectively, in a fluorimetric plate reader. Inhibitors were added in 5 µl increments and the quenching of fluorescence measured. The concentration of inhibitor added was varied to mimic the concentrations used to treat cell samples with the first 5 µl increment. Four additional increments were also added.

Measurement of formaldehyde levels in vitro

Formaldehyde concentrations were assayed using the method described by Nash [47]. Reactions of sobuzoxane (100 µl) were incubated at 37°C for 1 h in reaction buffer (90 mM NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 and pH 7) with or without porcine liver esterase (10 µg/ml, Sigma). Colorimetric reagent (2 M ammonium acetate, 50 mM acetic acid and 50 mM acetylacetone) was added to samples (1:1 v/v) and incubated at 50°C for 30 min and the absorbance determined at 412 nm. A formaldehyde standard curve was used to calculate the concentration of formaldehyde released in each sample.

Results

Catalytic inhibitors of topoisomerase II decrease doxorubicin induced dsDNA breaks

Recently we have shown that the topoisomerase II poison doxorubicin can undergo a non-topoisomerase II mediated mechanism of action that results in cell death. These studies established the role for formaldehyde mediated doxorubicin-DNA adducts in cell kill independently of doxorubicin-induced topoisomerase II-mediated cell effects [15]. To further characterise the role of classical topoisomerase II-mediated cell effects on formaldehyde-mediated doxorubicin-DNA adducts we employed a panel of known catalytic

inhibitors of topoisomerase II in order to investigate the effect of catalytic inhibitors of topoisomerase II on doxorubicin-DNA adduct induced cytotoxicity. These adducts were shown to be more cytotoxic than doxorubicin induced topoisomerase II mediated DNA dsb however the relative contribution of each mechanism to cell death was of considerable interest. The topoisomerase II catalytic inhibitors aclarubicin, maleimide, merbarone, suramin, staurosporine and sobuzoxane were investigated for their effects on cellular dsb and apoptosis when used prior to treatment with doxorubicin alone and on adduct forming combinations of doxorubicin and the formaldehyde releasing prodrug AN-9 (Fig. 2). As has been previously shown [15], doxorubicin alone (1 μM) induced topoisomerase II mediated dsb in HL-60 cells following a 4 h treatment as measured by an increase in OTM detected using the Comet assay, but no apoptosis was detected under these conditions. The level of dsb induced by a 4 h treatment with 1 μM doxorubicin in HL-60 cells resulted in an OTM of ~1.1 as measured by the Comet assay (Fig. 2a). This level of damage has been shown to equate to a treatment of 5 Gy of ionising radiation [15]. We have also previously shown that doxorubicin single agent treatment requires a longer timeframe to induce apoptosis, being ~12 h following a 4 h treatment (data not shown) with more significant levels of apoptosis and necrosis observed 24 and 48 h post-treatment, an effect that was attributed to the cell cycle dependent response to doxorubicin [15]. The adduct forming combination of doxorubicin (1 μM) and AN-9 (50 μM) did not induce topoisomerase II mediated dsb but did induce significant levels of apoptosis (~40%) following a 4 h treatment. Under the conditions employed the formaldehyde releasing prodrug AN-9 did not induce either dsb or apoptosis at significant levels as a single agent. Aclarubicin, maleimide, merbarone, suramin and staurosporine all decreased the dsb induced by doxorubicin as a single agent (Fig. 2), and had little effect on the levels of apoptosis induced. These five inhibitors did not alter the level of apoptosis induced by adduct forming combination treatments to a great extent, although pre-treatment with staurosporine induced an ~20% increase in doxorubicin/AN-9 apoptosis. Suramin, maleimide, aclarubicin and sobuzoxane had little effect on the dsb levels induced by doxorubicin/AN-9 combinations, while merbarone and staurosporine treatments lead to an increase in the dsb induced by combination treatments. Sobuzoxane decreased the dsb levels induced with doxorubicin alone, and that unexpectedly coincided with an increase in apoptosis. Interestingly, sobuzoxane as a single agent also induced a high level of dsb, similar to the level induced with doxorubicin single agent treatment. When sobuzoxane and doxorubicin treatments were combined, the level of dsb decreased to background levels and this response was accompanied by a substantial increase of apoptosis. Pre-treatment with

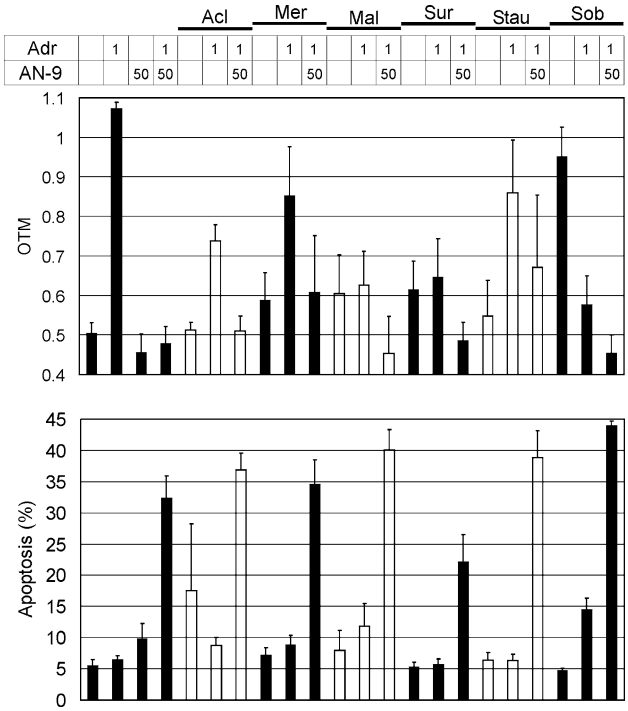

Fig. 2 Effect of topoisomerase II catalytic inhibitors on DNA-dsb and apoptosis induced by doxorubicin and doxorubicin/AN-9 combinations. HL-60 cells (2×10^5) were pre-treated with 100 nM aclarubicin (*Acl*), 100 μM merbarone (*Mer*), 2 μM maleimide (*Mal*), 100 μM suramin (*Sur*), 50 nM staurosporine (*Stau*) or 200 μM sobuzoxane (*Sob*) for 30 min followed by a 4 h treatment with either doxorubicin alone (1 μM) or doxorubicin in combination with AN-9 (50 μM). Samples were split and assessed for **a** dsb induction (OTM) by the Comet assay or **b** apoptosis by flow cytometery as described in the Materials and methods ($n = 3$)

sobuzoxane resulted in an increase in apoptosis for doxorubicin/AN-9 combination treatments also, suggesting a synergistic or possibly additive response for the combination of sobuzoxane with either doxorubicin alone or doxorubicin/AN-9 treatments.

Suramin and merbarone interact with doxorubicin

The exact mechanism of action of the catalytic inhibitors employed in this study is not fully understood. In order to establish if any of the inhibitors exerted a direct effect on doxorubicin activity, the quenching potential of inhibitors on doxorubicin fluorescence was assessed to establish if any of the inhibitors bound to the drug. All inhibitors were added in excess to ensure that, even at up to tenfold greater concentration than that employed in cell-based experiments, binding of inhibitor to doxorubicin did not occur. It was shown that suramin interacted with doxorubicin, decreasing the fluorescence of doxorubicin (Fig. 3). Merbarone was also shown to interact with doxorubicin, albeit to a lesser extent, requiring a tenfold greater concentration than that used in the cell-based assays (data not shown). It

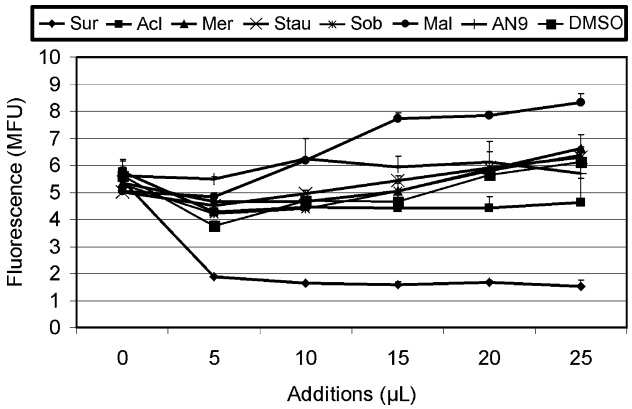

Fig. 3 Investigation of quenching of doxorubicin fluorescence by topoisomerase II catalytic inhibitors and formaldehyde-releasing prodrugs. The fluorescence of doxorubicin (10 µM) was measured after 5 µl additions of DMSO, the formaldehyde-releasing prodrug AN-9 (100 mM) and topoisomerase inhibitors Suramin (5 mM), Aclarubicin (50 nM), Merbarone (50 mM), Sobuzoxane (20 mM) and Staurosporine (50 µM)

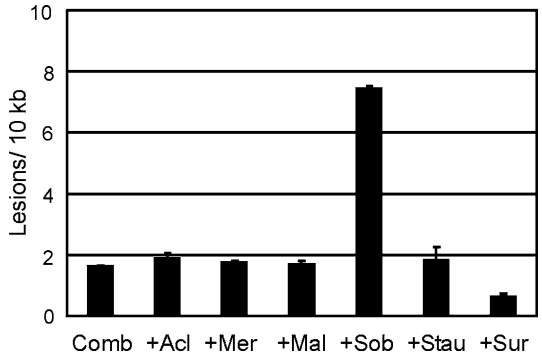

Fig. 4 Effect of topoisomerase II catalytic inhibitors on doxorubicin-DNA adducts in HL-60 cells. HL-60 cells were pre-treated with aclarubicin (*Acl*, 100 nM), merbarone (*Mer*, 200 µM), maleimide (*Mal*, 2 µM), sobuzoxane (*Sob*, 50 µM), staurosporine (*Stau*, 40 nM) or suramin (*Sur*, 200 µM) for 1 h then treated with [^{14}C]doxorubicin 2 µM (*Dox*) in combination with AN-9 100 µM (*Comb*) for a further 4 h in duplicate. DNA was extracted and [^{14}C]doxorubicin counts measured and quantitated as doxorubicin adducts per 10 kb DNA

is assumed that the interactions observed in this quenching study are due to direct binding of the catalytic inhibitors suramin and merbarone to doxorubicin, but the exact mechanism is not known. It was noted that AN-9 and the DMSO vehicle control did not quench the fluorescence of doxorubicin, however maleimide appeared to increase the fluorescence of doxorubicin.

Sobuzoxane enhances doxorubicin-DNA adduct formation

Because of the varied response to the topoisomerase II inhibitors observed in Fig. 2, the effect of these catalytic inhibitors on formaldehyde-mediated doxorubicin-DNA adducts was assessed. HL-60 cells were pre-treated with the catalytic inhibitors aclarubicin, merbarone, staurosporine and maleimide but they did not alter the level of doxorubicin-DNA adducts. Suramin decreased the level of adducts induced from ~2 lesions per 10 kb to ~0.7 lesions per 10 kb, consistent with the observed interaction of suramin with doxorubicin (Fig. 4). Sobuzoxane enhanced the level of adducts formed approximately fourfold, from 2 to 8 lesions per 10 kb. Given the increase in apoptosis observed by sobuzoxane pre-treatment of doxorubicin alone and adduct forming combination treatments, the increase in levels of adducts suggests a particularly strong potentiation of adduct formation with sobuzoxane that required further investigation.

Sobuzoxane mediated doxorubicin-DNA adduct formation

Analysis of the chemical structure of sobuzoxane indicated that upon hydrolysis and esterase degradation formaldehyde is released (Fig. 5a). This was tested using the assay described by Nash, a colorimetric assay used to determine aldehyde concentrations [47]. Following a 1 h incubation at 37°C in the presence of 10 µg/ml esterase, sobuzoxane was shown to release approximately two molecules of an aldehyde for every molecule of drug degraded (Fig. 5b). We have previously investigated the potential of other aldehydes to form doxorubicin-DNA adducts and discovered that acetaldehyde, butyraldehyde, glyceraldehyde, proprionaldehyde and a series of other aldehydes do not enhance the formation of doxorubicin-DNA adducts in vitro or in cells in culture (data not shown). Sobuzoxane was shown to induce concentration-dependent levels of doxorubicin-DNA adducts in HL-60 cells (Fig. 5c), supporting our model that the aldehyde released from esterase mediated sobuzoxane degradation is formaldehyde and thus indicating the role of sobuzoxane as a formaldehyde releasing prodrug that also inhibits the catalytic activity of topoisomerase II.

Glutathione abrogates doxorubicin-DNA adduct formation

As sobuzoxane releases several products when degraded, it was important to assess if any of the additional by-products (other than formaldehyde) contributed to doxorubicin-DNA adduct formation. Subsequent treatments with doxorubicin, sobuzoxane and AN-9 (or combinations thereof) were analysed for dsb induction by Comet OTM (Fig. 6a), apoptosis (Fig. 6b) and adduct formation (Fig. 6c). Both sobuzoxane and doxorubicin as single agents induced dsb but when combined the level of dsb was dramatically reduced (Fig. 6a). Pre-treatment of cells with glutathione was used to detoxify formaldehyde in HL-60 cells. By detoxifying formaldehyde, glutathione shifts the mode of cytotoxicity

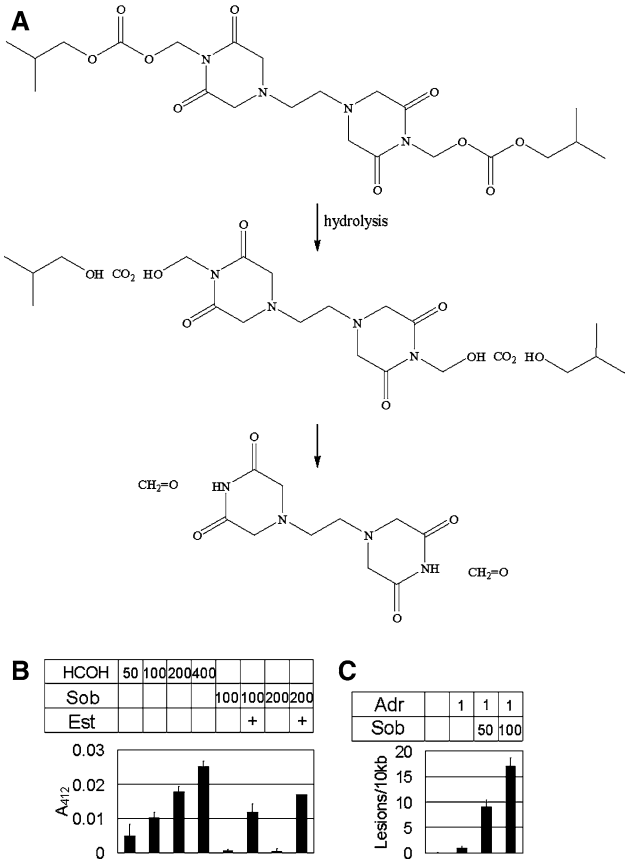

Fig. 5 Sobuzoxane is a formaldehyde-releasing drug. The proposed hydrolysis and esterase-dependent release of formaldehyde from sobuzoxane is shown in panel (**a**). The formaldehyde release from sobuzoxane assayed in vitro (**b**) results in a sobuzoxane dependent formation of [^{14}C]doxorubicin-DNA adducts in HL-60 cells in culture treated with doxorubicin (1 μM) and sobuzoxane (50 and 100 μM) for 4 h (**c**) ($n = 3$)

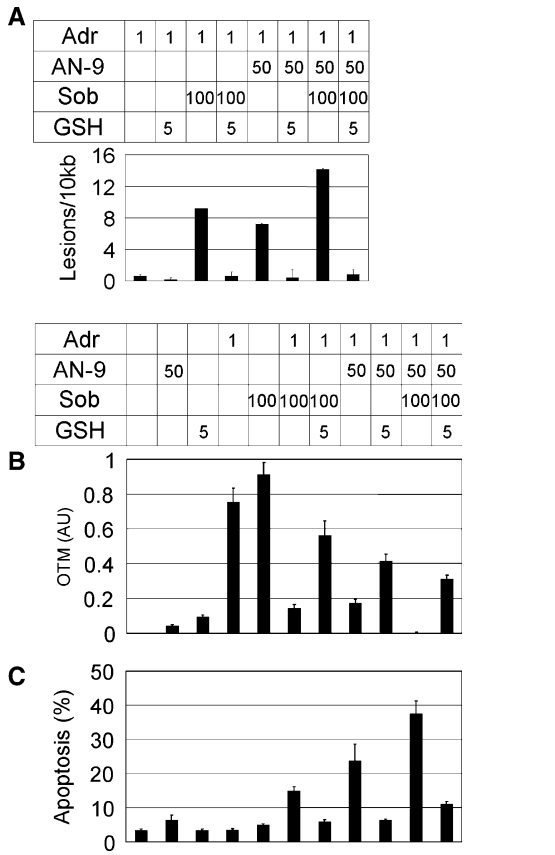

Fig. 6 Glutathione abrogates formaldehyde mediated doxorubicin-DNA adducts mediated by both sobuzoxane and AN-9. HL-60 cells were treated with sobuzoxane (100 μM, *Sob*) 30 min prior to combinations of glutathione (5 mM, *GSH*), doxorubicin (1 μM, *Dox*) and AN-9 (50 μM), and the resulting doxorubicin-DNA adducts (**a**), topoisomerase II mediated dsb (**b**) and the level of apoptosis induced (**c**) assessed ($n = 3$)

from adduct mediated, to topoisomerase II mediated. Significant doxorubicin-DNA adduct levels were only observed in the sobuzoxane/doxorubicin, doxorubicin/AN-9 and sobuzoxane/doxorubicin/AN-9 combinations, being 10, 6.5 and 14 lesions per 10 kb, respectively. Only background levels of adducts were detected in these combinations when pre-treated with the formaldehyde detoxifying agent glutathione (Fig. 6a), and no change in doxorubicin dsb or apoptosis was observed with glutathione pre-treatment (data not shown). This effect was further confirmed with an increase in dsb with glutathione pre-treatment in adduct forming treatments of doxorubicin/AN-9 (OTM 0.2 increased to 0.4), doxorubicin/sobuzoxane (OTM 0.2–0.6) and doxorubicin/sobuzoxane/AN-9 (OTM 0–0.3). Doxorubicin-DNA adducts are more cytotoxic lesions than topoisomerase mediated dsb under these conditions [15], hence it was expected that if glutathione detoxification of formaldehyde shifted the mechanism of doxorubicin from doxorubicin-DNA adduct formation to topoisomerase mediated dsb (as observed in Fig. 6a, b) a reduction in cytotoxicity would be observed. This was the case since glutathione pre-treatment of the adduct forming combinations resulted in a reduction of apoptosis from sobuzoxane/doxorubicin (15% reduced to 7%), doxorubicin/AN-9 (25–7%) and sobuzoxane/doxorubicin/AN-9 (37–11%) combinations. These results show a clear shift from adduct induced toxicity to topoisomerase mediated dsb formation with the formaldehyde detoxifying glutathione. The enhanced apoptotic potential of a combination of sobuzoxane, AN-9 and doxorubicin is evident in Fig. 6c. It should also be noted that glutathione could also potentially contribute to the responses observed (OTM and apoptosis) with doxorubicin as a single agent by its ability to detoxify free radicals [48–50]. However there appears to be little contribution from this mechanism in the present studies as the addition of glutathione (to doxorubicin as a single agent) did not result in any detectable change of doxorubicin-induced dsb (OTM) or apoptosis (data not shown).

Discussion

Clinical doxorubicin treatment is primarily limited by dose dependent cardiotoxicity and drug-induced resistance. Although it is thought that the topoisomerase mediated mechanism of action is the primary mechanism responsible for cell kill in doxorubicin treatments, we have previously shown that following the addition of the formaldehyde releasing prodrug AN-9, the mechanism of action of doxorubicin switches from topoisomerase II mediated lesions to that of formaldehyde-mediated doxorubicin-DNA adducts, and these adducts are more cytotoxic to cells in culture [15]. By switching the mechanism of action of doxorubicin to an adduct forming compound, a reduced concentration of drug is required to achieve similar cell kill as compared to doxorubicin as a single agent. The reduction of drug dosage required for equivalent cell kill opens up the possibility of using a reduced drug concentration to achieve adequate cell kill, or alternatively, provides a means of overcoming resistance to doxorubicin, as shown by the sensitivity to doxorubicin-DNA adducts in doxorubicin sensitive and resistant cell lines [51].

While investigating the contribution of topoisomerase II mediated cell effects on doxorubicin-DNA adducts, the topoisomerase II catalytic inhibitor sobuzoxane was observed to greatly potentiate the induction of apoptosis in response to adduct forming treatments. Further analysis established that it was the release of formaldehyde from sobuzoxane that resulted in enhancement of adduct formation and an enhanced cytotoxic response. The degradation of sobuzoxane provided two effects: the release of the active compound ICRF-154 [52], and the release of formaldehyde (Fig. 5b), thus providing an ideal compound for the inhibition of topoisomerase II mediated cell effects (due to release of ICRF-154) and potentiation of doxorubicin-DNA adduct formation (due to the formaldehyde release) in doxorubicin-based treatments.

Earlier analyses of combinations of doxorubicin and sobuzoxane suggested that the difference between the mechanisms of action of each of these compounds was likely to result in the enhanced doxorubicin cytotoxicity and reduced cross-resistance observed for combinations of these two drugs [53]. As an antitumour agent, sobuzoxane was found to be more toxic than the parent ICRF-154 compound due to increased bio-availability [52], which eventually aided the approval for clinical use in Japan in 1994 where it has since been used to treat adult T-cell leukemia and non-Hodgkin's lymphoma [26].

Sobuzoxane has also been shown to completely protect rats against the free radical induced cardiotoxicity of doxorubicin which is a major clinical limitation to doxorubicin-based treatments [28]. The present data shows that previous studies that have indicated the synergistic interaction between sobuzoxane and doxorubicin [27] may have been due to the formation of formaldehyde-mediated doxorubicin-DNA adducts, which explains not only the enhanced toxicity observed with sobuzoxane/doxorubicin combinations but also explains the ability of sobuzoxane/doxorubicin treatments to overcome resistance to doxorubicin (due to a shift to doxorubicin-DNA adduct formation) as these adducts are known to overcome resistance to doxorubicin [51]. Under the conditions employed (Fig. 6), doxorubicin was shifted from a topoisomerase II acting drug to a drug where DNA adducts were formed in the presence of sobuzoxane, suggesting only a minor role for topoisomerase II in doxorubicin/sobuzoxane combination treatments.

We have shown here that sobuzoxane and doxorubicin co-treatments induce the formation of doxorubicin-DNA adducts as the major lesion. These adducts have been shown to circumvent topoisomerase II and P-glycoprotein-based resistance mechanisms, major forms of anthracycline-induced resistance observed in the clinic. The sobuzoxane/doxorubicin combination has been shown to result in a decreased drug induced cardiotoxic response (due to the release from sobuzoxane of the cardio-protecting compound ICRF-154), resulting in circumvention of a major dose limiting side effect for doxorubicin based therapies. Sobuzoxane/doxorubicin combinations were also found to induce cell kill at lower drug concentrations than required when doxorubicin is used as single agent. These data suggest that treatments with the combination of doxorubicin and sobuzoxane may have clinical potential and may offer the prospect of improved responses for those tumours where doxorubicin is part of the standard treatment.

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