Doxorubicin-DNA Adducts Induce a Non-Topoisomerase II–Mediated Form of Cell Death


Abstract

Doxorubicin (Adriamycin) is one of the most commonly used chemotherapeutic drugs and exhibits a wide spectrum of activity against solid tumors, lymphomas, and leukemias. Doxorubicin is classified as a topoisomerase II poison, although other mechanisms of action have been characterized. Here, we show that doxorubicin-DNA adducts (formed by the coadministration of doxorubicin with non-toxic doses of formaldehyde-releasing prodrugs) induce a more cytotoxic response in HL-60 cells than doxorubicin as a single agent. Doxorubicin-DNA adducts seem to be independent of classic topoisomerase II–mediated cellular responses (as observed by employing topoisomerase II catalytic inhibitors and HL-60/MX2 cells). Apoptosis induced by doxorubicin-DNA adducts initiates a caspase cascade that can be blocked by overexpressed Bcl-2, suggesting that adducts induce a classic mode of apoptosis. A reduction in the level of topoisomerase II–mediated double-strand-breaks was also observed with increasing levels of doxorubicin-DNA adducts and increased levels of apoptosis, further confirming that adducts exhibit a separate mechanism of action compared with the classic topoisomerase II poison mode of cell death by doxorubicin alone. Collectively, these results indicate that the presence of formaldehyde transfers doxorubicin from topoisomerase II–mediated cellular damage to the formation of doxorubicin-DNA adducts, and that these adducts are more cytotoxic than topoisomerase II–mediated lesions. These results also show that doxorubicin can induce apoptosis by a non-topoisomerase II–dependent mechanism, and this provides exciting new prospects for enhancing the clinical use of this agent and for the development of new derivatives and new tumor-targeted therapies. (Cancer Res 2006; 66(9): 4863-71)

Introduction

The anthracyclines (including doxorubicin, daunomycin, epirubcin, and idarubicin) are one of the most clinically useful groups of anticancer chemotherapeutics. These drugs are routinely employed in combination regimes with other groups of drugs in which each drug generally exhibits a different mechanism of action to increase tumor cell kill and to minimize induced resistance to these drugs.

Although doxorubicin is one of the most widely used anticancer agents, the mechanism of action for this drug is not fully understood. Doxorubicin is normally described as a classic topoisomerase II poison (1, 2). The topoisomerase family of enzymes catalyze the unwinding of DNA for transcription and replication, involving the process of cleavage of one strand of DNA duplex and passing a second duplex through this transient cleavage. The intermediate formed is termed the “cleavable complex.” Doxorubicin poisons the cleavable complex, inhibiting religation of the cleaved duplex, a lesion that results in a DNA double-strand break (DSB; refs. 3, 4). Failure to repair DNA DSB results in an apoptotic response. However, the mechanism by which doxorubicin induces topoisomerase II–mediated DNA DSB does not fully explain the wide spectrum of cytotoxicity that doxorubicin exhibits (5). Several other mechanisms of action have been suggested as modes of doxorubicin-induced cell death (5, 6), including inhibition of DNA and RNA synthesis (7, 8), production of free radicals (9), and the formation of formaldehyde-mediated doxorubicin-DNA adducts (10, 11). None of these alternative modes of cell death have been previously shown to be independent of topoisomerase II–mediated cell effects.

Doxorubicin-DNA adducts were initially characterized in a cell-free activation system, where doxorubicin-induced transcriptional blockages were observed at 5' GpC sequences (12), indicating that doxorubicin formed covalent adducts with DNA at these sites. Doxorubicin-DNA lesions were observed to function as interstrand cross-links in strand renaturation assays (13) and have also been observed in the DNA of tumor cells in culture (14). A thorough analysis of the cell-free activation system used to form adducts revealed that formaldehyde was a byproduct of the reaction conditions, and that formaldehyde was responsible for the formation of an aminal linkage between the 3' amino group of doxorubicin and the N2 of guanine (15). Formaldehyde can also be produced in cells under Fe-catalyzed oxidative stress conditions induced by anthracyclines (16). Although the lesions are mono-adducts, they exhibit the characteristics of interstrand cross-links at GpC sequences, endowing strong duplex stabilization due to strong noncovalent interactions to the opposite strand (17, 18). Both nuclear magnetic resonance and X-ray crystal structures of the drug-DNA adduct have been solved (17, 19), and the formation of doxorubicin-DNA adducts has been studied in cells in culture (20). It has been shown that doxorubicin can be preactivated by formaldehyde to yield the compound doxoforin, which consists of two drug molecules bound together with three methylene groups (21). This preactivated form of doxorubicin displays an accelerated uptake by cells, is retained longer in the nucleus, and is substantially more cytotoxic than doxorubicin (22). Doxoforin exhibits >200-fold greater cytotoxicity than doxorubicin alone and is particularly cytotoxic to doxorubicin-resistant cell lines (21). Doxoforin studies have highlighted the importance of formaldehyde-mediated doxorubicin-DNA adducts in the mechanism of action of doxorubicin.

Formaldehyde-releasing prodrugs (such as AN-9) can be used to enhance the intracellular levels of formaldehyde. The prodrug AN-9 (pivaloxymethyl butyrate) is cleaved intracellularly by esterases,
liberating formaldehyde, butyric acid, and pivalic acid. Formaldehyde reacts with doxorubicin and induces doxorubicin-DNA adduct formation and a synergistic cytotoxic response in tumor cells in culture (20, 23). A combination of daunomycin (an anthracycline that is structurally similar to doxorubicin) and AN-9 was shown to increase the survival of mice inoculated with mouse mononcytic leukemic cells (24). AN-9 was initially synthesized as a butyric acid–releasing prodrug to function as a histone deacetylase inhibitor (25). Given the logistic problem of treating cells with formaldehyde (a highly reactive molecule), the butyric acid–releasing prodrugs were identified as a source of intracellular formaldehyde (20). The prodrug AN-9 has been examined as a single agent in phase I clinical trials (26) and has also been evaluated in phase II clinical trials. During phase I clinical trials, AN-9 doses given were limited by solubility of the drug and not by drug-related toxic side effects. The maximal feasible dose was determined to be 3.3 g/m²/d, indicating that AN-9 as a single agent is extremely well tolerated (26).

Here, we show that the cytotoxic potential of doxorubicin can be increased dramatically, resulting in the induction of apoptosis under conditions previously regarded as nontoxic. The cytotoxic lesion responsible for the enhancement of doxorubicin-induced cell death seems to be a formaldehyde-mediated doxorubicin-DNA adduct. We have shown that doxorubicin-induced topoisomerase II–mediated DNA damage does not contribute to adduct induced cell death, and that with increasing levels of adducts, there was a decrease of topoisomerase II–mediated DNA damage, indicating a preference for the formation of the more cytotoxic adduct lesion. To study doxorubicin-DNA adducts as separate lesions (compared with topoisomerase II–mediated DNA damage), we inhibited topoisomerase II–mediated DNA damage by employing various topoisomerase II catalytic inhibitors, and this did not seem to affect adduct formation and adduct-induced apoptosis. We have also shown that adduct formation and apoptosis can be efficiently induced in topoisomerase II–defective cells. The cell cycle effects of doxorubicin-DNA adducts were also different from the characteristic G2 block observed with doxorubicin as a single treatment, with a predominant S-phase cell cycle arrest. These data also show that doxorubicin can induce apoptosis by a non-topoisomerase II–dependent mechanism, and this provides exciting new prospects for enhancing the clinical use of this agent and for the development of new derivatives and new tumor-targeted therapies. The formation of doxorubicin-DNA adducts, therefore, provides potential advantages for reducing dose-limiting side effects and enhancing tumor cell kill.

Materials and Methods

Cell lines. The promyelocytic leukemic cell line HL-60 and the mitoxantrone resistant variant HL-60/MX2 (which exhibits decreased expression of topoisomerase IIβ; ref. 27) were obtained from the American Type Culture Collection (Rockville, MD). HL-60 cells overexpressing Bcl-2 (HL-60/Bcl2) and the parental control HL-60/Puro were generous gifts from Dr. Gino Vairo (CSL Limited, Melbourne, Australia; 28). HL-60/Puro and HL-60/Bcl2 cell lines contain a stably inserted plasmid expressing puromycin resistance and, in the case of the HL-60/Bcl2 cells, overexpress the Bcl-2 gene. These cells were routinely maintained by passaging in the presence of 2 μg/mL puromycin (Sigma, St. Louis, MO; ref. 28). All HL-60 cell lines were maintained in RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% FCS (Trace Scientific, Melbourne, Australia) at 37°C, 5% CO₂. Cells were counted using a Sysmex CDA-500 particle analyzer. Cell viability was assessed by trypan blue staining.

Detection of doxorubicin-DNA adducts. HL-60 cells (2 × 10⁶) were seeded in six-well plates and left overnight. The cells were incubated with varying concentrations of topoisomerase II inhibitors and/or formaldehyde–releasing prodrugs and [¹⁴C]doxorubicin (specific activity = 54 mCi/mmol; GE Healthcare, Bucks, United Kingdom) for desired times and then harvested (20). The genomic DNA was isolated using a QIAamp blood kit (Qiagen, Hilden, Germany) and subjected to two phenol extractions and one chloroform extraction before being precipitated in ammonium acetate and ethanol. Pellets were resuspended in TE buffer, and the DNA concentration was calculated at 260 nm. Samples were quantitated to determine the incorporation of [¹⁴C]doxorubicin into DNA using a Wallac 1410 Liquid Scintillation Counter and expressed as doxorubicin adducts per 10 kb.

Comet assay. The comet assay used was based on methods developed by Hartley and Salti (29, 30). Cell samples (1 × 10⁶) were treated in 10-cm Petri dishes for 4 hours unless otherwise stated. To generate DNA strand breaks by ionizing radiation, cells were irradiated in media with a ¹³⁷Cs Gammacell 1000 Elite (Nordion International, Inc., Ottawa, Canada). Standard microscope slides were precoated with low EEO agarose (Sigma) and allowed to dry. Treated cell samples in media (0.3-0.5 mL, depending on cell numbers) were mixed with 1 mL of type VII low gelling temperature agarose (molten and kept at 4°C) and 1 mL was transferred to the precoated slides and set with a 40 × 22 mm coverslip over the agarose. Once set, samples were subjected to a 1-hour lysis in ice-cold lysis buffer [ref. 29; 100 mmol/L Na₂EDTA, 2.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 10.5) plus 1% Triton X-100] then washed four times for 15 minutes in ice-cold Milli-Q water (lysis and wash step done on ice). Samples were transferred to a Horizon 2025 (Invitrogen, San Diego, CA) electrophoresis apparatus and allowed to sit in ice cold alkaline electrophoresis buffer (ref. 30; 300 mmol/L NaOH, 1 mmol/L EDTA) for 1 hour then subjected to electrophoresis (30 V) for 30 minutes at 4°C. The remaining cell sample was typically used to analyze sub-G1 events for quantitation of apoptosis as described below.

Following electrophoresis, each slide was flooded with 1 mL neutralization buffer [0.5 mol/L Tris-HCl (pH 7.5)] for 10 minutes and rinsed twice for 10 minutes with PBS. Milli-Q water was used to rinse and rehydrate slides (30 minutes) before staining twice with 1 mL of 2.5 μg/mL propidium iodide for 5 minutes. The stain was rinsed off with Milli-Q water, and comet tails were analyzed using a fluorescence microscope and Komet software (Kinetic Imaging, Nottingham, United Kingdom). For each sample, 50 comet tails were counted to yield the average olive tail moment (OTM; ref. 31). All comet results are representative of the average OTM of two or more individual experiments with the error being the SE.

Analysis of cell cycle distribution by flow cytometry. Analysis of cell cycle distribution and cellular events in the sub-G₁ phase was from a method used for cell cycle analysis as previously described (32). After drug treatment, cells (1 × 10⁶ in 10 mL) were pelleted and fixed by resuspension in 70% ethanol and incubated at room temperature for 30 minutes. After fixing, samples were pelleted at 2,000 rpm for 5 minutes, and pellets were washed once with ice-cold PBS and centrifuged for a further 5 minutes. Pellets were resuspended in 0.5 mL DNA staining solution (25 μg/mL propidium iodide, 100 μg/mL RNase A in PBS) and incubated at 37°C for 30 minutes in the dark. Samples were transferred to 5-mL Falcon tubes and stored on ice until assayed.

Samples were analyzed on a FACSCalibur employing CellQuest software (BD Biosciences, San Jose, CA). The FL2-H filter was used to measure event size (being a measure of cell size and therefore indicating cells with normal DNA content), or cells with fragmented DNA (sub-G₁ or apoptotic). Cells with normal DNA content were analyzed for G₁, S and G₂ based on the events that fell into the normal G₁, S and G₂ regions.

Analysis of DNA fragmentation. Treated cells (1 × 10⁶) were harvested, and DNA was extracted using a QIAamp blood kit (Qiagen) according to the manufacturer’s protocol. Extracted DNA was quantitated, and equal
amounts were loaded into a 1% agarose gel. Gels were electrophoresed for 2 to 3 hours at 70 V, and the resulting gel was stained with 2 μg/ml ethidium bromide and destained with Milli-Q water before visualization.

Results

Doxorubicin-DNA adduct formation compared with DNA strand breaks. We established conditions where formaldehyde-mediated doxorubicin-DNA adducts were observed by using the formaldehyde releasing prodrug AN-9 in the human promyelocytic leukemia cell line HL-60 and the topoisomerase II defective subline HL-60/MX2 (Fig. 1A). Only low levels of adducts were observed with doxorubicin as a single agent (Fig. 1A) and are thought to be due to endogenous levels of formaldehyde (33) and to formaldehyde released by redox cycling of doxorubicin (16). We observed a doxorubicin concentration-dependent increase in formation of adducts in the presence of formaldehyde from ~2 adducts per 10 kb DNA for 1 μmol/L doxorubicin and 100 μmol/L AN-9 to ~8.5 adducts per 10 kb for 4 μmol/L doxorubicin and 100 μmol/L AN-9. The ≥4-fold levels of doxorubicin-DNA adducts with doxorubicin and AN-9 combined treatments have also been detected in MCF-7 and IMR-32 cells (20). Doxorubicin-DNA adducts were also observed in the topoisomerase IIα-defective HL-60/MX2 cell line (27), where a 3.5-fold increase in adducts was detected with doxorubicin and AN-9 compared with doxorubicin as a single agent.

We then investigated the resulting DNA strand breakage induced by similar concentrations of doxorubicin alone or in combination with the formaldehyde releasing prodrug AN-9 (i.e., adduct forming treatments; Fig. 1B). The concentration of AN-9 was reduced in these studies compared with Fig. 1A, as the level of apoptosis induced was too high for accurate detection by the apoptosis (measured as sub-G1 events) assay. The level of AN-9 chosen was, therefore, 50 μmol/L, and under these conditions the dose-dependent increase in adduct formation was maintained (data not shown). The comet assay was used to measure drug DNA damage and results obtained as an OTM (34–36). An increase in DNA strand breakage was observed with doxorubicin as a single agent, but this was not observed when increasing concentrations of doxorubicin were combined with the formaldehyde-releasing prodrug AN-9. The background level of DNA damage observed is thought to reflect normal levels of damage associated with DNA repair and perhaps also to breaks induced during the experimental processes. Under identical treatment conditions, apoptosis was analyzed by sub-G1 DNA content analysis (Fig. 1C) and DNA fragmentation (Fig. 1D). The adduct-forming treatments of doxorubicin (1, 2, and 4 μmol/L) and AN-9 (50 μmol/L) induced a striking doxorubicin concentration-dependent increase in detectable apoptosis in HL-60 (30–50%), HL-60/MX2 (20–50%), and HL-60/Puro (25–50%) cell lines following 4 hours of treatment (Fig. 1C and D), but no apoptosis was observed for doxorubicin or AN-9 as single agents. Caspase-3/7 activity was also measured to verify apoptosis where the pattern of active caspase-3/7 (data not shown) matched the pattern of induction of apoptosis as measured by sub-G1 DNA content analysis and by DNA fragmentation. To assess whether apoptotic DNA fragmentation contributed to the OTM detected in HL-60 cells under adduct-inducing treatments, the cells were cotreated with 20 μmol/L of the apoptosis inhibitor Z-VAD-fmk. There was no detectable decrease of the OTM, whereas a substantial decrease of apoptosis was observed (data not shown).

Figure 1. Formation of formaldehyde-mediated doxorubicin-DNA adducts is independent of DNA strand breaks. HL-60 and topoisomerase-defective HL-60/MX2 cells were treated for 4 hours with doxorubicin (Dox) and AN-9, both as single agents and in combination at concentrations as indicated. A, adduct levels were detected in HL-60 cells following treatment with doxorubicin (1-4 μmol/L) in the absence and presence of 100 μmol/L AN-9 and in HL-60/MX2 cells following treatment with 4 μmol/L doxorubicin in the absence and presence of 100 μmol/L AN-9 (n = 4). B, the comet assay OTM was assessed in HL-60 (solid columns), HL-60/MX2 (horizontal lines), HL-60/Puro (diagonal lines), and HL-60/Bcl2 (vertical lines) cell lines following a 4-hour treatment with doxorubicin (1-4 μmol/L) in the absence and presence of AN-9 (50 μmol/L). Columns, average of three or more independent experiments where 50 cells per experiment were analyzed; bars, SE. The extent of apoptosis was determined as the sub-G1 cell population (C) and also by electrophoretic DNA fragmentation analysis (D) for HL-60, HL-60/MX2, HL-60/Puro, and HL-60/Bcl2 cell lines (column designation as in B) following 4-hour treatments with doxorubicin (0-4 μmol/L) alone or in combination with AN-9 (50 μmol/L), or a representative selection of treatments as indicated. Sub-G1 analysis represents the average of at least three individual experiments, while DNA fragmentation gels are representative of patterns that were reproduced at least three times. For HL-60, HL-60/MX2, and HL-60/Puro, the left hand lane is a 1-kb DNA molecular weight marker.
Furthermore, no apoptosis was detected in the antiapoptotic Bcl-2 overexpressing HL-60/Bcl2 cells (Fig. 1C and D), although formaldehyde-mediated doxorubicin-DNA adducts were induced in these cells (data not shown).

To further investigate the relationship between doxorubicin-DNA adducts and DNA strand breakage, we examined adduct formation at a constant doxorubicin concentration with increasing levels of formaldehyde (by increasing AN-9 concentrations from 0 to 30 μmol/L over 6 hours). As anticipated, increasing adducts were formed (Fig. 2A). Under these conditions, we noted a decrease in OTM (Fig. 2B) that was accompanied by an increase in apoptosis (Fig. 2C). This relationship suggests that by controlling the formaldehyde levels within a cell, doxorubicin can preferentially form drug-DNA adducts compared with mechanisms that induce DNA strand breaks.

To examine the DNA strand breaks induced by doxorubicin as a single agent in further detail (and to yield some insight into how they were formed), a time course analysis was undertaken (Fig. 3A). The topoisomerase II inhibitor etoposide was also examined under the same conditions for comparison. There was a time-dependent increase in the OTM values obtained with a plateau not being obtained within 4 hours; however, etoposide-induced OTM values remained essentially constant over the time period of the study.

As formaldehyde is a reactive species that can form protein-DNA cross-links, and doxorubicin-DNA adducts are DNA stabilizing lesions, we were interested to establish if either of these lesions inhibited the electrophoretic mobility of the DNA during the comet assay, hence resulting in artificially low comet OTM values. To investigate this possibility, we induced ionizing radiation-mediated DNA damage to HL-60 cells and compared the OTM obtained with untreated cells to the OTM obtained with doxorubicin and AN-9 as single agents and in combination. The increase in OTM with increasing ionizing radiation in the control samples (from ∼0.3 with 0 Gy to ∼0.9 with 5 Gy) were mimicked with all drug treatments (Fig. 3B). Both AN-9 (50 μmol/L) and AN-9 in combination with doxorubicin (4 μmol/L) resulted in similar increases of the OTM from ∼0.3 to 0.8. Doxorubicin (4 μmol/L) as a single agent increased the OTM from ∼0.95 with 0 Gy radiation to 1.6 at 5 Gy. It should be noted that there was no detectable increase of DNA fragmentation (assessed by sub-G1 analysis) accompanying increasing levels of irradiation for either doxorubicin treated cells, or for doxorubicin plus AN-9 combination treatments (data not shown).

**Catalytic inhibition of topoisomerase II damage does not affect adduct induced apoptosis.** To further characterize the DNA strand breaks induced by doxorubicin, and test whether inhibition of this damage also affected the magnitude of doxorubicin-DNA adducts, topoisomerase II catalytic inhibitors were employed (staurosporine, suramin, and maleimidate). All three inhibitors reduced doxorubicin-induced DNA strand breaks in single agent- and combination-treated samples, as evident by a reduction of OTM (Fig. 4A, C, and F). Significantly, under adduct-forming conditions, the level of adduct induced apoptosis was not reduced when topoisomerase catalytic inhibitors were present (Fig. 4B, D, and F).

**Cell growth and viability.** To assess the effect of different drug treatments on cell growth, cell numbers were determined at the end of the 4 hours of treatment following drug removal and again

![Figure 2](image-url)  
*Figure 2.* Increasing adduct levels results in reduced DNA strand breaks and increased apoptosis. HL-60 cells were treated with doxorubicin (1 μmol/L) and increasing concentrations of AN-9 (0, 5, 10, 20, and 30 μmol/L) for 6 hours, and the level of doxorubicin-DNA adducts (A), OTM (B), and sub-G1 apoptosis (C) was determined for each treatment. A and C, columns, average of two independent experiments; bars, SD. B, columns, average of two independent experiments; bars, SE (analysis two sets of 50 cells).

![Figure 3](image-url)  
*Figure 3.* DNA damage detected by the comet assay. HL-60 cells were subjected to drug and/or ionizing radiation treatment before determination of DNA strand breakage by the comet assay. A, comparative analysis of OTM induced by doxorubicin and etoposide (both at 1 μmol/L) for 0.5, 1, 2, and 4 hours. Columns, average of two independent experiments, where 50 cells were counted per experiment; bars, SE. B, cells were treated for 4 hours with either no drug as a control ( ), 4 μmol/L doxorubicin ( ), 50 μmol/L AN-9 ( ), or a combination of doxorubicin and AN-9 ( ). The cells were then exposed to 2.5 or 5 Gy of radiation before determination of the OTM (n = 3).
at 24 and 48 hours after treatment in HL-60 (Fig. 5A) and HL-60/MX2 cells (Fig. 5B). It was noted that the control and AN-9-treated cell populations exhibited linear increasing cell growth, whereas populations treated with doxorubicin as a single agent or in combination with AN-9 showed inhibition of cell growth. Given that doxorubicin-DNA adducts were shown to induce significant levels of apoptosis following 4 hours of incubation, the cell viability was investigated to ensure that data were not obtained for a predominantly necrotic cell population. All samples were assayed for levels of apoptosis induced following 4 hours of treatment as well as 24 and 48 hours after treatment (Fig. 5C and D). The resulting trypan-positive cells were also measured to assess cell viability (Fig. 5E and F). Although apoptosis was detected at 4 hours with combination treatments in both HL-60 and HL-60/MX2 cells, no apoptosis was detected with doxorubicin as a single agent until 24 hours after treatment in HL-60 cells and 48 hours after treatment in HL-60/MX2 cells. Very high levels of apoptosis were detected with doxorubicin (4 μmol/L) and AN-9 (50 μmol/L) at 24 and 48 hours after treatment, and these levels (>60%) are approaching the high end detection limit of this assay. However, apoptosis induced by doxorubicin as a single agent was much less pronounced at all time points, especially in HL-60/MX2 cells, where only ~20% apoptosis was detected at the 48-hour post-treatment time point. Trypan-positive cells indicate a measure of nonviable cells. The mode of death displayed by a cell that is measured as trypan blue positive is not indicated by this assay; however, it does reveal viability based on a number of modes of cell death (e.g., late apoptosis and necrosis). Trypan staining of these samples indicated that all cells assayed at the 4-hour time point were in fact viable cells (or consisted of a small proportion of early apoptotic cells as evidenced by lack of trypan blue staining of combination treated HL-60 cells at 4 hours). Following 24 hours after treatment, a high level of trypan-positive cells existed in the HL-60 line (~80%), whereas a lesser number were measured in HL-60/MX2 cells (~50%; Fig. 5E and F). These results correlate with the apoptosis data in Fig. 5C and D, showing a delay in progression through apoptosis before a cell becomes trypan positive.

**Alterations to cell cycle distribution by doxorubicin-DNA adducts.** The delayed cell growth data (Fig. 5A and B) suggest the possible involvement of cell cycle checkpoints in response to drug treatments. No alterations to the cell cycle profile were observed with AN-9 single agent treatments in either HL-60 cells or HL-60/MX2 cells (Fig. 6C and G, respectively). As expected, doxorubicin as a single agent induced a G2-phase cell cycle accumulation in HL-60 cells.

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**Figure 4.** Doxorubicin-DNA adducts are independent of topoisomerase II-mediated cell effects. Effect of 30 minutes of pretreatment with topoisomerase II catalytic inhibitors staurosporine (A and B), suramin (C and D), and maleimide (E and F) on comet OTM (A, C, and E) and apoptosis (B, D, and F) induced by 1 μmol/L doxorubicin as a single agent and in combination with 50 μmol/L AN-9 following a 4-hour exposure to drug(s) (n = 2).
cells (Fig. 6B) but did not induce an altered cell cycle profile in the topoisomerase II–deficient HL-60/MX2 cells (Fig. 6F) as has been previously described (37). In HL-60 and HL-60/MX2 cells, doxorubicin in combination with AN-9 induced a dramatically altered S-phase cell cycle accumulation. Apoptosis levels associated with these treatments were low in all single-agent treatments (percentages in Fig. 6A-H) but elevated for doxorubicin plus AN-9 treatments in both cell lines (Fig. 6D and H).

Discussion

Following the discovery that the topoisomerase II poison doxorubicin also induced formaldehyde-mediated doxorubicin-DNA adducts (10–13), we sought to investigate the role of topoisomerase II–mediated cell effects compared with drug-DNA adducts. Although it is well known that topoisomerase II is not the sole cytotoxic target of doxorubicin, numerous reports have highlighted the important role of topoisomerase II, predominantly in forming protein-associated DNA DSBs. Previous studies have assayed the formation of these topoisomerase-associated doxorubicin-induced DSB through alkaline elution and K-SDS precipitation (38, 39). Due to the experimentally demanding nature of the former assay and the potential nonspecific effects picked up by the latter assay, we sought to identify an alternative assay for the measurement of doxorubicin-mediated topoisomerase II damage.

Initially, we employed the in vivo complex of enzyme bioassay...
(a cesium chloride gradient isolation of DNA-protein complexes detected with an anti-topoisomerase II antibody); however, the complexes were not stable enough to survive this treatment (although complexes could be detected using the topoisomerase inhibitor etoposide). We, therefore, employed the comet or single cell gel electrophoresis assay to measure DNA strand break damage induced by doxorubicin. This assay has previously been shown to detect topoisomerase II-mediated effects induced by a range of drugs, including doxorubicin and mitoxantrone (30, 40). However, because it is known that doxorubicin can induce DNA strand breakage through other mechanisms of action (5), we validated the use of the comet assay to detect topoisomerase II-mediated damage by the use of topoisomerase II-deficient sublines and topoisomerase II catalytic inhibitors.

**Doxorubicin-mediated DNA damage detected using the comet assay.** The comet assay has been widely used to measure DNA damage in response to a variety of DNA-damaging agents (40). DNA damage in the form of single-strand breaks, DSB, and protein-associated strand breaks can all be measured by the comet assay (40). However, it is not possible to easily distinguish among these various forms of DNA damage. Because the comet assay is used to measure both single strand and DSB, it can potentially pick up a range of doxorubicin-mediated effects. For doxorubicin, the main forms of DNA damage that are likely to occur are topoisomerase II-mediated DSB and other secondary damage, free-radical-induced single-strand breaks, and apoptotic DNA fragmentation. The molecular basis for the DNA damage was, therefore, examined in further detail. We examined OTM values in HL-60 and topoisomerase II-deficient HL-60/MX2 cells and found high levels of strand breakage induced by doxorubicin in HL-60 cells but no strand breakage at any of the doses employed in the MX2 cells. Because the basis of the resistance in MX2 cells is topoisomerase II mediated (see below), this cell line should still be susceptible to free radical–mediated effects. Although we cannot rule out some contribution of free radical mediated effects, it seems that there is little free radical mediated strand breakage at the treatment conditions employed in our study.

Another potential contributor to DNA strand breakage is apoptotic DNA fragmentation. The conventional comet assay is not used to detect apoptosis, as these severely damaged cells normally harbor small pieces of DNA that disappear during lysis or electrophoresis, sometimes leaving ghost tails behind (40). However, we cannot completely rule out very early stages of apoptosis leading to long-range DNA strand breakages that may contribute to the OTM in comet assays. To inhibit apoptotic DNA fragmentation, the apoptosis inhibitor Z-VD-FMK was employed. Although Z-VD-FMK inhibited the apoptosis that was induced by both doxorubicin and combination treatments, there was no effect on the comet tails, indicating that the OTM measurements obtained are unlikely to include a significant contribution from apoptotic DNA fragmentation. This conclusion was further supported by the fact that 4 hours of drug treatments of HL-60/Bcl2 cells did not induce any apoptosis (assessed by sub-G1 analysis and DNA fragmentation gels) but yielded similar OTM values as in all other HL-60 cell lines.

It is likely that the majority of DNA damage detected by the comet assay in our experiments reflects topoisomerase II-mediated DNA damage. As alluded to above, the most convincing evidence is from experiments using the HL-60/MX2 cells. These cells exhibit varying degrees of resistance to a range of topoisomerase II inhibitors, including mitoxantrone, etoposide, and doxorubicin, but are not cross-resistant to a range of other drugs with non-topoisomerase II-mediated mechanisms of action (41). HL-60/MX2 cells exhibit reduced expression of the topoisomerase IIβ isoform and express a truncated α isoform that results in an altered subcellular distribution and decreased topoisomerase II activity and do not exhibit up-regulation of P-glycoprotein (27). No doxorubicin-mediated OTM was observed in the HL-60/MX2 cells, whereas comet tails were observed in HL-60, HL-60/Puro, and HL-60/Bcl2 cell lines (41). Moreover, three independent topoisomerase II catalytic inhibitors inhibited the doxorubicin OTM in a dose-dependent fashion. Although we cannot completely rule out some contribution from free radical–mediated and other forms of DNA damage, the major form of doxorubicin-induced DNA damage detected in these assays seems to be topoisomerase II mediated, fully consistent with the main documented mode of action of doxorubicin as a topoisomerase II inhibitor (2–5).

The exact nature of the topoisomerase-mediated damage detected by the comet assay is less clear. Although the primary lesion mediated by doxorubicin is a topoisomerase II–mediated DSB, due to other dynamic cellular processes, this may be quickly translated to secondary topoisomerase II–mediated lesions by stalled replication forks and transcriptional complexes with trapped topoisomerase II complexes, although these types of lesions have been better documented for topoisomerase I cleavage complexes (42). Most of the data generated were at a time point of 4 hours. To attempt to gain some insight into the nature of this topoisomerase II damage, a time course study was undertaken where the amount of doxorubicin-induced damage at 30 minutes was limited compared with more extensive damage (roughly 3-fold increase) at 4 hours. In contrast, etoposide exhibited a similar degree of damage at all time points with the exception of the 4-hour time point where repair processes may be commencing, thus resulting in a decreased OTM. Because early time points are appropriate for the measurement of strand breaks that derive from the inhibition of DNA religation in trapped cleavable complexes, it is likely that much of the damage detected at 4 hours reflects secondary forms of topoisomerase II-DNA lesions. This is also supported by reports that DNA lesions induced by doxorubicin persist and even increase following drug removal (43).

Topoisomerase IIα expression profile is found to correlate with a G2-phase doxorubicin cell cycle block, with an increase in expression predominantly in late S and early G2 phases of the cell cycle (44, 45). To confirm that HL-60 cells in this study displayed a typical topoisomerase II–mediated cell cycle response to doxorubicin, cell cycle analysis was undertaken. Doxorubicin treatment induced a G2-phase cell cycle arrest in HL-60, presumably due to DNA damage recognition at the G2 check point as has been previously described (45), whereas this check point was absent in HL-60/MX2 cells, consistent with absent or reduced topoisomerase II–mediated DNA damage in response to doxorubicin.

**Effect of formation of DNA adducts on topoisomerase II–mediated damage.** After validating the comet assay as a measure of topoisomerase II–mediated damage induced by doxorubicin as a single agent (where DNA adduct formation was negligible), we used AN-9 to provide the formaldehyde required for adduct formation and assess the effect of the combination on OTM values. The level of AN-9 chosen did not induce apoptosis, did not inhibit cell growth, and did not produce any OTM. However, a potential complication of the comet assay is that DNA cross-linking agents
may retard the movement of comet tails and thus can complicate interpretation of these results because the OTM would yield an underestimate of DNA strand breaks. Unlike conventional DNA cross-linking agents, such as cisplatin, it is unlikely that doxorubicin-DNA adducts have the ability to retard movement of comet tails because of the limited stability of these adducts under the alkaline conditions that were employed in the comet assay (46). This was confirmed by subjecting drug-treated HL-60 cells (with extensive comet tails) to increasing times (1 to 3 hours) in alkaline conditions (pH 12.5), and this treatment did not alter the observed comet OTM. Although formaldehyde-mediated DNA protein adducts can be detected using the alkali comet assay, higher formaldehyde concentrations than used in our experiments are usually required (47). Nevertheless, to verify whether DNA adduct formation by doxorubicin or DNA protein cross-links (a potential consequence of the formaldehyde released from AN-9) affected any of the comet tails formed, radiation experiments were done. Increasing doses of ionizing radiation resulted in similarly increased OTM values for control, AN-9, doxorubicin, and combination drug treatments, confirming that DNA adducts and DNA-protein cross-links formed in these assays do not lead to any significant under estimation of the observed OTM values.

OTM values for doxorubicin in the presence of AN-9 were reduced in magnitude compared with doxorubicin as a single agent, implying that topoisomerase-mediated damage is less significant in these drug combination treatments. Despite loss of the dose-dependent topoisomerase II–mediated damage, a dose-dependent increase in apoptosis was observed. A titration effect from predominantly topoisomerase II–mediated damage for doxorubicin alone to predominantly adduct formation for the AN-9 combination was illustrated in Fig. 2. It is apparent that the adduct-forming conditions are much more damaging in terms of induction of apoptosis than doxorubicin alone. However, this was observed at the relatively early time points of 4 to 6 hours. To assess longer-term effects of this damage, cells were monitored at various times after treatment. It is apparent that adduct-forming treatments induce apoptosis at earlier time points than with doxorubicin as a single agent. However, doxorubicin eventually causes apoptosis at later time points, suggesting that apoptosis is delayed compared with adduct-forming treatments, where apoptosis occurs rapidly. However, a large population of viable cells still remained for cells treated with doxorubicin as a single agent, even after 48 hours. The HL-60/MX2 cells displayed a similar short-term induction of apoptosis in response to DNA adducts but did not display delayed apoptosis in response to doxorubicin as a single agent, consistent with the lack of topoisomerase II–mediated damage in these cells.

Treatment with doxorubicin and AN-9 resulted in an S-phase cell cycle accumulation. As this was observed in both HL-60 and HL-60/MX2 cells, the observed S-phase accumulation seems to be independent of topoisomerase II. Presumably, S-phase accumulation following 24 hours of drug exposure indicates a cell cycle checkpoint block in response to doxorubicin-DNA adducts. The observation of two distinctly different cell cycle responses provides further support for the view that there are different mechanisms of action displayed by doxorubicin as a single agent, compared with doxorubicin-DNA adducts that form following combination treatments.

To further test any possible interdependence between the two types of lesions, topoisomerase II–mediated damage was inhibited in doxorubicin/AN-9 combination treatments. Three different topoisomerase II catalytic inhibitors were used to independently inhibit topoisomerase II–mediated damage. These treatments inhibited the damage induced by doxorubicin as a single agent and also combination treatments (although this was less dramatic than seen with doxorubicin alone as the initial OTM value was substantially less). However, the levels of apoptosis in combination treatments remained unaffected in the presence of each inhibitor. It should be noted that results obtained by the three catalytic inhibitors could be complicated by other mechanisms of action. For example, staurosporine is an inhibitor of the pre-strand passage step of the catalytic decatenation of topoisomerase II but is also a known inhibitor of protein kinases (48). Suramin inhibits binding of topoisomerase II to DNA but also inhibits the binding of certain growth factors to their receptors (49). Maleimide is thought to covalently modify topoisomerase II cysteine residues, thereby reducing the amount of catalytically active enzyme, but could also modify multiple cysteine-containing proteins (50). The similar results obtained with the three independent inhibitors provide good support for the view that the early apoptosis induced by adduct-forming treatments is independent of topoisomerase II–mediated effects.

We have shown that the apoptotic potential of doxorubicin can be increased dramatically, resulting in the induction of apoptosis under conditions previously regard ed as non-toxic. The mechanism underlying the enhancement of doxorubicin-induced cell death seems to be the formation of doxorubicin-DNA adducts. We have shown that doxorubicin-induced topoisomerase II–mediated DNA damage does not contribute to adduct-induced cell death, and that with increasing levels of adducts, there was a decrease of topoisomerase II–mediated DNA damage, indicating a preference for the formation of adducts when sufficient formaldehyde is available.

In clinical trials of advanced breast cancer patients, increased topoisomerase II expression resulted in a greater response to doxorubicin treatments (51), strongly linking single-agent doxorubicin to topoisomerase-mediated cell death. However, treatments that preferentially induce doxorubicin-DNA adducts may prove to be useful where molecular profiling of topoisomerase II status reveals patients who would not be good candidates for therapy with doxorubicin as a single agent (11). There is now considerable potential to enhance the cytotoxic response of doxorubicin, particularly in doxorubicin-resistant tumors or tumors that express low levels of topoisomerase II, thereby providing a specific strategy for the treatment of these tumors. The activation process also provides the potential to develop new targeting strategies to localize doxorubicin-induced cytotoxicity to tumors by localization of the formaldehyde-releasing prodrug.
References


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