Activation of clinically used anthracyclines by the formaldehyde-releasing prodrug pivaloyloxymethyl butyrate

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Abstract

The anthracycline group of compounds is extensively used in current cancer chemotherapy regimens and is classified as topoisomerase II inhibitor. However, previous work has shown that doxorubicin can be activated to form DNA adducts in the presence of formaldehyde-releasing prodrugs and that this leads to apoptosis independently of topoisomerase II–mediated damage. To determine which anthracyclines would be useful in combination with formaldehyde-releasing prodrugs, a series of clinically relevant anthracyclines (doxorubicin, daunorubicin, idarubicin, and epirubicin) were examined for their capacity to form DNA adducts in MCF7 and MCF7/Dx (P-glycoprotein overexpressing) cells in the presence of the formaldehyde-releasing drug pivaloyloxymethyl butyrate (AN-9). All anthracyclines, with the exception of epirubicin, efficiently yielded adducts in both sensitive and resistant cell lines, and levels of adducts were similar in mitochondrial and nuclear genomes. Idarubicin was the most active compound in both sensitive and resistant cell lines, whereas adducts formed by doxorubicin and daunorubicin were consistently lower in the resistant compared with sensitive cells. The adducts formed by doxorubicin, daunorubicin, and idarubicin showed the same DNA sequence specificity in sensitive and resistant cells as assessed by λ-exonuclease–based sequencing of α-satellite DNA extracted from drug-treated cells. Growth inhibition assays were used to show that doxorubicin, daunorubicin, and idarubicin were all synergistic in combination with AN-9, whereas the combination of epirubicin with AN-9 was additive. Although apoptosis assays indicated a greater than additive effect for epirubicin/AN-9 combinations, this effect was much more pronounced for doxorubicin/AN-9 combinations. [Mol Cancer Ther 2007;6(4):1450–9]

Introduction

Anthracyclines represent a mainstay of treatment for neoplastic diseases. The major clinically used anthracyclines consist of doxorubicin, daunorubicin, idarubicin, and epirubicin. Doxorubicin is a broad-spectrum antitumor antibiotic, and the tumors most commonly responsive to this drug include breast and esophageal carcinomas, osteosarcoma, soft tissue sarcoma, Kaposi’s sarcoma, and Hodgkin’s and non–Hodgkin’s lymphoma. However, daunorubicin (which differs from doxorubicin only by the lack of a hydroxyl group; see Fig. 1) is used mainly for the treatment of acute myelocytic and lymphocytic leukemias. Idarubicin is used for the treatment of acute myelogenous leukemias but is also active against multiple myeloma, non–Hodgkin’s lymphoma, and breast cancer, whereas epirubicin has found use in advanced breast cancer (1–3).

Daunorubicin was the first anthracycline to show antitumor activity and was isolated from the soil bacterium Streptomyces peucetius. Chemical mutagenesis of this bacterium leads to a variant strain, which produced doxorubicin (S. peucetius var. caesius; ref. 4). These anthracyclines proved useful in the treatment of various neoplasms but suffered from limitations, such as a severe dose-dependent cardiomyopathy. In a concerted effort to yield new and improved anthracyclines (an effort that has largely failed), epirubicin and idarubicin were identified as semisynthetic derivatives of doxorubicin and daunorubicin, respectively (5, 6). Epirubicin may offer some advantages over doxorubicin because it exhibits a lower cardiotoxicity, and idarubicin is unique because it may be administered orally due to its increased lipophilicity (1, 7). The structures of the various clinically used anthracyclines are shown in Fig. 1.

The anthracyclines are classified as topoisomerase II inhibitors and can evoke apoptosis via several key pathways (8). In addition, many other possible modes of action are being increasingly documented (1, 9). It has been known for several years that anthracyclines, such as doxorubicin, can also form adducts with DNA. These adducts have been termed virtual DNA cross-links because...
there is a covalent bond to only one strand of the DNA, and hydrogen bonding of the drug to the other strand of DNA endows adducts with cross-link-like characteristics, such as double-strand stabilization (10–14). However, it is only recently that this knowledge of DNA adduct formation has been exploited in the form of new drug derivatives and new approaches to therapy. Because the formation of DNA adducts requires the involvement of formaldehyde, derivatives of anthracyclines, which have been preactivated by formaldehyde (doxofom, daunoform, and epidoxoform), have provided an enormous improvement in activity against various cell lines. In particular, these drugs have activity against P-glycoprotein–overexpressing cells because they are less efficient substrates of the pump, and also, DNA adduct formation renders drugs unavailable for P-glycoprotein–mediated efflux (15–18). To overcome deleterious effects on normal tissues, these drugs have been tethered to the molecules 4-hydroxytamoxifen and cyanonilutamide, which target estrogen receptor–positive breast cancer cells and androgen-sensitive prostate cancer cells, respectively (19, 20).

An alternative approach is to combine anthracyclines with intracellular formaldehyde-releasing drugs. Pivaloyloxymethyl butyrate (AN-9) releases formaldehyde as well as butyric acid and has been used as a histone deacetylase inhibitor (21–23). The combination of daunorubicin with pivaloyloxymethyl butyrate is synergistic in various cell lines and mouse models of leukemia (24, 25). Although initially thought to be due to an interaction of the butyric acid with the anthracycline, the synergistic interaction with doxorubicin has been subsequently found to be primarily due to formaldehyde release, which results in efficient DNA adduct formation (26). These DNA adducts can initiate cell death in the absence of any topoisomerase II–mediated DNA damage (27). Combination of the formaldehyde-releasing drug hexamethylenetetramine with doxorubicin also seems promising and is not complicated by the release of other products with anticancer activity (28). The capacity of anthracyclines to form DNA adducts, and how this can be achieved in terms of formaldehyde activation strategies, has recently been reviewed (29, 30). If anthracycline/formaldehyde prodrug combinations eventually prove clinically viable, they may be able to be used simply as an extension of existing anthracycline treatment protocols.

To date, the above studies of formaldehyde-releasing drugs have focused mainly on combination with doxorubicin. In this study, we have attempted to determine the viability of combining AN-9 with the various clinically used anthracyclines in terms of efficiency of drug-DNA adduct formation. The combinations were also compared in terms of apoptosis and interaction in growth inhibition assays. Assays were also conducted in P-glycoprotein–overexpressing cells to determine whether such cell lines were particularly susceptible to combination treatments.

**Materials and Methods**

**Radiochemicals and Molecular Biology Reagents**

QIAamp blood kits for genomic DNA isolation were obtained from Qiagen (Hilden, Germany). The radionucleotides \([\alpha^{32}\text{P}]\text{dCTP}\) and \([\alpha^{32}\text{P}]\text{dATP}\) were purchased from GE Healthcare (Bucks, Switzerland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was from Sigma Diagnostics (Basil, Switzerland). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was from Sigma-Aldrich (St. Louis, MO).

**Cell Lines**

MCF7 and MCF7/Dx cells were cultured in RPMI 1640 (Invitrogen, San Diego, CA) containing 10% FCS (Thermo-Trace, Melbourne, Victoria, Australia). The MCF7/Dx cells were also supplemented with doxorubicin (300 nmol/L) during passaging. No antibiotics were used in the growth medium, and growth of cell lines was maintained at 37°C in a humidified atmosphere of 5% CO₂.

**Compounds**

Doxorubicin and daunorubicin were provided by Farmitalia Carlo Erba (now Pfizer, New York, NY), and idarubicin and epirubicin were purchased as the clinical formulations. Pivaloyloxymethyl butyrate (AN-9) was synthesized as described previously (21). Z-VAD-FMK was purchased from Promega (Madison, WI).

**Southern-Based Cross-Linking Assay**

**Preparation of Probes for Southern Hybridization.**

The plasmid pBH31R1.8 was provided by Dr. V.A. Bohr (National Institute of Aging, NIH, Baltimore, MD). A 1.8-kb EcoRI fragment containing exons I and II of the human DHFR gene (31) was isolated from pBH31R1.8 and radiolabeled with \([\alpha^{32}\text{P}]\text{dCTP}\) using a random-primed labeling kit. The mitochondrial probe pCRII-H1 was a gift from Dr. C.A. Filburn (National Institute of Aging). An EcoRI fragment containing the human mitochondrial probe (corresponding to nucleotides 652–3,226) was also prepared by random priming.
Drug Treatment of Cells for Southern Analysis. Cells were seeded in 10-cm Petri dishes at a density of 2.5 × 10^6 per dish. Cells were incubated with differing concentrations of anthracyclines (dissolved in H_2O) or AN-9 (dissolved in DMSO) in 10 mL complete medium for 4 h. The final concentration of DMSO in the medium did not exceed 0.5%. Cells were harvested and genomic DNA was isolated as described previously (26). All experiments were done in duplicate.

Detection of adducts by cross-linking assay. Genomic DNA was quantitated, restriction digested, extracted, and ethanol precipitated as described previously (26). Pellets were resuspended in 10 μL Tris-EDTA and 20 μL loading buffer containing 90% formamide, 10 mmol/L EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue (final formamide concentration of 60%). Samples were denatured at 60°C for 5 min, quenched on ice, and resolved on 0.5% agarose gels in 1× Tris-acetic acid-EDTA by overnight electrophoresis at 30 V.

The DNA was then probed using a Southern hybridization procedure as described previously (26). However, for detection of the mitochondrial genome, membranes were hybridized overnight at 65°C in 5× Denhardt’s solution, 0.5% SDS, 5× saline-sodium phosphate-EDTA, and 100 μg/mL salmon sperm DNA. Membranes were washed in 2× saline-sodium phosphate-EDTA at room temperature for 5 min and then in 2× saline-sodium phosphate-EDTA/0.1% SDS at room temperature for 15 min. The 15-min wash was repeated and membranes were washed in 0.1× saline-sodium phosphate-EDTA/0.1% SDS at room temperature for 15 min. Then, a final wash in 0.1× saline-sodium phosphate-EDTA/0.1% SDS at 60°C for 30 min was done. Membranes were exposed to phosphor plates and images were captured and the data were quantitated using a PhosphorImager (model 400B, Molecular Dynamics, Sunnyvale, CA). The interstrand DNA cross-linking frequency was calculated from the zero class of the Poisson distribution as described previously (32).

Drug Treatment and Isolation of 296-bp α-Satellite DNA Fragment

On the day before experiments, cells were seeded at a density of 2.5 × 10^6 per 10-cm Petri dish and allowed to attach overnight. Cells were treated with 4 μmol/L anthracycline and 500 μmol/L AN-9 for a total of 4 h and then harvested and washed twice with PBS. Total genomic DNA was extracted using a QIAamp blood kit, where the lysis procedure was carried out at 50°C for 30 min to minimize loss of adducts. DNA was then processed as described previously to isolate the 296-bp α-satellite DNA fragment (33). Briefly, DNA was restriction digested with EcoRI, and the 340-bp α-satellite repeat was isolated by PAGE. DNA fragments were 3’ end labeled with [γ-32P]dATP using the Klenow fragment of DNA polymerase and then restriction digested using HincII. The 296-bp band was isolated using a 5% nondenaturing polyacrylamide gel. Samples were exposed to phenol/chloroform extraction, ethanol precipitated, and resuspended in Tris-EDTA buffer. The radiolabeled 296-bp fragments were digested at 37°C for 1.5 h using λ-exonuclease to progressively release 5’ nucleotides. Maxam-Gilbert G sequencing was conducted as described previously (34). Samples were resolved by denaturing electrophoresis through 8% sequencing gels.

Growth Inhibition Assays

On the day before drug addition, cells were seeded into 96-well plates using 5 × 10^3 per well. On the following day, serial dilutions of both AN-9 and various anthracyclines were done and added to the plates to a final volume of 200 μL. For anthracycline growth inhibition assays, the maximum concentration was 10 μmol/L and this was diluted serially in medium to the lowest concentration of 0.316 nmol/L. To determine growth inhibition for AN-9, an initial concentration of 500 μmol/L was diluted serially to the lowest concentration of 15.8 nmol/L. Drugs were added to the plates alone or in combination (anthracycline to AN-9 ratio of 1:50), and additional medium was added where required to maintain the same concentrations. Cells were incubated with drug for 72 h. Cell viability was assessed by incubating cells with 100 μL of 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 3 h at 37°C, after which all liquid was removed. DMSO (100 μL) was then added to the plates that were then shaken for 10 min before absorbance readings at 570 nm. The extent of synergy was analyzed by determining the combination index (CI) as described previously (26). From this analysis, the combined effects of two drugs were assessed as either additive (zero) interaction indicated by CI = 1, synergism as indicated by CI < 1, or antagonism as indicated by CI > 1.

Apoptotic Morphology Assay

MCF7 cells were seeded in six-well plates at a density of 1 × 10^5/2 mL well. On the following day, drugs were added at the indicated concentrations and cells were incubated for 4 h. The medium was then replaced and cells were incubated for a further 44 h. The medium was collected, and cells were harvested by trypsinization and added back to the collected medium. Cells were pelleted, fixed in 3.7% paraformaldehyde, washed in PBS, and applied to polylysine-coated cover slips (35). Cells were permeabilized using 0.1% Triton X-100 and stained using Hoechst 33258 (1 μg/mL). Slides were then examined using an Olympus BX-50 fluorescence microscope (Olympus, Tokyo, Japan). Cells were scored for apoptotic morphology where at least 200 cells per treatment were analyzed. Apoptotic cells were scored as those with hypercondensed and fragmented nuclei.

Analysis of Apoptosis by Flow Cytometry

MCF7/Dx cells were seeded and treated with drugs as described for MCF7 cells in the apoptotic morphology assay. Analysis of apoptosis was done by assaying the sub-G1 cell population as described previously (27). Briefly, cells were pelleted and fixed by resuspension in 70% ethanol and incubated at room temperature for 30 min. Fixed cells were pelleted, washed once with PBS, and resuspended in DNA staining solution (25 mg/mL, 570 nm). The extent of synergy was analyzed by determining the combination index (CI) as described previously (26). From this analysis, the combined effects of two drugs were assessed as either additive (zero) interaction indicated by CI = 1, synergism as indicated by CI < 1, or antagonism as indicated by CI > 1.
propidium iodide and 100 mg/mL RNase A in PBS) and incubated at 37°C for 30 min in the dark. Samples (30,000 events) were analyzed on a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA) and gated to distinguish doublets and small debris.

Results

Southern-Based Cross-Linking Assay

To assess the capacity of the various anthracyclines to form drug-DNA adducts (or virtual DNA cross-links), a Southern-based cross-linking assay was used (36). The basis of this assay is that adduct formation renders DNA resistant to thermal denaturation in the presence of formamide, and thus, DNA migrates as a double-strand band (dsDNA) when detected by Southern hybridization. On the other hand, non–drug-reacted DNA is susceptible to denaturation and migrates as ssDNA. The percentage of dsDNA observed is then used in the Poisson distribution to calculate the number of DNA adducts per 10 kb. Drug treatment concentrations were established so that the number of adducts induced by AN-9–activated doxorubicin could be reliably quantitated in a linear region of adduct formation in this assay. Because the peak of doxorubicin adduct formation begins at 4 h and reaches a plateau thereafter (26), this time point was chosen for remaining assays. The other anthracyclines were then used under these same drug reaction conditions for comparison with doxorubicin. The result of this study is presented in Fig. 2A for both MCF7 cells and their resistant subline MCF7/Dx. Two different gene-specific probes were used: DHFR to detect adduct formation in the nuclear genome and a mitochondrial-specific probe to detect adduct formation in the mitochondrial genome. Idarubicin and daunorubicin were both efficient at inducing adducts in the presence of AN-9; however, epirubicin produced no detectable adducts. Results for epirubicin are only presented at the highest drug concentrations used because adducts were also not detected at lower levels. To further assess this reaction, a cell-free system was used to compare adduct formation by doxorubicin and epirubicin. In this assay, a linearized plasmid was reacted with drug in the presence of 2 mmol/L formaldehyde before being denatured and characterized by agarose electrophoresis. Adducts (detected as dsDNA) were detected with doxorubicin at concentrations as low as 500 nmol/L, whereas adducts were not detected with epirubicin even up to 20 μmol/L, the highest concentration used (data not shown).

Quantitation of the results shown in Fig. 2A is presented in Fig. 2C and D. In the MCF7 cells, both idarubicin and daunorubicin were superior to doxorubicin in inducing adducts in both nuclear and mitochondrial genomes, as these drugs produced ~4-fold higher levels of adducts at equimolar concentrations. In the MCF7/Dx cells, daunorubicin was superior to doxorubicin (~2-fold higher levels of adducts), whereas idarubicin produced the greatest levels of adducts (up to 10-fold higher than doxorubicin). However, because there were large errors apparent in calculating adduct levels via the Poisson distribution

![Figure 2. Southern-based cross-linking assay of anthracyclines in combination with AN-9 in MCF7 and MCF7/Dx cells. A, cells were treated with anthracyclines doxorubicin (DOX), idarubicin (IDA), daunorubicin (DAUNO), and epirubicin (EPI) at the indicated concentrations (0–3 μmol/L in MCF7 cells; 0–6 μmol/L in MCF7/Dx cells) in the presence of 200 μmol/L AN-9. Cells were harvested, and DNA was extracted for Southern analysis. Results from the mitochondrial genome and DHFR gene are shown where migration of drug-stabilized dsDNA (DS) and non–drug-stabilized ssDNA (SS) is indicated. B, the anthracyclines indicated (0–2 μmol/L) were used to treat cells in the presence of 200 μmol/L AN-9. Cells were then harvested and processed for Southern analysis. C, dose response of adduct formation. Quantitation of adducts in MCF7 cells (A, left). Data were quantitated to calculate the number of drug adducts per 10 kb. Closed symbols, mitochondrial genomes; open symbols, DHFR gene. Doxorubicin results are shown by dotted lines with the symbols (∆) or (Δ) representing mitochondrial genomes and the DHFR gene, respectively. Idarubicin results are represented by dashed lines with the symbols (▲) or (△), and daunorubicin results are represented by solid lines with the symbols (●) or (○). D, quantitation of adducts in MCF7/Dx cells (A, right). E, quantitation of adducts in MCF7 cells (B). F, quantitation of adducts in MCF7/Dx cells (B).]
Table 1. Summary table showing concentrations (μmol/L) required to achieve adduct levels of 0.2 and 0.5 adduct per 10 kb from various anthracyclines in nuclear and mitochondrial genomes

<table>
<thead>
<tr>
<th>Drug (μmol/L)</th>
<th>DHFR gene</th>
<th>Mitochondrial genome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 adduct/10 kb</td>
<td>0.5 adduct/10 kb</td>
</tr>
<tr>
<td></td>
<td>MCF7</td>
<td>MCF7/Dx</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>0.24</td>
<td>0.44</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.37</td>
<td>2.0</td>
</tr>
</tbody>
</table>

NOTE: Data were generated from Fig. 2.

(where cross-link levels approach 100%), drug treatment levels were decreased so that adduct levels would be in the linear detectable region of the Poisson distribution. These results are presented in Fig. 2B, and quantitation is presented in Fig. 2E and F. Daunorubicin and idarubicin produced adducts at a similar efficiency in the MCF7 cells, and unlike the other anthracyclines used, idarubicin was only slightly less efficient in forming adducts in the MCF7/Dx cells compared with the MCF7 cells. To provide a rigorous comparison of these drugs and produce an efficiency ranking, drug concentrations required to achieve adduct levels of 0.2 and 0.5 adduct per 10 kb are presented in Table 1. Several conclusions can be drawn from the values in this table. (a) Slightly higher levels of anthracycline are generally needed to form adducts in the mitochondrial genome versus the DHFR gene. (b) Although idarubicin and daunorubicin have similar adduct-forming capacity in MCF7 cells, idarubicin is by far the most effective compound in the MCF7/Dx cells. For instance, the concentration required to form 0.5 adduct/10 kb in the DHFR gene in the MCF7 versus MCF7/Dx cells is similar, and in other examples, although the concentration of idarubicin required to form similar adduct levels in the MCF7 versus MCF7/Dx/Dx cells is higher for the MCF7/Dx cells, this value is always less than a 2-fold difference in concentration. (c) Both doxorubicin and daunorubicin are less effective in MCF7/Dx cells. (d) Doxorubicin is the least efficient compound overall (of the compounds where adducts can be detected); however, daunorubicin yields the greatest difference in adduct-forming ability when the MCF7 cells are compared with the MCF7/Dx cells, and therefore, it seems that the adducts formed by this agent are most affected by P-glycoprotein overexpression.

Sequence-Specific Drug Binding Assay

To analyze the DNA sequence specificity of various anthracyclines in comparison with doxorubicin, the 296-bp α-satellite fragment was isolated from MCF7 and MCF7/Dx cells treated with each of the anthracyclines and AN-9 and was subjected to λ-exonuclease digestion. Adducts cause direct blockages to the stepwise digestion of λ-exonuclease, and the remaining lengths of radiolabeled fragment therefore reveal the location of adduct binding sites. The digestion patterns of λ-exonuclease are shown in Fig. 3A. Doxorubicin produced a characteristic pattern of digestion by the exonuclease as the truncated digestion products occurred at GC and GG sequences as previously observed (33). The same sequence specificity was also observed for doxorubicin in the resistant cells, albeit at a reduced frequency. Idarubicin and daunorubicin also produced a very similar sequence specificity pattern to doxorubicin; however, the level of idarubicin adducts was not significantly reduced in the resistant cells, whereas daunorubicin adduct levels were lowered. The epirubicin-treated cells exhibited a completely different pattern of sequence specificity, and this pattern was also reduced in the resistant cells. However, this same pattern of specificity was also present in control lanes (AN-9 only) at a lowered frequency. Replicate experiments revealed that these blockages were often observed at a higher frequency in control lanes or not produced significantly in epirubicin-treated lanes (see Fig. 3B for example). A qualitative comparison was made between the sequences modified by doxorubicin compared with daunorubicin and idarubicin (Supplementary Data 1), where it is apparent that all three drugs yield a virtually identical pattern of adduct formation (mainly at 5'-GG and GC sequences); however, idarubicin was more selective for GG sequences because the two high-intensity blockages before 5'-GC sequences (at positions 241 and 273) observed for doxorubicin and daunorubicin were absent in idarubicin-reacted samples.

To establish whether the drug-DNA adducts formed by the anthracyclines exhibited similar characteristics, their melting profile was assessed. Doxorubicin is known to form thermally labile, sequence-specific adducts as evidenced in Fig. 3B by a decline with increasing temperature. Idarubicin and daunorubicin also exhibited a similar melting profile. The band at 120 was quantitated to permit a comparison of melting profiles at a single adduct site corresponding to midpoint melting temperatures of 74.4°C, 70.2°C, and 69.2°C for doxorubicin, idarubicin, and daunorubicin, respectively. The nonspecific blockages produced in epirubicin-reacted samples and control lanes (also in the 80°C lanes of doxorubicin- and idarubicin-reacted samples).
idarubicin-reacted samples after thermal denaturation of adducts) persisted after high temperature treatment, thus distinguishing them from anthracycline adducts. Interestingly, epirubicin-reacted samples exhibited three low-intensity blockage sites, which were lost at higher temperature (at locations 120, 190, and 225 and indicated by arrows in Fig. 3B), implying a minor degree of adduct formation compared with the other anthracyclines. These blockages were all before GG sequences, although a GC was also present at the blockage at position 120.

**Drug Interaction in Growth Inhibition Assays**

It is known that the combination of doxorubicin and AN-9 is synergistic in several cell lines, including MCF7. To compare the various anthracyclines in combination, and to establish if this synergy was maintained or improved in resistant cells, the combinations were used in growth inhibition assays. The results of this study are presented in Table 2. The anthracycline values for growth inhibition in the MCF7 cells were similar; however, the drugs were less efficient in the MCF7/Dx cells as expected. The resistance indices obtained for doxorubicin, daunorubicin, and epirubicin were all \( \geq 30 \). Although idarubicin was less active against the MCF7/Dx cells, the resistance index was reduced to 6.8. AN-9 was similarly active against both sensitive and resistant cell lines. When used in combination, it is apparent that epirubicin was the only drug that was not synergistic in combination with AN-9 in either the sensitive or resistant cells. Although combinations involving doxorubicin, idarubicin, and daunorubicin were all synergistic in the resistant cells, anthracycline activity was never restored to the equivalent degree observed in the MCF7 cells, indicating that the combinations would not fully overcome the resistance phenotype.

Because doxorubicin adduct levels were optimal at 4 h, the growth inhibition assay was repeated for doxorubicin and epirubicin in MCF7 cells using this short-term 4-h treatment, and then, cells were incubated in drug-free medium for the remainder of the assay. These conditions have been previously shown to reduce the effectiveness of doxorubicin as a single agent presumably because topoisomerase II–mediated damage can be minimized or more effectively repaired (27). The results of this short-term treatment are presented in Table 3, where it is apparent that indeed doxorubicin was markedly less effective as a single agent compared with the 72-h drug incubation presented in Table 2 and that synergy between doxorubicin and AN-9 was more striking under these conditions. However, even under these conditions, epirubicin remained ineffective in combination with AN-9.

**Apoptosis Assays**

The combination of doxorubicin and AN-9 is a potent inducer of apoptosis in HL60 cells (27). The extent of apoptosis in doxorubicin/AN-9 versus epirubicin/AN-9 treatments was monitored to test if synergistic growth inhibition was mediated through apoptosis in the MCF7 cells. MCF7 cells do not have functional caspase-3; hence, apoptosis cannot be easily assessed by DNA fragmentation or caspase-3 activity assays. Therefore, simple morphology assays were used to measure apoptosis. The drug concentrations chosen for doxorubicin and AN-9 were based on their IC\(_{50}\) values (Table 3). These same concentrations were then used for epirubicin to maximize any possible interaction with AN-9. The apoptosis morphology data are presented in Fig. 4A, where the doxorubicin combination resulted in very high levels of apoptosis compared with minimal levels with either agent alone.
Epirubicin as a single agent induced ~5-fold higher levels of apoptosis than doxorubicin (as expected based on its greater inhibitory effect presented in Table 3), whereas the epirubicin/AN-9 combination seemed to induce a greater than additive effect. Although the epirubicin combination was greater than additive, the degree of epirubicin potentiation by AN-9 was not as pronounced as the degree of doxorubicin potentiation by AN-9 (3.2-fold compared with 40-fold, respectively). Z-VAD-FMK is a broad-spectrum inhibitor of caspases and was used to verify that the morphology assay was indeed detecting apoptosis and not other forms of cell death. The failure of apoptosis to revert to background levels was addressed by using higher concentrations of Z-VAD-FMK, but this did not decrease the level of apoptosis further (data not shown). It is probable that this level of apoptosis reflects apoptosis that has already progressed during the initial 4-h drug incubation.

The extent of apoptosis was also examined in MCF7/Dx cells. The apoptosis morphology assay was initially used to measure apoptosis; however, MCF7/Dx cells undergo far more extensive nuclear fragmentation than MCF7 cells, and because nuclei became highly fragmented, it was not possible to attribute these dispersed fragments to individual cells. Flow cytometry was therefore used as an alternative procedure to measure apoptosis in these cells. Because such different methods were used to assess apoptosis in the two different cell lines, absolute levels of apoptosis cannot be compared between the two methods. Regardless, as shown in Fig. 4B, the doxorubicin-resistant cells were very similar to MCF7 cells in their pattern of response to the various drugs with the doxorubicin/AN-9 combination, again leading to the highest levels of apoptosis.

### Discussion

#### Formation of Adducts by Anthracyclines

The studies relating to efficiency of adduct formation revealed a clear hierarchy among the four clinical anthracyclines, where daunorubicin and idarubicin were the best adduct-forming compounds in drug-sensitive cells, whereas idarubicin was by far the most effective in resistant cells. It is unlikely that differing affinities for cellular DNA are responsible for these effects. In previous studies of adduct formation by these anthracyclines in a cell-free system, doxorubicin, daunorubicin, and idarubicin displayed similar reaction kinetics (28). Furthermore, the relative DNA binding of daunorubicin, idarubicin, and doxorubicin using isolated calf thymus DNA does not correlate with our results (37). However, these results may be explained in terms of the higher lipophilicity of both daunorubicin and idarubicin in comparison with doxorubicin. The lipophilicity, as determined by octanol/aqueous partition coefficients (fluorescence of octanol phase divided by fluorescence of NaCl/phosphate buffer phase), was 0.8 for doxorubicin, 3.5 for daunorubicin, and 15 for idarubicin (38), potentially indicative of a higher cellular uptake of anthracyclines, where daunorubicin and idarubicin were the best adduct-forming compounds in drug-sensitive cells, whereas idarubicin was by far the most effective in resistant cells.

#### Table 2. Activity of anthracyclines and their interaction with AN-9 in MCF7 and MCF7/Dx cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCF7 IC₅₀</th>
<th>MCF7 CI</th>
<th>MCF7/Dx IC₅₀</th>
<th>MCF7/Dx CI</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>329 ± 75</td>
<td>0.62</td>
<td>9,184 ± 1,375</td>
<td>0.79</td>
<td>27.9</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>141 ± 39</td>
<td>0.62</td>
<td>964 ± 283</td>
<td>0.69</td>
<td>6.8</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>223 ± 31</td>
<td>0.62</td>
<td>6,849 ± 2,719</td>
<td>0.75</td>
<td>30.7</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>328 ± 78</td>
<td>0.62</td>
<td>9,709 ± 546</td>
<td>0.75</td>
<td>29.6</td>
</tr>
<tr>
<td>AN-9</td>
<td>35.5 ± 12.3*</td>
<td>0.74</td>
<td>53.0 ± 12.8*</td>
<td>0.79</td>
<td>4</td>
</tr>
<tr>
<td>AN-9 + doxorubicin</td>
<td>142 ± 61</td>
<td>0.74</td>
<td>566 ± 164</td>
<td>0.79</td>
<td>4</td>
</tr>
<tr>
<td>AN-9 + idarubicin</td>
<td>53.2 ± 16</td>
<td>0.74</td>
<td>360 ± 76</td>
<td>0.69</td>
<td>6.8</td>
</tr>
<tr>
<td>AN-9 + daunorubicin</td>
<td>212 ± 41</td>
<td>0.74</td>
<td>507 ± 56</td>
<td>0.7</td>
<td>2.4</td>
</tr>
<tr>
<td>AN-9 + epirubicin</td>
<td>308 ± 45</td>
<td>0.74</td>
<td>1,495 ± 302</td>
<td>1.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Note: Cells were treated as described in Materials and Methods. Growth inhibition values are shown in nmol/L, except for AN-9 values, which are shown in μmol/L (*), and where combined treatments are described, only the anthracycline growth inhibition value is shown (the corresponding AN-9 concentration is 50-fold higher). Growth inhibition of 50% (IC₅₀) of cells and their combination index values are presented. The figures obtained represent the average of three to five independent experiments, each consisting of four replicates. Errors represent SE. The resistance index is a measure of the difference of activity of the indicated anthracycline against MCF7 versus MCF7/Dx cells.

**Abbreviations:** CI, combination index; RI, resistance index.

#### Table 3. Activity of anthracyclines and their interaction with AN-9 in MCF7 cells after short-term drug treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>810 ± 220</td>
<td></td>
</tr>
<tr>
<td>Epirubicin</td>
<td>370 ± 100</td>
<td></td>
</tr>
<tr>
<td>AN-9</td>
<td>93 ± 1*</td>
<td></td>
</tr>
<tr>
<td>AN-9 + doxorubicin</td>
<td>166 ± 1</td>
<td>0.3</td>
</tr>
<tr>
<td>AN-9 + epirubicin</td>
<td>304 ± 83</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Note: Cells were treated as described in Materials and Methods, except that drug treatment was for 4 h, and then, cells were incubated in drug-free medium for the remainder of the assay. Growth inhibition values are shown in nmol/L, except for AN-9 values, which are shown in μmol/L (*), and where combined treatments are described, only the anthracycline growth inhibition value is shown (the corresponding AN-9 concentration is 50-fold higher). Growth inhibition of 50% (IC₅₀) of cells and their combination index values are presented. The figures obtained represent the average of two independent experiments, each consisting of eight replicates. Errors represent the SD.
daunorubicin and idarubicin in the sensitive MCF7 cells. However, the vast improvement in adduct formation displayed by idarubicin in MCF7/Dx cells may be explained in terms of a reduced propensity of this drug to be removed effectively by P-glycoprotein–overexpressing cells, and there have been many reports in the literature describing the greater effectiveness of idarubicin versus other anthracyclines, such as daunorubicin and doxorubicin, in P-glycoprotein–overexpressing cells. However, rather than representing a poor substrate for P-glycoprotein efflux, most of these studies have concluded that idarubicin is in fact a good substrate for P-glycoprotein and its increased effectiveness against these cells is due to favorable cell uptake kinetics (39–42). When a study was conducted on a group of 98 acute myelogenous leukemia patients to assess why idarubicin may be superior to daunorubicin in acute myelogenous leukemia treatment, no correlation of P-glycoprotein with improved response rate was found (43).

In clear contrast to the other anthracyclines studied, epirubicin showed little evidence of adduct formation. This agrees with a previous study in a cell-free system where epirubicin showed only background levels of adduct formation in comparison with other anthracyclines where the formaldehyde was provided using the pH-dependent formaldehyde-releasing compound hexamethylenetetramine (28). However, in other studies, epirubicin has been observed to form DNA adducts (16, 17, 44, 45). It is important to consider the different experimental conditions used and the characteristics of the observed adducts to attempt to reconcile this discrepancy. The adducts formed by epidoxorform and epirubicin plus formaldehyde were analyzed by high-performance liquid chromatography detection of drug bound to (GC)₄ oligonucleotides. Five structurally different adducts were found (16). In a subsequent study, the cellular retention of doxoform and epidoxorform fluorescence was used to calculate adduct half-lives. It was concluded that drug was released slower from virtual cross-linking sites in the case of doxoform compared with epidoxorform due to a decreased stability of epidoxorform adducts (17). The crystal structure of the epirubicin-formaldehyde virtual cross-link of DNA has been solved (45), and in this structure, it was shown that (as observed with other anthracycline-DNA complexes) the aglycone of epirubicin intercalates at CpG, the D ring reaches into the major groove, and the sugar moiety lies in the minor groove (12). However, compared with the daunorubicin-formaldehyde virtual cross-link, there were differences in hydrogen bonding. Overall, these differences suggest that daunorubicin is bound more effectively to the covalently bonded strand, whereas epirubicin is held more tightly to the opposing strand (17). Overall, there are several differences between epirubicin and doxorubicin/daunorubicin adducts that may explain the apparent discrepancy with our observation. (a) If the epirubicin adduct exhibits an altered structure and is less stable, it may be less amenable to the rigorous isolation conditions required in the current study to do the Southern and sequence-specific analyses. These conditions require DNA isolation at 50°C, phenol/chloroform extraction, and ethanol precipitation. Furthermore, for Southern analyses, DNA must be denatured in formamide, and for the sequence-specific analyses, isolation of the DNA required takes over 2 days where the DNA is subjected to extended incubations at 4°C. Although these conditions suit the isolation of doxorubicin adducts, this may not be appropriate for epirubicin. (b) The initial reaction of doxorubicin and daunorubicin with DNA may be more efficient when an initial cyclization to a five-membered mono-oxazolidine ring forms (16). However, this cannot occur in the case of epirubicin due to the equatorial position of the hydroxyl group.
group at C-4' (Fig. 5). The different structures of the anthracycline prodrugs daunoform and doxoform, in comparison with epidoxoform, illustrate this point (16).

(c) Epidoxoform exhibits an ~70-fold lower potency against MCF7 and MCF7/ADR cells compared with doxoform, and this may at least in part be due to reduced efficiency of adduct formation or altered structure of the DNA adducts.

Drug Interactions
In previous studies, doxorubicin and daunorubicin are synergistic in combination with AN-9 (24–26). The combination is synergistic when doxorubicin treatment is simultaneous with (or before) AN-9; however, it is antagonistic when AN-9 treatment precedes doxorubicin. These observations correlate well with the levels of DNA adduct formation (i.e., levels of DNA adducts are much higher under synergistic conditions). The critical involvement of formaldehyde has been established by sequestration of formaldehyde with semicarbazide, which ablated both adduct formation and cell sensitivity to the combination treatment (26). Furthermore, a series of AN-9 derivatives were used to confirm that formaldehyde was absolutely required for these effects (26, 46). In the present study, we have assessed the suitability of various clinically used anthracyclines to achieve these types of effects in combination with AN-9. It is apparent that doxorubicin, daunorubicin, and idarubicin all exhibit synergy in combination with AN-9, and this is accompanied by adduct formation. However, epirubicin does not display this synergy, and this is consistent with a lack of observed adduct formation. Idarubicin seems particularly useful in combination with AN-9, and its enhanced cellular uptake may render it available for P-glycoprotein–mediated cellular efflux. The mode of cell death induced by the combinations seems to be apoptosis as indicated using the apoptosis morphology assay and the Z-VAD-FMK apoptosis inhibitor. This is consistent with our previous observations in HL60 cells where cell death was blocked by Bcl-2 overexpression (27). We are currently investigating the DNA damage response pathways that initiate this apoptotic response.

In summary, we have identified combinations of anthracyclines and AN-9 that synergistically inhibit the growth of sensitive and resistant tumor cells. These combinations represent conditions where adduct formation can be achieved, and this is consistent with our previous studies of doxorubicin and AN-9 where adduct formation always accompanies the synergy observed. This now extends the repertoire of drugs that can be used to achieve such effects. The current results suggest that idarubicin would seem to be the most favorable drug to use in combination with formaldehyde-releasing drugs in anthracycline-sensitive and anthracycline-resistant tumors. Daunorubicin and doxorubicin are also useful alternatives, but the use of epirubicin in such circumstances would be predicted to be less effective. Confirmation of these recommendations would require testing in suitable animal models.

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References


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Activation of clinically used anthracyclines by the formaldehyde-releasing prodrug pivaloyloxymethyl butyrate


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