Doxoform and Daunoform: Anthracycline−Formaldehyde Conjugates Toxic to Resistant Tumor Cells

David J. Fenick, Dylan J. Taatjes, and Tad H. Koch*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received April 8, 1997[⊗]

The recent discovery that the clinically important antitumor drugs doxorubicin and daunorubicin alkylate DNA *via* catalytic production of formaldehyde prompted the synthesis of derivatives bearing formaldehyde. Reaction of the parent drugs with aqueous formaldehyde at pH 6 produced in 40−50% yield conjugates consisting of two molecules of the parent drug as oxazolidine derivatives bound together at their 3′-nitrogens by a methylene group. The structures were established as bis(3′-*N*-(3′-*N*,4′-*O*-methylenedoxorubicinyl))methane (Doxoform) and bis(3′-*N*-(3′-*N*,4′-*O*-methylenedaunorubicinyl))methane (Daunoform) from spectroscopic data. Both derivatives are labile with respect to hydrolysis to the parent drugs. 3′-*N*,4′-*O*-Methylenedoxorubicin and 3′-*N*,4′-*O*-methylenedaunorubicin are intermediates in the hydrolysis. Daunoform reacts with the self-complementary deoxyoligonucleotide $(GC)_4$ faster than the combination of daunorubicin and formaldehyde at an equivalent concentration to give drug−DNA adducts. In spite of hydrolytic instability, Doxoform is 150-fold more toxic to MCF-7 human breast cancer cells and 10000-fold more toxic to MCF-7/ADR resistant cells. Toxicity to resistant cancer cells is interpreted in terms of higher lipophilicity of the derivatives and circumvention of catalytic formaldehyde production.

Introduction

Doxorubicin (adriamycin) continues to be one of the most important antitumor drugs in the clinic. It is a broad spectrum drug particularly useful in the treatment of Hodgkin's disease, non-Hodgkin lymphomas, acute leukemias, sarcomas, and solid tumors of the breast, lung, and ovary.[1] The closely related drug daunorubicin (daunomycin) is used primarily for the treatment of acute leukemia. A major problem associated with doxorubicin and daunorubicin chemotherapy is multidrug resistance. Multidrug resistance is characterized by resistance to several drugs developed by tumor cells upon treatment with one drug. Mechanisms proposed for tumor cell multidrug resistance include overexpression of cell membrane proteins which enhance efflux of the drug and overexpression of glutathione transferase which transforms xenobiotics to glutathione conjugates for excretion.[2−5] Glutathione itself is also thought to be involved in resistance in a variety of tumors.[6] Resistance to anthracycline antitumor antibiotics has been shown to involve a lower concentration of drug-produced reactive oxygen species, presumably resulting from overexpression of enzymes which destroy superoxide and hydrogen peroxide.[7]

In spite of intensive investigation of the mode of action of doxorubicin and daunorubicin, the events leading to cell death and differential cytotoxicity are not totally understood. This has hindered the development of new analogs which both are more effective and overcome multidrug resistance. Both drugs are excellent DNA intercalators[8] and have been shown to concentrate in the cell nucleus.[9,10] Crystallographic data have established specific sequences as the sites of drug intercalation.[11,12] The drugs are redox active through the quinone functionality and are substrates for one-electron redox enzymes such as xanthine oxidase,[13,14] cytochrome P450 reductase,[14] and mitochondrial NADH dehydrogenase.[15] Furthermore, reduction in the presence of molecular oxygen results in catalytic production of superoxide and hydrogen peroxide.[16−18] In an anaerobic environment, reduction leads to glycosidic cleavage to produce a quinone methide transient,[19−21] long thought to be an alkylating agent for DNA.[22] Currently, the most popular explanation for cytotoxicity is induction of topoisomerase-mediated DNA strand breaks through intercalation,[23] with modulation through a signaling cascade involving a cell membrane receptor for doxorubicin.[24]

Recent reports from several laboratories have rekindled interest in the concept of drug alkylation of DNA *via* a redox pathway as an important cytotoxic event. Phillips and co-workers reported in a series of papers that *in vitro* reductive activation of doxorubicin and daunorubicin in the presence of DNA led to transcription blockages.[25−29] These transcription blockages were attributed to the alkylation and cross-linking of DNA by reductively activated drug, possibly involving a quinone methide transient. The site of alkylation and cross-linking was proposed to be the 2-amino substituents of 2′-deoxyguanosines at the location 5′-GpC-3′ in DNA. At about the same time, Skladanowski and Konopa established cross-linking of DNA by doxorubicin in HeLa S_3 cells using a mild DNA denaturation−renaturation assay.[30,31] They concluded that DNA cross-links, although unstable to isolation, induced tumor cell apoptosis.[32] We have recently demonstrated that the reported DNA alkylation and cross-linking does not involve the intermediacy of the quinone methide. The primary purpose of reductive activation of doxorubicin and daunorubicin is the production of superoxide and hydrogen peroxide.[33,34] These two reduced dioxygen species oxidize constituents in the medium to formaldehyde via Fenton chemistry.[35] The resulting formaldehyde couples the 3′-amino group of intercalated doxorubicin or daunorubicin to the 2-amino group of

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1997.

S0022-2623(97)00237-9 CCC: $14.00

Scheme 1

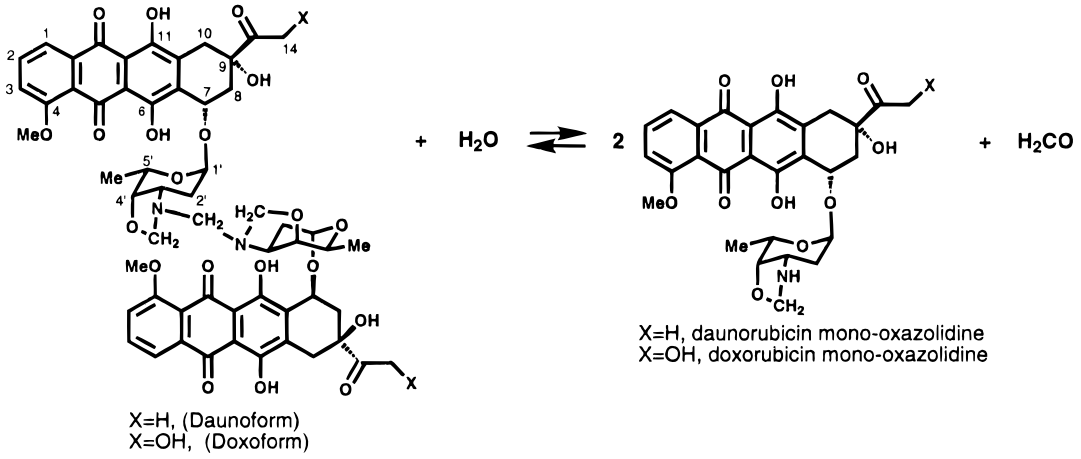
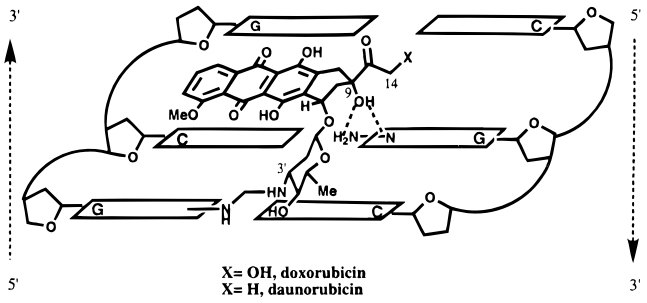


Chart 1



deoxyguanosine via Schiff base chemistry. Thus, what Phillips and co-workers call a DNA "cross-link" by drug at 5′-GpC-3′ [25] we describe as a *virtual cross-link* involving one covalent bond from formaldehyde and one intercalative hydrogen-bonding interaction with the opposing strand.[34,36] This *virtual cross-link* is shown in Chart 1 for the DNA sequence 5′-CpGpC-3′.

This new mechanism for the alkylation of DNA prompted a study of the reaction of doxorubicin and daunorubicin with formaldehyde. In this paper, we report the synthesis, characterization, and cytotoxicity of two new derivatives which result from reaction of formaldehyde at the amino sugar groups of doxorubicin and daunorubicin. These products, denoted Doxoform and Daunoform, respectively, are dimeric oxazolidines which rapidly hydrolyze back to the parent drugs and formaldehyde in aqueous medium. In spite of this lability, these derivatives show enhanced cytotoxicity toward tumor cells, especially resistant tumor cells, presumably because they do not require drug-induced intracellular production of formaldehyde.

Results and Discussion

Preparation and Characterization of Daunoform and Doxoform. Reaction of daunorubicin with an excess of formaldehyde in pH 6 buffer results in the production of a new derivative, assigned the trivial name Daunoform, in 70% yield (Scheme 1). The product was initially isolated as an amorphous solid and subsequently crystallized yielding crystalline Daunoform in 51% overall yield. The yield of Daunoform was found to be independent of the amount of excess formaldehyde used during the reaction (70% yield of amorphous material using 50 or 10 equiv of formaldehyde). An analogous derivative, Doxoform (Scheme 1), was obtained in 70% yield as an amorphous material from reaction of doxorubicin with an excess of formaldehyde. Crystalline Doxoform was obtained in 37% overall yield.

The structures of both derivatives were determined from spectroscopic data. Positive ion electrospray mass spectra of Daunoform and Doxoform exhibited intense M + 1 ions at m/z 1091 and 1123, respectively. The molecular weights indicate structures consisting of two drug molecules bound together with three methylene groups. ^{1}H NMR spectra established the structures as bis(3′-*N*-(3′-*N*,4′-*O*-methylenedaunorubicinyl))methane and bis(3′-*N*-(3′-*N*,4′-*O*-methylenedoxorubicinyl))methane (Daunoform and Doxoform, respectively; Scheme 1) with the assignments shown in Table 1. The assignments were made using detailed splitting patterns, two-dimensional homonuclear COSY spectra, and a reference spectrum of the free base of daunorubicin (Table 3, Supporting Information). Of particular significance in the one-dimensional spectrum are the two doublets at δ 4.18 and 4.71 with a small geminal coupling constant of 4 Hz. These chemical shifts and coupling constant are characteristic of a methylene group in a five-membered ring flanked by electron-withdrawing heteroatoms.[37] A feature indicative of two oxazolidines coupled at their nitrogens by a methylene group is the singlet at δ 3.48 which integrates to only one proton. Also of note in the spectrum of Daunoform is the size of the vicinal coupling constant for one of the 2′-protons with the 3′-proton relative to the analogous coupling constant for daunorubicin: 6 Hz versus 13 Hz. This reduction in the coupling constant is consistent with the anticipated reduction in the 2′-H/3-′H dihedral angle due to formation of an oxazolidine ring. The formation of these bis-oxazolidinylmethanes is due solely to the interaction of the amino alcohol functionality of the parent drugs with formaldehyde. Such reactions are precedented in the reaction of the much simpler *trans*-2-aminocycloheptanol and *trans*-2-aminocyclooctanol with formaldehyde.[38]

Hydrolysis. Daunoform was stable for a period of days in dry deuteriochloroform. Addition of 1 drop of deuterium oxide to the deuteriochloroform solution resulted in the formation of a mixture of Daunoform with the mono-oxazolidine (3′-*N*,4′-*O*-methylenedaunorubicin, Scheme 1) as established by periodic monitoring of the ^{1}H NMR spectrum. The structure for the mono-oxazolidine was determined by the ^{1}H NMR spectrum (Table 1). Of particular significance was the disappear-

Table 1. 400 MHz ^{1}H NMR Data for Daunoform, 3′-*N*,4′-*O*-Methylenedaunorubicin, Doxoform, and 3′-*N*,4′-*O*-Methylenedoxorubicin

chemical shifts for protons at positions (patterns, *J* coupling constants in Hz)																	
																CH_2	
1	2	3	4	6/11	7	8	9	10	14	1′	2′	3′	4′	5′	5′-Me	O,N	N,N
Bis(3′-*N*-(3′-*N*,4′-*O*-methylenedaunorubicinyl))methane (Daunoform) ($DCCl_3$, acidic protons identified by D_2O exchange)																	
7.84 (d, 8)	7.65 (t, 8)	7.20 (d, 8)	3.88 (s)	13.80 (s) 12.99 (s)	5.10 (bs)	2.46 (d, 15) 1.84 (dd, 15, 4)	4.77 (s)	3.04 (d, 18) 2.82 (d, 18)	2.39 (s)	5.47 (t, 6)	2.12 (dt, 15, 6) 1.75 (dt, 15, 6)	3.37 (q, 7)	3.93 (d, 7)	4.05 (q, 6)	1.34 (d, 6)	4.71 (d, 4) 4.18 (d, 4)	3.48 (s)
3′-*N*,4′-*O*-Methylenedaunorubicin ($DCCl_3$, acidic protons identified by D_2O exchange)																	
8.02 (d, 8)	7.78 (t, 8)	7.38 (d, 8)	4.07 (s)	13.96 (s) 13.29 (s)	5.28 (dd, 1.5, 4)	2.33 (dt, 15, 1.5) 2.07 (dd, 15, 4)	4.55 (s)	3.22 (dd, 1.5, 18) 2.96 (d, 18)	2.40 (s)	5.49 (t, 5)	1.81 (ddd, 4, 6, 17) 1.73 (ddd, 5, 7, 17)	3.61 (q, 7)	4.21 (dd, 2, 6)	4.16 (qd, 6, 2)	1.40 (d, 6)	4.93 (d, 7.5) 4.65 (d, 7.5)	
Bis(3′-*N*-(3′-*N*,4′-*O*-methylenedaunorubicinyl))methane (Daunoform) (DMSO-d_6, acidic protons identified by D_2O exchange)																	
7.58 (d, 8)	7.71 (t, 8)	7.37 (d, 8)	3.77 (s)	13.83 (s) 12.94 (s)	4.94 (dd, 5, 2)	2.29 (dd, 12, 2) 1.98 (dd, 12, 5)	5.38 (s)	2.93 (d, 17) 2.81 (d, 17)	2.23 (s)	5.26 (t, 5)	1.67 (dt, 14, 5) 1.90 (bdd, 14, 5)	3.37 (~t, 5) next to CH_2	3.85 (d, 6)	4.06 under CH_2	1.22 (d, 6)	4.54 (d, 4) 4.06 (d, 4)	3.35 (s)
Bis(3′-*N*-(3′-*N*,4′-*O*-methylenedoxorubicinyl))methane (Doxoform) ($DCCl_3$)																	
7.86 (d, 8)	7.69 (t, 8)	~7.2 under $HCCl_3$	3.89 (s)	13.78 (s) 12.93 (s)	5.09 (bs)	2.48 (d, 15) 1.89 (dd, 15, 3)		3.05 (d, 19) 2.87 (d, 19)	4.74 (s)	5.47 (t, 6)	2.15 (m) 1.75 (m)	3.37 (bq, 7)	3.95 (d, 7)	4.00 (q?, 7)	1.34 (d, 7)	4.71 (d, 4) 4.18 (d, 4)	3.49 (s)
3′-*N*,4′-*O*-Methylenedoxorubicin ($DCCl_3$)																	
8.01 (d, 8)	7.77 (t, 8)	7.38 (d, 8)	4.07 (s)	13.94 (s) 13.23 (s)	5.30 (bs)	2.42 (d, 14.5) 2.13 (dd, 14.5, 2)		3.26 (dd, 19, 1) 3.03 (d, 19)	4.74 (s)	5.49 (t, 2)	1.78 (m)	3.58 (q, 6.5)	4.21 (dd, 7, 1)	4.1 under 4-OMe	1.39 (d, 6.5)	4.92 (d, 7) 4.62 (d, 7)	

ance of the singlet at δ 3.48 and the appearance of a singlet at δ 9.71 for formaldehyde. The two doublets for the CH_2 group of the oxazolidine shifted slightly, and the geminal coupling constant increased slightly. The vicinal coupling constant between one of the 2′-protons and the 3′-proton remained 6 Hz. The presence of mono-oxazolidine is also evident in the electrospray mass spectrum of a chloroform/methanol solution of Daunoform, producing a strong M + 1 ion at m/z 540.

Additional experiments established that the ratio of mono- to bis-oxazolidine in deuteriochloroform was also a function of Daunoform concentration, with more mono-oxazolidine present at lower Daunoform concentrations. These experiments were performed in deuteriochloroform not dried by distillation from phosphorous pentoxide. The concentration effect is explained in terms of trace amounts of water dissolved in the deuteriochloroform and an equilibrium between a dimeric species and a monomeric species. The composition, as determined by integration of the phenolic OH signals relative to the chloroform signal, followed the anticipated mathematical relationship (eq 1) with an average deviation from the mean of 16%:

$$\text{constant} = (\text{monomer signal area})^2/[(\text{dimer signal area})(\text{chloroform signal area})] \quad (1)$$

Addition of deuterium oxide to a solution of Doxoform resulted in hydrolysis to the respective mono-oxazolidine (3′-*N*,4′-*O*-methylenedoxorubicin, Scheme 1) as established by ^{1}H NMR spectroscopy (Table 1). Continued addition of D_2O to the Doxoform solution eventually resulted in hydrolysis to doxorubicin. Extraction of doxorubicin into the deuterium oxide was evident by a separate ^{1}H NMR spectrum of the deuterium oxide layer. The presence of mono-oxazolidine was also evident in the electrospray mass spectrum of a chloroform/methanol solution of Doxoform, with an intense M + 1 ion at m/z 556. Furthermore, the ratio of mono- to bis-oxazolidines in wet deuteriochloroform showed the same concentration dependence as observed for Daunoform.

Because DMSO was used as the solvent for introducing Daunoform and Doxoform to cell cultures, the stability of the compounds was also explored in dimethyl sulfoxide. A solution of Daunoform in dry DMSO-d_6 was stable for at least 4 days as indicated by ^{1}H NMR spectroscopy. Addition of a small amount of deuterium oxide led to the formation of an equilibrium mixture of Daunoform and an intermediate on the pathway to daunorubicin. An unambiguous structural assignment for the intermediate could not be made because of the complexity of the NMR spectrum. The intermediate was formed without release of formaldehyde, and its spectrum showed an AX pattern characteristic of the geminal protons of an oxazolidine ring. Complete hydrolysis to daunorubicin and formaldehyde resulted from the addition of 3 equiv of hydrochloric acid in deuterium oxide. Formaldehyde appeared as its hydrate, characterized by a signal at δ 4.56.

Reaction of Daunoform with DNA. We have previously demonstrated that daunorubicin and formaldehyde react with the self-complementary DNA, $(GC)_4$, to yield four drug−DNA adducts, denoted adducts 1−4.[33,34] UV−vis absorption and electrospray mass spectrometry indicated that adducts 1 and 2 contained

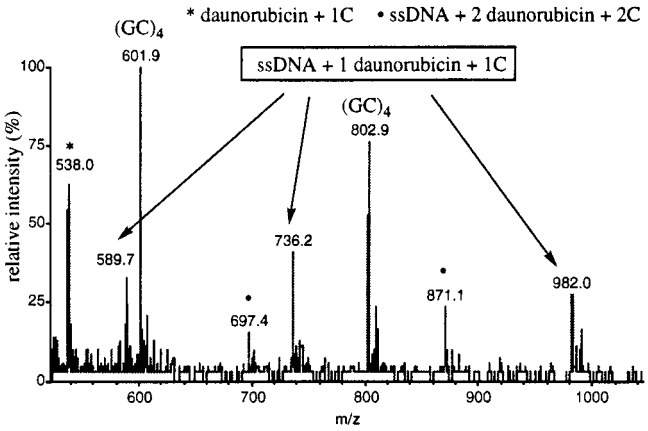


Figure 1. Negative ion, electrospray mass spectrum (ESMS) of DNA−daunorubicin adduct 5 from reaction of $(GC)_4$ with Daunoform in pH 7.4 phosphate buffer at 25 °C. Peaks representing assigned ions bearing one or more sodium ions are not labeled to simplify the figure. The calculated mass-to-charge ratios are as follows: 538.2, 589.1, 601.8, 697.0, 736.6, 802.8, 871.4, 982.5.

one molecule of daunorubicin per double-stranded DNA (dsDNA) and adducts 3 and 4 two molecules per dsDNA. Reaction of $(GC)_4$ with Daunoform in pH 7.4 phosphate buffer gave the same four drug−DNA adducts plus a significant amount of a fifth adduct which appeared at longer retention time in the reverse phase HPLC. Adduct 5 was observed in the earlier experiments with daunorubicin and formaldehyde but was not produced in sufficient quantities for characterization. UV−vis absorption indicated that adduct 5 contains three molecules of daunorubicin per dsDNA. The electrospray mass spectrum (Figure 1) shows peaks for ssDNA bound to one molecule of daunorubicin and for ssDNA bound to two molecules of daunorubicin. This is also consistent with the assignment of three molecules of daunorubicin per dsDNA for the structure of adduct 5.

The rate of reaction of $(GC)_4$ with Daunoform in pH 7.4 phosphate buffer was also compared to the rate of its reaction with a mixture of daunorubicin and formaldehyde. These experiments were performed at much lower concentrations of Daunoform (55 μM) such that the major adducts were 1 and 2. The amount of daunorubicin and formaldehyde employed was equivalent to that present in the Daunoform experiment. Formation of the DNA adducts as a function of time for the two reactions at 25 °C is shown in Figure 2. After approximately 1 min, Daunoform had reacted with 4 times as much $(GC)_4$ as the equivalent amount of daunorubicin and formaldehyde. The difference in rate of adduct formation in these two experiments most likely reflects a difference in the overall mechanism. Three possible reaction sequences for the formation of adducts from daunomycin + dsDNA + formaldehyde are outlined in Figure 3. For all of these mechanisms, two sequential bimolecular reactions must occur. In the case of Daunoform + dsDNA, the mechanism of adduct formation probably involves a first-order decay followed by one bimolecular reaction (Figure 3). Thus, the enhanced rate of adduct formation for the Daunoform + dsDNA system is presumably a reflection of the fact that one less bimolecular reaction needs to occur.

Cell Experiments. The biological activities of Daunoform and Doxoform were determined by their *in vitro* cytotoxicity against MCF-7 and MCF-7/ADR cell lines.

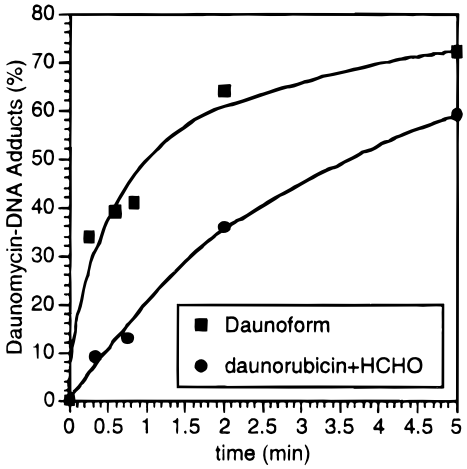
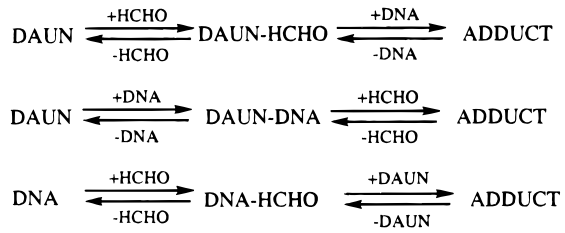


Figure 2. Formation of drug−DNA adducts from reaction of 33 μM $(GC)_4$ with either 55 μM Daunoform or 110 μM daunorubicin plus 165 μM formaldehyde in pH 7.4 phosphate buffer at 25 °C as a function of time. Adduct formation represents the sum of adducts 1−4 and is shown as a percent of the initial $(GC)_4$ concentration. It was calculated from HPLC peak areas with detection at 260 nm and is uncorrected for absorbance by drug.

daunorubicin + formaldehyde:



Daunoform:

Figure 3. Possible sequence of steps for drug−DNA adduct formation with daunorubicin (DAUN) plus formaldehyde and with Daunoform (DAUNF). DAUN-HCHO may be the mono-oxazolidine shown in Scheme 1.

Table 2. IC_{50} Values for Drugs versus MCF-7 and MCF-7/ADR Cells[a]

	IC_{50} values (nmol equiv/L) for			
cell type	doxorubicin	doxoform	daunorubicin	daunoform
MCF-7	300	2	60	8
MCF-7/ADR	10000	1	2000	10

[a] Cells were incubated with drug for 3 h in RPMI 1640 media containing 10% fetal bovine serum and 1% DMSO. Plates were developed using the crystal violet assay. The units are nmol equiv/L because Doxoform and Daunoform each contain two anthracycline equivalents. Dose−response curves and raw data are provided in Supporting Information.

MCF-7 is a sensitive human breast carcinoma line, whereas MCF-7/ADR is its doxorubicin resistant counterpart.[39] IC_{50} values for Daunoform, Doxoform, daunorubicin, and doxorubicin are shown in Table 2. In all cases, drug treatments were for 3 h and percent survival was determined by the extent of colony formation 6 days after drug treatment. Daunoform and Doxoform were 7 and 150 times more cytotoxic against the MCF-7 cell line than daunorubicin and doxorubicin, respectively. Doxoform showed a dramatic 10000-fold higher cytotoxicity against the MCF-7/ADR cell line relative to

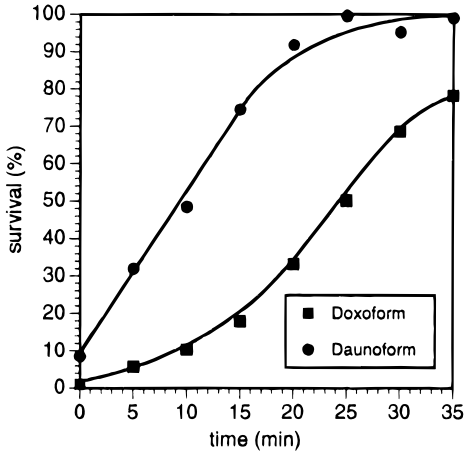


Figure 4. Cytotoxicity of 1 μmol equiv/L Daunoform and Doxoform to MCF-7/ADR cells as a function of the time allowed for drug hydrolysis, in serum-free media containing 10% DMSO, prior to treatment. The stock drug solution was maintained at 25 °C between additions to the cells. Cells were incubated with drug in serum-free media for 3 h and in drug-free growth media for 6 days. Cells were assayed by crystal violet staining.

doxorubicin, and Daunoform showed a 200-fold higher cytotoxicity relative to daunorubicin. Control experiments established that neither formaldehyde nor 1% DMSO were cytotoxic to MCF-7 or MCF-7/ADR cells in the concentration ranges employed in the experiments.

The observed cytotoxicity of Daunoform and Doxoform represents lower limits due to their rapid hydrolysis to the parent compounds in an aqueous environment. To demonstrate this point, the survival of Doxoform- and Daunoform-treated MCF-7/ADR cells was measured as a function of time where the drugs were allowed to stand in an aqueous enviornment at 25 °C for various time periods prior to addition to the cells. The results of these experiments are illustrated in Figure 4. For both drugs at $t = 0$ min, cells showed a survival rate of less than 10%. However, Daunoform completely lost its effectiveness within 25 min of standing in an aqueous solution. Doxoform showed a somewhat longer lifetime and a curious biphasic survival curve. This biphasic curve is probably due to the decay mechanism of Doxoform to doxorubicin in aqueous solution, but any detailed discussion would be speculative. Although the IC_{50} values reported in Table 2 were obtained using a 3 h incubation time, the actual exposure time to the cytotoxic component of Daunoform and Doxoform was much shorter.

Mechanism of Cytotoxicity and Circumvention of Drug Resistance. Doxoform is 150-fold more toxic to MCF-7 cells and 10000-fold more toxic to MCF-7/ADR cells. The results of our work [33,34] and that of others [25,30] point to the following sequence of events for cytotoxicity of daunorubicin and doxorubicin. The drugs catalyze the production of superoxide and hydrogen peroxide through the redox machinery of the quinone functionality. These reactive oxygen species, through an iron-catalyzed Fenton reaction, oxidize cellular constituents to produce formaldehyde. Iron is available because of its strong association with the drugs.[40] The resulting formaldehyde reacts with the drug to produce Daunoform and Doxoform or at least the respective mono-oxazolidines, 3′-*N*,4′-*O*-methylenedaunorubicin and 3′-*N*,4′-*O*-methylenedoxorubicin. Daunoform and Doxo-

form (or their mono-oxazolidines) react with DNA to form virtual cross-links (Chart 1) which trigger apoptosis.[32] Consequently, Daunoform and Doxoform, which carry their own formaldehyde into the cells, are more effective against sensitive cells than their parent compounds.

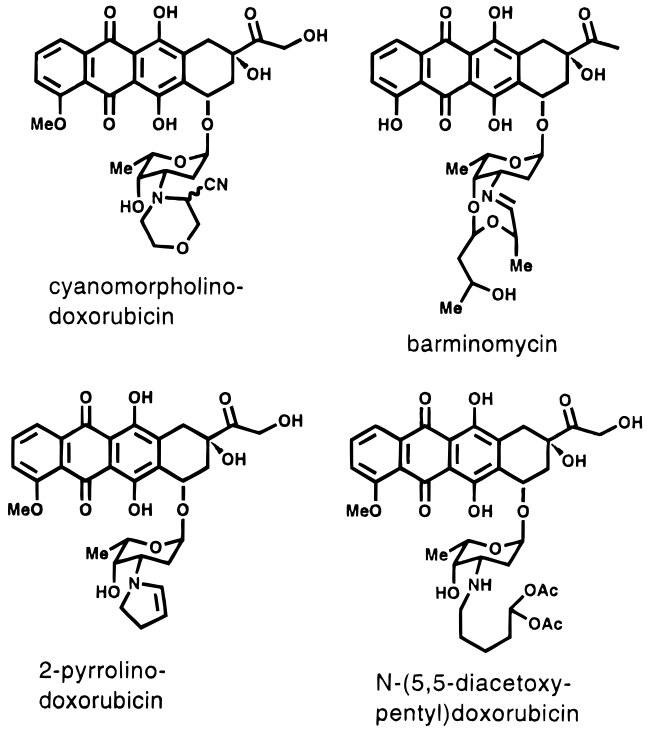
At least two mechanisms of drug resistance are operative in the MCF-7/ADR cell line: P-glycoprotein overexpression and decreased intracellular accumulation of reactive oxygen species.[7] We propose that Daunoform and Doxoform are more cytotoxic to resistant tumor cells because they effectively bypass both of these resistance mechanisms. The MCF-7/ADR cell line exhibits decreased intracellular accumulation of doxorubicin compared to its parental line (MCF-7) due to P-glycoprotein overexpression. However, recent work has established that doxorubicin anologs with increased lipophilicity are retained by cells overexpressing P-glycoprotein, presumably because they are poorer substrates for the efflux pump.[41] Because Doxoform and Daunoform are more lipophilic than their parent compounds due to their tertiary amine functionality, they can effectively bypass this resistance mechanism. However, P-glycoprotein overexpression is not the dominant mechanism of drug resistance in MCF-7/ADR cells. This cell line only shows a 2−3-fold difference in drug uptake as compared to the parental line, which is not sufficient to explain the observed drug resistance of up to 200-fold relative to MCF-7.[42] Thus, the mechanism thought to be largely responsible for anthracycline drug resistance in MCF-7/ADR cells is reduced production and/or increased scavenging of reactive oxygen species.[7] We propose that a consequence of this resistance mechanism is the slow production of formaldehyde through oxidation of intracellular components.[35] Consequently, Daunoform and Doxoform, which can skip formaldehyde production, effectively bypass this resistance mechanism as well.

An additional mechanism for tumor cell resistance is overexpression of glutathione transferase and formation of glutathione−drug conjugates.[5] In fact, overexpression of glutathione transferase has been observed in MCF-7/ADR cells.[42] Because Doxoform and Daunoform hydrolyze to their parent drugs so rapidly, they must be reaching their intracellular target within a very short period of time. Hence, glutathione transferase may not have sufficient time to transform Doxoform and Daunoform into the less toxic drug−glutathione conjugates.

MCF-7/ADR cells have a mutated p53 gene.[43] The p53 protein is part of a complex signaling pathway which slows the growth of normal cells with damaged DNA to allow for DNA repair.[44] It also appears to trigger apoptosis in tumor cells with damaged DNA. Consequently, the prognosis is poor for treatment of patients with malignancies having mutated p53 genes using chemotherapeutic agents which attack DNA.[45] Because MCF-7/ADR cells have a mutated p53 gene, Doxoform must be causing tumor cell death by a mechanism which does not require a functioning p53 protein.

Relevance to Cancer Chemotherapy. Tumor cell cytotoxicity alone is not the answer. High selectivity for sensitive and resistant tumor cells is required. Other DNA-alkylating anthracyclines which bear a masked aldehyde group on the 3′-amino substituent

Chart 2



have been described and include cyanomorpholinodoxorubicin,[46,47] barminomycin,[48,49] 2-pyrrolinodoxorubicin,[50] and *N*-(5,5-diacetoxypentyl)doxorubicin (Chart 2).[51,52] Some of these also show cytotoxicity to resistant tumor cells.[52] A critical difference between the derivatives shown in Chart 2 and Doxoform and Daunoform is the potential for release of the aldehyde functional group from the anthracycline and release of the anthracycline from the DNA. The derivatives in Chart 2 all have the masked aldehyde irreversibly attached to the 3′-amino group of the anthracycline, with the possible exception of barminomycin. Hence, DNA lesions will hydrolyze, at best, reversibly. Doxoform, Daunoform, and their associated DNA lesions[27,33,34,53] are all labile with respect to hydrolysis. Further, the hydrolysis results in irreversible escape of the formaldehyde through dilution. As a result, Doxoform and Daunoform have a potential for higher selectivity for resistant tumor cells than doxorubicin and daunorubicin through a kinetic advantage and a higher selectivity for sensitive and resistant tumor cells than the derivatives in Chart 2 through a thermodynamic advantage. The kinetic advantage results from the drugs' ability to alkylate DNA rapidly. The thermodynamic advantage comes from the instability of the DNA lesion and the ability of the normal cells to slow their growth. During slow growth the DNA lesions might hydrolyze without DNA damage. Further, drug clearance will be rapid because of hydrolysis to the less toxic parent drugs. This latter advantage should be more important with Doxoform than with Daunoform because Doxoform is 150 times more cytotoxic to sensitive cells than doxorubicin, whereas Daunoform is only 7 times more cytotoxic to sensitive cells than daunorubicin. Hence, Doxoform could possibly be given at a lower dose than is now used for doxorubicin.

Conclusions

Doxorubicin and daunorubicin react with aqueous formaldehyde to form bis-oxazolidinylmethane derivatives, denoted Doxoform and Daunoform, respectively. In spite of being hydrolytically unstable, Doxoform and Daunoform show enhanced cytotoxicity to both sensitive (MCF-7) and resistant (MCF-7/ADR) human breast cancer cells. A rationale for the enhanced cytotoxicity may be that Doxoform and Daunoform or, more likely, the mono-oxazolidines resulting from their initial hydrolysis are actually active metabolites of doxorubicin and daunorubicin.

Experimental Section

General Remarks. UV−vis spectra were recorded with a Hewlett-Packard 8452A diode array spectrometer and ^{1}H NMR spectra with a Bruker Model AM-400 spectrometer. Mass spectra of Daunoform and Doxoform were obtained with a Hewlett-Packard 5989B single quadrupole electrospray mass spectrometer; samples were introduced by direct infusion of chloroform/methanol solutions. Mass spectra of drug−DNA adducts were obtained with an API-III triple quadrupole mass spectrometer (Sciex) equipped with a nebulization-assisted electrospray (ES) ion source and a high-pressure collision cell; samples were introduced by direct infusion of water/methanol (75/25, v/v) solutions. Daunorubicin and doxorubicin as their hydrochloride salts or as clinical samples were received as gifts from Nexstar Pharmaceuticals, Inc., San Dimas, CA, and Pharmacia-Upjohn-Farmitalia, Milan, Italy. Formaldehyde was obtained from Mallinckrodt as a 37% by weight solution in water containing 10−15% methanol. Water was distilled and purified with a Millipore Q-UF Plus purification system to 18 MΩ·cm. Phosphate buffer, pH 7.4, was 77.4 mM Na_2HPO_4 and 22.6 mM NaH_2PO_4. Triethylamine for preparation of triethylammonium acetate buffer was 99$^+$% from Aldrich, and tris(hydroxymethyl)aminomethane (Tris) was from Boehringer Mannheim. HPLC analyses were performed with a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array UV−vis detector and workstation; chromatographies were performed with a Hewlett-Packard 5-μm C_{18} microbore column, 2.1 mm i.d. × 100 mm, eluting at 0.5 mL/min with gradients of pH 6 triethylammonium acetate (Et_3NHOAc) (0.02 M)/acetic acid (AcOH) buffer and acetonitrile and detecting at 260 and 480 nm.

Synthesis of Daunoform: Amorphous Daunoform. A solution containing 1 mM daunorubicin and 50 mM formaldehyde in 100 mL of pH 6 triethylammonium acetate (20 mM)/acetic acid buffer was allowed to react in the dark at 25 °C for 21 h. The buffer was then removed by high-vacuum rotary evaporation at 0.1 Torr for 5 h. The dry product was dissolved in chloroform and placed in a 15 mL centrifuge tube. The solvent was removed by rotary evaporation, and the dry sample was washed with 3 × 10 mL of water by centrifugation at 1550 rpm and removal of the water with a pipet. After the final wash, the sample was dried under vacuum at 0.02 Torr for 10 h to yield an amorphous red solid. The positive ion ESI mass spectrum with the sample in chloroform/methanol (5/1, v/v) showed the following major ions: m/z 1113 (M + Na^+, 49), 1101 (100), 926 (46), 540 (62), 528 (54), 399 (27), and 381 (49). The ^{1}H NMR spectrum in deuteriochloroform solvent (Table 1) established that the product was pure and, together with the MS spectrum, indicated that it had the structure bis(3′-*N*-(3′-*N*,4′-*O*-methylenedaunorubicinyl))methane (Daunoform). The yield of Daunoform was established by visible absorption at 480 nm because the sample could not be accurately weighed. A solution was prepared by dissolving the entire product in 5 mL of chloroform followed by a 400× serial dilution. The absorbance at 480 nm established a yield of 49 μmol (67%), using the molar extinction coefficient 9900 M^{-1} cm^{-1} for the daunorubicin chromophore. A similar experiment using 10 equiv of formaldehyde generated the same product in 70% yield. In this case the reaction time was 67 h.

Crystalline Daunoform. To a solution of 40 mg (70.9 μmol) of daunorubicin hydrochloride in 40 mL of pH 6 triethylammonium acetate buffer were added 80 mL of chloroform and 0.8 mL of aqueous formaldehyde (37% formaldehyde). The solution was stirred vigorously until extraction of the aqueous phase was complete (about 30 min). The organic layer was collected, and the aqueous layer was extracted a second time with 80 mL of chloroform in a similar manner. The organic layers were pooled and dried over sodium sulfate and the solvent removed by rotary evaporation yielding crude Daunoform as a red film. The crude product was redissolved in 0.8 mL of chloroform. The solution volume was brought to 16 mL by addition of ethyl acetate and the solution mixed thoroughly. The solution was dispensed in 3 mL aliquots into five 5 mL vials and allowed to stand undisturbed, stoppered, and in the dark for 3 days. The resulting crystals grew as red needles on the glass. The crystals were washed with *n*-hexane, collected, and dried under vacuum (0.1 Torr) to yield 20 mg (51%) of Daunoform. Anal. ($C_{57}H_{58}N_2O_{18}$) C, H, N. The crystalline material showed the same ^{1}H NMR spectral properties as the amorphous material prepared as described above (Table 1) and the following ^{13}C NMR resonances: δ ($CDCl_3$) 16.5, 24.8, 29.1, 33.0, 34.7, 55.5, 56.4, 65.7, 69.3, 72.4, 75.6, 77.2, 85.2, 100.3, 110.9, 111.0, 118.0, 119.5, 120.5, 134.1, 134.4, 135.1, 135.3, 155.5, 156.3, 160.6, 186.0, 186.1, 212.3. The positive ion ESI mass spectrum with the sample in chloroform/methanol (5/1, v/v) showed the following major ions: m/z 1091 (M + H^+, 100), 1079 (52), 552 (26), and 540 (59). The UV−vis spectrum of Daunoform appeared as follows: λ_{max} (ϵ, M^{-1} cm^{-1}) CH_2Cl_2 236 (27 300), 252 (20 000), 290 (7600), 482 (9700); $CHCl_3$ 252 (20 800), 290 (8000), 488 (10 100), 500 (10 200). A significant difference between the amorphous and crystalline materials was the rate at which each dissolved in dimethyl sulfoxide: the amorphous material dissolved instantly to at least 20 mg/mL, whereas the crystalline material dissolved very slowly.

Synthesis of Doxoform: Amorphous Doxoform. A clinical sample containing doxorubicin hydrochloride and lactose was used as the starting material. Most of the lactose was removed by extraction with chloroform. A doxorubicin/lactose mixture containing 40 mg of doxorubicin was dissolved in 75 mL of pH 8, 0.1 M potassium chloride, 40 mM Tris, 100 mM ethylenediaminetetraacetic acid (EDTA) buffer. To this was added 100 mL of chloroform, and the mixture was stirred vigorously for 30 min. The chloroform layer was then removed and a second extraction using 50 mL of chloroform performed. The chloroform extracts were combined, and the chloroform was removed by rotary evaporation yielding doxorubicin as the free base. A solution of 1 mM doxorubicin and 50 mM formaldehyde in 68 mL of pH 6, triethylammonium acetate (20 mM)/acetic acid buffer was allowed to react in the dark at 25 °C for 15 h. The buffer was then removed by high-vacuum (0.1 Torr) rotary evaporation for 5 h. The product was dissolved in chloroform and placed in a 15 mL centrifuge tube. The solvent was removed by rotary evaporation, and the dry sample was washed with 3 × 10 mL portions of water by centrifugation at 1550 rpm. After the final wash, the sample was again dried under vacuum at 0.02 Torr for 15 h. The ^{1}H NMR spectrum in deuteriochloroform solvent (Table 1) established that the product was pure and, together with the MS spectrum of crystalline material (*vide infra*), indicated that it had the structure bis(3′-*N*-(3′-*N*,4′-*O*-methylenedoxorubicinyl))-methane (Doxoform). The isolated yield was determined to be 70% by visible absorption at 480 nm of a chloroform solution as described above for Daunoform.

Crystalline Doxoform. Crystalline Doxoform was prepared exactly the same as crystalline Daunoform except for the crystallization step. For a procedure starting with 40 mg, the crude product was dissolved in 1.3 mL of chloroform diluted with 27 mL of 3:1 ethyl acetate:hexane. The resulting solution was dispensed in 3 mL aliquots into nine 5 mL vials and allowed to stand undisturbed and stoppered in the dark for 3 days. The resulting crystals grew as red hexagonal tubes on the glass. The crystals were washed with ethyl acetate, collected, and dried under vacuum (0.1 Torr) to yield 14.5 mg (37%) of pure Doxoform. Anal. ($C_{57}H_{58}N_2O_{22}$) C, H, N. The crystalline material showed the same ^{1}H NMR spectral properties as the amorphous material. The positive ion ESI

mass spectrum of a sample in chloroform/methanol (5/1, v/v) showed the following major ions: *m*/*z* 1123 (100), 1111 (45), 568 (19), and 556 (91).

Dilution of a Deuteriochloroform Solution of Daunoform. A sample of Daunoform was dissolved in 500 μL of deuteriochloroform, and the solution was analyzed by ^{1}H NMR spectroscopy. The sample was then diluted to volumes of 750, 1000, 1250, and 1500 μL with deuteriochloroform. At each new volume the ^{1}H NMR spectrum was obtained and integrated using the chloroform signal as an internal standard. The spectra showed conversion of Daunoform to an equilibrium mixture of Daunoform with 3′-*N*,4′-*O*-methylenedaunorubicin. The spectral assignment for 3′-*N*,4′-*O*-methylenedaunorubicin appears in Table 1. The composition of the solutions at each dilution was tabulated in terms of the areas for the phenolic OH signals relative to the area for the chloroform signal.

Daunoform Stability in DMSO. Crystalline Daunoform (2.8 mg, 2.6 μmol) was dissolved in 500 μL of DMSO-d_6 (stored over 3 Å molecular sieves) and analyzed by 400 MHz ^{1}H NMR (Table 1). NMR analysis showed that the Daunoform was stable in DMSO for at least 14 h. Upon addition of 25 μL of D_2O to the 500 μL DMSO-d_6 sample, the Daunoform reacted over a 24 h period to form an equilibrium mixture consisting of 67% Daunoform and 32% intermediate. Because of the complexity of the spectrum, a structure could not be assigned to the intermediate; however, the intermediate appeared to have an oxazolidine ring as indicated by the appearance of an AX pattern at δ 4.39 and 4.49 (J = 4 Hz). Upon addition of 3 equiv of hydrochloric acid in 130 μL of deuterium oxide, hydrolysis to daunorubicin hydrochloride was complete. The hydrochloric acid solution was prepared by the addition of 10 μL of concentrated HCl to 990 μL of D_2O. The formaldehyde released from the Daunoform was detected as its hydrate by a singlet at δ 4.56. The final product was identified as daunorubicin by comparison of the NMR spectrum with that of a sample of daunorubicin plus 3 equiv of HCl. Further, the final product was isolated by removal of all volatile components under vacuum (0.1 Torr), and its ^{1}H NMR spectrum was identical to that of daunorubicin hydrochloride.

Amorphous Daunoform, when worked up in methylene chloride, was stable in DMSO for at least 4 days. It hydrolyzed with added D_2O and hydrochloric acid in a manner similar to that of the crystalline material. When worked up in chloroform, amorphous Daunoform had less stability in DMSO, presumably due to traces of HCl from the chloroform.

Reaction of Daunoform with $(GC)_4$. A solution containing 317 μM $(GC)_4$ and 357 μM Daunoform in 150 μL of pH 7.4 phosphate buffer was allowed to react for 41 h in the dark at 25 °C. The DNA had completely reacted to form drug−DNA adducts 1, 2 + 3, 4, and 5 in a 2:19:68:11 peak area ratio as indicated by HPLC. The C18 HPLC column was eluted with the following gradient created with A = CH_3CN, B = 20 mM aqueous triethylammonium acetate: A:B, 0:100 to 7:93 at 1 min, to 9:91 at 10 min, to 70:30 at 13 min, isocratic until 15 min, and to 0:100 at 17 min. The retention times for adducts 1−5 were 7.4, 8.3, 8.6, 9.7, and 12.3 min. HPLC peaks for adducts 2 and 3 were not resolved and were integrated together. Adducts 1−4 were isolated and characterized by UV−vis absorption and negative ion ESMS; the spectral data showed them to be identical to adducts 1−4 from reaction of daunorubicin with $(GC)_4$ and formaldehyde described previously.[34] Adduct 5 was isolated by preparative reverse phase HPLC as described earlier for adducts 1−4. The UV−vis spectrum of adduct 5 indicated that it was a drug−DNA adduct containing three molecules of covalently bound, intercalated drug per dsDNA ($A_{510} > A_{480}$; A_{260}:A_{480} = 8:1). The negative ion, electrospray mass spectrum (Figure 1) confirmed this structural assignment.

Drug−DNA Adduct Formation in the Presence of Daunoform or Daunorubicin plus Formaldehyde as a Function of Time. Reaction mixtures containing 33 μM $(GC)_4$ and 55 μM Daunoform in pH 7.4 phosphate buffer were monitored periodically by HPLC over a 1 h time period. The reaction mixtures were maintained at 25 °C and contained 2% DMSO to facilitate Daunoform solubilization. The amount of available formaldehyde in this reaction was 165 μM (3 × 55 μM). Similar reaction mixtures containing 33 μM $(GC)_4$, 110 μM daunorubicin, and 165 μM formaldehyde in pH 7.4 phosphate buffer were prepared and monitored periodically by HPLC as described above. The reaction mixtures were maintained at 25 °C and contained 2% DMSO. The formation of drug−DNA adducts with respect to time for the Daunoform and daunorubicin reactions is compared in Figure 2.

Cell Experiments. All tissue culture materials were obtained from Gibco Life Technologies (Grand Island, NY) unless otherwise stated. MCF-7 breast cancer cells were obtained from American Type Culture Collection (Rockville, Maryland). MCF-7/ADR adriamycin resistant breast cancer cells[42] were a gift of Dr. William W. Wells (Michigan State University). Both cell lines were maintained *in vitro* by serial culture in phenol red-free RPMI media 1640 supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calbasas, CA), L-glutamine (2 mM), HEPES buffer (10 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). The MCF-7/ADR cell line medium was additionally supplemented with 5 μM adriamycin (Nexstar Pharmaceuticals, San Dimas, CA). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Cells were dissociated with trypsin-EDTA, counted, and suspended in growth media to a concentration of 5 × 10^3 cells/mL. Cell suspensions were dispensed in 200 μL aliquots into 96-well tissue culture plates. Plates were then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced with 180 μL of growth medium prior to addition of the cytotoxic agents. Cytotoxic agents (daunorubicin, doxorubicin, Daunoform, Doxoform) were dissolved in DMSO at a concentration of 10 mM, sonicated to facilitate solvation, and sterile-filtered through a 0.2 μm nylon syringe filter. Concentrations were then corrected by measuring the solution absorbance at 480 nm (ϵ = 11 500/mol of anthracycline). The solutions were then serially diluted in DMSO to give a series of 100× working concentration solutions. For each concentration, the 100× solution was diluted 1:10 in serum-free RPMI media 1640; 20 μL of the resulting 10× solution was *immediately* added to the appropriate lane. Additionally, one lane was treated with 20 μL of 10% DMSO in serum-free RPMI (no drug) and one lane replaced with 200 μL of 1.5 M Tris buffer (no cells). The cells were incubated at 37 °C for 3 h. The drug solutions were removed, and 200 μL of fresh growth medium was added to each well. The cells were then incubated for 6 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air (approximately 60% confluence for the no drug lane).

The extent of colony formation was determined by use of a crystal violet staining assay.[54,55] Cells were treated with 200 μL of 1% glutaraldehyde in Hank's balanced salt solution for 15 min. The cells were then stained with 75 μL of 0.1% crystal violet in deionized water for 30 min. The plates were then rinsed with deionized water and allowed to soak in a beaker of running deionized water (1 L/min) for 15 min. The plates were blotted dry, and 200 μL of 70% ethanol in water was added to each well to solubilize the dye. The plates were stored at 4 °C until solubilization was complete (about 4 h). The optical density of each well was measured on an ELISA plate reader at 588 nm. Relative colony sizes were determined by comparison of the drug-treated lanes to the control lanes.

Control experiments established that the IC_{50} value for formaldehyde was in excess of 200 μM with MCF-7 and MCF-7/ADR cells. Additional control experiments established that 3 h incubation of cells with 1% DMSO in growth media did not alter the survival of MCF-7 or MCF-7/ADR cells.

Cytotoxicity of Daunoform and Doxoform versus Time of Addition. Cells were dissociated with trypsin-EDTA, counted, and suspended in growth media to a concentration of 5 × 10^3 cells/mL. Cell suspensions were dispensed in 200 μL aliquots into 96-well tissue culture plates. Plates were then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced with 180 μL of growth medium prior to addition of the cytotoxic agents. The drug being investigated (Doxoform or Daunoform) was dissolved in DMSO at a concentration of 0.5 mM (1 mmol equiv/L), sonicated to facilitate solvation, and sterile-filtered

through a 0.2 μm nylon syringe filter. Concentrations were then corrected by measuring the solution absorbance at 480 nm (ϵ = 11 500/mol of anthracycline). The drug solution was diluted 1:100 with DMSO and then 1:10 in serum-free RPMI media 1640. A volume of 20 μL of the resulting 0.5 μM (1 μmol equiv/L) drug solution was immediately added to the t = 0 min lane. The remaining lanes were treated at 5 min intervals with 20 μL of the remaining drug solution, which was held at 25 °C between additions. Additionally, one lane was treated with 20 μL/well of serum-free RPMI containing 10% DMSO (no drug) and one lane replaced with 200 μL/well of 1.5 M Tris buffer (no cells). Each lane was incubated at 37 °C for 3 h. The drug solutions were removed, and 200 μL of fresh growth medium was added to each well. The cells were then incubated for 6 days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The extent of colony formation was determined by use of a crystal violet staining assay described above, and relative colony sizes were determined by comparison of the drug-treated lanes to the control lanes.

Acknowledgment. We are grateful to the National Cancer Institute of the NIH (Grant CA24665) for financial assistance. We thank Nexstar Pharmaceuticals, San Dimas, CA, and Pharmacia-Upjohn-Farmitalia, Milan, Italy, for generous samples of adriamycin and daunomycin. We also thank Dr. William W. Wells for MCF-7/ADR cells.

Supporting Information Available: Table 3 (reference ^{1}H NMR data for daunorubicin in $DCCl_3$ and DMSO-d_6), ^{1}H NMR spectrum for Daunoform, and dose−response curves and raw data for cell experiments (6 pages). Ordering information is given on any current masthead page.

References

(1) Young, R. C.; Ozols, R. F.; Myers, C. E. Medical Progress: The anthracycline antineoplastic drugs. *New Engl. J. Med.* **1981**, *305*, 139−153.
(2) Volm, M.; Mattern, J.; Samsel, B. Overexpression of P-glycoprotein and glutathione S-transferase-π in resistant non-small cell lung carcinomas of smokers. *Br. J. Cancer* **1991**, *64*, 700−704.
(3) Giai, M.; Biglia, N.; Sismondi, P. Chemoresistance in breast tumors. *Eur. J. Gynaecol. Oncol.* **1991**, *12*, 359−373.
(4) Black, S. M.; Wolf, C. R. The role of glutathione-dependent enzymes in drug resistance. *Pharmacol. Ther.* **1991**, *51*, 139−154.
(5) Serafino, A.; Sinibaldi-Vallebona, P.; Gaudiano, G.; Koch, T. H.; Rasi, G.; Ravagnan, G. *Int. J. Cancer* **1997**, *57*, submitted for publication.
(6) Blair, S. L.; Heerdt, P.; Sachar, S.; Abolhoda, A.; Hochwald, S.; Cheng, H.; Burt, M. Glutathione metabolism in patients with non-small cell lung cancers. *Cancer Res.* **1997**, *57*, 152−155.
(7) Sinha, B. K.; Mimnaugh, E. G. Free radicals and anticancer drug resistance: oxygen free radicals in the mechanisms of drug cytotoxicity and resistance by certain tumors. *Free Radical Biol. Med.* **1990**, *8*, 567−581.
(8) Chaires, J. B.; Satyanarayana, S.; Suh, D.; Fokt, I.; Przewloka, T.; Priebe, W. Parsing the free energy of anthracycline antibiotic binding to DNA. *Biochemistry* **1996**, *35*, 2047−2053.
(9) Egorin, M. J.; Hildebrand, R. C.; Cimono, E. F.; Bachur, N. R. Cytofluorescence localization of Adriamycin and Daunorubicin. *Cancer Res.* **1974**, *34*, 2243−2245.
(10) Coley, H. M.; Amos, W. B.; Twintyman, P. R.; Workman, P. Examination by laser confocal fluorescence imaging microscopy of the subcellular localisation of anthracyclines in parent and multidrug resistant cell lines. *Br. J. Cancer* **1993**, *67*, 1316−1323.
(11) Wang, A. H.-J.; Ughetto, G.; Quigley, G. J.; Rich, A. Interaction between an anthracycline antibiotic and DNA: molecular structure of daunomycin complexed to d(CpGpTpApCpG) at 1.2 Å resolution. *Biochemistry* **1987**, *26*, 1152−1163.
(12) Frederick, C. A.; Williams, L. D.; Ughetto, G.; Vander Marel, G. A.; Van Boom, J.; Rich, A.; Wang, A. H. J. Structural comparison of anticancer drug-DNA complexes: Adriamycin and Daunomycin. *Biochemistry* **1990**, *29*, 2538−2549.
(13) Pan, S.; Pedersen, L.; Bachur, N. R. Comparative flavoprotein catalysis of anthracycline antibiotic reductive cleavage and oxygen consumption. *Mol. Pharmacol.* **1981**, *19*, 184−186.
(14) Schreiber, J.; Mottley, C.; Sinha, B. H.; Kalyanaraman, B.; Mason, R. One-electron reduction of Daunomycin, daunomycinone, and 7-deoxydaunomycinone by the xanthine/xanthine oxidase system: detection of semiquinone free radicals by electron spin resonance. *J. Am. Chem. Soc.* **1987**, *109*, 348−351.
(15) Kappus, H. Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. *Biochem. Pharmacol.* **1986**, *35*, 1−6.
(16) Lown, W. J.; Chen, J. A.; Plambeck, J. A.; Acton, E. M. Further studies on the generation of reactive oxygen species from activated anthracyclines and the relationship to cytotoxic action and cardiotoxic effects. *Biochem. Pharmacol.* **1982**, *31*, 575−581.
(17) Doroshow, J. H. Anthracycline antibiotic-stimulated superoxide, hydrogen peroxide, and hydroxyl radical production by NADH dehydrogenase. *Cancer Res.* **1983**, *43*, 4543−4551.
(18) Sinha, B. K. Free radicals in anticancer drug pharmacology. *Chem. Biol. Interact.* **1989**, *69*, 293−317.
(19) Kleyer, D.; Koch, T. H. Mechanistic investigation of reduction of Daunomycin and 7-deoxydaunomycinone with bi(3,5,5-trimethyl-2-oxomorpholin-3-yl). *J. Am. Chem. Soc.* **1984**, *106*, 2380−2387.
(20) Abdella, B. R. J.; Fisher, J. A chemical perspective on the anthracycline antitumor antibiotics. *Environ. Health Perspect.* **1985**, *64*, 3−18.
(21) Gaudiano, G.; Resing, K.; Koch, T. H. Reaction of anthracycline antitumor drugs with reduced glutathione. Formation of aglycon conjugates. *J. Am. Chem. Soc.* **1994**, *116*, 6537−6544.
(22) Moore, H. W.; Czerniak, R. Naturally occurring quinones as potential bioreductive alkylating agents. *Med. Res. Rev.* **1981**, *1*, 249−280.
(23) Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **1989**, *58*, 351−375.
(24) Tritton, T. R. Cell surface actions of Adriamycin. *Pharmacol. Ther.* **1991**, *49*, 293−301.
(25) Cullinane, C.; van Rosmalen, A.; Phillips, D. R. Does adriamycin induce interstrand cross-links in DNA? *Biochemistry* **1994**, *33*, 4632−4638.
(26) Cullinane, C.; Cutts, M. S.; van Rosmalen, A.; Phillips, D. R. Formation of Adriamycin-DNA adducts in vitro. *Nucleic Acids Res.* **1994**, *22*, 2296−2303.
(27) van Rosmalen, A.; Cullinane, C.; Cutts, S. M.; Phillips, D. R. Stability of adriamycin-induced DNA adducts and interstrand crosslinks. *Nucleic Acids Res.* **1995**, *23*, 42−50.
(28) Cutts, S. M.; Phillips, D. R. Use of oligonucleotides to define the site of interstrand cross-links induced by Adriamycin. *Nucleic Acids Res.* **1995**, *23*, 2450−2456.
(29) Cutts, S. M.; Parsons, P. G.; Sturm, R. A.; Phillips, D. R. Adriamycin-induced DNA adducts inhibit the DNA interactions of transcription factors and RNA polymerase. *J. Biol. Chem.* **1996**, *271*, 5422−5429.
(30) Skladanowski, A.; Konopa, J. Relevance of interstrand DNA crosslinking induced by anthracyclines for their biological activity. *Biochem. Pharmacol.* **1994**, *47*, 2279−2287.
(31) Skladanowski, A.; Konopa, J. Interstrand DNA crosslinking induced by anthracyclines in tumour cells. *Biochem. Pharmacol.* **1994**, *47*, 2269−2278.
(32) Skladanowski, A.; Konopa, J. Adriamycin and Daunomycin induce programmed cell death (apoptosis) in tumour cells. *Biochem. Pharmacol.* **1993**, *46*, 375−382.
(33) Taatjes, D. J.; Gaudiano, G.; Resing, K.; Koch, T. H. Alkylation of DNA by the anthracycline, antitumor drugs adriamycin and daunomycin. *J. Med. Chem.* **1996**, *39*, 4135−4138.
(34) Taatjes, D. J.; Gaudiano, G.; Resing, K.; Koch, T. A redox pathway leading to the alkylation of DNA by the anthracycline, anti-tumor drugs, adrimaycin and daunomycin. *J. Med. Chem.* **1997**, *40*, 1276−1286.
(35) Taatjes, D. J.; Gaudiano, G.; Koch, T. H. Production of formaldehyde and DNA-adriamycin or -daunomycin adducts, initiated through redox chemistry of DTT/iron, xanthine oxidase/NADH/iron, or glutathione/iron. *Chem. Res. Toxicol.* **1997**, in press.
(36) Wang, A. H. J.; Gao, Y. G.; Liaw, Y. C.; Li, Y. k. Formaldehyde cross-links daunorubicin and DNA efficiently: HPLC and X-ray diffraction studies. *Biochemistry* **1991**, *30*, 3812−3815.
(37) Lambert, J. B.; Majchrzak, M. W. Ring-chain tautomerism in 1,3-diaza and 1,3-oxaza heterocycles. *J. Am. Chem. Soc.* **1980**, *102*, 3588−3591.
(38) Barkworth, P. M. R.; Crabb, T. A. Compounds with bridgehead nitrogens. Part 41. The reaction between trans-2-aminocycloalkanols and formaldehyde. *J. Chem. Soc. Perkin Trans. II* **1982**, 91−94.
(39) Cowan, K. H.; Batist, G.; Tulpule, A.; Sinha, B. K.; Myers, C. E. Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9328−9332.
(40) Myers, C. E.; Gianni, L.; Simone, C. B.; Klecker, R.; Greene, R. Oxidative destruction of erythrocyte ghost membranes catalyzed by the doxorubicin-iron complex. *Biochemistry* **1982**, *21*, 1707−1712.

(41) Lampidis, T. J.; Kolonias, D.; Podona, T.; Israel, M.; Safa, A. R.; Lothstein, L.; Savaraj, N.; Tapiero, H.; Priebe, W. Circumvention of P-GP MDR as a function of anthracycline lipophilicity and charge. *Biochemistry* **1997**, *36*, 2679−2685.
(42) Batist, G.; Tulpule, A.; Sinha, B. K.; Katki, A. G.; Myers, C. E.; Cowan, K. H. Overexpression of a novel anionic glutathione transferase in multi-drug-resistant human breast cancer cells. *J. Biol. Chem.* **1986**, *261*, 15544−15549.
(43) Gudas, J. M.; Nguyen, H.; Li, T.; Sadzewicz, L.; Robey, R.; Wosikowski, K.; Cowan, K. H. Drug-resistant breast cancer cells frequently retain expression of a functional wild-type p53 protein. *Carcinogenesis* **1996**, *17*, 1417−1427.
(44) Hartwell, L. H.; Kastan, M. B. Cell cycle control and cancer. *Science* **1994**, *266*, 1821−1828.
(45) Linn, S. C.; Honkoop, A. H.; Hoekman, K.; Valk, P. v. d.; Pinedo, H. M.; Giaccone, G. p53 and P-glycoprotein are often co-expressed and are associated with poor prognosis in breast cancer. *Br. J. Cancer* **1996**, *74*, 63−68.
(46) Acton, E. M.; Tong, G. L.; Mosher, C. W.; Wolgemuth, R. L. Intensely Potent Morpholinyl Anthracyclines. *J. Med. Chem.* **1984**, *27*, 638−645.
(47) Cullinane, C.; Phillips, D. R. Thermal stability of DNA adducts induced by cyanomorpholinoadriamycin in vitro. *Nucleic Acids Res.* **1993**, *21*, 1857−1862.
(48) Uchida, T.; Imoto, M.; Takahashi, Y.; Odagawa, A.; Sawa, T.; Tatsuta, K.; Naganawa, H.; Hamada, M.; Takeuchi, T.; Umezawa, H. New potent anthracyclines, barminomycins I and II. *J. Antibiot.* **1988**, *41*, 404−408.
(49) Ikeda, D.; Ajito, K.; Kondo, S.; Takeuchi, T. Synthesis of 3″-dehydro-4-O-methylbarminomycin II having an eight-membered acetal-azomethine ring. *Chem. Lett.* **1990**, 1431−1432.
(50) Nagy, A.; Armatis, P.; Schally, A. V. High yield conversion of doxorubicin to 2-pyrrolinodoxorubicin, an analog 500−1000 times more potent: Structure-activity relationship of daunosamine-modified derivatives of doxorubicin. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2464−2469.
(51) Zwelling, L. A.; Altschuler, E.; Cherif, A.; Farquhar, D. N-(5,5-Diacetoxypentyl)doxorubicin: A novel anthracycline producing DNA interstrand cross-linking and rapid endonucleolytic cleavage in human leukemia cells. *Cancer Res.* **1991**, *51*, 6704−6707.
(52) Cherif, A.; Farquhar, D. N-(5,5-Diacetoxypent-1-yl)doxorubicin: A New Intensely Potent Doxorubicin Analogue. *J. Med. Chem.* **1992**, *35*, 3208−3214.
(53) Leng, F.; Savkur, R.; Fokt, I.; Przewloka, T.; Priebe, W.; Chaires, J. B. Base specific and regiospecific chemical cross-linking of Daunorubicin to DNA. *J. Am. Chem. Soc.* **1996**, *118*, 4731−4738.
(54) Gilles, R. J.; Didier, N.; Denton, M. Determination of cell number in monolayer cultures. *Anal. Biochem.* **1986**, *159*, 109−113.
(55) Reile, H.; Birnböck, F.; Bernhardt, G. T.; Spruss, H. S. Computerized determination of growth kinetic curves and doubling times from cells in microcultures. *Anal. Biochem.* **1990**, *187*, 262−267.

JM970237E