# Epidoxoform: A Hydrolytically More Stable Anthracycline−Formaldehyde Conjugate Toxic to Resistant Tumor Cells

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The recent discovery that the formaldehyde conjugates of doxorubicin and daunorubicin, Doxoform and Daunoform, are cytotoxic to resistant human breast cancer cells prompted the search for hydrolytically more stable anthracycline−formaldehyde conjugates. Doxoform and Daunoform consist of two molecules of the parent drug bound together with three methylene groups, two forming oxazolidine rings and one binding the oxazolidines together at their 3′-amino nitrogens. The 4′-epimer of doxorubicin, epidoxorubicin, reacts with formaldehyde at its amino alcohol functionality to produce a conjugate, Epidoxoform, in 59% yield whose structure consists of two molecules of epidoxorubicin bound together with three methylene groups in a 1,6-diaza-4,9-dioxabicyclo[4.4.1]undecane ring system. The structure was established from spectroscopic data and is consistent with products from reaction of simpler vicinal *trans*-amino alcohols with formaldehyde. Epidoxoform hydrolyzes at pH 7.3 to an equilibrium mixture with dimeric and monomeric epidoxorubicin−formaldehyde conjugates without release of formaldehyde or epidoxorubicin. The hydrolysis follows the rate law (A ⇔ B) ⇔ C + D where A (Epidoxoform) is in rapid equilibrium with B, and B is in slow equilibrium with C and D. The forward rate constant for A/B going to C+D gives a half-life of approximately 2 h at 37 °C. At equilibrium the mixture is stable for at least 2 days. At pH 6.0, hydrolysis proceeds with first-order kinetics to epidoxorubicin and formaldehyde with a half-life of 15 min at 37 °C. Epidoxoform and epidoxorubicin plus formaldehyde react with the self-complementary DNA octamer $(GC)_4$ to yield five drug−DNA adducts which have structures analogous to the doxorubicin−DNA adducts from reaction of Doxoform with $(GC)_4$. Epidoxoform is 3-fold more toxic to MCF-7 human breast cancer cells and greater than 120-fold more toxic to MCF-7/ADR resistant cells than epidoxorubicin. Epidoxoform in equilibrium with its hydrolysis products is greater than 25-fold more toxic to resistant cells with respect to epidoxorubicin.

## Introduction

The anthracycline antitumor drugs doxorubicin (adriamycin) and daunorubicin (daunomycin) alkylate DNA through iron-catalyzed production of formaldehyde.[1] The formaldehyde links the 3′-amino group of the drug to the 2-amino group of a deoxyguanosine nucleotide.[2] At a 5′-CGC-3′ site the combination of intercalation, covalent bonding, and hydrogen bonding function as a *virtual DNA cross-link*.[3] Further, doxorubicin and daunorubicin react with formaldehyde at the amino alcohol functionality of their daunosaminyl group to form dimeric conjugates assigned the trivial names, Doxoform and Daunoform, respectively.[4] The conjugates are bis-oxazolindylmethanes which exist in equilibrium with mono-oxazolidines and the parent drugs in aqueous media as shown in Scheme 1. Doxoform and Daunoform also react with DNA, most likely as the mono-oxazolidines, to form the virtual cross-links. Doxoform and Daunoform are significantly more toxic to MCF-7 human breast cancer cells than doxorubicin and daunorubicin and dramatically more toxic to doxorubicin-resistant breast cancer cells (MCF-7/ADR cells). In fact, Doxoform and Daunoform are each equally toxic to both sensitive and resistant cells. The MCF-7/ADR cells are specifically doxorubicin resistant and multidrug resistant[5] and bear a nonfunctional p53 gene.[6] Toxicity to resistant tumor cells is proposed to result in part from the conjugate not requiring the production of formaldehyde for alkylation of DNA.[4] Formaldehyde production results from oxidative stress in the presence of iron,[1] which is available because of doxorubicin's chelation of iron.[7] Resistant tumor cells have lower levels of reactive oxygen species presumably because of overexpression of enzymes which scavenge reactive oxygen species.[5] Resistance also appears to result from overproduction of glutathione[8] which scavenges reactive oxygen species and formaldehyde.[9]

4′-Epidoxorubicin (epirubicin) is the diastereoisomer of doxorubicin with the alcohol at the 4′-position epimerized. It is a broad spectrum drug similar to doxorubicin and is marketed worldwide except in the United States.[10] The primary pharmacological difference is conversion to a glucuronide derivative at the 4′-hydroxyl group. Glucuronides have been observed in the urine of patients receiving epidoxorubicin but not in the urine of patients receiving doxorubicin.[11] Glucuronide conjugation leads to faster drug clearance. Like doxorubicin, epidoxorubicin intercalates in DNA,[12] forms DNA cross-links in HeLa S3 cells,[13] and causes topoisomerase II-mediated strand breaks.[14]

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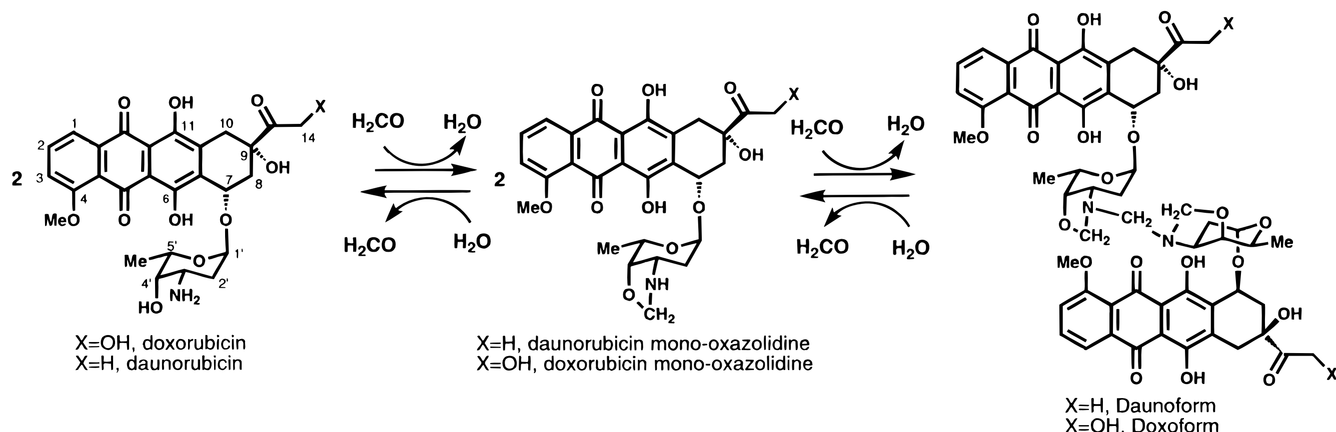
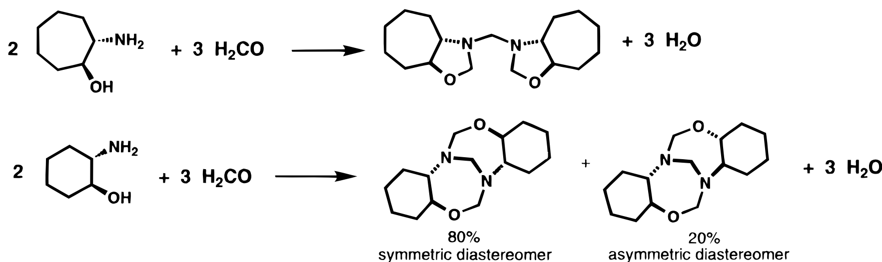
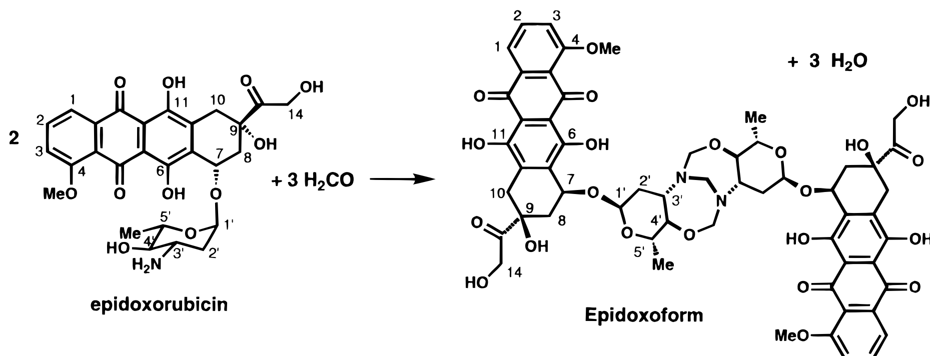
**Scheme 1.** Synthesis and Hydrolysis of Daunoform and Doxoform




**Scheme 2.** Analogy for the Syntheses of Daunoform, Doxoform and Epidoxoform




**Scheme 3.** Synthesis and Structure of Epidoxoform




Epidoxorubicin is of interest with respect to conjugation with formaldehyde because model studies predicted a different structure for the conjugate with respect to doxorubicin. The relevant models in the literature are *trans*-2-aminocycloheptanol and *trans*-2-aminocyclohexanol. *trans*-2-Aminocycloheptanol forms a bis-oxazolidinylmethane analogous to doxorubicin; however, *trans*-2-aminocyclohexanol forms a 1,6-diaza-4,9-dioxabicyclo[4.4.1]undecane ring system as shown in Scheme 2.[15-17] The chemistry appears to be controlled by the distance between the amino and alcohol functional groups.

We now describe the synthesis of the epidoxorubicin–formaldehyde conjugate Epidoxoform, its unusual hydrolysis, its reactivity with DNA, and its toxicity toward MCF-7 and MCF-7/ADR cells. The cytotoxicity of the Epidoxoform hydrolysis products is also reported.

## Results and Discussion

**Preparation and Characterization of Epidoxoform.** Reaction of epidoxorubicin with an excess of formaldehyde in pH 6 buffer followed by extraction into chloroform gave a new derivative, assigned the trivial name Epidoxoform, in 59% yield (Scheme 3). A methanol/chloroform solution of Epidoxoform showed a positive ion electrospray mass spectrum with an M + 1 peak for two epidoxorubicins bound together by three methylene groups with an intensity of 30% of the base peak. The base peak was assigned to the M + 1 for epidoxorubicin Schiff base. The $^{1}$H NMR spectrum indicates the symmetrical 1,6-diaza-4,9-dioxabicyclo[4.4.1]undecane ring system (Scheme 3) as predicted from the model reaction of *trans*-2-aminocyclohexanol with formaldehyde (Scheme 2) and is assigned relative to the $^{1}$H NMR spectrum of epidoxorubicin in $DCCl_3$ and DMSO-$d_6$ solvents in Table 1. The assignments were facilitated by a homonuclear COSY spectrum. Of particular significance are the patterns for the methylene groups from formaldehyde, $NCH_2O$ and $NCH_2N$, in contrast to the patterns for the methylene groups in Doxoform. The methylenes of Epidoxoform and Doxoform appeared as a 4-proton AX pattern and a 2-proton singlet in both molecules. The primary difference is $J_{AX}$ which is 4 Hz in Doxoform, characteristic of a methylene in a five-membered ring between two electronegative hetero-

**Table 1.** 500 MHz $^1H$ NMR Data for Epidoxoform and Epidoxorubicin (See Scheme 3 for Numbering System)

Chemical Shifts for Protons at Positions (Patterns, J Coupling Constants in Hz)

| | | | | | | | | | | | | | | | | CH2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 6/11 | 7 | 8 | 9 | 10 | 14 | 1′ | 2′ | 3′ | 4′ | 5′ | 5′-Me | O,N | N,N |
| Epidoxorubicin ($DCCl_3$) | | | | | | | | | | | | | | | | | |
| 8.02<br>(d, 8) | 7.77<br>(t, 8) | 7.38<br>(d, 8) | 4.07<br>(s) | | 5.29<br>(bs) | 2.37<br>(bd, 15)<br>2.14<br>(dd, 15, 4) | | 3.26<br>(d, 18)<br>3.01<br>(d, 18) | 4.76<br>(d, 20)<br>4.75<br>(d, 20) | 5.44<br>(d, 4) | 1.99<br>(dd, 13, 4)<br>1.56<br>(td, 13, 4) | 2.78<br>(ddd, 13, 10, 4) | 2.93<br>(t, 10) | 3.76<br>(dq, 10, 6) | 1.33<br>(d, 6) | | |
| Epidoxoform ($DCCl_3$) | | | | | | | | | | | | | | | | | |
| 8.04<br>(dd, 8, 1) | 7.77<br>(t, 8) | 7.38<br>(dd, 8, 1) | 4.07<br>(s) | 14.02<br>(s)<br>13.28<br>(s) | 5.21<br>(dd, 2, 4) | 2.37<br>(dt, 15, 2)<br>2.10<br>(dd, 15, 4) | 4.58<br>(s) | 3.29<br>(dd, 18, 2)<br>3.01<br>(d, 18) | 4.76<br>(bs) | 5.35<br>(d, 4) | 1.98<br>(dd, 14, 4)<br>1.57[a]<br>(ddd, 12, 14, 4) | 2.99<br>(ddd, 12, 9, 4) | 3.05<br>(t, 9) | 3.77<br>(dq, 9, 6) | 1.26<br>(d, 6) | 4.28<br>(d, 11)<br>3.92<br>(d, 11) | 4.16<br>(s) |
| Epidoxorubicin-HCl (DMSO-$d_6$)[b] | | | | | | | | | | | | | | | | | |
| 7.929[c]<br>(1, 5) | 7.93[c]<br>(5, 6) | 7.67[c]<br>(1, 5) | 3.99<br>(s) | 14.02<br>(s)<br>13.22<br>(s) | 5.27<br>(d, 3) | 2.15<br>(m) | 5.47<br>(s) | 2.99<br>(d, 16)<br>2.95<br>(d, 16) | 4.56<br>(d, 6)<br>4.86<br>(t, 6) | 4.96<br>(t, 4) | 2.04<br>(bd, 12)<br>1.74<br>(dt, 12, 4) | 3.07–<br>3.12 (m) | 3.07–<br>3.12 (m) | 3.93<br>(dq, 8, 6) | 1.20<br>(d, 6) | | |
| Epidoxoform (DMSO-$d_6$) | | | | | | | | | | | | | | | | | |
| 7.93[c]<br>(1, 7) | 7.924[c]<br>(5, 7) | 7.66[c]<br>(1, 5) | 3.98<br>(s) | 14.02<br>(s)<br>13.28<br>(s) | 5.13<br>(d, 3) | 2.16<br>(dd, 14, 3)<br>2.07<br>(dd, 14, 6) | 5.54<br>(s) | 2.96<br>(d, 18)<br>2.94<br>(d, 18) | 4.47<br>(d, 6)<br>4.77<br>(t, 6) | 4.90<br>(t, 5) | 1.91<br>(dd, 13, 5)<br>1.46<br>(td, 13, 5) | 2.91<br>(ddd, 13, 9, 5) | 3.18<br>(t, 9) | 3.91<br>(dq, 9, 6) | 1.14<br>(d, 6) | 4.14<br>(d, 11)<br>3.95<br>(d, 11) | 4.19<br>(bs) |

[a] The spectrum shows a large signal for $H_2O$ at $\delta$ 1.55 because Epidoxoform is a dihydrate. The $H_2O$ signal partially conceals the signal for one of the 2′-protons; the downfield doublet of the predicted triplet of doublets pattern is visible and has a splitting of 3 Hz. [b] The signal for the $NH_3+$ protons appears as a broad pattern centered at $\delta$ 7.85. [c] The aromatic protons appear as second-order patterns; chemical shifts and coupling constants were determined by computer simulation.

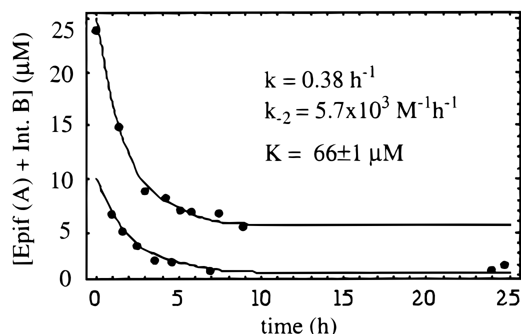



**Figure 1.** Disappearance of the equilibrium mixture of Epidoxoform and Intermediate B as a function of time and initial concentration of Epidoxoform in pH 7.3 phosphate buffer at 37 °C. The dots represent the data and the solid lines represent the fit of the data to the kinetic mechanism (A ⇔ B)/C + D as shown in Scheme 4 using the program Mathematica for numerical integration. Note that Mathematica created the figure with a small offset for time 0.

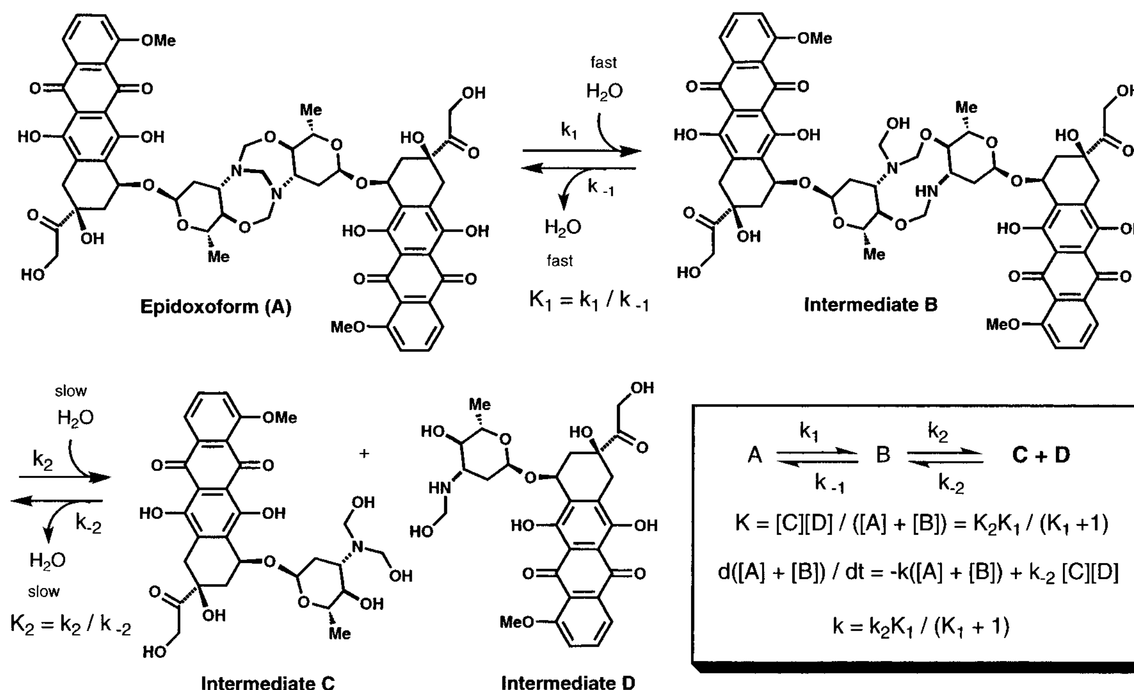
atoms, and 11 Hz in Epidoxoform, characteristic of a methylene between two electronegative heteroatoms in a larger ring system.[17,18] For comparison, the $^1$H NMR spectrum of the symmetric diastereomer from reaction of *trans*-2-aminocyclohexanol with formaldehyde (Scheme 2) shows an AX pattern for the $NCH_2O$ protons at $\delta$ 4.48 and 4.05 ppm with $J_{AX}$ = 11.5 Hz and a singlet for the $NCH_2N$ protons at $\delta$ 4.28 ppm.[16] Also of note is the observation of little change in the patterns for the protons at the 2′- and 3′-positions between epidoxorubicin and Epidoxoform. The same comparison of daunorubicin with Daunoform showed a factor of 2 change in one of the 2′−3′ coupling constants resulting from a dihedral angle change with formation of the five-membered oxazolidine rings. The NMR spectra also show no evidence of the diastereoisomer of Epidoxoform with the asymmetric, 1,6-diaza-4,9-dioxabicyclo[4.4.1]-undecane ring system shown in Scheme 2. The diastereomer would show an AB pattern for the methylene group bridging the two nitrogens.[17] The $^1$H NMR spectra of Epidoxoform and epidoxorubicin in $DCCl_3$ are provided in the Supporting Information.

**Hydrolysis.** Hydrolysis of Epidoxoform at 37 °C in pH 7.3 phosphate buffer containing 4% DMSO was monitored by reverse phase HPLC. The DMSO was present to increase solubility. The disappearance as a function of time is shown in Figure 1 starting with 25 and 10 $\mu$M Epidoxoform. Surprisingly, the reaction does not proceed to completion but comes to an equilibrium. Initially, we assigned the product to epidoxorubicin based upon retention time; however, co-injection of the hydrolysis products with epidoxorubicin showed that the products, in fact, have a slightly different retention time from that of epidoxorubicin. Further, the position of the equilibrium in the two experiments is consistent with one species going to two species, not one species going to five species as would be required if epidoxorubicin and formaldehyde were the products. The two sets of data were fit simultaneously to the rate law for one species reversibly forming two species using the computer program Mathematica to perform the numerical integration. The solid lines in Figure 1 represent the calculated fit. The kinetic mechanism was further substantiated by the agreement of calculated equilibrium constants for the two experiments (64.9 ± 0.4 $\mu$M) with the equilibrium constant $K$ obtained from the ratio of the forward and reverse rate constants (67 $\mu$M). The average value of these three determinations of $K$ is shown in Figure 1.

The hydrolysis was also examined by $^1$H NMR spectroscopy in DMSO-$d_6$. Epidoxoform was indefinitely stable in dry DMSO-$d_6$; however, upon addition of 7% $D_2O$, it rapidly formed a 2:1 equilibrium mixture with an intermediate with loss of no more than a trace of formaldehyde. The most prominent signals for the intermediate were three sharp singlets at $\delta$ 4.63, 4.76, and 4.78. The other signals for the intermediate appeared with the same chemical shifts as those of epidoxorubicin except for the signal for the protons at the 14-position. The protons at the 14-position appeared as two singlets, one at $\delta$ 4.45 and one at 4.56. When a trace of $H_2O$ containing 0.6 equiv of HCl was used in place of $D_2O$, the OH proton at the 9-position of the intermediate was also distinct, appearing at $\delta$ 5.75. The structure proposed for the intermediate is the 10-membered-ring structure (intermediate B) shown in Scheme 4 on the basis of the NMR spectral data and the kinetic data which require a mechanism with one species slowly equilibrating with two species. In this case, the one species is intermediate B which is in rapid equilibrium with Epidoxoform, and the two species are intermediates C and D. In the kinetics experiment the HPLC displayed only a single peak for the equilibrium mixture of intermediate B and Epidoxoform. Further, this equilibrium mixture in 93% DMSO-$d_6$/7% $D_2O$ was stable for days as indicated by $^1$H NMR monitoring. The three singlets in the $^1$H NMR spectrum of intermediate B are assigned to the three methylenes from the formaldehyde. The appearance of singlet patterns is potentially inconsistent with the proposed structure because the protons of each methylene are diastereotopic as a result of stereocenters on the daunosaminyl groups. However, they have the same apparent chemical shift because they are separated from the stereocenters by heteroatoms. A further contributing factor to the nuclei being isochronous is the conformational mobility of the 10-membered ring, as indicated by molecular models.

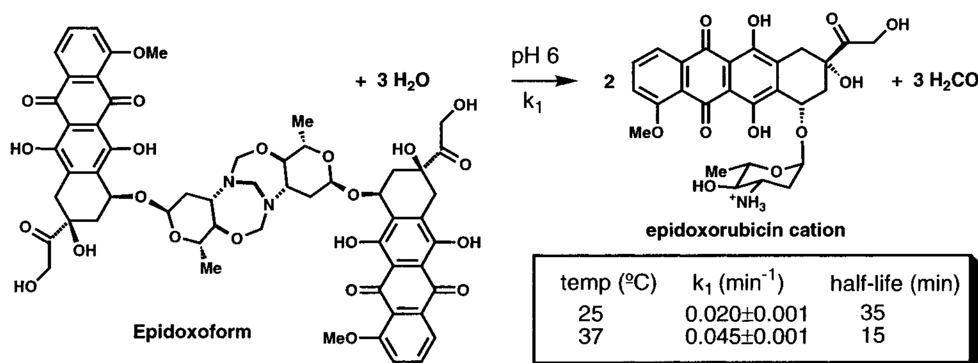
The $^1$H NMR spectrum of the hydrolysis reaction mixture resulting from equilibration of approximately 25 $\mu$M Epidoxoform in 96% $D_2O$/4% DMSO at 37 °C was also observed. This reaction mixture was analogous to the mixture present at equilibrium in the kinetic experiment shown in Figure 1, except for deuterium isotope effects and the effect of no buffer. The NMR spectrum was complex but was very informative in the region where the 5′-methyl groups resonate. In this region five methyl doublets were apparent, one for Epidoxoform ($\delta$ 1.33, relative intensity 2), two for intermediate B ($\delta$ 1.31 and 1.32, relative intensity 1 each), one for intermediate C ($\delta$ 1.20, relative intensity 4), and one for intermediate D ($\delta$ 1.38, relative intensity 4). The region where the methylenes resonate was completely obscured by the HOD signal, even with solvent suppression. Structures for intermediates C and D are proposed in Scheme 4. They are actually the products of the reaction but are called intermediates because they still bear formaldehyde. Intermediate C must be in equilibrium with D and formaldehyde, and

**Scheme 4.** Kinetic Mechanism for the Hydrolysis of Epidoxoform at pH 7.3[a]




[a] Derivations of the expressions for the equilibrium constant $K$ and the forward rate constant $k$ are provided in the supporting Information.

**Scheme 5.** Kinetic Mechanism for the Hydrolysis of Epidoxoform at pH 6.0[a]




| temp (ºC) | $k_1$ (min$^{-1}$) | half-life (min) |
|---|---|---|
| 25 | 0.020±0.001 | 35 |
| 37 | 0.045±0.001 | 15 |

[a] Kinetic data and analysis are provided in the Supporting Information.

D must be in equilibrium with epidoxorubicin and formaldehyde. These latter equilibria must favor C and D, respectively, at the experimental concentrations because epidoxorubicin was not apparent.

The kinetics of hydrolysis of Epidoxoform were also observed in pH 7.4 RPMI 1640 media containing 10% fetal bovine serum and in pH 8.1 fetal bovine serum, both at 37 °C with HPLC monitoring. In both cases the reaction kinetics were similar to those shown in Figure 1 for the reaction in pH 7.3 phosphate buffer. The data suggest that the half-life of the equilibrium mixture of Epidoxoform and intermediate B will be at least 2 h in serum at 37 °C with respect to formation of intermediates C and D. The half-life of C and D may be even longer but could not be determined with the available techniques. This is substantial when compared with an estimated half-life for Daunoform and Doxoform of less than 10 min.

The effect of acid on the hydrolysis of Epidoxoform was also studied. First, the equilibrium mixture of Epidoxoform, and intermediate B in 93% DMSO-$d_6$/7% $D_2O$ was titrated with aliquots of HCl, ranging from 0.5 to 2.0 molar equiv. Upon each addition of HCl, equivalent amounts of epidoxorubicin and formaldehyde were released as indicated by the $^1H$ NMR spectrum and no additional intermediates were observed. Second, the kinetics of hydrolysis were measured in pH 6.0 phosphate buffer. Under these conditions the reaction proceeded to completion with formation of epidoxorubicin with simple first-order kinetics as shown in Scheme 5. The half-life at 37 °C is only 15 min. In both experiments the reaction is driven toward epidoxorubicin and formaldehyde most likely because of the protonation of the 3′-amino group of epidoxorubicin. The kinetic data and data analysis are provided in the Supporting Information.

**Reaction of Epidoxoform and Epidoxorubicin with DNA.** The self-complementary 2′-deoxyoligonucleotide $(GC)_4$ was used for reactions with Epidoxoform and epidoxorubicin plus formaldehyde. Reaction of $(GC)_4$ with Epidoxoform yielded five drug−DNA adducts as indicated by reverse phase HPLC. UV−visible absorption indicated that the drug chromophore was intercalated between base pairs and that adducts

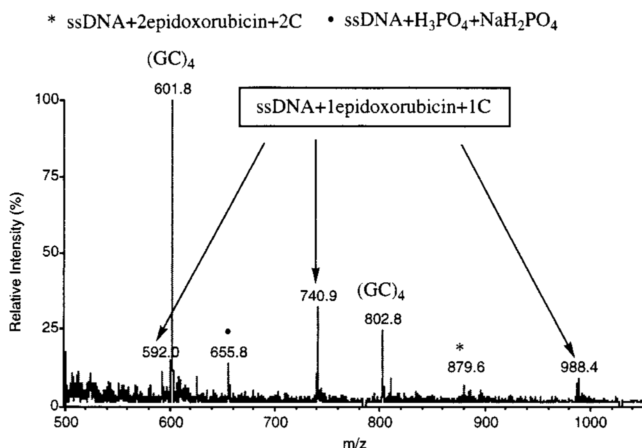



**Figure 2.** Negative ion, electrospray mass spectrum (ESMS) of DNA–epidoxorubicin adduct **5** from reaction of $(GC)_4$ with Epidoxoform in pH 7.3 phosphate buffer at 25 °C. Peaks representing assigned ions bearing one or more sodium ions are not labeled to simplify the figure. The calculated mass-to-charge ratios are as follows: 592.3, 601.8, 656.4, 740.6, 802.8, 879.4, and 987.9.

with shorter HPLC retention times contained one drug molecule per dsDNA (adducts 1 and 2) and that adducts with longer retention times contained two drug molecules per dsDNA (adducts 3, 4, and 5). Like doxorubicin– and daunorubicin–DNA adducts,[3,19–21] all of the epidoxorubicin–DNA adducts were hydrolytically unstable with respect to release of the drug from the DNA. With careful control of pH, the adducts were collected individually as they eluted from the HPLC column and analyzed by negative ion electrospray mass spectrometry. The mass spectra showed the appearance of peaks corresponding to one drug molecule bound to a single strand of DNA via one methylene group as well as peaks for DNA. The mass spectrum of the major product, adduct 5, appears in Figure 2. It also shows a small peak for ssDNA bound to two molecules of epidoxorubicin with methylene groups. The same five drug–DNA adducts were observed from reaction of $(GC)_4$ with epidoxorubicin plus an equivalent amount of formaldehyde. Structures analogous to those proposed for doxorubicin and daunorubicin bound to $(GC)_4$ are proposed for the adducts of epidoxorubicin bound to $(GC)_4$. In these structures, the chromophore of the drug is intercalated between 5′-CpG-3′ of 5′-CpGpC-3′, and the amino sugar in the minor groove is covalently linked via a methylene group from its 3′-amino substituent to the 2-amino substituent of the third G base of the opposing strand. Possible structures are shown schematically in Chart 1 of the Supporting Information.

The rates of reaction of Epidoxoform and epidoxorubicin plus formaldehyde with $(GC)_4$ are shown in Figure 3. At pH 7.3 epidoxorubicin plus formaldehyde reacts faster than Epidoxoform, and at pH 6.0 Epidoxoform reacts faster than epidoxorubicin plus formaldehyde. These relative rates are interpreted in terms of the rates of hydrolysis of Epidoxoform to drug–formaldehyde intermediates C and/or D (Scheme 4), the species which must be reactive with DNA. These intermediates form slowly at pH 7.3, and consequently, the hydrolysis of Epidoxoform must be the rate-limiting step. At pH 6.0, hydrolysis of Epidoxoform is much faster, and the rate-limiting step must be drug–DNA adduct formation. Starting with epidoxorubicin plus formaldehyde at pH 6.0 or pH 7.3, the rate-limiting step must be formation of the drug–formaldehyde intermediates.

**Cell Experiments.** Toxicity of Epidoxoform to MCF-7 human breast cancer cells and their doxorubicin resistant counterpart (MCF-7/ADR cells) relative to the toxicity of epidoxorubicin was determined as described previously for Doxoform and Daunoform.[4] The resulting $IC_{50}$ values are compared in Table 2. Epidoxoform is approximately 3-fold more toxic to sensitive cells (MCF-7) than is epidoxorubicin and greater than 120-fold more toxic to resistant cells (MCF-7/ADR). The $IC_{50}$ values show Epidoxoform to be about equally toxic to sensitive and resistant cells. These toxicity levels place Epidoxoform 7-fold less toxic than Daunoform which is 7-fold less toxic than Doxoform to both sensitive and resistant breast cancer cells. All three drug–formaldehyde conjugates are more toxic to sensitive cells than the bench mark, doxorubicin, and significantly more toxic to resistant cells than doxorubicin.

Because Epidoxoform hydrolyzes to an equilibrium mixture with intermediates B, C, and D in $H_2O$/DMSO, an $IC_{50}$ value versus resistant MCF-7/ADR cells was also determined with this equilibrium mixture of compounds. The mixture was established by preincubation of Epidoxoform in 90% $H_2O$/10% DMSO at 37 °C for 20 h. As an additional control, MCF-7/ADR cells were also treated with Epidoxoform stored in DMSO for 20 h at ambient temperature. Both of these solutions gave $IC_{50}$ values only 5-fold higher than a fresh solution of Epidoxoform, as shown in Table 2. The DMSO used in the additional control experiment was not dried. Thus, the DMSO probably contained sufficient $H_2O$ to hydrolyze a significant percentage of the Epidoxoform. The lower $IC_{50}$ values may have resulted from some hydrolysis of intermediate C to intermediate D and of intermediate D to epidoxorubicin at the lower concentrations of Epidoxoform. The lower concentrations of Epidoxoform in the cell experiment were significantly lower than those used in the hydrolysis experiments described above. Alternatively, the lower $IC_{50}$ values may have resulted from lower cell uptake of intermediates C and D relative to uptake of Epidoxoform and intermediate B. Comparing the cytotoxicity of the three drugs after being subjected to hydrolysis places them in the following order of activity: Epidoxoform $\gg$ Doxoform > Daunoform. This ordering suggests that the stereochemistry at the 4′-position and the presence of a hydroxyl at the 14-position are relevant to the stabilization of drug–formaldehyde conjugates. Thus, Epidoxoform, in contrast with Doxoform and Daunoform, might provide an active agent against resistant cancer with a relatively long lifetime in the vascular system.

Epidoxoform and intermediate epidoxorubicin–formaldehyde conjugates from its partial hydrolysis show significant toxicity to resistant breast cancer cells (MCF-7/ADR). Resistance in these cells is proposed to result from a combination of overexpression of enzymes which scavenge reactive oxygen species,[5] overexpression of glutathione transferase,[22] and overexpression of the P-170 glycoprotein efflux pump.[23] We propose that epidoxorubicin–formaldehyde conjugates can overcome at least some of these resistance mechanisms. Because

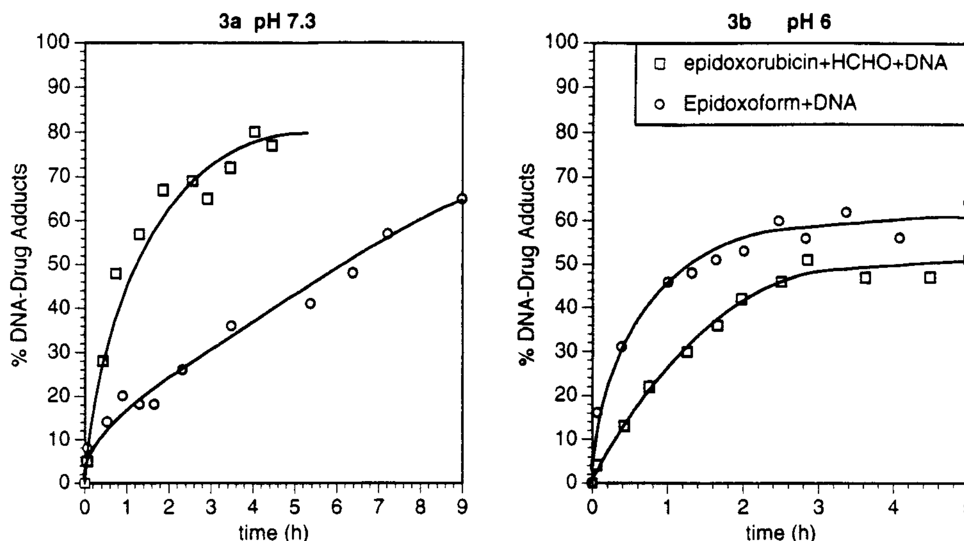



**Figure 3.** Formation of drug−DNA adducts from reaction of 33 $\mu$M $(GC)_4$ with either 33 $\mu$M Epidoxoform or 66 $\mu$M epidoxorubicin plus 99 $\mu$M formaldehyde in pH 7.3 (Figure 3a) and pH 6.0 (Figure 3b) phosphate buffer containing 4% DMSO at 25 °C as a function of time. Adduct formation represents the sum of adducts **1**−**5** 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.

**Table 2.** $IC_{50}$ Values for Epidoxorubicin (epi) and Epidoxoform (Epif) versus MCF-7 and MCF-7/ADR Cells Compared with the Values for Doxorubicin (dox)/Doxoform (Doxf) and Daunorubicin (daun)/Daunoform (Daunf)[4] and Values for Epidoxoform Stored in DMSO (Not Dried) for 20 h and for Epidoxoform Preincubated at 37 °C for 20 h in 90% $H_2O$/10% DMSO versus MCF-7/ADR Cells[a]

| | $IC_{50}$ values (nmolar equiv/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| cell type | epi | Epif | Epif/ DMSO | Epif/ $H_2O$ | dox | Doxf | daun | Daunf |
| MCF-7 | 200 | 65[b] | | | 300 | 2 | 60 | 8 |
| MCF-7 /ADR | >10000 | 70[b] | 300 | 400 | 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 nmolar equiv/L because Epidoxoform, Doxoform, and Daunoform each contain two anthracycline equivalents. Dose/response curves and raw data for epidoxorubicin and Epidoxoform experiments are provided in the Supporting Information. [b] Average of two determinations by two different investigators.

the conjugates carry their own formaldehyde, reactive oxygen species are not required for the production of formaldehyde. P-glycoprotein works best on positively charged anthracyclines.[24] Empirical rules for predicting $pK_a$'s of the conjugate acids of amino alcohols give $pK_a$'s = 8.5, 5.8, 5.8, and 3.1 for epidoxorubicin, intermediate B, intermediate D, and intermediate C, respectively.[25] The rules do not provide enough parameters for predicting the $pK_a$ of Epidoxoform but suggest that its $pK_a$ will be about 3. Hence, at physiological pH, only epidoxorubicin will be significantly protonated. Correspondingly, P-glycoprotein will be most effective against epidoxorubicin and significantly less effective against Epidoxoform and intermediates B, C, and D.

**Summary.** Reaction of epidoxorubicin with formaldehyde produces a formaldehyde conjugate (Epidoxoform) consisting of two molecules of epidoxorubicin held together with three methylene groups in a diazadioxabicyclic structure. Epidoxoform slowly hydrolyzes at pH 7.3 to an equilibrium mixture with intermediate drug−formaldehyde conjugates which are relatively stable even in fetal bovine serum. It reacts with DNA to yield drug−DNA adducts which are analogous to those formed from DNA and Doxoform. Epidoxoform is 3-fold and 120-fold more toxic than epidoxorubicin to MCF-7 and MCF-7/ADR cells, respectively. Additionally, the hydrolysis products of Epidoxoform are 25-fold more toxic to resistant breast cancer cells than epidoxorubicin.

## Experimental Section

**General Remarks.** UV−vis spectra were recorded with a Hewlett-Packard 8452A diode array spectrometer and $^1H$ NMR spectra, with a Bruker Am-400 or Varian Unity Inova 500 spectrometer. Mass spectra of Epidoxoform and 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 unless stated otherwise. Epidoxorubicin was received as gifts from Pharmacia-Upjohn-Farmitalia (Milan, Italy) and Sicor, Inc. (Milan, Italy). DNA oligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA, and purified as described earlier;[3] concentrations are reported as single stranded DNA (ssDNA). Formaldehyde was obtained from Mallinckrodt as a 37 wt % solution in water containing 10−15% methanol. Water was distilled and purified with a Millipore Q-UF Plus purification system to 18 Mohm-cm. Phosphate buffer, pH 7.3, was 77.4 mM $Na_2HPO_4$ and 22.6 mM $NaH_2PO_4$; phosphate buffer, pH 6.0, was 12 mM $Na_2HPO_4$ and 88 mM $NaH_2PO_4$. Triethylamine for preparation of triethylammonium acetate buffer was $99^+$% from Aldrich. Deuterium oxide ("100%") was obtained from Cambridge Isotope Laboratories; DMSO-$d_6$ was also obtained from Cambridge Isotope Labs and was stored over 3 Å molecular sieves. 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-$\mu$m $C_{18}$ microbore column, 2.1 mm i.d. × 100 mm, eluting at 0.5 mL/min with gradients of triethylammonium acetate ($Et_3NHOAc$) (20 mM)/acetic acid (AcOH) buffer and acetonitrile and detecting at 260 and 480 nm. The methods employed were method I: A = $CH_3CN$, B = pH 7.4 buffer, A:B, 0:100 to 70:30 at 10 min, isocratic until 12 min, 0:100 at 15 min, and method II: A = $CH_3CN$, B = pH 6 buffer, 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.

**Epidoxoform.** A pH 6 triethylammonium acetate solution containing 13.1 $\mu$mol of epidoxorubicin−HCl and 6.6 mmol of formaldehyde was allowed to react in the dark at 25 °C for 30 min. The mixture was then extracted with chloroform with vigorous stirring for 20 min. The chloroform solution was dried over sodium sulfate and the chloroform removed by rotary evaporation. The resulting dry solid was redissolved in 1.0 mL of chloroform. To this was added 10 mL of *n*-hexane. After 2 days, a large amount of precipitate had formed, which was washed with 3 × 5 mL of *n*-hexane, followed by 4 × 1 mL of water. The material was then placed under vacuum (0.01 Torr) for 6 h to remove residual water to yield 3.83 $\mu$mol (59%) of Epidoxoform. Anal. as the dihydrate ($C_{57}H_{58}N_2O_{22}\cdot 2H_2O$) C, H, N. Epidoxoform was characterized from $^1$H NMR data assigned in Table 1 and positive ion electrospray mass spectrometry. The sample for mass spectrometry was prepared by dissolving Epidoxoform in chloroform and diluting an aliquot of the chloroform solution with 200 parts of methanol v/v. The solution gave the following mass spectral peaks at *m*/*z* (assignment, relative intensity) 1219.5 (M + 1 + 3MeOH, 15), 1155.3 (M + 1 + MeOH, 27), 1143.4 (M + 1−1C + MeOH, 23), 1123.3 (M + 1, 30), 1111.3 (M + 1−1C, 33), 1099.3 (M + 1−2C, 20), 1086.5 (M + 1−3C, 12), 698.3 (27), 654.2 (35), 600.3 (epidoxorubicin + 1 + 2C + MeOH, 70), 588.3 (epidoxorubicin + 1 + 1C + MeOH, 55), 572.3 (55), 556.3 (epidoxorubicin + 1 + 1C, 100), 544.3 (epidoxorubicin + 1, 73). Epidoxoform was stable in dry DMSO-$d_6$ for at least 4 days, as determined by $^1$H NMR analysis. Epidoxoform was stored as a solid at ambient temperature in a desiccator.

**Stability of Epidoxoform in Aqueous Media.** A 960 $\mu$L solution of pH 7.3 phosphate buffer was heated to 37 °C. Epidoxoform was then introduced (40 $\mu$L in DMSO) such that the final concentration was 25 $\mu$M in 96% buffer/4% DMSO. The degradation of Epidoxoform to epidoxorubicin was monitored by HPLC using method I. The triethylammonium acetate/acetic acid (TEAA) buffer was adjusted to pH 7.4 to eliminate sample degradation on the column during analysis. No change in the chromatography was observed if pH 7.3 phosphate buffer was used as the eluent in place of pH 7.4 TEAA buffer. The retention times for epidoxorubicin and Epidoxoform were 7.4 and 11.1 min, respectively. The above procedure was also carried out with 10 $\mu$M Epidoxoform. Additional experiments were run in pH 6.0 phosphate buffer (25 °C and 37 °C), pH 7.4 RPMI Media 1640 containing 10% fetal bovine serum (37 °C), and pH 8.1 fetal bovine serum (37 °C), all with 25 $\mu$M Epidoxoform.

Hydrolysis of Epidoxoform was also observed by $^1$H NMR spectroscopy. A sample containing 3.6 mM Epidoxoform in 93% DMSO-$d_6$/7% $D_2O$ (v/v) was prepared. It showed 32% intermediate B in equilibrium with Epidoxoform within 5 min based upon integration of characteristic resonances described in Results and Discussion. The sample was maintained at ambient temperature for 2 h; after this period no change in the spectrum was observed. The amount of $D_2O$ was then increased to 10% (v/v) and the amount of intermediate B increased to 45%; this solution showed no additional change during a subsequent 2-day period. Another sample containing 3.9 mM Epidoxoform was prepared in DMSO-$d_6$. A stock 122 mM solution of HCl in $D_2O$ was prepared by adding concentrated hydrochloric acid to $D_2O$. The HCl solution was periodically added in 0.5 molar equiv aliquots to the DMSO-$d_6$ solution of Epidoxoform. Hydrolysis to epidoxorubicin and formaldehyde occurred within 5 min upon each addition. Complete hydrolysis occurred upon addition of ca. 2−3 equiv of HCl. A similar experiment was performed using HCl in $H_2O$. A third sample of Epidoxoform was prepared in $D_2O$ ("100%") containing 4% DMSO-$d_6$ to a concentration of ca. 25 $\mu$M. This solution was incubated at 37 °C for 24 h prior to NMR analysis. The spectrum showed evidence for Epidoxoform in equilibrium with intermediates B, C, and D (Scheme 4) as described in Results and Discussion. A control spectrum of epidoxorubicin in 96% $D_2O$/4% DMSO-$d_6$ was also obtained.

**DNA Adduct Formation with Epidoxoform.** A mixture containing 33 $\mu$M $(GC)_4$ and 33 $\mu$M Epidoxoform in pH 7.3 phosphate buffer containing 4% DMSO to facilitate Epidoxoform solubilization was prepared. The amount of available formaldehyde was 99 $\mu$M (3 × 33 $\mu$M). The formation of drug−DNA adducts at 25 °C was monitored by HPLC (method II). After 24 h, 68% of the DNA had reacted to form a mixture of five drug−DNA adducts. The ratio of the adducts, based upon HPLC peak areas, was 7:20:12:11:18, respectively. The experiment was also run at 25 °C in pH 6.0 phosphate buffer containing 4% DMSO. After 5 h, 64% of the DNA reacted to form drug−DNA adducts. The ratio of adducts **1**−**5** was 8:18:5:10:23, respectively. The results for both experiments are shown graphically in Figure 3.

For all Epidoxoform and epidoxorubicin−$(GC)_4$ reactions, the $A_{260}$:$A_{480}$ ratios for the drug−DNA adducts **2**−**5** were ca. 24:1, 15:1, 12:1, and 13:1, respectively. The ratio for adduct **1** was assumed to be 24:1, based upon the value for adduct **2**. Adduct **1** was often poorly resolved as a shoulder on the DNA peak. Under these circumstances, the adduct **1** area was estimated based upon its 480 nm absorbance, assuming the 24:1 ratio for $A_{260}$:$A_{480}$. For each of the adducts **1**−**5**, $A_{510} > A_{480}$. The retention times for the HPLC peaks were 6.4 min (DNA), 6.7 min (adduct 1), 7.9 min (adduct 2), 9.1 min (adduct 3), 11.0 min (adduct 4), and 12.1 min (adduct 5). Adduct 4 contained a shoulder which was included as part of the adduct 4 peak area.

**Large-Scale Reaction of Epidoxform with $(GC)_4$ for Electrospray Mass Spectral (ESMS) Analysis.** A reaction mixture containing 398 $\mu$M $(GC)_4$ and 796 $\mu$M Epidoxoform in pH 7.3 phosphate buffer was allowed to react in the dark at 25 °C for 5 h. At this time, the DNA had formed 91% drug−DNA adducts, according to HPLC analysis (method II). The ratios of the adducts were 1:5:10:22:53 for adducts **1**−**5**, respectively. Some Epidoxoform precipitated out of solution, as it was present in high concentration. The adducts were isolated by HPLC as described previously for DNA-daunomycin and -doxorubicin adducts [3] and analyzed by ESMS.

**DNA Adduct Formation with Epidoxorubicin and Formaldehyde.** A reaction mixture containing 33 $\mu$M $(GC)_4$, 66 $\mu$M epidoxorubicin, and 99 $\mu$M formaldehyde in pH 7.3 phosphate buffer containing 4% DMSO was prepared. The formation of drug−DNA adducts at 25 °C was monitored by HPLC (method II). After 5 h, 75% of the DNA had reacted to form drug−DNA adducts. The ratios of adducts **1**−**5** were 11:24:8:8:26, respectively. The experiment was also run in pH 6.0 phosphate buffer containing 4% DMSO. After 5 h, 51% of the DNA reacted to form drug−DNA adducts. The ratios for adducts **1**−**5** were 11:16:4:7:13.

**Large-Scale Reaction of Epidoxorubicin and Formaldehyde with $(GC)_4$ for ESMS Analysis.** A reaction mixture containing 166 $\mu$M $(GC)_4$, 664 $\mu$M epidoxorubicin, and 996 $\mu$M HCHO in pH 7.3 phosphate buffer (4% DMSO) was allowed to react in the dark at 25 °C for 5 h. At this time 93% of the DNA had reacted to form drug−DNA adducts. The adduct ratios were 1:6:5:31:49 for adducts **1**−**5**, respectively. The adducts were isolated by HPLC as described previously for DNA−daunomycin and −doxorubicin adducts[3] and analyzed by ESMS.

**Cell Experiments.** MCF-7 breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). MCF-7/ADR adriamycin resistant breast cancer cells [22] 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 (25 mM), penicillin (100 units/mL), and streptomycin (100 $\mu$g/mL). The MCF-7/ADR cell line media was additionally supplemented with 5 $\mu$M doxorubicin (Nexstar Pharmaceuticals, San Dimas, CA). Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. Determination of the cytotoxicity of epidoxorubicin and Epidoxoform was accomplished using experimental exactly as described earlier with doxorubicin and Doxoform.[4]

Control experiments established that the $IC_{50}$ value for formaldehyde was in excess of 200 $\mu$M 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.

**Toxicity of Hydrolyzed Epidoxoform.** The following stock solutions of Epidoxoform in DMSO were prepared: 1 mM, 500 $\mu$M, 100 $\mu$M, 50 $\mu$M, 10 $\mu$M, 5 $\mu$M, 1 $\mu$M, and 100 nM. These were then diluted 10× with autoclaved Millipore water. The resulting solutions in 90% water/10% DMSO (v/v) were incubated at 37 °C for 20 h. A 20 $\mu$L aliquot of each solution containing hydrolyzed Epidoxoform was then added to each of the appropriate wells of a 96-well plate each containing 1000 cells in 180 $\mu$L of RPMI 1640 media. At this point the experiment was conducted as previously described for $IC_{50}$ measurements with Doxoform and Daunoform.[4] As an additional control, the $IC_{50}$ value for Epidoxoform, stored for 20 h in DMSO (not dried) at ambient temperature, was simultaneously determined. The results are reported in Table 2, and the raw data and dose response curves are provided in the Supporting Information.

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**Supporting Information Available:** Supporting Information Available: $^1H$ NMR spectra for epidoxorubicin and Epidoxoform in $DCCl_3$, derivation of the kinetic and equilibrium expressions for the hydrolysis at pH 7.3, kinetic analysis of the hydrolysis of Epidoxoform at pH 6, schematic diagrams for proposed drug–DNA adducts, and dose/response curves and raw data for cell experiments (9 pages). Ordering information is given on any current masthead page.

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