Doxorubicin Analogues Incorporating Chemically Reactive Substituents

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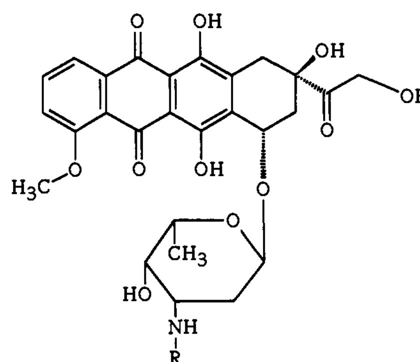
Doxorubicin (1) analogues 2–5, incorporating the following alkylating or latent alkylating substituents, R, on the 3′-position of the daunosamine sugar have been synthesized as potential antitumor agents: 2, R = $NHCOC_6H_4(p)SO_2F$; 3, R = $NHCOCH_2Br$; 4, R = $NHCOCH_2Cl$; 5, R = $NHCON(NO)CH_2CH_2Cl$. These compounds were designed on the premise that alkylating anthracyclines might bind covalently to critical intracellular target macromolecules and overcome resistance to the parent agent attributable to reduced cellular drug accumulation. Growth inhibitory studies of the analogues were conducted in vitro against mouse leukemia cells (L1210 and P388) and human uterine sarcoma cells that are sensitive (MES-SA) and resistant (MES-SA/DOX) to doxorubicin. The analogues were 5–100-fold less potent than doxorubicin against the sensitive cell lines. However, they were only marginally cross-resistant with doxorubicin against MES-SA/DOX. Compounds 3 and 5 were also evaluated against a human myelocytic cell line (KBM-3) and a subline (KBM-3/DOX) resistant to doxorubicin. They were equally potent against both cell lines, indicating a complete lack of cross-resistance with doxorubicin. Alkylating anthracyclines may have potential for the treatment of tumors resistant to the parent agents.

The anthracycline antibiotic doxorubicin is effective in the palliative management of a wide variety of human malignancies.[1] However, its clinical use is limited by a number of problems, including intrinsic and acquired drug resistance and dose-dependent cardiomyopathy. Numerous doxorubicin analogues have been synthesized in an attempt to overcome these shortcomings.[2–4] An interesting series of derivatives in which the 3′-amino group of the daunosamine sugar is replaced with a morpholino substituent has been reported by Acton and co-workers.[5–7] One of these analogues, 3′-deamino-3′-(3-cyano-4-morpholinyl)-adriamycin (MRA-CN), is 100–1000 times more cytotoxic than doxorubicin in vitro[7–10] and in vivo[6,11] and retains its potency against several tumor cell lines with acquired resistance to doxorubicin.[9,12,13] Moreover, MRA-CN is as effective as doxorubicin against a variety of transplantable animal tumors and, unlike the parent compound, is noncardiotoxic at optimal antitumor dosages.[6,7,9,14] Although its precise mechanism of cytocidal action is unknown, MRA-CN possesses both DNA intercalating[6] and DNA cross-linking[15–18] properties; the latter has not been observed for other anthracycline analogues and suggests that the drug kills cells by a unique mechanism. In view of the pronounced cytotoxicity of MRA-CN, it was of interest to evaluate other doxorubicin analogues bearing an alkylating substituent in the 3′-position of the sugar moiety. Such derivatives might bind covalently to critical intracellular target sites, thereby leading to increased intracellular drug accumulation and retention. A theoretical advantage of this approach is that it might overcome resistance to doxorubicin attributable to enhanced drug efflux[19–22] or reduced intracellular drug binding.[23] Preliminary accounts of this work have appeared.[24,25]

Chart I



1, R = H (Doxorubicin)
2, R = $COC_6H_5(p)SO_2F$
3, R = $COCH_2Br$
4, R = $COCH_2Cl$
5, R = $CON(NO)CH_2CH_2Cl$

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Table I. Growth Inhibition of L1210 and P388 Leukemia Cells in Vitro by Doxorubicin Analogues[a]

compd	IC_{50},[b] μM L1210	IC_{50},[b] μM P388
doxorubicin	0.2	1.2
2	6.9	7.7
3	2.1	1.5
4	2.1	2.9
5	2.9	2.9

[a] Exponentially growing cells were exposed to varying drug concentrations for 1 h at 37 °C. The cells were then centrifuged, resuspended, and cultured in drug-free medium for 72 h. [b] The drug concentration that inhibited cell growth by 50% compared to untreated control cultures.

Table II. Growth Inhibition of Human Sarcoma Cells (MES-SA) and a Subline (MES-SA/DOX) with Acquired Resistance to Doxorubicin in Vitro by Doxorubicin Analogues[a]

compd	IC_{50},[b] μM MES-SA	IC_{50},[b] μM MES-SA/DOX	resistance index[c]
doxorubicin	0.04	4.2	105.0
2	3.8	6.9	1.8
3	0.2	0.8	4.0
4	0.4	2.7	6.8
5	0.8	7.7	9.6

[a] See legend to Table I and "Materials and Methods". [b] The drug concentration required for 50% inhibition of cell growth compared to untreated control cultures. [c] Resistance index = IC_{50} for resistant cells/IC_{50} for sensitive cells.

Table III. Growth Inhibition of Human Myelocytic Cells (KBM-3) and a Subline Resistant to Doxorubicin (KBM-3/DOX) in Vitro by Doxorubicin Analogues[a]

compd	IC_{50},[b] μM KBM-3	IC_{50},[b] μM KBM-3/DOX	resistance index[c]
doxorubicin	0.026	0.49	18.8
3	0.14	0.16	1.1
5	0.21	0.25	1.2

[a] See legend to Table I and "Materials and Methods". [b] The drug concentration required for 50% inhibition of cell growth compared to untreated control cultures. [c] Resistance index = IC_{50} for resistant cells/IC_{50} for sensitive cells.

Chemistry

The structures of the analogues prepared are shown in Chart I. Compound **2** can theoretically form covalent bonds with nucleophilic sites in target macromolecules by displacement of the fluorine atom from the fluorosulfonyl moiety. The bromoacetamido and the chloroacetamido groups of **3** and **4** can similarly form covalent bonds by nucleophile displacement of the halogen atoms. The *N*-nitrosourea **5** is expected to be unstable in aqueous media and spontaneously decompose with elimination of the *N*-nitroso-*N*-(chloroethyl)amine moiety (as chloroethanediazohydroxide) to form an isocyanate intermediate that can carbamoylate nucleophilic sites.

Compound **2** was prepared from doxorubicin by reaction with 4-(fluorosulfonyl)benzoyl chloride in chloroform in the presence of diisopropylamine as proton acceptor. The bromoacetamide, **3**, and the chloro analogue, **4**, were prepared similarly by reaction of doxorubicin with bromoacetyl bromide and chloroacetic anhydride, respectively. The nitrosourea analogue, **5**, was synthesized by reaction of doxorubicin with *N*-nitroso-*N*-(2-chloroethyl)carbamoyl azide; this approach assures that the nitroso group is located on the chloroethyl-bearing nitrogen atom and avoids structural ambiguities (the formation of isomeric nitrosourea mixtures) associated with direct nitrosation of a urea precursor.

The analogues were purified to homogeneity by column chromatography on silica and characterized by NMR spectroscopy and mass spectrometry. NMR spectral features indicative of substitution at the 3′-amino group included the replacement of the NH_2 resonance of the parent compound with a one-proton doublet at δ 6.6–7.3 ($J_{NH-3'H}$ = 8.5 Hz) attributable to the amido (NH–acyl) proton. The mass spectra of the analogues showed well-defined parent molecular ions with the anticipated halogen isotope abundance ratios, as well as prominent fragmentation ions (e.g. *m/z* 414, 396, 378) characteristic of the unsubstituted aglycone portion of the molecule. However, because of poorly reproducible elemental analytical data,[26] the compounds were also analyzed by high-resolution mass spectrometry. Compounds **2**, **4**, and **5** gave the anticipated "exact mass" parent molecular ions. Compound **3**, on the other hand, decomposed during attempted volatilization precluding further characterization by this approach; however, the low-resolution MS and NMR data taken in conjunction with the chemical homogeneity of the compound leaves little doubt that it has the assigned structure.

Results

The effects of the analogues on the growth of P388 and L1210 murine leukemia cells during a 1-h incubation at 37 °C are shown in Table I. Compounds **3**, **4**, and **5** were 10–15-fold less potent that doxorubicin against the L1210 cells, whereas compound **2** was 35-fold less potent. A similar spectrum of activity was observed against P388 cells; however, the IC_{50} values compared more favorably with the IC_{50} value for doxorubicin because the parent drug was 6-fold less cytotoxic to P388 cells than to L1210 cells. The most toxic analogue, **3**, was only slightly less growth inhibitory than doxorubicin at equimolar concentration.

The analogues were also screened in vitro against a human uterine sarcoma cell line (MES-SA) and a subline (MES-SA/DOX) with over 100-fold resistance to doxorubicin (Table II). All of the analogues were less cytotoxic than doxorubicin against the sensitive cell line with the bromoacetyl compound, **3**, again being the most potent. Interestingly, the compounds retained much of their activity against the doxorubicin-resistant cell line. The fluorosulfonyl compound, **2**, possessed the most favorable resistance index, but the bromoacetyl compound, **3**, was the most toxic.

Compounds **3** and **5** were additionally screened against a human myelocytic cell line (KBM-3) and a subline (KBM-3/DOX) that had a 20-fold resistance to doxorubicin (Table III). Although the IC_{50} values for **3** and **5** were 5- and 8-fold higher, respectively, than that of doxorubicin against KBM cells, the activity of each compound against the resistant subline was nearly identical with that against the sensitive cell line, indicating a complete absence of cross-resistance with doxorubicin.

Discussion

Several hundred doxorubicin derivatives have been reported in an effort to develop analogues with enhanced antitumor efficacy and reduced host toxocity. Prior to initiation of the present studies,[24,25] however, there were

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(26) Despite their high degree of purity as evidenced by TLC, HPLC, and NMR analyses, all four compounds gave variant and poorly reproducible elemental analytical data. This finding is not unexpected, however, because of the amorphous character of the compounds, their intrinsic chemical reactivity, and susceptibility to degradation by atmospheric oxygen.

no reports of anthracyclines designed to bind covalently to biologic macromolecules in an effort to enhance cellular drug retention. This approach, inspired by the studies of Acton and colleagues[6,7] on MRA-CN, was devised to overcome pleiotropic resistance to anthracycline antibiotics, a phenomenon documented extensively in both experimental and human tumors.[27,28] Elevated expression of the multidrug resistance gene *mdr1* has been reported for the MES-SA cells used in this study.[29]

The reduced cytotoxicity of compounds **2** through **5** compared with doxorubicin in both mouse and human cell lines is consistent with previous reports that anthracycline analogues bearing *N*-acyl substituents are considerably less toxic than those with a free amino group.[30-38] The relatively low potency of N-acylated anthracyclines have been attributed to markedly decreased DNA binding affinity[37,38] a consequence of the loss of electrostatic binding energy between the protonated amino group and the negatively charged polynucleotide phosphate groups;[30,34,37,39,40] steric factors may also play a role. The pronounced cytotoxicity of MRA-CN, on the other hand, is consistent with the ability of the drug to form DNA intercalation complexes[6] and suggests that the 3′-nitrogen atom becomes basic before or during intercalation. One mechanism by which this could occur is by solvolysis of the cyano group followed by ring-opening of the resulting hydroxymorpholino intermediate to give the *N*-[2-(2-oxoethyl)ethyl] tautomer, i.e., **1**, R = $CH_2CH_2OCH_2CHO$. Covalent binding to DNA could then occur by interaction of the terminal aldehydo group with proximate nucleophiles, such as amino or sulfhydryl groups.

Although intriguing from a mechanistic viewpoint, pronounced cytocidal potency may not be predictive of the in vivo antitumor efficacy of doxorubicin analogues. Thus, MRA-CN has about the same therapeutic efficacy as doxorubicin in a number of experimental tumor systems.[7] In addition, the ability of anthracyclines to form DNA intercalation complexes does not appear to be a prerequisite for antitumor activity; AD 32, a nonintercalating *N*-acyl doxorubicin derivative, possesses good therapeutic properties.[41] Although the in vivo antitumor activity of the new alkylating analogues has not been established, their low degree of cross-resistance against doxorubicin-resistant cell lines derived from human tumors encourages further investigation. In this regard, compound **3** appears to be the most promising because it combines a low degree of cross-resistance with good cytocidal activity.

Whereas the activity of the new compounds against doxorubicin-resistant tumor cells in vitro is consistent with the rationale invoked in their design, it is emphasized that the mechanism of action of these analogues has not been established. Other mechanisms, such as increased drug uptake consequent upon enhanced lipophilicity or altered intracellular distribution, may equally account for the biologic findings.

In summary, several new doxorubicin analogues incorporating alkylating substituents have been synthesized and characterized. Although less potent than doxorubicin they show little or no cross-resistance with doxorubicin in human sarcoma and leukemic cell lines resistant to doxorubicin. Further studies of these compounds against drug-resistant tumors are in progress.

Experimental Section

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded at ambient temperature on an IBM-Bruker Model NR/200 AF spectrometer in the Fourier transform mode in $CDCl_3$ with tetramethylsilane as an internal reference. Chemical shifts (δ) are reported in parts per million and coupling constants (J) in hertz. Mass spectral analyses were conducted at the Midwest Center for Mass Spectrometry, Lincoln, NE. Samples were formulated in a 3-nitrobenzyl alcohol matrix and analyzed by fast-atom bombardment on a Kratos MS 50 TA instrument. All chemical reactions were carried out in dry glassware and were protected from atmospheric moisture. Solvents were dried over freshly activated (300 °C, 1 h) molecular sieves (type 4 A). Evaporations were carried out on a rotary evaporator under aspirator vacuum at a bath temperature of <25 °C. The homogeneity of the products was determined by ascending TLC on silica-coated glass plates (silica gel 60 F 254, Merck) with mixtures of $CHCl_3$–MeOH as the eluting solvent. Preparative separations were obtained by column chromatography on silica gel (Merck, 230–400 mesh) with mixtures of $CHCl_3$–MeOH as eluent.

Analogue Syntheses. Doxorubicin free base was prepared from the commerically available hydrochloride salt (Adriamycin) as follows: the salt (0.5 g), admixed with 2.5 g lactose as inert diluent, was dissolved in water (15 mL) by stirring for 30 min at room temperature under a nitrogen atmosphere. The solution was then diluted to a final volume of 25 mL with 0.2 M phosphate buffer, pH 10, and extracted repeatedly (8–10 times) with $CHCl_3$–MeOH, 95:5 (25 mL), until the organic layer was lightly colored. The extracts were combined, dried over Na_2SO_4, filtered, and evaporated. The amorphous red solid that remained was dried in vacuo over P_2O_5 at ambient temperature for 24 h. It was stored in the dark in a tightly stoppered vessel under a nitrogen atmosphere at −20 °C.

***N*-[4-(Fluorosulfonyl)benzoyl]doxorubicin (2).** Doxorubicin (40 mg, 0.0736 mmol) was dissolved in dry chloroform (5 mL), and diisopropylethylamine (9.51 mg, 12.82 μL, 0.0736 mmol) was added. The solution was cooled to 0 °C in an ice bath, and a solution of 4-(fluorosulfonyl)benzoyl chloride (17.2 mg, 0.0773 mmol) in dry chloroform (1 mL) was added with stirring. The reaction mixture was allowed to warm to room temperature and then stirred for 1 h. It was washed sequentially with 0.1 M phosphate buffer, pH 7.0 (3 mL), and water (2 × 3 mL), and the organic layer was dried over anhydrous sodium sulfate. The solution was concentrated to a volume of about 1 mL and applied to a column (1 × 30 cm) of silica (Merck, 230–400 mesh); the products were eluted with chloroform–methanol (98:2). Two minor forerunner bands were discarded, and the third, slower moving band was collected and evaporated to give **2** as a red solid. It was dried in vacuo over P_2O_5 at ambient temperature for 48 h: yield 33.3 mg (62%). TLC (silica) R_f 0.38 in $CHCl_3$–MeOH, 197:3; NMR ($CDCl_3$) δ 13.99 (s, 1 H, 6-OH), 13.22 (s, 1 H, 11-OH), 7.92–8.05 (m, 5 H, H-1, H-2″, H-3″, H-5″, H-6″), 7.77 (t, 1 H, H-2), 7.39 (d, 1 H, H-3), 6.69 (d, 1 H, NHCO), 5.54 (br s, 1 H, H-1′), 5.29 (br s, 1 H, H-7), 4.77 (s, 2 H, 2 × H-14), 4.55 (s, 1 H, 9-OH),

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4.25 (m, 2 H, H-3′, H-5′), 4.07 (s, 3 H, 4-OCH_3), 3.75 (m, 1 H, H-4′), 3.28 (d, 1 H, H-10B), 2.98 (d, 1 H, H-10A), 2.36 (d, 1 H, H-8B), 2.19 (dd, 1 H, H-8A), 1.90 (m, 2 H, H-2′), 1.25 (d, 3 H, 3 × H-6′); MS *m/z* 729 (M), 669 (M − $HCOCH_2OH$), 414 (aglycone-7-OH), 398, 396 (aglycone-7-OH − H_2O), 378 (aglycone-7-OH − $2H_2O$); HRMS calcd for $C_{34}H_{33}FNO_{14}S$ $(M + H)^+$ 730.1606, found 730.1580.

***N*-(Bromoacetyl)doxorubicin (3).** The compound was prepared from doxorubicin (40 mg, 0.0736 mmol) and bromoacetyl bromide (15.60 mg, 6.7 μL, 0.0773 mmol) according to the procedure described for 2: yield 24.4 mg (50%); TLC (silica) R_f 0.27 in $CHCl_3$–MeOH, 197.3; NMR ($CDCl_3$) δ 14.02 (s, 1 H, 6-OH), 13.29 (s, 1 H, 11-OH), 8.01 (d, 1 H, H-1), 7.78 (t, 1 H, H-2), 7.39 (d, 1 H, H-3), 6.95 (d, 1 H, NHCO) 5.51 (br s, 1 H, H-1′), 5.27 (br s, 1 H, H-7), 4.76 (s, 2 H, 2 × H-14), 4.17 (m, 2 H, H-3′, H-5′) 4.07 (s, 3 H, 4-OCH_3), 4.00 (s, 2 H, $COCH_2Cl$) 3.70 (m, 1 H, H-4′), 3.25 (d, 1 H, H-10B), 2.96 (d, 1 H, H-10A), 2.34 (d, 1 H, H-8B), 2.17 (dd, 1 H, H-8A), 1.85 (m, 2 H, H-2′), 1.23 (d, 3 H, 3 × H-6′); MS *m/z* 665 (^{81}Br), 663 (^{79}Br) (M), 647, 645 (M − H_2O), 605, 603 (M − $HCOCH_2OH$), 414 (aglycone-7-OH), 398 (aglycone-7-H − H_2O), 396 (aglycone-7-OH − H_2O), 378 (aglycone-7-OH − $2H_2O$).

***N*-(Chloroacetyl)doxorubicin (4).** The compound was prepared from doxorubicin (40 mg) according to the general procedure described for 2 except that chloroacetic anhydride (13.2 mg, 0.0773 mmol) was used in place of bromoacetyl bromide: yield 26.0 mg (57%); TLC (silica) R_f 0.28 in $CHCl_3$–MeOH, 197:3. NMR ($CDCl_3$) δ 13.98 (s, 1 H, 6-OH), 13.28 (s, 1 H, 11-OH), 8.01 (d, 1 H, H-1), 7.78 (t, 1 H, H-2), 7.39 (d, 1 H, H-3), 6.95 (d, 1 H, NHCO) 5.51 (br s, 1 H, H-1′), 5.27 (br s, 1 H, H-7), 4.76 (s, 2 H, 2 × H-14), 4.17 (m, 2 H, H-3′, H-5′) 4.07 (s, 3 H, 4-OCH_3), 4.00 (s, 2 H, $COCH_2Cl$) 3.70 (m, 1 H, H-4′), 3.25 (d, 1 H, H-10B), 2.96 (d, 1 H, H-10A), 2.34 (d, 1 H, H-8B), 2.17 (dd, 1 H, H-8A), 1.85 (m, 2 H, H-2′), 1.23 (d, 3 H, 3 × H-6′); MS *m/z* 619 (M), 601 (M − H_2O), 559 (M − $HCOCH_2OH$), 414 (aglycone-7-OH), 398 (aglycone-7-H − H_2O), 396 (aglycone-7-OH − H_2O), 378 (aglycone-7-OH − $2H_2O$); HRMS calcd for $C_{29}H_{31}{}^{35}ClNO_{12}$ $(M + H^+)$ 620.1535, found 620.1565.

***N*-[*N*′-(2-Chloroethyl)-*N*′-nitrosocarbamoyl]doxorubicin (5).** The compound was prepared from doxorubicin (40 mg) and *N*-nitroso-*N*-(2-chloroethyl)carbamoyl azide[42] (13.7 mg, 0.0773 mmol) by the general procedure described for 2: yield 14.5 (29%); TLC (silica) R_f 0.46 in $CHCl_3$–MeOH, 197:3; NMR ($CDCl_3$) δ 13.96 (s, 1 H, 6-OH), 13.18 (s, 1 H, 11-OH), 8.01 (d, 1 H, H-1), 7.78 (t, 1 H, H-2), 7.39 (d, 1 H, H-3), 7.27 (d, 1 H, NHCO), 5.56 (br s, 1 H, H-1′), 5.30 (br s, 1 H, H-7), 4.78 (s, 2 H, 2 × H-14), 4.56 (br s, 1 H, 9-OH), 4.26 (m, 2 H, H-3′, H-5′), 4.10 (t, 2 H, $N(NO)CH_2$), 4.07 (s, 3 H, 4-OCH_3), 3.65 (m, 1 H, H-4′), 3.43 (t, 2 H, CH_2Cl), 3.25 (d, 1 H, H-10B), 2.95 (d, 1 H, H-10A), 2.37 (d, 1 H, H-8B), 2.20 (dd, 1 H, H-8A), 1.97 (m, 2 H, H-2′), 1.34 (d, 3 H, 3 × H-6′); MS *m/z* 677 (M), 569 (M − $ClCH_2CH_2N.NOH$), 509 (M − $ClCH_2CH_2N.NOH$ − $HCOCH_2OH$), 414 (aglycone-7-OH), 398 (aglycone-7-H − H_2O) 396 (aglycone-7-OH − H_2O), 378 (aglycone-7-OH − $2H_2O$); HRMS calcd for $C_{30}H_{33}{}^{35}ClN_3O_{13}$ $(M - H^+)$ 678.1702; found 678.1702.

Drug Solutions. Since the analogues were only sparingly soluble in water, they were dissolved in dimethyl sulfoxide (DMSO) immediately before use. For growth inhibition studies, the stock solutions were diluted in the culture medium to a final DMSO concentration of 0.5%. Drug-free control cultures likewise contained 0.5% DMSO.

Cytotoxicity Assays. Growth inhibition of cultured tumor cells was used as a measure of the relative cytotoxicities of the analogues.

Cell Lines. P388 or L1210. P388 or L1210 murine leukemia cells were maintained in vitro by serial culture in RMPI medium 1640 containing 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 μmol/mL), 2-mercaptoethanol (10 μM), penicillin (50 units/mL), streptomycin (50 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells in exponential growth at a density of 10^6 cells/mL were exposed to varying drug concentrations for 1 h at 37 °C. They were then harvested by centrifugation for 5 min at 1500 rpm, washed twice with ice-cold phosphate-buffered saline (PBS) (2 mL), resuspended in drug-free medium at a concentration of 2 × 10^5/mL, and cultured for 72 h. Cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.[43]

MES-SA and MES-SA/DOX. The doxorubicin-sensitive (MES-SA) and doxorubicin-resistant (MESSA/DOX) uterine sarcoma cell lines were kindly provided by Dr. W. Graydon Harker, VA Medical Center, Salt Lake City, UT; their biologic characteristics have been described.[44] The cells were maintained in monolayer culture in Waymouth's/McCoy's 5A media (Gibco, Grand Island, NY) supplemented with penicillin (100 μg/mL), streptomycin (1000 μg/mL), insulin (5 μg/mL), and 15% FCS.

Drug sensitivity assays were performed by using a modification of the MTT colorimetric assay.[45] MES-SA and MES-SA/DOX cells were seeded at 4 × 10^3 and 6 × 10^3 cells per well, respectively, in 96-well microliter plates that were incubated at 37 °C in a 5% CO_2-humified atmosphere for 16–24 h to ensure cell attachment. The medium was then removed by inverting and flicking the plate. Fresh medium (100 μL) containing various drug concentrations was then added to each well. The plates were incubated for 72 h, then 15 μL of a solution of MTT in PBS (5 mg/mL) was added to each well. The plates were further incubated for 3 h at 37 °C. The unreduced MTT was reduced by inverting, flicking, and blotting the plate. DMSO (50 μL per well) was added, and the plates were placed on a shaker for 2 min to solubilize the reduced dye crystals. The optical density of each well was measured with use of an automated microplate reader (Biotek, Winooski, VT) with a 570-nm test wavelength. The resistance index was calculated by comparing the IC_{50} values (drug concentration required for 50% inhibition of cell growth compared to untreated control cultures) of the resistant cell line with that of the sensitive cell line.

KBM-3 and KBM-3/DOX. The human myelogenous leukemia cell line KBM-3 and a variant, KBM/DOX, with approximately 20-fold resistance to doxorubicin, as determined by clonogenic assay, were developed in our laboratory (B. S. Andersson, unpublished results) from a patient with monocytic leukemia. The KBM-3/DOX cell line was obtained by serial exposure of KBM-3 cells over 20 generations to progressively increasing concentrations of doxorubicin. Both cell lines were propagated in IMDM (Iscove's modification of Dulbecco's minimum essential medium; Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS.

Cells in exponential growth were suspended in 1 mL of enriched PBS (pH 7.4, supplemented with glucose, Ca^{2+}, and 5% FCS) at a density of 1 × 10^7/mL and incubated with the drugs over a range of concentrations for 1 h at 37 °C. The cells were then washed twice with ice-cold PBS and pelleted by centrifugation for 10 min at 150*g*. They were resuspended at a density of 1 × 10^5/mL in IMDM and assayed for surviving clonogenic cells by using a single-layer semisolid agar medium (IMDM, 15% FCS, and 0.3% agar). After 8 days at 37 °C in a fully humified atmosphere of 5% CO_2 in air, clones containing 50 or more cells were scored under an inverted phase-contrast microscope. The surviving fractions were calculated, dose–response curves constructed, and the IC_{50} values were determined. Results are expressed as the mean of triplicate cultures. The resistance index was calculated as before by comparing the IC_{50} values of the resistant cell line with that of the sensitive cell line.

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