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Removal of the basic center from doxorubicin partially overcomes multidrug resistance and decreases cardiotoxicity

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Hydroxyrubicin, a synthetic doxorubicin analog in which the basic amino group at C-3′ is replaced by a hydroxyl group, was used as a prototype compound to study the effects of basicity of the sugar moiety on the toxicity and antitumor activity of anthracycline antibiotics. Compared with doxorubicin, hydroxyrubicin showed similar or superior *in vitro* cytotoxicity against P388, L1210, and M5076 cells, as determined by an MTT assay, and against 8226 and CEM cells, as determined by a growth inhibition assay. Hydroxyrubicin was 5 and 13 times more effective than doxorubicin in inhibiting the growth of multidrug-resistant CEM (CEM_{vbl}) and 8226 (8226R) cells, respectively. Hydroxyrubicin was not cross-resistant with doxorubicin in a cytotoxicity assay against KB 3-1 and KB V1 cells (resistance index 1.1 for hydroxyrubicin versus > 15.6 for doxorubicin). Cellular uptake and retention of hydroxyrubicin were studied by flow cytometry in parent and multidrug-resistant 8226 cells, and compared with those of doxorubicin. In 8226 sensitive cells, 2 h uptake and retention of doxorubicin were similar or higher than those of hydroxyrubicin. In 8226R cells, uptake and retention of hydroxyrubicin were about 3-fold higher than those of doxorubicin. In mice, the acute LD_{50} of hydroxyrubicin was about 3-fold higher than that of doxorubicin (79.1 versus 25.7 mg/kg). At equitoxic doses, hydroxyrubicin was as myelosuppressive as doxorubicin but less cardiotoxic, as assessed by the Bertazzoli test. In contrast to doxorubicin, hydroxyrubicin, due to the lack of basic amine function, showed no selective interaction with negatively-charged cardiolipin (CL). The observed decrease of affinity to CL might be responsible for the reduced cardiotoxicity of hydroxyrubicin. In *in vivo* antitumor activity studies, hydroxyrubicin at the optimal dose (37.5 mg/kg, i.p., on day 1) had significant activity against intraperitoneal P388 leukemia resistant to doxorubicin, whereas doxorubicin (10 mg/kg, i.p., on day 1) was inactive (%T/C 163–200 versus 118–120). These studies indicate that: (i) the amino group at position 3′ is not essential for doxorubicin to exert its biological activity, (ii) removal of the basic center (deamination at the C-3′) results in an increased cellular uptake and retention, (iii) the increased cellular uptake and retention of hydroxyrubicin in multidrug-resistant cells correlate with a partial or total lack of cross-resistance of this analog with the parent compound, doxorubicin, and (iv) deamination at position 3′ confers a reduced cardiotoxicity and diminished affinity for CL.

Key words: Anthracycline antibiotics, cardiotoxicity, doxorubicin, hydroxyrubicin, multidrug resistance (MDR).

Introduction

Doxorubicin, owing to its marked activity against certain solid tumors and hematological malignancies, is one of the most widely used chemotherapeutic agents.[1] However, its clinical use is limited by toxic side effects, such as acute myelosuppression and chronic cumulative cardiotoxicity, and *de novo* or acquired resistance.[1,2] *De novo* (intrinsic) resistance is observed in solid tumors such as colon carcinoma, prostate carcinoma or non-small cell lung cancer.[3] Acquired resistance to doxorubicin, known as multidrug resistance (MDR) or pleiotropic drug resistance,[1,2] is a phenomenon characterized by the development of resistance not only to doxorubicin but also to a wide spectrum of structurally unrelated antitumor drugs.[2,4–6] The clinical relevance of MDR is being actively and widely investigated at present.

The doxorubicin molecule consists of two parts: the chromophore aglycone (adriamycinone, ADR) and the carbohydrate moiety (daunosamine, DSM), which are connected by a glycosidic bond. The

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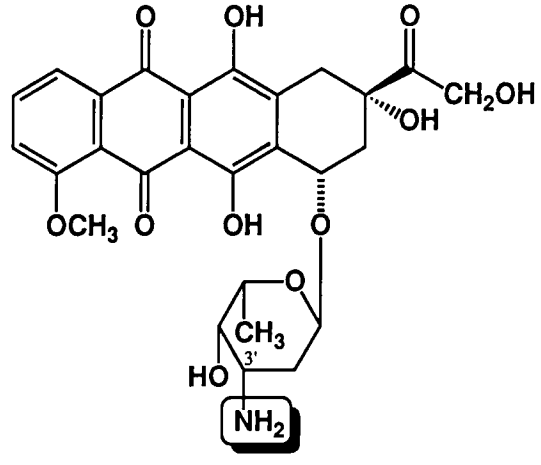
chromophore part, common to all anthracyclines, is believed to be responsible for the intercalating and cardiotoxic properties of this class of drugs. However, the ADR or related anthracyclinones have very limited antitumour activity by themselves.[7] The role of the carbohydrate moiety is less clear. It is generally accepted that the sugar moiety might be an important determinant of tissue and intracellular kinetics. Its importance is confirmed by the observation that sometimes even small chemical changes in the sugar moiety can result in a total loss of cytotoxicity. DSM, the carbohydrate portion of doxorubicin, is a monosaccharide that was first isolated as a component of anthracycline antibiotics. The most characteristic feature of this deoxy sugar is the presence of a basic amino group at position C-3′, which is protonated at physiological pH.

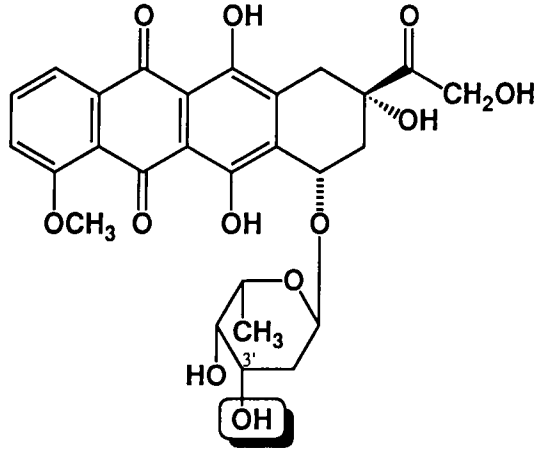
The amino group has been implicated by different authors: (i) as an important factor stabilizing the complex between anthracyclines and DNA through electrostatic interactions of positively charged amino group with the phosphate oxygen of DNA; (ii) as a determinant of the affinity of anthracyclines for negatively charged phospholipids, the doxorubicin complex with cardiolipin (CL) is strongly stabilized by interaction between the positively charged sugar amine and the negatively charged phosphate groups;[8–15] (iii) as a factor playing an important role in determining membrane transport and cytotoxicity;[16,17] and (iv) as a functionality associated with the mutagenic activity of anthracyclines.[18,19] Some reports have indicated that derivatization of the amino group reduces mutagenicity without altering the antitumor properties.[18]

Interestingly, the amino group has also been recognized as a common structural feature of different chemotherapeutic drugs exhibiting cross-resistance in MDR cells, as well as of some chemosensitizers that are able to reverse MDR *in vitro* by inhibiting the function of P-glycoprotein (PGP).[20,21]

The above observations stress the importance of the amino group in the interactions of anthracyclines with different macromolecules. Specifically, we have hypothesized that anthracycline binding is significantly affected by the basicity of the sugar part of anthracycline antibiotics.[22,23] Consequently, altering the basicity should noticeably affect the antitumor activity and toxicity of doxorubicin. As an example, if the amine group plays a role in the binding of the drug to PGP, reduction of basicity might increase the accumulation of the drug in MDR cells and, consequently, increase the cytotoxicity against these cells. 3′-Hydroxy-3′-deaminodoxorubicin (hydroxyrubicin), the compound configurationally identical to doxorubicin (Figure 1), was selected as a model to test this hypothesis, since the replacement of amino function at C-3′ by a hydroxyl group drastically affects the basicity of the sugar portion. Some of anthracycline analogs previously identified as non-cross-resistant with doxorubicin were highly modified in the sugar portion, in the aglycone part or in both.[24–29] Studies of such analogs are very interesting; however, it is difficult to draw conclusions concerning the effects of specific chemical modifications on the activity against MDR cells as well as on the interaction with other



DOXORUBICIN



HYDROXYRUBICIN

Figure 1. Chemical structures of doxorubicin and its 3′-deaminated congener, hydroxyrubicin.

biologically important macromolecules. Therefore, hydroxyrubicin seemed to be the most appropriate model for the proposed studies.

Here we describe studies of *in vitro* cytotoxicity, cellular uptake and efflux against a battery of sensitive and MDR, murine and human, cell lines with hydroxyrubicin and doxorubicin. In addition, we evaluated the *in vivo* mouse toxicity and antitumor activity against P388/Dox of both compounds. Our results indicate that deamination at position 3′ of the sugar portion of doxorubicin confers a partial or total lack of cross-resistance and decreased cardiotoxic potential. The reduced cardiotoxicity of hydroxyrubicin might be related to its significantly diminished affinity for CL.[14]

Materials and methods

Anthracyclines

Hydroxyrubicin was synthesized using procedures previously described by us,[30] employing daunomycinone and L-fucose as substrates. Material used for this study was purified by column chromatography on silica gel 60 (200–400 mesh, Merck AG, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on precoated plastic sheets (0.2 mm) of silica gel 60 F-254 (Merck AG); compounds were detected by first spraying with 10% sulfuric acid and subsequently heating the plates. Nuclear magnetic resonance (NMR) spectra were recorded for solution in chloroform-*d* (internal standard Me_4Si) with a QE 300 spectrometer operating at 300 and 75 MHz for ^1H and ^{13}C nuclei, respectively.

Doxorubicin was obtained from the Hospital Pharmacy (Adriamycin, Adria Laboratories, Columbus, OH).

Cell lines

P388 and L1210 leukemia cells were obtained from the Tumor Repository, National Cancer Institute (Frederick, MD) and were kept in culture in RPMI 1640 medium (Cellgro, Mediatech, Washington, DC) supplemented with 15 and 10% fetal calf serum (FCS), respectively.

M5076 cells were obtained from the Department of Cell Biology, The University of Texas MD Anderson Cancer Center (Houston, TX) and kept in culture in RPMI 1640 medium supplemented with 16% horse serum.

KB 3-1 human carcinoma and KB V1 were obtained from Dr Michael M Gottesman at the National Cancer Institute. KB V1 cells have the MDR phenotype and express glycoprotein P-170 in their membrane.[31,32] KB 3-1 and KB V1 cells were grown in monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For KB V1 cells, vinblastine was added to the culture medium (final concentration 0.5 μg/ml).

Cell line 8226 is a human myeloma cell line. Its resistant variant, 8226R, was selected in medium containing 1 mg/ml doxorubicin[33] and has been shown to express the MDR gene.[34] The 8226R cells were obtained from Dr Josh Epstein, currently at The University of Arkansas Medical School (Little Rock, AK).

The lymphoblastic leukemia cell line CCRF-CEM was obtained from the American Type Culture Collection (Rockville, MD). CEM_{vbl} cells have been shown to express the MDR gene both at the nucleic acid and at the protein product levels.[24] CEM_{vbl} cells were obtained from Dr Josh Epstein. CEM and 8226 cells were grown in RPMI 1640 medium supplemented with 10% FCS. Absence of mycoplasma was monitored using the B-M cyclin test (Boehringer Mannheim, Indianapolis, IN).

All cell lines were kept in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

In vitro cytotoxicity against murine tumor cells and KB cells

In vitro drug cytotoxicities against P388, L1210, M5076, KB 3-1 and KB V1 cells were assessed by using the MTT reduction assay, as previously reported.[35] The MTT dye was obtained from Sigma (St Louis, MO). Cells were plated in 96-well microassay culture plates (10^4 cells/well) and grown for 24 h at 37°C in a 5% CO_2 incubator. Drugs were then added to the wells to achieve a final drug concentration ranging from 0.1 to 50 μg/ml (eight wells were used for each different concentration). The same volume of 0.9% NaCl solution in water with 1% dimethyl sulfoxide was added to control wells. Wells containing culture medium alone without cells were used as blanks. The plates were incubated at 37°C in a 5% CO_2 incubator for 1 h (KB cells), 24 h (M5076 cells) or 72 h (P388 and L1210 cells). When incubation was complete, 15 μl of stock solution of MTT dye in 0.9% NaCl solution in water was added to each well to achieve a final dye concentration of 0.5 mg/ml. The plate was incubated at 37°C in a 5% CO_2 incubator for

4 h. Subsequently, 100 μl of medium was removed from each well from the upper microwell layer and 100 μl of DMSO was added to solubilize the MTT formazan. Complete solubilization was achieved by placing the plate in a mechanical shaker for 30 min at room temperature. The optical density of each well was then measured with a microplate spectrophotometer at a wavelength of 600 nm. The percent cell viability was calculated by the following equation:

$$\% \text{ cell viability} = \frac{\text{mean optical density of treated wells}}{\text{mean optical density of control wells}} \times 100$$

The percent cell viability values obtained were plotted against the drug concentrations used and the ID_{50} was calculated from the curve. Experiments were repeated at least three times.

In vitro cytotoxicity against 8226 and CEM cells

In vitro drug cytotoxicities against 8226, 8226R, CEM and CEM_{vbl} cells were assessed by using a growth inhibition assay. Exponentially growing cells were seeded in triplicate at 3×10^4 cells/ml in 24-well titer plates containing various concentrations of drugs. Control wells without drug were run in parallel. Cell numbers were determined by hemocytometry in the presence of Trypan Blue on the seventh or eighth day and expressed as a percentage of control.

Cellular drug uptake assay (8226 and 8226R)

Cells were washed once in phosphate buffered saline (PBS), resuspended in complete RPMI-1640 medium containing 10% FCS, then counted in a hemocytometer. Cell number was adjusted to $0.5–1 \times 10^6$ cells/ml. Drugs were added to achieve a desired concentration between 3 and 10 μg/ml. Both doxorubicin and hydroxyrubicin can be excited at 488 nm giving an emission at 580 nm, which makes them suitable for flow cytometric assay using a FACScan (Becton-Dickinson, Mountain View, CA). Flow cytometry technology allows the measurement of drug uptake under conditions of perfect equilibrium, e.g. the free drug does not need to be separated from the cells prior to measurement. For each sample, 10 000 events were collected and five parameters, including forward scatters, side scatters, green, orange and red fluorescence, were collected in list mode. Data were analyzed using FACScan Research Software with the data gated on light scatters to minimize cell debris or cell death. By splitting the signal from the orange photomultiplier to one of the unused amplifiers (e.g. the PMT tube amplifier monitoring green fluorescence), it is possible to record drug-derived fluorescence in both linear and logarithmic scales. The median channel number of both log and linear scales were recorded.

Cellular drug retention assay (8226 and 8226R)

After an incubation period of about 2 h at 37°C, cells were washed once with 3 volumes of normal saline, then immediately (30 s) resuspended in RPMI supplemented with 10% FCS and analyzed in the FACScan, utilizing the same set-up conditions as for the measurement of drug uptake at equilibrium.

Calibration of the FACScan orange fluorescence scale for drug content

To obtain a more accurate quantitation of drug retention, $5–20 \times 10^6$ cells were incubated with 3–30 μg/ml of drug for 3 h and then washed twice with RPMI supplemented with 10% FCS. Only drug sensitive cells were used because they exhibit low efflux. Cells were then analyzed by flow cytometry under standard conditions to determine the median channel number for log orange fluorescence. Subsequently, 5×10^6 cells were extracted with 2 ml of a mixture of ethanol–0.01 N HCl at 0°C to solubilize all bound anthracyclines. Cell extracts after neutralization to pH 7.5 by addition of a volume of 1 M NaH_2PO_4 were centrifuged at 3000 *g* for 10 min to remove particulate materials and then assayed fluorometrically in a Perkin–Elmer MRP60 spectrofluorometer, using known concentrations of free drug to build a calibration curve for drug content.

Subacute mouse toxicity studies in mice

Groups of eight to ten ICR Swiss mice obtained from Harlan Sprague–Dawley (Indianapolis, IN) were given different intravenous doses of hydr-

oxyrubicin and doxorubicin. Animals were observed on a daily basis and deaths recorded up to day 30. The LD_{50} was determined from the curve obtained by plotting the survival rates against the logarithm of the dose.

Mouse myelosuppression studies

Myelosuppression was assessed by determining the white blood cell count, granulocyte count, hemoglobin (Hgb) and platelet count in ICR Swiss mice 4 days after the intravenous administration of the LD_{50} of hydroxyrubicin and doxorubicin.

Mouse cardiotoxicity studies

Cardiotoxicity was evaluated using the Bertazzoli test[36] with slight modifications. Groups of ten ICR Swiss mice (Harlan) were given equitoxic intravenous doses of doxorubicin (4 mg/kg) and hydroxyrubicin (12 mg/kg) weekly for 11 weeks. Equitoxic doses were calculated based on the LD_{50} values obtained from the subacute toxicity studies described above. On week 11, animals were sacrificed, and the hearts were removed and placed in formaldehyde. After fixation, the hearts were dehydrated and embedded in glycerol methacrylate. Two microsections of the heart were stained with toluidine blue and examined by light microscopy for characteristic doxorubicin vacuolization of the myocardial fibers in a blind fashion. The hearts were scored for severity and extent of myocardial damage using a scale of 0–4 by two independent pathologists. The evaluation was coordinated by Dr LC Stephens in the Section of Veterinary Pathology at the MD Anderson Cancer Center. Comparisons of the frequency of heart lesions at equitoxic doses allowed us to draw conclusions on the cardiotoxic potential of the drugs.

In vivo antitumor activity against P388/Dox leukemia

BDF_1 male mice weighing 18–22 g were obtained from Harlan Sprague–Dawley. P388/Dox cells for *in vivo* experiments were obtained from the Tumor Repository, National Cancer Institute, and kept as an ascitic tumor in BDF_1 mice with weekly transplants. P388/Dox cells (1×10^6) were inoculated intraperitoneally on day 0. Treatment was administered intraperitoneally on day 1 in volumes ranging from 0.1 to 0.8 ml. Doxorubicin and hydroxyrubicin were dissolved in 5% dextrose in water at a concentration of 1 mg/ml before usage. Groups of five animals each were used. Animals were monitored on a daily basis and the median survival for each group was recorded. A wide dose range was used in the initial experiments to identify the dose levels that resulted in an optimal activity without deaths due to toxicity. All animal experiments were approved by the Institutional Committee for Animal Use and Care. Results were expressed as %T/C (median survival of treated animals/median survival of control animals $\times 100$).

Affinity of hydroxyrubicin for CL-containing membranes

L-α-Dimyristoyl phosphatidylcholine (DMPC) and CL were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. All other chemicals were reagent grade and were used without further purification.

Small unilamellar vesicle suspensions composed of a binary mixture of DMPC and CL (molecular ratio of 3:1) were prepared the day of an experiment as reported previously.[37,38] Briefly, stock lipid suspensions containing 0.075 M lipid in PBS were prepared by Vortex mixing. The PBS buffer contained 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 3 mM KCl and 137 mM NaCl (pH 7.4). The dispersions were then sonicated using a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY) until they became optically clear. Vesicle suspensions were annealed for 30 min at 37°C prior to use in an experiment.

Steady-state fluorescence measurements were obtained on a SLM Model 4800C spectrofluorometer with a thermostated cuvette compartment. This instrument was interfaced with an IBM PS/2 Model 55 SX computer. Excitation and emission spectra were recorded with an excitation resolution of 8 nm and an emission resolution of 4 nm. Steady-state anisotropy measurements were made in the absence of polarizer. Steady-state anisotropy (a) measurements were determined with the instrument in the 'T-format' for simultaneous measurement of two polarized intensities. The alignment of polarizers was routinely checked by using a 2 mg/ml suspension of glycogen in water (anisotropy values of 0.98 were obtained). For both intensity and anisotropy measurements, excitation light of 470 nm was used and fluorescence emission for the anthracyclines was observed through 550 nm long

wave pass filters (Schott). These filters allowed us to adequately separate fluorescence from scattered light. The contribution of background fluorescence, together with scattered light, was less than 2% of the total intensity. All experiments were conducted in 1 cm quartz cuvettes.

As we have described previously,[37] the method of fluorescence anisotropy titration was employed in order to determine the concentrations of free and bound species of drug in liposome suspensions containing a total drug concentration of 2×10^{-6} M and varying lipid concentrations. All experiments were conducted in glass tubes pretreated with silating agent. The overall association constants are defined as $K = [A_B]/[A_F][L]$ where $[A_B]$ represents the concentrations of bound drug, $[A_F]$ represents the concentration of free drug and [L] represents the total lipid concentration of the sample. Double-reciprocal plots of the binding isotherms were linear and K values were determined from their slopes.[37]

Results

In vitro cytotoxicity studies: L1210, P388 and M5076 murine cells

Table 1 shows the results of cytotoxicity of hydroxyrubicin and doxorubicin against three conventional cell lines, P388, L1210 and M5076, as determined using the standard MTT colorimetric assay. Against P388 and L1210, both anthracyclines showed very similar cytotoxicity using a 72 h incubation period (ID_{50} P388: 0.33 ± 0.07 μg/ml for hydroxyrubicin versus 0.35 ± 0.08 μg/ml for doxorubicin; ID_{50} L210: 0.54 ± 0.04 μg/ml for hydroxyrubicin versus 0.53 ± 0.09 μg/ml for doxorubicin). Against M5076 reticulosarcoma cells, hydroxyrubicin was significantly more cytotoxic than doxorubicin (ID_{50} 0.60 ± 0.20 μg/ml versus 1.28 ± 0.15 μg/ml, $p \leq 0.05$).

Table 1. *In vitro* cytotoxicity of hydroxyrubicin against P388, L1210 and M5076 cells[a]

Drug	ID_{50} (μg/ml)		
	P388	L1210	M5076
Hydroxyrubicin	0.33 ± 0.07	0.54 ± 0.04	0.60 ± 0.20
Doxorubicin	0.35 ± 0.08	0.53 ± 0.09	1.28 ± 0.15

[a] MTT assay; 10 000 cells/well; drug incubation 72 h (P388, L1210), 24 h (M5076); ID_{50} = 50% inhibition dose (mean value from at least three experiments ± SD).

Table 2. *In vitro* cytotoxicity of hydroxyrubicin against KB 3-1 and KB V1 cells[a]

Drug	ID_{50} (μg/ml)		
	KB 3-1	KB V1	RI
Hydroxyrubicin	18.9 ± 2.4	21.3 ± 2.7	1.1
Doxorubicin	3.2 ± 0.6	>50.0	>15.6

[a] MTT assay; ID_{50} = 50% inhibition dose; resistance index (RI) = ID_{50} for resistant cells/ID_{50} for sensitive cells.

In vitro cytotoxicity studies: KB 3-1/KB V1 human cells

Table 2 shows the results of cytotoxicity of both agents against human KB 3-1 and KB V1 carcinoma cells, as determined by an MTT assay using an incubation period of 1 h. Doxorubicin was approximately six times more cytotoxic than hydroxyrubicin against the parent KB 3-1 cell line (ID_{50} 3.2 ± 0.6 μg/ml versus 18.9 ± 2.4 μg/ml). However, hydroxyrubicin was equally cytotoxic for the parent and MDR cells (ID_{50} 18.9 ± 2.4 μg/ml for KB 3-1 versus 21.3 ± 2.7 μg/ml for KB V1), while the ID_{50} of doxorubicin against the MDR cells was not reached at a concentration of 50 μg/ml. In accordance with these results, the calculated resistance indices (RI = ID_{50} KB V1/ID_{50} KB 3-1) for hydroxyrubicin and doxorubicin were 1.1 and >15.6, respectively. Hydroxyrubicin showed a lower cytotoxic potential than doxorubicin against wild-type KB cells. However, hydroxyrubicin was equally cytotoxic against sensitive and resistant KB cells. Therefore, it showed complete lack of cross-resistance in this cell system.

In vitro cytotoxicity studies: 8226/8226R and CEM/CEM_{vbl} human cells

The results of the cytotoxicity studies with hydroxyrubicin and doxorubicin against sensitive and MDR human myeloma (8226, 8226R) and human lymphoblastic leukemia (CEM, CEM_{vbl}) cells are shown in Table 3. In these studies, cells were continuously exposed to different concentrations of the agents and cell growth inhibition was

Table 3. *In vitro* cytotoxicity of hydroxyrubicin against myeloma (8226, 8226R) and leukemic (CEM, CEM_{vbl}) cells[a]

Drug	ID_{50} (ng/ml)			ID_{50} (ng/ml)		
	8226	8226R	RI	CEM	CEM_{vbl}	RI
Hydroxyrubicin	4.2 ± 0.8	53.0 ± 0.7	13	5.0 ± 1.0	300 ± 40	60
Doxorubicin	2.6 ± 0.3	700.0 ± 3.3	269	4.5 ± 1.0	1600 ± 49	356

[a] Resistance index (RI) = ID_{50} for resistant cells/ID_{50} for sensitive cells.

assessed by counting the number of viable cells on day 7. Against the parent cell lines, hydroxyrubicin and doxorubicin displayed similar cytotoxicities (ID_{50} 8226 cells: 4.2 ± 0.8 ng/ml for hydroxyrubicin versus 2.6 ± 0.3 ng/ml for doxorubicin; ID_{50} CEM cells: 5.0 ± 1.0 ng/ml for hydroxyrubicin versus 4.5 ± 1.0 ng/ml for doxorubicin). By contrast, hydroxyrubicin was 13 times more cytotoxic than doxorubicin against 8226R cells (ID_{50} 53 ± 0.7 ng/ml versus 700 ± 3.3 ng/ml) and five times more cytotoxic than doxorubicin against CEM_{vbl} cells (ID_{50} 300 ± 40 ng/ml versus 1600 ± 49 ng/ml). The calculated resistance indices for hydroxyrubicin and doxorubicin were 13 and 269 for the 8226 cell system, and 60 and 356 for the CEM cell system. Hydroxyrubicin was, therefore, as potent as doxorubicin against the wild-type cells and partially not cross-resistant with doxorubicin against the MDR counterparts.

Cellular drug uptake and retention studies

Figures 2 and 3 show the drug uptake and retention in 8226 and 8226R cells exposed to the same concentrations of hydroxyrubicin (Figure 2) and doxorubicin (Figure 3) for 2 h at different time points during the drug incubation period and immediately after removing the drug-containing medium and exposing the cells to drug-free medium.

The kinetics of cellular uptake and retention of hydroxyrubicin (Figure 2) was similar for both cell lines; however, cellular accumulation in sensitive cells was higher than that in resistant cells at all time points during the incubation period, with the resistance uptake factor S/R varying between 1.3 and 2.0 (S/R = uptake by sensitive cells/uptake by resistant cells). The cellular drug retention after washing was 2-fold higher in the sensitive cells than in the resistant cells. The decay in cellular accumulation after washing was similar for both cell types.

In the case of doxorubicin (Figure 3), cellular uptake in sensitive cells was directly related to the time of incubation, the peak cellular accumulation being observed at 2 h. By contrast, cellular uptake

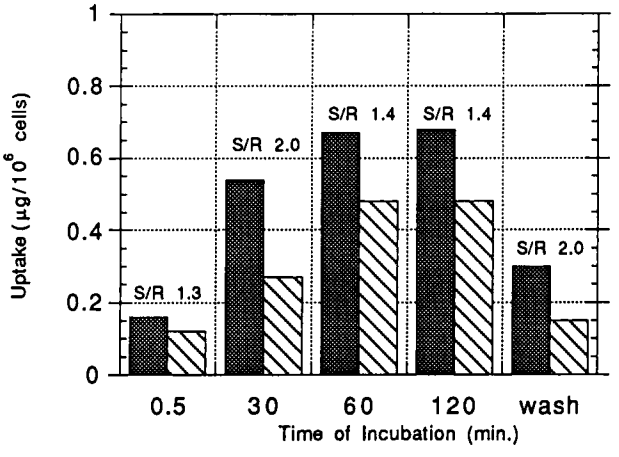


Figure 2. Uptake of hydroxyrubicin by (■) 8226 and (▨) 8226R cells.

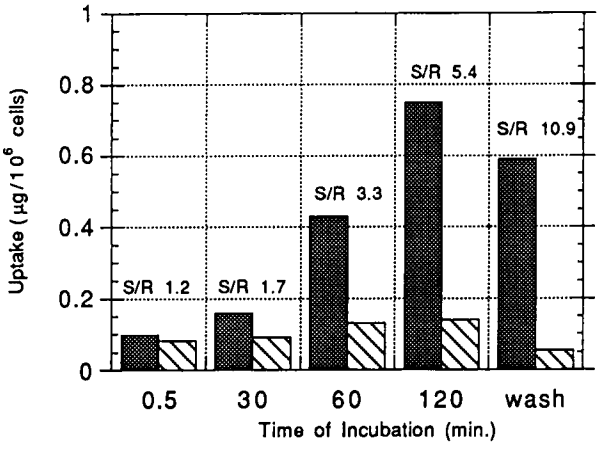


Figure 3. Uptake of doxorubicin by (■) 8226 and (▨) 8226R cells.

in resistant cells remained basically unchanged during the incubation period and was several-fold lower than that in sensitive cells. At 2 h, cellular drug accumulation was 5.4 times higher in sensitive cells than in resistant cells. Immediately after washing, this difference was increased by 2-fold, the cellular retention being 10.9 times greater in sensitive cells than in resistant cells. The decay in cellular accumulation was different in both cell lines: about 20% in sensitive cells and 60% in resistant cells.

The analysis of the results of cellular uptake and retention of both drugs by cell lines showed the following results. In sensitive cells (dark bars of Figures 2 and 3), the accumulation of hydroxyrubicin was faster than that of doxorubicin (maximal accumulation at 60 and 120 min, respectively); however, the cellular accumulation at 2 h was very similar for both drugs. After washing, the decay in cellular accumulation was greater for hydroxyrubicin than for doxorubicin, 56% versus 20%. As a result, the cellular retention of doxorubicin was 2-fold higher than that of hydroxyrubicin.

In resistant cells (light bars of Figures 2 and 3), the accumulation of hydroxyrubicin was about 3.5-fold higher during the period of maximal accumulation of both drugs, between 60 and 120 min. After washing, the decay in cellular accumulation was about 60–70% for both drugs. However, owing to the higher accumulation of hydroxyrubicin, the cellular retention of hydroxyrubicin was about 3-fold higher than that of doxorubicin.

In vivo antitumor activity against P388/Dox

Hydroxyrubicin showed significant activity *in vivo* against intraperitoneal P388 murine leukemia resistant to doxorubicin (P388/Dox) when administered as a single intraperitoneal injection on day 1 (Table 4). In two separate experiments, the %T/C values for hydroxyrubicin at a dose 37.5 mg/kg were 163 and 200, whereas doxorubicin at the maximum tolerated dose of 10 mg/kg showed no antitumor activity in the same experiments (%T/C 118 and 120).

Table 4. *In vivo* antitumor activity of hydroxyrubicin against P388/Dox leukemia resistant to doxorubicin[a]

Drug	Dose (mg/kg)	TC (%)	
		experiment 1	experiment 2
Hydroxyrubin	25.0	136	130
	37.5	163	200
	50.0	154	160
Doxorubicin	10.0	118	120

[a]Treatment intraperitoneal on day 1; T/C = median survival time of treated animals expressed as percent of control.

Subacute toxicity after a single intravenous bolus injection

Results of subacute toxicity studies are shown in Table 5. In two separate experiments, the LD_{50} of hydroxyrubicin after a single intravenous bolus injection was 79.1 mg/kg. In the same experiments, the LD_{50} of doxorubicin was 25.7 mg/kg. Most deaths occurred within the first week after drug administration. Hydroxyrubicin is, therefore, about three times less toxic than doxorubicin.

Acute myelosuppression studies

Table 5 shows the granulocyte count, platelet count and Hgb values of ICR Swiss mice obtained 4 days after the administration of the predetermined LD_{50} dose of hydroxyrubicin (79.1 mg/kg) and doxorubicin (25.7 mg/kg) as a single intravenous bolus

Table 5. Subacute toxicity of hydroxyrubicin in ICR Swiss mice

Drug	LD_{50}[a] (mg/kg)	RBC[b] ($\times 10^6/mm^3$)	Granulocytes[b] (mm^3)	Platelets[b] ($\times 10^6/mm^3$)
Control	—		1945	1.28
Hydroxyrubicin	79.1	7.46 ± 0.85	110 ± 52	1.09 ± 0.19
Doxorubicin	25.7	7.43 ± 0.67	102 ± 45	1.09 ± 0.79

[a] Single intravenous bolus
[b] Determined 4 days after administration of LD_{50}.

injection and also of untreated control animals. Both anthracyclines resulted in similarly profound granulocytopenia (granulocyte count 102 ± 45, 110 ± 52 and $1945/mm^3$ for hydroxyrubicin, doxorubicin and control, respectively). Thrombocytopenia was also similar and very mild for both drugs. These experiments indicate that myelosuppression affecting mainly the white cell lineage is an equally important form of toxicity for both compounds.

Chronic cardiotoxicity studies

Table 6 shows the results of the chronic cardiotoxicity studies conducted in ICR Swiss mice treated weekly for 11 weeks with equitoxic doses of hydroxyrubicin and doxorubicin (12 and 4 mg/kg, respectively) as determined from the subacute toxicity studies described above. Ten animals were included in each group. In the group of animals treated with hydroxyrubicin, five animals showed grade 1 or 2 lesions (two grade 1 and three grade 2). In the group of animals treated with doxorubicin, all animals showed grade 1 or 2 lesions (four grade 1 and six grade 2). These results suggest that hydroxyrubicin is less cardiotoxic than doxorubicin.

Binding of hydroxyrubicin to CL

We have previously exploited the intrinsic fluorescence emission of anthracyclines to gather information concerning the selective interactions of doxorubicin for CL.[38] Whereas the overall association constant (K) of doxorubicin for vesicles composed of the electroneutral lipid DMPC was $200\ M^{-1}$, incorporation of small amounts of CL into the vesicles (at a DMPC:CL ratio of 3:1) resulted in a 5.5-fold enhancement in K to a value of $1100\ M^{-1}$.

In this study we have assessed the impact of the removal of doxorubicin's basic center on drug associations with CL. To do so we employed the same fluorescence spectroscopic methodologies used in the previous studies.[38] The overall association constants of hydroxyrubicin interacting with DMPC and DMPC/CL vesicles were both determined to be $200\ M^{-1}$, with no selective interactions of drug with DMPC/CL vesicles being observed due to the presence of the negatively-charged lipid. In light of the data discussed above, it can be concluded unequivocally that removal of the basic center of doxorubicin abrogates the drug's selective interactions with the negatively-charged phospholipid CL.

Discussion

This study demonstrates that the reduction of basicity of doxorubicin due to the replacement of the amino function by a hydroxyl group confers partial or total lack of cross-resistance (Tables 1–3). The resistance indices of hydroxyrubicin in different pairs of parental and MDR *in vitro* cell lines ranged from 1.1 to 60, whereas the resistance indices for doxorubicin were 6 to 20 times higher. The ability of hydroxyrubicin to overcome *MDR1*-mediated resistance *in vitro* was also confirmed in *in vivo* experiments against P388/Dox (Table 4). This finding may lead to compounds of clinical importance, capable of overcoming drug resistance in humans.

The non-cross-resistance properties of hydroxyrubicin can in part be explained by its increased cellular uptake compared with doxorubicin. The uptake of hydroxyrubicin by 8226R cells was 3-fold higher than that of doxorubicin, whereas the

Table 6. Cardiotoxicity study[a] of hydroxyrubicin

Drug	Dose (mg/kg)	No. of mice with lesions of grade[b]				Total no. of mice with lesions/ total examined
		1	2	3	4	
Hydroxyrubicin	12	2	3	0	0	5/10
Doxorubicin	4	4	6	0	0	10/10

[a] Twelve Cox ICR Swiss mice in the group, 10 injections, mouse sacrificed at 11 weeks; doses of 4 mg/kg for doxorubicin and 12 mg/kg for hydroxyrubicin represent equitoxic doses based on LD_{50} studies.
[b] Lesion severity: grade 1 = mild; grade 4 = severe.

uptakes of both drugs in sensitive 8226 cells were similar. After a 2 h wash, the retention of doxorubicin was 11-fold lower in MDR cells than in sensitive cells (S/R = 10.9, Figure 3), whereas for hydroxyrubicin the S/R value was only 2.0 (Figure 2).

The reduced accumulation doxorubicin in MDR cells may be due to efflux of the drug mediated by membrane PGP, which is overexpressed in MDR cells.[39] The relatively higher accumulation of hydroxyrubicin in 8226R cells and the efflux profile suggest that this anthracycline analog might not be a good substrate for PGP and, as a result, is not effluxed by PGP as effectively as doxorubicin. The percent of intracellular drug effluxed after resuspension of cells in drug-free medium was similar in resistant and sensitive cells incubated with hydroxyrubicin (30–40%). This suggests but does not prove that the efflux of hydroxyrubicin is not PGP related since sensitive cells are PGP negative. The similar drug efflux in both cell types may be the result of passive reequilibration of drug across the cell membrane since hydroxyrubicin is more lipophilic than doxorubicin. On the other hand, the percent of intracellular doxorubicin effluxed was three times higher in resistant cells than in sensitive cells (60 versus 20%). The increased efflux in resistant cells is consistent with PGP mediated efflux.

The only structural difference between doxorubicin and hydroxyrubicin is the substitution of the basic amino group at the C-3′ in doxorubicin for a hydroxyl group in hydroxyrubicin. Both compounds are also configurationally identical. A basic amino group is present in many of the drugs that are effluxed by or inhibit PGP, including vinca alkaloids, verapamil, doxorubicin[20,21] and more recently certain analogs of camptothecin.[40] The association of removal of the amino group with lack of cross-resistance found in our studies suggests that the amino group is a stabilizing factor in the binding of PGP with anthracyclines. It also supports our hypothesis that the amino group (basicity) is an important determinant of the binding of anthracycline antibiotics to PGP and other macromolecules. However, it is not possible from our study to assess the contribution of other factors to the increased cellular accumulation of hydroxyrubicin. [^{3}H]Azidopine photoaffinity labeling studies, transmembrane transport studies aimed at distinguishing between passive diffusion and active transport, as well as detailed drug efflux studies are planned in order to better understand the non-cross-resistance properties of hydroxyrubicin and to further elucidate the mechanism of resistance associated with *MDR1*.

Hydroxyrubicin was also found to be three times less toxic than doxorubicin in mice (Table 5). However, optimal doses of hydroxyrubicin *in vivo* are also about three times higher, which suggests no significant change in the therapeutic index. However, the spectrum of toxicities of both drugs appears somewhat different. At equitoxic doses, both drugs appeared to be equally myelosuppressive but hydroxyrubicin was less cardiotoxic. A similar explanation centered on the alteration of basicity can be offered for the reduced cardiotoxic potential of hydroxyrubicin (Table 6). Damage to mitochondrial membranes is widely accepted to be a prominent morphological characteristic of doxorubicin's dose-limiting side effect of cardiotoxicity. The specific disturbances of cardiac mitochondrial function apparently involve the formation of stable complexes between doxorubicin and CL,[8,10,12,14] a negatively-charged phospholipid abundant in the mitochondrial membrane.[8–10] The binding between doxorubicin and cardiolipin is stabilized by the electrostatic interaction of the positively charged amino group of doxorubicin and the negatively charged phosphate oxygen of CL. Deamination (reduction of basicity) of doxorubicin led to elimination of the electrostatic share of drug–CL complex stabilization and reduction of affinity of the drug for CL. It is unlikely that the decreased affinity for CL associated with reduction of basicity at C-3′ could completely suppress the cardiotoxic potential of the drug since the mechanism of cardiotoxicity involves reactions at the chromophore part. However, it is reasonable to conclude that the reduction of basicity in the sugar portion contributed to the reduction of cardiotoxicity as a result of drastically reduced affinity to CL.

Conclusion

In summary, our results demonstrate that a small modification in the chemical structure of anthracyclines that results in reduced basicity is associated with partial or total lack of cross-resistance and reduced cardiotoxicity in mice. Whether such favorable modulation of biological activity is the result of an altered affinity of hydroxyrubicin for PGP alone, or is also related to increased lipophilicity, altered topoisomerase activity or interaction with DNA, is being further investigated.

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