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IN VITRO CIRCUMVENTION OF ANTHRACYCLINE—RESISTANCE IN EHRLICH ASCITES TUMOUR BY ANTHRACYCLINE ANALOGUES

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Abstract—In previously reported studies, acquired experimental resistance and cross resistance to anthracyclines are related to decreased drug accumulation and retention. The decreased accumulation seems to depend on a cellular mechanism for active drug efflux. *N*-Acetyl-daunorubicin (*N*-acetyl-DNR) has demonstrated the ability to increase drug accumulation and to overcome experimental resistance to daunorubicin (DNR) in resistant cells. In the present *in vitro* study 25 different anthracycline analogues were tested for their influence on [^{3}H]DNR accumulation in resistant cells. At equimolar concentrations (5 μM) four of the analogues enhanced [^{3}H]DNR accumulation more than 200%. Increasing the concentration of the analogues 3–20-fold, 12 of the compounds could enhance [^{3}H]DNR accumulation above 200%. No specific structural changes separated those 12 compounds from the 13 analogues with no or minor effect. The lipid solubility of the 25 analogues was examined by measuring the partition coefficient in octanol/phosphate and pentanol/phosphate buffer (pH 7.45). A good correlation was demonstrated between increased lipid solubility of the analogues and their effect on [^{3}H]DNR accumulation in resistant cells. Further studies demonstrated that *N,N*-dibenzyl-DNR was able to potentiate cytotoxicity of DNR in resistant cells. It is concluded that several anthracycline analogues are able to reverse resistance, but it is not possible from the chemical structure to predict which analogue results in enhanced [^{3}H]DNR accumulation in resistant cells.

Decreased steady-state drug accumulation by tumour cells is the most common finding in experimental resistance to anthracyclines [1–5]. Attempts to overcome resistance have therefore been directed to increase cellular drug accumulation. The anthracycline analogue *N*-acetyl-daunorubicin (*N*-acetyl-DNR) has previously demonstrated the ability to increase drug accumulation and to overcome experimental resistance to DNR [6]. In the present study we have examined whether 25 different DNR and Adriamycin® (ADM) analogues were able to influence [^{3}H]DNR accumulation in resistant cells. The influence of chemical structure, the drug concentration and the lipid solubility of the analogues on DNR accumulation was examined. Two of these anthracyclines were also examined for the ability to act as chemosenzitizer using clonogenic assay.

MATERIALS AND METHODS

Chemicals. Daunorubicin (DNR); Adriamycin® (ADM); 4′-epi-ADM; *N*-acetyl-ADM; *N*-acetyl-13-dihydro-ADM; 4′-deoxy-ADM; 3′,4′-diepi-ADM; 11-deoxy-4-*O*-demethyl-ADM; 4′-epi-DNR; *N,N*-dimethyl-DNR; 4-*O*-methyl-ADM; 7R,9R-4-demethoxy-DNR; 4-demethoxy-4′-*O*-methyl-DNR; *N*-trifluoroacetyl-ADM; 4-demethoxy-DNR; 4′-*O*-(2,6-dideoxy-2-L-arabino)-DNR; *N*-acetyl-3′,4′-isopropyliden-DNR and 4-demethoxy-*N*-trifluoroacetyl-DNR were kindly supplied from Farmitalia, Carlo Erba (Milan, Italy). Carubicin from Bristol-Myers Co. (New York, U.S.A.). Aclacinomycin A from Lundbeck & Co A/S (Copenhagen, Denmark). Rubidazone, 9-(formylhydrazone-1-ethyl)-DNR and 9-(hydroxy-1-ethyl)-DNR from Rhone Poulenc Co. (Paris, France). Mitoxantrone from Lederle (New Jersey, U.S.A.). *N,N*-Dibenzyl-DNR was a special gift from Dr Nicholas Bachur, U.S.A. *N*-Acetyl-DNR was synthesized according to the method described by Yamamoto *et al.* [7] and T. Skovsgaard [6]. [^{3}H]DNR (sp. act. 1.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of analytical grade.

Tumour cells. The cells investigated were of the wild-type Ehrlich ascites tumour line (EHR 2) and from the corresponding DNR-resistant line (EHR2/DNR+) [8]. Resistance to DNR was developed and maintained *in vivo* by treatment with 1.6 mg/kg × 4 i.p. corresponding to LD_{10} [8]. Ascites fluid was removed 6 to 8 days after inoculation of the tumour. No drug treatment was given to the resistant tumour during the last passage before an *in vitro* experiment.

A test with methylene blue demonstrated that 94% (92–95%) of the tumour cells were intact. Packed cell volume was determined by centrifugation of samples at 5500 *g* for 8 min in a haematocrit centrifuge, and the final cell suspension was adjusted to 0.5% (v/v). Samples of 2.0 mL were withdrawn and the pellets were washed twice at 4° with Ringer solution to remove the extracellular drug.

Experimental. Cells were incubated for 60 min at

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37° in a buffer containing 60 mM sodium phosphate (pH adjusted to 7.45); 57.0 mM NaCl; 5.0 mM KCl; 1.3 mM $MgSO_4$; 9.0 mM NaH_2PO_4; 51.0 mM Na_2HPO_4; 10.0 mM glucose. Dialysed calf serum, 5% (v/v), was added to the medium in all trials. The analogues were added at time zero immediately before [^{3}H]DNR. After 60 min 2.0 mL cell suspension was withdrawn to an ice-cold Ringer solution to stop the reaction, pelleted at 3000 *g* and washed twice with ice-cold Ringer at 4°. Each value represents a mean of three determinations. The influence of all anthracyclines on [^{3}H]DNR accumulation was determined at 60 min, when steady-state levels of DNR were reached [9].

Determination of cellular [^{3}H]*DNR content*. The drug content of intact cells was extracted with 0.3 N HCl–50% ethanol solution from the drained pellet [10]. Aliquots of 200 μL cell extract were transferred to the scintillation solution (Packard Insta-Gel; Packard Instrument Co., Downers Grove, IL) and the vials were counted in a Beckman LS-250 liquid scintillation spectrometer. The influence of quenching was negligible.

Clonogenic assay. For use in clonogenic assay, aclacinomycin and DNR were dissolved in sterile water. *N,N*-Dibenzyl-DNR was dissolved in DMSO. Drug toxicity was assessed by colony formation in soft agar on a feeder layer containing sheep red blood cells as previously described [11]. Single-cell suspensions (2×10^4 cells/mL) in RPMI-1640 supplemented with 10% fetal calf serum were exposed to the drugs for 1 hr and then washed twice with PBS (150 mM NaCl, 50 mM phosphate, pH 7.2) at 20°. Cells (2×10^4) were plated to obtain 2000–3000 colonies in the control dishes. In each experiment the drug combinations were tested on the same batch of cells to reduce the inter-experimental variation [12]. The DMSO concentrations never exceeded 1% and had no influence on cell survival. The colonies were counted after 3 weeks of incubation.

Lipophilicity. Partitioning of the anthracyclines was measured by adding 100 μL 1 mM (the analogue dissolved in buffer) in 5 mL phosphate buffer (pH 7.45) and 5.1 mL octanol and pentanol, respectively. After separation of the phases by centrifugation drug levels in the aqueous phase were determined spectrophotometrically. Drug concentration was determined in all cases by comparison with spectrophotometrically adjusted standards [6].

RESULTS

DNR-sensitive and -resistant Ehrlich ascites tumour cells were incubated for 60 min, in the presence of equimolar concentrations of [^{3}H]DNR and various anthracyclines or mitoxantrone, and the results are shown in Table 1. The effect of the analogues are compared to control accumulation of [^{3}H]DNR in resistant and sensitive cells which is considered as 100% (corresponding values in pmol/μL packed cells are indicated in square brackets). The analogues are arranged according to their increasing effect on DNR accumulation in resistant cells. These experiments demonstrate that only four analogues at equimolar concentrations enhanced the accumulation of [^{3}H]DNR more than 200%. Their influence on [^{3}H]DNR accumulation in sensitive cells was in most cases modest and the maximal effect was only 138%.

We examined concentration dependence of those drugs that enhanced uptake of [^{3}H]DNR by more than 200%: Aclacinomycin A, 4-demethyoxy-*N*-trifluoroacetyl-DNR, *N*-acetyl-3′,4′-isopropyliden-DNR, and *N,N*-dibenzyl-DNR. Figure 1 showed that increasing concentrations of all four analogues resulted in increasing accumulation of [^{3}H]DNR. Very high concentrations of aclacinomycin and *N*-acetyl-3′,4′-isopropyliden-DNR resulted in some reduction of [^{3}H]DNR uptake. A possible explanation might be that in very high concentration, these analogues compete for binding sites inside the cell. For most analogues, an optimal concentration could be defined, but for 4-demethoxy-*N*-trifluoroacetyl-DNR its poor solubility in water became the limiting factor.

To further characterize the 26 anthracyclines their lipid solubility was examined (Table 2). The distribution of the drugs between octanol/phosphate buffer and pentanol/phosphate buffer shows that the compounds cover a wide range of lipophilicity. Despite their great similarity in chemical structure, these compounds displayed an appreciable difference in partition coefficients. Thus, the *o/p* ratio for DOX was 1.22, while the *o/p* ratios for aclacinomycin, 4-demethoxy-*N*-trifluoroacetyl-DNR and *N,N*-dibenzyl-DNR all exceeded 200. A common structural feature of these four analogues is that they all are heavily substituted in the glycosidic amino group in position 3′.

We examined whether there was a relationship between DNR accumulation (Table 1) and lipophilicity (Table 2). Figure 2 shows clearly that there is a significant correlation (correlation coefficient 0.89) between the lipid solubility of the anthracycline analogue and its ability to increase [^{3}H]DNR accumulation by the resistant cells. Thus, with regard to the four most potent analogues, lipophilicity combined with substitution in the amino group might be important factors for analogues modulating anthracycline resistance.

Two of the analogues that enhanced DNR accumulation more than 200% were chosen to be tested in a clonogenic assay to clarify if they were able to increase the sensitivity to DNR in resistant cells. The effect on cytotoxicity of combining DNR and aclacinomycin or *N,N*-dibenzyl-DNR are shown in Fig. 3. EHR/DNR+ cells were incubated with either 5 μM *N,N*-dibenzyl-DNR or with 2.5 or 5.0 μM aclacinomycin and increasing concentrations of DNR. After 1 hr, drugs were removed by washing the cells twice in drug free PBS, and the cells were then seeded in soft agar to assess colony forming ability. DNR in concentrations up to 10 μM was without effect, and *N,N*-dibenzyl-DNR alone reduced the survival to only 90% (SD = 2%) of untreated controls. However, combining *N,N*-dibenzyl-DNR with increasing concentrations of DNR resulted in increasing cell kill. Aclacinomycin alone was very toxic to the resistant cells; at 2.5 μM it reduced the survival to 67% (SD = 3%), and at 5.0 μM it reduced the survival to 5% (SD = 1%). In contrast to *N,N*-dibenzyl-DNR, aclacinomycin could not modulate

Table 1. Comparison of the effect of 25 anthracyclines on DNR-accumulation

Treatment		Steady-state accumulation of [^{3}H]DNR (% of control, [pmol/μL packed cells]) EHR/DNR+		EHR/S	
1.	Daunorubicin (control)	100	[116]	100	[612]
2.	Doxorubicin (DOX)	74	[87]	94	[575]
3.	4′-Epi-DOX	80	[93]	101	[618]
4.	Mitoxantrone	86	[99]	93	[569]
5.	*N*-Acetyl-DOX	90	[104]	100	[612]
6.	*N*-Acetyl-13-dihydro-DOX	97	[112]	99	[606]
7.	Rubidazon	98	[114]	108	[661]
8.	4′-Deoxy-DOX	100	[116]	107	[655]
9.	3′,4′-Diepi-DOX	103	[119]	99	[606]
10.	9-(Formylhydrazone-1-ethyl)-DNR	103	[119]	99	[605]
11.	11-Deoxy-4-*O*-demethyl-DOX	105	[122]	105	[643]
12.	4′-Epi-DNR	106	[123]	106	[649]
13.	9-(Hydroxy-1-ethyl)-DNR	107	[124]	131	[802]
14.	*N*,*N*-Dimethyl-DNR	109	[126]	122	[747]
15.	4-*O*-Methyl-DOX	112	[130]	90	[551]
16.	7*R*,9*R*-4-demethoxy-DNR	118	[137]	114	[698]
17.	*N*-Acetyl-DNR	124	[144]	104	[636]
18.	4-Demethoxy-4′-*O*-methyl-DNR	127	[147]	138	[845]
19.	*N*-Trifluoroacetyl-DOX	127	[147]	105	[643]
20.	Carminomycin	128	[148]	110	[673]
21.	4-Demethoxy-DNR	129	[150]	97	[594]
22.	4′-*O*-(2,6-Dideoxy-2-L-arabino)-DNR	129	[150]	113	[692]
23.	*N*-Acetyl-3′,4′-isopropyliden-DNR	224	[260]	105	[643]
24.	Aclacinomycin A	306	[355]	100	[612]
25.	4-Demethoxy-*N*-trifluoroacetyl-DNR	341	[396]	106	[649]
26.	*N*,*N*-Dibenzyl-DNR	413	[478]	136	[832]

Effect of various anthracyclines on steady-state accumulation of [^{3}H]DNR in sensitive and DNR-resistant Ehrlich ascites tumour cells. Suspension of cells (5 μL packed volume/mL) were incubated for 1 hr at 37° with 5 μM [^{3}H]DNR plus 5 μM of an anthracycline derivative. In the controls, cells were incubated with 5 μM [^{3}H]DNR. In all cases a steady-state was reached within 60 min. Each value represents the mean of three determinations.

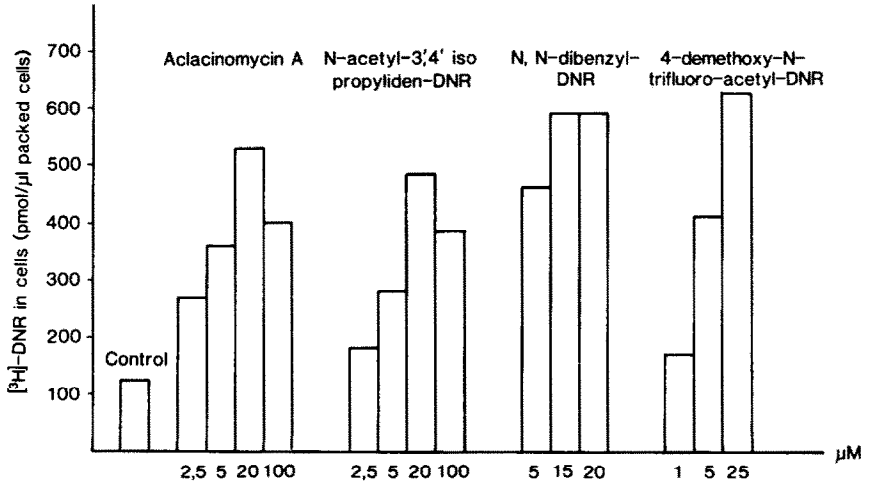

Fig. 1. The effect of different concentrations of four anthracycline analogues on [^{3}H]DNR accumulation at steady state (60 min) in DNR-resistant Ehrlich ascites tumour cells. Cells were incubated in standard medium, pH 7.45 at 37° with 5 μM of [^{3}H]DNR plus varying concentrations of the respective analogues as indicated at time zero. The cellular content of [^{3}H]DNR was determined at a mean of three samples as described in Materials and Methods.

Table 2. Partition coefficient for the anthracyclines

	Drug	Octanol/buffer	Pentanol/buffer
1.	Doxorubicin (DOX)	1.22	3.08
2.	11-Deoxy-4-*O*-demethyl-DOX	1.95	2.80
3.	9-(Formylhydrazone-1-ethyl)-DNR	2.28	5.67
4.	9-(Hydroxy-1-ethyl)-DNR	4.88	9.75
4.	Mitoxantrone	5.06	9.10
6.	4'-Deoxy-DOX	6.30	14.87
7.	4'-Epi-DOX	6.41	10.49
8.	Daunorubicin (DNR)	7.85	13.93
9.	3'-4'-Diepi-DOX	8.09	18.23
10.	4-*O*-Methyl-DOX	8.80	11.82
11.	4-Demethoxy-DNR	13.08	33.10
12.	4'-Epi-DNR, β-anomer	13.93	28.41
13.	*N*-Acetyl-DOX	18.61	28.41
14.	4'-*O*-(2,6-Dideoxy-2-L-arabino)DNR	21.73	62.13
15.	Rubidazon	23.39	61.50
16.	*N*,*N*-Dimethyl-DNR	23.39	69.30
17.	*N*-Trifluoroacetyl-DOX	30.25	77.18
18.	*N*-Acetyl-13-dihydro-DOX	36.04	82.33
19.	7*R*,9*R*-4-Demethoxy-DNR	42.48	75.92
20.	*N*-Acetyl-DNR	70.43	110.11
21.	Carminomycin	75.92	104.52
22.	4-Demethoxy-4'-*O*-methyl-DNR	110.11	>200
23.	*N*-Acetyl-3',4'-isopropyliden-DNR	>200	>200
24.	Aclacinomycin A	>200	>200
25.	4-Demethoxy-*N*-trifluoroacetyl-DNR	>200	>200
26.	*N*,*N*-Dibenzyl-DNR	>200	>200

Lipid solubility was determined by measuring the partition of the drugs between an aqueous phase and a lipophilic solvent, octanol. One hundred μL 1 mM drug in 5 mL phosphate buffer at pH 7.45 was shaken with an equal volume of octanol at 25°. The concentration in the buffer was determined spectrophotometrically. The concentration in chloroform was calculated by subtraction of drug in buffer from the total amount added to the solvent. The values indicate mean of triplicate determinations.

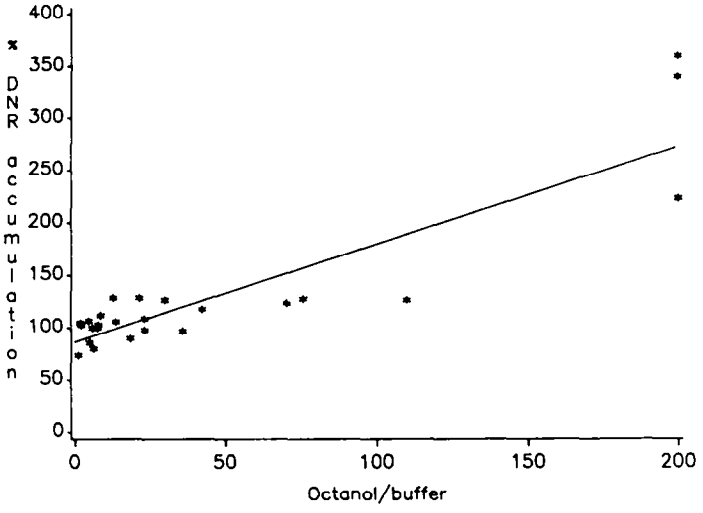

Fig. 2. Data compiled from Tables 1 and 2. The per cent accumulation of DNR is plotted against the partition coefficients of the analogues in octanol/buffer. The correlation coefficient for this relationship is 0.89 ($P < 0.001$).

the DNR toxicity and an additive cell kill was obtained with all tested DNR concentrations.

In subsequent experiments the concentration of *N*,*N*-dibenzyl-DNR was varied with or without combination with DNR. As seen in Fig. 4, *N*,*N*-dibenzyl-DNR is almost non-toxic at concentrations up to 25 μM. DNR alone results in a survival of 92% (SD = 2%). The combination with *N*,*N*-dibenzyl-DNR results in increased cell kill. No further cytotoxicity is obtained beyond 5 μM *N*,*N*-dibenzyl-DNR, in accordance with its effects on DNR accumulation in resistant cells.

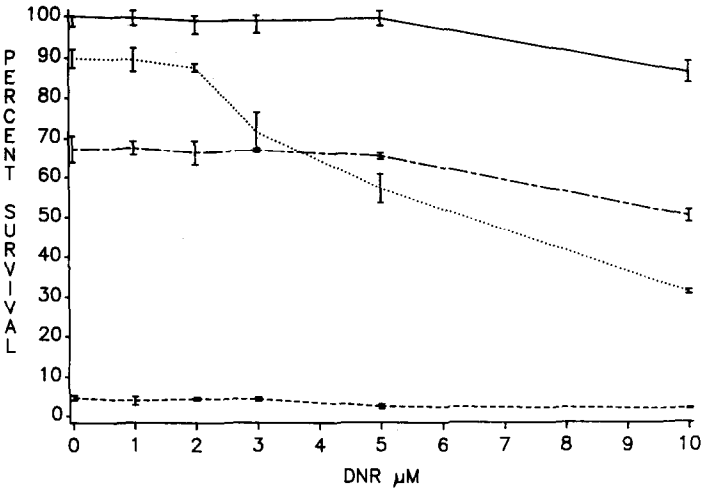

Fig. 3. DNR dose–response curves obtained in a clonogenic assay with cell line EHR/DNR+. The cells were incubated for 60 min with DNR alone (——) and combined with 5 μM aclarubicin (– – –), 2.5 μM aclarubicin (·—·) and 5 μM *N,N*-dibenzyl-DNR (····). Bars represent standard errors from triplicate cultures.

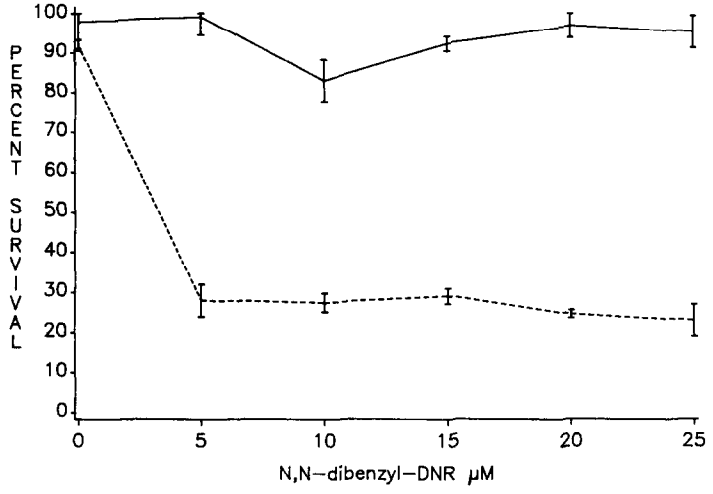

Fig. 4. *N,N*-Dibenzyl-DNR dose–response curves obtained in a clonogenic assay with cell line EHR/DNR+. The cells were incubated for 60 min with *N,N*-dibenzyl-DNR alone (———) and combined with 5 μM DNR (– – –). Bars represent standard errors from triplicate cultures.

DISCUSSION

It was shown earlier that the non-toxic analogue *N*-acetyl-DNR [6] can circumvent DNR resistance by enhancing DNR uptake to sensitive levels. Also three synthetic anthracycline analogues [13] and several vinca alkaloids [14] lacking antitumor activity, significantly reversed resistance to VCR and DNR by increasing their intracellular accumulation. These findings suggested that examination of other anthracycline analogues with a low cytotoxicity may be able to increase DNR accumulation and cytotoxicity in resistant cells. In order to predict the ability of a compound to reverse drug resistance, examination of structure–activity relationships may be helpful, as we showed earlier [15]. The present study demonstrated that small chemical modifications in the anthracycline molecule had a significant effect on its ability to increase cellular accumulation of [^{3}H]DNR. 4-demethoxy-*N*-trifluoroacetyl-DNR and *N,N*-dibenzyl-DNR were the analogues that on a molar basis caused the most pronounced increase in [^{3}H]DNR accumulation.

The effect of the analogues on [^{3}H]DNR accumulation could be a result of a competitive inhibition of a carrier mediated efflux of [^{3}H]DNR. However, no specific chemical groups could be related to the capacity of the analogue to increase the accumulation of [^{3}H]DNR in resistant cells. The effect of these compounds also appeared to be concentration dependent even if it was not always possible to define an optimal concentration because of solubility limitations. Conversely the ability to increase [^{3}H]DNR accumulation showed a good correlation with the

degree of lipid solubility of the analogue. It is of interest to note that analogues that are most lipophilic are all substituted in the amino group. However, comparing the different analogues, several, such as *N*-acetyl-DOX and *N,N*-dimethyl-DNR, are substituted at the amino group without resulting in enhancement of DNR uptake, but their lipophilicity is low compared to *N*-acetyl-3′,4′-isopropyliden-DNR, Aclacinomycin A, 4-demethoxy-*N*-trifluoroacetyl-DNR and *N,N*-dibenzyl-DNR. Therefore it appears that lipophilicity is the major determinant of the ability of an analogue to increase [^{3}H]DNR accumulation. This conclusion does not support the hypothesis that these compounds inhibit carrier-mediated DNR transport. In fact, that we do not see enhanced DNR accumulation in DNR-sensitive cells, also suggests that these anthracycline analogues are not merely perturbing the lipid bilayer. Based on work of Pastan and Gottesman [16] and Safa *et al.* [17], it is likely that the effective analogues block binding of DNR to P-glycoprotein which is present in our DNR-resistant cell line (unpublished data).

To test whether increased drug accumulation also results in reversal of drug resistance, (in a separate experiment, accumulation of a DNR concentration of about 20 μM corresponds to the accumulation of the combination of 5 μM DNR + 5 μM *N,N*-dibenzyl-DNR), cytotoxicity experiments were performed. We showed that *N,N*-dibenzyl-DNR is able to modulate DNR resistance. Thus, the combination of 5 μM DNR with 5 μM *N,N*-dibenzyl-DNR was more toxic than 10 μM alone and corresponds to the effect of 20 μM alone (30–45% survival), and thereby shows a good correlation with accumulation results. With regard to aclacinomycin, it must be concluded that it is not suitable as a modulator of DNR cytoxicity as it is toxic in itself in the resistant cells, and combination of the two drugs does not result in a further cell kill. Thus, even if *N,N*-dibenzyl-DNR *in vitro* acts as a good modulator, *in vivo* experiments are required to ensure that *N,N*-dibenzyl-DNR in combination with DNR will not result in enhanced toxicity and thereby be unacceptable as a treatment.

Considering these results, a continued effort must be undertaken to identify anthracyclines which more effectively and especially in nontoxic doses, in combination are able to circumvent resistance to well known cytotoxic drugs.

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