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9-Alkyl, Morpholinyl Anthracyclines in the Circumvention of Multidrug Resistance

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The intramolecular combination of 9-alkyl substitution in the anthracyline A-ring plus incorporation of the amino group of the daunosamine sugar within a morpholinyl ring led to the retention of almost complete activity against P-glycoprotein positive, multidrug resistant variants of a mouse mammary tumour line and a human small cell lung cancer line. Resistance factors were close to unity. These structural elements may prevent efflux by the P-glycoprotein multidrug transporter. The use of 9-alkyl, morpholinyl anthracyclines with resistance circumvention properties may have clinical application.
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INTRODUCTION

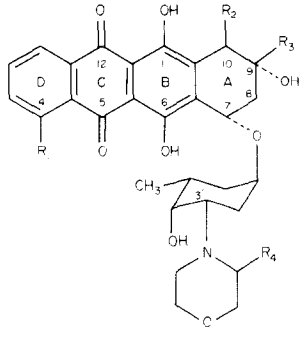
MULTIDRUG RESISTANCE (MDR) and its circumvention continues to attract interest [1]. Many MDR lines hyperexpress the membrane P-glycoprotein that is believed to act as an efflux pump for the wide range of natural products to which such lines are cross-resistant [1, 2]. Increased expression of P-glycoprotein has also been correlated with clinical resistance [3]. The major approach to circumvention of MDR is the use of 'reversal compounds', such as verapamil and cyclosporin [1], which may bind to P-glycoprotein and inhibit its pump function [4]. Antibodies to P-glycoprotein may also be of value in the blockade of P-glycoprotein or for targeting of toxins or radionuclides to resistant cells [5].

An alternative involves the identification of analogues of MDR drugs that retain activity in resistant cells. Such derivatives may be less efficient substrates for the P-glycoprotein pump. Studies of specific modifications to the anthracycline molecule (Fig. 1) highlighted the importance of an alkyl substitution in the 9-position of the A ring, and also of certain sugar modifications, including the incorporation of the 3'-amino group of the daunosamine sugar into a morpholinyl ring (Fig. 1) [6–9]. To clarify the importance of these two types of substitution and to elucidate any advantage of incorporating both changes, we evaluated 9-alkyl, morpholinyl doxurubicin analogues *in vitro* against mouse and human MDR cell lines that hyperexpress P-glycoprotein. Preliminary results have appeared in abstract form [10].

MATERIALS AND METHODS

We used the mouse mammary tumour parent line EMT6/Ca/VJAC, referred to as EMT6(P), with its MDR counterpart EMT6/AR1.0 and the human small cell lung cancer parent line NCI-H69, referred to as H69(P), with its MDR variant H69/LX4 [6, 11]. Both MDR lines were produced by exposure to doxorubicin *in vitro*, resulting in a 30-fold and 200-fold resistance for EMT6/AR1.0 and H69/LX4, respectively; each shows a typical cross-resistance profile and hyperexpression of P-glycoprotein [11–13].

Doxorubicin was obtained from Farmitalia Carlo Erba and from Sigma. Morpholinyl and cyanomorpholinyl doxorubicin were from the M.D. Anderson Hospital and Tumor Institute, Houston, and the National Cancer Institute, Bethesda. Ro 31-3294 and Ro 31-1215 were from Roche. MX2 was from the Kirin Brewing Company, Tokyo. Aclacinomycin A was from Lundbeck.



	Morpholinyl doxorubicin	Cyanomorpholinyl doxorubicin	Ro31-3294	MX2
R_1	CH_3O	CH_3O	H	OH
R_2	H	H	H	OH
R_3	$COCH_2OH$	$COCH_2OH$	CH_2CH_3	CH_2CH_3
R_4	H	CN	H	H

Fig. 1. Structures of morpholinyl anthracyclines. Doxorubicin CH_3O at R_1, H at R_2, $COCH_2OH$ at R_3 and unsubstituted NH_2 at 3' of daunosamine sugar.

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Table 1. Activity of doxorubicin and four morpholinyl derivatives, together with aclacinomycin A and Ro 31-1215, in EMT6 and H69 parent lines and MDR variants*

Compound	EMT6 ID_{50} (μg/ml) EMT6/P	EMT6/AR1.0	RF	H69 ID_{50} (μg/ml) H69/P	H69/LX4	RF
Doxorubicin	0.083	2.4	33.9 (±4.1)	0.0063	0.96	190 (±31.5)
Morpholinyl doxorubicin	0.033	0.072	2.7 (±1.1)	0.028	0.070	2.5 (2.6,2.4)
Cyanomorpholinyl doxorubicin	0.000029	0.000065	2.2 (2.6,1.8)	0.000087	0.00032	3.5 (±0.3)
Ro 31-3294	0.42	0.46	1.1 (1.0,1.1)	0.52	1.00	2.0 (2.0,1.9)
MX2	0.027	0.026	1.0 (0.9,1.0)	0.021	0.049	2.3 (1.9,2.7)
Aclacinomycin A	0.028	0.090	4.7 (±1.1)	0.034	0.14	5.8 (±1.4)
Ro 31-1215	0.20	0.37	8.1 (±1.5)	0.024	0.075	12.4 (±1.6)

*Aclacinomycin A and Ro 31-1215 lack the morpholinyl substituent, but do have the 9-alkyl group.
Values of both ID_{50} and RF were calculated as mean values from at least two independent experiments. Also shown in parentheses for RF are S.E. values ($n > 2$) or results for individual experiments ($n = 2$).

In vitro cytotoxicity testing was done in multiwell plates with the semi-automated MTT tetrazolium dye reduction assay in which optical density is proportional to viable cell number [7, 14]. Parent and resistant lines were always compared at the same time and doxorubicin was run as a quality control standard throughout. Within each experiment dose–response curves were constructed with four replicate wells per dose. The ID_{50} was calculated as the dose of drug required to reduce optical density to 50% of control and the resistance factor (RF) as the ratio of the ID_{50} of the resistant line to that of the control line. Replicate experiments were done independently.

RESULTS

Dose–response curves for doxorubicin and the morpholinyl-containing analogues in H69 cells are shown in Fig. 2. All four morpholinyl anthracyclines had low RFs in both pairs of cell lines (Table 1). In all cases the RFs for the morpholinyl derivatives were lower than those for analogues lacking the morpholinyl substituent but possessing the 9-alkyl group (aclacinomycin A and Ro 31–1215). The low RF values were not related to cytotoxic potency. Thus, for example, morpholinyl doxorubicin and MX2 had similar (EMT6) or slightly lower (H69) potency compared with doxorubicin, while Ro 31-3294 was less potent and cyanomorpholinyl doxorubicin was more potent.

We confirmed that the cyano substitution in the morpholinyl ring conferred potency but was not essential for circumvention of MDR. Agents containing both the morpholinyl sugar and the 9-alkyl molecular substitutions had the lowest RFs. Values were close to unity in EMT6 and around 2 in H69, indicating little difference in activity between parent and MDR lines.

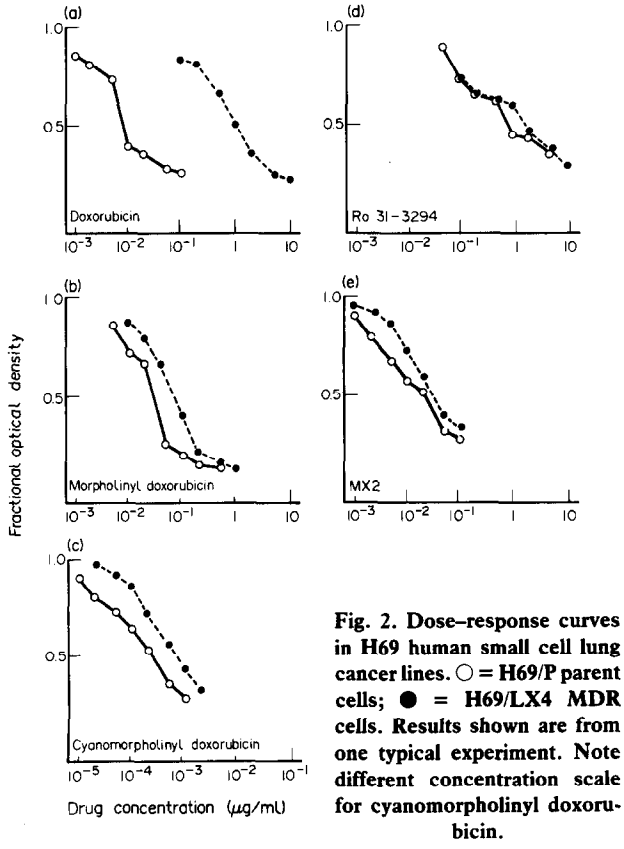

Fig. 2. Dose–response curves in H69 human small cell lung cancer lines. ○ = H69/P parent cells; ● = H69/LX4 MDR cells. Results shown are from one typical experiment. Note different concentration scale for cyanomorpholinyl doxorubicin.

DISCUSSION

Identification of anthracyclines that retain activity in MDR cells is an interesting alternative to the use of reversal compounds. We had pinpointed the 9-alkyl and morpholinyl sugar substitutions as key features for retention of activity [6, 7]. Other studies supported this conclusion [8, 9, 15]. We have now shown that resistance factors are close to unity when both features are combined within the same molecule. The excellent results reported for one of the 9-alkyl, morpholinyl analogues, MX2, in doxorubicin-resistant mouse P388 leukaemia cells [16] can now be explained by the simultaneous presence of the two active moieties. These compounds were active in MDR variants of both a mouse mammary tumour line and a human small cell lung cancer line. It would be useful to obtain further data on the 9 alkyl, morpholinyl anthracyclines in additional MDR cell lines, particularly where different mechanisms are thought to predominate (see later).

Each of the resistant lines we used hyperexpresses P-glycoprotein [12, 13]. We hypothesise that the 9-alkyl and morpholinyl substituents confer reduced affinity for the putative drug efflux pump. Consistent with this proposal, our studies (data not shown) with morpholinyl doxorubicin have shown improved accumulation compared with doxorubicin in the EMT6 and H69 MDR variants, as in a P388 MDR line [9]. However, the improved accumulation of this morpholinyl agent in MDR lines was not superior to that of the non-morpholinyl but 9-alkyl-containing agents aclacinomycin A and Ro 31-1215 [17], both of which had higher RFs. The morpholinyl group may confer

additional advantageous properties, possibly related to altered subcellular disposition or to DNA damage [18].

The morpholinyl doxorubicins were originally developed in the early 1980s as potent and potentially less cardiotoxic anthracyclines *in vivo* [19–22]. The high potency of the cyanomorpholino derivative is due to DNA cross-linking [18, 23–26]. Hepatic microsomal metabolism of the morpholinyl ring in morpholinyl doxorubicin [27] and probably in a related methoxy analogue [15] will confer *in vivo* potency but is likely not a major factor for MDR cells *in vitro*.

Both MDR variants we studied have shown substantial modification of doxorubicin response by the reversal compounds verapamil and cyclosporin [7, 11, 13, 28]. We previously proposed [17] that the activity of substituted anthracyclines with intrinsic activity in MDR was related mechanistically to reversal, since they may avoid the attention of P-glycoprotein to which reversal compounds generally bind. However, it is likely that additional mechanisms operate for the 9-alkyl, morpholinyl compounds, since we found that these agents are active in an MDR line that fails to express P-glycoprotein and which is subject to minimal verapamil reversal (data not shown).

The resistance factors we observed for the 9-alkyl, morpholinyl compounds were lower than those obtained by combining doxorubicin with clinically relevant concentrations of verapamil or cyclosporin in the EMT6 and H69 MDR variants [7]. The use of substituted anthracyclines with intrinsic MDR circumvention properties may also have the advantage of avoiding the potential side-effects of reversal compounds and pharmacokinetic complications. The evaluation of morpholinyl anthracyclines in the circumvention of clinical MDR will depend on the demonstration of favourable *in vivo* activity and toxicity. Early results with MX2 in mice appear promising [16] and this agent is now in clinical trial in Japan.

The 9-alkyl substitution and also the morpholinyl sugar modification in the anthracycline structure are key elements in the circumvention of MDR. Their intramolecular combination is especially valuable. Reduced efficiency for the P-glycoprotein multidrug transporter is likely to be involved. Identification of these critical substitutions should enhance understanding of the molecular topography required to avoid efflux by P-glycoprotein. It may be relevant that both 9-alkyl and morpholinyl substitution will increase lipophilicity and the morpholinyl substitution will modify basicity; however, steric factors may also be involved.

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