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Identification of anthracycline analogues with enhanced cytotoxicity and lack of cross-resistance to adriamycin using a series of mammalian cell lines in vitro

Bridget T. Hill, Lorraine Y. Dennis, Xue-Teng Li, and Richard D. H. Whelan

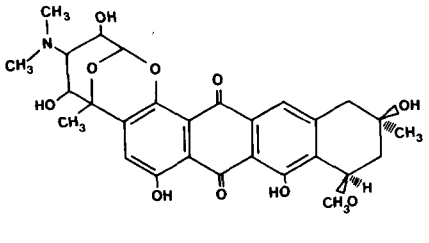
Laboratory of Cellular Chemotherapy, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

Summary. *Clinical resistance to adriamycin (ADR) develops readily, and cardiotoxicity is a major dose-limiting side effect. A range of anthracycline derivatives have been synthesized recently, and a number reported to exhibit significantly reduced cardiotoxicity in experimental animals. Using NIL 8 Syrian hamster ovary cells and two continuous human tumour cell lines derived from colon carcinomas we have screened a series of 11 anthracycline analogues, determining their in vitro cytotoxic effects by colony-forming assays. Five agents proved significantly more cytotoxic than ADR: dihydroxyanthraquinone (DHAQ), mitoxantrone (DHAD), 4-demethoxydaunorubicin (4-DNR), 4'-0-tetrahydropyranyl-adriamycin (THP-ADR), and 4'-deoxyadriamycin (4-ADR). We have also established in vitro a subline of the L5178Y murine lymphoma resistant to ADR and have used this model to identify derivatives with potential value for overcoming ADR resistance. We have observed three patterns of response: (i) complete cross-resistance with 4'-epiadriamycin and daunorubicin; (ii) slight cross-resistance with 4-DNR, THP-ADR, 7-con-0-methyl-nogarol and aclacinomycin A; and (iii) complete absence of cross-resistance with 4-ADR, 4'-0-methyladriamycin, DHAQ, DHAD, and methylhydroxyellipticinium. These straightforward preclinical screens thus identify three drugs which may merit clinical evaluation, since they not only show an increased level of cytotoxicity in vitro to ADR at equivalent concentrations but also overcome resistance to ADR in this murine model system.*

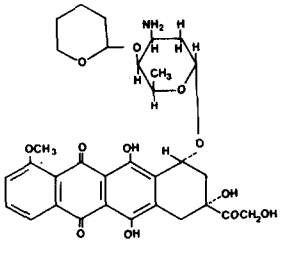
Introduction

Since its introduction to clinical practice in 1968, adriamycin (ADR) has proven one of the most effective single antitumour agents, with an impressively broad spectrum of activity encompassing many of the 'solid' tumours [44], and it is now widely used in combination chemotherapy protocols, as reviewed recently [30]. However, myelosuppression and cardiotoxicity have proved to be dose-limiting toxic side effects [4, 29], and in several malignancies, including for example ovarian cancer, ADR has proved ineffective as second-line treatment, suggesting that clinical resistance may develop readily [22].

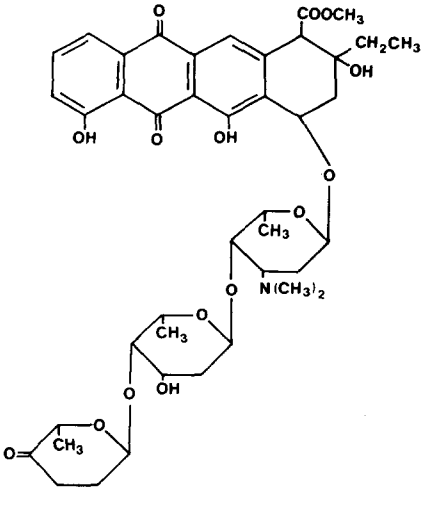
Recently a large number of adriamycin derivatives and related DNA-intercalating agents (Fig. 1) have been synthesized and various experimental model systems have been used to evaluate their antitumour effectiveness. These studies initially concentrated on experimental murine tumours in vivo includ-



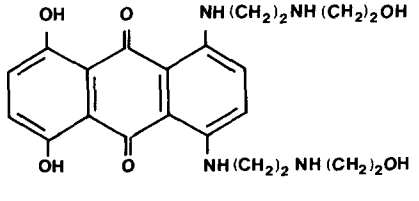
7-CON-O-METHYLNOGAROL (7-OMEN)



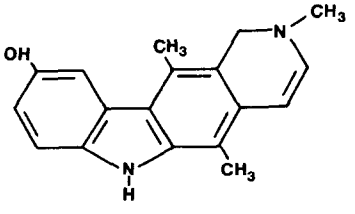
4'-O-TETRAHYDROPYRANYLADRIAMYCIN (THP-ADR)



ACLACINOMYCIN-A (ACM)



DIHYDROXYANTHRAQUINONE (DHAQ)



2-CH_3-9-OH-ELLIPTICINIUM (NMHE)

Fig. 1. Structures of anthracycline derivatives and related compounds

Offprint requests to: B.T. Hill

ing the L1210 and P388 leukaemias [6, 12, 15, 24, 40, 41], but more recently the use of nude mouse xenografts has been reported [17, 38, 45]. In these latter studies, comparisons between in vitro and in vivo sensitivities have been attempted, with conflicting conclusions as to whether in vitro screening of drugs can predict in vivo activity accurately. However, Salmon et al.[34] have concluded that the in vitro human tumour stem cell assay is clinically predictive and provides a rapid and relatively inexpensive means of simultaneously testing a large number of analogues of a parent compound against a spectrum of human tumours. This approach has also been used by a few other groups [39, 42, 43]. However, in view of the low plating efficiencies (i.e., $< 0.01\%$) frequently obtained with this clonogenic assay method on fresh human tumour biopsy material, we have opted to use continuous human tumour cell lines for screening purposes. Having adopted this procedure, we are encouraged by the recent report by Minna et al. [28] that such lines derived from small cell lung cancer appear as useful as a screening system for clinical correlations as the original clinical specimens. We have therefore been able to evaluate a wider range of these newer analogues than previously reported using clonogenic assays and a series of mammalian cell lines. Two human colon carcinoma lines were included, since some activity has been reported for certain analogues against human colon tumour xenografts [38, 45]. In addition, we have used an in vitro drug-induced ADR-resistant murine lymphoma cell line to identify analogues particularly effective at overcoming ADR resistance.

Materials and methods

Drugs and chemicals. The following drugs were kindly donated for our studies: ADR, 4′-epiadriamycin (EPI-ADR), 4′-deoxyadriamycin (4-ADR), 4′-0-methyladriamycin (40-ADR), daunomycin (DNR), and 4-demethoxydaunomycin (4-DNR) by Farmitalia Carlo Erba, Barnet, Herts., England and Milan, Italy; 4′-0-tetrahydropyranyladriamycin (THP-ADR) by Prof. G. Mathé, Institut de Cancérologie et d′ Immunogénétique, Villejuif, France; 2-methyl-9-hydroxyellipticinium (NMHE) by Sanofi Ltd., The Wirral, Ches., England; 7-con-0-methylnogarol (7-OMEN) by Dr B. K. Bhuyan, Upjohn Company, Kalamazoo, Michigan, USA; dihydroxyanthraquinone (DHAQ) by Dr B. Kimler, Kansas City, Mo, USA; aclacinomycin A (ACM-A) by Lundbeck, Luton, Beds., England; mitoxantrone (DHAD) by Lederle Labs, Gosport, Hants., England. Media and sera were obtained from Gibco Bio-Cult, Renfrewshire, Scotland. All drug dilutions were made in medium or phosphate-buffered saline. Low-gelling-temperature agarose was purchased from Uniscience Ltd, Cambridge, England.

Cell culture. Some details from the cell lines used are provided in Table 1 and full information is available in the references cited. The two human tumour cell lines were kindly donated by the laboratories where they were first established.

The drug-resistant L5178Y subline was derived from the parent line by exposure of logarithmically growing cells to 100 ng/ml ADR for 24 h and then cloning in soft agarose as described previously [20], with the modifications cited [19], in the continuous presence of 1 ng/ml ADR. Cells from surviving colonies were re-exposed to ADR (200 ng/ml) for 24 h and recloned. Individual colonies were used to establish cell lines which were designated ADR-resistant cells, and their response to ADR was established. The characteristics of the L5178Y cell lines used in this study are listed in Table 2.

Cell counts and volumes were determined by means of a Coulter Counter Model ZBI. Cellular DNA, RNA, and protein contents were measured after extraction by the Scott procedure [36] as described earlier [20]. Cell-cycle distributions were estimated by flow microfluorimetric analyses of mithramycin-stained whole cells with the aid of a FACS-1 machine (Becton-Dickinson, California, USA) as described elsewhere [18]. DNA histogram evaluation was performed according to the procedure recommended by Barfod [2]. Modal chromosome numbers were estimated from metaphase spreads. Chromosomes were stained with Giemsa.

Clonogenic assays. NIL 8 and LOVO cells were cloned directly on plastic using the methods described earlier [18, 21], and plating efficiencies of 50%–65% and 40%–60%, respectively, were routinely obtained. Details of the soft agarose (0.17%) assay used with the COLO 205 cells, resulting in a 20%–30% colony-forming efficiency, have been reported previously [21],

Table 1. Characteristics of mammalian cell lines

Cell line	Origin (ref.)	Media/ sera	PDT[a] (h)	CFE[b] (%)	Cloning conditions
NIL 8	Syrian hamater ovary [11]	Eagle's +10% CS[c]	11.5	50	On plastic
COLO 205	Human colon carcinoma [37]	RPMI 1640 +10% FCS[d]	24	34	In 0.17% agarose
LOVO	Human colon carcinoma [13]	Ham's F12 +10% FCS	30	43	On plastic *or* in 0.17% agarose

[a] Population doubling time
[b] Colony forming efficiency
[c] Calf serum
[d] Fetal calf serum

Table 2. Characteristics of murine L5178Y cell lines

	Parent	ADR-resistant
Population doubling time (h)	22.5 ± 1.2	23.9 ± 1.1
Cell cycle distribution (%)[a]		
G_1	41.1	40.3
S	39.2	38.5
G_2 + M	19.7	21.3
Cell volume (μm^3)	886 ± 37	943 ± 59
DNA content ($\mu g/10^6$ cells)	12.7 ± 1.2	11.7 ± 0.6
RNA content ($\mu g/10^6$ cells)	25.0 ± 1.3	27.0 ± 2.6
Protein content ($\mu g/10^6$ cells)	130 ± 3.0	135 ± 4.8
Modal chromosome number	40	40
Colony-forming efficiency (%)[b]	44 ± 3	38 ± 4

[a] As judged by flow microfluorimetry and calculated by the method of Barfod [2]. The overall scatter never exceeded 5%
[b] In 0.17% agarose

and the modified procedure adopted for the L5178Y cell lines has recently been detailed [19].

Drug assays. Cells in logarithmic growth were exposed to a range of drug concentrations for a fixed period of 24 h. Drug-treated cells were removed from suspension culture by centrifugation and washed twice in drug-free medium, whilst monolayer cultures were trypsinized after being washed twice in drug-free medium, and cell numbers were determined. Appropriate numbers of cells were then used to produce approximately 200 colonies per assay dish or tube. Colony formation of drug-treated cultures was expressed as a percentage of the control untreated or solvent-treated cells, and survival curves were constructed, being fitted by eye to the data points.

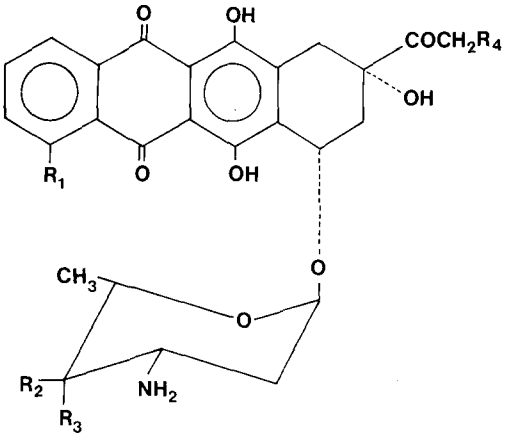
Results

Comparative in vitro lethal effects of 11 anthracycline derivatives

The cytotoxic effects of a 24-h exposure to a range of anthracycline derivatives (Figs. 1, 2) were determined by colony-forming assays on three established mammalian cell lines. The dose-response curves are shown in Fig. 3. In general, the NIL 8 cells proved least sensitive to the drugs, whilst LOVO cells were more sensitive than the COLO 205 cells. The patterns of response to the 11 agents under evaluation were remarkably consistant between the three cell lines, with six agents proving significantly more cytotoxic than ADR, three proving less cytotoxic and EPI-ADR exhibiting comparable effects. Table 3 summerizes these comparative in vitro cytotoxic effects in terms of ratios of IC_{50} values. It can be seen that five drugs consistently proved significantly more cytotoxic in vitro (i.e., > twofold) than ADR at equivalent drug concentrations (ng/ml) against all three lines: DHAQ (14- to 22-fold), DHAD (5- to 23-fold), 4-DNR (5- to 15-fold), THP-ADR (3- to 4.6-fold), and 4-ADR (2.5- to 3-fold).

Identification of anthracycline derivatives which overcome resistance to ADR in the murine L5178Y model system

Figure 4 illustrates the cytotoxic effects of a 24-h exposure to ADR, as judged by clonogenic assay, on the parent and



COMPOUND (abbreviation)	R_1	R_2	R_3	R_4
ADRIAMYCIN (ADR)	OCH_3	H	OH	OH
4'-EPI-ADR (EPI-ADR)	OCH_3	OH	H	OH
4'-DEOXY-ADR (4-ADR)	OCH_3	H	H	OH
4'-O-METHYL-ADR (4O-ADR)	OCH_3	H	OCH_3	OH
DAUNORUBICIN (DNR)	OCH_3	H	OH	H
4'-DEMETHOXY-DNR (4-DNR)	H	H	OH	H

Fig. 2. Structures of ADR analogues

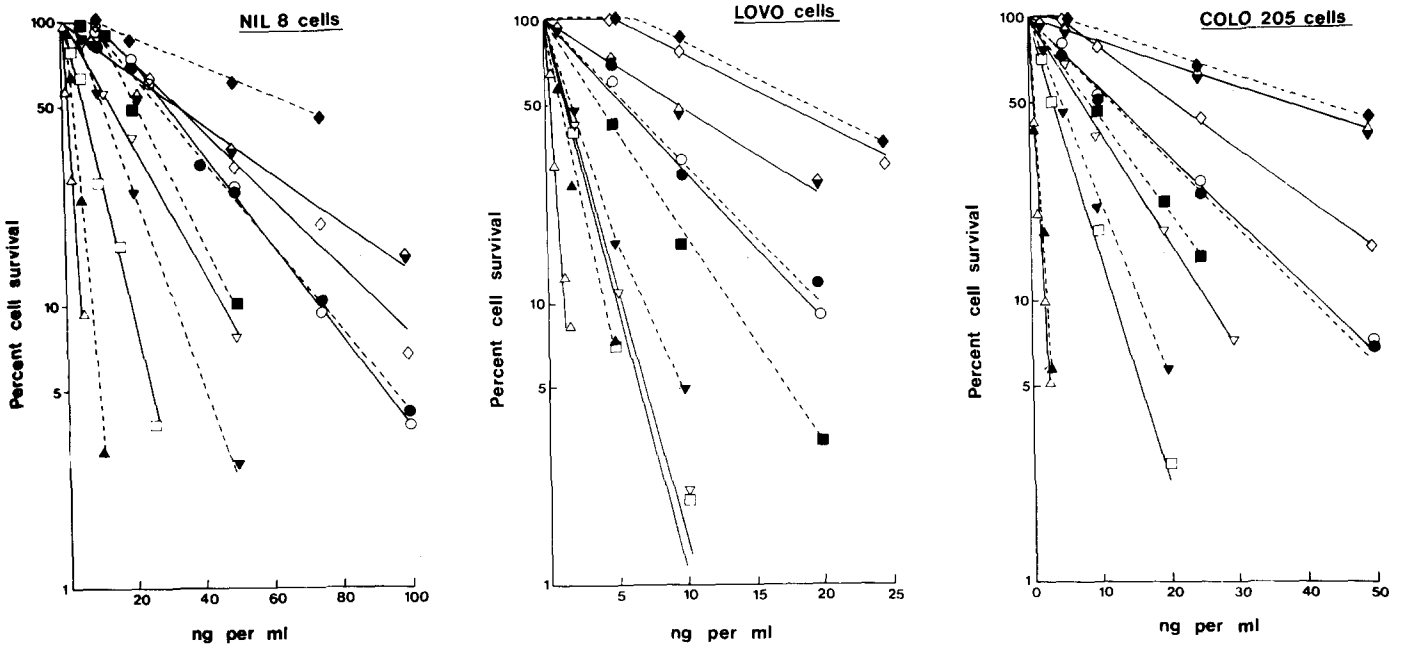


Fig. 3. Comparative lethal effects of the anthracycline derivatives, as determined by colony-forming assays following a 24-h drug exposure of logarithmically growing NIL 8, COLO 205 or LOVO cells: ○ ADR; △ DHAQ; ▽ 40-ADR; □ THP-ADR; ◇ 7-OMEN; ● EPI-ADR; ▲ 4-DNR; ▼ 4-ADR; ■ DNR; ♦ NMHE; ◆ ACL-A. Each point represents the mean of at least four determinations. The overall scatter at any point never exceeded 10%

ADR-resistant sublines of the L5178Y lymphoma: IC_{50} values differ by a factor of 2.5. The dose-response curves obtained when similarly evaluating the series of derivatives are shown in Fig. 5–7, and Table 4 summarizes the results in terms of ratios of IC_{50} values. Two agents, EPI-ADR und DNR (see Fig. 5) proved completely cross-resistant with ADR, some slight cross-resistance was noted with four other agents, 4-DNR, THP-ADR, and 7-OMEN (see Figure 6) and ACA-A (full data not shown but see Table 4), whilst the other five drugs showed no cross-resistance. Indeed these last agents 4-ADR, 40-ADR, DHAQ, NMHE (see Fig. 7), and DHAD (full data not provided, but see Table 4) proved more cytotoxic to the ADR-resistant subline than the parent line under these exposure conditions, thus providing evidence of collateral sensitivity.

Table 3. Comparative in vitro effects of anthracycline derivatives

Derivative tested	IC_{50} value for ADR[a]		
	IC_{50} value for derivative[a]		
	NIL 8 cells	COLO 205 cells	LOVO cells
ADR	1	1	1
EPI-ADR	1.05	1.06	0.93
DNR	1.41	1.41	1.43
40-ADR	2.14	1.59	3.56
4-ADR	2.57	2.45	2.95
THP-ADR	4.59	4.00	3.35
4-DNR	10.71	15.20	4.83
DHAQ	22.50	19.00	14.25
DHAD	23.45	12.65	4.73
NMHE	0.45	0.31	0.30
7-OMEN	0.92	0.62	0.32
ACL-A	0.88	0.39	0.66

[a] Data derived from Fig. 3, except for DHAD

These in vitro data therefore suggest that DHAQ, DHAD, and 4-ADR merit further study, since they not only show enhanced cytotoxicity to ADR at equivalent drug concentrations but also overcome resistance to ADR in this murine model system.

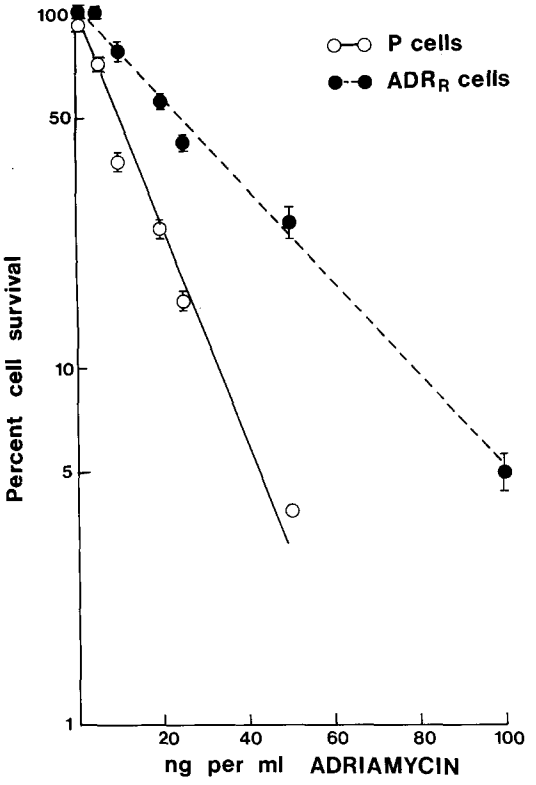


Fig. 4. The lethal effects of ADR following a 24-h drug exposure on the colony-forming abilities of logarithmically-growing L5178Y lymphoma parent (*P*) and ADR-resistant (ADR_R) cells. Each *point* represents the mean and each *bar* the SEM of four assays

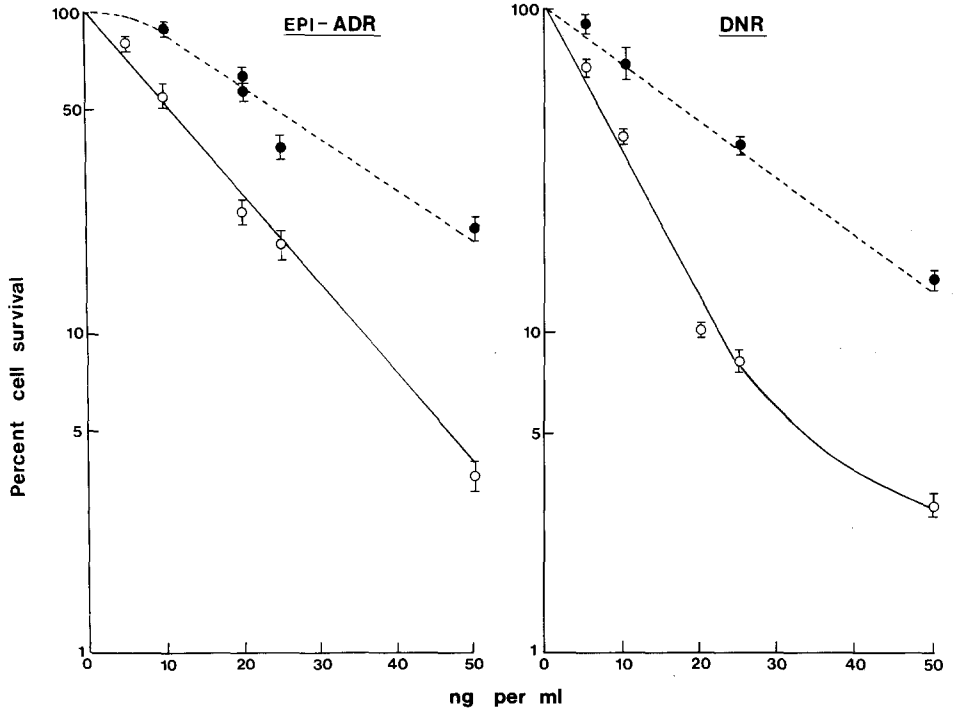


Fig. 5. The lethal effects of a 24-h exposure to EPI-ADR or DNR on the parent (○———○) or ADR-resistant (●– – –●) L5178Y cell lines, which exhibit complete cross-resistance with ADR. Each *point* represents the mean and each *bar* the SEM of at least four assays

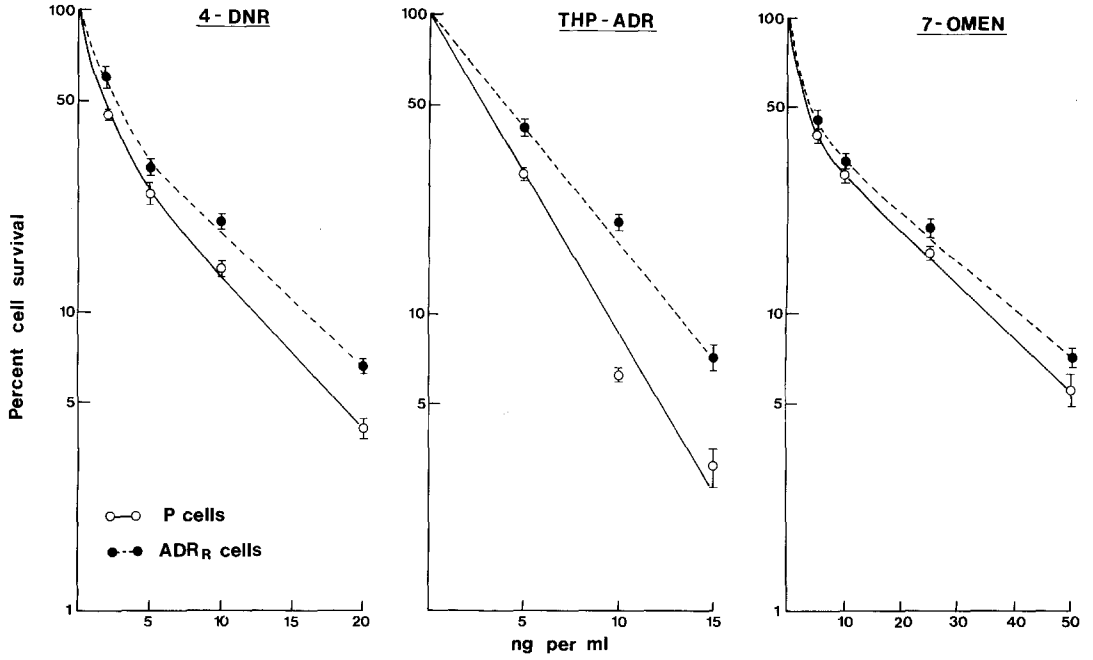


Fig. 6. The lethal effects of a 24-h exposure to 4-DNR, THP-ADR, or 7-OMEN on the parent (○———○) and ADR-resistant (●– – –●) L5178Y cell lines, which exhibit some slight cross-resistance with ADR. Each *point* represents the mean and each *bar* the SEM of at least four assays

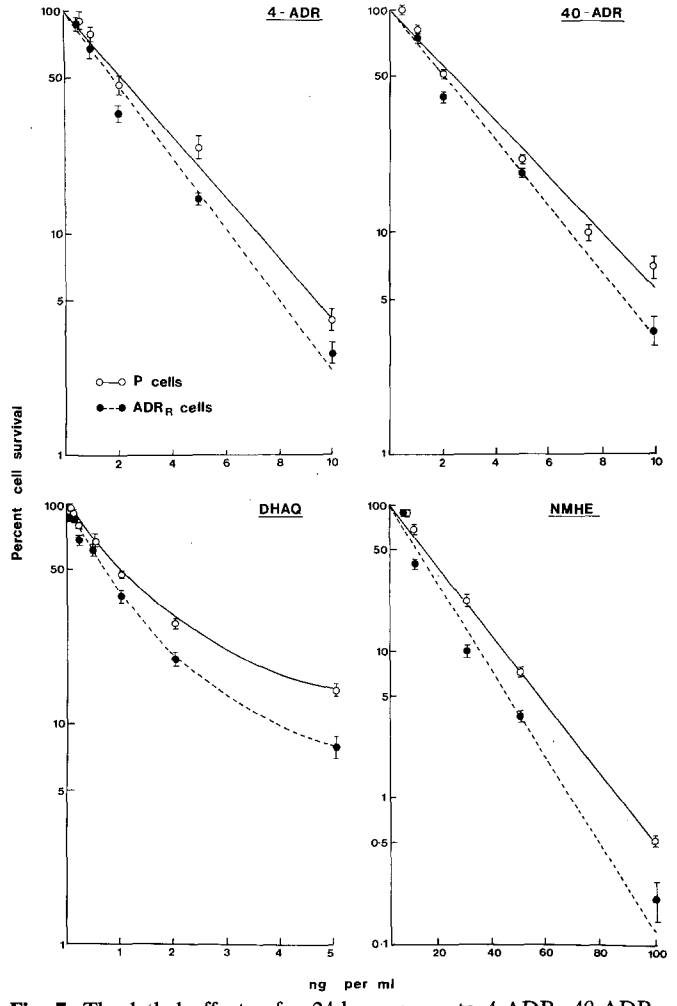


Fig. 7. The lethal effects of a 24-h exposure to 4-ADR, 40-ADR, DHAQ, or NMHE on the parent (○———○) and ADR-resistant (●– – –●) L5178Y cell lines, which exhibit collateral sensitivity to ADR. Each *point* represents the mean and each *bar* the SEM of at least four assays

Table 4. Comparative in vitro effects of anthracycline derivatives against L5178Y lymphoma cells

Derivative tested	IC_{50} values for ADR-resistant cells[a] / IC_{50} values for parent cells[a]
ADR	2.50
EPI-ADR	2.23
4-ADR	0.82
40-ADR	0.84
DNR	2.50
4-DNR	1.31
THP-ADR	1.37
7-OMEN	1.20
DHAQ	0.77
DHAD	0.90
NMHE	0.84
ACL-A	1.15

[a] Data derived from Figs. 4–6 except for DHAD

Table 5. Comparison of clinical and in vitro drug levels

Analogue	Clinical doses recommended per course for phase II studies on 'solid' tumours	[Ref.]	Clinical: ADR DOSAGE derivative dose	In vitro: ADR IC_{50} derivative IC_{50}
ADR	60–75 mg/m^2	[7]	1	1
EPI-ADR	70–90 mg/m^2	[32]	0.8 –0.9	0.9– 1.1
DNR	60–75 mg/m^2	[7]	1	1.4
4-DNR	12.5–15 mg/m^2	[5]	4.8 –5	5 –15
THP-ADR	40 mg/m^2	[31]	1.5–1.9	3 – 4.6
4-ADR	30 mg/m^2	[33]	2 –2.5	2.5– 3
DHAD	12 mg/m^2	[1]	5 –6.3	5 –23
ACA-A	65–80 mg/m^2	[26]	0.75–0.9	0.4– 0.9
NMHE	80–160 mg/m^2	[10]	0.5 –0.75	0.3– 0.4

Discussion

With the synthesis and development of large numbers of analogues of 'standard' antitumour agents it is important to establish screening procedures to identify the particular agents which merit clinical evaluation. In this respect it is necessary that the new agents show (i) enhanced cytotoxic effects; (ii) increased selectivity against the tumour; (iii) reduced toxic side effects; and/or (iv) a lack of cross-resistance with the parent compound. We report here the use of a series of mammalian cell lines in vitro as a straighforward initial screen to evaluate two of these factors. We have assesed the relative cytotoxicity of a series of anthracycline derivatives and analysed their potential value for overcoming specific resistance to ADR in a murine L5178Y lymphoma model system.

Using the NIL 8 Syrian hamster ovary cells and two continuous cell lines derived from human colon carcinomas (COLO 205 and LOVO) we have consistently identified five drugs as significantly more cytotoxic in vitro than ADR. This enhanced cytotoxicity of DHAQ and DHAD confirms reports from other laboratories where DHAQ proved five- to ten-fold more cytotoxic against CHO cells [27], DHAD was more effective against a series of murine experimental leukemias [15], and DHAD showed enhanced lethal effects against LOVO cells [14]. However, in the last study quoted equivalent cytotoxic effects were reported for DHAQ and ADR, in contrast to our results. There was a major difference in the drug concentrations used in these studies, with our LOVO cells proving at least 10 times more sensitive to all the anthracyclines tested. In agreement with our results, 4-DNR was reported as more effective than ADR in the L1210 leukemia [8] and 10-fold more cytotoxic against CCRF-CEM human leukaemic cells [35]. Similarly, THP-ADR was shown to be equally effective as or more effective than ADR in a series of murine tumour cell lines in vitro [41], and 4-ADR and 40-ADR proved significantly better than ADR and EPI-ADR in certain murine colon tumours in vitro [38]. However, in this last study a word of caution was introduced, since this enhanced activity was not confirmed in vivo although another group demonstrated more activity for 40-ADR than ADR in the L1210 and P388 leukemias in vitro [3].

The consensus from most studies is that EPI-ADR and ADR have comparable activity [3, 16, 24, 30, 34, 35]. In our study three agents, namely NMHE, 7-OMEN, and ACA-A proved less cytotoxic than ADR, a finding confirmed for the last two drugs in murine experimental tumours, including the B16 melanoma and L1210 leukemia [6]. Therefore, in general, the patterns of response noted in our straightforward initial screen are comparable with those reported by other workers using predominantly murine experimental tumours either in vitro or in vivo.

These data on the two human tumour cell lines are also consistent with those derived from in vitro phase-II studies using a human tumour cloning system for 4-ADR, THP-ADR, and 7-OMEN, with the two last compounds proving more cytotoxic and the first showing no advantage to ADR [23, 34, 42]. However, we have not confirmed the enhanced activity for ACA-A reported in the human tumour cloning assay study of Tihon and Issell [39]. Salmon et al. [34] reported some positive correlations between results obtained using the human tumour stem cell assay on tumour biopsy material and a continuous line of HeLa cells, but they favoured the use of a panel of human tumours for testing analogues. We suggest that since such studies are more readily feasible with continuous human tumour cell lines this possibility should be more fully evaluated. Indeed, these two types of study may complement each other, with cell line work preceding in vitro phase-II studies using the human tumour cloning system on biopsy specimens.

Furthermore, although we have demonstrated enhanced in vitro cytotoxicity at equivalent concentrations with certain of these derivatives compared with ADR, if this information is to have any clinical relevance it is important that their usage results in an improved therapeutic index. At present, comparative effects of new drugs and analogues on normal tissues are provided from screening studies with experimental laboratory animals, followed by phase-I clinical trials, and clearly cannot be derived from in vitro experiments. However, in an attempt to put these in vitro evaluations of cytotoxicity into perspective it is important to establish that the clinically tolerated dose levels of these analogues are not markedly different. The available information for eight of these anthracycline derivatives which have entered phase-I/II clinical trials is listed in Table 5, and a correlation is attempted between in vitro and in vivo data. This simplified comparison suggests that benefit may indeed accrue in terms of enhanced cytotoxicity for only four of these derivatives, namely 4-DNR, THP-ADR, 4-ADR, and DHAD, and that it is most marked for DHAD. Of course it remains to be proven clinically whether these compounds have superior antitumour activity in human tumours without enhanced toxic side effects. Another favourable consideration would be a lack of cross-resistance to ADR.

Resistance and cross-resistance experimental studies with anthracyclines have predominantly centred on the use of drug-resistant sublines derived in vivo from the P388 or L1210 leukaemias. In the P388-ADR-resistant subline in vivo studies have shown almost universal cross-resistance between the other anthracyclines tested, which have included EPI-ADR, DNR, 4-DNR, DHAD, 40-ADR, 7-OMEN, NMHE, and ACA-A [24]. However, using the L1210 model system, which is considered naturally to show partial resistance to ADR in vivo [9], 40-ADR was reported as active [12] and in a daunomycin-resistant L1210 subline moderate activity was shown by DHAD [15]. Therefor apart from the EPI-ADR and DNR data, our results with the in vitro ADR-resistant subline of the L5178Y cells are completely at variance with the P388 data, but show some concordance with the limited studies using the L1210 system.

Differences in drug responses between the P388 model in vivo and the L5178Y system in vitro have been noted previously in evaluation of the vinca alkaloids [19]. Whilst the resistant cell lines induced in vitro may not represent a valid in vivo model and resistance mechanisms may differ, it should be noted again that the drug concentrations used in vitro with the L5178Y cells are markedly lower (at least 100-fold) than those used with the P388 cells [25]. It remains of course to be shown which murine model system more accurately predicts clinical experience. We are currently developing ADR-resistant human tumour cell lines in vitro so as to establish patterns of cross-resistance in this model and relate them to our murine model studies and compare them with the limited data available from in vitro phase-II studies in the human tumour cloning system. In this last area results from two recent studies provide some evidence that (i) ACA-A has some positive activity against six tumours considered resistant to ADR [39], confirming our findings, but (ii) that specimens from patients who had received prior treatment with ADR tend to be less

sensitive in vitro to THP-ADM [23], contrasting with our findings. Clearly further studies in this area are needed. On the basis of our work it will be interesting to see DHAD, DHAQ, and 4-ADR evaluated in this manner. Indeed we are encouraged to pursue these compounds following the clinical demonstration that prior therapy with ADR did not predispose to resistance to DHAD [1].

In summary, using continuous human tumour cell lines in vitro and an in vitro murine model system for evaluating ADR resistance, we have been readily able to screen a series of 11 anthracycline analogues and identify three agents which have enhanced cytotoxicity and lack cross-resistance with the parent compound. This straightforward screening procedure can be readily carried out and may prove a valuable initial preclinical test system, perhaps complementing in vitro phase-II studies with the human tumour cloning system using fresh tumour material.

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