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Comparative effectiveness of mitoxantrone and doxorubicin in overcoming experimentally induced drug resistance in murine and human tumour cell lines in vitro

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Summary. Using a range of cell lines of murine and human tumour origin in which relatively modest levels (2- to 17-fold) of drug resistance have been selected in vitro by exposure to a range of standard antitumour drugs, we compared the cytotoxic effects of doxorubicin (DOX) and mitoxantrone (MITO). In general, significantly lower concentrations of MITO than of DOX were required to achieve comparable cytotoxicity, confirming previously published data. MITO appears more generally effective against the murine L5178Y drug-resistant sublines than DOX, although there was no expression of collateral sensitivity to this newer agent. In the various human tumour lines there was a lack of cross-resistance to both DOX and MITO in two 5-fluorouracil (FU)-resistant lines and one of two cisplatin (CDDP)-resistant cells, but cross-resistance was expressed in one subline resistant to vincristine (VCR) and two etoposide (VP-16)-resistant sublines. One murine and two human DOX-resistant sublines were effectively killed by MITO, whilst DOX proved effective against the human MITO-resistant subline. This apparent lack of cross-resistance between DOX and MITO in these resistant sublines expressing low levels of resistance in vitro therefore appears to contrast with previous reports involving highly multidrug-resistant DOX-selected sublines. However, since the latter lines generally exhibited profound cross-resistance to VCR and definite cross-resistance to VP-16, this may at least in part dictate their responses to MITO. Therefore, attempts to use experimentally derived drug-resistant sublines for preclinical drug screening should be approached with caution, since patterns of drug response appear to be influenced by the level of drug resistance expressed. The need remains to determine which type of model system provides the most relevant clinical information.

Introduction

Identification of antitumour drugs that will effectively kill drug-resistant tumour cells is one of the major objectives of pre-clinical screening programmes. With the definition of the phenomenon of multidrug resistance (MDR), characterized by the simultaneous expression of resistance to a number of structurally unrelated compounds [2, 3, 6, 25, 26], the search for drugs specifically effective against such MDR cells has intensified. Many of these MDR cell lines have been experimentally derived such that high levels of resistance (frequently greater than 100-fold) are expressed to the selecting agent [3, 6, 12, 26].

Although there is some preliminary evidence that the MDR phenotype may be expressed in certain tumour samples taken directly from patients [5, 11], the need remains to conclusively demonstrate that tumour cells expressing lower levels of resistance, which may be more frequently encountered clinically, express identical patterns of drug response to these highly resistant MDR cells. The drugs generally implicated in the MDR phenotype include the anthracyclines, dactinomycin, the Vinca alkaloids and epipodophyllotoxins [25]. Certain recent publications have also included the newer agent mitoxantrone (MITO) in this category, reporting cross-resistance between doxorubicin (DOX) and MITO [12, 23, 24, 30]. However, in other publications [7, 29, 31] and in initial studies from our laboratory [10, 20], we found a certain lack of cross-resistance between these two anthraquinone compounds. Since MITO is now undergoing active clinical evaluation, including testing of patients previously treated with DOX [1, 8, 30, 32], we considered it worthwhile to identify any differential effectiveness of MITO or DOX in overcoming experimentally induced drug resistance in a series of cell lines expressing relatively modest levels (i.e. <20-fold) of drug resistance, which we consider to be clinically relevant.

Materials and methods

Drugs and chemicals. The following drugs were kindly donated for our studies: DOX by Farmitalia Carlo Erba (St. Albans, Hertfordshire, UK); methotrexate (MTX), vincristine (VCR) and MITO (Novantrone) by Lederle Laboratories (Gosport, Hampshire, UK); 5-fluorouracil (FU) from Roche Products (Welwyn Garden City, Hertfordshire, UK); vindesine (VDS) by Eli Lilly and Company Limited (Basingstoke, Hampshire, UK); and etoposide (VP-16) by Bristol-Myers Products (Slough, UK). Cisplatin (CDDP) and the tetrazolium salt (MTT) were purchased from Sigma Chemical Company (Poole, Dorset, UK). Media and sera were obtained from Gibco-Biocult (Renfrewshire, Scotland). Low-gelling-temperature agarose was obtained from Uniscience Limited (Cambridge, UK), and Bactoagar, from Difco Laboratories (Michigan, USA).

Cell culture. The cell lines used and the orders of resistance expressed to each selective agent are listed in Table 1. The

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Table 1. Comparative in vitro cytotoxic effects of mitoxantrone and doxorubicin

Cell line origin	Subline used (Resistance Index[a])		References	IC_{50} values (ng per m)[c] (Resistance Index)[a]	
				MITO	DOX
Murine L5178Y lymphoblasts:	Parental			0.095	8.5
	DOX-resistant	(× 2.5)	[17]	0.090 (× 0.95)	21 (× 2.5)
	MTX-resistant	(× 17)	[16]	0.077 (× 0.8)	0.65 (× 0.1)
	FU-resistant	(× 8)	[13]	0.090 (× 0.95)	3.3 (× 0.4)
	VCR-resistant	(× 4)	[14]	0.130 (× 1.4)	50 (× 6)
	VDS-resistant	(× 7)	[14]	0.150 (× 1.6)	86 (× 10)
	CDDP-resistant	(× 3)	[15]	0.090 (× 0.95)	8.5 (× 1)
Human breast carcinoma:	Parental		[15]	8.1	12
MCF-7	DOX-resistant	(× 4)[b]		14.2 (× 1.8)	50.5 (× 4.2)
	VCR-resistant	(× 14)	[22]	50 (× 6.2)	50 (× 4.2)
Human ovarian carcinoma:	Parental			*0.53	9
TR170	DOX-resistant	(× 2)	[21]	*0.52 (× 1)	21 (× 2.3)
	CDDP-resistant	(× 2)	[21]	*0.53 (× 1)	8 (× 0.9)
Human colon carcinoma:	Parental			2.2	6
LoVo	FU-resistant	(× 7)	[15]	1.3 (× 0.6)	6 (× 1)
Human colon carcinoma:	Parental		[18]	2.2	10
COLO 205	FU-resistant	(× 4)[b]		2.5 (× 1.1)	13 (× 1.3)
Human tongue carcinoma:	Parental			0.43	8.7
HN-1	VP 16-resistant	(× 4)	[22]	2.5 (× 5.8)	23.4 (× 2.7)
Human teratoma of the testis:	Parental			0.60	1.9
SuSa	VP 16-resistant	(× 9)	[22]	1.08 (× 1.8)	2.9 (× 1.5)
Human bladder carcinoma:	Parental			*2.75	*32.5
RT112	CDDP-resistant	(× 3)	[4]	*7.5 (× 2.7)	*45 (× 1.4)

[a] The Resistance Index is defined as the ratio of the IC_{50} values of the parental and resistant sublines for each drug tested

[b] See *Materials and methods*

[c] These values were derived from full dose-response curves following 24-h drug exposures. Cell survival was assayed by clonogenic assay, except for those values preceded by an asterisk, where growth inhibition assays were used

sources of the MCF-7 and COLO 205 cells kindly provided for this study are detailed in previous publications [15, 18]. The parental RT112 and SuSa cells were obtained from Dr. J. R. W. Masters, Institute of Urology (London, UK). Details of growth conditions and the colony-forming assay methodologies adopted for determining the effects of drugs on cell survival are provided in the references cited in Table 1.

The DOX-resistant subline of MCF-7 cells was derived by exposure of the parental line to six 24 h pulsed treatments with 50 ng/ml (an IC_{90} drug concentration that reduced survival by 90% as judged by clonogenic assay). Cell populations were permitted to repopulate and resume logarithmic growth between each drug treatment. The FU-resistant subline of COLO 205 cells was established by the continuous exposure of cells in vitro to increasing concentrations of FU, commencing at 0.2 μg/ml and reaching a final concentration of 0.5 μg/ml; resistant cells were then cloned and subsequently maintained in 0.2 μg/ml FU. The VP-16-resistant subline (designated VPC2) of SuSa cells was established by the continuous exposure of cells in vitro to increasing concentrations of VP-16, ranging from 50 to 80 μg/ml. Resistant sublines were cultured for at least 2 weeks in drug-free medium before their drug responses were assessed.

All of the data listed in Table 1 were derived after the exposure of cells in vitro to a range of drug concentrations for 24 h only. The IC_{50} (drug concentration required to reduce cell survival by 50%) values quoted were derived from full dose-response curves. These were constructed with each point derived from triplicate cultures, with the overall scatter of values required to produce a ≤ 1 log cell kill never exceeding 10%. The Resistance Index (RI) is defined as the ratio of the IC_{50} values of the parental and resistant sublines for each drug tested. In general, since the overall scatter of data points did not exceed 10%, RI values of < 1.3 were considered indicative of a lack of cross-resistance. Since we had previously established that results derived from both clonogenic and growth inhibition assays were comparable for TR170 cells [21], this simpler procedure was used when evaluating MITO and a similar assay was carried out on the RT112 cell lines for both drugs.

A MITO-resistant subline of the MCF-7 cells was established by exposure in vitro to six 24-h pulsed exposures to 20 ng/ml, an IC_{90} drug concentration, as described above for the DOX-resistant subline. The MTT assay [27] was used to establish patterns of in vitro drug sensitivities, with a 4-day continuous exposure to the drug, followed by a 2-h incubation with 0.25 mg/ml MTT.

Results

The results are summarized in Table 1. In the murine cell lines, as previously reported [13, 16], DOX was highly effective at overcoming resistance to both MTX and FU,

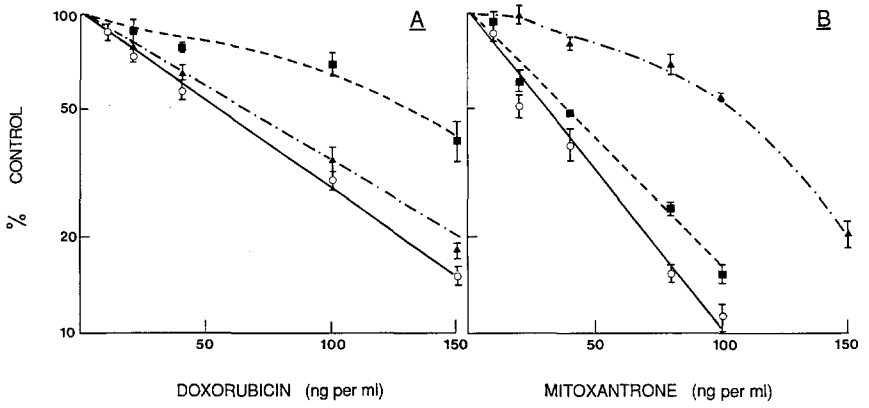

Fig. 1. The cytotoxic effects, as judged by MTT assay, of continuous exposure to a range of concentrations of DOX or MITO on drug-resistant sublines of the MCF-7 human breast tumour cell line. Each point represents the mean of at least 16 estimations ± SEM, with the experiment repeated twice. O——O parental MCF-7 cells; ■-----■ DOX-resistant subline; ▲——▲ MITO-resistant subline

with both of these resistant sublines expressing collateral sensitivity to the drug. However, DOX was ineffective against VCR- and VDS-resistant cells, with an apparent expression of significant cross-resistance between DOX and both of these Vinca alkaloids [14]. We now report a lack of cross-resistance between DOX and CDDP in this murine model system. Evaluation of MITO against this same panel of murine cell lines showed that (a) to achieve equivalent cytotoxicity against the parental L5178Y cells, approximately 100 times less MITO than DOX was required, and (b) the range of IC_{50} values for MITO in these seven sublines was very narrow, i.e. 2-fold, contrasting with the approximate 132-fold range observed with DOX, suggesting that MITO is generally more effective against a series of different drug-resistant L5178Y sublines than DOX. Cross-resistance to MITO was not observed in the DOX-resistant subline.

We next carried out a similar comparison in a series of human tumour sublines, derived such that they expressed relatively low levels of drug resistance. Amongst the different tumour types, the parental lines exhibited a 17-fold range of sensitivity to DOX, with the SuSa cells proving particularly sensitive to this drug, and a 19-fold sensitivity range to MITO. In general, significantly lower concentrations of MITO than of DOX were required to achieve comparable cytotoxic effects, as reported using the murine sublines. A general lack of significant cross-resistance was observed when DOX was tested against two FU-resistant colon carcinoma cell lines and two lines expressing CDDP resistance, one derived from an ovarian carcinoma (TR170), and the other, from a transitional bladder carcinoma (RT112). MITO was also effective against the two FU-resistant cell lines, with collateral sensitivity being expressed in the case of the LoVo subline as well as against the CDDP-resistant TR170 subline. However, significant cross-resistance between MITO and CDDP was evident in the CDDP-resistant RT112 line. Both MITO and DOX proved ineffective against VCR-resistant MCF-7 cells and the VP-16-resistant HN-1 subline, and there was some cross-resistance expressed in the VP-16-resistant SuSa subline. However, a certain lack of cross-resistance between MITO and DOX was again observed, since MITO was highly effective against a DOX-resistant TR170 subline and the resistance index to MITO was only 1.8 in the 4-fold DOX-resistant MCF-7 subline.

To investigate further this certain lack of cross-resistance between these two drugs, we developed a MITO-resistant MCF-7 subline. Using the MTT assay, we showed (Fig. 1) not only that DOX-resistant cells were effectively killed by MITO, but also that MITO-resistant cells were susceptible to DOX.

Discussion

We evaluated the in vitro cytotoxic effects of DOX and MITO in a series of experimentally induced drug-resistant murine and human tumour cell lines. We showed that, in general, significantly lower concentrations of MITO than of DOX were required to achieve comparable cytotoxic effects. This was particularly true of certain murine cell lines, where IC_{50} values for the two drugs differed by factors as great as 100- to 400-fold. However, in the human tumour cell lines, differences were more modest, ranging from approximately 3-fold in the LoVo cells, confirming a previous observation by Drewinko et al. [9] using 1-h drug exposures, to 20-fold in the HN-1 cells. The main exception appeared to be the MCF-7 cells, which exhibited very similar sensitivities to both drugs, as judged by both clonogenic and MTT assays. These differences in the human lines are therefore more comparable with the approximately 4- to 6-fold lower levels of MITO (12–14 mg/m^2) than of DOX (50–75 mg/m^2) that are currently used in clinical studies [1, 8, 28, 32].

In the series of murine L5178Y resistant sublines, it is particularly noticeable that the IC_{50} values for MITO fell within a very narrow range, suggesting that this drug was generally effective against all of these drug-resistant cells, although there was no expression of collateral sensitivity to this newer agent. These observations were in marked contrast to the wide range of responses to DOX. However, the same pattern was not evident amongst the series of human cell lines derived from different tumour types. The range of IC_{50} values for DOX was approximately 27-fold, but if the hypersensitive SuSa cells are excluded the range becomes relatively narrow, i.e. 8-fold, comparable with the 6-fold figure previously reported for a larger series of 18 human tumour cell lines [19]. However, the range of IC_{50} values was much larger for MITO, being approximately 120-fold.

As in the murine sublines, in the human sublines MITO was effective in killing FU-resistant cells and both DOX- and CDDP-resistant ovarian carcinoma cells (TR170), but this was not the case for the CDDP-resistant bladder carcinoma cells (RT112). MITO also proved more

Table 2. Degrees of cross-resistance to vincristine, VP-16 and mitoxantrone expressed by certain DOX-selected multidrug-resistant human tumour cell lines

Cell line (origin)	Resistance Indices				Reference
	DOX	VCR	VP 16	MITO	
MES-SA(Dx5) (sarcoma)	100	240	30	60	[12]
K562/ADM (myelogenous leukemia)	134	630	44	79	[29]
NCI-H69/LX4 (small-cell lung cancer)	85	1060	88	15	[30]

cytotoxic against the DOX-resistant MCF-7 subline than DOX itself, appearing to confirm our previous report that MITO overcame DOX-resistance in an SK-OV-3 human ovarian carcinoma cell line [20]. We also provided evidence that there is a certain lack of cross-resistance between DOX and MITO in human MCF-7 sublines expressing relatively modest levels of resistance to either DOX (x 4) or MITO (x 5). However, cross-resistance was noted between MITO and the Vinca alkaloids; it was only slight in the two murine sublines but was marked in the more highly VCR-resistant human MCF-7 subline. Two human VP-16-resistant sublines also proved to be cross-resistant to MITO.

These results therefore indicate that, working with a series of cell lines derived from either the murine L5178Y lymphoma or a range of human tumours expressing relatively modest levels of drug resistance, certain generalisations can be made: (a) tumour cells in which resistance to VCR has been induced by VCR exposure generally appear cross-resistant to both DOX and MITO; (b) tumour cells in which resistance to VP-16 has been induced by exposure to VP-16 generally express some cross-resistance to both agents; and (c) tumour cells in which resistance to DOX has been induced by DOX exposure appear less cross-resistant to MITO. One of the clinical implications suggested by these findings is that, whilst pre-treatment with VCR or VP-16 might argue against the subsequent use of MITO, prior exposure to DOX should not automatically preclude the use of MITO as second-line treatment, and vice versa. The latter observation appears to be borne out by recently reported clinical cross-over studies using these two drugs [1, 28].

These data therefore appear to argue against automatically including MITO in the list of drugs to which MDR cells are likely to express cross-resistance. The fact that others have reported cross-resistance to MITO in their human MDR cells [12, 29, 30] may be not directly associated with the MDR phenotype, but could perhaps be related to the fact that these DOX-selected MDR sublines generally express significant cross-resistance to VCR and VP-16 (see Table 2). This use of human tumour cell lines expressing modest orders of drug resistance may therefore provide rather different information relating to patterns of drug resistance and collateral sensitivity than that obtained using very highly drug-resistant sublines. However, the need remains to determine which system provides the most clinically relevant information.

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