L1210 cells selected for resistance to methoxymorpholinyl doxorubicin appear specifically resistant to this class of morpholinyl derivatives

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Summary We investigated the mechanism of resistance in murine L1210 leukaemia cells selected after treatment with FCE 23762 methoxymorpholinyl doxorubicin; (MMRDX), a methoxymorpholinyl derivative of doxorubicin active *in vitro* and *in vivo* on multidrug-resistant (mdr) cells, currently undergoing phase I clinical trials. The resistant subline obtained after repeated *in vitro* treatments, L1210/MMRDX, is resistant *in vitro* and *in vivo* to all tested methoxymorpholinyl derivatives and to cyanomorpholinyl doxorubicin, but shows resistance to morpholinyl derivatives only *in vivo* or following their activation with rat S9-liver fractions *in vitro*. L1210/MMRDX cells are sensitive to classic mdr- and altered topoisomerase (AT)-mdr-associated drugs. These cells do not appear to overexpress the *mdr*1 gene, nor do they exhibit impaired intracellular drug accumulation and efflux or altered levels of glutathione and glutathione S-transferase. The extent of DNA single-strand break formation and, after microsomal activation, of DNA interstrand cross-links after treatment with MMRDX was similar in the parent and the resistant subline. The mechanism of resistance in L1210/ MMRDX cells remains to be identified but may prove a novel one, highly specific for this class of mdr-active anthracyclines.

Treatment with doxorubicin (DX) or daunorubicin commonly selects resistant tumour cells that express the multidrug resistant (mdr) phenotype, characterised by enhanced drug efflux mediated by a high molecular weight membrane glycoprotein (Endicott & Ling, 1989; Hayes & Wolf, 1990; Roninson, 1992). Among the several classes of anthracyclines synthesised in the past 20 years, the morpholinyl anthracyclines are of particular interest since they appear to be active *in vitro* and *in vivo* against mdr tumour cells (Watanabe *et al.*, 1988; Coley *et al.*, 1990; Ripamonti *et al.*, 1992).

Methoxymorpholinyl doxorubicin (MMRDX) is a lipophilic compound, able to reach high intracellular levels in sensitive and mdr tumour cells (Grandi *et al.*, 1990*a*; Ripamonti *et al.*, 1992). In addition, compounds of this class appear to be effective against tumour cells expressing the altered topoisomerase-mdr phenotype (AT-mdr) (Grandi *et al.*, 1990*b*). MMRDX is currently under investigation in phase I clinical trials. It was thus of interest to determine whether resistant tumour cells could be selected for resistance to MMRDX following *in vitro* exposure to the drug and, if so, to identify the mechanisms involved. Murine leukaemia L1210 cells resistant to MMRDX (L1210/MMRDX) were isolated after repeated *in vitro* treatments with the drug and characterised for their pattern of cross-resistance *in vitro* and *in vivo* to a panel of antineoplastic drugs and to selected anthracyclines bearing either the methoxymorpholinyl or the morpholinyl substitution on the 3′ position of the sugar moiety.

Materials and methods

Chemicals

3′-Deamino-3′-(2-methoxy-4-morpholinyl)doxorubicin (MM-RDX), 3′-deamino-3′-(4-morpholinyl)doxorubicin (MRDX), 4′-epi-3′-deamino-3′-(2-methoxy-4-morpholinyl)doxorubicin (4′-epi-MMRDX), 4′-epi-3′-deamino-3′-(4-morpholinyl) doxorubicin (4′-epi-MRDX), 4-demethoxy-3′-deamino-3′-(2-methoxy-4-morpholinyl) doxorubicin (4-dm-MMRDX), 4-demethoxy-3′-deamino-3′-(2-methoxy-4-morpholinyl) daunorubicin (4-dm-MMRDNR), 4-demethoxy-3′-deamino-3′-(4-

morpholinyl) daunorubicin (4-dm-MRDNR), 3′-deamino-3′-(3-cyano-4-morpholinyl) doxorubicin (CN-MRDX) (Figure 1) and doxorubicin (DX) were obtained from Farmitalia Carlo Erba (Milan, Italy).

The following compounds were pharmaceutical preparations: vinblastine (Eli Lilly, Indianapolis, IN, USA); melphalan (L-PAM), camptothecin and 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase, reduced glutathione (GSH), NADPH, NADP and 5-5′-dithiobis,2-nitrobenzoic acid (DTNB) (Sigma, St Louis, MO, USA); mitomycin C (Kyowa Hakko, Tokyo, Japan); 5-fluorouracil (5-FU) (Roche, Milan, Italy); 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) (Nitrumon) (Simes, Vicenza, Italy); cisplatin (Bristol Myers, Syracuse, NY, USA). DX, MMRDX, MRDX, 4′-epi-MMRDX, 4′-epi-MRDX, 4-dm-MMRDX, 4-dm-MMRDNR, 4-dm-MRDNR and CN-MRDX were dissolved immediately
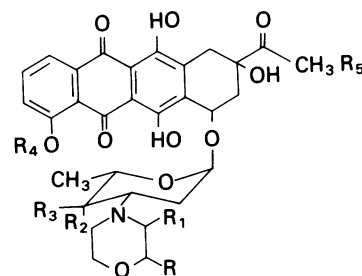
Compound	R	R_1	R_2	R_3	R_4	R_5
MMRDX	OCH_3	H_2	OH	H	CH_3	OH
4′-epi-MMRDX	OCH_3	H_2	H	OH	CH_3	OH
4-dm-MMRDX	OCH_3	H_2	OH	H	OH	OH
4-dm-MMRDNR	OCH_3	H_2	OH	H	OH	H
MRDX	H_2	H_2	OH	H	CH_3	OH
4′-epi-MRDX	H_2	H_2	H	OH	CH_3	OH
4-dm-MRDNR	H_2	H_2	OH	H	OH	H
CN-MRDX	H_2	CN	OH	H	CH_3	OH

Figure 1 Chemical structures of morpholinyl derivatives.

before use and the concentrations were checked spectrophotometrically.

DX	$\lambda_{max} = 496$ nm	E 1% 200	(H₂O)
MMRDX	$\lambda_{max} = 495$ nm	E 1% 173	(CH₃OH)
MRDX	$\lambda_{max} = 495$ nm	E 1% 199	(CH₃OH)
4'-epi-MMRDX	$\lambda_{max} = 496$ nm	E 1% 172.5	(CH₃OH)
4'-epi-MRDX	$\lambda_{max} = 496$ nm	E 1% 188.29	(CH₃OH)
4-dm-MMRDX	$\lambda_{max} = 482$ nm	E 1% 168.4	(CH₃OH)
4-dm-MMRDNR	$\lambda_{max} = 432$ nm	E 1% 178	(CH₃OH)
4-dm-MRDNR	$\lambda_{max} = 482$ nm	E 1% 165.2	(CH₃OH)
CN-MRDX	$\lambda_{max} = 496$ nm	E 1% 136	(CH₃OH)

Cell cultures

The murine lymphocytic leukaemia cell lines (L1210 and L1210/MMRDX) were grown *in vitro* as a stationary suspension culture in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Flow, Irvine, UK), 2 mM L-glutamine (Gibco Europe, Glasgow, UK) 10 µM β-mercaptoethanol, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin.

Isolation of L1210/MMRDX cells

The MMRDX-resistant cell subline (L1210/MMRDX) was selected *in vitro* by continuous exposure to 20 ng ml⁻¹ MMRDX, and after 20 passages cloned by limiting dilution (Norman & Thompson, 1977) in the presence of 20 ng ml⁻¹ MMRDX.

A clone resistant to MMRDX was selected and propagated *in vitro* in the absence of the drug. Resistance was stable for at least 1 year. The doubling time was determined by seeding the cells at the concentrations of 5×10^4 and 10^5 cells ml⁻¹ (1 ml per well, 12-well plates; Costar, Cambridge, MA, USA). Every 24 h two replicate samples were harvested and the cell number was determined by a ZBI Coulter counter (Hialeah, FL, USA).

RNA analysis

Total cellular RNA was extracted by guanidinium isothiocyanate–caesium chloride centrifugation (Maniatis *et al.*, 1982).

For Northern blot analysis 20 µg of total RNA was fractionated on 1% agarose gel containing 6.7% formaldehyde and transferred to nylon membranes (Gene-Screen Plus, NEN, Boston, MA, USA). The filters were hybridised for 16 h at 42°C in 50% formamide, 10% dextran sulphate, 1 M sodium chloride, 1% SDS, 100 µg ml⁻¹ of denatured salmon sperm DNA and 10⁶ c.p.m. ml⁻¹ denatured ³²P-labelled probe. After hybridisation, the filters were washed sequentially in 2 × SSC at room temperature and in 2 × SSC, 1% SDS, at 65°C. The probes utilised were the 1.3-kb *Eco*RI/*Sal*I insert of pcDR.3 (Gros *et al.*, 1986) containing the human *mdr* gene (Gros *et al.*, 1986) and the 1.8-kb *Pst*I insert of the murine action gene. Both probes were ³²P-labelled using the multiprime DNA labelling system and [³²P]dCTP (Amersham, Aylesbury, UK).

Glutathione-S-transferase (GST) determination

Expontentially growing cells were lysed by sonication in distilled water, the cell lysate was centrifuged (10,000 r.p.m. for 15 min) and the supernatant was used for enzyme assay according to the method of Habig and Jakoby (1981) using CDNB as substrate.

Glutathione (GSH) determination

Cells were analysed during the exponential phase of growth. GSH total content was measured as described by Tietze (1969).

Alkaline elution

DNA damage was detected by the alkaline elution technique described by Kohn *et al.* (1981). Briefly, [³H]thymidine-prelabelled cells were layered and lysed on polycàrbonate filters (0.8 µm pore size, Nucleopore, Pleasanton, CA, USA) at room temperature with 5 ml of lysis solution containing 0.1 M glycine, 0.025 M EDTA and 2% SDS (pH 10). After proteinase K (Merck, Darmstadt, Germany) digestion, alkaline elution was carried out using a solution containing 0.02 M EDTA, 0.1% SDS and tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY, USA) to give a pH of 12.2. The pumping rate was 0.04 ml min⁻¹ and fractions were collected at 180 min intervals for 15 h. ³H-labelled DNA was quantitated by liquid scintillation β counter.

In experiments carried out to identify DNA damage after microsomal activation of MMRDX, cells were incubated with the drug in the presence of the S9 fraction of rat liver (1 mg of protein per ml), glucose 6-phosphate 1 mg ml⁻¹ and NADP 2 mg ml⁻¹ in a final volume of 1 ml for 1 h before being processed as for DNA single-stand break assay.

Intracellular drug accumulation and retention

Intracellular drug content was determined in L1210 and L1210/MMRDX cells treated with 10 and 100 nM MMRDX and incubated at 37°C for up to 4 h.

For drug efflux determination, cells incubated with MMRDX for 1 h were washed in PBS, then resuspended in drug-free medium and reincubated at 37°C.

Drug was extracted from the cells with 0.6 M hydrochloric acid–ethanol (1:1 mixture) and samples were analysed in an HPLC system using a C₁₈ reversed-phase column and a spectrofluorimeter as detector (excitation and emission wavelengths were 479 and 593 nm respectively).

The intracellular accumulation of the drug at various time intervals is reported as ng per 10⁶ cells.

In vitro *drug sensitivity*

Exponentially growing L1210 and L1210/MMRDX cells were exposed to various concentrations of drugs continuously for 48 h. The antiproliferative activity of the drugs was evaluated by counting surviving cells with a Coulter counter and results were expressed as IC₅₀ (dose causing 50% inhibition of cell growth in treated cultures relative to untreated controls).

The cytotoxicity of morpholinyl derivatives on L1210 and L1210/MMRDX cells with and without microsomal activation was determined on cells incubated at 37°C in aerobic conditions for 1 h in the presence of various concentrations of drugs with or without an incubation mixture consisting of 0.33 mg ml⁻¹ protein of S9 fraction of rat liver homogenate, 0.33 mg ml⁻¹ NADP and 0.16 mg ml⁻¹ glucose 6-phosphate (Boehringer Mannheim Italia, Milan, Italy).

S9 was prepared according to the method of Hilton and Sartorelli (1970). The incubation was stopped by washing cells with ice-cold RPMI-1640 medium. The cells were then incubated for 48 h in drug-free medium and cell growth was assessed as described above.

In vivo *studies*

Inbred DBA2 and CD2F1 adult female mice (Charles River, Calco, Italy), 2–3 months old, weighing 20–24 g, were kept under standard laboratory conditions.

The L1210, obtained from the National Cancer Institute (NIH, Bethesda, MD, USA), and L1210/MMRDX mouse leukaemias were maintained by weekly i.p. passages of 10⁶ cells in DBA2 mice; in the case of the L1210/MMRDX subline mice were treated weekly with 0.05 mg kg⁻¹ MMRDX i.p. For experimental studies i.p. inocula of 10⁵ cells into CD2F1 mice were used. Drugs were administered i.p. on day 1, control animals receiving vehicle alone. Drug activity was determined by comparing the median survival

time (MST) of the treated group with that of the control group and results are expressed as %T/C where:

$$\%T/C \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100$$

Toxicity was evaluated on tumour-bearing mice on the basis of the gross autopsy findings and weight loss.

Results

Selection and characterisation of L1210/MMRDX cells

L1210/MMRDX cells are 8.5-fold resistant *in vitro* to the selecting agent (Table I), and maintain resistance after > 100 passages in drug-free medium. L1210/MMRDX cells have the same doubling time (9 h) and *in vivo* tumorigenicity (8–10 days) as the parent line.

The levels of GSH (7.76 and 7.83 fmol per cell) and of glutathione *S*-transferase (100 ± 14 and 111 ± 8 relative units) are also similar in L1210 and L1210/MMRDX cells.

Results obtained comparing the levels of *mdr-1* mRNA in L1210, L1210/MMRDX and L1210/DX cells a subline 20-fold resistant to DX (not shown) as the positive control clearly indicate that L1210/MMRDX cells do not over-express *mdr-1* mRNA gene.

Intracellular accumulation and efflux

The kinetics of accumulation and efflux in L1210 and L1210/MMRDX cells exposed to 10 and 100 nM MMRDX is presented in Figure 2a and b. At all time points the intracellular levels of MMRDX are similar in both cell lines.

Alkaline elution studies

The frequency of single-strand breaks (DNA-SSBs) and formation of DNA interstrand cross-links (DNA-ISCs) in response to MMRDX treatment is reported in Table II. No differences were observed between L1210 and L1210/MMRDX cells. DNA-SSB levels after exposure to 1 μg ml^{-1} were similar to those found with DX at the same concentration (not shown). The repair of DNA-SSBs appeared very quick as no breaks were detectable after 1 h drug washout in both L1210 and L1210/MMRDX cells.
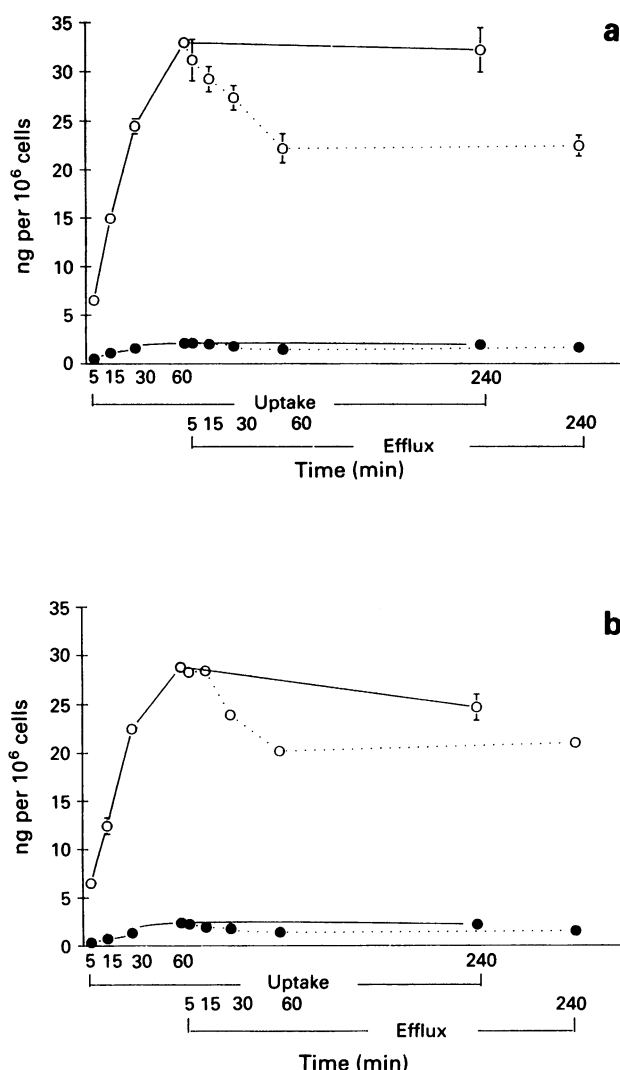

Figure 2 Time response of MMRDX accumulation (——) and efflux (. . .). a, L1210 cells; b, L1210/MMRDX cells treated with 10 nM (filled symbols) and 100 nM (open symbols) MMRDX. Values represent the average of six determinations. Bars = s.e.; when bars are not shown they are smaller than the symbol.

Table I *In vitro* and *in vivo* activity of different antineoplastic agents against L1210 and L1210/MMRDX leukaemias

Compound	In vitro[a] RI[b]	Dose (mg kg⁻¹)	In vivo anti-tumour activity L1210[c] %T/C[d]	TOX[e]	L1210/MMRDX[c] %T/C[d]	TOX[e]
MMRDX	8.5	0.11	138	0/10	96	0/40
		0.13	163	0/10	100	10/20
DX	0.9	10	–	–	208	0/20
		13	200	0/10	229	3/20
Vinblastine	1.2	3	156	0/10	163	0/10
		5.9	63	9/10	63	8/10
Camptothecin	1.3	20	194	0/10	225	0/10
		30	88	7/10	75	10/10
L-PAM	0.7	10	169	0/30	289	0/10
		15	211	1/30	>667	1/10
Cisplatin	0.5	7.7	175	0/10	311	0/10
		10	100	7/10	>667	1/10
BCNU	0.7	30	394	0/10	367	0/10
5-FU	0.5	200	175	0/10	172	0/10
		300	188	0/10	178	2/10
Mitomycin C	0.3	5	125	0/10	>750	0/10
		9	156	0/10	719	0/10

[a]Drug sensitivity was determined by counting surviving cells after 48 h of continuous exposure to at least four concentrations of each drug. [b]Resistance index: ratio between IC$_{50}$ values on resistant cells and sensitive cells. [c]CD2F1 mice were given an injection of 10^5 cells i.p. and treated i.p. on day 1. [d]Median survival time of treated mice/median survival time of controls × 100. [e]Number of toxic deaths/number of mice.

MMRDX did not cause DNA-ISCs in either cell line when incubated without rat liver S9 fraction; conversely, after 1 h incubation in the presence of rat liver S9 fraction the number of DNA-ISCs appeared similar in L1210 and L1210/MMRDX cells (Table II). In L1210 cells 30% and 55% of DNA-ISCs were repaired after 1 and 4 h respectively; in L1210/MMRDX 20% and 64%.

In both cell lines DNA-ISCs were no longer detectable at 24 h. These data suggest that the mechanism of resistance is not related to differences in DNA damage produced by MMRDX.

Pattern of in vitro and in vivo sensitivity to different anti-tumour compounds

The activity of different anti-tumour molecules tested in vitro and in vivo on L1210/MMRDX cells in comparison with L1210 cells is reported in Table I. The subline is resistant to MMRDX and sensitive to all other tested drugs, including

Table II DNA-SSBs and DNA-ISCs in rad equivalents induced by MMRDX treatment

DNA damage	Dose ($\mu g\ ml^{-1}$)	Cell line L1210	Cell line L1210/MMRDX
DNA-SSBs[a]	1	111.4 ± 36	105.6 ± 16
	1	220.6 ± 76	208.8 ± 45
DNA-ISCs[b]	0.5[c]	70 ± 7	91 ± 7
	1	169.6 ± 30	170.7 ± 39

[a]Cells were treated with MMRDX for 1 h. Each value is the mean ± s.e. of five experiments. [b]Cells were treated with MMRDX for 1 h in the presence of S9 fraction of rat liver homogenate. Each value is the mean ± s.e. of at least three experiments. [c]Results from one experiment ± s.e. of three replicate samples.

mdr-inactive drugs such as DX and vinblastine. Moreover, the anti-tumour activity of L-PAM, cisplatin and mitomycin C is markedly higher in the resistant than in the parent line.

Pattern of in vitro and in vivo sensitivity to morpholinyl anthracyclines

It is reported that most morpholinylanthracyclines are activated to highly cytotoxic metabolites when administered in vivo and in vitro in the presence of liver microsomes (Lau et al., 1989; 1991; Duran et al., 1991; Lewis et al., 1992). The relative sensitivity of L1210/MMRDX cells to a series of molecules of the same chemical class (Figure 1) was thus assayed with and without rat liver homogenate (S9) (Table III). L1210/MMRDX cells are resistant to all tested methoxymorpholinyl anthracyclines with resistance indexes (RI) ranging between 10.5 and 4.2. The RI values are unaltered after treatment with S9. Conversely, L1210/MMRDX cells are sensitive to the analogues bearing the morpholinyl instead of the methoxymorpholinyl group but become resistant after treatment with S9. In particular, the cytotoxicity of MRDX and 4'-epi-MRDX is increased 50-fold after microsomal activation.

The only cyanomorpholinyl derivative tested, CN-MRDX, was inactive, independently of the treatment with microsomes, which also did not augment its cytotoxic activity.

Results reported in Table IV indicate that, when administered in vivo to mice bearing ascitic L1210 and L1210/MMRDX cells, all tested compounds have anti-tumour activity against the sensitive line, and are inactive against the resistant one. These results confirm the data obtained in vitro, which indicate that L1210/MMRDX cells are resistant to methoxymorpholinyl derivatives, but only show resistance to morpholinyl derivatives after activation in the presence of liver microsomes.

Table III Cytotoxic activity of different morpholinyl derivatives against L1210 and L1210/MMRDX cells with and without rat liver microsomes (S9)

Compound	IC_{50} (ng ml^{-1}) − S9[a] L1210	IC_{50} (ng ml^{-1}) − S9[a] L1210/MMRDX	RI[b]	IC_{50} (ng ml^{-1}) + S9[a] L1210	IC_{50} (ng ml^{-1}) + S9[a] L1210/MMRDX	RI[b]
MMRDX	9.8 ± 3	103.6 ± 20	10.5	3.5 ± 0.7	26.5 ± 4	7.5
4'-epi-MMRDX	11.7 ± 2	49.7 ± 11	4.2	2 ± 0.5	6.8 ± 2	3.4
4-dm-MMRDNR	13.5 ± 0.6	108.8 ± 8	8	12.2 ± 0.8	83.2 ± 5	6.8
4-dm-MMRDX	10.2 ± 2.4	93 ± 20	9.1	2.2 ± 0.5	11.6 ± 2	5.2
MRDX	60 ± 11	83.4 ± 16	1.3	1.1 ± 0.3	14.7 ± 4	13.3
4'-epi-MRDX	130 ± 19	122.2 ± 24	0.9	2.8 ± 0.5	29.4 ± 8	10.5
4-dm-MRDNR	94.8 ± 5	181 ± 12	1.9	17.1 ± 3	67.1 ± 27	3.9
CN-MRDX	0.25 ± 0.08	7.7 ± 0.2	30.8	0.26 ± 0.06	7.9 ± 0.5	30.3

[a]50% inhibitory concentration (IC_{50}) represents the mean ± s.e. from dose-response curves of at least three experiments. Cells were treated with the drug for 1 h in the presence (+ S9) and in the absence (− S9) of rat liver homogenate; S9 fraction. [b]Resistance index: ratio between IC_{50} values on resistant cells and sensitive cells.

Table IV Anti-tumour activity of different morpholinyl derivatives against ascitic L1210 and L1210/MMRDX leukaemias

Compound	Dose (mg kg^{-1})	L1210[a] %T/C[b]	L1210[a] TOX[c]	L1210/MMRDX[a] %T/C[b]	L1210/MMRDX[a] TOX[c]
MMRDX	0.11	138	0/10	96	0/40
	0.13	163	0/10	100	10/20
4-dm-MMRDNR	0.24	150	0/10	100	0/10
	0.36	175	0/10	106	7/10
MRDX	0.075	150	0/10	100	0/10
	0.11	150	7/10	113	9/10
4'-epi-MMRDX	0.3	156	0/10	100	0/10
	0.45	125	9/10	100	1/10
4-dm-MRDNR	0.51	188	0/10	129	0/10
	0.66	206	1/10	104	7/10
CN-MRDX	0.0063	150	0/10	113	0/10
	0.0125	178	2/10	113	0/10

[a]CD2F1 mice were given an injection of 10^5 leukaemia cells i.p. and were treated i.p. on day 1. [b]Median survival time of treated mice/median survival time of controls × 100. [c]Number of toxic deaths/number of mice.

Discussion

We describe the isolation and characterisation of a murine L1210 cell line selected for resistance to MMRDX, a new anthracycline derivative undergoing phase 1 clinical studies. The mode of action of MMRDX appears to differ from that of anthracyclines (Ripamonti et al., 1992), since it is active against mdr and AT-mdr cells (Grandi et al., 1990b), and forms DNA-ISCs when tested in the presence of liver microsomes (Duran et al., 1991; Lau et al., 1991).

Our results using L1210 cells indicate that exposure to MMRDX selects a stable cell population specifically resistant in vitro and in vivo to the selecting agent and compounds of the same chemical class, the morpholinyl anthracyclines.

L1210/MMRDX cells were found to be sensitive to anti-tumour drugs associated with the classic mdr phenotype and to topoisomerase II inhibitors. No overexpression of the mdr1 gene, or any alteration in drug accumulation or efflux was identified in these resistant cells. The levels of GSH and GST were found to be unchanged in L1210/MMRDX cells, suggesting that GSH-mediated detoxification is not the basis of resistance to this compound.

As regards the DNA damage induced by MMRDX treatment, we found that the number of DNA-SSBs and, after microsomal activation, of DNA-ISCs was similar in L1210 and L1210/MMRDX cells.

Although these data suggest that the mechanism of resistance selected by treatment with MMRDX is not related to differences in DNA damage or in repair mechanisms, the drug concentrations required to obtain a number of DNA lesions detectable by alkaline elution are far greater than the minimal cytotoxic concentrations. Therefore, we cannot exclude the possibility that the different drug sensitivity is due to a more efficient repair of a lower number of DNA lesions.

The pattern of cross-resistance to morpholinylanthracyclines is an interesting finding: in fact, all compounds bearing the methoxymorpholinyl group are inactive in vitro and in vivo against L1210/MMRDX cells, as well as cyanomorpholinyl doxorubicin, whereas the morpholinyl derivatives are inactive only in vivo, and in vitro when tested in the presence of rat liver S9 fraction. Such results can be interpreted assuming that: (a) the active metabolite(s) of MMRDX and MRDX have similar modes of action and mechanisms of resistance; (b) the mechanism of resistance to MMRDX and its active metabolite is the same, as demonstrated by the evidence that the RIs before and after metabolic activation of MMRDX are equivalent; (c) CN-MRDX is inactive against L1210/MMRDX cells without requiring metabolic activation. Therefore is seems plausible that MMRDX, as well as its active metabolite(s), MRDX active metabolite(s) and CN-MRDX act by the same mechanism.

CN-MRDX is known to alkylate DNA very efficiently, causing many DNA adducts and DNA-ISCs (Westendorf et al., 1985; Jesson et al., 1989).

The anti-tumour activity of alkylating agents such as L-PAM, BCNU, cisplatin and mitomycin C against L1210/MMRDX cells suggests that, if MMRDX activity is associated with alkylating species, its mechanism of interaction with macromolecules is different from that of the classical alkylating agents.

In conclusion, L1210/MMRDX is a cell line which may be a useful tool for investigating the mechanism of in vitro and in vivo resistance to MMRDX and its analogues.

The results obtained so far strongly support the view that this drug is profoundly different from previously investigated anthracyclines and should not be considered as one of the various DX analogues, but as a new type of anti-tumour drug.

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