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ORIGINAL ARTICLE

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Comparative resistance of idarubicin, doxorubicin and their C-13 alcohol metabolites in human MDR1 transfected NIH-3T3 Cells

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Abstract The anthracycline analog idarubicin (ID) is useful in the treatment of leukemias, and is of further interest because of the unique activity of its major circulating metabolite idarubicinol (IDOL). In vitro studies have shown that ID retains activity against tumor cells made resistant by prolonged exposure to substrates of the p-glycoprotein energy-dependent efflux pump. To selectively investigate multidrug resistance to ID in tumor cells, ID, IDOL, doxorubicin (DX) and doxorubicinol (DXOL) were evaluated for growth inhibitory activity when incubated with NIH-MDR1-G185 (MDR) cells or with the parent NIH-3T3 (3T3) cells. The MDR cells are transfected with the human multidrug gene mdr_1, and express a functional p-glycoprotein. ID growth inhibitory activity was much less affected by p-glycoprotein-mediated efflux than was DX. ID IC_{50}values were only 1.8-fold greater in the MDR cell line than in the parental 3T3 cell line, while the IC_{50} value for DX was 12.3-fold greater in the transfected cell line. Verapamil (VRP) fully restored drug sensitivity of the MDR cell line to ID and DX. In studies with the alcohol metabolites, IDOL and DXOL IC_{50} values were 7.8- and 18.9-fold greater, respectively, for the MDR cell line than for the parental cell line. Intracellular concentrations of DX and DXOL, but not ID and IDOL, were substantially increased in the MDR cells when VRP was present in the incubation mixtures. ID and IDOL retain substantial growth inhibitory activity in mdr_1-transfected cells,and ID may be of value in clinical settings where multidrug resistance mediated by p-glycoprotein is a potential limitation of therapy.

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Introduction

Idarubicin (ID) is an anthracycline analog with substantial activity in adult and pediatric leukemias (5). In comparison with more widely used anthracyclines such as daunorubicin (DN) and doxorubicin (DX), ID has greater growth inhibitory activity against human tumor cells in culture [2,12], possesses superior activity in DNA damage assays [12], and may be less cardiotoxic [6]. The pharmacology and biology of the major circulating metabolite, idarubicinol (IDOL), appears to be important in the antitumor activity of ID. We and others have shown that IDOL possesses significant growth inhibitory activity when incubated with tumor cell lines [12, 21]. IDOL is much more potent than anthracycline alcohols such as doxorubicinol (DXOL), and daunorubicinol (DNOL), and in fact is equipotent with the parent drug, ID [12,21]. Following administration to adult and pediatric cancer patients, high plasma concentrations and prolonged elimination of IDOL are observed, resulting in systemic exposure much greater than that to the parent drug [2,5,23]. In pediatric studies, we have detected IDOL in the cerebrospinal fluid (CSF) following intravenous administration of ID [18].

The development of resistance to anthracyclines in cancer patients is a common phenomenon. While a number of resistance mechanisms have been characterized in laboratory studies, expression of the multidrug resistance gene mdr_1 has been associated with exposure to anthracyclines as well as other naturally occurring chemotherapeutic agents [11]. Expression of this gene yields a 170-kDa transmembrane protein known as p-glycoprotein [24]. The presence of this protein results in an active efflux pump which reduces intracellular drug concentrations [9].

ID activity has been evaluated in a number of tumor cell lines made drug-resistant by prolonged exposure to anthracyclines and other agents. ID growth inhibitory activity (and thus resistance to ID) varies considerably among those cell lines [3,8,13,15,22], although all express the mdr_1 gene product, p-glycoprotein. Prolonged exposure to these drugs may lead to other mechanisms of resistance to anthracyclines, including ID. Thus, the role of p-glycoprotein-mediated drug efflux is not clear in studies using drug-induced resistant cell lines. We utilized a cell line made resistant solely by transfection with the human mdr_1 gene which encodes a wild-type p-glycoprotein (p170) with glycine at amino acid residue 185, resulting in a functional multidrug efflux pump [7]. We further assessed IC_{50} values and intracellular drug concentrations in the presence or absence of verapamil (VRP). This agent effectively blocks drug efflux by binding directly to p-glycoprotein, resulting in increased intracellular drug concentrations [4,19,25]

Materials and methods

Cells and culture

The mouse fibroblast cell lines, NIH-3T3 (3T3) and NIH-MDR1-G185 (MDR) were kindly provided by Dr. M. Gottesman (NCI, Bethesda, M.). Both lines were maintained in Dulbecco's minimum essential medium (DMEM) (Gibco Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (Gibco) and supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). The transfected line was also grown in the presence of 60 ng/ml colchicine (Gibco) and removed from the growth medium 24–48 h prior to use. All cultures were incubated at 37°C in an atmosphere containing 5% CO_2.

Drugs

ID, IDOL, and DXOL were generously provided by Pharmacia-Farmitalia Carlo Erba (Milan, Italy) and Pharmacia Inc. (Columbus, Ohio). DX and VRP were purchased from Sigma Chemical Company (St. Louis, Miss.). The anthracyclines were dissolved in dimethylsulfoxide (DMSO) and stored at -20°C. VRP was dissolved in 50% DMSO just prior to use. Light exposure was kept to a minimum for all drugs used.

Microculture tetrazolium assay

A microculture tetrazolium (MTT) assay was performed according to the method of Alley et al. [1] with slight modifications. In brief, exponentially growing cells were harvested and plated at 1000 cells/well in 96-well cell culture plates in a volume of 100 μl minimum essential medium (MEM) containing Earle's salts without phenol red (Flow laboratories, McLean, Va.) and supplemented with 10% fetal calf serum (Gibco), 2 mM l-glutamine (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Drugs were diluted in medium such that 100 μl added to each well gave the following concentrations. (0.0001 –1.0 μM) ID, (0.001–10 μM) IDOL, (0.0001–5 μM) DX, and (0.01–100 μM) DXOL. For each experiment seven to nine drug concentrations were assessed which gave a broad range of survival. In experiments using VRP, drug was added to anthracycline-containing medium prior to addition to wells to a final concentration of 10 μM. Controls of DMSO or VRP alone were conducted for all experiments. Plates were incubated for 72 h prior to development with the MTT assay. Absorbance was measured with a Dynatec MR 5000 microplate reader at 570 nm. Each drug concentration or control vehicle was plated in six wells for each experiment. An average absorbance was calculated for the six wells. The control absorbance value was taken as 100% cell survival, and reductions in that value as toxicity due to drug. IC_{50} values were calculated from dose-response plots. Reported data are the average of at least three experiments.

Intracellular drug concentration

Exponentially growing cells were harvested and plated at 1×10^6 cells per 60-mm culture dish in complete medium (3 ml) 18–24 h prior to drug exposure. ID and IDOL were added to a final concentration of 1 μg/ml. DX and DXOL were added to a final concentration of 10 μg/ml in order to achieve a measurable fluorescence signal. Drug exposure was for 1,2, or 3 h in the presence or absence of 10 μM VRP. Controls of DMSO or VRP alone were conducted for each experiment. After drug exposure, the cells were washed twice with ice-cold PBS, treated with trypsin-EDTA (Gibco), then suspended in cold medium and kept on ice prior to flow analysis.

Intracellular drug fluorescence was measured by flow cytometry with a FACS Vantage (Becton-Dickinson) equipped with an argon laser (488 nm excitation, emission collected with a 530–30 filter). Lysis II software (Becton-Dickinson) was used for data analysis. Fluorescence histograms were generated and mean channel numbers of the linear fluorescence intensity distribution (FL1) were calculated. Forward angle light scatter (FALS) histograms were also generated to calculate relative cell volume. Results are expressed as the normalized mean fluorescence index (NMFI) according to the procedure of Luk and Tannock [14] using the following relationship:

$$NMFI = (FL1/FALS) - (FL1_0/FALS_0)$$

that is, NMFI = (mean fluorescence channel number/mean FALS channel number) with drug − (mean fluorescence channel number/mean FALS channel number) without drug.

NMFI values were used as a measure of relative intracellular drug accumulation. Reported data are the average of two experiments.

Results

Growth inhibitory activity of ID and DX following exposure to the non-transfected 3T3 and to the transfected MDR cell lines are summarized in Table 1. ID was much more potent than DX when incubated with both cell lines, consistent with our earlier in vitro studies with ID and related anthracyclines [12]. Of particular interest were IC_{50} values for ID and DX when incubated with 3T3 cells as compared with the values obtained with MDR cells. The ID IC_{50} value was only 1.8-fold greater for the MDR cell line than for the 3T3 cell line under identical incubation conditions, whereas the IC_{50} value for DX was 12.3-fold greater for the MDR cell line than for the 3T3 cell line. VRP had little effect on IC_{50} values for either agent in experiments with 3T3 cells. When VRP was present in incubations with the MDR cells, the IC_{50} value for ID decreased approximately two-fold, and the IC_{50} value for DX decreased 18-fold. Thus, VRP reversed mdr_1-mediated resistance to both agents.

The growth inhibitory activity of the alcohol metabolite IDOL was much greater than that of DXOL in both cell lines (Table 1), as previously observed in human tumor cell lines [12]. When incubated with the

Table 1 Growth inhibition following a 72 h exposure to idarubicin and doxorubicin and their alcohol metabolites, idarubicinol and doxorubicinol. Values are mean IC_{50} values ± SEM of three experiments

	IC_{50} (nM)		
	NIH-3T3	NIH-MDR1-G185	Ratio[a]
ID	10.1 ± 3.6	18.5 ± 2.0	1.8
ID + 10 μM VRP	13.6 ± 9.2	9.0 ± 3.1	0.7
IDOL	24.2 ± 6.7	188.2 ± 98.9	7.8
DX	58.4 ± 19.3	717.8 ± 169.3	12.3
DX + 10μ MVRP	27.1 ± 8.6	39.8 ± 8.1	1.5
DXOL	2260.8 ± 1382.5	42 620[b]	18.9

[a]NIH-MDRI-G185 value divided by NIH-3T3 value
[b]Average of two experiments.

Table 2 Effect of 10 μM VRP on intracellular drug concentration analyzed in NIH-3T3 and NIH-MDR1-G185 cell lines. Values are the NMFI for 3-h drug exposure calculated as described in Materials and methods, and are the average of two experiments

	NIH-3T3 Cells			NIH-MDR1-G185 Cells		
	Drug alone	Drug + VRP	Ratio[a]	Drug alone	Drug + VRP	Ratio[a]
DX	0.469	0.478	1.02	0.204	0.423	2.07
DXOL	0.160	0.152	0.95	0.099	0.146	1.47
ID	1.132	1.124	0.99	1.501	1.340	0.89
IDOL	1.287	1.213	0.94	1.287	1.364	1.06

[a]NMFI value for cells treated with drug in the presence of 10 μM VRP divided by NMFI value of cells treated with drug alone. A value of one or less indicates that VRP had no effect on intracellular drug concentration. A value greater than one indicates that VRP increased intracellular drug concentration

MDR cells, the IC_{50} value of IDOL was 7.8-fold greater than the value obtained in experiments with the parental 3T3 cells. The IC_{50} value for DXOL was 18.9-fold greater for the MDR cell line than for the 3T3 cell line. Limited availability of IDOL and DXOL precluded growth inhibition studies with VRP.

The relative intracellular drug concentrations of ID, DX, IDOL and DXOL were determined in the two cell lines in the presence and absence of VRP (Table 2). VRP had no significant effect on drug concentrations in the 3T3 cells. When present in MDR cell incubations, VRP increased the intracellular concentrations of DX and DXOL to values similar to these observed in the 3T3 cells. VRP had little effect on drug concentrations of ID and IDOL in the MDR cells.

Discussion

A major problem in the clinical application of anthracycline antitumor agents is the development of drug resistance by tumors. The most thoroughly characterized mechanism of anthracycline resistance is active efflux from tumor cells mediated by the mdr_1 gene product p-glycoprotein. Tumor cell lines made resistant by prolonged exposure to a variety of p-glycoprotein substrates (e.g., anthracyclines, vinca alkaloids, etc.) vary in their degree of cross-resistance to ID [3,8,13,15,22]. Mechanisms other than mdr_1 gene expression can also be important in resistance to anthracyclines, including topoisomerase II alterations [17,20] and increased concentrations of reduced glutathione [10,16]. Since prolonged exposure of tumor cell lines to mdr_1 substrates may induce any of these mechanisms of resistance as well as expression of the p-glycoprotein, we chose for our studies a cell line (MDR) which is specifically transfected with the human mdr_1 gene and which expresses a functional p-glycoprotein.

Based on IC_{50} values, ID was only two-fold less potent when incubated with MDR cells expressing the p-glycoprotein, while DX was 12-fold less potent in cells of the same cell line as compared with the results with the parental 3T3 cells. These data are consistent with earlier reports [3,8,13,15,22] that ID is relatively active in cell lines made resistant by exposure to drugs rather than by transfection with the human mdr_1 gene as in our study. Co-incubation of VRP restored the sensitivity of MDR cells to ID and DX. Further, when VRP was present in MDR cell incubations, intracellular concentrations of DX were increased to values approximating those in the parental 3T3 cells. ID concentrations differed only slightly between the two cell lines, and thus VRP had little effect on concentrations in the MDR cell line. These data suggest that ID is a relatively poor substrate for the p-glycoprotein efflux pump. This is somewhat surprising since the structures of ID and DX are identical except for two features. ID lacks the methoxy moiety found in DX at the 4 position of the D ring, and ID contains a hydroxyl moiety at carbon 14 of the side chain not found in DX. ID is more lipophilic than DX, and the more lipophilic analogs among a series of related compounds usually have a greater affinity for p-glycoprotein (11).

IDOL was approximately eight-fold less potent when incubated with the MDR cell line than with the 3T3 cell line. DXOL was almost 19-fold less potent in the analogous experiments. While IDOL appeared to be more affected by the presence of p-glycoprotein than was the parent drug, the metabolite was still a potent inhibitor of growth in the MDR cell line (IC_{50} 24.2 nM). Thus, both ID and IDOL had significant activity in this model system of drug resistance. These IDOL data are in contrast to a study utilizing rat glioblastoma cells made resistant to anthracyclines through exposure to DX, in which IDOL was 220-fold less active in the resistant cells (21). This may have been due to other mechanism of resistance operative in that cell line. It is possible that another mechanism of resistance to IDOL may be operative in the transfected MDR line. There was little difference between IDOL concentrations in the 3T3 and MDR cells at the times measured despite the eight-fold difference in IC_{50} values, and VRP had no effect on those concentrations. However, it may also be that the 3-hour time course of intracellular drug concentration measurements was not adequate to detect concentration differences for the alcohol.

The current interest in ID, primarily for the treatment of leukemias, is due in part to the increased antitumor activity of this agent in comparison with other anthracyclines, and the unique antitumor activity of the major circulating metabolite IDOL. Data obtained in this study confirm that ID, and the alcohol metabolite IDOL, both possess substantial activity in *mdr*$_1$-transfected tumor cells. While other mechanisms of resistance may be important in the clinical use of ID, this agent may be active in settings where p-glycoprotein-mediated resistance is a potential factor in the effectiveness of therapy.

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