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COMPARISON OF MECHANISMS RESPONSIBLE FOR RESISTANCE TO IDARUBICIN AND DAUNORUBICIN IN MULTIDRUG RESISTANT LoVo CELL LINES

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Abstract—Two human colon carcinoma drug resistant clones (LoVo-IDA-1 and LoVo-IDA-2) were selected by continuous pressure of LoVo parent cell lines to idarubicin (IDA). Both cell sublines exhibited a typical multidrug resistance (MDR) phenotype but, despite IDA selection, the resistance index (RIext) was higher for daunorubicin (DAU) (RIext = 101–112) than for IDA (RIext = 20–23). A similar pattern of cross-resistance was also observed in two (DOX) doxorubicin-selected LoVo cell lines (LoVo-DOX-1 and LoVo-DOX-2). All the MDR cell lines exhibited decreased drug accumulation and increased intracellular drug tolerance as evidenced by the greater intracellular amount of drug required to cause a 50% growth inhibition (IC_{50}int) compared to their parent cell line. The differences between DAU and IDA RIext exhibited by MDR cells were a function of intracellular resistance. DAU IC_{50}int was 13.9 and 14.9 times higher in LoVo-IDA-1,2 and 6.4 and 6.2 in LoVo-DOX-1,2 cell lines, respectively, than in LoVo-sensitive cells, whereas IDA IC_{50}int was only 3.6 and 3.2 times higher in LoVo-IDA-1,2 and 2.2 and 2.3 in LoVo-DOX-1,2 cell lines, respectively. Conversely, variations in IDA accumulation between resistant and sensitive cells were similar to those observed for DAU [the ratios between DAU uptake in sensitive and resistant cells were almost identical (P = NS) to those observed for IDA]. Differences between IDA and DAU intracellular distribution accounted for the relatively higher DAU intracellular resistance. In fact nuclear/cytoplasmic (N/C) DAU fluorescence ratio was higher ($P < 0.01$) in sensitive ($N/C = 3.4 \pm 2.7$) than in MDR cells (N/C ranging from 0.31 ± 0.2 to 0.41 ± 0.1). In contrast, no significant (P = NS) differences were observed in IDA N/C ratios between sensitive and MDR cells (N/C ranging from 0.16 ± 0.1 to 0.20 ± 0.1). In MDR cells, 1-hr VER (10 μM) treatment partially reverted both DAU N/C ratios and intracellular DAU resistance but neither changes in IDA N/C ratios nor variation in intracellular IDA resistance were observed following VER exposure. In conclusion, the greater intracellular drug tolerance that MDR cells show for DAU compared to IDA makes IDA more effective than DAU in MDR cells overexpressing P-glycoprotein (P-gp).

Key words: idarubicin; daunorubicin; multidrug resistance

Anthracyclines are among the most valuable cytostatic agents in clinical use. Their usefulness is, however, limited by tumor cell drug resistance. One of the most important biochemical mechanisms by which tumor cells develop resistance to anthracyclines is the increased activity of P-gp†. However, overexpression of the P-gp affects differentially drug resistance of tumor cells to the various classes of anthracyclines [1–3]. P-gp determines two major biochemical phenomena. One acts at the cellular plasma membrane level and affects drug-transmembrane equilibria determining reduced intracellular drug accumulation [4, 5]. The other works at the intracellular level [6–9], possibly preventing drugs from reaching the cellular target sites of drug cytotoxic activity and, consequently, allowing MDR cells to tolerate intracellular drug concentrations higher than those tolerated by their drug-sensitive parent cells.

It is known that P-gp cannot fully account for anthracycline resistance in many MDR cells (see review No. 10). Other mechanisms contribute to the MDR phenotype: e.g. glutathione transferase and glutathione peroxidase or changes in levels and/or activity of topoisomerase II. Moreover, the interaction with the cell membrane may be an important aspect of anthracycline action which does not depend on intracellular drug accumulation [11].

IDA (4-demethoxydaunorubicin hydrochloride) is a new DAU analog which is being used to treat a variety of human malignancies [12–14]. IDA is endowed with greater biological activity and lower cardiotoxicity than the parent compound [12–14]. *In vitro* studies indicate that it may be more effective than DAU in tumor cell lines that display the MDR phenotype, thus suggesting that it could be useful in

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† Abbreviations: IDA, idarubicin or 4-demethoxydaunorubicin hydrochloride; DAU, daunorubicin; VER, verapamil; MDR, multidrug resistance; IC_{50}ext, indicates the extracellular drug concentration inhibiting cell growth by 50% and IC_{50}int indicates the intracellular drug content necessary to obtain the same cytotoxic effect; RIext and RIint, resistance index, indicate how many times IC_{50}ext and IC_{50}int, respectively, were higher in MDR than in sensitive cells; N/C ratio, nuclear/cytoplasmic fluorescence ratio; P-gp, P-glycoprotein.

circumventing, at least partially, MDR [1, 2, 15, 16]. At present little is known about the biochemical mechanisms that make MDR cells more susceptible to IDA than to DAU. It has been supposed that this might depend on the high lipophilicity of IDA, which enhances intracellular accumulation of this drug [1, 15, 17]. In addition, it has been proposed that IDA could be less affected by P-gp activity [2]. Aims of the present work were: (a) to investigate the biochemical bases of the lower resistance of MDR cells to IDA compared to DAU; and (b) to clarify further the biochemical mechanisms by which tumor cells develop resistance to IDA. For this purpose we selected drug-resistant clones by continuous exposure of the parent LoVo human colon carcinoma cell line to IDA. All the resistant clones exhibited a typical MDR phenotype but showed a RIext for DAU greater than IDA.

MATERIALS AND METHODS

Drugs. IDA, DAU and doxorubicin were obtained from Farmitalia-Carlo Erba (Milan, Italy); etoposide and teniposide from Bristol-Myers (Rome, Italy); vincristine (Oncovin®) from Lilly (Florence, Italy); actinomycin-D (Cosmegen®) from Merck Sharp (Rome, Italy), verapamil from Knoll (Ludwigshafen, Germany). Drugs were dissolved in saline under sterile conditions just before use, except for epipodophillotoxins which were dissolved in dimethyl sulfoxide under the same sterile conditions (<0.1% final concentration).

Cell lines. LoVo cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were propagated in Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Seralab, Sussex, U.K.), 50 μg/mL streptomycin and 50 U/mL of penicillin G. Cell cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 and 95% air.

Induction/selection of IDA resistant clones. Drug-resistant sublines were obtained by treating exponentially growing cell cultures with 5 ng/mL IDA, as described [18]. The medium was replaced twice a week with fresh medium containing drug at the same concentrations. After 5–10 weeks, single colonies of drug-resistant cell variants were harvested with 0.25% trypsin and 0.02% EDTA and were singularly cultured for more than 6 months in the continuous presence of drug at the selected concentrations. Drug-resistant sublines were maintained in the presence of drug until 24 hr before testing.

Drug cytotoxicity. Cytotostatic effects were assessed as described previously [19]. Cells were plated in 6-well tissue culture plates (Falcon, NJ, U.S.A.) and incubated in the presence of cytotoxic agents for 1 or 24 hr. After drug treatment cells were washed twice with saline and incubated in drug-free medium for at least three doubling times. They were then washed with saline, trypsinized and counted with a ZM coulter counter (Coulter Electronics Ltd, Luton, U.K.). In the experiments with VER, cells were co-incubated with antineoplastic agents plus 10 μM VER for 1 hr. A post-incubation of 24 hr with VER was performed in order to increase its modulating effect, as suggested previously [7].

In some experiments, data obtained by cell counting were compared with those obtained by clonogenic assay in liquid medium, as described [18], but no significant differences were noted.

Northern and Southern blot analyses of MDR1 gene and determination of P-gp levels. Total cellular RNA and high-molecular mass DNA were extracted from exponentially growing cells by the guanidine chloride and proteinase/phenol-chloroform methods, respectively. Northern and Southern blot analyses were performed as described previously [18]. The probes used were: the 1.2-Kb *Eco*RI fragment derived from plasmid pHuP170 #1 representing a cDNA, covering the 3′ portion of the human MDR1 gene specific for MDR1 gene [20]; the 0.7-Kb *Eco*RI-*Bam*HI fragment derived from plasmid pHF β-3′UT specific for β-actin gene [21]; and the 1.6-kb *Cla*I-*Eco*RI fragment derived from plasmid pHSR-1 specific for *c*-myc gene [22]. The β-actin mRNA level and *c*-myc signal were used as internal standards for MDR1 gene mRNA expression and amplification, respectively.

P-gp expression was determined by indirect immunofluorescence and cytofluorimetric analysis as described [6], using the MRK16 specific anti-P-gp monoclonal antibody.

Drug uptake and drug efflux analyses. Exponentially growing cells, 3–10 × 10^6 in 10 mL medium, were seeded in 90-mm Petri dishes (Falcon, Italy) and incubated overnight at 37°. After the removal of the culture medium, cells were incubated for 1 hr at 37° with fresh medium containing different concentrations of drugs. The medium was subsequently withdrawn and Petri dishes were chilled on ice and quickly washed three times with ice-cold saline solution. $AgNO_3$ in distilled water (3.3% final concentration) was then added and drugs were extracted with water-saturated normal butyl alcohol. The fluorescence intensity of extracts was measured by a fluorescence spectrophotometer (SFM25, Kontron, Italy) at the following excitation and emission wavelengths: 470 and 565 nm for IDA and 490 and 590 nm for DAU. The average efficiency of the extraction procedure was over 90%. Cellular drug uptake was quantified by means of calibration curves and expressed as ng drug/10^6 cells. To estimate the cell number in each experiment, cells were seeded in additional triplicate Petri dishes, incubated overnight at 37°, treated with drug for 1 hr and washed as described above. They were then trypsinized and counted with a coulter counter (Coulter Mod. ZM) apparatus.

For efflux studies, cells were treated with drugs for 1 hr. At the end of incubation the medium was discarded and the cells were quickly washed in a saline solution prewarmed to 37°; prewarmed drug-free medium was then added and the plates were incubated at 37°. Retention of drug in the cells was determined at various time points as described for uptake studies.

DAU and IDA subcellular location. Drug subcellular distribution was visualized with a Leitz Orthoplan fluorescence microscope using a band pass filter of 530–560 nm, a long wave pass filter of 580 nm and a chromatic beam splitter of 580 nm. Cells grown on chamber slides were incubated for

1 hr with drugs. Microscopy observation was performed with the microscopic slide kept at the specified temperatures. When the effect of VER was being tested, the drug was added 15 min before and throughout DAU or IDA treatment. Fluorescence photographs were taken with Ektachrome 400 film (Kodak, Germany). Exposure times were 120 sec when the fluorescence signal was weak; otherwise, they were automatically determined by the photograph system.

For quantification of the nuclear/cytoplasmic ratio (N/C ratio) of IDA and DAU, cells grown on chamber slides were incubated for 1 hr at 37° with the appropriate drug. Then, with chamber slides kept at 37°, analysis was performed using a Nikon Diaphot-TMD fluorescence microscope (Nikon, Japan) equipped with a high pressure HBO 100 W DC mercury lamp, appropriate filters, a C2400-87 intensified CCD Hamamatsu camera (Hamamatsu, Japan) and a custom-made microscope image analysis system (TGA, Italy). Experimental data are the means of 30–50 cell measurements for each treatment.

Plasma membrane preparation and photoaffinity labeling analysis. Cells in the midlog stage were washed in cold phosphate-buffered saline solution, and hypotonic lysis buffer [10 mM NaCl, 1.5 mM $MgCl_2$ 10 mM Tris–HCl pH 7.4, 2 mM phenylmethylsulfonylfluoride (PMSF)] was added. Under sterile conditions, cells were harvested with a rubber policeman, placed into 50-mL sterile centrifuge tubes, and centrifuged at 300 *g* at 4° for 10 min. The supernatant was discarded and the pellet was transferred to a homogenizer tube and placed on ice for approximately 40 min. Cells were disrupted utilizing at least 30 strokes. The homogenate was transferred to sterile centrifuge tubes and centrifuged at 1000 *g* for 10 min. The supernatant was placed into ultra-clear centrifuge tubes and spun at 34,000 rpm in SW55Ti (Beckman, CA, U.S.A.) rotor for 1 hr. The pellet was resuspended in 0.250 M sucrose in Tris–HCl at pH 7.5 and stored at −80° until use [23]. Protein determination was performed with the BioRad assay system (BioRad Laboratories, Richmond, CA, U.S.A.), with bovine serum albumin as standard.

For photoaffinity labeling analysis, 100 μg of plasma membrane protein were reacted with 0.4 μM [^{3}H]azidopine in 0.250 M sucrose, 10 mM Tris–HCl pH 7.5, in a final volume of 75 μL at room temperature for 30 min in the presence or absence of competitors. The reaction mixture on ice was then irradiated with a UV lamp at 366 nm for 20 min. Labeled proteins were separated by 7% SDS–PAGE. The gel was fixed, treated with the fluorographic reagent EN^{3}HANCER (DuPont), dried and then exposed for 14 days at −80° using XAR-5 film (Eastman Kodak Co., Rochester, NY). Quantitation of [^{3}H]azidopine-labeled P-gp was carried out by densitometric tracings of fluorograms [24].

DNA strand-passing activity of topoisomerase II. The strand-passing activity of topoisomerase II was monitored by the P4-unknotting assay, as previously described [24]. Cell nuclei were isolated from exponentially growing cell culture by gentle detergent lysis (0.1% Triton X-100) in an isotonic nucleus buffer [25]. Trypan blue was used to confirm the loss of cell membrane integrity. Nuclei were then extracted for 30 min at 4° by adding nucleus buffer containing 2 M NaCl so as to bring the final NaCl concentration to 350 mM. The preparation was then centrifuged at 100,000 *g* for 1 hr. The supernatant was the topoisomerase II-containing nuclear extract. Protein concentrations were determined using the BioRad assay system. Nuclear extracts were brought to a 20-μg/mL protein concentration, and 5 μL of undiluted or 1:2 serial dilution were added to 20 μL of P4 reaction mixture (5 mM Tris–HCl, pH 7.5; 100 mM NaCl, 10 mM $MgCl_2$ 0.5 EDTA; 30 μg/mL bovine serum albumin, 0.5 mM dithiothreitol, 1 mM ATP, 20 μg/mL of P4 knotted DNA) as indicated by Zwelling *et al.* [26]. After incubation at 37° for 30 min, the reaction was terminated by the addition of 5 mL of prewarmed stopping buffer (50% glycerol, 5% sodium dodecyl sulphate, 20 mM EDTA, 0.25% bromophenol blue) and DNA analysed on a 0.9% agarose gel in 80 mM Tris–HCL, pH 8.0, 40 mM boric acid, and 2 mM tetrasodium EDTA. Photographic negatives from ethidium bromide-stained gels were scanned by a densitometer for the unknotted P4 DNA band. Topoisomerase II activities were expressed as the ratio between unknotted and knotted forms after control background subtraction, and 1 U of enzyme activity was defined as the concentration capable of unknotting 50% of P4 knotted DNA in 30 min under the conditions described.

Statistical analysis. For statistical evaluation the unpaired *t*-test was used.

RESULTS

Selection and characterization of LoVo-IDA resistant clones

Exponentially growing LoVo HCC cells (0.5–1×10^6 cells/plate in 10 mL medium) were exposed to 2 × serial IDA concentrations ranging from 1.25 to 20 ng/mL for approx. 8 weeks. Ten drug resistant clones (LoVo-IDA-$_{1-10}$) were collected at the selecting dose of 5 ng/mL IDA. This was the highest IDA concentration at which drug selection generated drug resistant mutants with a single step. All of the 10 LoVo-IDA resistant clones expressed P-gp at higher levels (without significant clonal heterogeneity) than the parent LoVo cell lines (data not shown). Two of the 10 resistant mutants (LoVo-IDA-1 and LoVo-IDA-2) were cultured in the presence of the selecting drug for 12 months and were characterized for MDR1 gene product expression and pattern of cross resistance. Both LoVo-IDA-1 and LoVo-IDA-2 expressed levels of MDR1 mRNA approx. 30 times higher than those exhibited by LoVo sensitive cells. The increase in MDR1 mRNA expression was associated with MDR1 gene amplification (six copies for aploid genomes). LoVo-IDA-1,2 resistant clones showed a typical MDR phenotype: they were cross-resistant to DAU, doxorubicin, vincristine, actinomycin-D, etoposide, and teniposide but not to cis-platinum or methotrexate. In Table 1, LoVo-IDA-1,2 clones are compared with MDR LoVo-DOX-1,2 cell lines for

Table 1. Drug resistance, MDR1 mRNA expression levels and MDR1 gene copy number in LoVo-IDA-1,2 and LoVo-DOX-1,2 cell lines

Cell line	LoVo	LoVo-IDA-1	LoVo-IDA-2	LoVo-DOX-1	LoVo-DOX-2
Relative resistance to:*					
Idarubicin	1 (2.05 ± 0.5)†	23	20	9	10
Daunorubicin	1 (11.2 ± 2.3)	101	112	45	40
Doxorubicin	1 (21.4 ± 6.0)	97	94	59	53
Vincristine	1 (38.4 ± 5.3)	104	130	49	52
Actinomycin-D	1 (10.3 ± 2.1)	82	78	44	42
Etoposide	1 (30.2 ± 1.4)	74	80	52	50
Teniposide	1 (44.9 ± 2.4)	56	57	36	31
Cis-platinum	1 (480 ± 75)	0.7	0.8	0.9	0.8
Methotrexate	1 (49.1 ± 7)	0.9	0.8	1.1	1.0
MDR1 mRNA‡	1	32	27	18	15
MDR1 gene copy number	1	6	6	5	4

* Relative resistance is expressed as the ratio between the IC_{50}ext of the cell lines considered and the IC_{50}ext of the parent cell line. Cells were exposed to drugs for 24 hr. Data were obtained from the mean of at least three independent experiments.

† Value in brackets is the IC_{50}ext (ng/mL).

‡ MDR1 mRNA is expressed in arbitrary units.

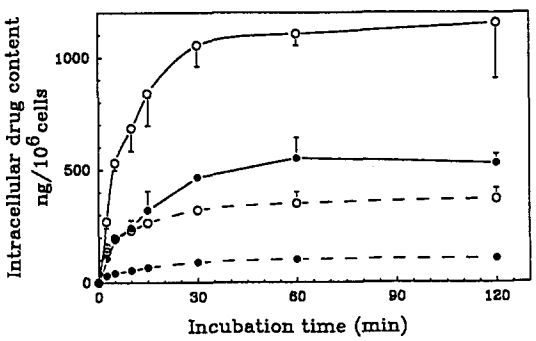

Fig. 1. Intracellular accumulation of IDA and DAU plotted against time in LoVo-sensitive (dark line) and LoVo-IDA-1 resistant cells (dotted line). Cells were incubated with 3000 ng/mL of IDA and DAU. Accumulation is expressed as the amount of drug in 10×10^6 cells (○—○), IDA; (●—●) DAU. Data obtained from three experiments. Bars, SD.

MDR1 gene product expression and RIext. LoVo-DOX-1,2 cell lines were obtained by prolonged exposure (more than 24 months) to 100 ng/mL DOX. Despite the drug used for selection, MDR cell lines were less resistant to IDA than to DAU (Table 1).

Idarubicin and daunorubicin transmembrane transport

Parent LoVo and MDR cells (LoVo-IDA-1 and LoVo-DOX-1 cell lines) were analysed for transmembrane equilibria of IDA and DAU. Intracellular anthracycline accumulations were determined after 1 hr of continuous cell exposure to the drugs, when the steady state in transmembrane drug equilibria was completely established (Fig. 1). After exposure to extracellular drug equi-concentrations, IDA intracellular content was greater than that of DAU in both sensitive and resistant cells. However, both IDA and DAU uptakes were decreased in resistant cells as compared to the parent sensitive cells ($P < 0.01$) (Table 2). The relative decrease in IDA uptake, as determined by the ratio between IDA uptake in sensitive and resistant cells, was similar (P = NS) to the relative decrease in DAU uptake between resistant and sensitive cells (Table 2). Efflux kinetic analysis showed that the decrease in intracellular accumulations was due to an increased drug efflux (Fig. 2). However, according to the data on drug uptakes the IDA efflux rate was slower than that of DAU, both in sensitive and resistant cells. As a consequence, the ratio between the percentage of IDA retained in resistant and sensitive cells at specific times during efflux kinetics was similar (P = NS) to that observed for DAU (Fig. 2).

Treatment of LoVo-IDA-1 and LoVo-DOX-1 cells with VER (10 μM) showed an increased cytotoxic effect of the 2 anthracyclines (see Tables 3 and 4). Verapamil increased IDA and DAU uptake by reducing drug efflux (Fig. 3). Moreover, the relative increase of IDA uptake after VER exposure compared to IDA uptake in the absence of VER was not significantly (P = NS) different from the relative increase in DAU uptake in the presence of VER compared to DAU uptake in the absence of VER (Table 2).

Inhibitory effect of IDA, and DAU on [^{3}H]azidopine photolabeling of P-gp

Photolabeling of proteins in intact LoVo and LoVo-IDA-1 cells with [^{3}H]azidopine and the effect of IDA and DAU on labeling is shown in Fig. 4. Doxorubicin was used as internal control. Doxorubicin is less affected than IDA and DAU by P-gp activity in LoVo-IDA-1 and LoVo-DOX-1 cells: the ratio between DOX uptake in sensitive and resistant cells is lower than that of IDA or DAU (data not shown).

Table 2. Intracellular accumulation of IDA and DAU after 1-hr exposure to the same extracellular drug concentration in the presence or absence of 10 μM verapamil

Drug	[ng/mL]* ext	LoVo			LoVo-IDA-1			LoVo-DOX-1		
		−VER†	+VER‡	Increase§	−VER	+VER	Increase	−VER	+VER	Increase
IDA	300	106 ± 19	110 ± 10	1.0	26 ± 5 (4.1)‖	87 ± 9	3.3	37 ± 5 (2.9)	86 ± 6	2.3
IDA	1000	302 ± 17	341 ± 25	1.1	72 ± 4 (4.4)	272 ± 47	3.8	123 ± 22 (2.8)	292 ± 51	2.4
IDA	3000	1164 ± 168	1326 ± 112	1.1	304 ± 24 (3.8)	969 ± 131	3.2	398 ± 38 (3.1)	887 ± 134	2.2
DAU	300	55 ± 8	69 ± 11	1.3	14 ± 2 (3.7)	38 ± 7	2.7	20 ± 4 (2.8)	38 ± 3	1.9
DAU	1000	177 ± 21	192 ± 23	1.1	48 ± 10 (3.8)	156 ± 20	3.3	76 ± 13 (2.7)	152 ± 12	2.0
DAU	3000	531 ± 70	611 ± 104	1.1	98 ± 20 (5.4)	340 ± 43	3.5	173 ± 28 (3.3)	377 ± 44	2.2

* Extracellular drug concentration.
† Intracellular drug concentration expressed as ng/10^6 cells in the absence or presence of 10 μM VER (‡).
§ Ratio between ‡ and †.
‖ In brackets relative decrease in IDA and DAU uptake in resistant cells as determined by the ratio between drug uptake in sensitive and resistant cells.

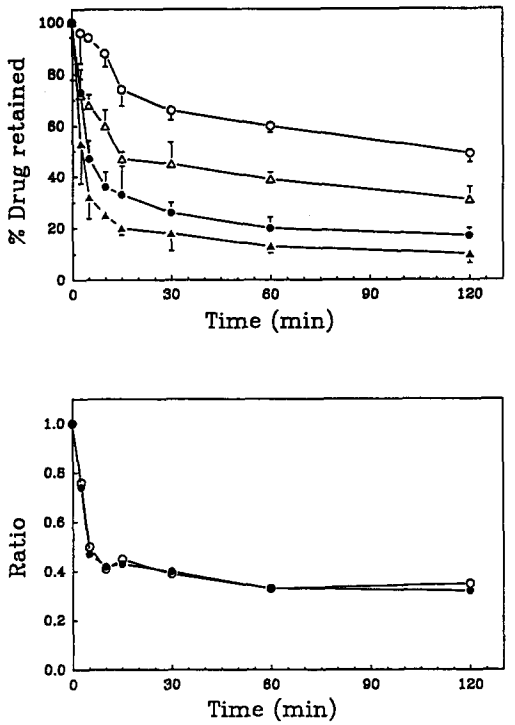

Fig. 2. (Top) Retention of IDA and DAU in LoVo-sensitive (△—△, DAU; ○—○, IDA) and in LoVo-IDA-1 (▲—▲, DAU; ●—●, IDA) cells. Cells were incubated with 3000 ng/mL of IDA and DAU, respectively, for 1 hr. They were then washed and cultured in drug-free medium for the times indicated. Retention of drug is expressed as the percentage of drug retained as compared to time 0. Data obtained from triplicate experiments. Bars, SD. (Bottom) ratio between the percentage of drug retained in resistant and sensitive cells (●—●, DAU; ○—○, IDA).

In LoVo-IDA-1 cells, a distinct M_r 170,000 band at the position of P-gp was labeled with 0.4 μM [^{3}H]-azidopine (Fig. 4). The specific labeling of P-gp by [^{3}H]azidopine was inhibited by the anthracyclines in a dose-dependent manner when the cells were preincubated for 30 min. In LoVo-IDA-1 cells, the inhibitory effect was similar for IDA and DAU. Forty micromolar IDA and 40 μM DAU resulted in 57 and 50% labeling inhibition, respectively, whereas 40 μM DOX inhibited [^{3}H]azidopine photolabeling by approx. 18%, indicating that DOX has less affinity than IDA and DAU for P-gp, as suggested by experimental data on DOX uptake.

Intracellular equitoxic anthracycline contents

The relationship between intracellular concentrations of IDA and DAU, and the corresponding cytotoxic effect, was analysed in LoVo-IDA-1,2 and LoVo-DOX-1,2 resistant cells. As shown in Table 3, all the resistant cell lines tolerated higher intracellular contents of both anthracyclines compared to LoVo sensitive cells. IDA intracellular content inhibiting cell growth by 50% (IC_{50}int) was 3.2 and 3.6 times higher in LoVo-IDA-1 and LoVo-IDA-2 cells, respectively, than in LoVo parental

Table 3. Extracellular and intracellular IDA and DAU concentrations inhibiting cell growth by 50% (IC_{50}int) in LoVo sensitive and resistant cell lines

Cell line	IC_{50}ext (ng/mL)*		RIext†		IC_{50}int (ng/10^6 cells)		RIint‡	
	IDA	DAU	IDA	DAU	IDA	DAU	IDA	DAU
LoVo	10.4 ± 1.3	47.8 ± 3.5	1	1	5.5 ± 0.9	11.6 ± 2.4	1.0	1.0
LoVo-IDA-1	239.2 ± 25.7	5216.7 ± 1056	23	109	19.9 ± 1.3	161.2 ± 22.7	3.6	13.9
LoVo-IDA-2	217.4 ± 17.8	5788.3 ± 521	21	121	17.4 ± 1.5	169.0 ± 31.7	3.2	14.6
LoVo-DOX-1	106.1 ± 20.8	1709.4 ± 398	10	36	12.2 ± 2.7	74.2 ± 15.1	2.2	6.4
LoVo-DOX-2	116.5 ± 11.0	1514.7 ± 203	11	32	12.6 ± 1.6	71.3 ± 8.4	2.3	6.2

* Cell exposure was 1 hr. Data obtained from at least three independent experiments.
† IC_{50} ext resistant cells/IC_{50}ext sensitive cells.
‡ IC_{50} int resistant cells/IC_{50} int sensitive cells.

Table 4. Extracellular and intracellular concentrations of IDA and DAU inhibiting cell growth by 50% after verapamil exposure

Cell line	IDA			DAU		
	IC_{50}ext*	IC_{50}int†	RIint‡	IC_{50}ext	IC_{50}int	RIint
LoVo	9.8 ± 2.8	4.1 ± 1.6	1.0	38.6 ± 6.7	13.5 ± 2.4	1.0
LoVo-IDA-1	47.7 ± 2.0	14.5 ± 3.7	3.5	340.0 ± 53.6	27.9 ± 4.4	2.1
LoVo-IDA-2	42.6 ± 5.1	15.3 ± 4.1	3.7	362.3 ± 21.9	31.8 ± 3.5	2.4

* Extracellular drug concentration (ng/mL) inhibiting cell growth by 50%.
† Corresponding intracellular content inhibiting cell growth by 50% (expressed as ng-drug/10^6 cells).
‡ Ratio between IC_{50} int in resistant and sensitive cells. VER treatment was performed for 1 hr during exposure to the antineoplastic drug and prolonged 24 hr after.

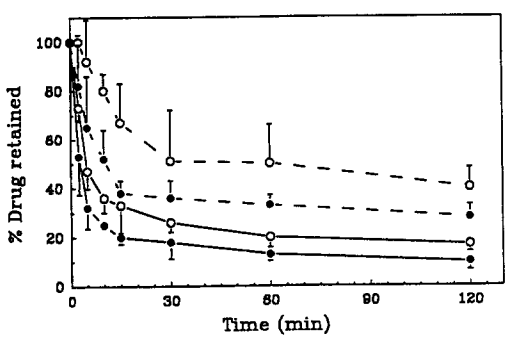

Fig. 3. Efflux of IDA and DAU from LoVo-IDA-1 resistant cells in drug medium with or without VER. Cells were incubated for 1 hr with 3000 ng/mL DAU or IDA in the presence or absence of 10 μM VER. They were then washed with saline containing or not containing VER and were cultured in medium with or without VER. Retention of drug is expressed as the percentage of drug retained as compared to time 0. Data obtained from at least triplicate experiments. Bars, SD. Cells treated with VER (dotted line) or without VER (dark line). (●—●) DAU; (○—○) IDA.

cells, whereas DAU IC_{50}int was 13.9 and 14.6 times higher in LoVo-IDA-1 and LoVo-IDA-2, respectively. Compared to LoVo sensitive cells, LoVo-DOX-1 and LoVo-DOX-2 cells showed an increase in IDA IC_{50}int of 2.2 and 2.3 times, respectively, whereas DAU IC_{50}int increase was of 6.4 and 6.2. Ten micromolar VER treatment (for 1 hr during cell exposure to the antineoplastic drug and prolonged 24 hr after) had a different effect on IDA and DAU RIint of MDR cell lines. While no significant differences (P = NS) in IDA RIint were observed in the presence or absence of VER, DAU RIint was significantly ($P < 0.01$) reduced after VER treatment (see Tables 3 and 4). In LoVo-IDA-1 cells, DAU RIint was 13.9 in the absence of VER and 2.1 after VER exposure ($P < 0.01$).

IDA and DAU subcellular distribution and N/C fluorescence ratios in sensitive and resistant cells

LoVo-MDR (LoVo-IDA-1,2 and LoVo-DOX -1,2) cells exposed to DAU for 1 hr displayed a predominantly intracytoplasmic drug accumulation. Intracytoplasmic DAU compartmentation was not observed in drug-sensitive LoVo cells where DAU predominantly achieved nuclear location. Competitive inhibition of P-gp cells by VER treatment (10 μM) or by lowering cell temperature

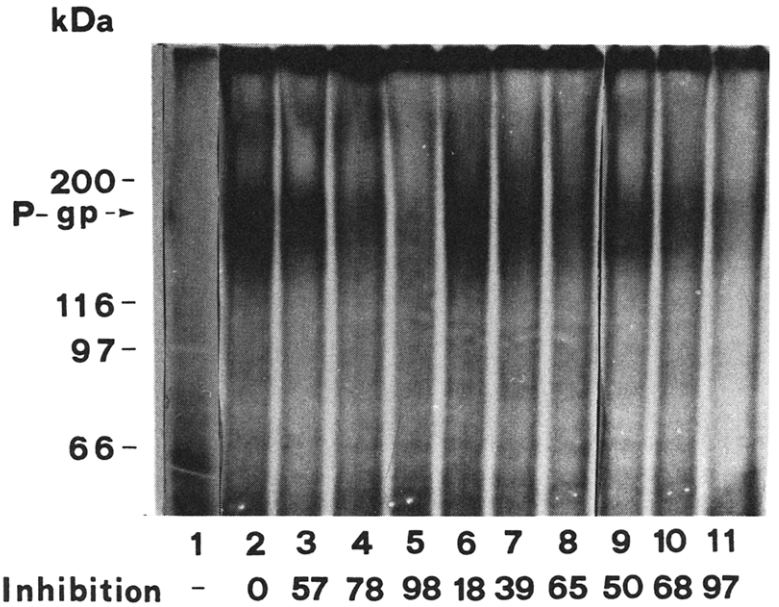

Fig. 4. SDS–PAGE fluorography and inhibitory effect of [^{3}H]azidopine photoaffinity labeled plasma membranes of LoVo (lane 1) and LoVo-IDA (lanes 2–11) cells. Photoaffinity labeling was carried out with 0.4 μM [^{3}H]azidopine using 100 μg of protein in the absence (lane 1 and 2) or presence of 40, 120 and 240 μM IDA (lanes 3–5), doxorubicin (lanes 6–8) and DAU (lanes 9–11), respectively.

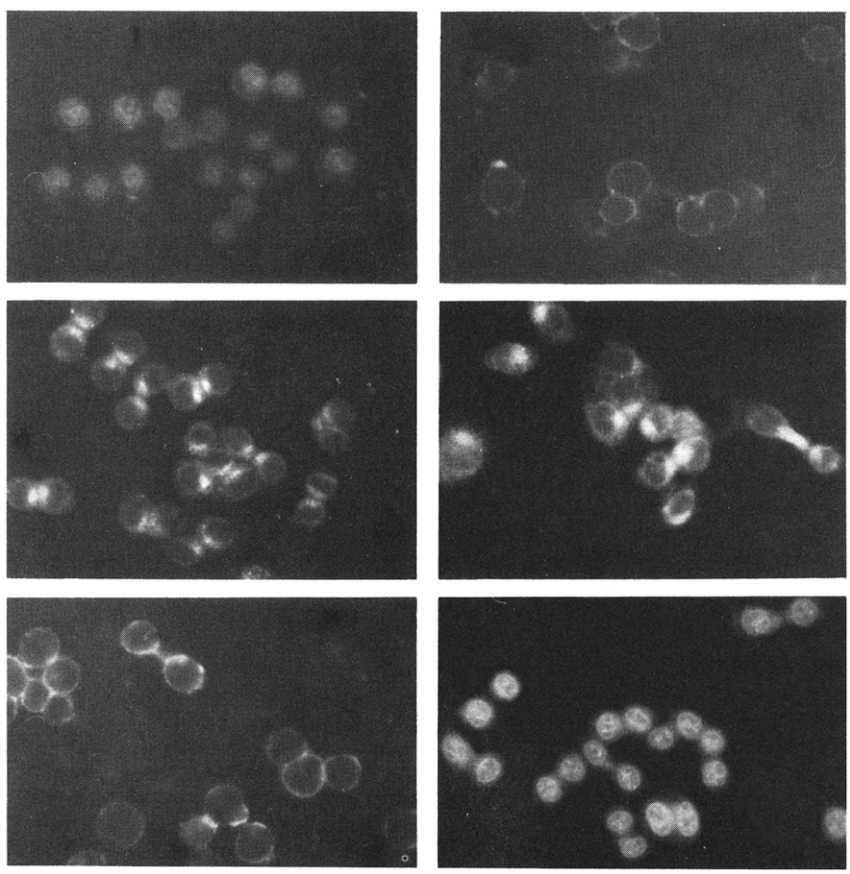

Fig. 5. Subcellular location of IDA and DAU in LoVo sensitive and LoVo-IDA-1 resistant cells. IDA and DAU concentrations used were in all cases five times higher than the IC_{50} ext values. (Top) DAU (left) and IDA (right) distribution in LoVo sensitive cells. (Middle) IDA distribution in resistant cells in the absence (left) or presence of VER (right). (Bottom) DAU distribution in resistant cells in the absence (left) or presence of VER (right).

Table 5. Subcellular idarubicin and daunorubicin distribution in LoVo sensitive and MDR cell lines

Cell line	N/C ratio*			
	IDA		DAU	
	−VER	+VER	−VER	+VER
LoVo	0.19 ± 0.1	0.20 ± 0.10	3.40 ± 2.7	3.7 ± 1.9
LoVo-IDA-1	0.16 ± 0.1	0.19 ± 0.04	0.31 ± 0.2	1.3 ± 0.5
LoVo-DOX-1	0.20 ± 0.1	0.17 ± 0.12	0.41 ± 0.1	1.1 ± 0.3

* N/C ratio was determined at the IC_{50} ext value in LoVo-IDA-1 and LoVo-DOX-1 cells. In sensitive cells, or when VER was used, 1000 ng/mL of IDA or DAU were used since at lower concentrations background noise was too high.

Table 6. P4-unknotting activity in nuclear extract of LoVo sensitive and MDR cell lines

Cell line	Activity* (U/ug)
LoVo	14.1 ± 2.7
LoVo-IDA-1	7.9 ± 1.2†
LoVo-IDA-2	7.1 ± 2.0†
LoVo-DOX-1	9.0 ± 1.2†
LoVo-DOX-2	8.6 ± 1.4†

* The values reported represent the means of a triplicate experiment.

† $P < 0.05$ as compared to sensitive cells.

(4°) allowed DAU to be redistributed in the nucleus of MDR cells. Unlike DAU, IDA localized in the cytoplasm of both LoVo sensitive and LoVo MDR cell lines, with IDA intracellular distribution not being affected by VER treatment (Fig. 5) or lowering of cell temperature (data not shown). In Table 5, N/C IDA ratios and N/C DAU ratios, determined by fluorescence microscopy and image analysis, are shown. The IDA N/C ratio was 0.19 ± 0.1 in LoVo sensitive cells, 0.16 ± 0.1 in LoVo-IDA-1 cells and 0.20 ± 0.1 in LoVo-DOX-1 cells (P = NS, when comparing the N/C ratio in sensitive and resistant cells). The DAU N/C ratio was 3.4 ± 2.7 in LoVo sensitive cells, 0.31 ± 0.1 in LoVo-IDA-1 cells and 0.41 ± 0.2 in LoVo-DOX-1 cells ($P < 0.01$). Simultaneous treatment with 10 μM VER did not significantly affect IDA N/C ratios (P = NS) in either sensitive or resistant cells, whereas VER treatment caused an increase in DAU N/C ratios, which was of 4.2 and 2.7 times in LoVo-IDA-1 and LoVo-DOX-1 cells, respectively ($P < 0.01$) (Table 5).

Topoisomerase II activity in nuclear extracts

Topoisomerase II activity present in nuclear extracts of LoVo and LoVo-DOX-1,2 and LoVo-IDA-1,2 cells was determined by using the P4 DNA-unknotting assay. Results are reported in Table 6. MDR sublines exhibited a significant ($P < 0.05$) reduction in enzymatic activity (about 40% in LoVo-DOX-1,2 cells and about 50% in LoVo-IDA-1,2 cells) compared to the parent cell line.

DISCUSSION

Our data indicate that IDA is a drug involved in MDR. In fact, all the resistant clones obtained by continuous exposure of the LoVo human colon carcinoma cell line to IDA exhibited an increase in expression of MDR1 gene products. Despite IDA selection, LoVo-IDA-1,2 resistant clones were less resistant to IDA than to DAU or to drugs included in the MDR phenotype. This pattern of resistance was also observed in other LoVo MDR cell lines selected with doxorubicin (LoVo-DOX-1,2). These data strongly agree with previous reports [1, 2, 15, 16] and indicate that such a phenomenon is common in MDR cells and independent of the anthracycline used for the selection of MDR mutants. It has previously been supposed that the greater efficacy in MDR cells of IDA compared to DAU might be due to specific physico-chemical characteristics of IDA, especially its high lipophilic coefficient, which allows MDR cells to reach IDA intracellular accumulations higher than those of DAU when exposed to equimolecular extracellular drug concentrations [1, 2, 16, 17]. Nevertheless we believe that such a difference in drug accumulation is not the main factor in determining the variations in resistance index that LoVo MDR cell lines displayed for both anthracyclines. In fact, in LoVo sensitive cells, intracellular drug accumulations were greater for IDA than DAU and the ratio between IDA and DAU uptakes was very similar to that observed in LoVo MDR cells. Increased intracellular drug accumulation due to the hydrophobic characteristics of IDA could determine variations in the relative potency (expressed as the ratio between IDA IC_{50}ext and DAU IC_{50}ext), rather than have a selective effect on P-gp activity. In both LoVo sensitive and LoVo MDR cells the IC_{50}ext of IDA was lower than that of DAU, indicating that IDA has greater potency. Conversely, azidopine-binding experiments showed that there were no differences between IDA and DAU in the inhibition of P-gp photolabeling, thus excluding the possibility that variations in anthracycline lipophilicity could alter their affinity

for P-gp, as previously suggested by Friche *et al.* [27].

Reduction of drug incorporation has often been considered as the primary cause of MDR [4, 5]. In both LoVo-IDA-1 and LoVo-DOX-1 cell lines, resistance to IDA and DAU was accompanied by an active efflux of these drugs out of the cells, resulting in a reduced drug intracellular level. However, from our experimental models it clearly appears that reduction of drug incorporation by itself cannot explain resistance since MDR cells require much higher intracellular levels of both anthracyclines than sensitive cells for the same growth inhibition effect. In LoVo-IDA-1,2 cells, RIint was approx. 14 and 3.5 for DAU and IDA, respectively, and in LoVo-DOX the RIint was approx. 6 and 2 for DAU and IDA, respectively. These results are in favor of the development of mechanisms in resistant cells acting at the intracellular level, supplementary to the reduced drug incorporation.

It is worth considering whether biochemical mechanisms acting at the intracellular level affect intracellular resistance to DAU and IDA differently. In fact, LoVo MDR cells showed a lower RIint for IDA than for DAU. In contrast, the P-gp localized at the transmembrane level affected IDA uptake as much as DAU uptake: (a) ratios between IDA uptakes in sensitive and resistant cells were not significantly (P = NS) different from the ratios of DAU uptakes in the parent and resistant cell line; and (b) inhibition of P-gp activity by VER treatment had a similar effect on DAU and IDA uptakes. These data suggest that the reduced cross-resistance to IDA compared to DAU displayed by MDR cells is due to a differential effect of the biochemical mechanisms acting at intracellular rather than at transmembrane levels. It is possible that biochemical mechanisms acting at the transmembrane and intracellular levels act synergistically, resulting in the wide differences in IDA RIext compared to DAU RIext showed by MDR cells.

Several explanations could account for the increased intracellular tolerance towards anthracyclines and multiple resistance factors could be involved in MDR: (a) differences in intracellular drug metabolism [28]; (b) abnormal interaction with the ultimate target, especially with topoisomerase II [29]; (c) decreased ability to activate the drugs to reactive semiquinone-free radical species [30]; and (d) abnormal cytoplasmic-drug compartmentation that prevents anthracyclines from reaching the nucleus [7, 9, 19]. Recent evidence indicates that in MDR cells overexpressing P-gp, changes in subcellular drug distribution may have a crucial role in resistance to several anthracyclines [9, 19]. In LoVo MDR cells DAU was predominantly localized in the cytoplasm, whereas it was mainly distributed in the nucleus in sensitive parent cells; DAU N/C ratios were lower in resistant cells. Determination of N/C ratios could be inaccurate, especially at low intracellular drug contents: background noise was too high, part of the cytoplasmic fluorescence overlapped the nuclear fluorescence, and cytoplasmic and nuclear fluorescence quenching could occur if nuclear anthracyclines intercalated into the DNA strands, resulting in fluorescence quenching. Nevertheless, DAU N/C ratio analysis suggested that resistant cells required higher intracellular DAU contents than sensitive cells in order to approach the same nuclear contents, as recently suggested by Schuurhuis *et al.* [19]. After treatment of MDR cells with 10 μM VER, DAU was partially redistributed in the nucleus and intracellular DAU resistance reverted. This indicates that the shift of DAU from the nucleus to the cytoplasm is the major biochemical phenomenon responsible for intracellular resistance of MDR cells to DAU.

In contrast to DAU, IDA intracellular resistance was not related to a different N/C drug ratio between sensitive and resistant cells. IDA was distributed predominantly in the cytoplasm of both MDR cells (LoVo-IDA-1,2 and LoVo-DOX-1,2) and LoVo sensitive cells. Therefore, IDA intranuclear accumulation was the same function of IDA intracytoplasmic concentration in both sensitive and resistant cells. VER treatment increased drug uptake but did not significantly affect the IDA N/C ratio; consequently, in LoVo MDR cells, the IC_{50}int in the presence of VER was not different from the IC_{50}int in the absence of VER.

Intracellular compartmentation of anthracyclines might depend on drug- physico-chemical peculiarities [31] (especially hydrophobicity [32], net charge [33], and steric configuration [34]) and/or specific characteristics of the cells, (an increase in the vesicular compartment [35], an increase in cytoplasmic pH [36] and more importantly, an increased expression of P-gp localized in subcellular structures such as membranes of cytosolic vesicles [9, 37]). Chemico-physical peculiarities, such as hydrophobicity, might confer to IDA a relatively higher affinity for cytoplasmic structures than for nuclear ones, whereas the opposite occurs with DAU. P-gp localized in subcellular structures significantly affects intracellular compartmentation of anthracyclines, such as DAU, which localized mainly in the nucleus. In contrast, the high lipophilicity of IDA determines an intracellular cytoplasmic distribution of this drug that makes the activity of intracellular P-gp useless.

The precise mechanism responsible for the increased intracellular resistance to IDA exhibited by LoVo MDR cells is not known at present. Capranico *et al.* [38] have previously reported that topoisomerase II could be involved in increased intracellular resistance to IDA. IDA has a much stronger activity than DAU in inducing DNA strand breaks [16, 38]. This may also account for the lower intracellular nuclear contents of IDA required to have the same cytotoxic effect of DAU [16, 38]. It is interesting to note that in our LoVo MDR cells topoisomerase II enzymatic activity was reduced as compared to the LoVo parent cell line; moreover, it is well known that resistance to the cytotoxic effects of topoisomerase II inhibition depends on the low levels of topoisomerase II activity [39]. It is likely that in LoVo-IDA and LoVo-DAU cell lines drug resistance is not due to "pure" MDR1 mRNA expression. Topoisomerase II could account, to various degrees, for intracellular tolerance towards IDA and DAU; in the case of DAU it could be marginal, while it could play a major role in IDA intracellular resistance [40].

In summary, this report suggests that the reduced resistance displayed by MDR cell lines to IDA compared to DAU may be due to the differential N/C distribution of this drug. This allows cells with the MDR phenotype to tolerate intracellular levels of IDA relatively lower than those of DAU, as compared to sensitive cells. Therefore, IDA is a useful cytostatic agent with MDR cells.

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