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INTRACELLULAR DOXORUBICIN CONCENTRATIONS AND DRUG-INDUCED DNA DAMAGE IN A HUMAN COLON ADENOCARCINOMA CELL LINE AND IN A DRUG-RESISTANT SUBLINE*

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Abstract—The mechanisms of resistance to doxorubicin (DX) were investigated using a human colon adenocarcinoma cell line (LoVo) and a subline approximately 30 times less sensitive to doxorubicin. LoVo and LoVo/DX were similar in terms of DNA and protein content, cell volume, duration of S phase and the generation time, and proportion of cycling cells. LoVo/DX showed cross-resistance to other anthracyclines, to vinca alkaloids, epipodophyllotoxin derivatives, 4'-(9-acridinylamino-methanesulfon-*m*-aniside) and actinomycin D. LoVo/DX was equally sensitive to melphalan and showed collateral sensitivity to *cis*-platinum and 1-β-D-arabinofuranosylcytosine.

On exposing LoVo and LoVo/DX to 1.25 and 40 μg/ml DX respectively, for 4 hr, similar DX intracellular concentrations were reached in the two cell lines. In these treatment conditions protein associated DNA-single strand breaks or DNA-double strand breaks, assessed by alkaline elution methods were only slightly less in LoVo/DX than in LoVo cells. In LoVo/DX cells, however, DNA breaks disappeared very quickly after drug removal whereas they persisted longer in LoVo cells. This persistance is probably related to the much slower DX efflux from LoVo than LoVo/DX. When verapamil was combined with DX it inhibited the rapid DX efflux from LoVo/DX and reversed the resistance in this cell line, but it had no significant activity on LoVo cells. Verapamil also increased DX-induced DNA-single strand breaks and DNA-double strand breaks in LoVo/DX cells, but not in LoVo cells.

DX‖ is one of the most effective drugs for the therapy of several human malignancies [1]. Its mode of action is still debated, but recently the hypothesis that this intercalator inhibits DNA topoisomerase II by stabilizing a DNA-topoisomerase II complex (which is cleavable upon treatment with denaturants or digestion with proteinase K) has gained ground over other proposed mechanisms [2, 3].

As for many other effective anticancer agents, DX's clinical efficacy is limited by the inherent or acquired drug resistance frequent in cancer cells [4, 5]. A better knowledge of the molecular basis of drug resistance is needed to find methods to counteract it or to design new, non-cross-resistant drugs.

A number of studies on several cell types, mostly of murine origin [6–10] indicate that cells selected for resistance to anthracyclines (after prolonged exposure to increasing DX concentrations) are also resistant to other compounds such as vinca alkaloids, epipodophyllotoxins and m-AMSA. It has been proposed that this "pleiotropic" resistance to drugs with different chemical structures, which in some cases act presumably by different mechanisms, is due to decreased intracellular drug retention [11–14]. On the other hand in a Chinese hamster ovary cell subline resistant to epipodophyllotoxin derivatives or to other topoisomerase II inhibitors qualitative or quantitative changes in topoisomerase II (or some as yet unidentified modulating factor) has been recently suggested [15, 16].

Using a human adenocarcinoma cell line (LoVo) and a subline recently selected in our laboratories for resistance to DX (LoVo/DX) [17], we studied the cytotoxicity of DX and other structurally related and unrelated drugs, intracellular DX concentrations and drug-induced DNA damage. The different sensitivity to DX of the two cell lines appears mainly related to differences in drug efflux which is much more efficient in LoVo/DX.

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‖ Abbreviations used: DX, doxorubicin; DNR, daunorubicin; m-AMSA, 4'-(9-acridinylamino) methanesulfon-m-aniside; Ara-C, 1-β-D-arabino-furanosylcytosine; DMSO, dimethylsulfoxide; VP-16, etoposide; PBS, phosphate buffered saline; PI, propidium iodide; FITC, fluorescine isothiocyanate; T_D, doubling time; GF, growth fraction; BrdU, bromodeoxyuridine; T_{G1} duration of G1; T_S, duration of S; T_{G2M}, duration of G2M; TC, duration of a cell cycle; SDS, sodium dodecyl sulfate; DNA-SSB, DNA-single strand breaks; DPC, DNA-protein cross links; DNA-DSB, DNA-double strand breaks.

MATERIALS AND METHODS

Cell culture. The DX-resistant LoVo cell line (referred as LoVo/DX) was derived from the human colon adenocarcinoma cell line LoVo [18] as previously described [17]. Monolayer cultures of LoVo/DX were maintained in Ham's F12 medium (Grand Island Biological Company, Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories), 1% vitamins (BME vitamin solution, 100×), 1% glutamine (200 mM), 100 U/ml penicillin and 100 μg/ml streptomycin.

For LoVo/DX, 100 ng/ml DX were added at every passage. Both cell lines were passaged twice a week and maintained at 37° in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay. The drug concentrations required for 50% inhibition of colony formation were determined using a single-cell plating technique. Exponentially growing cultures were dispersed with 0.25% trypsin (Flow Laboratories) to produce a single-cell suspension; 400 cells/dish (Falcon, 36 mmØ) were seeded 24 hr or 48 hr before treatment, and cells were exposed to drugs by replacing growth medium with drug-containing medium. After 4 or 24 hr, medium was withdrawn, cells were rinsed with saline, and fresh growth medium was added; colonies containing 50 cells/colony were counted under an inverted microscope after 8–10 days incubation at 37° in 5% CO_2.

The antiproliferative activity of the drugs was calculated from dose–response curves and expressed as ID_{50} (dose causing 50% inhibition of growth of colonies in treated cultures relative to untreated controls).

Intracellular content and efflux. Exponentially growing cells were seeded at the concentration of 3×10^5 cells/ml into 60 mm ∅ plastic dishes (Falcon, 4 ml/dish). After incubation for 48 hr at 37° in 5% CO_2, they were exposed to varying concentrations of DX and daunorubicin (DNR) and incubated another 4 hr at 37° in 5% CO_2; then medium was withdrawn and cells were quickly washed twice with ice-cold saline. For evaluation of drug content at different times during treatment, cells were harvested with a few drops of 0.25% trypsin at room temperature, suspended in 3 ml ice-cold saline, collected by low-speed centrifugation at 4° and resuspended in 1 ml of a 1:1 mixture of ethanol: 0.3 N HCl for drug extraction. For evaluation of drug efflux, after washing with ice-cold saline fresh growth medium was added to cells, which were further incubated at 37° for different intervals, then processed as reported.

Intracellular drug content was determined by fluorescence spectrophotometry at excitation and emission wavelengths of respectively 479 and 593 nm and is reported as ng DX or DNR $\times 10^{-6}$ cells. Each sample was run in triplicate. Cell number was determined with a Coulter Counter [Kontron (Mod. Z M)] on an aliquot of the cell suspension before centrifugation.

Drugs. Doxorubicin and Daunorubicin were from Farmitalia Carlo Erba, Milan, Italy. Mitomycin C, vincristine sulphate and *cis*-platinum were pharmaceutical preparations obtained respectively from Kyowa Hakko, Tokyo, Japan; Eli Lilly, Indianapolis, U.S.A., and Bristol Myers, New Haven, U.S.A.; VP-16 was from Bristol Myers; m-AMSA was obtained from DTP, NCI, Bethesda, U.S.A. Actinomycin D, Ara-C and melphalan were from Sigma Chemical Co., St. Louis, MO. Verapamil (Isoptin) was purchased from Knoll AG, Liestal, Switzerland.

Compounds were dissolved at the concentration of 0.1 mg/ml in water (DX, mitomycin C, Ara-C and vincristine) or in 10% DMSO and water (actinomycin D, aclacinomycin A, VP-16 and m-AMSA) or in 0.01 N HCl (melphalan). Further dilutions were made in growth medium.

Flow cytometry and cell counts. At 0, 16, 24, 48 and 90 hr of recovery after DX treatment triplicate culture plates were harvested using 0.25% trypsin (Eurobio, Paris) in Dulbecco's PBS without Ca^{2+} and Mg^{2+}, containing 0.5 mM EDTA, and centrifuged with the supernatant medium. For counting, an aliquot of each sample was separated before centrifugation and analysed using a Coulter counter (Coulter Electronics Ltd., Harpenden, Herts, U.K.) equipped with a cell analyzer (Coulter Channalyzer C-1000) for size measurements.

DNA was stained with propidium iodide (PI) (Calbiochem Behring Co., San Diego, CA, U.S.A.) by adding to the cell pellet 2 ml of PI solution (50 μg/ml in 0.1% sodium citrate), 30 μl 1% Nonidet P40 detergent (Sigma Chemical Co., St Louis, MO.) and 30 μl of RNAse (Calbiochem) 0.5 mg/ml stock solution. Flow cytometric analysis was performed using a 30 L cytofluorograph (Ortho Instruments, U.S.A.). Percentages of cells in cell cycle phases were computed using a "sum of Gaussians" method as previously described [19]. Triplicate control plates were processed the same way at the same times.

After cells were fixed in 70% ethanol in PBS + 0.5 mM EDTA, DNA and proteins were stained by adding to the cell pellet a cocktail (1 ml per 10^6 cells) containing 50 μg/ml of PI, 0.1 μg/ml FITC (Sigma) and 7.5 μg/ml RNAse in PBS. Biparametric analysis was done with a FACS IV (Becton Dickinson, Sunnyvale, CA) fitted with an HP-85 microcomputer (Hewlett–Packard Co., Corvallis Co., Corvallis, OR) for data display and storage.

Kinetic measurements. The exponential phase of the growth curve was determined in a preliminary experiment, whose results were used to select the time for beginning treatment and the time points to be fitted in order to calculate the T_D. For computing the GF, the flow cytometric technique of double staining with PI and fluorescinated anti-BrdU antibody (Anti-BrdU) [20] was adopted, after continuous exposure to BrdU. The fraction of cycling cells was calculated by the difference from the number of (resting) cells that do not incorporate BrdU during an interval (30 hr) longer than the doubling time.

On the basis of the GF and T_D, and of %G1, %S and %G2M during exponential growth, the durations of the cell cycle phases (T_{G1}, T_S and T_{G2M}) can be estimated according to Dosik *et al.* [21].

Alkaline elution. DNA-SSB, DNA-DSB and DPC were quantitated by alkaline elution [22]. Cells were

seeded at a concentration of 5×10^4/ml and incubated for 24 hr. Then they were labelled for 24 hr with [^{3}H]-thymidine (specific activity 20 Ci/mmol; New England Nuclear) at a concentration of 0.1 μCi/ml in a medium containing 10^{-6} M unlabeled thymidine. Before treatment, postlabeling chasing was performed in medium without [^{3}H]-thymidine for 16–24 hr. After 4 hr treatment or at different intervals post-incubation, approximately 10^6 cells were resuspended in PBS and layered on polycarbonate filters, 0.8 μm pore size and 25 mm diameter (Nucleopore Corp., Pleasanton, CA). Cells were then lysed with a solution containing 2% SDS, 0.02 M EDTA, 0.1 M glycine, pH 10.0 (lysis solution), which was allowed to flow through the filter by gravity.

The outlet of the filter holders was connected to the pumping system and 2 ml of 0.5 mg/ml proteinase K (EM Laboratories, Darmstadt, F.R.G.), dissolved in the lysis solution were added to reservoir over the filters and pumped for approximately 1 hr at 0.35 ml/min. DNA was eluted from the filters by pumping 20 mM EDTA solution adjusted to pH 12.1 with tetrapropylammonium hydroxide (Fluka, F.R.G.) containing 0.1% SDS through the filters at approximately 2 ml/hr. Three-hour fractions were collected and processed as described [22].

For the DPC assays, cells were layered on Metricel Membrane filters (Gelman Sciences Inc., Ann Arbor, MI, U.S.A.) 0.8 μm pore size and 25 mm diameter and then lysed with 5 ml of the lysis solution. The detergent was washed away with 10 ml of 20 mM EDTA pH 10.0. The elution buffer was the same as that used for DNA-SSB, except that no SDS was added.

For DNA-DSB assays, cells (300,000–400,000 cells/sample) were lysed with only 2 ml of the lysis solution containing proteinase K and the elution buffer was adjusted to pH 9.6 ('neutral elution').

In initial experiments 300 rads irradiated [^{14}C]-labeled L1210 cells (exposed to 0.01 μCi/ml) of [^{14}C]-thymidine) were added to each sample as internal standard. As the flow rate of each elution channel is virtually the same in our experimental conditions addition of this internal standard did not improve the reproducibility. We therefore did further experiments without the internal standard cells. All data have been plotted as relative retention of [^{3}H]-DNA on filter vs time of elution.

Table 1. Cross resistance of LoVo/DX cells to different drugs

Compound	R.I.*
Doxorubicin	29.4
Daunorubicin	28.8
Vincristine	26.6
VP-16	20.8
m-AMSA	25.0
Actinomycin D	11.6
Aclacinomycin A	3.1
Mitomycin C	2.8
Melphalan	1.0
cis-Platinum	0.3
Ara-C	0.3

* R.I. (Resistance index): ratio between ID_{50} values on LoVo/DX and LoVo after 4 hr exposure.

RESULTS

Cytotoxicity

LoVo/DX cells were approximately 30 times less sensitive to DX than LoVo cells as assessed from the inhibition of colony formation of cells exposed to the drug for 24 hr (Table 1). Cross resistance was found not only for the structurally related analogue daunorubicin but also for other compounds such as m-AMSA, vincristine, VP-16 and actinomycin D. Aclacinomycin A and mitomycin also showed less activity against LoVo/DX than LoVo whereas melphalan was equally effective on both cell lines. Collateral sensitivity was seen for *cis*-platinum and Ara-C which were about three times more effective in LoVo/DX than in LoVo cells.

Studies of reversion of resistance in the presence of verapamil were made after 4 hr treatment. When verapamil (20 μg/ml) was given in combination with DX it markedly potentiated DX's cytotoxic effect in LoVo/DX cells, whereas in LoVo cells there was a mild but statistically insignificant increase of activity (Table 2), when the antiproliferative effect of verapamil was also taken into account (Table 2).

Flow cytometry

LoVo and LoVo/DX cells did not differ in DNA content (DNA index = 1.3) or protein content (Fig. 1). The size of both cell lines, assessed by the mode of size distributions, was also similar, the diameter being 14.9 μm for LoVo cells and 14.2 μm for LoVo/DX cells. Table 3 shows the growth fraction and the duration of each phase of the cell cycle in LoVo and LoVo/DX cells during exponential growth. The growth fraction was 89 and 86% of the total cell populations and the cell cycle phases lasted virtually the same time in the two cell lines.

DX-induced cell cycle perturbations were investigated at different intervals after drug exposure. Figure 2 refers to experiments in which LoVo and LoVo/DX were treated for 4 hr with respectively 1.25 and 40 μg/ml DX which caused proportionally approximately the same growth inhibition in the two cell lines. In both cases DX treatment caused an accumulation of cells in the late phases of the cell cycle. The percentages of cells in G1, S and G2-M were established after DX treatment using a G1/G2-M plan where "iso-S" lines are drawn [19]. In LoVo cells (Fig. 2, panel A) in the first 16 hr post-

Table 2. Cytotoxic activity of doxorubicin on LoVo and LoVo/DX cells

Cell line	Verapamil	ID_{50} (ng/ml)
LoVo	—	52.5 (49.3–56.0)
LoVo	20 μg/ml	36.0 (31.9–41.2)
LoVo/DX	—	1815.0 (1657–2034)
LoVo/DX	20 μg/ml	130.0 (115–152)

LoVo and LoVo/DX cells were treated with DX for 4 hr. Verapamil alone had no cytotoxic effect.

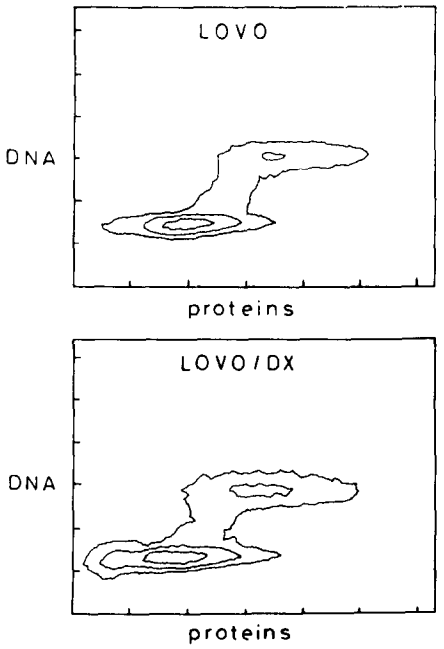

Fig. 1. Comparison of biparametric (DNA and protein content) flow cytometric distributions derived from LoVo (upper panel) and LoVo/DX cell populations. DNA and protein content are expressed in the same (arbitrary) units in the two panels.

Table 3. Kinetic parameters of LoVo and LoVo-DX cell lines

Cell line	GF	Duration of phase (hr)				
		T_D	T_C	T_{G1}	T_S	T_{G2M}
LoVo	0.89	27	25	11	9	5
LoVo/DX	0.86	27	24	11	8	5

Table 4. Intracellular content of doxorubicin in LoVo cells after 4 hr treatment and 1 and 4 hr recovery in drug-free medium

Doxorubicin concentration (ng/ml)	Intracellular drug content ($ng/10^6$ cells)		
	Treatment (4 hr)	Post-treatment incubation in drug-free medium	
		(1 hr)	(4 hr)
75	21 ± 0.8	19 ± 2.6	9 ± 1
150	40 ± 1.7	27 ± 2.8	16 ± 1
625	170 ± 5.0	85 ± 3.0	65 ± 5
1250*	201 ± 21.0	138 ± 30.0	94 ± 31
2500*	454 ± 32.0	293 ± 39.0	168 ± 5

* ± SD calculated from two replicate experiments (three points/experiment). Other values were from three replicate points in a single experiment.

treatment in drug-free medium there was a clear change in the proportion of cells in the different phases of the cell cycle. A decrease of cells in G1 was balanced by an increase in S and, to a lesser extent, in G2-M. Beyond 16 hr (at 24 and 48 hr) the input of (relatively few) newborn cells in G1 equalled the output to S, while slackened S cells progressively reached G2-M and stopped there. After 48 hr the situation was nearly static in all phases with about 25% of G1 cells, 25% of S cells and 50% of G2-M cells.

In LoVo/DX cells (Fig. 2, panel B) a similar cell cycle perturbation was observed after DX treatment, with a larger increase in the percentage of cells in G2-M in the first 16 hr and a lower output of G1 cells. The sweep-out of S cells seemed easier than in the LoVo cells and by 48 hr only 12% of cells remained in S while about 48% were still in G1 or were newborn.

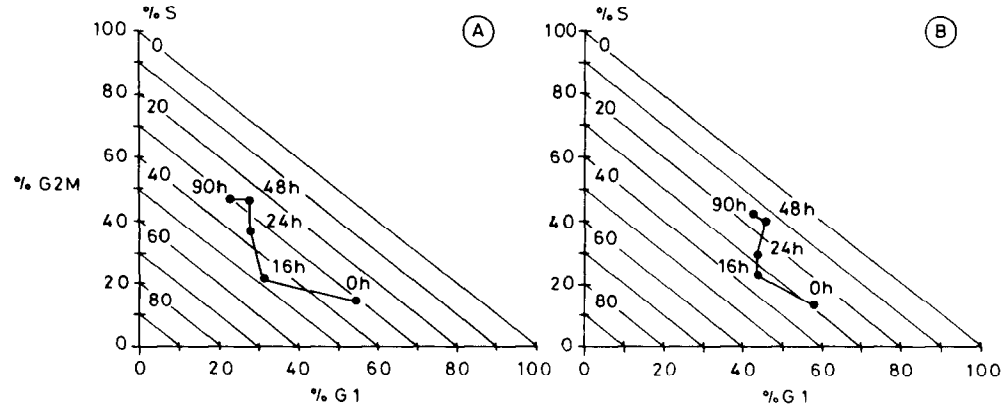

Fig. 2. Percentages of cells in cell cycle phases during post-drug incubation. Data points give simultaneously the percentage of cells in phase G1 (abscissa), in phase G2M (ordinate) and in phase S (by extrapolation using the drawn iso-S straight lines) 0, 16, 24, 48, 90 hr from the end of treatment. Panel A: LoVo cells treated for 4 hr with 1.25 μg/ml DX. Panel B: LoVo/DX cells treated for 4 hr with 40 μg/ml DX.

Table 5. Intracellular content of doxorubicin in LoVo/DX cells after 4 hr treatment and 1 and 4 hr recovery in drug-free medium

Doxorubicin concentration (ng/ml)	Intracellular drug content ($ng/10^6$ cells)		
	Treatment (4 hr)	Post-treatment incubation in drug-free medium	
		(1 hr)	4 hr)
1250†	30.0 ± 7.0	9.1 ± 3.0	8.2 ± 3.0
2500*	44.5 ± 0.7	9.0 ± 1.4	9.5 ± 2.0
10000*	107.0 ± 25.0	12.3 ± 0.5	10.5 ± 2.0
20000†	166.0 ± 39.0	33.7 ± 13.0	29.8 ± 13.0
40000†	257.0 ± 116.0	58.0 ± 32.0	44.6 ± 14.0

* ± SD calculated from two replicate experiments (three points/experiment).

† ± SD calculated from three replicate experiments (three points/experiment).

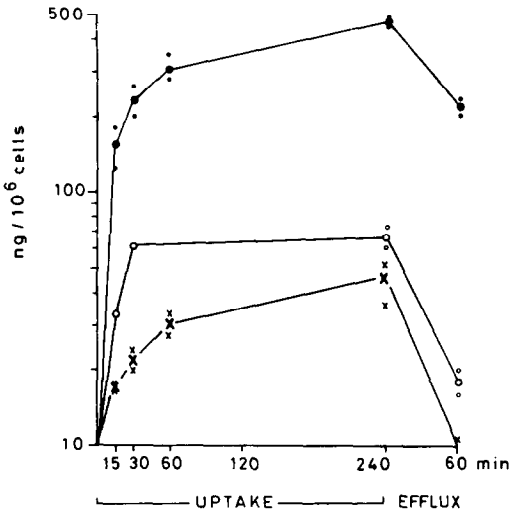

Fig. 4. Uptake and efflux of DNR in LoVo and LoVo/DX cells. ●——●, DNR 2.5 μg/ml in LoVo cells; ×——×, DNR 2.5 μg/ml in LoVo/DX cells; ○——○, DNR 10 μg/ml in LoVo/DX cells; experimental values and mean are shown.

Drug intracellular concentration

Tables 4 and 5 show intracellular DX concentrations in LoVo and LoVo/DX cells after 4 hr exposure and after 1 or 4 hr of post-treatment incubation in drug-free medium. When LoVo and LoVo/DX cells were exposed for 4 hr to the same DX concentrations there were very clear differences in the intracellular drug concentrations. The intracellular DX concentration was similar when LoVo cells were treated for 4 hr with 1.25 μg/ml and LoVo/DX cells with 40 μg/ml.

Figure 3 shows the profiles of uptake and efflux of DX in LoVo and LoVo/DX cells treated with 1.25 and 40 μg/ml. Up to 1 hr DX concentrations were higher in LoVo/DX cells, becoming similar at 2 and 4 hr. The efflux of DX from LoVo/DX cells appeared to be much faster than from LoVo cells. Efflux appeared to be biphasic with half lives of 8.6 and 401 min for LoVo and 5.0 and 111 min for LoVo/DX.

Figure 4 shows that intracellular DNR concentrations were also much higher in LoVo than in LoVo/DX cells exposed for 4 hr to 2.5 μg/ml and to 2.5 or 10 μg/ml, respectively. DNR efflux assessed by determining the drug concentrations 1 hr after drug treatment, was much faster in LoVo/DX than in LoVo. Table 6 shows the influence of verapamil

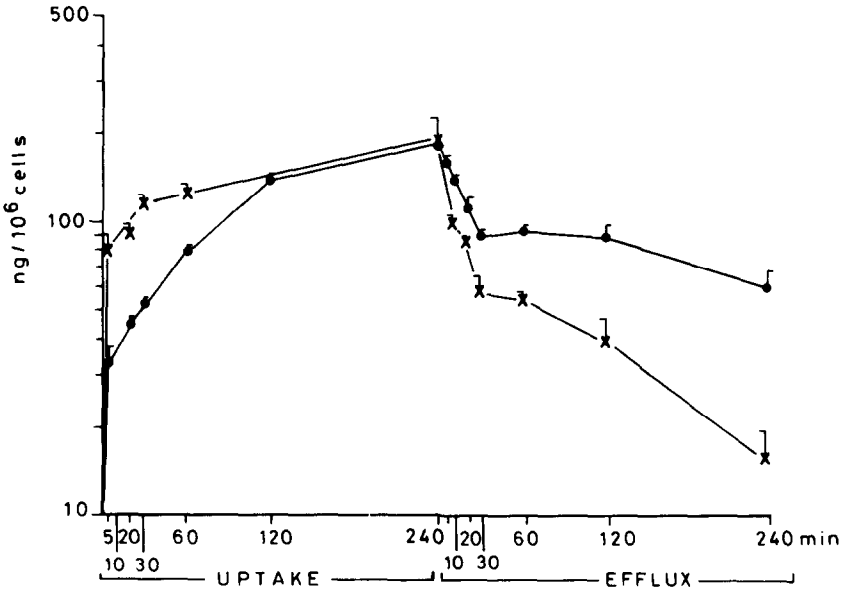

Fig. 3. Uptake and efflux of DX in LoVo and LoVo/DX cells. ●——●, DX 1.25 μg/ml in LoVo cells; ×——×, DX 40 μg/ml in LoVo/DX cells; values are mean ± SD.

Table 6. Intracellular content of doxorubicin in LoVo and LoVo/DX cells in the absence and presence of 20 μg/ml verapamil after 4 hr treatment and 1 hr recovery in drug-free medium

Drug concentration	Intracellular drug content (ng/10^6 cells)		
		1 hr recovery in drug-free medium	
	4 hr treatment	Medium alone	Medium + verapamil 20 μg/ml
LoVo cells			
Doxorubicin 1250 ng/ml	201.0 ± 23.0	124.0 ± 9.0	154.4 ± 8.0
Doxorubicin 1250 ng/ml + verapamil 20 μg/ml	270.6 ± 19.0	194.0 ± 4.0	212.0 ± 3.5
LoVo/DX cells			
Doxorubicin 1250 ng/ml	24.6 ± 1.7	8.5 ± 0.6	18.2 ± 2.0
Doxorubicin 1250 ng/ml + verapamil 20 μg/ml	152.0 ± 13.0	28.0 ± 7.5	143.0 ± 17.0

± SD calculated from two replicate experiments (three points/experiment).

(a calcium antagonist) on intracellular DX concentrations in LoVo/DX cells.

In LoVo cells verapamil caused only a slight rise in DX concentration and during the first hour post-DX incubation it did not modify drug efflux. In LoVo/DX cells verapamil dramatically raised the intracellular DX concentration; by the end of treatment the DX concentration in cells concomitantly treated with verapamil was more than six times that in the same cells exposed only to DX. In addition verapamil clearly prevented the rapid efflux from LoVo/DX cells during the first hour of post-drug incubation.

DNA damage

DX 1.25 μg/ml for 4 hr caused a number of DNA-SSB and DPC in LoVo cells, slightly more than were produced by 40 μg/ml DX in LoVo/DX cells (Figs 5 and 6). In addition the kinetics of repair of DNA

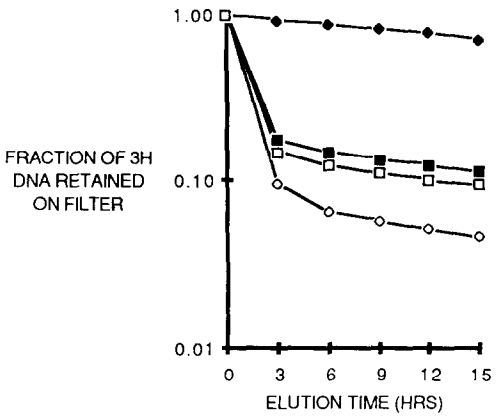

Fig. 6. DX-induced DPC in LoVo (■——■) and LoVo/DX cells (□——□) after 4 hr treatment with 1.25 μg/ml (LoVo) or 40 μg/ml (LoVo/DX). ◆——◆, Controls; ◇——◇, 3000 rads. DPC were determined by the alkaline elution method (see Materials and Methods).

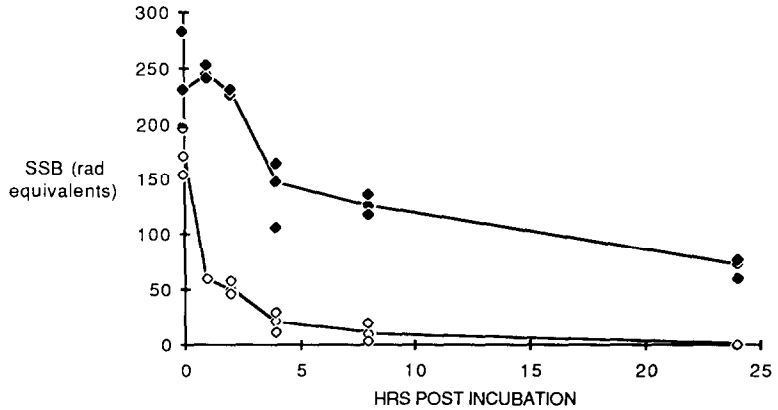

Fig. 5. Kinetics of repair of DX-induced DNA-SSB in LoVo cells (◆——◆) and LoVo/DX cells (◇——◇) after 4 hr treatment with 1.25 μg/ml and 40 μg/ml, respectively. DNA-SSB were determined by the alkaline elution method (see Materials and Methods).

Table 7. DNA-SSB in RAD equivalent induced by doxorubicin

Dose (ng/ml)	Cell line	SSB (RAD equivalent)
75	LoVo	13 (12–14)
1250	LoVo	231 (197–282)
1250 + verapamil	LoVo	220 (200–237)
1250	LoVo/DX	22 (14–36)
1250 + verapamil	LoVo/DX	154 (153–155)
20800	LoVo/DX	82 (65–101)
40000	LoVo/DX	172 (155–196)

LoVo and LoVo/DX cells were treated with DX for 4 hr. Verapamil was added at the concentration of 20 μg/ml during DX treatment. Verapamil alone did not produce any DNA-SSB.

damage were different in the two cell lines. In LoVo/DX cells 1 hr after drug removal, DNA-SSB were already less than one-third of those present at the end of treatment. In LoVo cells DNA-SSB were the same for at least 2 hr post-treatment incubation in drug-free medium, then declined slowly to one-third of their initial amount by 24 hr.

Verapamil increased DNA-SSB induced by DX in LoVo/DX cells but not in LoVo cells (Table 7). In LoVo/DX cells 1.25 μg/ml DX associated with verapamil produced the same amount of DNA-SSB as DX alone at the dose of 40 μg/ml. Verapamil did not influence the DX-induced production of DNA-DSB in LoVo cells but it significantly increased them in LoVo/DX cells (Fig. 7). After exposure of LoVo/DX cells to 1.25 μg/ml DX no DNA-DSB were detectable whereas the same dose combined with verapamil induced DNA-DSB to the same extent as after 40 μg/ml of DX alone.

DISCUSSION

One approach to elucidating the biochemical mechanisms underlying resistance to anticancer agents is to investigate comparatively tumor cell lines which are as similar as possible except for their different sensitivity to a certain drug.

We selected a human adenocarcinoma cell line (LoVo/DX) which is 30 times less sensitive to DX than the original cell line (LoVo). LoVo and LoVo/DX cells appear similar in their DNA index and protein content as assessed by flow cytometry. Their growth rate is also similar, the fraction of cycling cells and the duration of each phase of the cell cycle being virtually the same. The similar growth kinetics of these human tumor cell lines thus makes them a good tool for investigating mechanisms of drug resistance. A different growth rate, for example, would be a confounding factor since differences in drug effects could be simply due to different proportions of cells being in a more sensitive phase of the cycle.

LoVo/DX cells are also resistant to DNR, vinca alkaloids, epipodophyllotoxins, m-AMSA and, though to a lesser extent, to actinomycin D. This pattern of cross-resistance has already been observed in studies mainly in mouse [7, 8, 11] or hamster [23] tumor cells but recently on some human cell lines too [9, 10, 24]. The cross-resistance involves drugs with different chemical structures and in some cases

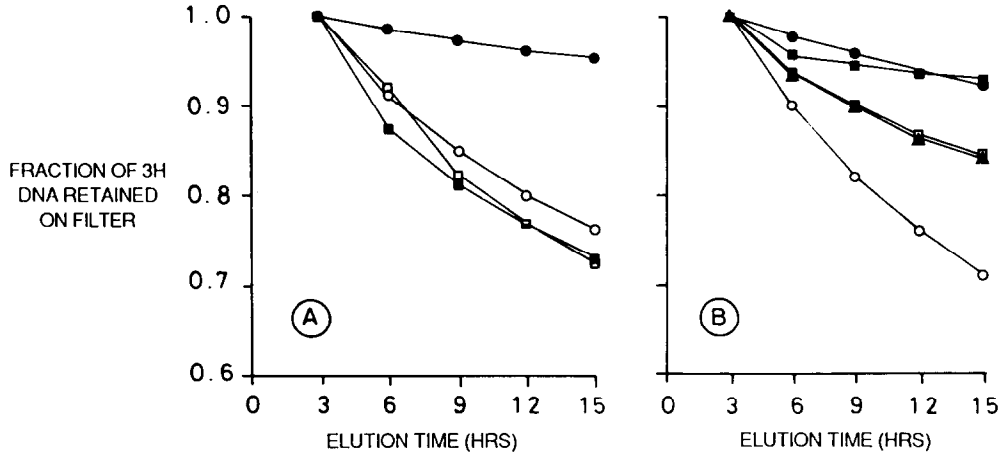

Fig. 7. DX-induced DNA-DSB in LoVo (panel A) and LoVo/DX (panel B) cells. ●——●, Controls; ○——○, 1500 rads; ■——■, DX 1.25 μg/ml; □——□, DX 1.25 μg/ml + verapamil 20 μg/ml; ▲——▲, DX 40 μg/ml. DNA-DSB were determined by the neutral elution (pH 9.6) (see Materials and Methods). Verapamil alone did not produce any DNA-DSB.

different modes of action and appears mainly associated with lower net drug accumulation in these cells [11, 25]. In the cells we investigated there were also clear differences in intracellular drug concentration and in the rate of drug efflux.

To achieve similar DX concentrations in LoVo and LoVo/DX cells in 4 hr the drug exposure needed was respectively 1.25 and 40 μg/ml. After 1 hr wash-out the intracellular drug concentration had dropped by about 32% in LoVo cells and 78% in LoVo/DX cells. It appears therefore that LoVo/DX are resistant to DX mainly because of rapid drug extrusion from the cell before crucial interactions can occur between the drug and the intracellular (probably intranuclear) target. DNR intracellular concentrations suggest that the cross resistance pattern here is also due to rapid drug extrusion, and this is consistent with findings in other cell lines [11–14].

As previously described on other cell lines [26–29] the calcium antagonist verapamil raises DX concentrations in LoVo/DX cells but has no such effect on LoVo cells. Verapamil also partially reversed the resistance to DX in LoVo/DX cells but did not markedly change the response to DX in LoVo cells.

The mechanism by which verapamil increases net drug accumulation has still to be established. The data obtained in the present study suggest that it reduces the rapid efflux of DX from the resistant cells, thus producing greater drug accumulation. Since verapamil is a calcium channel blocker, it has been proposed that its ability to reverse multi-drug resistance is associated with a decreased intracellular Ca concentration. An argument against this hypothesis is that verapamil acts as a voltage-dependent calcium channel blocker at concentrations around 10^{-7} M [30]. To achieve reversal of drug resistance in our cell line or cell lines investigated in previous studies, verapamil concentrations between 10^{-4} and 10^{-5} M were required. These high concentrations, which cannot be achieved in patients' plasma at non-toxic doses, have many unspecific effects unrelated to the Ca channel action (e.g. on Na transport, or phosphoinositol breakdown, etc. see ref. [30] for a review).

However, the verapamil receptor is a glycoprotein and changes in glycoprotein content are reportedly associated with multi-drug resistance [12, 13]. A recent paper on the vinblastine-resistant KB cell line indicates that in-vitro verapamil affects the binding of vinblastine to proteins in the vesicles of resistant cells [31].

Therefore though at present it cannot be excluded that Ca plays a role in the mechanism of reversal of drug resistance induced by verapamil, it also appears worthwhile to study changes in cell membrane structure when multi-drug resistant cells are exposed to verapamil. Preliminary studies in progress in these laboratories suggest that verapamil induces changes in the ultrastructural features of resistant cells, the anionic sites on the external cell surface being increased (Bellini *et al.* unpublished results).

The finding that LoVo/DX cells were equally sensitive to the alkylating agent melphalan and even were more sensitive to ara-C and *cis*-platinum suggests potential clinical utility of these drugs in patients resistant to anthracyclines, epipodophyllotoxins or vinca alkaloids.

In order to ascertain whether there were differences in DX-induced DNA damage in sensitive and resistant cells we exposed LoVo and LoVo/DX cells to DX doses which gave similar intracellular drug concentrations (i.e. 1.25 μg/ml in LoVo cells and 40 μg/ml in LoVo/DX cells). In both lines we observed DX-induced protein associated DNA-SSB and DNA-DSB though there were some quantitative differences. At the end of drug treatment, in LoVo cells only a slightly larger number of DNA-SSB was found.

Similar slight differences in DNA-SSB in LoVo and LoVo/DX were seen when the two cell lines were exposed to the same DX concentrations in the presence of verapamil, which virtually equalized intracellular DX content. In addition, the recovery of DNA integrity, differed greatly, being much more prompt in LoVo/DX (in which most DNA-SSB disappeared after 1 hr wash-out) than in LoVo cells, in which there was still considerable persistant DNA damage several hours after drug removal. The more rapid recovery of DNA-SSB is probably due to faster drug efflux from these cells than from the parent cell line.

DNA-DSB were also slightly greater in LoVo than in LoVo/DX cells exposed to 1.25 and 40 μg/ml DX, respectively. Similar differences were found when the intracellular DX concentration was made similar by the concomitant use of verapamil.

Though further studies are needed to characterize better the molecular basis of drug resistance in these cells, the overall picture from these investigations is that multi-drug resistance is mainly due to more efficient drug extrusion from cells with consequent less DNA damage, this probably being the cytotoxic event. Further studies are needed to cast light on the mechanism by which verapamil reverses drug resistance, with a view to setting up pharmacological treatments of potential clinical use. Even though the mechanism of resistance appears mainly due to a low intracellular drug concentration, other mechanisms mainly associated with the induction of DNA damage have been suggested in other cell lines [15, 32–34] and, though probably not predominant in LoVo/DX, these offer a further explanation of the lower sensitivity of this cell line to DX.

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