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Mechanism of Multidrug Resistance in Human Tumour Cell Lines and Complete Reversion of Cellular Resistance

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The biochemical basis of the multidrug-resistant (MDR) phenotype has been investigated in drug-resistant sublines derived from LoVo and SW984 human colon carcinoma cell lines by doxorubicin selection. Besides drug extrusion through the plasma membrane, two further observations, both ascribable to the drug transport property of the gp170 glycoprotein, were made. First drug deposition into cytoplasmic membranous structures which allows cells to tolerate a high intracellular drug concentration since it prevents drugs from reaching their cellular target site(s). Secondly drug removal from the complexes formed by interaction of drug with target cellular macromolecules, a phenomenon which extends its an effect that continues after treatment and appears to be the most important resistance mechanism in MDR cells. Treatments based on the gp170 inhibitory property of verapamil were developed that allowed abrogation of resistance in MDR cell lines, a strategy that may be applicable to therapy treatments.
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INTRODUCTION

IN THE chemotherapy of human cancer, the effectiveness of anticancer agents is often limited by the emergence of multidrug-resistant (MDR) [1–4] tumour cell populations. Since toxicity for non-tumour tissues limits the clinical use of chemotherapeutic agents to a narrow dose range, tumours acquiring even low-level of multidrug-resistance (MDR) may become refractory to further clinical treatments. To design more effective chemotherapeutic regimens for MDR tumours, it requires a better understanding of the biochemical mechanisms which confer cell the MDR phenotype.

Previous studies have shown that the MDR phenotype [5,6] is consequent upon the overexpression of the p-glycoprotein (gp170) [7] and is sustained by two major biochemical phenomena [8–10]. The first phenomenon, which works at the plasma-membrane level and affects drug-transmembrane equilibria determining reduced intracellular drug accumulation [3,11] can be ascribed to the biochemical activity of the mdr1 gene product, the p-glycoprotein [7]. The second, which works at intracellular level [10] possibly prevents the drugs from reaching the cellular site targets of drug cytotoxic activity and, consequently, allows MDR cells to tolerate higher intracellular drug concentrations than those tolerated by their drug-sensitive parent cells [10]. Identification of the molecular bases of the latter phenomenon and development of chemotherapeutic regimens capable of a complete *in vitro* reversion of the MDR phenotype were the aims of the present study.

MATERIALS AND METHODS

Drugs

Doxorubicin (Farmitalia-Carlo Erba), vincristine (Lilly), actinomycin D (Merck, Sharp), and cisplatin (Bristol-Myers) were dissolved in saline in sterile conditions just before their use.

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Cell lines

SW948 and LoVo human colon carcinoma (HCC) cell lines were obtained from the American Type Culture Collection (Rockville). Cell lines were propagated in F12 (LoVo) or RPMI 1640 (SW948) supplemented with 10% heat-inactivated fetal calf serum (Seralab, Sussex) 1 mmol/l Na-pyruvate, 50 μg/ml streptomycin and 50 units/ml penicillin G. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 air.

Selection of drug-resistant sublines

Drug-resistant sublines were obtained by treating in continuous exponentially growing cell cultures with specific doxorubicin concentrations as described [10,12]. Medium-containing doxorubicin was substituted twice a week. After 5–10 weeks single colonies of drug-resistant cells were harvested with 0.25% trypsin/0.02% EDTA and cultured for more than 6 months in the continuous presence of doxorubicin, at the same concentration used during selection. Drug-resistant sublines were maintained in the presence of doxorubicin until 24 h before the experiments. SW948 and LoVo cell line were first selected at 100 ng/ml doxorubicin. SW948-R-100 subline was then selected at 300 ng/ml doxorubicin and SW948-R-300 subline at 500 ng/ml doxorubicin. Drug-resistant sublines were termed with the monogram of the parent cell line followed by -R- and the level of doxorubicin concentration used for their selection.

Cytotoxic test

The cytotoxic effects of pharmacological treatments were determined by clonogenic assay in liquid medium as previously described [13]. Drug cytotoxicity was calculated as the percentage of cell survival in drug-treated cultures compared with that in untreated controls. Results are reported as drug concentrations (ng/ml) inhibiting cell clonogenicity of 50% (IC_{50}). IC_{50} was extrapolated by linear regression of experimental data.

Northern analysis

Total cellular RNA was extracted from exponentially growing cells by the guanidine chloride method [14]. Northern blot were performed as described [10,15]. Autoradiographic signals were quantified by densitometry using β-actin mRNA level as internal

standard. mdrl mRNA expression levels were reported in arbitrary units. A value of 1 unit was assigned to the quantity of mdrl mRNA in 10 μg total RNA from SW948 HCC cell line.

Probes used were: a 1.2 Kb *Eco*RI fragment derived from plasmid pHu P1701 representing a cDNA covering the 3′ portion of the human mdrl gene [16]; a human β-actin fragment (0.7 Kb) derived from *Bam*HI-*Eco*RI-digested plasmid pHRβA-3′UT [17]. Probes were ^{32}P labelled by multiprime labelling system (Amersham,) at specific activity > 10^9 cpm per 1 μg DNA.

gp170 expression analysis

The gp170 expression was determined by indirect immunofluorescence and cytofluorimetric or fluorescence-microscopy analyses.

Cytofluorimetric analyses were performed either on living cells or on cells formaldehyde-fixed (3.7% in PBS for 10 min at 20°C) and saponin-permeabilised (0.1% in PBS for 30 min at 20°C)[18] labelled with MRK 16 (MRK16 Mab) [19] or C219 (C219 Mab) [20] (Centocor) anti gp170-specific monoclonal antibody (Mab) and fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG_s (Becton Dickinson) [10].

Fluorescence microscopy analyses were performed on cells grown on chamber slide (mod. 177372, Nunc) for at least 24 h and subconfluent. Living cells or cells fixed with formaldehyde both permeabilised and not with saponin were labelled with the MRK16 in C219 Mab and FITC-conjugated goat antimouse IgG_s, then observed at the fluorescence microscope.

Doxorubicin-subcellular location

Doxorubicin-subcellular distribution was visualised 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 [21].

Cells grown on chamber slides were incubated with doxorubicin containing medium at the concentration, for the time and at the temperature described in the Results. Microscopic observation was performed keeping the microscopic slide at the specified temperatures. Cells were metabolically blocked by 30 min incubation with 15 mmol/l Sodium azide/50 mmol/l deoxyglucose [22] before and during drug treatment.

When testing the effect of verapamil, this drug was added 15 min before and during doxorubicin treatment and continuously maintained or not in doxorubicin-free culture medium as specified in the Results.

Doxorubicin-uptake analyses and determination of IC_{50} int *doxorubicin*

Exponentially growing cells, 3×10^6 in 10 ml medium, were seeded in 90 mm petri dishes (Falcon) and incubated overnight at 37°C, then treated for 1 h at 37°C with fresh medium containing specific concentrations of ^{14}C doxorubicin (Amersham). Radioactive medium was subsequently withdrawn and petri dishes were chilled on ice and quickly washed 3 times with ice-cold saline solution. Cells were harvested by trypsin treatment and counted using an haemotocytometer. ^{14}C doxorubicin uptake was determined by liquid scintillation counting. Cytotoxic effect of drug treatments was determined on replicate petri dishes.

Cell size was determined using ^3H_2O and D-1-^{14}C mannitol (Amersham) [23]. Since cell volume of MDR cells was not significantly different from that of the corresponding drug-sensitive parent cells, intracellular drug concentration was expressed as pmol/10^6 cells. The intracellular doxorubicin concentration inhibiting cell growth of 50% (IC_{50} int doxorubicin) was determined after exposing the cells for 1 h to the external-drug concentration inhibiting cell growth of 50% (IC_{50} ext doxorubicin).

RESULTS

mdrl gene products expression in MDR sublines

SW948 and LoVo drug-resistant sublines displayed both the classical MDR phenotype, being cross-resistant to doxorubicin, vincristine and actinomycin D but not to cisplatin, and a mdrl mRNA expression level (EL) higher than that expressed by their parent cell line (Table 1). Moreover, SW948 MDR sublines showed enhancements in drug resistance and in mdr1 mRNA EL roughly proportional to the drug selective pressure at which they arose (Table 1) [12]. The increased mdrl mRNA EL in LoVo-R-100 subline might be consequent upon gene amplification (4–5 mdrl gene copies per aploid genome) (data not shown) [10]. On the contraty, SW948 MDR sublines exhibited neither mdr1 gene amplification nor rearrangements (data not shown) [10].

In every MDR subline, the global amount (cytoplasmic plus plasma membrane) of gp170 glycoprotein molecules was proportional to the mdrl mRNA EL as shown by indirect immunofluorescence labelling, with either MRK16 Mab (Fig.1) or C219 Mab (data not shown), and cytofluorimetric analyses performed on formaldehyde-fixed saponin permeabilised cells (Fig 1). Cytofluorimetric analysis of immunofluorescence-labelled (MRK16 Mab) living cells showed that LoVo MDR cells increased plasma membrane gp170 density, relative to LoVo drug-sensitive parent cell line (Fig 1). SW948 MDR sublines, on the contrary, were unable to increase gp170 molecules at the plasma membrane level (Fig 1).

Disconnection between gp170-expression level and drug-extrusion activity in SW948 MDR sublines

In a previous paper [10] we reported that SW948-R-100 sublines did not display energy-dependent drug-extrusion activity. The present study has extended such observation to SW948-R-300 and SW948-R-500 sublines which, despite their high gp170 EL, did not differ for this property from the SW948-R-100 cell line. The incapability of SW948 MDR sublines to alter drug-transmembrane equilibria as compared to parent cell line (Fig. 2) was possibly consequent upon their inability to assemble gp170 molecules at the plasma membrane level.

gp170-subcellular location and doxorubicin-intracellular compartimentation

gp170-immunofluorescence labelling of saponin-permeabilised LoVo and SW948 MDR cells showed that the intracytoplasmic gp170 molecules are not uniformly diffused but localised in spots and possibly associated with cytoplasmic membranous structures.

Metabolically-active MDR cells, exposed to doxorubicin at $IC_{50\ ext}$ level for 1 h, displayed a punctate intracytoplasmic, but not nuclear, doxorubicin accumulation (Fig. 3). Such intracytoplasmic-doxorubicin distribution resemble the intracytoplasmic location of the gp170 molecules (Fig. 3). Intracytoplasmic-doxorubicin compartmentation was never observed in drug-sensitive LoVo and SW948 cells in which doxorubicin achieved nuclear location (data not shown), as previously described by Schuurhuis *et al* [24].

Intracytoplasmic-doxorubicin accumulation and compart-

Table 1. Drug resistance and mdr1 mRNA expression levels in SW948 and LoVo parent cell lines and derived drug-resistant sublines

Cell line	Relative resistance to:* Doxorubicin‡	Vincristine	Actino-mycin D	Cisplatin	mdrl mRNA† EL
SW948	1[285(45)]§ 1[22(4)]	1[60(13)]	1[15(4)]	1[518(71)]	1
SW948-R-100	5.7 5.9	3.5	5.3	1.2	4.8
SW948-R-300	12.1 15.5	7.5	8.6	1.2	18.7
SW948-R-500	22.4 26.7	10.8	12.2	1.2	26.3
LoVo	1[291(46)] 1[26(4)]	1[71(20)]	1[14(3)]	1[601(107)]	2.1
LoVo-R-100	11.8 14.9	8.5	6.4	1.0	22.0

*Relative resistance is expressed as the ratio of $IC_{50\ ext}$ of the considered cell line to the $IC_{50\ ext}$ of the parent cell line treated with the same drug for the same period of time.
†mdrl mRNA expression level (EL) is expressed in arbitrary units.
‡Drug treatments were for 1 h (upper value) or 24 h (lower value) for doxorubicin; 24 h for the other drugs.
§Values in brackets are the $IC_{50\ ext}$ expressed in ng/ml (S.D.)

mentation, may account for the enhanced cellular resistance to high intracellular-drug concentrations displayed by LoVo and SW948 MDR sublines. Their $IC_{50\ int}$ doxorubicin were, in fact 3.2, 3.1, 7.8 and 14.0 (LoVo-R-100; SW948-R-100; SW948-R-300, SW948-R-500, respectively) fold greater than that of the corresponding parent cell line.

Differential subcellular-doxorubicin location in metabolically-active and inactive and in verapamil-treated MDR cells

LoVo and SW948 MDR cells, either metabolically-poisoned by sodium azide/deoxyglucose treatment or fixed with formaldehyde, lost the capability to compart doxorubicin intracytoplasmically and allowed the drug to reach the nucleus where doxorubicin accumulated (Fig. 4). Doxorubicin nuclear accumulations is consequent upon strong chemical–physical interactions occurring between the drug and nuclear macromolecules [25]. As consequence of such interactions doxorubicin was unable to diffuse out of the nucleus when the metabolically-blocked cells were incubated (up to 12 h) in doxorubicin-free medium (Fig. 4 a).

A reversible metabolic block, obtained by lowering cell temperature (up to 4°C), produced an effect similar to that observed in poisoned or fixed MDR cells: lack of intracytoplasmic-doxorubicin compartmentation and drug-nuclear accumulation (Fig.4 b). By returning the cells to physiological temperature, doxorubicin-nuclear accumulation quickly (less that 15 min) disappeared and the drug accumulated again into the cytoplasmic membranous structures (Fig. 4 b).

Lack of intracytoplasmic-doxorubicin compartmentation and drug-nuclear accumulation were also observed when doxorubicin "pumping factor" was competitively inhibited with verapamil 2 or 6 μmol/l (Fig. 4 c). Removal of doxorubicin but not verapamil from the culture medium did not affect the described drug accumulation and compartmentation (Fig. 4 c) which lasted qualitatively and quantitatively uncharged for at least 12 h. Removal of verapamil from the culture medium allowed a quick (less than 15 min) intracellular relocation of the drug resulting in the disappearance of drug-nuclear accumulation and reappearance of cytoplasmic compartmentation (Fig 4 c).

Inhibition of doxorubicin removal from the nucleus causes a complete reversal of drug resistance

The cytotoxic enhancing effect of doxorubicin nuclear accumulation caused by cell cooling was analysed by incubating cells for 1 h at 37°C and for 30 min at 4°C with appropriate extracellular-doxorubicin concentrations. Cells were subsequently washed with doxorubicin-free medium and returned at 37°C. Colonies were counted about 10 days after drug treatment. In no cell line the temporary nuclear drug accumulation caused by cell cooling affected cellular sensitivity to doxorubicin (Table 2; lane doxorubicin + cooling versus lane doxorubicin).

The cytotoxic enhancing effects of nuclear drug accumulation caused by various verapamil treatments were analysed as follows: cells, pretreated for 15 min with verapamil, were incubated at 37°C for 1 h with appropriate doxorubicin concentrations in the presence of either 2 or 6 μmol/l verapamil. Doxorubicin was removed from the plates by three washings and 15 min incubation with prewarmed culture medium (37°C) both containing verapamil and not. Thereafter plates washed with verapamil-containing medium were incubated at 37°C in the continuous presence of verapamil. Plates washed with verapramil-free medium were incubated either with verapamil-containing medium or verapamil-free medium.

The sensitising effect of verapamil exposure, performed before and during doxorubicin treatment caused a decrease of about 30%–60% of the $IC_{50\ ext}$ doxorubicin of the MDR cell lines as compared to treatment with doxorubicin alone (Table 2; lane doxorubicin + verapamil, wash verapamil−, cult verapa-

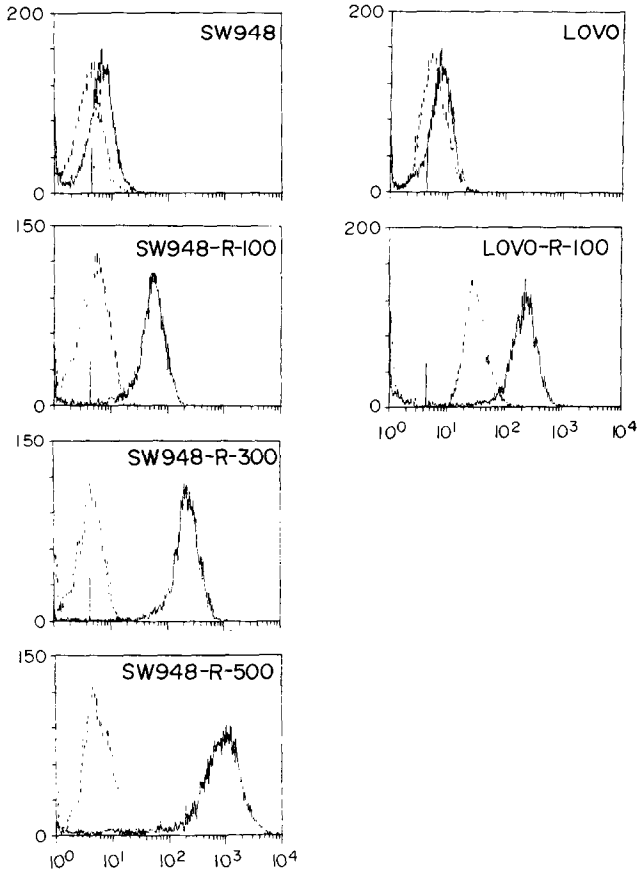

Fig. 1. Flow cytometric profile of living (broken line) and formaldehyde-fixed saponin-permeabilised (continuous line) drug-sensitive and MDR sublines. Cells were incubated with MRK16 Mab plus FITC-goat anti mouse IgGs. The log fluorescence intensity was plotted on the *x*-axis against the relative cell number of the *y*-axis. Bars at 5×10^0 log indicates the maximum fluorescence itensity of the same cells incubated with non-immune IgG_s, as negative control, and FITC-goat anti mouse IgG_s.

mil− vs. lane doxorubicin). Notwithstanding verapamil exposure, MDR cells still resulted more drug-resistant than their parent cell line.

A very deep sensitising effect was obtained by maintaining verapamil in the culture medium after the end of doxorubicin treatment: MDR cell sublines completely lost drug resistance and acquired drug sensitivity similar to that of the drug-sensitive

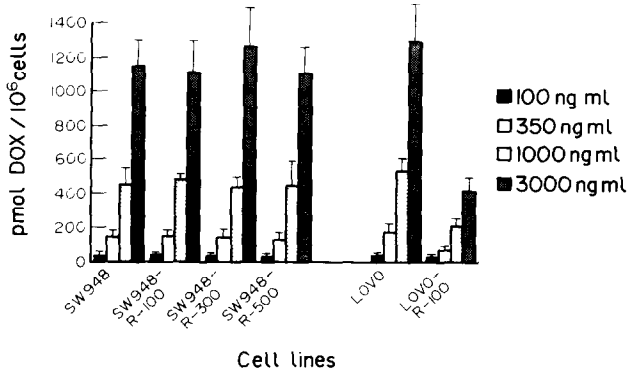

Fig. 2. Intracellular doxorubicin concentrations, expressed as pmol doxorubicin/10^6 cells, in SW948 and LoVo drug-sensitive and MDR cells. Cells were exposed for 1 h at the extracellular doxorubicin concentrations indicated. Bars, S.D.

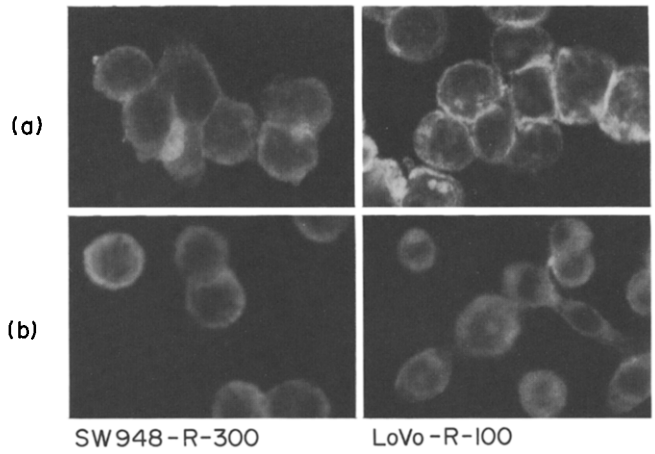

Fig. 3. gp170 subcellular location and doxorubicin intracellular compartmentation in SW948-R-300 (left) and LoVo-R-100 (right) sublines. SW948-R-100 and SW948-R-500 sublines gave qualitatively similar results (data not shown). Immunofluorescence staining with MRK16 Mab of cells permeabilised with saponin (a). Intracytoplasmic location of doxorubicin molecules in cell incubated for 1 h with doxorubicin at the respective $IC_{50\,ext}$ level (b).

parent cell lines (Table 2; lane doxorubicin, wash verapamil+, cult verapamil+ vs. lane doxorubicin). The complete inhibition of drug resistance caused by the incontinuous verapamil treatment required verapamil to be uninterruptedly present in the culture medium. A very short incubation (15 min) in verapamil-free medium, performed at the end of the pharmacological treatment (doxorubicin+verapamil) made, the subsequent discontinuous-verapamil treatment ineffective (Table 2; lane doxorubicin+verapamil, wash verapamil−, cult verapamil+ vs. lane doxorubicin+verapamil, wash verapamil+, cult verapamil+).

DISCUSSION

Our results indicate that overexpression of mdrl gene products confers cells multidrug resistance (MDR) as a result of three major biochemical phenomena: (1) drug extrusion from the cell through the plasma-membrane; (2) drug compartmentation into cytoplasmic membranous structures; and, (3) drug removal from the molecular complexes originated by physico-chemical interactions between the drug and its target cellular macromolecules. Conclusive biochemical elucidations of such phenomena are still lacking, however we believe that all of them have to be ascribed to the drug-transporting activity of the gp170 glycoprotein, since: (1) all the phenomena are energy-dependant, being all blocked by the inhibition of cellular metabolic activity; (2) they are similarly inhibited by a chemical compound, verapamil, which is able to interact with the gp170 glycoprotein [26–28]; and, (3) their entity is directly related to mdrl gene expression levels and, more specifically, to the gp170 density at the specific subcellular site(s) where each phenomenon takes place. The gp170 glycoprotein location, in fact, is not restricted to plasma membrane but it is undoubtedly, associated with some cytoplasmic membranous structures [present results, 24, 26, 29] and possibly, with perinuclear and/or intranuclear membranous structures (our preliminary results).

Contrary to previous assumptions [3, 11, 19, 21, 29], drug extrusion from the cell through the plasma membrane does not seem to be the major phenomenon responsible for establishing of drug-resistance in MDR cells. Some cells, and specifically our SW948 MDR series, in fact, acquire the gp170-associated

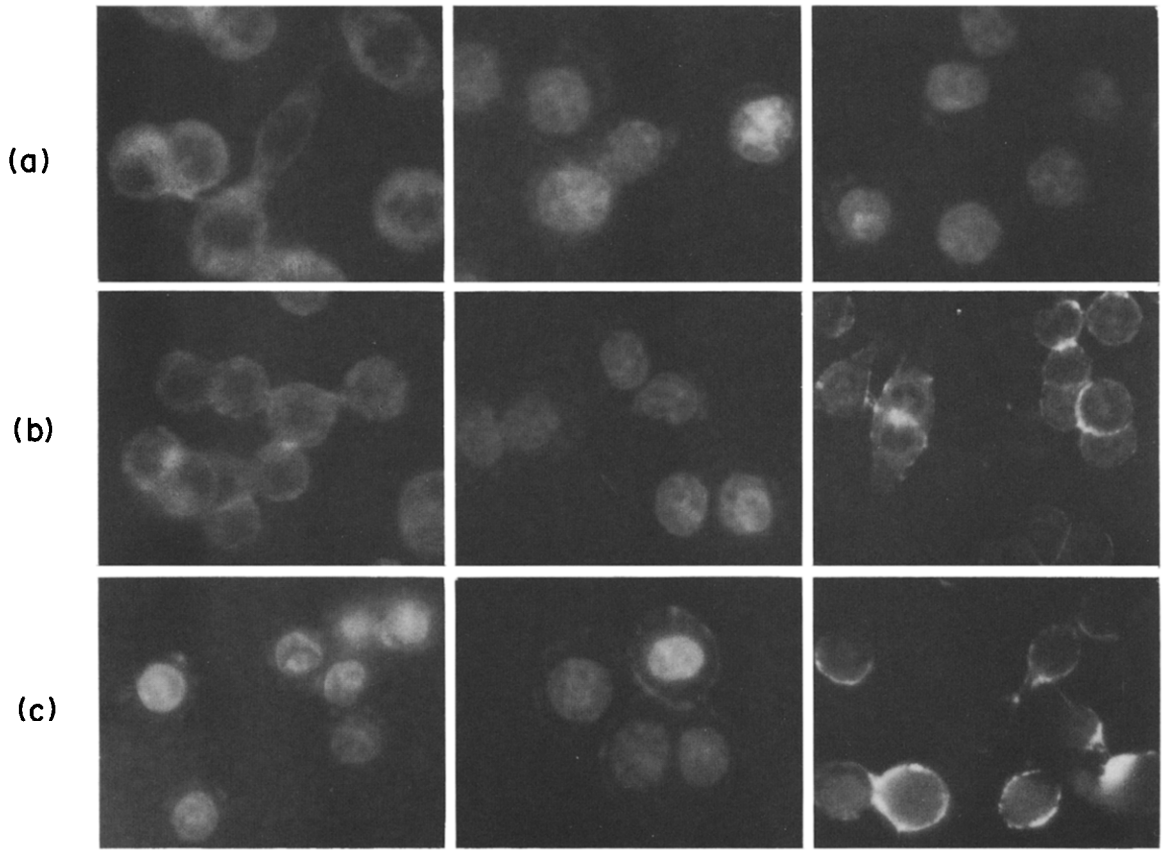

Fig. 4. Subcellular doxorubicin location in LoVo-R-100 cells treated as follows. (a)Metabolically-active cells (left); Na azide/deoxyglucose poisoned cells incubated with doxorubicin at the $ic_{50\ ext}$ level (middle) and, cells incubated as A-middle then incubated in drug-free medium for up to 12 h (right). (b) Metabolically-active cells incubated for 1 h at 37°C with doxorubicin at the $ic_{50\ ext}$ level (left); then cooled at 4°C for 30 min (middle); and, subsequently warmed at 37°C for 15 min (right). (c) Metabolically-active cells incubated at 1 h at 37°C with doxorubicin at the $ic_{50\ ext}$ level in the presence of verapamil 6 μmol/l (left); then incubated for 15 min in verapamil 6 μmol/l containing medium (middle) or in verapamil-free medium (right).

MDR phenotype without assembling gp170 molecules at the plasma-membrane level. These findings must be carefully considered when attempting to infer multidrug-resistance from the gp170 plasma-membrane density.

Drug compartmentation, into cytoplasmic membranous structures possibly accounts for the capability of the MDR cells to tolerate much higher intracellular drug concentrations than those tolerated by their drug-sensitive parent cell lines [8 – 10]. We hypothesise that drug compartmentation into cytoplasmic membranous structures is a dynamic process consisting of two opposite phenomena: (1) gp170-driven drug introduction into cytoplasmic membranous structures, and (2) drug exit from cytoplasmic membranous structures by passive diffusion. As a consequence of such a dynamic process, an equilibrium is established between drug concentrations into cytoplasmic membranous structures and in the surrounding cytoplasmic fluid. Drug molecules dynamically confined in the cytoplasmic fluid establish a concentration barrier which hinders extracellular drug molecules to passively diffuse into the cells and reach their target macromolecules.

Said drug-resistance mechanism may be overcome either by exceeding drug-extracellular concentrations or metabolic/competitive inhibition of the gp170 drug-pumping activity. This allows the drug to reach its target cellular macromolecules and interact with them. However, the drug can exert its cytotoxic effect(s) only as long as the drug pumping activity of the gp170 glycoprotein is inhibited. In cells not irreversibly injured, re-establishment of the gp170 drug-pumping activity, either by decreasing extracellular drug concentration or by removing the gp170 inhibitor factors, allows, in fact, the drug complexed with its target cellular macromolecules to be displaced, recompartmented into cytoplasmic membranous structures, and, finally, extruded through the plasma membrane.

Removal of the drug from drug-cellular macromolecule complexes must be the major drug-resistance mechanisms in MDR cells. Inhibition of this phenomenon by discontinuous-verapamil treatment resulted in a dramatic increase in doxorubicin cytotoxic effect relative to verapamil treatment performed only during doxorubicin exposure. MDR cell sublines treated in the continuous presence of verapamil irrespectively of their mdrl gene products EL, completely lose their drug resistance, becoming as sensitive to doxorubicin as their parent cell line. It must be stressed that, because of the high affinity for doxorubicin of the pumping factor and its quick effect in removing and extruding doxorubicin from the nucleus, it is mandatory that the inhibitory pressure of verapamil be maintained uninterruptedly in order to prevent nuclear doxorubicin clearance. A short interruption (less than 15 min), of verapamil inhibitory pressure performed at the end of the pharmacological treatment allowed the total extrusion of doxorubicin from the nucleus (Fig. 4 c) and, consequently the abrogration of the cytotoxic enhancing effect of the dis-continuous-verapamil treatment (Table 2 lane doxorubicin-+verapamil, wash verapamil−, cult verapamil+ vs. lane doxorubicin+verapamil, wash verapamil+, cult verapamil+).

Table 2. Drug resistance of parent and MDR cell lines as consequence of pharmacologic treatments

Cell line	(A) Verapamil (μmol/l)	(B) Doxorubicin	(C) Doxorubicin + cooling	(D) Doxorubicin + verapamil, wash verapamil cult. verapamil − (D vs.B)	(E) Doxorubicin + verapamil, wash verapamil + cult. verapamil + (E vs.D)	(F) Doxorubicin + verapamil, wash verapamil− cult. verapamil+ (F vs.D)
	0	286(45)(G)	321(51)	—	—	—
SW948	2	—	—	328(59)	217(23)†	ND
	6	—	—	275(34)	189(37)*	261(37)
	0	1706(343)	2015(391)	—	—	—
SW948-R-100	2	—	—	1356(108)*	282(53)‡	ND
	6	—	—	1169(158)†	173(69)‡	1057(202)
	0	3476(546)	3898(616)	—	—	—
SW948-R-300	2	—	—	2129(312)‡	327(111)‡	ND
	6	—	—	1930(216)‡	116(112)‡	1778(280)
	0	6428(923)	7230(916)	—	—	—
SW948-R-500	2	—	—	3388(542)‡	341(151)‡	ND
	6	—	—	2980(374)‡	244(60)‡	2473(402)
	0	291(46)	373(41)	—	—	—
LoVo	2	—	—	323(53)	212(31)*	ND
	6	—	—	306(28)	131(27)‡	281(32)
	0	3585(489)	3631(295)	—	—	—
LoVO-R-100	2	—	—	1786(240)‡	266(85)‡	ND
	6	—	—	1480(346)‡	206(90)‡	1250(266)

(A) Verapamil concentration used in the chemosensitivity test; (B)-(F):pharmacological treatments: (B) doxorubicin, 1 h 37°C; (C) doxorubicin, 1 h, 37°C plus doxorubicin 30 min, 4°C;(D) doxorubicin + verapamil, 1 h 37°C; (E) doxorubicin + verapamil, 1 h 37°C plus verapamil discontinuous; (F) doxorubicin + verapamil, 1 h 37°C then 15 min dis-verapamil free medium and verapamil in discontinuous; (G) drug-sensitivity expressed as $IC_{50\,ext}$ in ng/ml (S.D.)

ND = not done.

Statistical analyses were performed by one way ANOVA test. Three comparisons were performed D vs. treatment B; E vs. treatment D; and F vs. treatment D. *$P<0.05$; †$P < 0.01$; and ‡$P<0.005$.

In conclusion, our studies contribute to a better understanding of the molecular phenomena responsible for the multidrug-resistance. As consequence of such knowledge, we were able to develop pharmacological treatments extremely effective in suppressing drug-resistance *in vitro*. Such treatments have a sole mandatory requirement: the gp170 inhibitory pressure must be continuously maintained. Since a similar gp170 continuative inhibitory pressure will be quickly obtained *in vivo*, it will be possible to make the pharmacological treatment of human tumours more effective. Our expectation is supported by a recently published clinical protocol [30] which tested a chemotherapeutic regimen associated to an discontinuous-verapamil treatment in patients with drug-resistant gp170 positive malignant lymphomas, achieving extremely valid clinical results [31].

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Characterisation of Insulin-like Growth Factor I Receptors of Human Acute Lymphoblastic Leukaemia (ALL) Cell Lines and Primary ALL Cells

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The expression of insulin-like growth factor I (IGF-I) receptors (IGF-IR) on human B-lineage and T-lineage acute lymphoblastic leukaemias (ALL) representing different maturational stages has been studied. Immature (stage I) and mature (stage II) T ALL as well as pre-B ALL cell lines expressed high numbers of IGF-IR with high affinity for IGF-I. In contrast, on T ALL, stage II and B ALL only low specific binding of ^{125}I-IGF-I was detected. No binding of ^{125}I-IGF-I to Burkitt lymphoma cells was found. Primary human T, pre-B and cALL cells also expressed IGF-IR with K_d for IGF-I and IGF-IR number per cell in the same range as the investigated cell lines. Crosslinking of ^{125}I-IGF-I to T and pre-B ALL cells revealed IGF-IR alpha-subunits of 135 and 116 kD for HSB2. Gene expression of IGF-IR could be detected in all T ALL cell lines but was indetectable in SKW6, a B ALL cell line.
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INTRODUCTION

INSULIN-LIKE growth factor (IGF)-I is related to insulin and produced by liver cells after stimulation by growth hormone (GH). It promotes skeletal growth by inducing proliferation of chondrocytes. Further, this peptide acts as a paracrine or autocrine growth factor of many non-transformed and transformed cells and cell lines (for review, see [1]). Physiological actions of IGF-I and IGF-II are mediated by specific interactions with cell surface receptors. IGF-I and IGF-II are ligands for the IGF-I and IGF-II receptors. From both related peptides IGF-I has a crossreactivity with the insulin receptor and IGF-II with the IGF-I receptor. Insulin, with lower affinity, also binds to the IGF-I receptor. Extensive homology exists between the insulin and IGF-I receptor [2], whereas the IGF-II receptor, which