

Changes in subcellular doxorubicin distribution and cellular accumulation alone can largely account for doxorubicin resistance in SW-1573 lung cancer and MCF-7 breast cancer multidrug resistant tumour cells

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Summary Doxorubicin accumulation defects in multidrug resistant tumour cells are generally small in comparison to the resistance factors. Therefore additional mechanisms must be operative. In this paper we show by a quantitative approach that doxorubicin resistance in several P-glycoprotein-positive non-small cell lung cancer and breast cancer multidrug resistant cell lines can be explained by a summation of accumulation defect and alterations in the efficacy of the drug once present in the cell. This alteration of efficacy was partly due to changes in intracellular drug localisation, characterised by decreased nuclear/cytoplasmic doxorubicin fluorescence ratios (N/C-ratios). N/C-ratios were 2.8–3.6 in sensitive cells, 0.1–0.4 in cells with high (> 70-fold) levels of doxorubicin resistance and 1.2 and 1.9 in cells with low or intermediate (7.5 and 24-fold, respectively) levels of doxorubicin resistance. The change of drug efficacy was reflected by an increase in the total amount of doxorubicin present in the cell at equitoxic (IC₅₀) concentrations. N/C ratios in highly resistant P-glycoprotein-containing cells could be increased with the resistance modifier verapamil to values of 1.3–2.7, a process that was paralleled by a decrease of the cellular doxorubicin amounts present at IC₅₀. At the low to moderate residual levels of resistance, obtained with different concentrations of verapamil, a linear relationship between IC₅₀ and cellular doxorubicin amounts determined at IC₅₀ was found. This shows that at this stage of residual resistance, extra reversal by verapamil should be explained by further increase of drug efficacy rather than by increase of cellular drug accumulation. A similar relationship was found for P-glycoprotein-negative MDR cells with low levels of resistance. Since in these cells N/C ratios could not be altered, verapamil-induced decrease of IC₅₀ must be due to increased drug efficacy by action on as yet unidentified targets. Although the IC₅₀ of sensitive human cells cannot be reached with resistance modifiers, when using these relationships it can be shown by extrapolation that cellular and nuclear doxorubicin amounts at IC₅₀ at complete reversal of resistance were the same as in sensitive cells. It is concluded that doxorubicin resistance factors for multidrug resistant cells can for a large part, and in the case of P-glycoprotein-containing cells probably fully, be accounted for by decreased amounts of drug at nuclear targets, which in turn is characterised by two processes only: decreased cellular accumulation and a shift in the ratio nuclear drug/cytoplasmic drug.

Expression of the putative drug efflux pump P-glycoprotein in multidrug resistant (MDR) cells results in lower drug accumulation than in the corresponding parent cells (Bradley *et al.*, 1988). Reduced drug accumulation may also be a parameter of drug resistance for some (Coley *et al.*, 1991; Haber *et al.*, 1989; Hindenburg *et al.*, 1989; Kuiper *et al.*, 1990; McGrath & Center, 1988; Slapak *et al.*, 1990; Slovak *et al.*, 1988; Taylor *et al.*, 1991) but not for all (Cole *et al.*, 1991; Danks *et al.*, 1987; Harker *et al.*, 1989; McGrath *et al.*, 1989) MDR cells which do not overexpress P-glycoprotein. Effective modulation of multidrug resistance is possible in P-glycoprotein containing cells with compounds which exhibit different structural features (Zamora *et al.*, 1988) and it is attributed to increases in cellular drug accumulation resulting from inhibition of drug efflux (Bradley *et al.*, 1988). Modulation of non-P-glycoprotein mediated MDR and accumulation defects by verapamil and other Pgp modulators, however, seems to be less efficient than for P-glycoprotein mediated MDR (Cole *et al.*, 1989; Coley *et al.*, 1991; Harker *et al.*, 1989; Kuiper *et al.*, 1990; Schuurhuis *et al.*, 1991; Slovak *et al.*, 1988; Taylor *et al.*, 1991).

It is known that changes in drug accumulation can not fully account for anthracycline resistance in many MDR cells (evidence reviewed by Schuurhuis *et al.*, 1989a). Such con-

siderations have led to the suggestion that other mechanisms must contribute to the MDR phenotype. One of the possibilities would be that enzymes, involved in the detoxification of oxygen-derived free radicals generated by semiquinone compounds like doxorubicin, such as glutathione transferase and glutathione peroxidase, contribute to anthracycline resistance in MDR cells (Batist *et al.*, 1986; Cowan *et al.*, 1986). MDR human breast cancer MCF-7^{ADR} cells have been studied in depth as to this and it was found that doxorubicin-induced oxygen free radical formation was strongly reduced compared to sensitive cells (Sinha *et al.*, 1987). Further, changes in levels and/or activity of topoisomerase II may contribute to anthracycline and VP-16-213 resistance (Beck, 1989). Another important phenomenon associated with MDR is an altered intracellular drug distribution. Several studies now have shown that the development of MDR is associated with a relative shift of doxorubicin or daunorubicin fluorescence from the nucleus to the cytoplasm (Broxterman *et al.*, 1990; Gervasoni *et al.*, 1991; Gigli *et al.*, 1989; Hindenburg *et al.*, 1987, 1989; Keizer *et al.*, 1989; Schuurhuis *et al.*, 1989a, 1991; Willingham *et al.*, 1986). In previous work with Chinese hamster ovarian cells we have argued that this shift may contribute for an important part to the ineffectiveness of anthracyclines in MDR cells (Schuurhuis *et al.*, 1989a).

We now extend these drug distribution studies using various human MDR cells, including MCF-7^{ADR} cells. With a simple mathematical approach we show that doxorubicin resistance in these P-glycoprotein-containing cells is determined mainly by reduced access to nuclear targets which in turn is characterised by two factors only: reduced drug accumulation and altered subcellular drug distribution. Thereby the need is excluded to postulate that other mechanisms contribute significantly to resistance. In addition,

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reversal of resistance in P-glycoprotein/MDR by verapamil is related to effects on these factors. It is further shown that in non-P-glycoprotein/MDR cell lines verapamil exerts part of its effects via a third mechanism different from drug accumulation or distribution.

Part of this work has been presented in preliminary form at the 80th Annual Meeting of the American Association for Cancer Research (Schuurhuis *et al.*, 1989b).

Materials and methods

Drugs

Verapamil. HCl and colchicine were obtained from Sigma (St. Louis, MO) and doxorubicin from Farmitalia (Italy). [^{14}C]doxorubicin (50 Ci Mol $^{-1}$) was purchased from Amersham (Little Chalfont, UK). Drugs were added from concentrated solutions in 0.9% sodium chloride.

Cells and cell culture

The Human non small cell lung cancer cell line SW-1573 (originally established by Dr A. Leibowitz, Scott and White Clinic, Temple, TX) was exposed to increasing concentrations of doxorubicin resulting in the MDR variants SW-1573/1R50 and SW-1573/1R500 (Keizer *et al.*, 1989) and the SW-1573/2R30 cells, which were selected independently. SW-1573/2R30 cells showed instable resistance and finally resulted in the SW-1573/2R50 cells described earlier (Baas *et al.*, 1990; Kuiper *et al.*, 1990). The partially revertant cell line SW-1573/1R500-0 was obtained by culturing SW-1573/1R500 cells in drug-free medium as described (Keizer *et al.*, 1989). Resistance factors for doxorubicin as measured in a 2 h incubation assay (see below) were 345 (SW-1573/1R500), 24 (SW-1573/1R500-0), 7.5 (SW-1573/1R50) and 4.8 (SW-1573/2R30). The Chinese hamster ovarian cell line AUXB1 and its MDR cell line CH $^{\text{R}}$ C5 were a gift from Dr V. Ling (Ontario Cancer Institute, Ontario, Canada) and were cultured in α MEM. The human breast cancer cell line MCF-7 and its MDR subline MCF-7 $^{\text{ADR}}$ were kindly provided by Dr K. Cowan (National Cancer Institute, Bethesda, MD). The human ovarian cancer A2780 cells were from the National Cancer Institute (Dr R.F. Ozols). Lung, breast and ovarian cancer cells were grown in Dulbecco's modification of Eagle's medium (DMEM, Gibco, Europe Ltd, UK), containing 20 mM HEPES and supplemented with 10% foetal bovine serum (Flow Laboratories, UK). MDR cells were grown in the presence of drug until 1–2 weeks before experiments: 0.5 μM (SW-1573/1R500), 0.05 μM (SW-1573/1R50), 0.03 μM (SW-1573/2R30), 10 μM (MCF-7 $^{\text{ADR}}$) and 2 μM (2780 $^{\text{AD}}$) doxorubicin and 10 $\mu\text{g ml}^{-1}$ colchicine (CH $^{\text{R}}$ C5). Cell doubling times (standard deviations: <15%) were: 24 h (SW-1573), 27 h (SW-1573/1R50 and SW-1573/2R30), 30 h (SW-1573/1R500 and SW-1573/1R500-0), 14 h (AUXB1), 24 h (CH $^{\text{R}}$ C $_3$), 24 h (MCF-7), 36 h (MCF-7 $^{\text{ADR}}$), 17 h (A2780) and 24 h (2780 $^{\text{AD}}$). All the MDR cell lines except SW-1573/1R50 and SW-1573/2R30 were P-glycoprotein positive (Baas *et al.*, 1990; Fairchild *et al.*, 1987; Keizer *et al.*, 1989; Kuiper *et al.*, 1990; Scheper *et al.*, 1988).

Drug cytotoxicity

Cytostatic effects were assessed essentially as described previously (Schuurhuis *et al.*, 1987). Cells were plated in 6-well tissue clusters (Costar, USA) and incubated in the presence of doxorubicin with or without verapamil for 2 h at 37°C. A post-incubation of 24 h with verapamil was applied in order to increase its modulating effect by inhibition of doxorubicin efflux. After that treatment cells were incubated for at least three doubling times and counted. Verapamil, at the highest concentration used (128 μM), inhibited cell growth at 30% maximally. In all cases modulating effects of different concentrations of verapamil on doxorubicin cytotoxicity were determined using cell growth in the presence of verapamil, but without doxorubicin present, as the 100% control.

Drug accumulation

Log-phase trypsinised cells were suspended in growth medium without NaHCO $_3$ and phenol red but containing 20 mM HEPES, pH 7.4. Cellular doxorubicin accumulation was measured with [^{14}C] doxorubicin after a 2 h incubation period at 37°C as described (Schuurhuis *et al.*, 1987). No corrections were made for direct binding of doxorubicin to the cells (less than 20% at concentrations up to at least 2 μM). In the case of CH $^{\text{R}}$ C5 and AUXB1 cells doxorubicin accumulation was measured on cells adhered to 6-well culture clusters. In those cases cells were washed three times with 0.1 M phosphate buffered saline, pH 7.4 after the incubation period and thereafter trypsinised and counted. Controls included wells without cells, but incubated with drugs.

Determination of cellular and nuclear diameters

Diameters of trypsinised cells were determined using a Elzone Electrozone/Celoscope, type 80XY (Particle Data, Inc., Elmhurst, Ill.) Orifice diameter was 120 μM . Nuclei from different SW-1573 cells were isolated by homogenising in 10 mM Tris.HCl, pH 7.6, containing 0.2 mM MgCl $_2$. Isolated nuclei (about 90% of the large particles present) were quickly diluted in isoton and particle diameter was determined as described above for cell diameters.

Cellular doxorubicin amounts present at different IC $_{50}$ values in SW-1573/MDR cells were corrected for their differences in cell volume compared to SW-1573 parent cells using the following formula:

$$\text{dox}_i = \text{dox}_n + \frac{V_c(\text{SW-1573})}{V_c(\text{SW-1573/MDR})} \times \text{dox}_c(\text{SW-1573/MDR})$$

Do x_i is the corrected cellular doxorubicin amount in SW-1573/MDR cells. Nuclear doxorubicin (do x_n) at the IC $_{50}$ was estimated to be about 8 pmol 10 $^{-6}$ cells and assumed to be a constant value for all SW-1573 cells as explained under Results (first section). V_c means cytoplasmic volume and was calculated from nuclear and cellular diameters (see text Results section + Table I). Cytoplasmic doxorubicin (do x_c) was calculated by subtracting the fixed value of 8 pmol 10 $^{-6}$ cells from the total cellular doxorubicin amounts measured at each IC $_{50}$ value using radiolabelled doxorubicin (shown under Results in Figure 6).

Quantification of drug distribution with laser scan microscopy

Quantification of ratios of nuclear doxorubicin fluorescence/cytoplasmic doxorubicin fluorescence (N/C ratio) with laser scan microscopy and image analysis was performed as described (De Lange *et al.*, 1992; Schuurhuis *et al.*, 1989a). Adhered MDR and sensitive cells were incubated for 2 h at 37°C at the same concentrations of doxorubicin and/or verapamil that would result in the different IC $_{50}$ values found in the cytotoxicity assay. For each treatment in a particular experiment 30–50 cells were measured unless indicated otherwise. Total nuclear amounts of doxorubicin were calculated from ratios nuclear doxorubicin/cytoplasmic doxorubicin (N/C fluorescence ratios, but now corrected for doxorubicin fluorescence quenching by DNA as outlined in the next paragraph) and from [^{14}C] doxorubicin accumulation experiments (giving total cellular doxorubicin amounts) at the doxorubicin and verapamil concentrations of interest.

For direct comparison of relative fluorescence signals in AUXB1 and CH $^{\text{R}}$ C5 cells, these cells were allowed to grow on petri dishes in close proximity. This enabled fluorescence recording under the same optical and instrumental conditions. Fluorescence was recorded in at least ten cells in each experiment.

Quenching of doxorubicin fluorescence

In order to be able to calculate total nuclear amounts of doxorubicin in intact cells using N/C doxorubicin fluorescence ratios and total cellular amounts as determined with radiolabelled doxorubicin (see previous paragraph), the

percentage fluorescence quenching in nuclei needs to be determined. For that purpose isolated nuclei of AUXB1 and CH^RC5 cells were prepared by incubating intact cells in hypotonic medium (10 mM Tris.HCl containing 2 mM MgCl₂, pH 7.6) for 30 min on ice and subsequent homogenising by pottering. The nuclei were resuspended in growth medium (see under Drug accumulation). The fluorescence signal of 2 μM doxorubicin in medium of 37°C in a 1 ml cuvet (I₀) was determined using a spectrofluorometer (FluoroMaxTM from SPEX Industries, Edison, NJ). Aliquots of nuclei (3.10⁷ ml⁻¹) were added to the cuvet. The fluorescence signal decreased as a result of quenching due to intercalation into DNA. Extra aliquots of nuclei were added until the fluorescence signal (originating partly from nuclei-associated and partly from some remaining extra-nuclear fluorescence) had stabilised (I₁). The percentage quenching of doxorubicin in the nuclei could be calculated from the decrease of the initial fluorescence of doxorubicin. The fluorescence signal was corrected for (i) the autofluorescence of the particular number of nuclei used (I₂) and (ii) the remaining extranuclear doxorubicin fluorescence (I₃), which was determined by centrifuging the nuclei in the cuvet (without washing) and measuring the fluorescence of the supernatant. Nuclear doxorubicin fluorescence is now I₁-I₂-I₃, while total nuclear doxorubicin (fluorescent plus non-fluorescent) is I₀-I₃. Fluorescent doxorubicin as a percentage of the total amount of doxorubicin in the nuclei can thus be calculated from:

$[(I_1 - I_2) - I_3] / [I_0 - I_3] \times 100$. The ratio was $8.5 \pm 1.4\%$ (mean \pm s.e.m. of two independent experiments, each determined in triplo, for nuclei of both cell lines. No quenching of doxorubicin in non-nuclear cellular compartments was assumed to occur (Tarasiuk *et al.*, 1989).

Results

Relationship between IC₅₀ and drug accumulation at IC₅₀

As for many MDR cells with P-glycoprotein, verapamil caused a dose-dependent decrease of IC₅₀ in the human non-small cell lung cancer MDR cells SW-1573/1R500 as illustrated in Figure 1 (ordinate). Doxorubicin resistance could not be reversed completely (residual level of resistance was about 7) at least partly because concentrations of verapamil higher than 128 μM could not be used due to unacceptable toxicity in the 24 h incubation assay with verapamil. When cellular doxorubicin accumulation was determined in a 2 h incubation assay at the actual IC₅₀ values obtained (e.g. 32 μM doxorubicin with no verapamil present and 1 μM doxorubicin with 24 μM verapamil present), a linear relationship was found when IC₅₀ values were plotted on a logarithmic scale against cellular doxorubicin amounts measured at these IC₅₀ values (Figure 1). This shows that reversal of resistance can be described by a function of the type

$$IC_{50} = e^{c[\text{doxi at } IC_{50}]}$$

in which *c* is a constant and doxi is the cellular amount of doxorubicin at each IC₅₀ value. The figure shows that (i) intracellular doxorubicin is very ineffective in inhibiting cell growth in SW-1573/1R500 cells when no verapamil is present (490 pmol 10⁻⁶ cells were necessary to reach IC₅₀ compared to about 14 pmol 10⁻⁶ cells in the parent cell line SW-1573) and (ii) verapamil drastically increases the efficacy of doxorubicin in the MDR cells (only about 80 pmol 10⁻⁶ cells are necessary to reach IC₅₀ when 128 μM verapamil is present). For the human breast cancer cell line MCF-7^{ADR} similar results were obtained (Figure 2). The ability of verapamil to increase the efficacy of doxorubicin must be added to its well-known action on drug accumulation perse at a fixed doxorubicin concentration and which is illustrated in Figure 3 for MCF-7^{ADR} cells. The verapamil-induced change of efficacy predominates at low residual levels of resistance (> 32 μM verapamil) where IC₅₀ decreases (Figure 2) despite the fact that doxorubicin accumulation is already maximal at 32 μM verapamil (Figure 3). It should be noticed that extra-

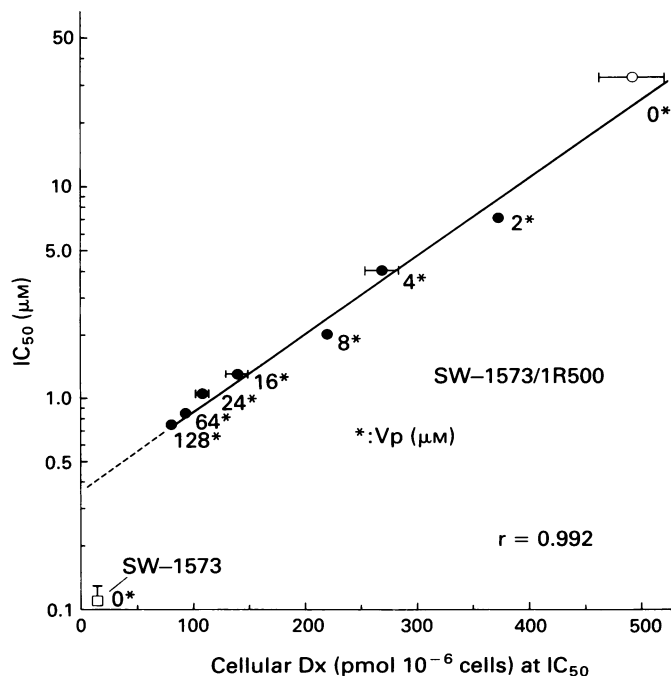


Figure 1 Relationship between logIC₅₀ and cellular doxorubicin at IC₅₀ in SW-1573/1R500 cells. Doxorubicin IC₅₀ was determined in a 2 h incubation with doxorubicin in the presence of the indicated concentrations (0–128 μM) of verapamil (Vp) as described under Materials and methods. Cellular amounts of doxorubicin at the doxorubicin IC₅₀ values thus found were measured under the same conditions in a 2 h incubation assay (see Materials and methods). O, SW-1573/1R500 minus verapamil; ●—●, SW-1573/1R500 plus verapamil. The parent cell line SW-1573 is indicated by an open square. The figure shows a representative cytotoxicity experiment (carried out in duplicate) and accumulation experiment (carried out in triplicate). Bars represent s.e.; no bars are present if s.e. is smaller than the symbol. Correlation coefficient is 0.992 for data obtained with 0–128 μM verapamil. Dx, doxorubicin; Vp, verapamil.

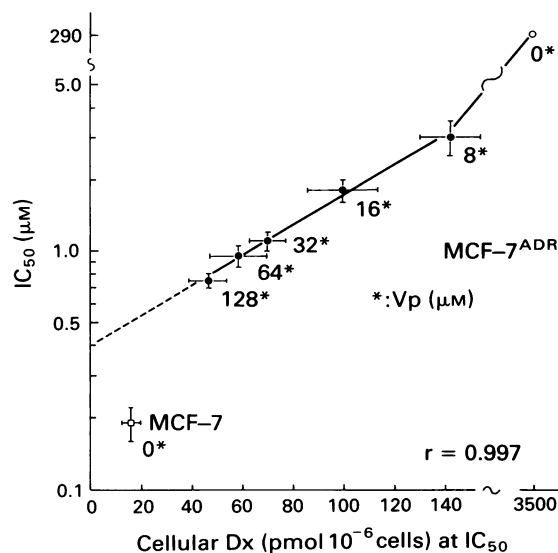


Figure 2 Relationship between logIC₅₀ and cellular doxorubicin at IC₅₀ in MCF-7^{ADR} cells. Doxorubicin IC₅₀ and accumulation data were obtained as described for SW-1573/1R500 cells in Figure 1. O, MCF-7^{ADR} minus verapamil; ●—●, MCF-7^{ADR} plus verapamil; □, the parent cell line MCF-7. Data shown are means \pm s.e. for two independent cytotoxicity and accumulation experiments each carried out in duplicate and triplicate, respectively. Correlation coefficient is 0.997 for data obtained with 8–128 μM verapamil and 0.98 with 0–128 μM verapamil. However, accumulation values at 0 μM verapamil likely have been overestimated as a result of excessive sticking of doxorubicin to the cells at the very high (290 μM) concentration used. Dx, doxorubicin; Vp, verapamil.

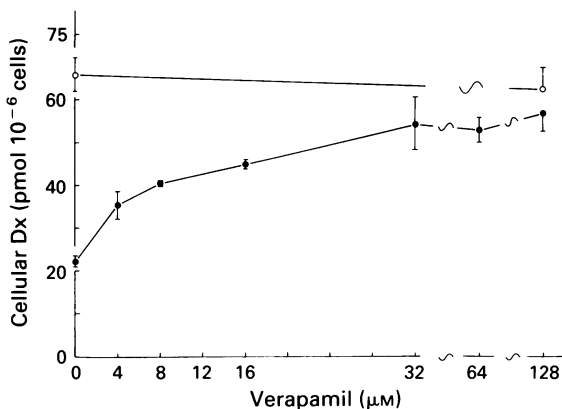


Figure 3 Verapamil-induced stimulation of doxorubicin accumulation in MCF-7^{ADR} cells. Doxorubicin accumulation was measured on trypsinised MCF-7^{ADR} and MCF-7 cells as described under Materials and methods at a fixed concentration of doxorubicin (0.5 μM) after a 2 h incubation period as a function of the verapamil concentration. ●—●, MCF-7^{ADR}, ○—○, MCF-7. Shown is a particular experiment in which each point represents mean \pm s.d. of triplicate samples. Vp, verapamil.

polated straight lines, which would predict results obtained with infinitely high concentrations of verapamil, did not cross the coordinates found for the sensitive cell lines (Figures 1 and 2).

The revertant cell line SW-1573/1R500-0, used because of its intermediate level of doxorubicin resistance (24-fold), shows similar characteristics (Figure 4). The use of 128 μM verapamil resulted in a residual level of doxorubicin resistance of a factor 2 (Figure 4). For this cell line, however, deviations from linearity in the semilogarithmic plot occurred at higher (8–128 μM) verapamil concentrations (see broken curve).

When the data shown in the Figures 1, 2 and 4 were plotted on a linear scale, part of the curve that describes the remaining doxorubicin resistance at high verapamil concentrations turned out to be a straight line (Figures 5 and 6). Strikingly, extrapolation to complete reversal of resistance showed that the line now almost crosses the origin as well as the coordinates of the sensitive SW-1573 and MCF-7 cells (Figures 5a and b, respectively). Thus at least the last part of reversal of resistance can be described by the simple function

$$\text{IC}_{50} = c \cdot [\text{dox}_i \text{ at } \text{IC}_{50}]$$

in which c is a constant and dox_i is the cellular amount of doxorubicin at each IC_{50} value. In SW-1573 variants that show a MDR phenotype without overexpression of P-glycoprotein (Baas *et al.*, 1990; Kuiper *et al.*, 1990) the same relationship was found (Figure 6).

The minimal cellular amount of doxorubicin necessary to reach IC_{50} was 8 $\text{pmol } 10^{-6}$ cells as found for SW-1573 cells in the presence of verapamil (see Figure 6). Since the majority of doxorubicin in sensitive cells is present in the nucleus (Seeber *et al.*, 1980), the nuclear amount of doxorubicin necessary to reach IC_{50} was approximately 8 $\text{pmol } 10^{-6}$ cells.

For the SW-1573/1R500-0 cells the curve did not cross the coordinates found for the sensitive SW-1573 cells (Figure 6). When, however, the intracellular doxorubicin amounts at IC_{50} were corrected for the cytoplasmic volume (which is relatively large in this cell line; see Table I) in a way described under Materials and methods and in the legends of Figure 6, a corrected line was obtained which now closely approaches the coordinates of the sensitive cell line. Only small corrections due to differences in cellular volume between sensitive and resistant cells were necessary for the other cell lines including MCF-7 cells (Table I). Corrected lines are therefore not shown in the Figures.

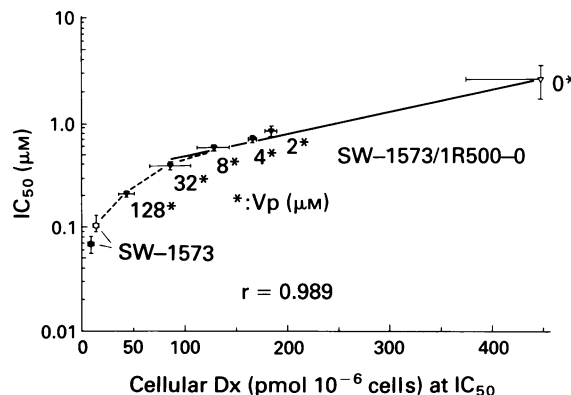


Figure 4 Relationship between $\log \text{IC}_{50}$ and cellular doxorubicin at IC_{50} in revertant SW-1573/1R500-0 cells. Doxorubicin IC_{50} and accumulation data were obtained as described for SW-1573/1R500 cells in Figure 1. Open symbols: no verapamil present; closed symbols: with verapamil. ▽, ▴: SW-1573/1R500-0 cells; ○, ●: the parent cell line SW-1573. For SW-1573 cells only 32 μM verapamil was used. Data are means \pm s.d. of three independent experiments each performed in duplicate or triplicate. Correlation coefficient is 0.989 for data obtained with 0–32 μM verapamil in SW-1573/1R500-0 cells. Broken curved line indicates possible extrapolation to sensitive (SW-1573) coordinates. Dx, doxorubicin; Vp, verapamil.

Doxorubicin N/C fluorescence ratios in sensitive and MDR cells

Previously we have presented evidence that the increased amounts of intracellular doxorubicin at IC_{50} in MDR cells and the verapamil-induced reversal of this process results from changes in intracellular distribution of doxorubicin (Schuurhuis *et al.*, 1989a). We now have measured similar resistance-related and/or verapamil-induced changes in the ratio nuclear doxorubicin fluorescence and cytoplasmic doxorubicin fluorescence in human MDR cancer cell lines (Table II). Figure 7 illustrates doxorubicin fluorescence distribution in the 2780 ovarian cancer cell lines in the presence of different concentrations of verapamil. In parental A2780 cells relatively little fluorescence is present in the cytoplasm (Figure 7a). This situation is not altered when verapamil is used (Figure 7b). In contrast, resistant 2780^{AD} cells contain a relatively large fraction of the total cellular fluorescence in the cytoplasm, either as a diffuse Golgi-like cloud or in a punctuate pattern throughout the cytoplasm (Figure 7c). Verapamil in increasing concentrations increases the percentage of cells with preferential nuclear fluorescence localisation (compare situation in the absence of verapamil in Figure 7c with d and e, which illustrate the effects of 4 and 8 μM verapamil, respectively). In the presence of 128 μM of verapamil almost all cells showed preferential nuclear fluorescence (illustrated in Figure 7f). The situation was not completely the same as in the sensitive cell line, since residual punctuate cytoplasmic fluorescence was still present. Qualitatively similar results were found for the other cell lines used (not shown here). Of particular interest doxorubicin fluorescence distribution in SW-1573/1R50 cells, which do not overexpress P-glycoprotein (Baas *et al.*, 1990), could not be modulated with verapamil similar to the situation for other non-P-glycoprotein/MDR cell lines (Schuurhuis *et al.*, 1991).

Nuclear amounts of doxorubicin at equitoxic concentrations

In Figure 8 it is shown that intracellular doxorubicin amounts at IC_{50} are increased in the MDR Chinese hamster cells and can be decreased by verapamil in a dose-dependent way as shown in this paper for other MDR cells. The photographs in Figure 9 further illustrate that this is most probably largely due to a verapamil-reversible increase of cytoplasmic doxorubicin during development of resistance. On

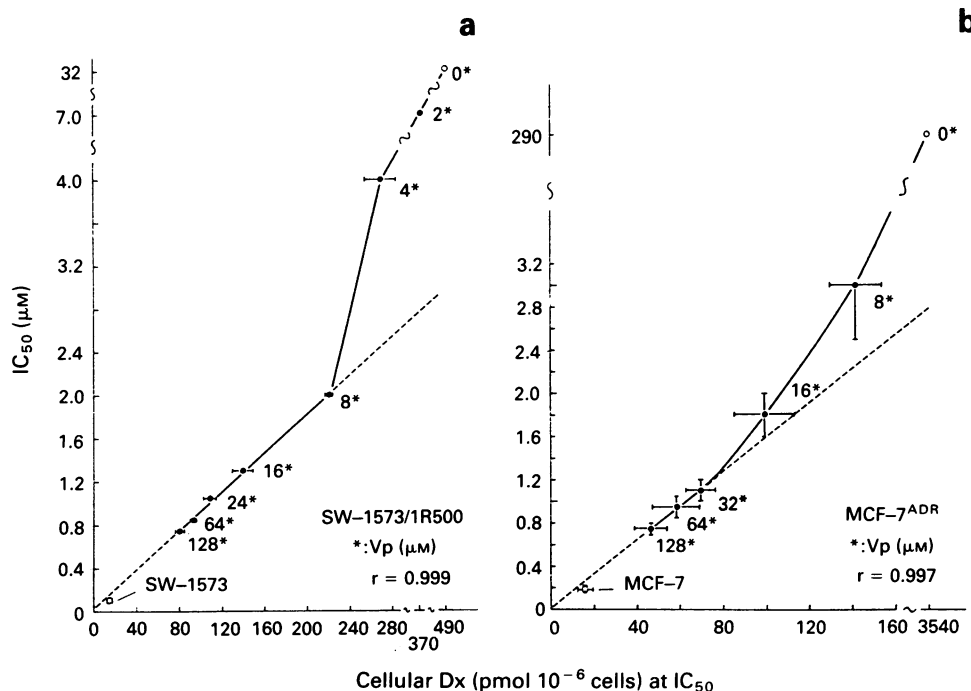


Figure 5 Relationship between IC_{50} and cellular doxorubicin at IC_{50} in SW-1573/1R500 and MCF-7^{ADR} cells. Data from the Figures 1 and 2 were replotted on a linear scale in Figure 5a (SW-1573/1R500 and SW-1573) and Figure 5b (MCF-7^{ADR} and MCF-7), respectively. For Figure 5a correlation coefficients were 0.999 for data obtained with 8–128 μM verapamil. The same was found when the data for the parent cell line SW-1573 and the origin (0,0) were included. \circ , SW-1573/1R500 minus verapamil; \bullet — \bullet , SW-1573/1R500 plus verapamil; \square , the parent cell line SW-1573. For Figure 5b the correlation coefficient was 0.997 for data obtained with 32–128 μM verapamil. The same was found when the data for the parent cell line MCF-7 and the origin were included. \circ , MCF-7^{ADR} minus verapamil; \bullet — \bullet , MCF-7^{ADR} plus verapamil; \square , the parent line MCF-7. Dx, doxorubicin; Vp, verapamil.

the other hand the photographs in Figure 9 also illustrate that the *nuclear* doxorubicin fluorescence, measured at equitoxic concentrations, may vary much less over the whole range of reversal of resistance. As a result of these processes total cellular fluorescence was much higher in CH^RC5 cells without verapamil than in AUXB1 cells without verapamil (factor >13), when measured under equitoxic conditions (700 μM and 1 μM , respectively), corresponding to the Figures 9f and a, respectively) and when using the method that enabled direct comparison of fluorescence signals (see Materials and methods under 'quantification of drug distribution with laser scan microscopy'). This difference was reduced to a factor 9.7 ± 4.3 ($n = 3$) under conditions illustrated in Figure 9e. A further reduction to 2.7 ± 1.4 ($n = 3$) was found using 8 μM doxorubicin plus 4 μM verapamil (Figure 9d). At complete reversal of resistance total cellular fluorescence was only a factor 1.2 ± 0.3 ($n = 3$) higher than in AUXB1 cells.

No quenching of cytoplasmic fluorescence has been assumed to occur (see Materials and methods; Tarasiuk *et al.*, 1989). Recently, however, a possible concentration-dependent quenching of daunorubicin in denucleated cells has been reported (Slapak *et al.*, 1992). Consequently, at the higher drug concentrations (e.g. >3 μM in Figure 8), C-values may have been underestimated. The measured N/C ratios and thereby the calculated N-values may thus have been overestimated. This would fit even better with the conclusion that N-values are constant irrespective residual resistance levels.

Unfortunately, determination of N/C ratios was inaccurate especially at higher levels of resistance because part of the cytoplasmic fluorescence overlapped the nuclear fluorescence. As a result the N value in the N/C ratio, and thereby the calculated total nuclear doxorubicin amounts, will be overestimated at the higher levels of resistance. Nevertheless Figure 8 shows that at complete reversal of resistance (32 μM verapamil) the calculated *nuclear* drug amounts are the same as in AUXB1 cells.

Discussion

From the literature it appears that there is almost consensus about the fact that multidrug resistance is multifactorial. For example, the factors mentioned to contribute to anthracycline or VP-16 resistance in MDR cells include decreased drug accumulation, caused by changes in drug influx and/or drug efflux (Bradley *et al.*, 1988) and mostly paralleled by changes in the intracellular distribution of the drug (Broxterman *et al.*, 1990; Gervasoni *et al.*, 1991; Gigli *et al.*, 1989; Hindenburg *et al.*, 1987, 1989; Keizer *et al.*, 1989; Schuurhuis *et al.*, 1989a, 1991; Willingham *et al.*, 1986), changes in topoisomerase II activity/levels (Beck, 1989) or alterations in drug activation or in the capacity to detoxify reactive drug-induced oxygen-derived free radicals (Sinha & Mimnaugh, 1990). In the present paper we have made an analysis of factors contributing to drug resistance in MDR cells by the use of a quantitative approach.

First we have shown that development of doxorubicin resistance in human lung cancer and human breast cancer cells is characterised by an increase in cellular amounts of drug measured at equitoxic drug concentrations. Apart from this decrease in drug efficacy it is known that these cells have a decreased drug accumulation compared to parent cells when exposed to a fixed external concentration of drug. This is due, at least for a part, to the presence of the putative drug P-glycoprotein efflux pump in these cells (Fairchild *et al.*, 1987; Keizer *et al.*, 1989). The decrease in drug efficacy might in principle be caused by factors such as alterations of glutathione transferase activity as mentioned above. However, an agent like verapamil, known for its potent stimulation of drug accumulation in such P-glycoprotein-containing cells (Bradley *et al.*, 1988; Ford *et al.*, 1988; Keizer *et al.*, 1989; Politi *et al.*, 1990) turned out to be active in reversing the decreased doxorubicin effectiveness as well (Figures 1, 2 and 4–6). As will be explained below it is unlikely that verapamil exerts this action via an effect on other factors

(such as glutathione transferase activity) mentioned earlier to contribute to doxorubicin resistance.

For the high level MDR cell lines it was found that reversal of resistance can be described by a function of the type

$$IC_{50} = e^{c[\text{dox}_i \text{ at } IC_{50}]}$$

in which c is a constant.

This relationship is applicable to moderate to high levels of residual resistance, i.e. resistance left upon verapamil exposure. At low to moderate residual levels of resistance the data fit into a simpler function:

$$IC_{50} = c \cdot [\text{dox}_i \text{ at } IC_{50}]$$

in which c is a constant.

In Figure 10 a simple model is depicted which may explain the relationships found between IC_{50} and $[\text{dox}_i \text{ at } IC_{50}]$. If

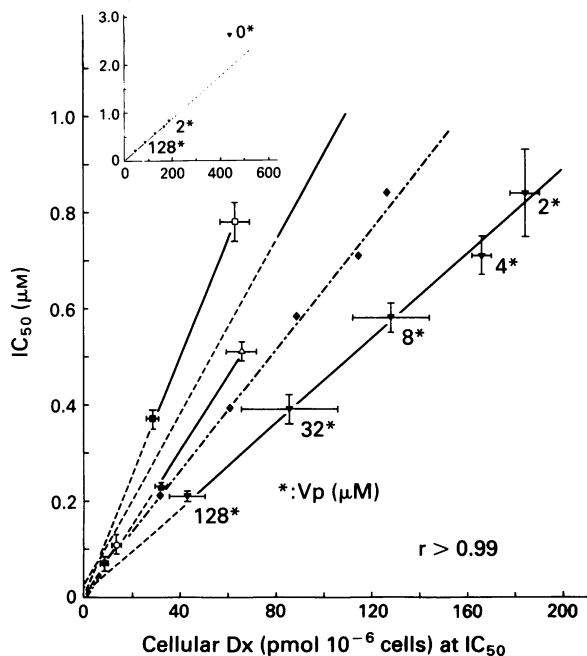


Figure 6 Relationship between IC_{50} and cellular doxorubicin at IC_{50} in SW-1573/1R500-0, SW-1573/1R50 and SW-1573/2R30 cells. Data from Figure 4 were replotted on a linear scale. In addition, data obtained for the non-P-glycoprotein/MDR cell lines SW-1573/2R30 and SW-1573/1R50 are added. Open symbols, no verapamil present; closed symbols, plus verapamil ($32 \mu\text{M}$ in SW-1573, SW-1573/1R50 and SW-1573/2R30 cells). ∇ , \blacktriangledown : SW-1573/1R500-0 cells; \square , \blacksquare : SW-1573/1R50 cells; Δ , \blacktriangle : SW-1573/2R30 cells; \circ , \bullet : parental SW-1573 cells. The correlation coefficient was >0.99 for SW-1573/1R500-0 cells for data obtained with 2–128 μM verapamil, also when the origin was included. The same was found for SW-1573/1R50 and SW-1573/2R30 cells. Note that coordinates of origin, SW-1573 cells minus verapamil (\circ) and the non-P-glycoprotein/MDR cells (\square , Δ) indicate a linear relationship between IC_{50} and cellular doxorubicin at IC_{50} in SW-1573/2R30 but not in SW-1573/1R50 cells.

The data obtained for SW-1573/1R500-0 cells when cellular amounts of doxorubicin had been corrected for cytoplasmic volume are shown too (\blacklozenge — \blacklozenge ; $r > 0.99$, when now also the coordinates of SW-1573 cells were included). Corrections were made using the formula shown under Materials and methods. It is assumed that nuclear amounts of doxorubicin are the same at all IC_{50} values found for SW-1573 and SW-1573/1R500-0 cells, which is highly likely as explained in the Results section under 'nuclear amounts of doxorubicin at equitoxic concentrations'. For reference to residual levels of resistance in the highly resistant cell line SW-1573/1R500, the last part of Figure 5a (at doxorubicin concentrations $<1 \mu\text{M}$) is indicated (— \cdots). Dx, doxorubicin; Vp, verapamil. In the inset it is shown that at verapamil concentrations $<2 \mu\text{M}$ deviations from linearity occur in SW-1573/1R500-0 cells similar to those seen at low verapamil concentrations in Figure 5.

reversal of resistance by verapamil would have been caused solely by stimulation of drug accumulation, $[\text{dox}_i \text{ at } IC_{50}]$ would have been constant in the presence or absence of different concentrations of verapamil (line I in Figure 10A). On the other hand, if hypothetically the verapamil-induced reversal of resistance would not be paralleled at all by a stimulation of drug accumulation, $[\text{dox}_i \text{ at } IC_{50}]$ should be lower in the presence of verapamil (line II in Figure 10A). With this in mind the shape of the curves as shown in the Figures 5 and 6 can easily be explained assuming that verapamil shows both effects at residual resistance levels of >17 (SW-1573/1R500), >6 (MCF-7^{ADR}) and >7.5 (SW-1573/1R500-0), while at residual resistance levels of 7–17 (SW-1573/1R500), 4–6 (MCF-7^{ADR}) and 2–7.5 (SW-1573/1R500-0) it acts almost exclusively via its accumulation-independent effect.

In the 7.4-fold resistant SW-1573/1R50 cells, which do not express P-glycoprotein (Baas *et al.*, 1990), an accumulation defect of 1.6 was found (not shown), indicating that emergence of resistance in these cells occurred both via a decrease of drug accumulation and a decrease of drug efficacy. This is compatible with the observation that a curve which would include the coordinates of the origin, SW-1573 (minus verapamil) and SW-1573/1R50 (minus verapamil) (see Figure 6) would be intermediate between lines I and II in Figure 10B. In contrast, the coordinates of origin, SW-1573 cells minus verapamil and SW-1573/2R30 cells minus verapamil (Figure 6) indicate a close-to-linear relationship between IC_{50} and $[\text{dox}_i \text{ at } IC_{50}]$. This shows that a change of doxorubicin efficacy may be the main or only factor responsible for the low degree of doxorubicin resistance in these cells

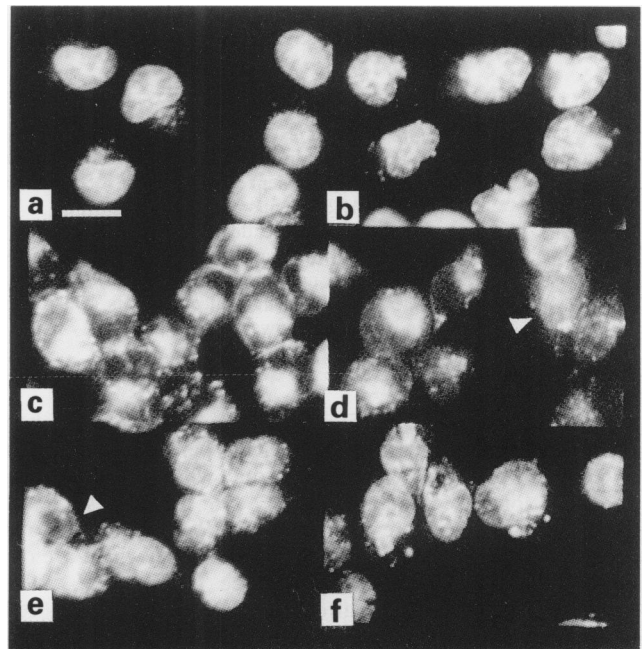


Figure 7 Subcellular doxorubicin fluorescence distribution in 2780 human ovarian cancer cells in the presence of different concentrations of verapamil. A2780 parental and 2780^{AD}MDR cells were incubated for 2 h at 37°C with doxorubicin and with different concentrations of verapamil. In order to improve detection of fluorescence in nucleus and cytoplasm, doxorubicin concentrations were used that resulted in about equal intracellular amounts at all combinations used. a, A2780; 4 μM doxorubicin, no verapamil; b, A2780; 4 μM doxorubicin, 32 μM verapamil. c, 2780^{AD}; 20 μM doxorubicin, no verapamil. d, 2780^{AD}; 15 μM doxorubicin, 4 μM verapamil. e, 2780^{AD}; 10 μM doxorubicin, 8 μM verapamil. f, 2780^{AD}; 5 μM doxorubicin, 32 μM verapamil. Note mainly nuclear localisation of fluorescence in a, b, e and f and mainly cytoplasmic localisation of fluorescence in c and d. Incidentally cells with mainly nuclear localisation can be observed under conditions illustrated in d (arrowhead); incidentally cells with mainly cytoplasmic fluorescence localisation can be seen under conditions illustrated in e (arrowhead). Bar in a, indicates 10 μm .

Table I Cellular and nuclear diameters of MDR and sensitive cells

Cell line	<i>d</i> cell (μm)	<i>d</i> nucleus (μm)	Calculated cytopl. vol. (μm^3)
SW-1573	16.15 \pm 1.20 ^a (9) ^b	10.47 \pm 0.84 (3)	1606
SW-1573/1R500	16.82 \pm 1.31 (9)	10.54 \pm 0.97 (4)	1877
SW-1573/1R500-0	17.92 \pm 0.70 ^c (7)	10.54 \pm 1.54 (2)	2399
AUXB1	12.96 \pm 0.31 (4)	–	–
CH ^{RC} ₅	13.69 \pm 1.35 (4)	–	–
MCF-7	16.20 \pm 1.64 (4)	–	–
MCF-7 ^{ADR}	16.41 \pm 0.83 (4)	–	–

^aMean \pm s.d. ^bValues in parenthesis indicate number of independent experiments. ^cSignificantly different from SW-1573 value ($P < 0.01$, Student's *t*-test). –, not determined.

Table II Subcellular doxorubicin fluorescence distribution in human breast, lung and ovarian cancer MDR and sensitive cell lines

Cell line	Modifier (μM)	IC ₅₀ ^a (μM)	[dox _i at IC ₅₀ ^a] (pmol 10 ⁻⁶ cells)	N/C ratio ^b
MCF-7	None	0.19	16	exp.1: 3.6 \pm 1.1 exp.2: 3.2 \pm 0.9
MCF-7 ^{ADR}	None	290	3542	exp.1: 0.1 \pm 0.1 exp.2: 0.1 \pm 0.1
	16 Vp	1.8	99	exp.1: 0.8 \pm 0.3
	128 Vp	0.75	46	exp.1: 2.5 \pm 0.8 exp.2: 1.3 \pm 0.7
SW-1573	None	0.11	13.5	2.8 \pm 0.1 ($n = 3$)
SW-1573/1R500	None	38	490	0.3 \pm 0.3
	8 Vp	2	220	1.7 \pm 1.5
	128 Vp	0.75	80	2.2 \pm 1.0
SW-1573/1R500-0	None	2.6	448	1.2 \pm 0.7
	128 Vp	0.21	43	2.5 \pm 1.1
SW-1573/1R50	None	0.78	63	1.9 \pm 0.1* ($n = 4$)
	32 Vp	0.37	28	1.8 \pm 0.1
A2780	None	0.24	24	2.9 \pm 1.2
2780 ^{AD}	None	17.4	128	0.4 \pm 0.2
	32 Vp	0.45	35	2.7 \pm 1.0

^aMean of 2–7 independent experiments (s.s. < 20%). ^bMean (\pm s.s.) of at least 30–50 cells in a representative experiment, except in the case of MCF-7 and MCF-7^{ADR} cells (two independent experiments as indicated; each 30–50 cells) and SW-1573 and SW-1573/1R50 cells (given are M \pm SD of 3–4 independent experiments). *Significantly different from N/C ratio of SW-1573 parent cells ($P < 0.01$, Student's *t*-test).

N/C ratios were determined at IC₅₀ values of doxorubicin except for the sensitive cells or when verapamil (Vp) was used (in those cases 2 μM doxorubicin was used since at lower concentrations background noise became too high). N/C ratios are independent of doxorubicin concentration in a large range of drug concentrations (De Lange *et al.*, 1992), suggesting that this is also the case at concentrations lower than 2 μM .

(compare with line II in Figure 10A and B. This has been confirmed in accumulation experiments performed at a fixed (0.5 μM) doxorubicin concentration which revealed no accumulation defect (not shown).

Continuous exposure of SW-1573/2R30 cells to doxorubicin led to isolation of SW-1573/2R50 cells, which are of a non-P-glycoprotein MDR phenotype (Baas *et al.*, 1990; Kuiper *et al.*, 1990) and after prolonged exposure to cells with an increased *mdr-1* mRNA and P-glycoprotein expression (Kuiper *et al.*, 1990). Similar results have been found for murine erythroleukaemia cells (Slapak *et al.*, 1990). It could thus be that doxorubicin resistance in low level MDR non-P-glycoprotein lung cancer cells as well as doxorubicin resistance of early selected cells of the high level MDR P-glycoprotein cell lines MCF-7^{ADR} and 2780^{AD}, is caused, at least partly, by the same P-glycoprotein-independent phenotypic change that occurs in P-glycoprotein-mediated resistance, i.e. a change in intracellular drug efficacy, which would, at least partly, be caused by a change in subcellular drug distribution.

In the present study it is shown that a partial reversal of doxorubicin resistance was accomplished in human MDR cells which was accompanied by a partial reversal of N/C ratio changes (Table II). Complete reversal of doxorubicin resistance in CH^{RC}₅ P-glycoprotein/MDR Chinese hamster ovarian cells to parental AUXB1 levels was accompanied by a complete reversal of the drug accumulation defect as well

as the drug distribution change (Schuurhuis *et al.*, 1989a). The consequence of these findings would be that it is mainly if not only the nuclear concentration of drug which determines sensitivity/resistance. For CH^{RC}₅ cells we confirmed this relationship (Figures 8, 9). It cannot be excluded, however, that verapamil acts via a mechanism comprising increment of nuclear doxorubicin efficacy. This mechanism of resistance reversal might prevail in non-P-glycoprotein MDR cells. The fact that considerable decrease of doxorubicin resistance in such cells by verapamil is possible with only small concomitant changes of drug accumulation or drug distribution (this paper; Schuurhuis *et al.*, 1991), are in favour of this idea. The results presented in the present paper for P-glycoprotein MDR cells are nevertheless in agreement with a paper of Gigli and colleagues, who did not study verapamil effects, but, making use of spectral properties of doxorubicin, showed that nuclear concentrations of doxorubicin were not significantly different at equitoxic drug concentrations in sensitive and resistant K562 human leukaemia cells (Gigli *et al.*, 1989). Also, in a study comparing cellular doxorubicin accumulation, cytotoxicity and DNA lesions in sensitive and multidrug-resistant human myeloma cells, Bellamy *et al.* showed that at low doxorubicin concentrations important for cytotoxicity, equal cellular concentrations in both sensitive and resistant cells, obtained by adjustment of extracellular drug concentrations, nevertheless caused less cytotoxicity in resistant cells (Bellamy *et al.*, 1988b). DNA

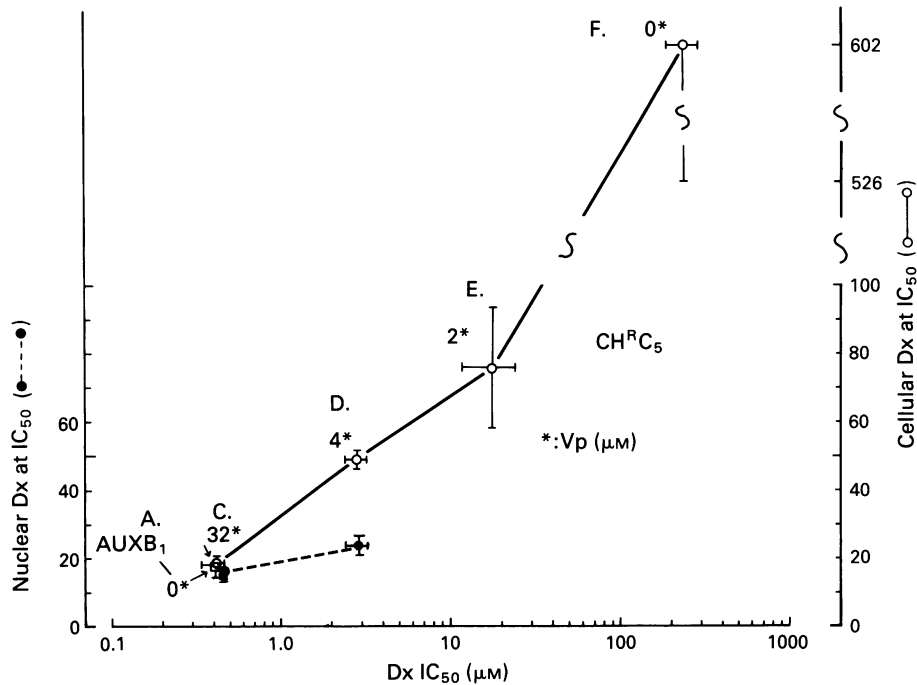
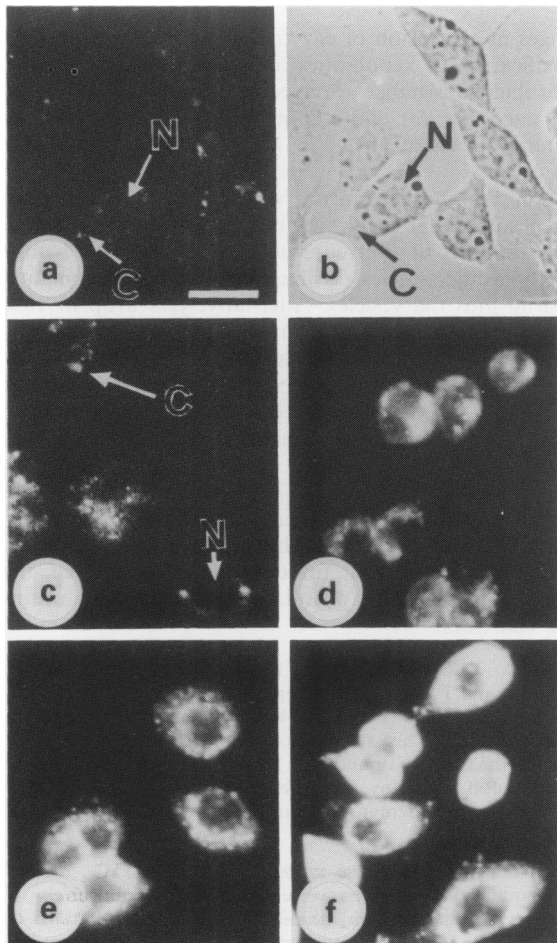


Figure 8 Interrelationship between IC_{50} , cellular doxorubicin at IC_{50} and nuclear doxorubicin at IC_{50} in adherent $CH^R C_5$ and $AUXB_1$ cells. IC_{50} values were obtained in an assay including a 2 h incubation with doxorubicin as described under Materials and methods. Total cellular amounts of doxorubicin at IC_{50} obtained with different concentrations of verapamil were determined in a 2 h incubation assay using [^{14}C]-labelled doxorubicin as described under Materials and methods. Nuclear amounts of doxorubicin were calculated from N/C fluorescence ratio measurements with laser scan microscopy (corrected for fluorescence quenching by DNA as described under Materials and methods) and from the accumulation experiments described above (giving total cellular amounts of doxorubicin). Open symbols: measured cellular amounts of doxorubicin in $CH^R C_5$ cells (○—○) and $AUXB_1$ cells (□); closed symbols: calculated nuclear amounts of doxorubicin in $CH^R C_5$ cells (●---●) and $AUXB_1$ cells (■). Doxorubicin amounts are in $pmol\ 10^{-6}$. No verapamil was used for $AUXB_1$ cells. Data are means \pm s.d. from 3–9 independent cytotoxicity, accumulation and N/C fluorescence ratio measurements. The numbers a, c-f refer to the photographs shown in Figure 9.



damage in the resistant cells was also less under these conditions (compare Bellamy *et al.*, 1988a and b). This is in good agreement with the observation that N/C ratios are lower in these resistant cells (De Lange *et al.*, 1992; Broxterman *et al.*, 1990). This suggests, as outlined extensively in the present paper, that higher cellular concentrations are needed in the resistant cells in order to obtain the same nuclear concentrations as in sensitive cells.

It has to be stated that other reversing agents behave in a way similar to verapamil: bepridil, Ro 11-2933/001 and Cremophor EL have been tested as to this (Schuurhuis *et al.*, 1989a, 1990a). None of the modifiers, even at high concentrations, was able to overcome completely accumulation defects in human MDR cells (see e.g. Schuurhuis *et al.*, 1990b). Verapamil has been used mainly because it is less toxic than several other reversing agents and because it is the most widely studied reverter.

Figure 9 Subcellular doxorubicin fluorescence distribution in the presence or absence of verapamil at equitoxic concentrations in $CH^R C_5$ and $AUXB_1$ cells. Adhered cells were incubated for 2 h at 37°C with combinations of different concentrations of doxorubicin and verapamil, as specified below, which all resulted in the same growth-inhibition. Doxorubicin concentrations used were in all cases 2.5 times higher than the IC_{50} values, shown in Figure 7, since otherwise the fluorescence in nucleus and/or cytoplasm could hardly be visualised and quantified. Viability as determined with the trypan blue exclusion test remained $>99\%$ for at least 12 h when cells were incubated without any drug present after the 2 h period in the presence of doxorubicin and/or verapamil. a and c–f: fluorescence microscopy; b: phase contrast microscopy. a, $AUXB_1$, 1 μM doxorubicin without Vp. b, $AUXB_1$, same cells as in a, phase contrast. c, $CH^R C_5$, 1 μM doxorubicin plus 32 μM Vp. d, $CH^R C_5$, 8 μM doxorubicin plus 4 μM Vp. e, $CH^R C_5$, 50 μM doxorubicin plus 2 μM Vp. f, $CH^R C_5$, 700 μM doxorubicin without Vp. N, nucleus; C, cytoplasm; Dx, doxorubicin; Vp, verapamil. Bar in a indicates 20 μm .

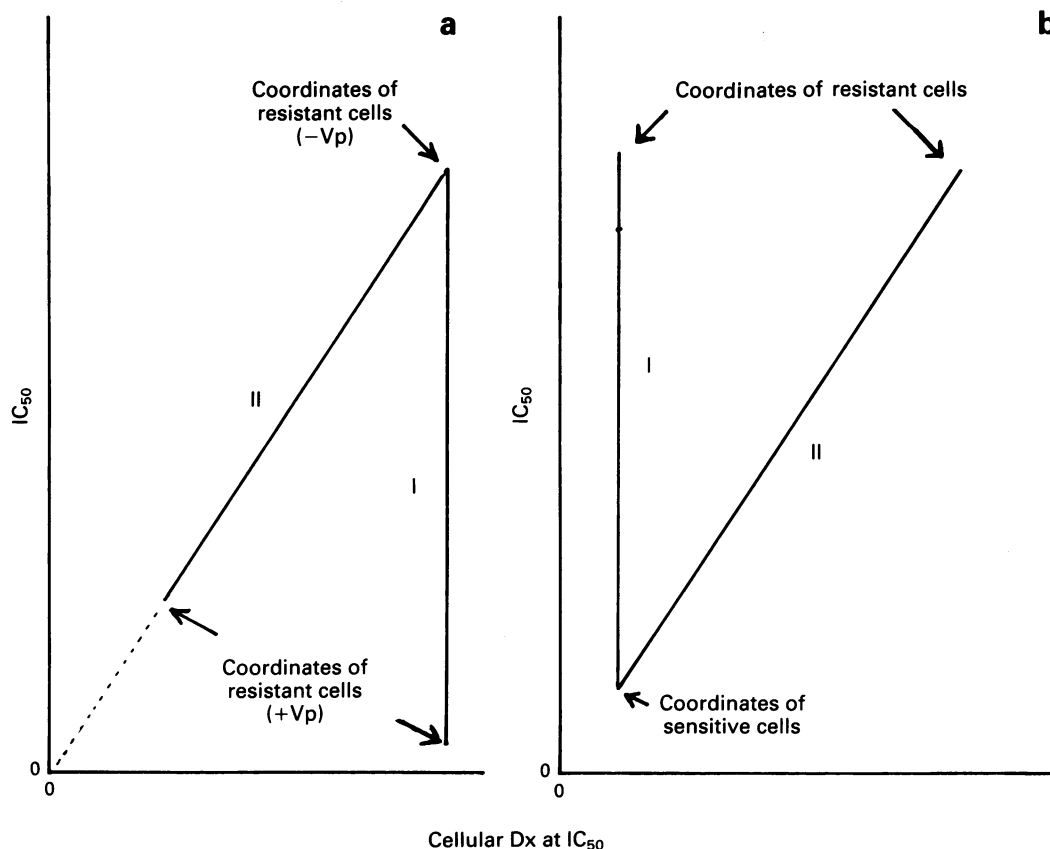


Figure 10 Theoretical relationship between IC_{50} and cellular doxorubicin measured at IC_{50} assuming that two factors contribute to doxorubicin resistance and that two modes of action of verapamil exist. **A**, Reversal of resistance by verapamil occurs solely by stimulation of drug accumulation (I) or solely by changing the efficacy of cellular doxorubicin (II). **B**, Emergence of resistance occurs solely by a decrease of drug accumulation (I) or solely by changes in efficacy of cellular doxorubicin (II).

Apart from MDR Chinese hamster ovarian (this paper; Schuurhuis *et al.*, 1989a), human ovarian cancer (this paper; Schuurhuis *et al.*, 1989a), human lung cancer (this paper; Keizer *et al.*, 1989) and human breast cancer (this paper; Gervasoni *et al.*, 1991) cells, also P-glycoprotein containing KB (Willingham *et al.*, 1986), human myeloma (Broxterman *et al.*, 1990) and human leukaemia (Gigli *et al.*, 1989) cells show changes in subcellular drug distribution in addition to changes in cellular drug accumulation. It is therefore tempting to speculate that the relatively simple explanation for doxorubicin resistance in P-glycoprotein/MDR cells, including the two factors depicted in this paper, is generally applicable.

It has been speculated that changes in free radical detoxifying enzymes such as glutathione transferase and glutathione peroxidase contribute to doxorubicin resistance in P-glycoprotein/MDR MCF-7^{ADR} cells (Batist *et al.*, 1986; Cowan *et al.*, 1986; Sinha *et al.*, 1987; Sinha & Mimnaugh, 1990). From our results, however, it appears that also in MCF-7 MDR cells decreases in drug accumulation together with changes in drug distribution can largely account for doxorubicin resistance. This conclusion is based on proven effects of verapamil on drug accumulation and drug distribution. We have found no anomalous effects of verapamil on glutathione transferase, which might have coincided with resistance reversal: this enzyme (48-fold increased in MCF-7^{ADR} in our hands) was not affected by verapamil concentrations as high as 1 mM (not shown). Such verapamil concentrations are beyond those calculated to occur in cells using effective verapamil concentrations (Broxterman *et al.*, 1988; Canogauci & Riordan, 1987; Yusa & Tsuruo, 1989). Verapamil also had no effect on glutathione peroxidase activity (Batist *et al.*, 1991). Lastly, it has been found that transfection of the gene encoding for glutathione transferase did not confer resistance to doxorubicin (Moscow *et al.*, 1989). Since

changes in expression of enzymes suggested to be involved in detoxification of xenobiotics apparently are not involved in doxorubicin resistance we speculate that transfection of the gene encoding for glutathione peroxidase will not confer resistance to doxorubicin in MCF-7 or other parental cell lines.

Upon the mechanism by which MDR cells acquire an altered subcellular drug distribution can only be speculated. Small amounts of P-glycoprotein present on the membranes of cytoplasmic vesicles (Willingham *et al.*, 1987) might force the drug into the vesicles. Alternatively, a drop in vesicular pH, probably paralleling an increase of the cytoplasmic pH under certain conditions (Keizer & Joenje, 1989; Thiebaut *et al.*, 1990) or an increase of the vesicular compartment (Sehested *et al.*, 1987) might explain higher drug accumulation in cytoplasmic compartments, resulting in decreased N/C ratios.

In conclusion, the results presented in this paper show that, at least in P-glycoprotein MDR cells, changes in drug accumulation together with changes in subcellular drug distribution largely account for doxorubicin resistance, thereby excluding the need to postulate additional mechanisms for these cell lines.

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Abbreviations: DMF, dose modifying factor = IC_{50} minus resistance modifier/ IC_{50} plus resistance modifier; Dx, doxorubicin; IC_{50} = drug concentration, which inhibits cell growth by 50% of control values; MDR, multidrug resistant/resistance; Vp, verapamil.

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