Anti-Cancer Drugs 1997, 8, pp. 610–617

Cellular pharmacology of lipophilic anthracyclines in human tumor cells in culture selected for resistance to doxorubicin

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We have studied the cytotoxicity and intracellular accumulation of two lipophilic anthracyclines, pirarubicin and idarubicin, as compared to doxorubicin, in two human tumor cell lines, MCF7 and K562, and in their doxorubicin-resistant counterparts, presenting the multidrug-resistant (MDR) phenotype. The new lipophilic anthracyclines were found to present a higher cytotoxicity and accumulation than the reference anthracycline, doxorubicin, and there was a significant inverse correlation between drug accumulation and IC_{50} in both cell types. With the aim of identifying the reasons for the higher cytotoxicity and accumulation of lipophilic anthracyclines, we used and compared the efficiency of three MDR modulators, verapamil, quinine and S-9788. We showed that all three were able to sensitize the resistant cells to the three anthracyclines, but with different efficiencies, S-9788 being the most active reverter and quinine the least active at equimolar doses. We also observed that there was no correlation between the abilities of a modulator to reverse resistance and to restore drug accumulation. In view of the sustained activity of the modulators to increase pirarubicin and idarubicin cytotoxicity and accumulation, as they do for doxorubicin, we conclude that the better efficiency of lipophilic anthracyclines is likely to be due to their high uptake rate rather than to a decreased activity of P-glycoprotein on these drug substrates.

Key words: Anthracyclines, *in vitro* cytotoxicity, multidrug resistance.

Introduction

Multidrug resistance in cancer is likely to be a major cause of treatment failure in the clinical setting.[1] It is characterized by a reduced accumulation of the anticancer agent, which is actively expelled out of the cells by a plasma membrane transporter, P-glycoprotein, whose poor specificity explains the pattern of cross-resistance featuring multidrug resistance.[2] An approach to overcome this problem is the development of drugs able to inhibit P-glycoprotein-mediated drug efflux (MDR modulators[3]); another one is the identification of anticancer drugs devoid of cross-resistance with the original compounds.

Anthracyclines are of special interest in circumventing the mechanism of multidrug resistance as the anthracyclines, doxorubicin and daunorubicin are good substrates of P-glycoprotein. Several new anthracyclines have been developed in the recent years and introduced in routine clinical use.[4,5] These drugs were mostly developed with the aim of reducing the cardiac toxicity of doxorubicin, a problem that has not been solved yet and still warrants research.[6] However, another point of interest in developing new anthracyclines lies in the search for molecules which do not display cross-resistance to the classical anthracyclines and would be able, therefore, to circumvent multidrug resistance.[7]

Increased lipophilicity appears to be a general character of the new anthracyclines recently developed. Idarubicin and pirarubicin are both characterized by an octanol/water partition coefficient much higher than that obtained for doxorubicin and daunorubicin.[8] These properties are known to strongly modify the interactions of drugs with membranes, especially at the level of drug transport. Anthracyclines are generally considered to enter the cells by passive diffusion through the plasma membrane as unionized forms and to be eventually expelled out of resistant cells by active transporters such as P-glycoprotein.[9] In theory, the new lipophilic anthracyclines could overcome multidrug resistance either because their influx in increased in such proportions that P-glycoprotein cannot expel the drug with sufficient efficacy or because these drugs cannot be recognized and transported by P-glycoprotein, resulting in higher steady-stade accumulation levels within the cells.[10]

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In this study, we have compared the pharmacological properties of idarubicin and pirarubicin as compared to the reference anthracycline, doxorubicin. Furthermore, we attempted to correlate the patterns of drug accumulation and cytotoxicity in two models of human tumor cells in culture and in their doxorubicin-resistant counterparts, presenting the MDR phenotype. Using P-glycoprotein modulators such as verapamil, we also investigated the degree of drug uptake and extrusion in the differential levels of resistance to doxorubicin and lipophilic anthracyclines, repectively.

Material and methods

Drugs and products

Doxorubicin and idarubicin were kindly provided by Pharmacia (Saint-Quentin-en-Yvelines, France) and pirarubicin by Bellon Rhône-Poulenc Rorer (Neuilly, France). Verapamil was used as clinical formulation (Isoptine®; Knoll, Levallois-Perret, France), S-9788 was provided by Laboratoire Servier (Courbevoie, France) and quinine was purchased from Sigma (Saint-Quentin-Fallavier, France). All other chemicals were of the better analytical grade commercially available.

Cell culture

The MCF7 human breast cancer cell line[11] and its doxorubicin-resistant variant, MCF7 doxR,[12] were obtained from Dr K. Cowan (Bethesda, MD). The K562 human leukemic cell line[13] and its doxorubicin-resistant variant, K562 doxR,[14] were obtained from Dr M. Manfait (Reims, France). They were routinely cultivated in Petri dishes (Nunc, Denmark) with RPMI 1640 medium (Seromed, Germany) supplemented with 10% fetal calf serum (Seromed) at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were replicated each week and culture medium was renewed once or twice in between. Resistant cells were continuously grown in the presence of doxorubicin to maintain the selection pressure: 10 μM for the MCF7 doxR cells, 0.34 μM for the K562 doxR cells.

Evaluation of growth inhibition

Appropriate numbers of cells were seeded in the wells of 96-well plates, such that exponential growth of the cells could last for 5 days after seeding. Incubations with the anthracyclines at doses ranging between 0.001 and 32 μg/ml lasted for 2 h, and were performed on day 1 or 2 after seeding for K562 and MCF7 cells, respectively. After rinsing and addition of fresh medium, the cells were allowed to regrow for 2 or 3 days for K562 and MCF7 cells, respectively. Cell survival was then evaluated using the MTT assay. Briefly, the cell culture medium was replaced by fresh medium containing 0.5 mg/ml MTT. After 4 h incubation, the MTT-formazan crystals were dissolved in DMSO by gentle stirring, and the absorbance of each well measured at 570 and 630 nm on a Biotek photometer. The absorbance in the treated cells was plotted as a percentage of that of untreated cells and the IC_{50} calculated by interpolation, as the drug concentration causing 50% reduction in absorbance. All measurements were made in triplicate and three to five independent experiments were always performed.

Evaluation of drug accumulation

Appropriate numbers of cells were seeded in 10 or 20 cm^2 Petri dishes with 3 or 5 ml medium, such that 4 days later, the cell numbers approximate 2×10^6 cells per Petri dish still in exponential phase of growth. Incubations were performed with two drug concentrations, 1 and 10 μg/ml, and for various periods of time (5 min to 24 h). Then, the cells were recovered, rinsed several times at 4°C as rapidly as possible to avoid drug efflux and pelleted at 3000 r.p.m. for 5 min; 0.5 ml water and 0.5 ml 40% trichloroacetic acid were successively added and the samples were kept at 4°C overnight, then centrifuged for 30 min at 3000 r.p.m. The acid-soluble supernatant was used to evaluate intracellular concentration of the anthracycline by fluorimetry using a spectrofluorometer, model SFM25 (Kontron Instruments), with excitation and emission wavelength, respectively, set at 468 and 592 nm. The acid-insoluble pellet was used to evaluate protein concentration by the Bradford assay.

Evaluation of the reversing properties of verapamil, quinine and S-9788

The MDR reversing properties of two well-known modulators, verapamil and quinine, and of a modulator under evaluation, S-9788, were evaluated on

the cytotoxicity of doxorubicin, pirarubin and idarubicin towards MCF7 doxR and K562 doxR, as well as on the intracellular accumulation of these drugs. For the study of resistance reversal, all three modulators were used at the concentrations of 0.1, 1, 10 and 50 μM, and the anthracyclines at the doses of 0.03, 0.1, 0.3, 1, 3, 10 and 30 μg/ml. Modulators were incubated simultaneously with the anthracycline for 2 h, according to the experimental schedule already described. For studying the restoration of drug accumulation, the same concentrations of modulators were used, and the doses of anthracyclines were set at 1.5 and 10 μg/ml.

Results

Anthracycline-induced growth inhibition

Figure 1 presents the cytotoxicity profiles of doxorubicin, pirarubicin and idarubicin against MCF7 and K562 cells, and Table 1 lists the IC_{50} of the three drugs and the degree of resistance of the resistant lines.

The MCF7 doxR cells were highly resistant to doxorubicin (more than 1000-fold), whereas the K562 doxR cells had a much lower resistance factor. For both cell types, pirarubicin and idarubicin

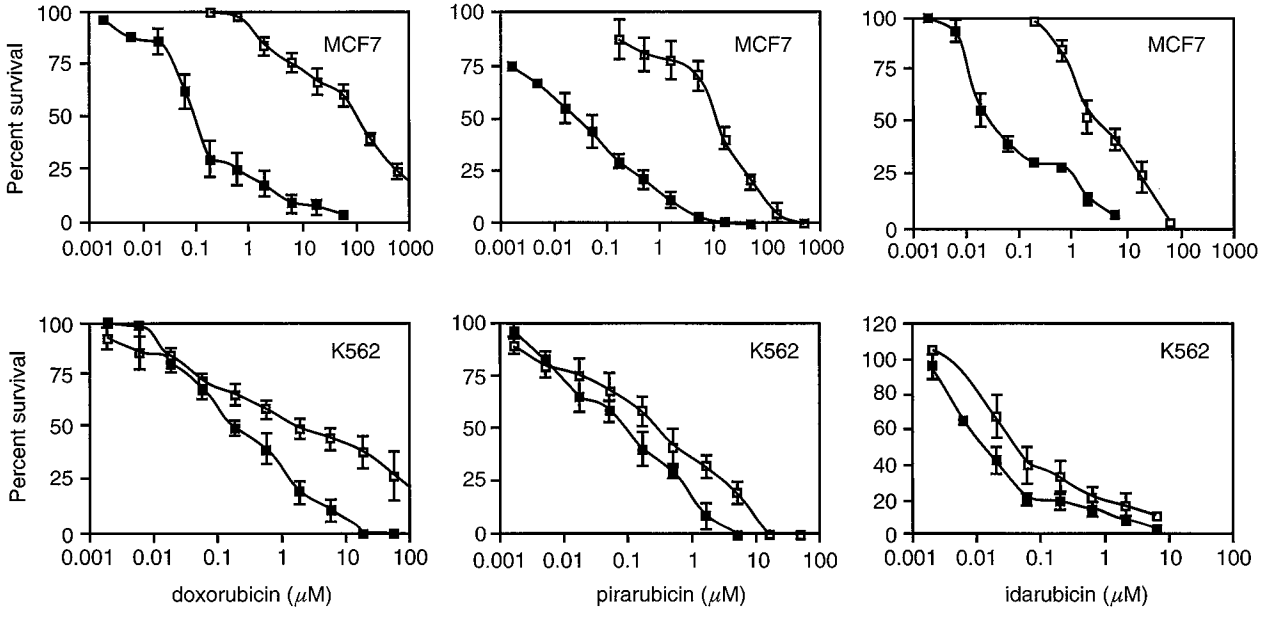

Figure 1. Cytotoxicity of doxorubicin (left), pirarubicin (center) and idarubicin (right) in MCF7 (upper diagrams) and K562 (lower diagrams) cells, and their doxorubicin-resistant counterparts. Cell survival was evaluated after 2 h incubation in the presence of various concentrations of each drug, followed by 2.5 cell cycles of regrowth. Results are means ± SD of at least three independent measurements. Sensitive cells, ■; resistant cells, □.

Table 1. Growth inhibition of sensitive and doxorubicin-resistant MCF7 and K562 cells by doxorubicin, pirarubicin and idarubicin

	MCF7 WT IC_{50} (μM)	MCF7 doxR IC_{50} (μM)	Resistance factor	K562 S IC_{50} (μM)	K562 doxR IC_{50} (μM)	Resistance factor
Doxorubicin	0.089 ± 0.004	109 ± 12	1225	0.17 ± 0.02	2.58 ± 0.60	15
Pirarubicin	0.029 ± 0.014	10.0 ± 0.6	345	0.082 ± 0.024	0.27 ± 0.01	3.29
Idarubicin	0.015 ± 0.009	2.73 ± 0.17	182	0.015 ± 0.004	0.043 ± 0.01	2.87

IC_{50} are the drug extracellular concentrations providing a decrease of 50% of cell survival, evaluated as described in Materials and methods. IC_{50} values are the means ± SD of at least three independent experiments performed in triplicate.

appeared more cytotoxic than doxorubicin, and the difference between doxorubicin and the lipophilic anthracyclines was more important in the resistant cells than in the sensitive ones. As a consequence, the resistance factor to pirarubicin and idarubicin was markedly less than the resistance factor to doxorubicin in both cell types.

Anthracycline accumulation in sensitive and resistant cells

When studied as a function of time at two concentrations (1 and 10 μg/ml) the accumulation of doxorubicin in both sensitive lines was relatively slow, having not yet reached a plateau after 4 h (Figure 2). In contrast, the accumulation of pirarubicin and idarubicin was much more rapid, the maximum concentration being reached after 1 h or even before. The level of accumulation reached by lipophilic anthracyclines was higher than that reached by doxorubicin. For all three drugs, there was a proportionality between the exposure dose and the intracellular accumulation of the drug.

In resistant cells incubated with 10 or 32 μg/ml of anthracyclines, the accumulation was always rapid (maximum level generally reached in 30 min). When the same exposure dose was used (10 μg/ml), resistant cells incorporated less drug than sensitive cells and the accumulation ratio sensitive/resistant cells was higher for MCF7 cells than for K562 cells, corresponding to the much higher resistance factor exhibited by MCF7 doxR cells as compared to K562 doxR cells. As in sensitive cells, there was a proportionality between the exposure dose and the level of intracellular accumulation, and, also, pirarubicin and idarubicin accumulated to a higher extent than doxorubicin. Table 2 summarizes the data on steady-state drug accumulation as measured after 2 h of drug exposure.

It should be emphasized that the ratio of drug accumulation in sensitive to that of resistant cells was higher for doxorubicin (31 in MCF7 cells and 5 in K562 cells for a 10 μg/ml exposure dose) than for lipophilic anthracyclines: for idarubicin (10 μg/ml exposure dose), it amounts, for instance, to 3.2 in MCF7 cells and to 1.5 in K562 cells.

Reversal of resistance and restoration of drug accumulation by MDR modulators

All three modulators were able to sensitize MCF7 doxR and K562 doxR cells, but with quite different efficiencies. Figure 3 shows the progressive decrease of the IC_{50} of each anthracycline when increasing the dose of modulator. S-9788 was the most potent

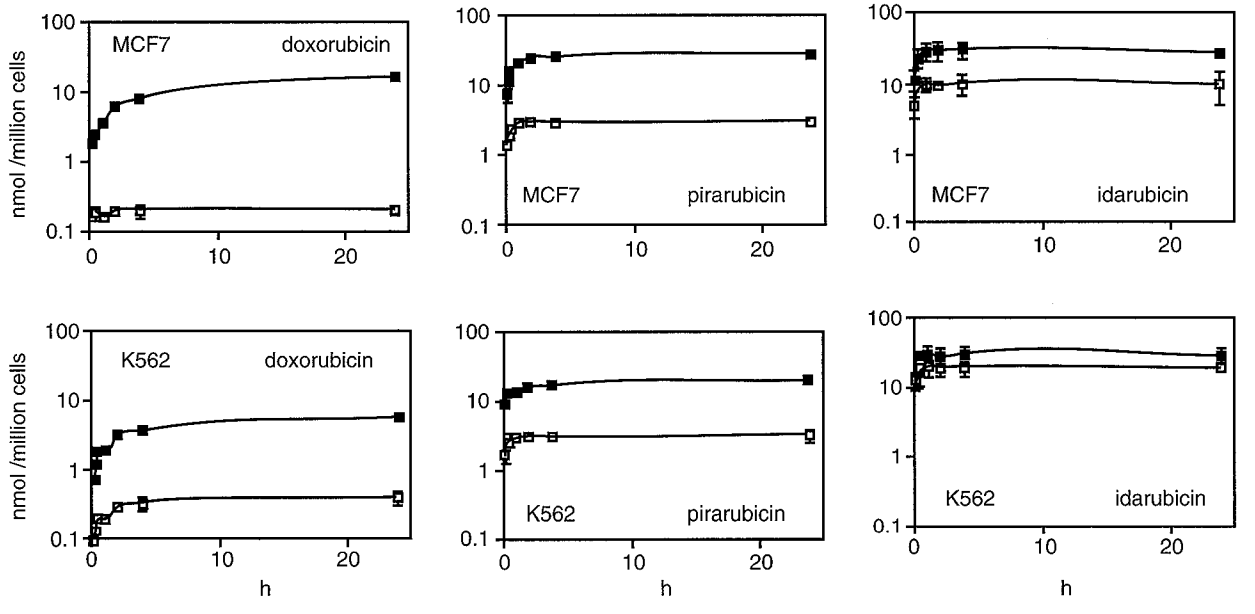

Figure 2. Accumulation of doxorubicin (left), pirarubicin (center) and idarubicin (right) in MCF7 (upper diagrams) and K562 (lower diagrams) cells, and their doxorubicin-resistant counterparts. Drug accumulation was evaluated after various exposure times to a constant amount of 10 μg/ml of each drug. Drug accumulation is expressed in nmol drug per million cells. Results are means ± SD of at least three independent experiments. Sensitive cells, ■; resistant cells, □.

Table 2. Intracellular accumulation of doxorubicin, pirarubicin and idarubicin in sensitive and doxorubicin-resistant MCF7 and K562 cells after 2 h exposure to these drugs

	Intracellular accumulation (nmol/10^6 cells)			
	MCF7 WT	MCF7 doxR	K562 S	K562 doxR
Doxorubicin				
1 μg/ml	0.62 ± 0.08	–	0.31 ± 0.03	–
10 μg/ml	6.20 ± 0.82	0.20 ± 0.04	3.10 ± 0.30	0.62 ± 0.09
32 μg/ml	–	0.65 ± 0.11	–	1.84 ± 0.17
Pirarubicin				
1 μg/ml	2.41 ± 0.30	–	1.46 ± 0.2	–
10 μg/ml	21.6 ± 1.8	2.91 ± 0.37	13.1 ± 1.9	2.99 ± 0.25
32 μg/ml	–	9.3 ± 1.4	–	8.45 ± 0.50
Idarubicin				
1 μg/ml	3.13 ± 0.22	–	4.32 ± 0.54	–
10 μg/ml	30.9 ± 9.5	9.65 ± 1.21	28.4 ± 6.5	19.1 ± 4.8
32 μg/ml	–	36.5 ± 7.3	–	36.0 ± 8.6

Intracellular accumulation was evaluated as described in Materials and methods after 2 h exposures to two drug concentrations. Values are means ± SD of at least three independent experiments performed in triplicate.

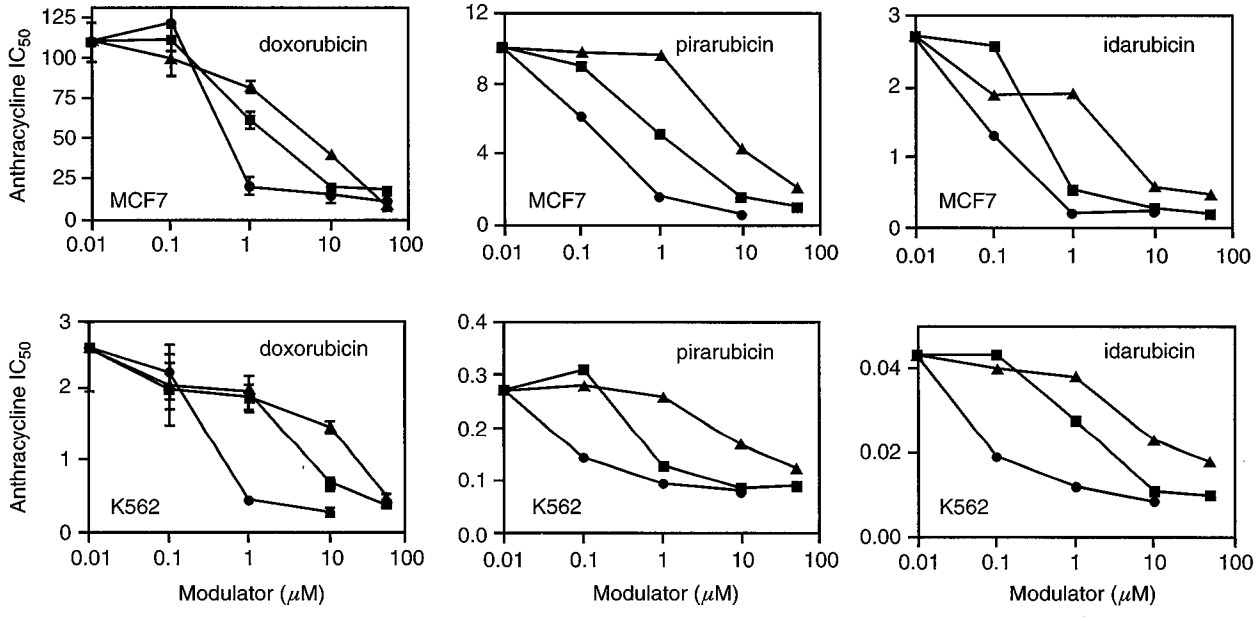

Figure 3. Variation of doxorubicin (left), pirarubicin (center) and idarubicin (right) IC_{50}s in MCF7 (upper diagrams) and K562 (lower diagrams) doxorubicin-resistant cells in the presence of various concentrations of several modulators: verapamil, ■; quinine, ▲; S-9788 ●. The IC_{50}s were evaluated from the cell survivals after 2 h incubations with various concentrations of each drug, at defined concentrations of the modulators, followed by 2.5 cell cycles of regrowth. Results are means ± SD of three independent experiments.

reversing agent and quinine the least active. The three modulators allowed us to reach a roughly similar degree of sensitization, which could not be further increased beyond this level. This maximal effect was reached with 1 μM S9788, 10 μM verapamil and 50 μM quinine. It was, however, impossible to reach, even with the highest concentration of modulator, the level of anthracycline cytotoxicity obtained in the corresponding sensitive cells. A residual degree of resistance remained present, which was markedly higher in MCF7 cells (135-fold for doxorubicin) that in K562 cells (1.7-fold for doxorubicin). There was no fundamental difference between the two resistant cell lines, apart from the fact that the basal anthracycline IC_{50} (i.e. with no modulator) was much higher for MCF7 doxR cells than for K562 doxR cells, as already mentioned. There was also no important difference between the

three anthracyclines, the IC_{50} of all them decreasing in parallel upon the addition of the modulators.

An increase in intracellular anthracycline accumulation upon incubation with MDR modulators was obtained with all three anthracyclines (Figure 4). There again, there were differences between the efficiencies of the three modulators, S-9788 being the most active drug and quinine the least active. It was remarkable, however, that marked discrepancies may exist between the restoration of drug accumulation and the reversal of drug resistance. With doxorubicin, S-9788 was the only modulator to restore drug accumulation to the level reached in sensitive cells under the same conditions; however, even with this level of drug accumulation, the reversal of the resistance was incomplete and there remained an important residual resistance, especially in MCF7 cells. Another important observation is the fact that the maximal degree of reversal of resistance occurred in fact for a dose of modulator which does not fully restore drug accumulation to its maximal degree. This is particularly clear for quinine, which was able, at 50 μM, to reduce doxorubicin resistance by a factor of 12 in MCF7 cells, whereas drug accumulation was increased in minimal proportions. Similarly, the reversal of resistance to doxorubicin by S-9788 was already maximum at 1 μM, a dose which did not allow an important increase of doxorubicin accumulation, whereas drug accumulation could further increase up to 3-fold more when the dose of modulator was 50 μM.

With the lipophilic anthracyclines, an increase of drug accumulation followed incubation with the modulators, which could reach the level obtained in sensitive cells. Verapamil was more efficient with pirarubicin and idarubicin than with doxorubicin, giving the same intracellular drug levels as those obtained with S-9788, but quinine remained barely active on this parameter, especially in K562 cells, despite its efficiency in resistance reversal at high dosage.

Discussion

We wanted in this study to compare the cellular pharmacology of three anthracyclines, in two models of human tumor cells in culture. It appeared that the lipophilic anthracyclines were accumulated more rapidly and to a higher extent than doxorubicin. Comparable results had been already obtained in other models of cultured cells[15–17] as well as in human leukemic cells.[18] This results in a higher efficiency of these drugs. There is a clear inverse relationship between the IC_{50} of the drugs and their degree of accumulation, and this is true both for MCF7 cells and K562 cells, with coefficients of correlation of -0.8 ($p < 0.01$). Drug accumulation

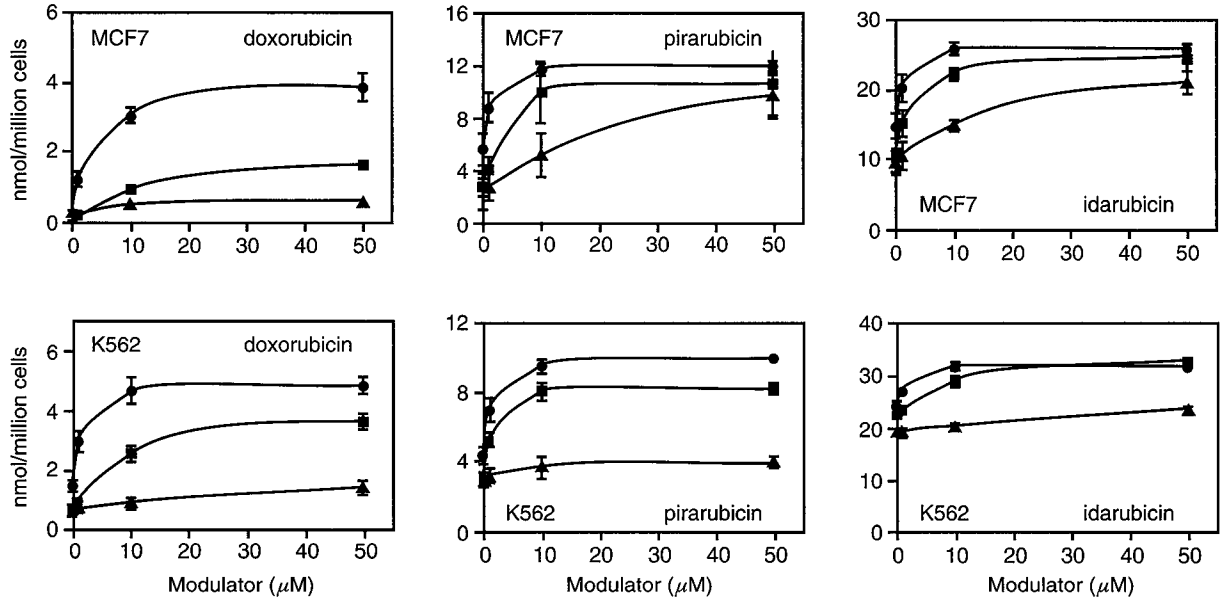

Figure 4. Restoration of doxorubicin (left), pirarubicin (center) and idarubicin (right) accumulation in MCF7 (upper diagrams) and K562 (lower diagrams) resistant cells in the presence of various concentrations of several modulators: verapamil, ■; quinine, ▲; S-9788, ●. Drug accumulation was evaluated after 2 h exposures to 10 μg/ml of each anthracycline, at defined concentrations of the modulators. Results are means ± SD of three independent experiments.

appears, therefore, as a major determinant of drug efficacy in the anthracycline series. This is clearly in favor of the existence of intracellular targets for these drugs, these targets being required for drug action. In the 1980s[19] and even recently,[20] the cytotoxicity of anthracyclines has been attributed to membrane interactions, generating a signal leading to cell death. Our results, as many others in the field of cellular pharmacology, argue against this type of mechanism of action for anthracyclines.

The lipophilicity of drugs is an obvious condition for their cellular uptake via passive diffusion through the plasma membrane.[8–10] Several anthracyclines among the more recently developed are characterized by an increased lipophilicity and have been rapidly shown to display only a partial cross-resistance with the reference anthracycline, doxorubicin. The question has been raised whether this was due to the characteristics of drug uptake or to a decreased P-glycoprotein-mediated drug efflux, and it was suggested by Berman *et al.*[21] that idarubicin was less affected than doxorubicin by P-glycoprotein-mediated efflux. However, a detailed study of the kinetics of anthracycline uptake and efflux was recently published by Mankhetkron *et al.*,[10] who concluded that the increased cytotoxicity of lipophilic anthracycline in MDR cells was due to an increased uptake of these drugs and that P-glycoprotein-mediated drug efflux was the same for all the anthracyclines tested. We approached this problem with MDR reverters, in order to show how the inhibition of P-glycoprotein could affect the cytotoxicity of lipophilic anthracyclines. Our results show unequivocally that MDR reverters increase the cytotoxicity of lipophilic anthracyclines in a similar way as they affect doxorubicin cytotoxicity. It can be concluded that the resistance to lipophilic anthracyclines, which is basically much lower than the resistance to doxorubicin, can be further reduced by the use of P-glycoprotein inhibitors. Such compounds can be included, therefore, in association to idarubicin or pirarubicin in the clinical trials of MDR reversal.

There is a clear difference in the relative activities of the three modulators in terms of reversal of resistance to any of the anthracyclines tested. Such differences have already been shown in various *in vitro* models. They do not mean, however, that the most active reverter *in vitro* should be the better one for use *in vivo*. Indeed, the clinical interest of a modulator is a compromise between its activity on the target and its toxicity. In this respect, S-9788 appeared to be unsuitable for clinical use because of its important cardiac toxicity, whereas quinine was shown to be potentially more useful, because it has much less general toxicity at the level required for MDR modulation. In addition, *in vitro* results cannot be translated to the clinical setting because the cell culture medium contains one-tenth of the protein content of the plasma and drug binding is consequently quite different in these two fluids. This has been shown to have decisive consequences for MDR reverters, in view of the fact that quinine has a low level of protein binding in comparison to other drugs.[22]

The dissociation between the resistance reversing effect of a modulator and its ability to restore drug accumulation has been observed in numerous cellular models and remains largely unexplained. It is especially puzzling to observe that S-9788 has reached its maximal reversing capacities of doxorubicin in resistance at a concentration of 0.1 μM despite the fact that drug accumulation can be further increased by at least 3-fold and that quinine can efficiently reverse doxorubicin resistance (provided that it is used at 50 μM) without significant increase in drug accumulation. The same is true for the lipophilic anthracyclines we used in this study. We have previously shown that quinine was able, in K562 resistant cells, to promote an intracellular redistribution of doxorubicin which could contribute to the reversal of resistance through an increase in the accessibility of its targets.[23]

Such a redistribution of drug within the cells has also been shown to occur with verapamil in other models.[24] It has been suggested that such drug redistribution might be mediated by intracellular P-glycoprotein, a localization that has been recently shown to occur,[25] but remains an important point of controversy.

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(Received 20 March 1997; revised form accepted 10 April 1997)