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AZELASTINE AND FLEZELASTINE AS REVERSING AGENTS OF MULTIDRUG RESISTANCE: PHARMACOLOGICAL AND MOLECULAR STUDIES

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Abstract—The effects of two new phthalazinone derivatives, azelastine (AZ) and flezelastine (FZ), on the reversal of resistance to doxorubicin (dox) were studied using two variants of the rat C6 glioblastoma cell line, selected with dox (C6 0.5) or with vincristine (C6 1V). Both lines presented a multidrug-resistant phenotype which was, in the case of C6 0.5 cells, likely to be accompanied by an additional mechanism leading to intracellular tolerance of the drug. Both AZ and FZ reversed dox resistance in a concentration-dependent manner, and FZ was shown to be at least three times more potent than AZ. FZ was able, at a relatively high concentration (30 μM), to completely restore dox sensitivity in both cell lines. Both drugs were able to virtually restore dox accumulation to the level reached in sensitive cells, and, interestingly, this complete restoration occurred at lower concentrations of modulator than required for complete reversal of resistance. FZ was able to reverse dox intracellular tolerance of C6 0.5 cells and to restore dox accumulation at the IC_{50} to the level observed in sensitive cells. AZ and FZ both inhibited azidopine binding to membrane preparations of C6 0.5 and C6 1V cells, although FZ was more potent. Both drugs more successfully inhibited azidopine binding to membranes prepared from C6 1V cells (which express the *mdr*1b gene product) than to membranes from C6 0.5 cells (which express the *mdr*1a gene product). In view of its potent activity on MDR, further preclinical evaluation of FZ is warranted.

Key words: azelastine; flezelastine; multidrug resistance; doxorubicin; MDR modulation

Resistance to treatment appears as one of the most frequent causes of failure in cancer chemotherapy. Cells grown in culture in the presence of increasing amounts of anticancer drugs have provided numerous *in vitro* models that have permitted the understanding of the basic mechanisms involved in resistance at the molecular level. Among the numerous potential mechanisms of cellular resistance against natural products used in cancer chemotherapy, MDR† appears as the most relevant in clinics, both because its occurrence has been proven in the clinical setting and because therapeutic approaches may exist. The main characteristics of tumour cells displaying the MDR phenotype are (i) cross-resistance to structurally unrelated cytotoxic drugs having different mechanisms of action, such as doxorubicin (dox), *vinca* alkaloids, and epipodophyllotoxins [1, 2]; (ii) decrease in drug accumulation in the resistant cells due to enhanced drug efflux; and (iii) overexpression of a particular transmembrane protein of 170 kDa, called P-gp. This protein is believed to function as an efflux pump for various lipophilic xenobiotics, including anticancer drugs, and is encoded by a gene called *mdr*1, which is overexpressed in resistant cells.

Because of the obvious role of P-gp in cytotoxic drug resistance, methods for circumventing such resistance are desirable. Many substances which interfere with P-gp function and are thus able to reverse MDR *in vitro* and *in vivo* have been identified: these include calcium channel blockers such as verapamil [3], calmodulin antagonists such as trifluoperazine [4], cyclosporines [5], quinolines [6] as well as many other compounds [7]. These compounds are able to increase the intracellular concentration of cytotoxic drugs to the level obtained in sensitive cells or tumors, probably through competitive or non-competitive inhibition of P-gp-mediated drug transport [8]. This is associated with a total or partial restoration of the cytotoxicity of the anticancer drugs. However, most of the compounds identified for their reversing capacities display other pharmacological properties, and, at doses efficient to reverse the MDR phenotype, have undesirable and sometimes dangerous side-effects. For example, the cardiovascular toxicity of verapamil [9] and the hepatic and renal toxicities of cyclosporin A [10] have hindered the clinical development of their association with anticancer drugs. A major approach for MDR reversal in the clinical setting is, therefore, to identify novel compounds which would be more potent and devoid of pharmacological activities other than MDR-reversing properties at the administered doses.

It is not clear at present whether all agents able to reverse MDR act by the same mechanism of P-gp inhibition; what is clear is that several of these

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† Abbrevations: MDR, multidrug resistance; P-gp, P-glycoprotein; AZ, azelastine; FZ, flezelastine; dox, doxorubicin; MTT, dimethylthiazol-diphenyltetrazolium bromide; SDS, sodium dodecyl sulphate.

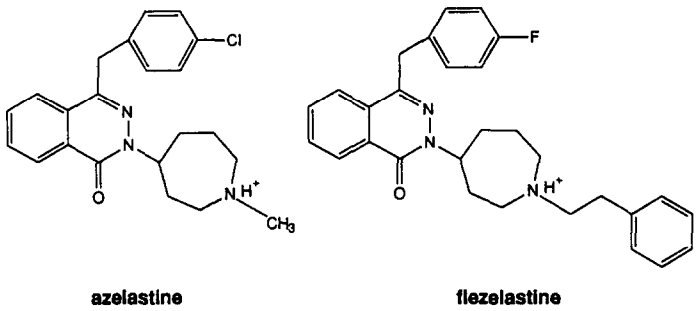

Fig. 1. Structural formulae of AZ and FZ.

modulators are able to compete with the 'natural substrates' of P-gp by inhibiting its photoaffinity labelling by azidopine or vinblastine analogues [8]. This is especially the case for verapamil and the cyclosporines. It has been thought that all MDR-reversing agents interact with P-gp [11], probably at several independent sites. However, it cannot be excluded that other targets of these modulators are involved [12].

AZ and FZ are two new phthalazinone derivatives (see Fig. 1 for structural formulae) which do not belong to any class of compounds already known to reverse MDR *in vitro* or *in vivo*. AZ is known for its activity as an antihistaminic and has been developed by ASTA-Medica for the treatment of allergic rhinitis. FZ, an analogue of AZ, has been developed for the same indication. Both compounds present the key features of MDR-reversing drugs as defined by Zamora *et al.* [13]: an important lipophilicity in relation to the presence of several aromatic rings, and a nitrogen atom ionized to a large extent at neutral pH. It was therefore of interest to detect and quantify the eventual reversing properties of these compounds in usual *in vitro* models. Preliminary results have indicated that AZ could partially reverse dox resistance in mouse and human leukaemia cell lines through an increase in dox accumulation [14]. The aim of the present study was to compare these properties to those of various other MDR reverters such as verapamil, quinine, cyclosporin A and S9788 in the rat glioblastoma model C6, rendered resistant either to dox (C6 0.5) or to vincristine (C6 1V). This model has already been used for such evaluations [15] as well as for the evaluation of the circumvention of MDR by drug encapsulation [16]. We studied the effect of these compounds on the decrease in dox IC_{50} and on the restoration of dox accumulation in the cells. We also verified the ability of AZ and FZ to inhibit [^{3}H]azidopine photolabelling of P-gp in plasma membrane-enriched preparations of resistant cells.

MATERIALS AND METHODS

Drugs and products. Dox was a gift from Pharmacia (Saint-Quentin-en-Yvelines, France). AZ and FZ were obtained from ASTA-Medica (Frankfurt, Germany). AZ and FZ were solubilized at 10 mM in DMSO and further diluted directly in the culture medium: ensuring that the amount of DMSO introduced during cell incubation had no effect on cell growth nor on dox cytotoxicity or accumulation. [^{3}H]Azidopine was purchased from Amersham (Les Ulis, France).

Cell culture. The C6 rat glioblastoma clone [17] and two variants selected with dox (C6 0.5, [18]) or vincristine (C6 1V) were routinely cultivated in Petri dishes (Nunc) with Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum (Seromed, Berlin, Germany) and antibiotic mixture, at 37°, in a humidified atomosphere containing 5% CO_2. The cultures were replated each week and the medium changed every 2 or 3 days, depending on cell density. Selection pressures of 0.5 μg/mL of doxorubicin and 1 μg/mL of vincristine were constantly maintained in the routine culture medium of the C6 0.5 and C6 1V cells, respectively, but were omitted 1 week before any experiment.

Dox cytotoxicity. A colorimetric assay using the tetrazolium salt MTT [19] was used to assess cytotoxicity after 2 hr exposure to dox in the presence or absence of the two MDR modulators. Briefly, 500 C6 cells or 2000 C6 0.5 cells or C6 1V cells were plated in 96-well plates (Nunc) in a volume of 200 μL of culture medium. After incubations of 24 hr for C6 cells and 48 hr for C6 0.5 cells or C6 1V cells, the cells were exposed for 2 hr at the appropriate concentrations of dox and AZ or FZ. The culture medium was then removed, the cell layers rinsed twice with phosphate-buffered saline, fresh culture medium added, and the cells allowed to grow for a further 4 (C6 cells) or 5 days (C6 0.5 and C6 1V cells). These conditions were established, after a careful study of the growth curves of the cells, in order to keep the cells in the exponential phase of growth over the 5 or 7 days of culture. At the end of the incubation, 200 μL of MTT-containing medium was added and maintained for 4 hr; after elimination of the medium, 200 μL of DMSO was added to dissolve the formazan crystals. Absorbance was then measured immediately on a two-wavelength microplate photometer (Bio-Tek Instruments) set at 570 and 630 nm. All experiments were performed at least twice in triplicate. The concentration of dox required for 50% growth inhibition (IC_{50}) was estimated as that giving a 50% decrease in absorbance as compared to controls incubated simultaneously without dox. The cytotoxicity of the modulator itself

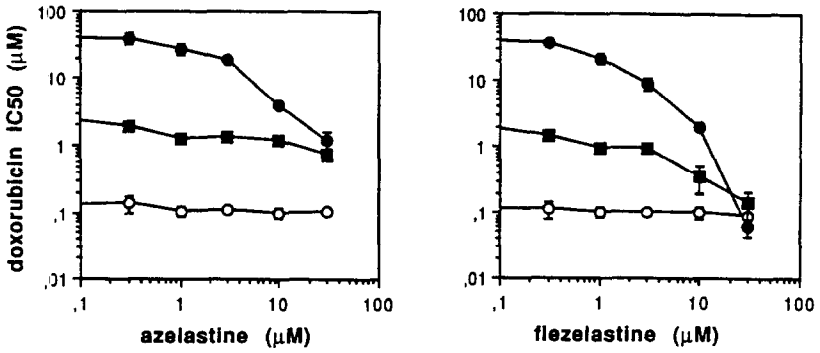

Fig. 2. Dox IC_{50} in C6 cell variants for various concentrations of AZ (left) or FZ (right). Cells were simultaneously incubated with dox and modulator for 2 hr; the medium was then renewed and the cells allowed to grow further for 2.5 cycles; cytotoxicity was then evaluated by the MTT assay. Dox IC_{50} values were calculated from each curve by interpolation and plotted as a function of modulator concentration. Values are means ± SD of three independent experiments performed in triplicate. (○) Sensitive cells; (●) C6 0.5 cells; (■) C6 1V cells.

was estimated by comparing the cell survival obtained with the various concentrations of the modulator in absence of dox.

The resistance factor was estimated from the ratio of the IC_{50} of a cytotoxic drug in the resistant line and in the sensitive one. The reversal factor was determined as the ratio of the IC_{50} of a cytotoxic drug in the absence and presence of the reverter.

Dox accumulation. The accumulation of dox was studied in the three cell lines under slightly different conditions given their different growth characteristics. C6 cells (8×10^4) were seeded in 10 cm^2 Petri dishes with 3 mL medium while 1.5×10^5 C6 1V cells and 5×10^5 C6 0.5 cells were seeded in 20 cm^2 Petri dishes with 5 mL drug-free medium. The medium was changed 3 days later; on the fourth day, the number of cells was approximately 2×10^6 cells per dish in all cases. The effect of AZ and FZ was then tested on dox accumulation at several concentrations of the modulator. Dox concentration was set at 17.2 μM in a first series of experiments, then it was equalled to the IC_{50} of the drug as evaluated in the presence of various concentrations of the modulator. All incubations with drugs were performed for 2 hr. At the end of this incubation, the monolayers were washed twice with 0.15 M NaCl, harvested mechanically, and pelleted at 1000 *g* for 5 min. This was done rapidly in order to avoid any significant drug efflux during these steps. Trichloroacetic acid (0.5 mL) and water (0.5 mL) were successively added and the samples kept at 4° overnight, then centrifuged for 30 min at 1000 *g*.

The acid-soluble fraction was used to evaluate the intracellular concentrations of non-covalently bound drug by fluorometry with a Kontron spectrofluorometer, model SFM 25, using excitation and emission wavelengths set at 480 and 590 nm, respectively.

The acid-insoluble pellet was solubilized with 1 M NaOH and used to evaluate the protein content [20]. All incubations were performed in triplicate and at least two independent experiments were performed for each drug.

Preparation of plasma membranes and photoaffinity labelling of P-gp. One to 5×10^8 C6 0.5 or C6 1V cells were washed and resuspended in 25 mL of 0.01 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and 0.2 mM $CaCl_2$ for 30 min at 4°. Cells were then disrupted with a Dounce homogenizer. After addition of EDTA to a final concentration of 1 mM and of 100 mL of Tris-HCl buffer, pH 7.5, containing 0.025 M sucrose, the homogenates were centrifuged at 1000 *g* for 10 min. Supernatants were overlaid on 0.01 M Tris-HCl (pH 7.5), 1 M sucrose, 1 mM EDTA and centrifuged at 11,000 *g* for 30 min. The membrane fraction, located at the interface, was then collected and centrifuged at 76,000 *g* for 75 min. Pellets enriched in plasma membranes were then resuspended in 4 mL of 0.01 M Tris-HCl, pH 7.5 containing 0.25 M sucrose and stored at −80°.

Proteins (20 μg) were incubated with various concentrations of AZ or FZ (12.5–100 μM) in 0.01 M Tris-HCl buffer (pH 7.5), 0.25 M sucrose, containing 5 μCi [^{3}H]azidopine (50 Ci/mmol) (final volume: 150 μL). After 30 min in the dark, samples were irradiated at 354 nm for 30 min. The reaction was stopped with SDS gel sample buffer and electrophoresed in 5–15% gradient SDS polyacrylamide gels. Proteins were stained with Coomassie blue and fixed. Gels were then treated with an amplifying solution (Amersham), dried, and exposed for 11 days to MP-Hyperfilm (Amersham) at −80°. Spots were then quantified from the autoradiograms using a densitometer (GS 300, Hoefer Scientific Instruments).

RESULTS

Effects of AZ and FZ on dox IC_{50}

The antiproliferative activity of dox was studied in the presence of AZ or FZ in the C6 variants after 2 hr exposures to the drugs. It was found that in the C6 sensitive line (wild-type cells), there was no

Table 1. Dox IC_{50}, reversal factors and residual dox resistance obtained with AZ or FZ at 10 μM

	Azelastine (AZ)			Flezelastine (FZ)		
	Dox IC_{50} (μM)	Reversal factor	Residual resistance	Dox IC_{50} (μM)	Reversal factor	Residual resistance
C6 S	0.10	NA*	NA	0.10	NA	NA
C6 0.5	3.92	11	37	1.98	22	19
C6 1V	1.22	3.8	12	0.77	5.9	7.4

* NA, not applicable.

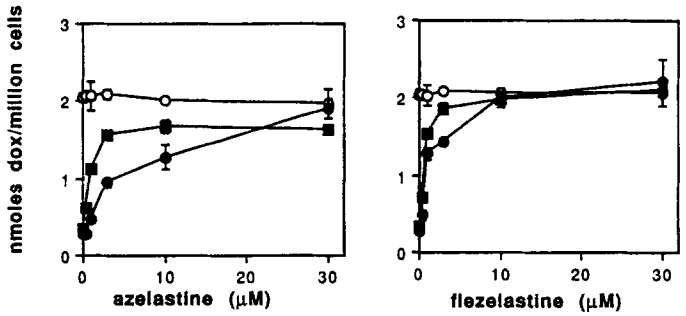

Fig. 3. Accumulation of dox in C6 cell variants for various concentrations of AZ (left) and FZ (right). Cells were exposed to 17.2 μM dox, then harvested, and dox extracted and quantified as described in the Materials and Methods section. Values are means ± SD of two independent experiments performed in triplicate. (○) Sensitive cells; (●) C6 0.5 cells; (■) C6 1V cells.

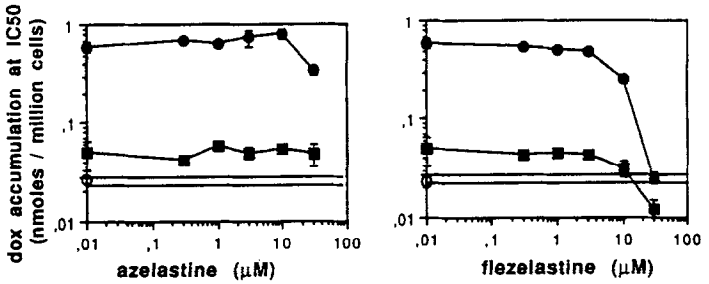

Fig. 4. Accumulation of dox in C6 cell variants exposed for 2 hr at the concentration providing 50% growth inhibition in the presence of the indicated concentration of modulator. Each point was thus obtained with a different combination of dox and modulator concentrations. After incubations, cells were harvested and dox extracted and quantified as described in the Materials and Methods section. Values are means ± SD of two independent experiments performed in triplicate. (○) Level obtained in sensitive cells in absence of modulator; (●) C6 0.5 cells; (■) C6 1V cells.

significant effect of AZ and FZ on the cytotoxicity of dox, up to a concentration of 30 μM; in the C6 0.5 and C6 1V cell lines (approx. 400- and 40-fold resistant to dox, respectively), there was a dose-dependent increase in dox cytotoxicity (Fig. 2). We calculated the ratio of the IC_{50} obtained with modulators in the C6 0.5 and C6 1V lines to the IC_{50} in the sensitive C6 line; this "residual resistance factor" is presented in Table 1 for one representative concentration of the modulator (10 μM). AZ had only a modest effect on resistance reversal, and both resistant cell lines remained at least 10-fold resistant to dox. FZ was much more active than AZ, and was able, at 30 μM, to completely restore the dox IC_{50} value of the resistant C6 0.5 and C6 1V lines to that observed in the sensitive line (no residual resistance). At lower concentrations, this reversal was only partial. It must be noted, however, that con-

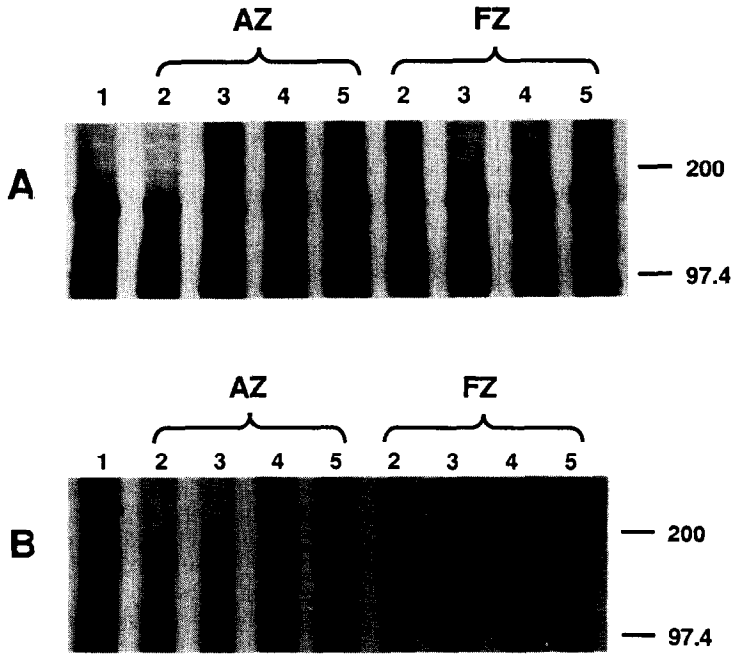

Fig. 5. Autoradiograms of protein electrophoreses of membrane extracts of C6 0.5 cells (A) and C6 1V cells (B) after photolabelling with [^{3}H]azidopine in the absence (lanes 1) or presence of AZ or FZ at decreasing concentrations: 100 μM (lanes 2), 50 μM (lanes 3), 25 μM (lanes 4) and 12.5 μM (lanes 5).

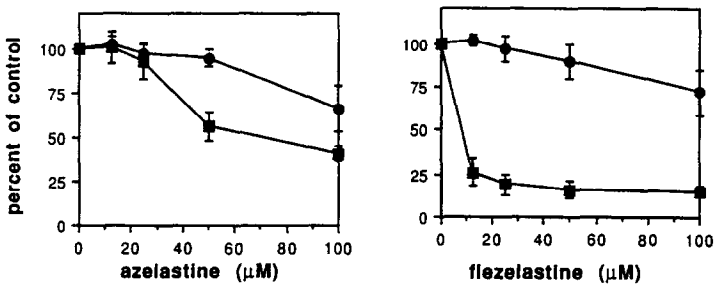

Fig. 6. Densitometric records of azidopine binding in the presence of AZ (left) and FZ (right) of membrane extracts of C6 1V cells (■) and C6 0.5 cells (●). Results are means ± SD of two independent experiments.

centrations of 30 μM resulted in a significant cytotoxicity of both compounds in absence of dox, slightly higher in the C6 0.5 than in the C6 1V line. Cell survival at 30 μM modulator was 70–75% for C6 0.5 cells, 80–85% for C6 1V cells and 85–90% for wild-type C6 cells.

Effects of AZ and FZ on dox accumulation

The effect of both modulators on dox accumulation after a 2 hr exposure at the concentration of 17.2 μM is presented in Fig. 3. The C6 0.5 and C6 1V resistant cells accumulated 10-fold and 6-fold less dox respectively than the sensitive cells in absence of modulators. Modulators had no significant effect on dox accumulation in the sensitive line. In the C6 1V line, the accumulation of dox was virtually restored with 10 μM AZ or 3 μM FZ to that measured in the sensitive line; in the C6 0.5 line, this level was restored with 30 μM AZ or 10 μM FZ. Using this parameter for the comparison of the modulators indicates that FZ can be considered three times more active than AZ.

We then measured dox accumulation in C6 0.5 and C6 1V cells after 2 hr exposures to the IC_{50} values obtained for dox at each concentration of the modulator ("intracellular IC_{50}"). As shown in Fig. 4, FZ was able at high concentrations (10 μM for C6 1V cells and 30 μM for C6 0.5 cells) to efficiently decrease this parameter low enough to reach the values observed in the sensitive C6 cells, but AZ

was unable to significantly decrease the amount of drug incorporated in C6 0.5 and C6 1V cells at IC_{50} exposure.

Effects of AZ and FZ on azidopine binding

Figure 5 shows the specific labelling of P-gp by [^{3}H]-azidopine in the plasma membrane fractions of C6 0.5 and C6 1V cells at varying concentrations of the modulators, while Fig. 6 presents the densitometric analysis of the autoradiograms. Both drugs were able to inhibit azidopine binding to the membrane preparation of both cell lines in a concentration-dependent manner. It is worth noting that there was a considerable difference between the two lines: 50% inhibition of azidopine binding was not obtained with membranes from the C6 0.5 line, even at 100 μM AZ or FZ, whereas it was obtained with 50 μM AZ and 10 μM FZ with membrane preparations from the C6 1V line.

DISCUSSION

Based upon their structural characteristics, AZ and FZ have been proposed by ASTA-Medica for the reversal of multidrug resistance. We have tested them in a model of rat glioblastoma cells aready used for the evaluation of chemical modulators [15] and drug encapsulation [16] for the reversal of dox resistance. In our experience, C6 1V cells appear to express a relatively pure MDR phenotype, whose resistance is only or mainly due to a defect in drug accumulation; the C6 0.5 cells, in contrast, are characterized by the presence of an MDR phenotype which only partially explains its resistance to dox [21]. This line in particular presents a phenomenon that we have called "drug tolerance": when sensitive and resistant cells are exposed to their corresponding IC_{50} values, the resistant cells accumulate, and therefore tolerate, 50-times more dox than the sensitive ones [18]. We have shown that some reverters such as quinine or cyclosporin A reverse this tolerance to dox, whereas others, such as verapamil or trifluoperazine, do not [15]. This tolerance phenomenon is characterized by the presence of high intracellular levels of dox in absence of marked cytotoxicity and can be attributed to special protection mechanisms of the nuclear target of dox, DNA-topoisomerase II, possibly by sequestration of the drug in compartments from which it cannot reach the nucleus [22]. Tolerance can also be attributed to a decrease in the number of DNA sites for topoisomerase II interaction, and cytotoxicity would then require much more dox at the DNA level [23].

In this study we have observed that AZ and FZ were able to increase dox cytotoxicity in C6 0.5 and C6 1V cells in a dose-dependent manner. The effect of AZ was qualitatively and quantitatively similar to that of verapamil [15], but the effect of FZ was much higher: it is the only compound, along with aminodarone [15], to completely restore dox cytotoxicity in the C6 0.5 model, although at a relatively high concentration (30 μM). The reversal of resistance can be partially attributed to the restoration of dox accumulation in the cells. However, complete restoration of this parameter was obtained at concentrations lower than those resulting in reversal of resistance. It was obtained with 10 μM FZ in the C6 0.5 line (instead of 30 μM for complete resistance reversal); it was also achieved in this line with 30 μM AZ, although a 10-fold residual resistance to dox was still present at this concentration. In C6 1V cells, complete restoration of dox accumulation occurred at lower concentrations of AZ or FZ than in C6 0.5 cells (10 and 3 μM, respectively, versus 30 and 10 μM). Similarly, dox resistance was reversed in this line at lower concentrations of AZ and FZ than in the C6 0.5 line. This can be attributed to the fact that the dox resistance of the C6 1V line appears to be almost entirely due to the overexpression of P-gp ("pure" MDR phenotype).

In addition to restoring dox accumulation, FZ also provided a reversal of dox tolerance within the cells. When C6 0.5 cells were exposed to the dox IC_{50} obtained with 30 μM FZ in the presence of this concentration of FZ, dox accumulation was restored to the value obtained in C6 sensitive cells. This was not apparent with AZ. With the C6 1V line, which presents only a marginal level of drug tolerance, this effect of FZ could be also detected. We can therefore conclude that FZ, like quinine and cyclosporine A, presents the property of reversing the intracellular tolerance to dox. However, it must be mentioned that this occurs only at the highest concentration of FZ tested (30 μM). It is not known whether the plasma concentration of FZ could reach such high values without concomitant toxicity. Indeed, the clinical interest of a modulator consists in its therapeutic index (ratio between active and toxic concentrations), rather than in the absolute concentrations required for effect on MDR phenotype.

AZ and FZ were able to inhibit azidopine binding to P-gp-enriched membrane fractions from C6 0.5 and C6 1V cells, suggesting that their mechanism of action involves, at least in part, a direct interaction with P-gp. In the C6 1V membrane preparations, 50% inhibition occurred at approx. 50 μM AZ or 10 μM FZ, thus confirming the higher activity of FZ as compared to AZ. In the C6 0.5 membrane preparations, however, 50% inhibition occurred for both compounds at higher concentrations, suggesting a weaker interaction of AZ and FZ with the P-gp of this cell line than with that of C6 1V cells. We have shown earlier [24] that the P-gp expressed by C6 0.5 cells is mainly the product of the *mdr*1a gene, and that expressed by C6 1V cells the product of the *mdr*1b gene. It has been shown by Devault and Gros [25] that there is some degree of drug specificity in P-gp-mediated resistance when originating from mouse *mdr*1a or *mdr*1b genes. It is thus conceivable that AZ and FZ would differently inhibit azidopine binding to the P-gps originating from the two *mdr*1 genes, with a stronger inhibition of the *mdr*1b than of the *mdr*1a gene product. The different amount of P-gp in the two cell lines might also contribute to the stronger effect seen in C6 1V as compared to C6 0.5 cells. It should be also mentioned that the possibly better activity of these drugs on the *mdr*1b gene product does not prevent them from reversing the resistance of the C6 0.5 line in an efficient manner. This can be explained by the fact that these

drugs, especially FZ, may have other targets than P-gp for resistance reversal. Because there is only one *MDR*1 gene in humans, this observation may not be relevant to the clinical situation; however, the *in vivo* experimentation of AZ and FZ as MDR reverters in mice should be considered for proper evaluation, because of the possibility that mouse tumours express one gene or the other.

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