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RIFAMPICIN ENHANCES ANTI-CANCER DRUG ACCUMULATION AND ACTIVITY IN MULTIDRUG-RESISTANT CELLS

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Abstract—Rifampicin, a semi-synthetic antibiotic used in the treatment of tuberculosis and belonging to the chemical class of rifamycins, was examined for its effect on anti-cancer drug accumulation and activity in multidrug resistant cells overexpressing P-glycoprotein (P-gp). Rifampicin was shown to strongly enhance vinblastine accumulation in both rat hepatoma RHC1 and human leukemia K562 R7 multidrug resistant cells, but had no effect in rat SDVI drug-sensitive liver cells. By contrast, two other rifamycins, rifamycins B and SV, had no or only minor effect on vinblastine accumulation in RHC1 cells. Efflux experiments revealed that rifampicin was able, like the well-known chemosensitizer agent verapamil, to decrease export of vinblastine out of resistant cells. Rifampicin, when used at a concentration close to plasma concentrations achievable in humans (25 μM), was able to increase sensitivity of RHC1 cells to both vinblastine and doxorubicin. Rifampicin was also demonstrated to inhibit P-gp radiolabeling by the photoactivable P-gp ligand azidopine, thereby suggesting that the anti-tuberculosis compound can interfere directly with P-gp drug binding sites. These results thus indicate that rifampicin was able to down-modulate P-gp-associated resistance through inhibition of P-gp function.

Key words: chemosensitizer, doxorubicin, multidrug resistance, P-glycoprotein, rifampicin, vinblastine

Resistance to chemotherapy is a major cause of failure in the treatment of many human cancers. One major mechanism of such drug resistance is the overexpression of a plasma membrane glycoprotein termed P-gp§ [1–3]. P-gp, encoded by mdr genes, is thought to act as an ATP-dependent efflux pump; it therefore contributes to reducing the intracellular accumulation of various hydrophobic antitumor compounds, including anthracyclins, vinca alkaloids, epipodophyllotoxins and taxol, thus conferring a MDR phenotype [4].

P-gp overexpression had initially been reported in cell lines selected *in vitro* with increased amounts of a single anti-tumor compound [5]. High levels of P-gp have also been found in many human tumor biopsies and have been correlated with lack of response to chemotherapeutic agents and poor survival [6–8]. P-gp overexpression has been demonstrated to occur during relapse after chemotherapy and even before treatment in some cancers derived from tissues physiologically expressing P-gp, such as colon and kidney [9].

A wide variety of drugs, including calcium channel blockers such as verapamil, calmodulin antagonists such as phenothiazines, steroids and immunosuppressive agents, have been shown to inhibit P-gp function and thus to restore sensitivity to cytotoxic drugs [10–12]. These reversing or chemosensitizer agents thus represent an interesting way to circumvent drug resistance in cancer cells although, for most, the high doses required to inhibit P-gp function lead to toxic effects as recently demonstrated by clinical human trials [13].

Analysis of structure–activity relationships among reversing agents has brought to light certain structural features important for their chemosensitizing potential, particularly hydrophobicity and the presence of two planar phenyl rings [14]. Such characteristics are also shared by rifampicin, a semi-synthetic antibiotic belonging to the chemical group of rifamycins and used in the treatment of tuberculosis [15]. Thus, the present study was designed to determine whether rifampicin has the capacity to affect anti-cancer drug accumulation and activity in multidrug-resistant cells.

MATERIAL AND METHODS

Drugs. Rifampicin and two other rifamycins, rifamycin B and rifamycin SV, were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.) and were prepared as stock solutions in DMSO. The structures of these compounds are given in Fig. 1. Vinblastine and doxorubicin were purchased from Roger Bellon laboratories (Neuilly, France) and verapamil was supplied by Biosedra laboratories

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§ Abbreviations: MDR, multidrug resistance; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; P-gp, P-glycoprotein.

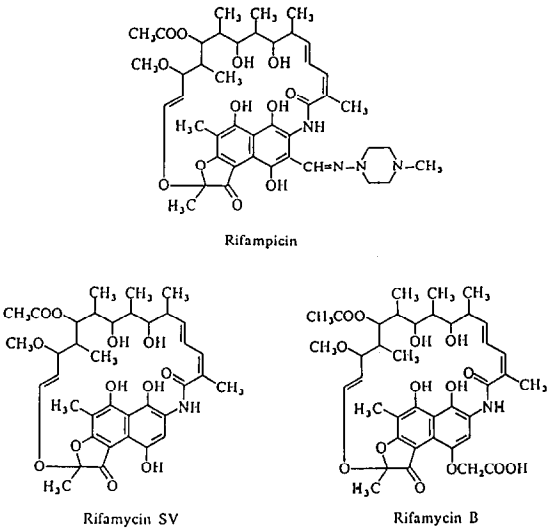

Fig. 1. Chemical structure of rifampicin, rifamycin SV and rifamycin B.

(Levallois-Perret, France). [^{3}H]vinblastine (8.3 Ci/mmol) and [^{3}H]azidopine (47 Ci/mmol) were obtained from Amersham (Bucks, U.K.).

Cell culture. RHC1 rat hepatoma cells, obtained from a chemically induced liver tumor [16], and SDVI normal rat liver epithelial cells [17] were cultured in Williams' medium supplemented with 10% foetal calf serum. As previously reported [16] and in contrast to SDVI cells [18], RHC1 cells displayed a constitutive overexpression of functional P-gp and a MDR phenotype and were thus 28-fold more resistant to vinblastine than SDVI cells as demonstrated by drug sensitivity assays.

Human leukemic K562 and the multidrug-resistant variant K562 R7 cell lines (kindly provided by Dr J. P. Marie, Hôtel Dieu, Paris, France) were cultured in RPMI medium supplemented with 10% foetal calf serum.

Normal rat hepatocytes were prepared by the two-step collagenase perfusion method and then maintained in conventional primary culture in a mixture of 75% minimal essential medium and 25% medium 199 [19]. Hepatocytes were used after 2 days of culture, at which time they displayed high levels of P-gp [20].

Intracellular vinblastine accumulation. Cells were washed with PBS and then incubated with 12.5 nM [^{3}H]vinblastine for 2 hr in the presence or absence of various concentrations of rifampicin, rifamycin B, rifamycin SV or verapamil. Cells were then washed three times with ice-cold PBS and lyzed in PBS by ultrasonication. These steps were performed quickly in order to avoid any drug efflux. Radioactivity of [^{3}H]vinblastine in the cell extract was then determined by a liquid scintillation counter. An aliquot of cell lyzate was used in parallel to determine cellular protein concentration by the Bio-Rad assay [21]. Preliminary studies showed no toxicity of vinblastine, rifampicin, rifamycin B, rifamycin SV or verapamil at the concentrations used over the 2 hr incubation period.

Cellular vinblastine efflux. Cells were washed with PBS and incubated with 12.5 nM [^{3}H] vinblastine for 2 hr. Cells were then rapidly washed with ice-cold PBS and reincubated in vinblastine-free medium for 4 hr in the presence or absence of rifampicin (200 μM) or verapamil (25 μM). Intracellular residual radioactivity of [^{3}H]vinblastine was further measured by scintillation counting. Results were expressed as percentages of initial intracellular [^{3}H]vinblastine accumulation values.

Drug-sensitivity assay. Anti-cancer drug effect on RHC1 cell proliferation was evaluated using the MTT dye assay [22]. Briefly, the cells were seeded at 20,000/mL in 96-well microplates and cultured with varied concentrations of vinblastine or doxorubicin in the absence or presence of rifampicin (25 μM). After 72 hr of incubation, 20 μL of a 5 mg/mL MTT solution were added to each well. The medium was then aspirated after a 2 hr incubation at 37° and replaced by 100 μL of DMSO. The blue formazan product formed was further quantified by its absorbance at 540 nm using a Titertek Multiskan MCC/340. Growth inhibition was evaluated as IC_{50}, i.e. the drug concentration providing a 50% reduction in cell number as compared to controls cultured in parallel without anti-cancer drug.

Photoaffinity radiolabeling of P-gp with [^{3}H]-azidopine. Photoaffinity of P-gp with [^{3}H]azidopine was performed as previously described [23]. Briefly, P-gp-containing membrane fractions were prepared from sensitive K562 and multidrug-resistant K562 R7 cells by differential centrifugation as described by Germann *et al.* [24]. Fifty micrograms of membrane proteins per assay was incubated in 40 mM phosphate potassium buffer, 10 μM $CaCl_2$, 4% DMSO with 5 μCi [^{3}H]azidopine in a final volume of 50 μL. The mixture was preincubated 1 hr at 25° in the dark in the absence or presence of various concentrations of rifampicin and then irradiated on ice for 10 mn with an ultraviolet lamp at 254 nm at a distance of 10 cm. The photolabeled membrane preparations were separated on a 7% SDS-PAGE. The gel was then fixed in 25% ethanol/10% acetic acid, treated with the fluorographic reagent Amplify (Amersham) and exposed to X-ray film at −80°.

Statistical analysis. The results of cellular [^{3}H]-vinblastine accumulation and efflux studies were analyzed by the Student's *t*-test. The criterion of significance of the differences between the means (± standard deviation) was $P < 0.05$.

RESULTS

In order to determine whether rifampicin could affect anti-cancer drug accumulation in multidrug-resistant cells, intracellular retention of [^{3}H]-vinblastine was determined in RCH1 multidrug-resistance cells in the presence or absence of various concentrations of rifampicin (Fig. 2). The antibiotic enhanced [^{3}H]vinblastine accumulation in RCH1 cells in a dose-dependent manner and its effect was maximal at 200 μM, a concentration at which cellular [^{3}H]vinblastine retention was increased by

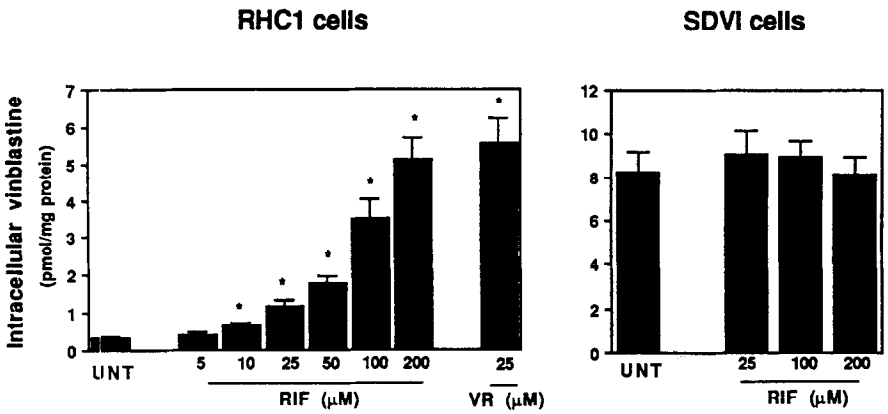

Fig. 2. Effects of various concentrations of rifampicin on [^{3}H]vinblastine accumulation in RHC1 and SDVI cells. Drug-resistant hepatoma RHC1 cells and drug-sensitive liver SDVI cells were incubated for 2 hr with 12.5 nM [^{3}H]vinblastine alone (UNT) or in the presence of rifampicin (RIF) at various concentrations or of 25 μM verapamil (VR). Intracellular [^{3}H]vinblastine retention was then determined by scintillation counting. The values are the means ± SD of three independent experiments in triplicate. * $P < 0.05$.

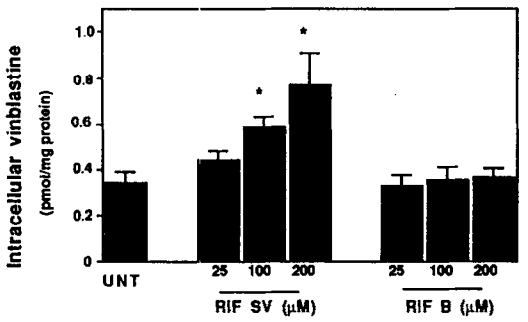

Fig. 3. Effects of rifamycin SV and rifamycin B on [^{3}H]-vinblastine accumulation in RHC1 cells. Drug-resistant RHC1 cells were incubated for 2 hr with 12.5 nM [^{3}H]-vinblastine alone (UNT) or in the presence of varied concentrations of rifamycin SV (RIF SV) or rifamycin B (RIF B). Intracellular [^{3}H]vinblastine retention was then determined by scintillation counting. The values are the means ± SD of three independent experiments in triplicate. * $P < 0.05$.

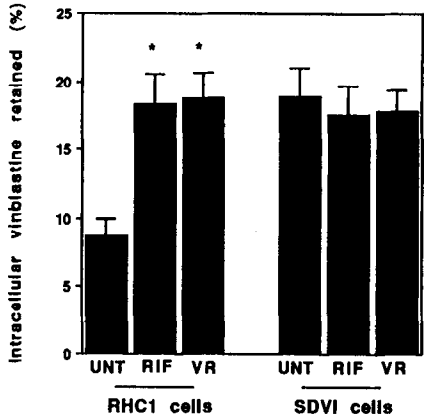

Fig. 4. Effect of rifampicin on [^{3}H]vinblastine efflux from RHC1 and SDVI cells. Drug-resistant hepatoma RHC1 cells and drug-sensitive liver SDVI cells were incubated for 2 hr with 12.5 nM [^{3}H]vinblastine. Cells were then washed three times with ice-cold phosphate-buffered saline and were reincubated in vinblastine-free medium in the absence (UNT) or presence of 200 μM rifampicin (RIF) or of 25 μM verapamil (VR). Intracellular residual [^{3}H]-vinblastine concentration was then determined by scintillation counting and expressed relative to initial drug accumulation values. The values are the means ± SD of three independent experiments in triplicate. * $P < 0.05$.

approximately 16-fold and was similar to that obtained with 25 μM of the well-known chemosensitizer agent verapamil (Fig. 2). Light microscopic examination of the cultures and determination of total cellular protein content revealed no alteration in RHC1 cell viability by the various tested concentrations of rifampicin over the 2 hr incubation period. In contrast to RHC1 cells, drug-sensitive SDVI cells did not show any alteration of [^{3}H]-vinblastine retention by rifampicin whatever the concentration used (Fig. 2).

In order to establish if other rifamycins could also modulate drug levels in resistant cells, [^{3}H]-vinblastine accumulation in RHC1 cells was analyzed in the presence of rifamycin SV and rifamycin B. Results indicated that rifamycin SV had only a weak effect, enhancing cellular vinblastine retention no more than 2-fold at a concentration of 200 μM, while rifamycin B had no effect whatever the concentration used (Fig. 3).

Efflux experiments showed low levels of residual [^{3}H]vinblastine in RHC1 cells when compared to SDVI cells after post-incubation in drug-free medium, thus reflecting the existence of an active

Table 1. Effect of rifampicin on RHC1 cell sensitivity to vinblastine and doxorubicin

Chemosensitizer	IC_{50} (nM)	
	Vinblastine	Doxorubicin
None	59.9 ± 11.6	3060 ± 490
Rifampicin	11.8 ± 2.3 (5)	690 ± 140 (4.4)
Verapamil	3.1 ± 0.8 (19.3)	148 ± 24 (20.7)

Drug-resistant RHC1 cells were cultured for 72 hr with various concentrations of vinblastine and doxorubicin in the absence or presence of either 25 μM rifampicin or 25 μM verapamil. Drug effects on cell proliferation were then determined by using the MTT dye assay as described in Materials and Methods. Growth inhibition is expressed as IC_{50}, i.e. the anti-cancer drug concentration providing a 50% reduction in cell number as compared to controls cultured in parallel without anti-cancer drug. The values are the means ± SD of at least three independent determinations. Numbers in parentheses indicate the sensitization factor, i.e. the ratio of IC_{50} in the absence of the chemosensitizer versus the IC_{50} in the presence of the chemosensitizer.

P-gp in RHC1 cells (Fig. 4). Addition of either 200 μM rifampicin or 25 μM verapamil during the 4 hr-efflux period enhanced intracellular vinblastine retention in RHC1 cells, but had no effect in SDVI cells.

The effect of prolonged rifampicin exposure (72 hr) on RHC1 cell proliferation was then monitored by the MTT dye assay. A rifampicin IC_{50} value of 91.2 ± 17.6 μM was determined, thus demonstrating that high concentrations of this antibiotic (100–200 μM) found to greatly increase intracellular accumulation of vinblastine (Fig. 2), affected RHC1 cell proliferation during long-term treatment. Therefore, we tested rifampicin at 25 μM in reversing experiments; at this concentration (i.e. in the range of the plasma concentrations observed after *in vivo* administration to patients [25]), the anti-tuberculosis compound had no effect on RHC1 cell growth, but still enhanced cellular vinblastine retention approx. 4-fold (Fig. 2). This rifampicin concentration strongly affected the sensitivity of RHC1 cells to vinblastine and doxorubicin as assessed by MTT dye assay (Table 1). Vinblastine and doxorubicin IC_{50} values were thus decreased by 5- and 4.4-fold, respectively. The use of verapamil at 25 μM also decreased the resistance of RHC1 cells to vinblastine and doxorubicin by 19.3- and 21.7-fold respectively (Table 1).

In order to determine whether rifampicin was also active in multidrug-resistant cells other than RHC1 cells, its effect on cellular [^{3}H]vinblastine accumulation was investigated in human leukemia K562 R7 cells and in 2-day-old primary cultures of normal rat hepatocytes, which both markedly overexpressed P-gp [20, 26]. Results demonstrated that 200 μM rifampicin, like 25 μM verapamil, strongly enhanced vinblastine retention in both leukemia K562 R7 cells and cultured normal hepatocytes (Fig. 5).

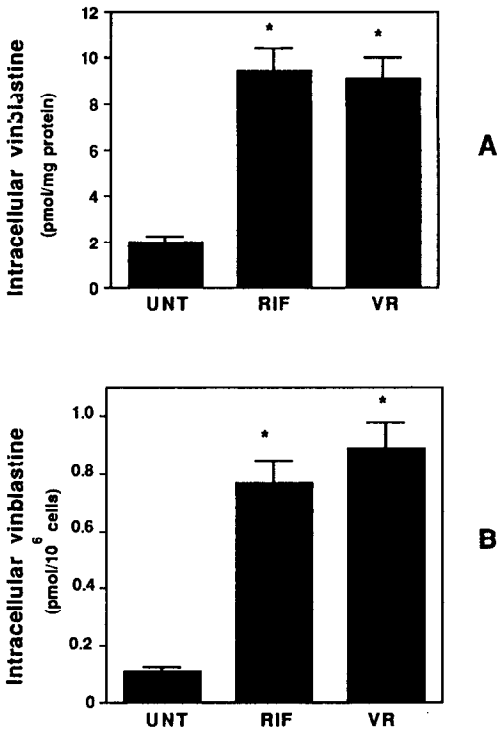

Fig. 5. Effects of rifampicin on [^{3}H]vinblastine accumulation in cultured rat hepatocytes and in K562 R7 cells. Two-days cultured rat hepatocytes (A) and human multidrug resistant K562 R7 cells (B) were incubated for 2 hr with 12.5 nM [^{3}H]vinblastine alone (UNT) or in the presence of 25 μM rifampicin (RIF) or of 25 μM verapamil (VR). Intracellular [^{3}H]vinblastine retention was then determined by scintillation counting. The values are the means ± SD of three independent experiments in triplicate. * $P < 0.05$.

The effect of rifampicin on drug-binding to P-gp was further analyzed with [^{3}H]azidopine. This compound is a photoaffinity ligand for P-gp [23] and evidenced the presence of a 170 kDa P-gp in drug-resistant K562 R7 cells, but not in parental drug-sensitive K562 cells (Fig. 6). This azidopine-mediated radiolabeling of P-gp was found to be inhibited by rifampicin in a dose-dependent manner (Fig. 6).

DISCUSSION

The results reported in the present study demonstrate for the first time to our knowledge that the anti-tuberculosis compound rifampicin affects anti-cancer drug accumulation and activity in tumoral cells overexpressing P-gp. Rifampicin enhanced both vinblastine retention and activity in rat RHC1 resistant cells; it also strongly increased cellular vinblastine levels in human multidrug-resistant leukemia cells, thereby demonstrating that the anti-tuberculosis drug is active in both rodent and human multidrug resistant cells. Moreover, rifampicin was shown to decrease doxorubicin resistance in RHC1 cells, thus demonstrating that the effect of this drug is not limited to vinblastine. By contrast, rifampicin

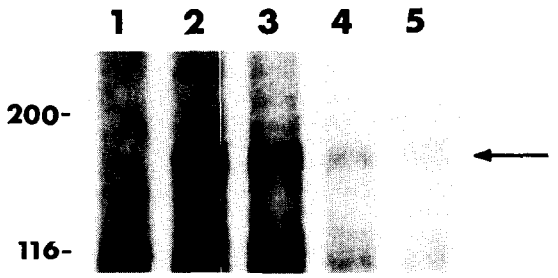

Fig. 6. Effect of rifampicin on [^{3}H]azidopine labeling of P-glycoprotein. Crude membrane proteins (50 μg/lane) prepared from multidrug resistant K562 R7 cells (lanes 2–5) and from drug-sensitive K562 cells (lane 1) were photolabeled with [^{3}H]azidopine in the absence (lanes 1 and 2) or presence of rifampicin at 20 μM (lane 3), 100 μM (lane 4) or 200 μM (lane 5). The size of molecular standards (in kDa) is indicated on the left. Arrow indicates the position of P-gp.

had no effect on vinblastine accumulation in SDVI drug-sensitive cells. These data therefore indicate that the rifampicin effect is restricted to multidrug resistant cells and thus could reflect a specific interaction of rifampicin with P-gp function. This conclusion is also supported by the results of efflux experiments, which demonstrated that rifampicin, like verapamil, decreased vinblastine export out of cells and thus could block P-gp-mediated drug transport in resistant cells. Moreover, rifampicin was demonstrated to inhibit the radiolabeling of P-gp by azidopine, thereby suggesting that its effect on P-gp function is probably related to direct interactions with drug-binding sites on P-gp. Such a mechanism of action has already been reported for many chemosensitizer agents [10].

Rifampicin appeared to be a less potent chemosensitizer than verapamil, since a 200 μM concentration of rifampicin was required to obtain an increase in vinblastine retention in RHC1 cells similar to that observed with only 25 μM verapamil. This conclusion is also supported by the data from drug-sensitivity assays. Indeed the use of verapamil resulted in a greater chemosensitization of RHC1 cells to vinblastine and doxorubicin than that obtained with rifampicin. However, it is noteworthy that a non-toxic concentration of rifampicin (25 μM) used in reversing experiments is in the range of the plasma concentrations usually observed after *in vivo* administration to humans [25]. This last point is likely to be significant since many potent chemosensitizer agents act at elevated concentrations which can usually not be obtained *in vivo* without the appearance of indesirable side-effects [10, 13]. Indeed the undesirable cardiac effects of verapamil at relevant anti-MDR concentrations have been well documented [27]. Similarly, administration of trifluoperazine as a reversing agent in combination with doxorubicin had led to severe neurologic effects [28]. In contrast, rifampicin can have some anti-MDR activity *in vitro* at concentrations close to non-toxic plasma concentrations usually observed during the treatment of tuberculosis [25], thereby suggesting that rifampicin could have a real effect *in vivo* on drug-resistant cells without major toxicity.

Comparing the effects of rifampicin, rifamycin B and rifamycin SV on intracellular vinblastine retention has revealed that rifampicin was the most efficient compound in enhancing drug accumulation in RHC1 cells. This result suggests that some structural features restricted to rifampicin could account for its anti-MDR activity. It is thus noteworthy that rifampicin differs from rifamycin B and rifamycin SV in the presence of a piperazinyl amino side group at position 3 of the naphthoquinone ring, which therefore could play a major role in the reversing potential of rifampicin. This conclusion agrees with previous studies investigating structure–activity relationships among chemosensitizers. Indeed, analysis of a series of phenothiazines demonstrated that phenothiazines containing a piperazinyl amine were potent reversing agents [29]. Similarly, thioxanthenes with a piperazinyl amino side group are particularly effective chemosensitizers [30]. These studies also indicated that some other structural features shared by rifampicin, in particular hydrophobicity and presence of cyclic rings, are required for a potent anti-MDR activity.

Rifampicin was demonstrated to strongly enhance vinblastine accumulation in cultured rat hepatocytes, thus suggesting that it could inhibit P-gp function in normal cells. Other chemosensitizer compounds such as verapamil or cyclosporin have already been shown to interact with P-gp in normal tissues [31]. Since the physiological substrates for P-gp remain to be determined, the exact consequences of the inhibition of P-gp function are difficult to predict. However, P-gp has been observed at the biliary pole of hepatocytes [32, 33]; it could thus be hypothesized that its inhibition by chemosensitizer agents could result in some alterations in hepatobiliary transport and could thereby account for hepatic side-effects observed during recent clinical human trials of some reversing drugs [13] or after administration of rifampicin [34].

In conclusion, our data demonstrated that the anti-tuberculosis compound rifampicin strongly enhances vinblastine accumulation in both rodent and human multidrug resistant cells, at least partly via an inhibition of cellular vinblastine efflux. Rifampicin also increases the sensitivity of resistant cells to vinblastine and doxorubicin and could therefore represent a new tool for overcoming multidrug resistance.

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