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In Vitro and *In Vivo* Reversal of Cancer Cell Multidrug Resistance by the Semi-Synthetic Antibiotic Tiamulin

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ABSTRACT. A large number of multidrug resistance (MDR) modulators, termed chemosensitizers, have been identified from a variety of chemicals, but most have been proven to be clinically toxic. Low concentrations of the pleuromutilin-derived semi-synthetic antibiotic tiamulin (0.1 to 10 μM) sensitized the three highly resistant P-glycoprotein (Pgp)-overexpressing tumor cell lines P388 (murine lymphoid leukemia), AS30-D (rat hepatoma), CEM (human lymphoblastic leukemia), and the barely resistant AS30-D/S cell lines to several MDR-related anticancer drugs. Flow cytometric analysis showed that tiamulin significantly increased the intracellular accumulation of daunomycin. When compared to reference modulating agents such as verapamil and cyclosporin A, tiamulin proved to be 1.1 to 8.3 times more efficient in sensitizing the resistant cell lines. Moreover, when given i.p. (1.6 μg/mg body weight), tiamulin increased the survival rate of adriamycin-treated mice bearing the P388/ADR25 tumor line by 29%. In the presence of an anticancer drug, tiamulin inhibited both ATPase and drug transport activities of Pgp in plasma membranes from tumor cells. Tiamulin is thus a potent chemosensitizer that antagonizes the Pgp-mediated chemoresistance in many tumor cell lines expressing the MDR phenotype at different levels and displays no toxic effects on contractile tissues at active doses, therefore providing the promise for potential clinical applications. BIOCHEM PHARMACOL **56**;9:1219–1228, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. multidrug resistance; tiamulin; P-glycoprotein; reversion; flow cytometry; pleuromutilin

In many cases, chemotherapeutic drugs used to treat hematological malignancies and solid tumors induce the emergence of resistant cancer cells leading to treatment failure. These drugs, such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, taxol, actinomycine D, topotecan, and COL,¶ share the same lipophilic structure. Resistance arises either at initial presentation or at the time of relapse. *In vitro*, sensitive tumor cells cultured in the presence of these drugs also lead to selection of resistant cells, the difference being that their resistance level can reach much higher levels due to resistance gene amplification. The mechanism underlying this phenomenon has been largely studied on *in vitro* models. In most cases, tumor cells selected for the MDR phenotype overexpress the Pgp [1], a 170 kDa membrane-bound energy-dependent drug efflux pump conferring resistance on a broad variety of commonly used chemotherapeutic drugs. If selected with one drug, these cells display cross-resistance to other drugs involved in the MDR phenotype, in which the most consistent alteration is the increased expression of Pgp encoded by the *mdr*1 class of genes [2]. Both Pgp and the resulting MDR phenotype were found to be inhibited by a variety of pharmacological products [3], suggesting the possibility for drug resistance modulation in human tumors. These inhibitors, called MDR modulators or chemosensitizers, belong to different classes such as calcium channel inhibitors (verapamil, tiapamil, nifedipine, and prenylamine), neuroleptics (trifluoperazine, chlorpromazine, and flupentixol), antidepressants (tricyclic and clomipramine), quinolines (chloroquine, quinine, and quinidine), plant alkaloids (vindoline), steroids (progesterone), antiestrogens (tamoxifen), surfactants (Tween-80 and cremophor-EL) [4], and cyclosporin A and its derivatives. They were shown to bind Pgp [5] and to restore intracellular drug retention in MDR cells [6]. Drugs used to inhibit the Pgp pump administered at doses necessary to be effective might be, or become, toxic themselves if they are not already intrinsically toxic, as are the calcium channel inhibitors. This is mostly due to

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¶ *Abbreviations:* COL, colchicine; ADR, adriamycin; ICaL, L-type calcium current; IR, index of resistance; MDR, multidrug resistance; MRP, multidrug-related resistance protein; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; Pgp, P-glycoprotein; and VBL, vinblastine.

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multifactorial resistance, which is frequently encountered with MDR-reversing agents. We describe here the *in vitro* and *in vivo* reversing effects of the semi-synthetic pleuromutilin-derivative antibiotic tiamulin on the MDR phenotype of cancer cells. This antibiotic, currently used in veterinary medicine to treat farm animals, may represent a valuable tool for the clinical reversion of the MDR phenotype.

MATERIALS AND METHODS

Chemicals

Cell culture media (Dulbecco's modified Eagle's medium and RPMI-1640) were from BioWhittaker and fetal bovine serum was from Sigma. ADR, COL, daunomycin, propidium iodide, antibiotic, and antimycotic solution (containing 10,000 units/mL of penicillin, 10 mg/mL of streptomycin, and 25 μg/mL of amphotericin B), were from Sigma. VBL was a gift from Roger Bellon Laboratories. Tiamulin (BM cyclin) was purchased from Boehringer Mannheim. All drugs were prepared in water under sterile conditions and sterile-filtered.

Absorbance Spectrum of Tiamulin

The absorbance spectrum was checked on a Uvikon 930 (Kontron Instruments) between 200 and 600 nm in a 1-cm path quartz cuvette containing 25 μg of tiamulin in 1 mL of solution.

Cell Culture and Selection of Highly Multidrug-Resistant, Pgp-Overproducing Cell Lines

Murine lymphoid leukemia P388 and rat hepatoma AS30-D cell lines were maintained in Dulbecco's modified Eagle's medium and the human lymphoblastic leukemia CEM cell line in RPMI-1640 medium. In all cases, the culture media were supplemented with fetal bovine serum (10%), penicillin (100 units/mL), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL). Cells were grown at 37° in a humidified atmosphere under 5% CO_2 in air. AS30-D cells were cultured in the presence of 0.5% glucose as described previously [7]. The highly drug-resistant cell lines P388/ADR25, AS30-D/COL3.2, AS30-D/COL5, AS30-D/COL10, CEM/VLB0.45, CEM/VLB3.6, CEM/VLB5, and CEM/VLB10 were established by growing the corresponding parental cell line in the medium containing stepwise-increased concentrations of ADR, COL, and VBL for 24 months, up to the indicated value. Finally, the cells thus selected were able to grow in the presence of the highest possible concentration of drug (25 μg/mL of ADR for P388/ADR25; 3.2, 5, and 10 μg/mL of COL for AS30-D/COL3.2, AS30-D/COL5, and AS30-D/COL10; and 0.45, 3.6, 5, and 10 μg/mL of VBL for CEM/VLB0.45, CEM/VLB3.6, CEM/VLB5, and CEM/VLB10, respectively); all these cell lines were cultured for at least eight months under the same conditions prior to use in sensitization assays and were regularly checked for mycoplasma contamination by using both MycoTect (GIBCO BRL) according to the manufacturer's instruction and fluorescence staining using Hoechst 33258 according to Adams [8]. We used COL as a means of comparison with other drugs studied for their ability to induce the MDR phenotype. Even though COL is never used in human anticancer therapy, it has been used *in vitro* to induce the MDR phenotype in several cell lines.

Cytotoxicity Index (IC_{50}) for Anticancer Drugs and Chemosensitizers, and Index of Resistance (IR)

Resistant and parental sensitive cells were passaged in fresh medium to a concentration of 0.2×10^5 mL and incubated for 24 hours before testing. Cell viability (measured by trypan blue exclusion) of each cell line in the presence of the selective anticancer drugs (for resistant cells) or in their absence (for sensitive parental cells) was considered as 100% growth.

Drug cytotoxicity was assessed by a colorimetric assay using the tetrazolium salt MTS (Cell Titer 96® AQ_{ueous}, Promega) 3 hours after cell exposure to either COL, doxorubicin, or VBL depending on the cell line (AS30-D, P388, or CEM, respectively). The assay was performed in the presence or absence of tiamulin or of other MDR modulators, according to Carmichael *et al.* (1987) [9] with the following modifications: 5000 cells from P388, AS30-D, and CEM tumor cell lines (sensitive and resistant for both lines) were plated in a 96-well plate (Costar) in a 100 μL volume of the appropriate culture medium. After 24 hr for P388 cells and 48 hr for AS30-D and CEM cells, the cells were passaged onto new fresh medium and exposed for 72 hr to the appropriate concentration of the inducing drug (COL, doxorubicin, or VBL, respectively at a concentration spanning several decades from zero to 100 μg/mL, depending on the cell line), or to the sensitizing agent (tiamulin, verapamil, or cyclosporin A), or to both the inducing drug and the reversing agent, diluted in the corresponding culture medium to a final volume of 200 μL in each well. These conditions kept the cells in an exponential growth phase. At the end of the incubation period, 20 μL of the reactive MTS salt was added and maintained for 4 hr. Cells were then centrifuged at 200 *g* for 1 min and 200 μL of DMSO was added to the pellet to dissolve the formazan crystals; the optical density was measured on a microplate spectrophotometer (Dynatek MR5000, BioTek) at 490 nm. Blanks containing both the culture medium and the corresponding drugs for each concentration were incubated in the same way and subtracted from the assays. IC_{50}, defined as the drug concentration necessary to induce 50% growth inhibition, was calculated from the dose-response curves obtained by plotting the measured growth (given by the optical density of assays compared to that of controls incubated under the

same conditions in the absence of any drug) versus the drug concentration, using the GraphPad Prism program.

We define the IR as the ratio of the IC_{50} for a given cytotoxic compound measured from a resistant cell line (IC_{50}^{R}) to that measured from its parental sensitive cell line (IC_{50}^{S}) so that IR = IC_{50}^{R}/IC_{50}^{S}.

Intracellular Retention of Daunomycin by Flow Cytometry

After a viability test performed by trypan blue exclusion, resistant or sensitive cells in exponential growth phase were washed twice in PBS (1.8 mM of KH_2PO_4, 10 mM of Na_2HPO_4, 138 mM of NaCl, 2.7 mM of KCl, pH 7.4), seeded to a concentration of 0.3×10^6/mL into fresh culture medium, incubated for 1 hour at 37° prior to the addition of 1.9 μM of daunomycin (final concentration) and incubated again for 3 hours at 37° in the adequate culture medium. For kinetic measurements of daunomycin retention, tiamulin was added at a 2 μM final concentration at the same time as daunomycin, and a 300 μL aliquot containing 1.0×10^5 cells was immediately withdrawn (for time zero) and thereafter at indicated times, and centrifuged for 1 min at 100 *g* at 4°. From then on, the cells were kept on ice. The cell pellet was washed twice in ice-cold PBS, suspended in 100 μL of ice-cold PBS, and immediately analyzed by flow cytometry using a FACSort flow cytometer with Cellquest software (Beckton-Dickinson FACS system). For daunomycin and propidium iodide, the excitation source was an argon ion laser emitting a 488 nm beam at 15 mW. The red fluorescence of daunomycin and propidium iodide was collected through a 585/42 nm band pass filter (FL2) and measured on a 4-decade logarithmic scale (10,000 events analyzed for each sample). Aliquots of the same cell batches as those used for the retention assays but in the absence of any drug were withdrawn for viability testing. Propidium iodide was added at a final concentration of 0.5 ng/mL and cells were immediately checked for viability, which typically exceeded 98%.

In Vivo *Sensitization by Tiamulin*

Intraperitoneal injections of 5×10^6 P388/ADR25 cells in 0.1 mL of PBS were performed in DBA/2 male mice (weighing 16 g, 4 weeks old) (IFFA CREDO). Cells were previously cultured in the absence of any drug 2 to 3 days prior to injection. The day following the injection, 30 mice were injected with ADR (8 mg/kg) and 30 with ADR (8 mg/kg) plus tiamulin (1.6 mg/kg). Drug treatment was repeated every 5 days. Survival of mice in each group was examined daily. The results were obtained from curves of the number of surviving mice versus days after tumor implantation. The experiment was repeated 5 times. Data were analyzed by a Wilcoxon signed rank test and the mean survival days were compared with a Student's *t*-test.

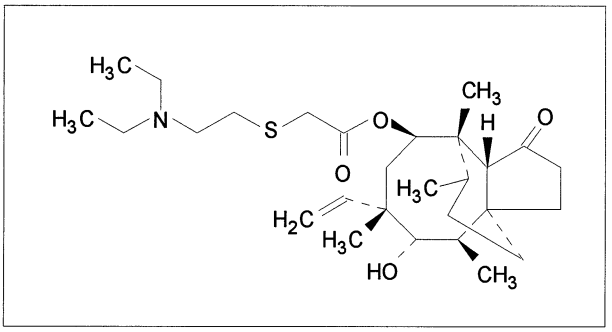

FIG. 1. Chemical structure of tiamulin [3aS-(3aα,4β,5α,6α, 8β,9α,9αβ,10S)] - [(2 - (diethylamino)ethyl)thio]acetic acid 6-ethenyldecahydro-5-hydroxy-4,6,9,10-tetramethyl-1-oxo-3a,9-propano-3aH-cyclopentacycloocten-8-yl ester].

Analyses of Plasma Membranes from Resistant and Sensitive Cells

Preparation of plasma membranes, assay for ATPase activity, and measurements of [^{3}H]-VBL uptake by plasma membranes were performed as previously reported [10].

Calcium Channel Activity Assay

Electrophysiological experiments were carried out on isolated rat right ventricular cells [11] with the whole-cell patch-clamp technique [12] under voltage clamp conditions. The external solution contained: 140 mM of NaCl, 5 mM of CsCl, 2 mM of $MgCl_2$, 2.5 mM of $CaCl_2$, 0.1 mM of thiamine, 10 mM of glucose, 0.4 mM of mannitol, and 10 mM of HEPES/NaOH, pH 7.2. The pipette solution contained: 110 mM of aspartic acid, 20 mM of CsCl, 7 mM of NaCl, 1.1 mM of $MgCl_2$, 0.2 mM of EGTA, and 5 mM of HEPES/CsOH, pH 7.2. Tiamulin was added to the external solution at concentrations of 1 and 10 μM.

RESULTS

Tiamulin

Tiamulin (Fig. 1) or [3aS-(3aα,4β,5α,6α,8β,9α,9αβ,10S)]-[(2-(diethylamino)ethyl)thio]acetic acid 6-ethenyldecahydro-5hydroxy-4,6,9,10-tetramethyl-1-oxo-3a,9-propano-3aH-cyclopentacycloocten-8-yl ester] ($C_{28}H_{47}NO_4S$, molecular weight 493.76) is a semi-synthetic derivative of the natural antibiotic pleuromutilin isolated from the Basidiomycete *Pleurotus mutilus* [13]. It is frequently used in veterinary medicine to treat Gram-positive and mycoplasma infections of farm animals and is known to have no toxicity at the usual doses of 35 mg/kg weight. While treating L1210-resistant cells for mycoplasma infection with tiamulin, we noticed that cells died when the anticancer drug was present in the culture medium, whereas they survived when the anticancer drug was removed. Therefore, we studied this antibiotic on three different tumor cell lines from different species and at different IRs for its capacity to restore drug-sensitivity of *in vitro* proliferating MDR cells.

TABLE 1. IC_{50} **and IR of MDR-related drugs in sensitive and resistant cancer cell lines, cross-resistance to several anticancer drugs**

	Daunomycin		Vinblastine		Adriamycin		Colchicine	
	IC_{50} (μg/mL)	IR	IC_{50} (μg/mL)	IR	IC_{50} (μg/mL)	IR	IC_{50} (μg/mL)	IR
AS30-D/S	0.055 ± 0.028	NA*	0.021 ± 0.006	NA	0.53 ± 0.2	NA	0.17 ± 0.08	NA
AS30-D/COL3.2	—	—	3.51 ± 0.6	165	—	—	6.23 ± 1.1	37
AS30-D/COL5	3.12 ± 1.7	56	—	—	—	—	9.43 ± 0.34	56
AS30-D/COL10	20.35 ± 7.52	370	—	—	—	—	17.48 ± 2.35	103
P388/S	0.0037 ± 0.0023	NA	0.0018 ± 0.0005	NA	0.0054 ± 0.003	NA	0.00064 ± 0.0004	NA
P388/ADR25	7.68 ± 2.01	2075	0.36 ± 0.1	194	45.5 ± 6.7	8426	3.33 ± 0.3	5203
CEM/S	0.0030 ± 0.0027	NA	0.00058 ± 0.00034	NA	0.0019 ± 0.001	NA	—	—
CEM/VLB0.45	—	—	2.6 ± 0.7	4483	—	—	0.00089 ± 0.00005	—
CEM/VLB3.6	1.80 ± 1.03	600	8.44 ± 3.7	14552	28.0 ± 10	15098	0.95 ± 0.2	1067
CEM/VLB5	3.25 ± 1.65	1083	10.9 ± 2.11	18793	—	—	1.62 ± 0.2	1820
CEM/VLB10	—	—	24.4 ± 1.6	42069	—	—	—	—

IC_{50}s of anticancer drugs used to induce the MDR phenotype (shown in italics) and of other anticancer drugs were obtained as described in Materials and Methods for different levels of resistance of the three cell lines CEM, AS30-D, and P388, with increasing concentrations of the anticancer drug. IC_{50}s are reported in μg/mL as means of six experiments ± SD. Indexes of resistance (IR = IC_{50} for a given anticancer drug in a resistant cell line/IC_{50} for the same drug in the parental sensitive cell line as defined in Materials and Methods) are calculated whenever possible.

*NA, not applied.

From its absorbance spectrum, tiamulin does not display any absorbance maximum in the visible range from 300 to 600 nm, and therefore does not interfere with flow cytometric analysis.

In Vitro *Chemosensitization by Tiamulin*

In order to determine whether tiamulin was able to affect anticancer drug accumulation in Pgp-overproducing cancer cells, its effect on the reversal of the MDR phenotype was tested on resistant and sensitive cells in culture.

ESTABLISHMENT OF RESISTANT CELL LINES. For this purpose, we established highly resistant cancer cell lines from different species by stepwise increasing the anticancer drug concentration over a 24-month period. Then, we verified that these cells were indeed overproducing Pgp by performing a Western blot of plasma membrane proteins separated by gel electrophoresis and transferred onto nitrocellulose, using the C219 monoclonal antibody, previously described by us in [10].

CYTOTOXICITY OF ANTICANCER DRUGS USED. The cytotoxicity effect that each drug which was used to induce the MDR phenotype exerts on a cell line was assessed by the IC_{50} and is given in Table 1. From these data, we can calculate the IR, which indicates how many times a resistant cell line is chemoresistant relative to its parental cell line, as defined in Materials and Methods. CEM/VLB0.45, CEM/VLB3.6, CEM/VLB5, and CEM/VLB10 were ca. 4,400, 14,500, 18,800, and 42,000 times more resistant than their sensitive parental CEM/S cells, respectively; P388/ADR25 cells were approximately 8400 times more resistant than their parental P388/S cells; and finally, AS30-D/COL3.2, AS30-D/COL5, and AS30-D/COL10 cells were 37, 56, and 100 times more resistant, respectively, than their parental AS30-D/S cells. It is interesting to note that, for cell lines such as CEM and AS30-D, IRs corresponding to different intermediate resistant levels of VBL (for CEM) and to COL (for AS30-D) were linearly correlated to the corresponding quantity of the same drug that is regularly used to maintain cellular drug resistance to the level these cells reached.

Cellular cross-resistance to cytotoxic drugs other than those used to induce the MDR phenotype was evaluated from the data presented in Table 1. Drugs such as daunomycin (an anthracyclin which is closely related to ADR and that we also used for cytometry experiments), VBL, and ADR were tested on both sensitive and resistant cell lines. All the resistant cell lines showed important resistance levels to the other MDR-related drugs, as shown by their respective IRs.

CYTOTOXIC EFFECT OF TIAMULIN AND CHEMOSENSITIZATION OF RESISTANT CANCER CELLS. Intrinsic cytotoxicity of tiamulin tested with the MTS salt on both sensitive and resistant cell lines in the absence of any other drugs is represented by IC_{50} values exceeding 400 μg/mL (0.8 mM), irrespective of the cell line considered.

Tiamulin-Induced Intracellular Retention of Daunomycin

One hypothesis for the observed sensitization effect is that tiamulin induces anticancer drug accumulation inside the cell. To verify this, we measured the intracellular retention of daunomycin, an anthracyclin closely related to doxorubicin. We first determined that the incubation time necessary to reach an equilibrium state for daunomycin in each resistant cell line on which the effects of tiamulin were studied was 3 hours. Figure 2 represents the cytograms obtained with the three cell lines studied in both their sensitive and resistant forms. All the results presented correspond to data collected 3 hours after the addition of 2

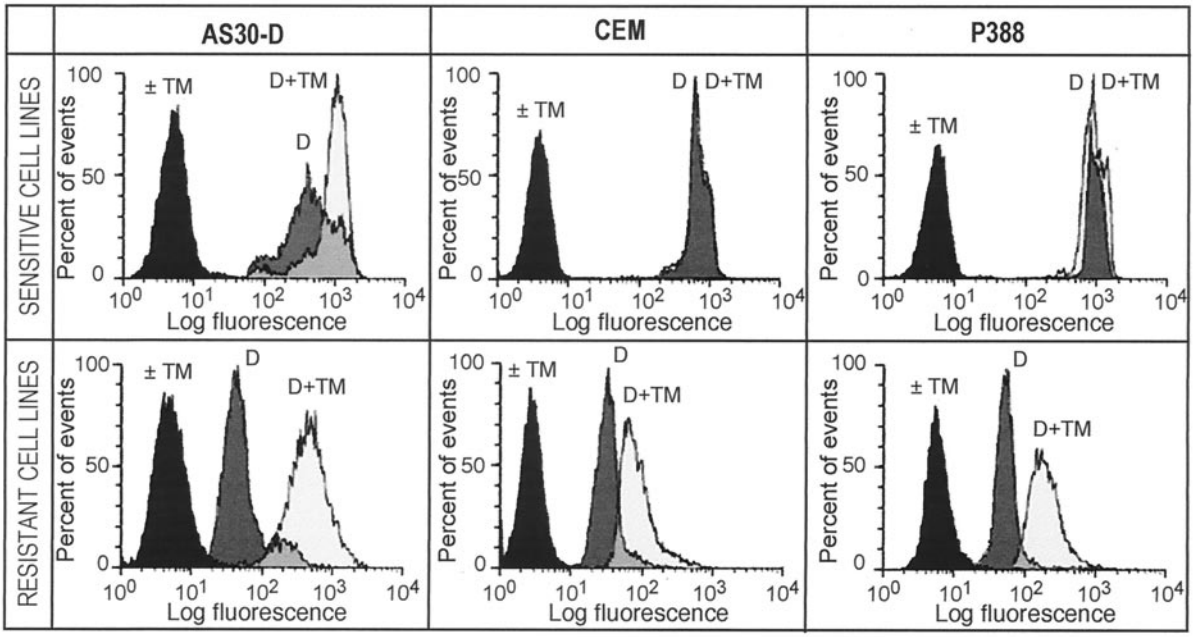

FIG. 2. Effect of tiamulin on the intracellular retention of daunomycin in parental (upper row) and resistant-derived (lower row) cell lines (AS30-D/COL10, CEM/VLB3.6, and P388/ADR25, from left to right). Cells were treated as described in Materials and Methods prior to the flow cytometry analysis. When indicated, cell samples were incubated for 3 hours in the presence of 2 μM of tiamulin. Cytograms represent the cell population as a function of the red-emitted fluorescence intensity. In each frame, the three overlaid cytograms represent, from left to right, the autofluorescence control (±TM), the peak of which is actually superimposed with the peak obtained for cells incubated with 2 μM of tiamulin (±TM), cells incubated with 1.9 μM of daunomycin (D), and cells incubated with both 1.9 μM of daunomycin and 2 μM of tiamulin (D + TM), respectively. One typical experiment is presented for each cell line.

μM of tiamulin to the culture medium. In the three cell lines considered, the presence of tiamulin did not change the fluorescence value indicated by the median of the autofluorescence (control) peak. Moreover, the P388 cell lines aside, the median autofluorescence values in the other two cell lines were identical in both sensitive and resistant cells from the same line.

AS30-D AND AS30-D/COL10. Addition of daunomycin to sensitive cells induced a rightshift of the control (±TM) peak, indicating an intracellular retention of the anticancer drug, which markedly increased (peak D + TM) when tiamulin was present in the medium. Intracellular retention of daunomycin in resistant cells was much less efficient than in sensitive cells (peak D); however, the presence of tiamulin induced a marked rightshift (peak D + TM).

CEM, CEM/VLB3.6, P388, AND P388/ADR25. The important rightshift of the fluorescence observed with sensitive cells incubated in the presence of daunomycin (peak D) was not changed when tiamulin was present in the incubation medium (peak D + TM). However, when resistant cells are considered, the intracellular retention of daunomycin was much less substantial (peak D) and was markedly increased in the presence of tiamulin (peak D + TM).

Chemosensitization Ability of Tiamulin and Comparison with that of Known Sensitizers

In order to estimate the chemosensitization effect of tiamulin on the resistant cell lines, the tiamulin concentration was kept constant while the anticancer drug concentration varied, as shown in Table 2. The presence of 1 μg/mL of tiamulin in resistant cells cultured in the presence of varying concentrations of MDR-related anticancer drugs makes it possible to calculate IC_{50}s for that anticancer drug, which are significantly decreased by the factor indicated between brackets when compared to IC_{50}s obtained under the same conditions but in the absence of tiamulin. Much greater decreasing factors were obtained when the concentration of tiamulin was elevated to 2.5 and 5 μM, as shown in Table 2.

All the resistant cells considered acquired a high level of resistance *in vitro*. However, AS30-D/S cells displayed a much higher IC_{50} for their resistance-inducing drug than that of the other two sensitive cell lines, i.e. 31 and 293 times higher than the IC_{50}s of CEM/S and P388/S, respectively. This is mainly due to the production of small quantities of Pgp by AS30-D/S cells, as we have previously shown [10]. In this cell line with a low level of resistance, 2 μM of tiamulin increased the sensitivity level to COL by a factor of 8.5, which is approximately twice the effect obtained when 2 μM of tiamulin was tested on AS30-D/

TABLE 2. IC_{50}s for the indicated anticancer drugs in the absence or the presence of a reversing agent and its efficacy index in sensitive and resistant cell lines

Cell line Anticancer drug	CEM/S VBL	CEM/VLB0.45 VBL	CEM/VLB5 VBL	CEM/VLB10 VBL	AS30-D/S COL	AS30-D/COL5 COL	P388/S ADR	P388/ADR25 ADR
IC_{50}, no TM	$5.8\ 10^{-4} \pm 3\ 10^{-5}$	2.6 ± 0.7	10.9 ± 3.0	24.4 ± 1.6	0.17 ± 0.08	9.4 ± 0.3	$5.4\ 10^{-3} \pm 4\ 10^{-4}$	48.3 ± 6.7
IC_{50}, TM 1 μg/mL	$5.6\ 10^{-4} \pm 4\ 10^{-5}$	0.4 ± 0.1	4.1 ± 1.7	ND	0.02 ± 0.005	2.1 ± 0.3	$5.1\ 10^{-3} \pm 5\ 10^{-4}$	13.1 ± 1.8
[efficacy index]		[6.5]	[2.7]		[8.5]	[4.5]		[3.7]
IC_{50}, TM 2.5 μg/mL	$5.4\ 10^{-4} \pm 5\ 10^{-5}$	0.2 ± 0.01	1.4 ± 0.01	3.7 ± 1.4	0.0085 ± 0.0005	1.6 ± 0.8	$5.3\ 10^{-3} \pm 5\ 10^{-4}$	1.6 ± 0.9
[efficacy index]		[13]	[7.8]	[6.6]	[20]	[5.9]		[30.2]
IC_{50}, TM 5 μg/mL	$5.3\ 10^{-4} \pm 5\ 10^{-5}$	0.1 ± 0.08	1.79 ± 1.46	1.23 ± 1.01	0.0046 ± 0.0002	1.81 ± 1.21	$5.1\ 10^{-3} \pm 9\ 10^{-5}$	1.6 ± 0.04
[efficacy index]		[26.0]	[6.1]	[19.8]	[37]	[5.2]		[30.2]
IC_{50}, VP 5 μg/mL	ND	0.14 ± 0.08	2.99 ± 2.37	8.44 ± 3.12	ND	1.97 ± 1.06	ND	2.4 ± 0.9
[efficacy index]		[18.6]	[3.6]	[2.9]		[4.8]		[20.1]
IC_{50}, CyA 2.5 μg/mL	ND	0.38 ± 0.06	4.38 ± 3.01	10.2 ± 4.1	ND	2.21 ± 1.04	ND	ND
[efficacy index]		[6.8]	[25]	[2.4]		[4.5]		

IC_{50}s of anticancer drugs used to induce the MDR phenotype (VLB, COL, and ADR) are given in μg/mL and were obtained as described in Materials and Methods for different levels of resistance of the three cell lines CEM, AS30-D, and P388, with increasing concentrations of the anticancer drug. Values in brackets represent the efficacy index, i.e. the number of times tiamulin may decrease the initial IC_{50} of the corresponding resistant cell line studied in the absence of tiamulin (first row). IC_{50}s are reported in μg/mL as means of six experiments ± SD. ND, not determined; TM, tiamulin; VP, verapamil; CyA, cyclosporin A.

COL5 cells. In conclusion, tiamulin efficiently sensitized resistant cells that express different levels of Pgp to the cytotoxic effects of an MDR-related anticancer drug.

In order to assess the efficiency of tiamulin in reversing the MDR phenotype and contrast it with other chemosensitizers, we compared the IC_{50}s of anticancer drugs used to induce the MDR phenotype in the three cell lines studied with the IC_{50}s obtained after addition of a chemosensitizer compound to the resistant cell lines. As shown in Table 2, the presence of a chemosensitizer decreased the IC_{50} of the inducing anticancer drug by values ranging from 5.2 to 30.2 with 5 μg/mL of tiamulin, from 2.9 to 20.1 with 5 μg/mL of verapamil, and from 2.4 to 25 with 2.4 μg/mL of cyclosporin A. Data in this table show that tiamulin was in all cases 1.1 to 6.8 times more efficient than verapamil and 1.2 to 8.3 times more efficient than cyclosporin A, all used at a same molar concentration, the latter being the least efficient of the three compounds.

Effect of Tiamulin on the ATPase Activity of Pgp

In order to determine the mechanism by which tiamulin chemosensitizes cells that express Pgp, we investigated its effects on the ATPase activity of Pgp in isolated plasma membrane vesicles of AS30-D/COL10 cells in the presence of a cocktail containing inhibitors of other known ATPases. We previously reported that for the three resistant cell lines considered, the remaining ATPase activity was specifically stimulated by verapamil and inhibited by orthovanadate [10], which is a characteristic of Pgp ATPase. As shown in Fig. 3A, when added alone, either 1.5 μM of COL or 20 μM of tiamulin did not exert any effect on the specific ATPase activity of Pgp. To obtain a 25% inhibition of that activity, tiamulin needed to reach a concentration of 120 μM in the assay medium. However, if the anticancer drug was present in the assay medium, i.e. COL at 1 μM, increasing tiamulin concentrations significantly inhibited the ATPase activity of Pgp so that the presence of 1.5 or 6 μM of tiamulin induced a 60% or a 70% inhibition, respectively. The same observation could be made with the ATPase activity of Pgp from CEM/VLB5 plasma membranes incubated in the presence of tiamulin alone (120 μM to reach 25% inhibition of the Pgp ATPase activity) or of tiamulin (1.5 μM) + VBL (1 μM) to reach 60% inhibition of the Pgp ATPase activity.

A more precise analysis of tiamulin-induced inhibition of the specific Pgp ATPase activity measured from AS30-D/COL10 plasma membrane vesicles as a function of tiamulin concentration in the presence of 1 μM of COL is presented in Fig. 3B. The curve obtained is a biphasic hyperbola with a rapid decrease at low tiamulin concentrations between 0 and 3 μM, followed by a slow decrease at higher tiamulin concentrations. Since the maximum specific Pgp ATPase activity measured corresponds to 586 mmol/min/mg of membrane proteins under our conditions, the concentration of tiamulin that induced a 50% inhibition is calculated to be 0.54 μM.

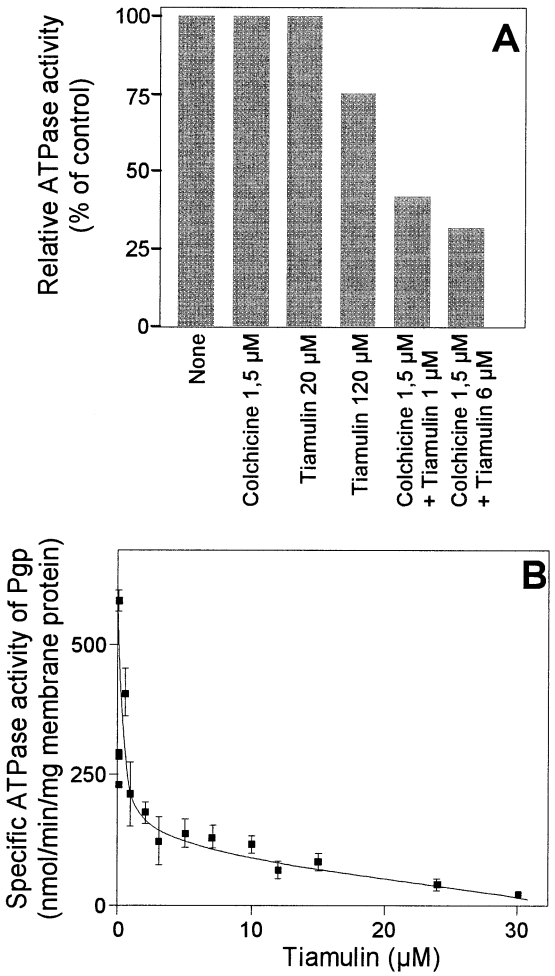

FIG. 3. Tiamulin-induced modulation of the specific ATPase activity of Pgp in AS30-D/COL10 plasma membranes. Membrane vesicles were prepared and Pgp ATPase activity (A) was measured as described in Materials and Methods in the absence of any drug (control = 586 nmol/min/mg proteins) and in the presence of COL or tiamulin, or both, as indicated. (B) Specific ATPase activity is plotted as a function of increasing tiamulin concentration in the presence of 1 µM of COL. Data are means of 5 experiments ± SD.

Effect of Tiamulin on [^{3}H]-VBL Transport by Plasma Membrane Pgp

Pgp is believed to function as an ATP-dependent drug efflux pump for which drug transport is coupled to ATP hydrolysis. We showed that, in the presence of an anticancer drug, tiamulin restored intracellular drug retention while the ATPase activity of Pgp was inhibited. To address drug transport impairment by tiamulin, we measured the transport of [^{3}H]-VBL by Pgp in isolated plasma membrane vesicles from AS30-D/COL10 cells. Figure 4 plots [^{3}H]-VBL uptake as a function of increasing concentrations of either tiamulin or verapamil. When 25 nM of [^{3}H]-VBL was added to the reaction medium, its uptake was inhibited by 50% with 6.2 µM of tiamulin, whereas lower tiamulin concentrations were needed (i.e. 2.1 µM) when the concentration of [^{3}H]-VBL was 100 nM. When we consider verapamil-induced [^{3}H]-VBL transport, the same inhibition pattern was observed; however, the concentration of verapamil that induced 50% inhibition of [^{3}H]-VBL uptake was lower (i.e. 1.9 µM) when 25 nM of [^{3}H]-VBL was added to the reaction medium. Therefore, when used at 25 nM, verapamil was 3-fold more efficient than tiamulin at inhibiting 50% of [^{3}H]-VBL uptake. The two products demonstrated comparable inhibitory patterns when tiamulin was 4 times more concentrated than verapamil. The insert panel of Fig. 4 shows a Dixon plot for tiamulin-induced inhibition of [^{3}H]-VBL transport through Pgp in AS30-D/COL10 plasma membrane vesicles depending on different substrate concentrations. The parallel plots obtained indicate that tiamulin exerted an uncompetitive type of inhibition from which we can calculate an inhibition constant of 0.07 µM with a V_{max} for [^{3}H]-VBL transport of 5.9 pmol/min/mg protein, as previously determined [10].

Effect of Tiamulin on the Calcium Channels of Myocardial Cells

Many chemosensitizers display some contractile tissue toxicity. We assayed the influence of tiamulin on the ICaL in rat right ventricular cells. One µM of tiamulin had a barely detectable effect on the ICaL, while 10 µM of tiamulin decreased it by about 25% in 2 minutes. Lower tiamulin concentrations did not have any effect on this channel. The block induced by high tiamulin concentrations was totally reversible when tiamulin was progressively removed from the medium.

In Vivo *Reversal Activity of Tiamulin*

In order to assess the activity of tiamulin *in vivo*, we transplanted the P388/ADR25 cell line into DBA/2 mice which had been treated with ADR in the presence or absence of tiamulin at doses directly extrapolated from *in vitro* conditions, as described in Materials and Methods. Figure 5 shows the survival curve of mice treated or not with tiamulin after tumor implantation. Mice treated with ADR alone survived 11.5 ± 1.1 days after tumor inoculation, whereas mice injected with both ADR and tiamulin survived 14.9 ± 1.0 days after tumor inoculation, which represents a 29% increase in mean survival ($P = 0.001$).

DISCUSSION

The results reported in the present study strongly support the notion that tiamulin, a semi-synthetic antibiotic used against Gram positive and mycoplasma infections in farm animals, promotes anticancer drug accumulation and modulates Pgp activity in Pgp-expressing or Pgp-overexpressing multidrug-resistant cancer cells *in vitro* and *in vivo*.

Although a common chemical basis for the modulating action of all chemosensitizers does not seem to exist, several

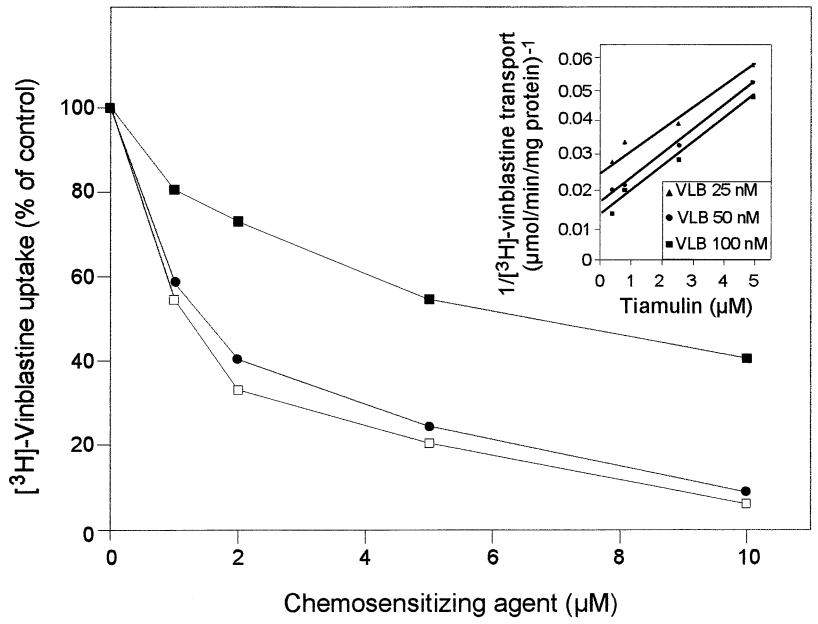

FIG. 4. Inhibition of [^{3}H]-VBL uptake by plasma membranes from AS30-D/COL10 cells induced by chemosensitizing agents. Plasma membranes from AS30-D/COL10 cells were prepared as described in Materials and Methods. ATP-dependent [^{3}H]-VBL uptake (25 nM, ■——■, □——□, or 100 nM ●——●) was measured after a 20-min incubation in the absence (controls) and in the presence of increasing amounts of either tiamulin (■——■ or ●——●) or verapamil (□——□) and calculated by subtracting blank values obtained in the presence of AMP instead of ATP. In each experiment, the amount of total membrane protein in which Pgp represented ca. 20% (w/w) was 50 μg. Data points represent the means of duplicate determinations; the deviation of data points from their respective absolute mean values varied from 0 to 2 ordinate units. The control value for [^{3}H]-VBL uptake by plasma membrane in the absence of chemosensitizing agent was 18.8 ± 0.9 pmol/mg protein/20 min and 67.4 ± 1.4 pmol/mg protein/20 min when 25 and 100 nM of [^{3}H]-VBL were used, respectively. Inset: Dixon plot for the inhibition of [^{3}H]-VBL transport by plasma membranes from AS30-D/COL10 cells. Inhibition of [^{3}H]-VBL uptake measured after a 20-min incubation was performed according to Materials and Methods and plotted as a function of tiamulin concentration in the presence of 25 nM (▲——▲), 50 nM (●——●), and 100 nM (■——■). Results are means of two experiments.

"rules" have been defined for the design of an ideal MDR modulator [14]. Tiamulin contains a tertiary nitrogen between 2 alkyl groups in a *trans* conformation, which has been described as the most statistically significant biophore with relevant reversal activity [15]. A quaternary nitrogen would most probably prevent the molecule from entering the cell, and thereby negate its reversal activity.

We assayed the chemosensitizing properties of tiamulin on highly resistant cancer cell lines (and their respective sensitive parental lines) from different species: AS30-D rat hepatoma, P388 murine lymphoid leukemia, and CEM human lymphoblastic leukemia. Resistant cells expressed or overexpressed Pgp in their plasma membrane and cross-resisted drugs known to be involved in the MDR phenotype.

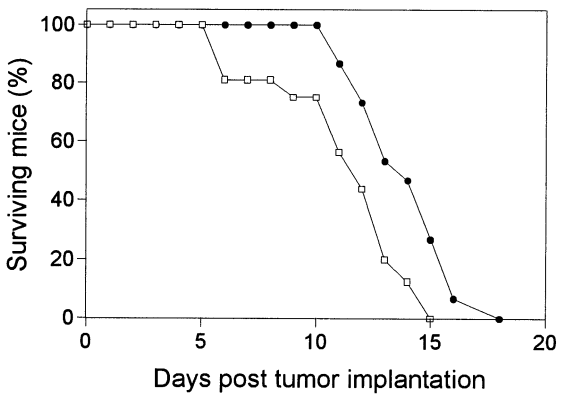

FIG. 5. Survival curves of P388/ADR25-bearing mice treated or not with tiamulin as a function of days after tumor implantation. Mice were injected i.p. with 5 × 10^6 resistant P388/ADR25 cells at day 0. The following day, they received ADR (8 mg/kg) in the absence (□——□) or in the presence of tiamulin (1.6 mg/kg) (●——●) as described in Materials and Methods.

We showed that tiamulin enhanced the intracellular retention of all anticancer drugs tested, i.e. COL, ADR, daunomycin, and VBL in AS30-D-, P388- and CEM-resistant cells, respectively. This indicates that it is active in highly resistant cancers from many mammalian species.

After ensuring that tiamulin did not display any excitation or emission maximum that could disturb flow cytometry data acquisition of daunomycin fluorescence, we showed that tiamulin enhanced the intracellular retention level of daunomycin by 5.3-, 2.3- and 4.0-fold in AS30-D/COL5, CEM/VLB3.6, and P388/ADR25 cells, respectively, demonstrating that the effect of tiamulin was not limited to the anticancer drug used to induce the MDR phenotype. By contrast, at doses employed to reverse the MDR phenotype, no changes were observed in the retention of daunomycin in sensitive cancer cells, except for AS30-D/S cells. These cells behaved like resistant cells with regard to tiamulin since: (1) the fluorescence spectrum corresponding to the cell population that received daunomycin was at least half a decade shifted to the left when compared to the other two sensitive cell populations, indicating that daunomycin is extruded; and (2) the addition of 2 μM of tiamulin induced a substantial rightshift of the fluorescence spectrum, indicating that intracellular daunomycin retention was en-

hanced. Therefore, these cells may not be strictly regarded as "sensitive" cells. In fact, they express low but significant amounts of Pgp on their plasma membranes as we previously showed [10]. Interestingly, the efficacy index of tiamulin used from 1 to 5 μg/mL in the same cells varied from 8.5 to 37, indicating its ability to reverse the MDR phenotype in "naturally" resistant cancers. Most of the reversing agents reported in the literature and used in a 1–10 μM range exhibit an efficacy index of approximately 5. When compared to the two known chemosensitizers, verapamil and cyclosporin A, tiamulin always showed 1.1- to 8.3-fold higher efficacy indices in the resistant cell lines from the three different species studied. When tiamulin was used at the sensitizing doses in the absence of any anticancer drug and up to at least 100 times these doses, no significant effect was observed in either sensitive or resistant cells. These results show that tiamulin synergizes the effects of an anticancer drug in resistant cancer cells that express the MDR phenotype.

MRP, the second important MDR-related ABC transporter, has been shown to be expressed in CEM-resistant cells [16]. However, in both our CEM- and AS30-D-resistant cells, we were not able to detect any expression of MRP when using MRP-specific antibodies by Western blotting, whereas a slight signal was detected in the plasma membranes of P388/ADR25 cells which was 1/100th below that of Pgp.* Co-expression of both Pgp and MRP is possible, since the inducing drug in this case is an anthracyclin which was also known to induce MRP expression [17]. Therefore, the data obtained with CEM and AS30-D cells are likely to be representative of Pgp-only-mediated drug resistance whereas, in the case of P388/ADR cells, the participation of MRP should not be very important, especially when we consider their cross-resistance profile that mostly agrees with a Pgp-mediated MDR phenotype.

We showed that tiamulin was able to restore intracellular anticancer drug accumulation. Several chemosensitizers act through a direct competitive inhibition of anticancer drug transport, or through a noncompetitive mechanism [18]. To address the mechanism of tiamulin action, we first measured its effect on the ATPase activity of membrane Pgp. We have previously reported that such activity in our resistant cell lines was stimulated by verapamil and inhibited by orthovanadate, a feature that is recognized as specific to Pgp ATPase activity. When incubated alone, tiamulin should be used at high concentrations (over 100 μM) to induce slight inhibitory effects (below 20%) on the ATPase activity of membrane Pgp. On the contrary, when used in the presence of an anticancer drug, low tiamulin concentrations induced relatively high inhibition levels of the same ATPase activity. For instance, 0.7 μM of tiamulin induced 50% inhibition of the ATPase activity. The inset of Fig. 4 shows that the higher the substrate concentration, the lower the inhibitor concentration needed to inhibit drug transport. The same relationship was observed in cultured resistant cells for the IC_{50} values for tiamulin obtained when the VBL concentration varied. This relationship suggests that the inhibition becomes more effective when enzyme–substrate complexes become more abundant, which is typical of uncompetitive inhibitors. Data obtained from Dixon plots of [^{3}H]-VBL transport under increasing tiamulin concentrations strongly support the existence of such a mechanism. We found an inhibition constant for the inhibition of VBL transport of 0.07 μM, which shows a 14-fold higher affinity than VBL pumping through Pgp in whole cells (K_m = 1 μM) as reported by Horio *et al.* [19], or 2- and 354-fold higher affinity than vincristine transport by plasma membrane vesicles (K_m = 0.14 and 24.8, respectively [20]). The uncompetitive mechanism is supported by our finding that tiamulin alone did not change the basic ATPase activity of plasma membrane Pgp when used at doses that are compatible with *in vitro* reversal of the MDR phenotype. In other words, tiamulin did not seem to possess any pharmacophore in the Pgp. Recent evidence supports the multisite-dependent inhibition of the ATPase activity of Pgp. A reconstituted purified Pgp system should be of interest in better understanding its target of interaction.

A serious problem encountered with many modulating agents is their degree of toxicity at the high doses needed to reach reversing responses in clinical trials. At the doses used to efficiently reverse the *in vitro* resistance of all our resistant cell lines (i.e. up to 1 μM), tiamulin did not exert any calcium channel blockage on excitable tissues such as rat myocardial fibers. Only a 25% decrease in the ICaL was observed when up to 20 μM of tiamulin was used, one totally reversed when tiamulin was withdrawn. This feature reinforces the interest of this compound. This result should be confirmed by toxicological tests in whole organs and animals, and studies should be extended to other targets since some inhibition of cytochrome P-450 in animals [21] and aortic and cardiac muscle electrical alterations in guinea-pigs has been reported [22].

We have shown that tiamulin was able to sensitize resistant tumors *in vivo* with, under our conditions, a 29% increase in the survival time for tumor-bearing mice injected with tiamulin as compared to controls. These results were obtained after extrapolation of *in vitro* conditions without optimization of *in vivo* conditions. Even though drug pharmacokinetics *in vitro* is different from that *in vivo*, our *in vivo* results allow us to believe that use of tiamulin in clinical situations is feasible. This will require extensive animal experimentation and *in vivo* optimization.

In conclusion, we showed for the first time that tiamulin is an efficient chemosensitizing agent for resistant cancer cells from different species that express the MDR phenotype, mainly through Pgp. Its mode of action consists of the increased intracellular retention of the anticancer drug used, likely through inhibition of the ATPase activity of Pgp and/or drug transport by Pgp through an uncompetitive mechanism, which is uncommon among chemosensitizers.

*Baggetto LG and Marthinet E, unpublished results.

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