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STRUCTURE-ACTIVITY RELATIONSHIP OF VERAPAMIL ANALOGS AND REVERSAL OF MULTIDRUG RESISTANCE

G. TOFFOLI, F. SIMONE, G. CORONA, M. RASCHACK,* B. CAPPELLETTO, M. GIGANTE and M. BOIOCCHI†

Division of Experimental Oncology 1, Centro di Riferimento Oncologico, via Pedemontana Occidentale 12, 33081 Aviano, Italy; and *Knoll AG, Department of Pharmacology, Postfach 21 0805, Ludwigshafen D-6700, Germany

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Abstract—We studied the relationship between the chemical structure and multidrug resistance (MDR) reversal activity of racemic verapamil (VER) and 14 VER analogs (VAs). The LoVo-R human colon carcinoma cell line was used as an experimental model. This cell line exhibited a typical MDR phenotype and overexpressed the MDR1 gene products. Key structural features were identified as being related to MDR reversal and cytotoxic activity. In particular, we demonstrated that the methoxy groups in the VER molecule structure [1.7-Bis-(3.4-dimethoxyphenyl)-3-methylaza-7-cyan-8-methyl-nonane] prevented cytotoxicity when the VAs were used alone, whereas the 7-cyan-8-methyl groups were important for MDR reversal activity and interaction with P-glycoprotein (P-gp). Among the VAs tested, the most active compounds were gallopamil, R-isomer of VER (R-VER), and nor-VER, which potentiated doxorubicin (DOX) cytotoxicity by 52.3 ± 7.2 ($n = 3 \pm$ SD), 38.9 ± 6.4 ($n = 4 \pm$ SD), and 35.4 ± 4.3 ($n = 3 \pm$ SD) times, respectively. The reversal activity of these compounds was similar to that of VER, which enhanced DOX cytotoxicity by 41.3 ± 5.0 ($n = 3 \pm$ SD) times. The potentiation of DOX cytotoxicity was associated with an increase in DOX uptake in LoVo-R cells and with an increased [^{3}H]azidopine P-gp photolabeling inhibition. Some compounds that had a high reversal potency (i.e. R-VER and nor-VER) showed a lower calcium antagonist activity than VER, and seem useful candidates for the treatment of MDR in cancer patients.

Key words: multidrug resistance; P-glycoprotein; verapamil

The development of cellular resistance to drugs is a major impediment to successful cancer therapy. MDR‡ implies that cells become simultaneously resistant to many structurally and functionally unrelated drugs, such as vinca alkaloids, anthracyclines, epipodophillotoxins, and actinomycin D [1]. In human cells, the MDR phenotype is thought to be primarily a consequence of the increased expression of the MDR1 gene. This encodes for a transmembrane glycoprotein of 170 kDa (P-gp) [2, 3] that acts as an energy-dependent, drug-transmembrane transporter [4–6].

A wide range of chemical compounds can inhibit the P-gp-mediated efflux of the drug from the cells [7, 8], and a number of studies have attempted to elucidate the structural features necessary for the compounds to reserve MDR [9–13]. It has been suggested that reversers have to be hydrophobic, contain two or more planar aromatic rings, a tertiary nitrogen and a positive net charge at physiological pH [9].

VER is an effective MDR reversing agent [8]. In humans, however, VER plasma concentrations necessary to reverse MDR may cause unacceptable cardiovascular side effects due to VER calcium antagonist activity [14–17]. *In vivo* and *in vitro* studies have shown that there is no correlation between reversal potency and calcium antagonist activity [10, 18, 19]. R-VER is, in fact, 10-fold less potent as a calcium antagonist than S-isomer [20, 21], but displays a similar reversal potency [22–24].

In an attempt to identify more suitable compounds for *in vivo* MDR reversal and to gain insight into the structure-activity relationship of chemical compounds in reverting MDR, we studied VER and 14 VAs. They were evaluated for their ability to reverse DOX resistance in the MDR LoVo human colon carcinoma cell line (LoVo-R) in relation to their cytotoxic and calcium antagonist activity. The aim was to provide further information for the design of new, more active MDR-reversing compounds with low cellular and/or cardiological toxicity.

MATERIALS AND METHODS

Drugs

The hydrochloride form of VER and all VA, with the exception of LU46605 (dihydrochloride) and LU46324 (amidosulfonate), were obtained from Knoll AG (Ludwigshafen, Germany). Purity of drugs was >95% by HPLC analysis. Each VA was dissolved in DMSO and kept at −20° as stock solution of 10–100 mg/mL. In this form, each VA was stable for more than 2 months. Final concentration of DMSO in the culture medium was ≤0.1%. No toxicity due to 0.1% DMSO alone was observed. Figure 1 shows the Knoll reference code for each VA, the molecular weight, generic name, enantiomer form tested, and the structure formula. The chemical group substitution introduced in the VER molecule (1.7-

† Corresponding author: Dr. Mauro Boiocchi, Division of Experimental Oncology 1, Centro di Riferimento Oncologico, via Pedemontana Occidentale 12, 33081 Aviano, Italy. Tel 434-659300; FAX 434-659428.

‡ Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; VER, racemic verapamil; VAs, verapamil analogs; DOX, doxorubicin; R-VER, R-isomer of verapamil; S-anipamil and R-anipamil, S- and R-isomer of anipamil, respectively; and IC_{50} and IC_{20}, cellular drug concentration inhibiting cell growth by 50% and 20%, respectively.

Reference code	(MW)	Generic name	Stereoisomers	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}
	491	verapamil	(R/S)	- H	- OCH_3	- OCH_3	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- OCH_3
LU 33925	491	verapamil	(R)	- H	- OCH_3	- OCH_3	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- OCH_3
LU 41723	477	nor-verapamil	(R/S)	- H	- OCH_3	- OCH_3	- H	- CN	- $CH(CH_3)_2$	- H	- $(CH_2)_2$ Ph	- OCH_3	- OCH_3
LU 30029	521	gallopamil	(R/S)	- OCH_3	- OCH_3	- OCH_3	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- OCH_3
LU 46605	530.5		(R/S)	- H	- OCH_3	- OCH_3	- H	- CH_2NH_2	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- OCH_3
LU 44494	461	devapamil	(R/S)	- H	- OCH_3	- OCH_3	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- H
LU 46324	560.5		(R/S)	- H	- OCH_3	- OCH_3	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- Cl	- Cl
LU 43918	500		(R/S)	- H	- Cl	- Cl	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- OCH_3
LU 49667	469		(R/S)	- H	- OCH_3	- OCH_3	- OCH_3	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- H	- H
LU 37076	371	emopamil	(R/S)	- H	- H	- H	- H	- CN	- $CH(CH_3)_2$	- CH_3	- $(CH_2)_2$ Ph	- H	- H
LU 52151	575	anipamil	(S)	- H	- OCH_3	- H	- H	- CN	- $(CH_2)_{11}CH_3$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- H
LU 52152	575	anipamil	(R)	- H	- OCH_3	- H	- H	- CN	- $(CH_2)_{11}CH_3$	- CH_3	- $(CH_2)_2$ Ph	- OCH_3	- H
LU 49940	469		(R/S)	- H	- OCH_3	- OCH_3	- OCH_3	- CN	- $CH(CH_3)_2$	- CH_3	- C_8H_{17}	-	-
LU 48895	497		(R/S)	- H	- OCH_3	- OCH_3	- OCH_3	- CN	- $CH(CH_3)_2$	- CH_3	- $C_{10}H_{21}$	-	-
LU 51903	526		(R/S)	- H	- OCH_3	- OCH_3	- OCH_3	- CN	- $CH(CH_3)_2$	- CH_3	- $C_{12}H_{25}$	-	-

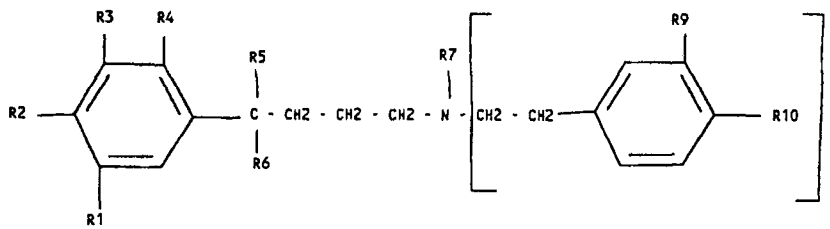

Fig. 1. The structure characteristics of verapamil analogs.

Bis-(3.4-dimethoxyphenyl)-3-methylaza-7-cyan-8-methyl-nonane) is also indicated. DOX was obtained from Pharmacia Farmitalia-Carlo Erba (Milan, Italy).

Cell lines

LoVo and DLD-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were propagated in Ham's F12 (LoVo) or RPMI 1640 (DLD-1) medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Seralab, Sussex, U.K.), 50 μg/mL streptomycin and 50 U/mL of penicillin G. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

LoVo and DLD-1 resistant sublines (LoVo-R and DLD-1-R) were obtained by continuous exposure of the parental cell lines to 100 ng/mL DOX, as described previously [25]. The LoVo-R and DLD-1-R cell lines were cultured for more than 48 and 24 months, respectively, in the continuous presence of 100 ng/mL DOX until 24 hr before testing. LoVo-R and DLD-1-R cells have an MDR phenotype. They expressed levels of MDR1 mRNA 18- and 10-fold higher than those of LoVo and DLD-1 sensitive cells, respectively, as shown by Northern and dot blot analysis [25, 26]. We previously demonstrated that LoVo and DLD-1 cells also expressed detectable levels of MDR1 mRNA, although at very low levels [25].

Drug cytotoxicity

Cytostatic effects of VER and VAs were assessed as previously described [25]. Cells were plated in 6-well tissue culture plates (Falcon, NJ) and incubated in the presence of VER or VAs for 24 hr. After drug treatment, the cells were washed with saline and incubated in drug-free medium for at least three doubling times (72 hr). Then they were washed with saline, trypsinized, and counted with a ZM coulter counter (Coulter Electronics Ltd., Luton, U.K.). Results are reported as IC_{50}. IC_{50} were interpolated by the best regression model, which exhibited the highest coefficient of correlation ($R > 0.91$) among 4 models (linear, exponential, logarithmic, and power). Within one entire set of data, all fitting was by the same model. Cells not treated with drugs were used as controls. The results were not significantly different from those obtained by clonogenic assay in liquid medium [25] (data not shown).

The sensitizing effect of VER and VAs on DOX cytotoxicity was determined by incubating cells with DOX, in the presence or not of VA, for 1 hr. A post-incubation of 24 hr with VER or VA, using the same concentration as in the coincubation with DOX, was performed to optimize its modulating effect, as previously demonstrated [27]. In the experiments with VAs, cells treated with VA alone were used as controls. Results are reported as DOX IC_{50} interpolated as described above.

DOX and VA uptake analyses

Exponentially growing cells, 3–10 × 10^6 in 10-mL medium, were seeded in 90-mm Petri dishes (Falcon, NJ) and incubated overnight at 37°C. After removing the culture medium, the cells were incubated for 1 hr at 37° with 15 mL fresh medium containing appropriate concentrations of DOX or VAs. The medium was subsequently withdrawn, and Petri dishes were chilled on ice and quickly washed three times in ice-cold saline solution. Under these experimental conditions, DOX concentration in the medium was decreased to <10% after 1 hr cell incubation, when DOX concentration was 6 μg/mL. To evaluate DOX content, $AgNO_3$ in distilled water (3.3% m/v final concentration) was added to the cultures. DOX was then extracted with water-saturated normal butyl alcohol. Intracellular content of DOX was determined by measuring the fluorescence intensity of the extracts at excitation and emission wavelengths of 500 and 590 nm, respectively. Calibration curves were obtained by measuring fluorescence intensity using standard concentrations of DOX in the range in which the intensity of fluorescence was linear (up to 250 ng/mL).

To determine the passive uptake of DOX, cells were preincubated at 37° for 15 min with 15 mM sodium azide (NaN_3) plus 10 mM deoxyglucose in PBS (pH 7.4) and 10% dialyzed FCS. They were then incubated at 37° for 1 hr with 6 μg/mL of DOX in the same buffer. The cells were then chilled on ice and washed as described above; the intracellular DOX content was quantified after extraction. Comparative uptakes were also performed on energy-supplied cells using PBS (pH 7.4) plus 10% dialyzed FCS added with 10 mM glucose as incubation buffer. Intracellular DOX content obtained under these conditions was not significantly different from that obtained using Ham's F12 plus 10% FCS as incubation medium.

To determine VER or VA cellular content, the cells were detached from Petri dishes with 2 mL of trypsin and lysed with 20 mL NaOH 1M. VER or VAs were then extracted and measured using the high-pressure liquid chromatographic assay described by Salama *et al.* [28], with slight modifications.

The average efficiency of extraction procedures was over 90%. Cellular drug uptake was quantified by means of calibration curves and expressed as ng drug/10^6 cells. To calculate the cell number in each experiment, cells were seeded in additional triplicate Petri dishes, incubated, and treated with drugs exactly as in the drug uptake assay. Finally, they were trypsinized and counted with a coulter counter (Coulter Mod ZM) apparatus. The ratio between the volume of resistant and sensitive cells was ~1, as determined by the coulter counter.

Plasma membrane preparation and photoaffinity labeling analysis

Cells in the midlog stage were washed in cold phosphate-buffered saline solution, and hypotonic lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, 2 mM phenylmethylsulfonylfluoride (PMSF)) was added. Under sterile conditions, cells were harvested with a rubber policeman, placed into 50-mL sterile centrifuge tubes, and centrifuged at 300 g at 4°C for 10 min. The supernatant was discarded and the pellet transferred to a homogenizer tube and placed on ice for approximately 40 min. Cells were disrupted utilizing at least 30 strokes. The homogenate was transferred to sterile centrifuge tubes and centrifuged at 1000 g for 10 min. The supernatant was placed into ultra-clear centrifuge tubes and spun at 100,000 g in an SW55Ti (Beckman, CA) rotor for 1 hr. The pellet was resuspended in 0.250 M sucrose in Tris-HCl at pH 7.5 and stored at −80° until use [29]. Protein determination was performed by the BioRad assay system (BioRad Laboratories, Richmond, CA), with bovine serum albumin as standard.

For photoaffinity labeling affinity, 100 μg of plasma

membrane protein were reacted with 0.4 μM [^{3}H]azidopine in 0.250 M sucrose, 10 mM Tris-HCl pH 7.5 in a final volume of 75 μL at room temperature for 30 min, with or without competitors. The reaction mixture on ice was then irradiated with a UV lamp at 366 nm for 20 min. Labeled proteins were separated by 7% SDS-PAGE. The gel was fixed, treated with the fluorographic reagent Amplify (Amersham, U.K.), dried, and then exposed for 14 days at −80° using XAR-5 film (Eastman Kodak, Rochester, NY). Quantitation of [^{3}H]azidopine-labeled P-gp was carried out by densitometric tracings of fluorograms.

Calcium antagonism in aortic preparations

Male rats (Sprague Dawley, Ivanovas or Charles River, KiBlegg/Allg. or Sulzfeld, Germany) weighing approximately 300 g were used. The animals were anesthetized with ether, and spiral aortic strips (approximately 2 mm × 2 cm) were prepared. Preload was 1.5 g, and tension was measured isometrically (transducer Harvard Bioscience 52–9545). The experiments were performed at 37°C and bath solutions were permanently gassed with a mixture of 95% O_2 and 5% CO_2, resulting in a pH of 7.4.

The strips were relaxed for 60 min in Tyrode solution of the following composition (mM): NaCl 112, KCL 5, $CaCl_2$ 1.25, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, glucose 11.2. Then the strips were rinsed three times for 5 min in a Ca-free Tyrode solution containing 0.2 mM EDTA to ensure calcium depletion. After depolarization for 10 min in potassium-rich Tyrode solution, in which 100 mM NaCl was replaced by KCl, a control contraction was induced by adding 0.5 mM $CaCl_2$. After 15 min the strips were washed out for 10 min with Ca-free Tyrode solution containing 0.2 mM EDTA. After 10-min depolarization in potassium-rich Tyrode solution, the test substance was added, and after 15 min of exposure, the induction of the calcium contraction was repeated.

The calcium-induced contractions were measured in mg, and the deviation of the second contraction from the first was calculated as a percent. The results obtained for several substance concentrations were used to calculate a linear dose-response relationship and the EC_{50} with 95% confidence limits. The EC_{50} shows the concentration at which there is a 50% inhibition of the second calcium-induced contraction.

Statistics

For statistical analyses, the unpaired *t*-test and the Pearson Product-Moment Correlation system were used. Results were considered statistically significant at $P \leq 0.05$.

RESULTS

Cytotoxicity of verapamil analogs (VAs) on LoVo and LoVo-R cell lines

The cytotoxic effect of VER and each VA after a 24-hr treatment is shown in Table 1. Data are reported as IC_{50}. With the exception of compound LU46605 (which will be considered separately), the VA IC_{50} was slightly but not significantly (P = NS) increased in resistant LoVo-R cells compared with parental LoVo cells. The VA IC_{50} ratio between LoVo-R and LoVo cells (R/S ratio) ranged from 1.0 to 1.6. Gallopamil was the least toxic analog (IC_{50} = 79.5 ± 7.9 μg/mL in LoVo-R cells), followed by VER, R-VER, and nor-VER, which exhibited an IC_{50} ranging from 29.7 ± 5.1 μg/mL to 34.6 ± 4.1 μg/mL in LoVo-R cells. In the entire series, the decrease in the number of methoxy groups on the phenyl rings or

Table 1. Cytotoxic effect, potency, inhibition of azidopine photolabeling, and calcium antagonist activity of VER analogs

Compound	Cytotoxicity: IC_{50} (μg/mL) LoVo	IC_{50} (μg/mL) LoVo-R	R/S	IC_{20} (μg/mL)† LoVo	IC_{20} (μg/mL)† LoVo-R	Potency* (μg/mL) LoVo-R	Calcium antagonist activity in aortic strip EC_{50} (ng/mL)	VA concentration required to inhibit [^{3}H]azidopine photolabeling by 50% in LoVo-R (μg/mL)
VER	29.08 ± 2.4‡	34.55 ± 4.1‡	1.2	9.01 ± 2.0‡	10.94 ± 1.5‡	0.11 ± 0.05‡	17.3	105.5 ± 37.9§
R-VER	29.70 ± 4.0	34.50 ± 1.9	1.2	10.41 ± 2.3	10.24 ± 2.2	0.15 ± 0.04	108.0	98.3
Nor-VER	20.56 ± 1.5	29.70 ± 5.1	1.4	10.10 ± 1.6	9.44 ± 3.9	0.15 ± 0.1	71.6	137.4
Gallopamil	66.14 ± 6.7	79.48 ± 7.9	1.2	28.40 ± 3.0	30.40 ± 5.0	0.07 ± 0.01	7.3	74.0 ± 10.9
LU46605	10.82 ± 1.4	87.21 ± 3.7	8.1	4.21 ± 1.1	36.41 ± 3.1	13.0 ± 1.50¶	2599.5	888.8 ± 291.1
Devapamil	13.61 ± 2.1	16.24 ± 1.6	1.2	5.28 ± 0.9	5.71 ± 0.2	0.13 ± 0.05	1.8	69.8 ± 13.0
LU46324	3.88 ± 0.2	4.02 ± 1.7	1.0	1.20 ± 0.5	1.28 ± 0.2	0.10 ± 0.04	55.5	160.4 ± 18.8
LU43918	3.10 ± 1.3	4.41 ± 1.6	1.3	1.25 ± 0.2	1.80 ± 0.7	0.09 ± 0.01	60.0	61.6
LU49667	10.28 ± 1.5	13.09 ± 0.8	1.3	4.32 ± 0.8	5.36 ± 0.7	0.24 ± 0.11‖	98.0	142.0
Emopamil	6.02 ± 0.6	7.10 ± 0.9	1.2	2.41 ± 0.4	3.04 ± 0.6	0.35 ± 0.10¶	137.3	115.2
S-anipamil	5.41 ± 1.8	8.44 ± 3.0	1.6	2.97 ± 0.4	2.99 ± 1.0	>MC¶	ND	392.0 ± 87.6
R-anipamil	5.42 ± 0.7	8.16 ± 0.8	1.5	2.63 ± 0.5	3.10 ± 0.9	>MC¶	ND	425.8
LU49940	3.99 ± 0.2	5.53 ± 1.7	1.4	1.03 ± 0.3	1.21 ± 0.4	0.13 ± 0.04	>46.9	65.7
LU48895	0.97 ± 0.2	1.36 ± 0.2	1.4	0.62 ± 0.1	0.79 ± 0.1	0.06 ± 0.01	69.5	46.8
LU51903	1.12 ± 0.3	1.50 ± 0.1	1.3	0.44 ± 0.2	0.46 ± 0.2	0.07 ± 0.01	ND	37.9 ± 11.8

* VA concentration required to decrease DOX IC_{50} by 5-fold in LoVo-R cells.
† IC_{20} represents the minimal cytotoxic concentration (see Results).
‡ Data were obtained from at least 3 independent experiments, each done in triplicate (mean ± SD).
§ Mean ± SD from triplicate experiments or mean without SD from duplicate experiments.
ND = not done; ‖$P \leq 0.05$; ¶$P \leq 0.01$ as compared to VER.

the replacement of a phenyl ring with aliphatic chains resulted in a considerable increase in cytotoxic activity (see Fig. 1 and Table 1).

Unlike the other VAs, the compound LU46605 had a much higher IC_{50} ($P < 0.01$) in LoVo-R (87.2 ± 3.7 μg/mL) than LoVo cells (10.8 ± 1.4 μg/mL) (LU46605 – IC_{50}, R/S = 8.1) (Table 1), behaving similarly to a P-gp-extruded drug such as DOX. After 1 hr of drug incubation, the DOX IC_{50} was 13.17 ± 2.6 μg/mL in LoVo-R and 0.23 ± 0.04 μg/mL in LoVo cells. The DOX resistance index of the LoVo-R cell line was 57. The peculiar behavior of LU46605 was also observed in another MDR human colon carcinoma cell line, the DLD-1-R, in which the IC_{50} in resistant cells was 2.64-fold higher than in sensitive DLD-1 cells ($P < 0.01$) (Table 2).

VAs uptake in LoVo and LoVo-R cells

To ascertain whether the unusual behavior of compound LU46605 was a consequence of variations in the transmembrane equilibria, we analyzed the cellular uptake of LU46605 in the LoVo-resistant and parental-sensitive counterparts. The intracellular uptake of other compounds (VER, R-VER, gallopamil, and LU48895) exhibiting no significant differences in the IC_{50} between sensitive and resistant cells was also investigated.

After 1 hr of exposure to the drug, when the steady-state transmembrane equilibria were reached (data not shown), the intracellular content of LU46605 was much higher ($P < 0.05$) in LoVo than in LoVo-R cells (Table 3). The ratio of intracellular content in sensitive and resistant cells (LS/LR intracellular content ratio) ranged from 4.1 (at 0.33 μg/mL extracellular concentration) to 1.7 (at 100 μg/mL). The decrease in the LS/LR intracellular content ratio when LU46605 extracellular concentration increased was consistent with the activity, in LoVo-R cells, of a saturable drug-extruding mechanism. In contrast, the content of VER, R-VER, gallopamil, and LU48895 was not significantly different in LoVo-R as compared to the sensitive cells (P = NS). A higher content of LU46605 ($P < 0.01$) but not of VER was also observed in DLD-1-R cells compared with the wild-type sensitive counterparts (Table 2).

Reversal of DOX resistance and enhancement of DOX uptake

For all the VAs tested, DOX cytotoxicity enhancement was VA dose-dependent. DOX IC_{50} decreased when the cells were exposed to DOX concomitantly with increased VA concentrations. The maximum DOX cytotoxicity enhancement achievable in LoVo-R and LoVo cells with the minimal cytotoxic VA concentration is shown in Fig. 2. Data are reported as the ratio between DOX IC_{50} interpolated from regression model analysis of data obtained by treating cells without VA and DOX IC_{50} in the presence of the VA concentration causing 20% cell death when reversers were used alone (IC_{20}). We assumed that such cytotoxicity of VA did not significantly affect the cytotoxic effect of DOX. The VA IC_{20} was interpolated from experimental data by the best regression model. The DOX IC_{50} corresponding to the VA IC_{20} was calculated by the best regression model from DOX IC_{50} obtained in the presence of different concentrations of VA (representative examples are shown in Fig. 3).

DOX cytotoxicity enhancement increased as the number of methoxy groups increased in the phenyl rings of the VER molecule structure. In contrast, the substitutions introduced in positions R_5, R_6, and R_8 of the molecule dramatically decreased the DOX chemosensitizing effect obtained by exposing LoVo-R cells to the minimal VA cytotoxic concentration (IC_{20}). The greatest en-

Table 2. Cytotoxic effect and drug uptake of LU46605, VER, and DOX in DLD-1 sensitive and DLD-1-R cells

	Cell line	
	DLD-1	DLD-1-R
IC_{50} LU46605*	15.60 ± 1.3 (7.6 ± 1.4)	41.20 ± 7.2 (20.0 ± 3.8)
IC_{50}VER†	33.7 ± 5.4 (10.4 + 2.1)	34.2 ± 4.5 (12.7 ± 3.2)
Uptake of LU46605‡	12.9 ± 2.5	6.1 ± 1.0
Uptake of VER§	9.6 ± 3.3	8.7 ± 2.6
IC_{50} DOX‖	279.9 ± 30.1	3702.0 ± 437.9
IC_{50} DOX + LU46605¶	167.6 ± 34.6	364.5 ± 54.3
IC_{50} DOX + VER#	155.1 ± 14.0	29.3 ± 30.9
Uptake of DOX + LU46605**	1.1 ± 0.1	1.5 ± 0.3
Uptake of DOX + VER††	1.2 ± 0.3	2.1 ± 0.1

*,† Cell treatment was for 24 hr. Data are reported as μg/mL. The minimal cytotoxic concentration (IC_{20}) is shown in parentheses.

‡,§ Cell were exposed to 1 μg/mL. Data are expressed as ng × 10^6 cells.

‖ Data are expressed as ng/mL. Drug exposure was 1 hr.

¶,# Data are expressed as ng/mL of DOX. Cell were incubated for 1 hr with DOX plus LU46605¶ or VER# followed by a 24-hr post-incubation with LU46605 or VER. The minimal cytotoxic concentration of chemosensitizer (IC_{20}) was used (see points * and †).

,†† Cell were exposed to 6 μg/mL of DOX plus subcytotoxic concentrations (IC_{20}) of LU46605 or VER†† for 1 hr. Data are reported as the ratio of DOX content in the presence or absence of chemosensitizer. In the absence of the chemosensitizer, DOX content was 1487 ± 306 and 782 ± 112 ng × 10^6 cells for DLD-1 and DLD-1-R cells, respectively.

Data were obtained from at least quadruplicate experiments.

Table 3. VER and VA uptake in LoVo and LoVo-R cells

Analog (μg/mL)	VER*			R-VER		
	LoVo	LoVo-R	LS/LR†	LoVo	LoVo-R	LS/LR
0.33	ND	ND	ND	7.5 ± 0.6	8.7 ± 1.1	0.9
1.0	8.0 ± 2.4	8.9 ± 3.3	0.9	15.8 ± 3.4	18.2 ± 2.4	0.9
3.3	16.3 ± 0.9	15.6 ± 1.5	1.1	27.4 ± 4.2	30.0 ± 2.8	0.9
10.0	29.6 ± 7.2	33.5 ± 0.7	0.9	48.3 ± 5.2	44.5 ± 7.1	1.1
33.0	75.7 ± 5.1	78.5 ± 9.2	1.0	98.2 ± 1.3	94.6 ± 12.4	1.0
100.0	ND	ND	ND	141.6 ± 20.1	168.9 ± 11.2	0.8

Analog (μg/mL)	Gallopamil			LU 46605			LU 48895		
	LoVo	LoVo-R	LS/LR	LoVo	LoVo-R	LS/LR	LoVo	LoVo-R	LS/LR
0.33	4.1 ± 0.5	3.96 ± 0.6	1.0	6.8 ± 1.4	1.7 ± 0.8	4.1§	ND	ND	ND
1.0	7.5 ± 1.0	7.53 ± 0.5	1.0	9.8 ± 3.6	3.6 ± 0.8	2.7§	ND	ND	ND
3.3	20.6 ± 3.0	23.8 ± 2.3	0.9	15.3 ± 4.4	6.8 ± 1.3	2.3§	13.5 ± 8.0	13.2 ± 1.6	1.0
10.0	53.6 ± 4.3	47.5 ± 2.5	1.1	29.7 ± 5.6	12.9 ± 3.2	2.2§	22.9 ± 3.3	28.8 ± 9.3	0.8
33.0	85.4 ± 12.9	78.8 ± 8.1	1.1	66.4 ± 7.2	30.7 ± 11.8	2.2‡	44.1 ± 6.5	53.5 ± 4.7	0.8
100.0	124.4 ± 29.1	158.2 ± 18.0	0.8	82.4 ± 12.1	48.2 ± 7.6	1.7‡	83.4 ± 4.4	99.1 ± 12.5	0.8

* Data obtained from at least triplicate independent experiments are expressed as ng × 10^6 cells. Cells were exposed for 1 hr to the drugs. The initial extracellular VA concentration is reported in the first column.

† Ratio: ng × 10^6 cells in LoVo/ng × 10^6 cells in LoVo-R.

ND, not done; ‡$P < 0.05$; §$P < 0.01$ as compared to VER.

hancement of DOX cytotoxicity after exposure of LoVo-R cells to the VA IC_{20} occurred with gallopamil (52.3 ± 7.2-fold), VER (41.3 ± 5.0-fold), R-VER (38.9 ± 6.4-fold), and nor-VER (35.4 ± 4.3-fold) (Fig. 2). In LoVo sensitive cells the enhancement after exposure to the VA IC_{20} was of 1.9 ± 0.2-fold, 1.6 ± 0.3-fold, 1.6 ± 0.3-fold, and 1.5 ± 0.2-fold for VER, gallopamil, R-VER, and nor-VER, respectively (Fig. 2). It is worth considering that LoVo sensitive cells expressed detectable levels of MDR1 mRNA, although at very low levels compared with those of resistant cells [25]. Therefore, the slight increase in DOX cytotoxicity was consistent with their low levels of MDR1 mRNA expression.

In LoVo-R cells, enhancement of DOX cytotoxicity was related to an increase in DOX uptake after treatment with VA. Similar results were observed in DLD-1-R cells (Table 2). The enhancement in the intracellular DOX content after exposure of LoVo-R cells to 0.5–100 μg/mL of VAs is shown in Table 4. When LoVo cells were exposed to the same VA concentrations, the enhancement was ≤1.4 (data not shown). The enhancement of DOX uptake was mainly associated with the inhibitory effect of VAs on P-gp activity. In fact, VAs did not affect the passive uptake of DOX in energy-depleted LoVo-R cells: LoVo-R cells treated with NaN_3/deoxyglucose, in the presence or absence of VAs, showed no significant difference in DOX content (P = NS). Moreover, the increase in DOX uptake when using the compounds with great reversal activity (i.e., gallopamil, VER, R-VER) was similar to that obtained in metabolically inactivated cells treated with NaN_3/deoxyglucose, where P-gp is not active (Fig. 4). Conversely, the VAs with low reversal activity (i.e. R-or S-anipamil and LU46605) slightly but not significantly increased DOX uptake (P = NS). Finally, VAs or NaN_3 only slightly increased DOX uptake in LoVo cells. Due to the large SD, these differences were not significant (P = NS) (Fig. 4).

Potency of VAs in reversing DOX resistance in LoVo-R

The potency of VAs in reversing DOX resistance in LoVo-R cells was determined by cytotoxic assay. Reversal potency was expressed as the extracellular VA concentration (μg/mL) required to decrease DOX IC_{50} in LoVo-R cells by 5 times (from 13.2 ± 2.6 μg/mL to 2.64 ± 0.38 μg/mL) (Table 1). DOX IC_{50} was calculated by the best regression model (see Materials and Methods and Fig. 3). Compared to VER, no significant (P = NS) effect in MDR reversal potency was consequent to the replacement of the phenyl ring at position R_8 with long aliphatic chains (LU49940, LU48895, and LU51903) (Fig. 1 and Table 1), or to replacement of the methoxy groups in the phenyl rings with Cl atoms (LU43918 and LU46324). A slight (approximately 2-fold) but significant decrease in MDR reversal potency (i.e. the VA concentration necessary to potentiate DOX cytotoxicity by 5-fold was higher than the VER concentration) was consequent to the replacement of both methoxy groups in the phenyl ring at positions R_9 and R_{10} with H atoms (emopamil, $P = 0.01$ and LU49667, $P = 0.05$), but not to the replacement of only one methoxy group (devapamil) (Table 1). Finally, a great decrease ($P < 0.01$) in reversal potency was consequent to the replacement of the –CN group at position R_5 with $-CH_2NH_2$ (LU46605) or to the replacement of $-CH\ (CH_3)_2$ with the $-(CH_2)_{11}\ CH_3$ group in R_6 position (R- and S-anipamil).

According to the data on reversal potency, the least potent VAs (i.e. LU46605, R-anipamil, and S-anipamil) also determined the lowest enhancement in DOX uptake when LoVo-R cells were exposed to the same extracellular concentration of VA (i.e. 1 μg/mL) (Table 4).

Finally, no significant association was observed by the Pearson Product Moment Correlation test (P = NS) between reversal potency (expressed as the VA concentration enhancing 5-fold DOX cytotoxicity) and the VA cytotoxic effect (expressed as VA IC_{50}), or between re-

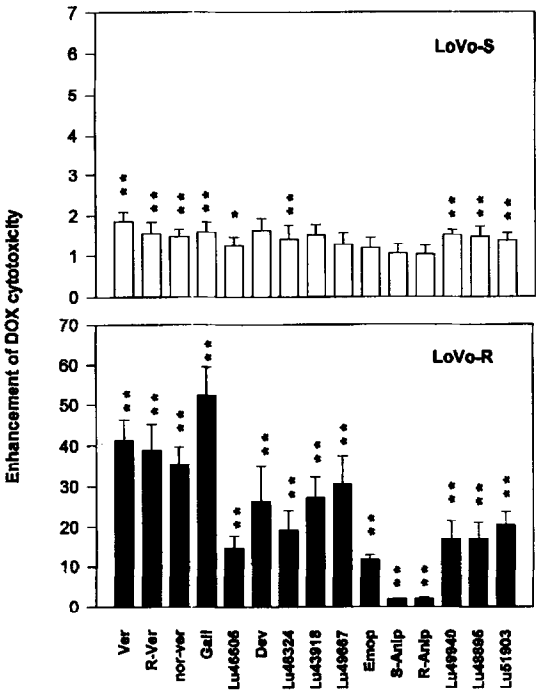

Fig. 2. Enhancement of DOX cytotoxicity in LoVo-R and LoVo sensitive (LoVo-S) cells by the Vas. The results are expressed as -fold increase in cytotoxicity represented by the ratio of DOX IC_{50} in the absence and in the presence of VAs. The VA concentrations used were the minimal cytotoxic (MC) concentrations (IC_{20}) (see Table 1). Data obtained from at least quadruplicate experiments. Bars, SD. * $P \leq 0.05$; ** $P \leq 0.01$ compared with cellular DOX content in the absence of VAs.

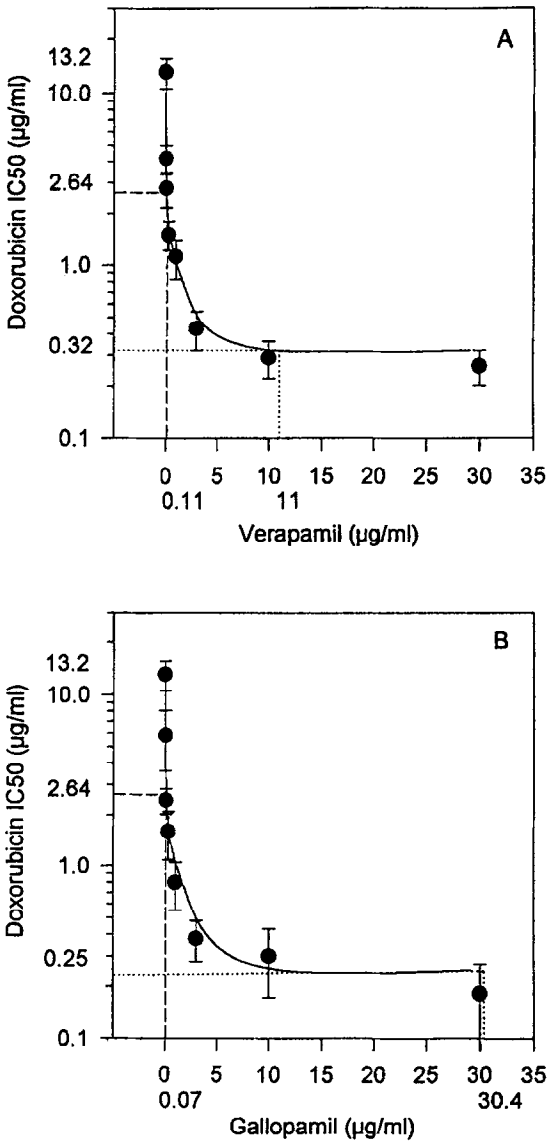

Fig. 3. DOX IC_{50} in LoVo-R cells after exposure to various concentrations of (A) VER and (B) gallopamil. (X axis) VER or gallopamil concentrations; (Y axis) DOX IC_{50}. The interpolated chemosensitizer concentration that decreased DOX IC_{50} by 5-fold (from 13.2 to 2.64 µg/mL) is shown on the X axis (· · ·); the interpolated DOX IC_{50} corresponding to the minimal cytotoxic concentration of the chemosensitizer (IC_{20}) is shown on the Y axis (— — —). Each point represents the mean ±SD of at least three independent experiments.

versal potency and calcium antagonist activity (Table 1 and data not shown).

Inhibitory activity of [^{3}H]azidopine P-gp photolabeling

The effect of VAs on photolabeling of P-gp in LoVo-R cells with [^{3}H]azidopine is shown in Table 1 and Fig. 5. Data are reported as the VA concentration required to inhibit azidopine P-gp photolabeling by 50%, as determined from regression analysis of experimental data. A strict relationship was observed between the VA concentration required to obtain 50% P-gp photolabeling inhibition and the VA potency expressed as VA concentration necessary to enhance DOX cytotoxic effect by 5-fold ($P < 0.01$ by Pearson Product Moment Correlation test) (Fig. 6). Photolabeling inhibition data also support the finding that substitution in the R_5 and R_6 positions profoundly affects the ability of the compound to interact with P-gp. In fact, replacement of the –CN group with $-CH_2-NH_2$ (LU46605) or replacement of $-CH(CH_3)_2$ with $-(CH_2)_{11}$ (R- and S-anipamil) decreased azidopine photolabeling inhibition (Table 1).

DOX inhibition of analog uptake in LoVo-R cells

LoVo-R cells were exposed to 0.3 µM VER, R-VER, gallopamil, LU48895, and LU46605, in the presence of various concentrations of DOX (0.3, 3, 30 µM). After a 60-min co-incubation, when the steady state was achieved, cells were lysed, and intracellular concentration of the VER analog determined by HPLC. No competitive effect was observed for any analog even at concentrations of DOX 100-fold higher (30 µM). The only exception concerned compound LU46605, whose uptake was not affected by 0.3 µM DOX, but was 1.30 ± 0.14 ($P = 0.05$, $n = 4$ ± SD) and 1.59 ± 0.12 ($P < 0.01$, $n = 4$ ± SD) times higher at 3 and 30 µM DOX, respectively.

DISCUSSION

In agreement with previous data [10, 22–24], the experiments reported here show that the calcium antagonist activity and reversal potency of a wide variety of VAs are not correlated properties, and confirm that reversal activity is not enantio-selective (at least for the enantio-

Table 4. DOX uptake enhancement after exposure of LoVo-R cells to reversers

Compound [μg/mL]	VER	R-VER	Nor-VER	Gallop.	LU46605	Devap.	LU46324	LU43918
0.5	2.02*	1.98	1.84	2.1	0.94	1.97	1.09	2.09
1.0	1.83	2.0	1.82	2.21	1.12	1.89	1.32	2.44
3.3	1.88	2.48	1.83	2.53	0.97	2.3	1.58	3.31
10.0	2.41	2.34	2.27	2.72	1.30	2.45	1.76	3.43
33.0	2.63	2.35	2.46	2.55	1.21	2.71	2.0	2.84
100.0	2.57	2.51	2.34	2.61	1.73	2.87	1.89	3.42

Compound [μg/mL]	LU49667	Emop.	S-anip.	R-anip.	LU49940	LU48895	LU51903
0.5	1.53	1.22	0.97	0.89	1.48	1.89	1.48
1.0	1.29	1.73	1.19	1.07	1.51	1.86	1.86
3.3	1.60	1.91	1.14	1.13	1.52	1.99	2.21
10.0	1.49	2.61	1.35	1.20	1.63	2.0	2.50
33.0	1.72	2.83	1.81	1.74	1.70	2.34	2.89
100.0	1.71	3.11	2.05	1.72	1.83	3.11	2.76

* Ratio of DOX content in LoVo-R cells in the absence and in the presence of VAs. Cells were exposed to an initial extracellular concentration of DOX of 6 μg/mL for 1 hr. At that time, the steady state in DOX uptake was almost completely established (>80%). The VA concentrations used are indicated in the first column. Data were obtained from the means of at least three experiments. SD was less than 25%. In the absence of VAs the DOX content in LoVo-R cells was 111 ± 21 ng/10^6 cells.

mers we tested, i.e. VER vs R-VER and S-anipamil vs R-anipamil).

The structure-activity relationship analysis of the 14 VAs provided the following information. In comparison with VER, no significant (P = NS) differences in reversal potency were observed (in the sense that the VA concentrations required to obtain a 5-fold potentiation of DOX cytotoxicity were similar to VER) when:

1. the methoxy groups in the phenyl rings were replaced with Cl atom (LU43918 and LU46324);
2. when the 1-phenyl ring was replaced with aliphatic chains (LU49940, LU48895, and LU51903); or
3. when a methoxy group in the phenyl ring was added (gallopamil).

A slight but significant ($P \leq 0.05$) decrease in reversal potency (approximately 2- to 4-fold) was consequent to the replacement of the methoxy group in the phenyl rings with H atoms (LU49667 and emopamil). In contrast, compared with VER, it was much more ($P < 0.01$) detrimental to reversal activity when:

1. the structure of the 7-cyan-8-methyl-nonane was altered by carbon extension (R-anipamil and S-anipamil); or
2. when the 7-cyan was replaced with the 7-methylamino group (LU46605).

Reversers must interfere with P-gp activity without causing cytotoxic side effects. Since the reversal activity of VER and the VAs analyzed was dose-dependent, it is crucial to define the maximum concentration that can be safely used without important cytotoxic effects. The analysis of the relationship between structural changes in functional groups indicated that the methoxy groups confer to the molecules low cytotoxic properties. Gallopamil, which has an additional methoxy group in the R_1 position, displayed the lowest cytotoxic activity of all the VAs. In contrast, replacement of the methoxy groups in the VER structure with either H or Cl atoms, or replacement of the 1-phenyl ring with aliphatic chains increased the cytotoxic activity of the VAs. When the compounds were used at the minimal cytotoxic concentrations (IC_{20}), the ones most active in reversing MDR were gallopamil, VER, R-VER, and nor-VER, which potentiated DOX cytotoxicity by 52.3 ± 7.2, 41.3 ± 5.0, 38.9 ±

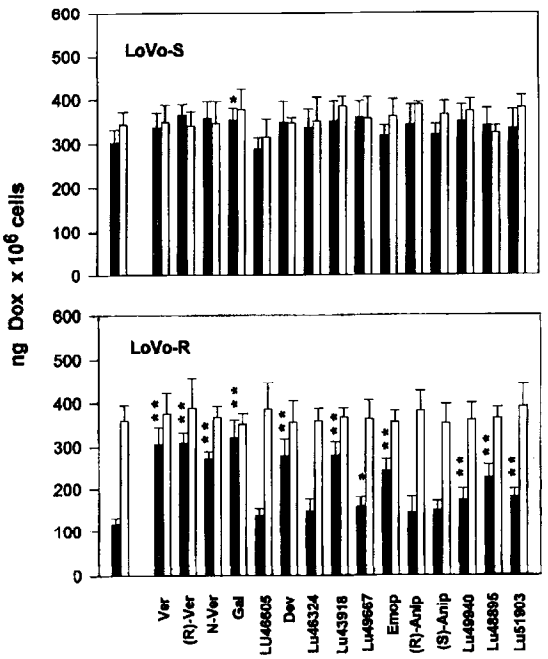

Fig. 4. Effect of VAs on the uptake of DOX in energy-depleted and non-depleted cells. (Top) LoVo-S; (bottom) LoVo-R cells. Cells were incubated with 6 μg/mL of DOX in the presence of VAs at the minimal cytotoxic (MC) concentration (IC_{20}) (see Table 1) at 37°C for 1 hr. (Hatched bars) metabolic active cells; (open bars) energy-depleted cells treated with 15 mM NaN_3 plus 10 mM deoxyglucose at pH 7.4. Control uptakes of DOX in LoVo-R cells without VAs are shown in the first two columns without label. Data obtained from quadruplicate experiments. Bars, SD. For the statistical analysis, the cellular DOX content in the absence or presence of VAs was compared either in metabolic active or in energy-depleted cells. **$P \leq 0.01$; *$P \leq 0.05$.

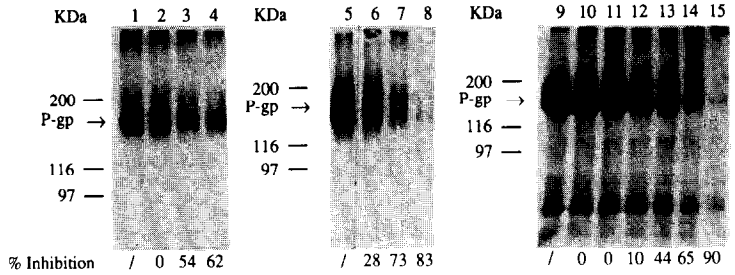

Fig. 5. Representative example of [^{3}H]azidopine photolabeling inhibition. The inhibition of the photolabeling by VER and two analogs, LU48895 and LU46605, is shown. Lines 1, 5, and 9 (controls): 0.4 μM [^{3}H]azidopine. Lines 2, 3, 4: inhibition by VER at concentrations of 40, 120, 360 μM. Lines 6, 7, 8: inhibition by LU48895 at the same concentration as VER. Lines 10, 11, 12, 13, 14, 15: inhibition by LU46605 at concentrations of 40, 120, 360, 1080, 3240, 10800 μM. The percentage of inhibition for each concentration is also reported.

6.4, and 35.4 ± 4.3 times, respectively. Cytotoxic effects and reversal activity did not appear to be strictly related phenomena, since changes in the functional groups affecting cytotoxicity did not significantly affect reversal potency.

In the majority of the compounds analyzed, slight differences in cytotoxic effect were observed between sensitive and resistant cells when they were used alone. Only compound LU46605 exhibited a peculiar behavior, showing a much higher IC_{50} (8.1-fold) in LoVo-R cells than in sensitive cells. Resistance to LU46605 of LoVo-R cells could be explained by the dramatically reduced steady-state accumulation in resistant cells compared with LoVo cells. This phenomenon was also observed in another MDR cell line, DLD-1-R, in which LU46605 IC_{50} and cellular content were significantly lower ($P < 0.01$) in resistant compared with sensitive cells. Conversely, only slight differences between sensitive and resistant cells were observed in drug uptake for the other compounds investigated. At present, it would be hasty to conclude that, among the compounds investigated, only LU46605 is a substrate for P-gp transport. The rapid passive membrane permeability of the other VAs may have led to an underestimation of their intracellular accumulation and, therefore, these VAs could be also transported by P-gp, as recently suggested for VER [30]. It is interesting to note that the peculiar behavior of LU46605 is a consequence of the replacement of the –CN group with a positive charged $-CH_2NH_2$ group in position R_5 of the VER molecule. Such a substitution confers a much higher hydrophilicity to this compound than that displayed by all the other VAs. Despite the great difference in intracellular uptake between sensitive and resistant cells, LU46605 was a less potent reverser, thus confirming previous suggestions that chemosensitizers with hydrophilic characteristics have a low reversal potency.

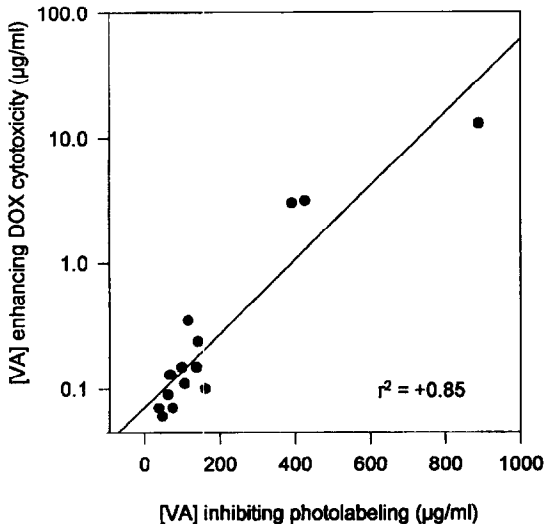

Fig. 6. Scatter diagram of VA concentration inhibiting [^{3}H]azidopine photolabeling by 50% (X axis) in relation to the VA concentration required to decrease DOX IC_{50} by 5-fold in LoVo-R cells (Y axis). *r*, coefficient of correlation. For R- and S-anipamil the minimal cytotoxic concentrations are reported on the X axis.

Several groups have used [^{3}H]azidopine to search for compounds capable of reversing MDR [31–33]. [^{3}H]azidopine photolabeling of P-gp can be inhibited by several reversing agents, including VER analogs [33, 34]. We found that the binding site(s) for [^{3}H]azidopine photolabeling of P-gp was inhibited by the VAs in a concentration-dependent manner. The structural motif of the –7-cyan-8-methyl group seems to play a key role in the interaction with P-gp. In fact, replacements in positions R_5 and R_6 greatly decreased [^{3}H]azidopine photolabeling inhibition. The issue is whether there are distinct drug binding sites of P-gp or whether there exists a single binding site with low specificity for all the VAs and DOX on this protein [33–36]. We found that all the VAs affected DOX uptake in LoVo-R cells, whereas even a 100-fold excess of DOX had no effect on transmembrane transport of the VAs, with the sole exception of LU46605. This suggests that the VAs interact with a site of P-gp different from the DOX binding site, as recently suggested for VER [30]. A peculiar behavior was exhibited by LU46605, whose uptake in LoVo-R cells significantly increased ($P < 0.01$) in the presence of 100 × excess of DOX. However, further studies are needed to determine whether LU46605 and DOX competitively interact with the same P-gp binding site.

Finally, the chemosensitizers could restore the sensitivity to antineoplastic drugs with mechanisms different from interaction with P-gp (see Ref. [8]). In particular, the chemosensitizers could increase DOX uptake either by altering the composition and fluidity of the plasma membrane or by changing intracellular pH. At present,

we cannot exclude the effect of VAs on these parameters. However, VAs did not affect the passive uptake of DOX. This suggests that the variation in DOX uptake after VA treatment is mainly due to the effect of VAs on P-gp activity. This suggestion was also confirmed by data on DOX cytotoxicity and DOX uptake in wild-type LoVo sensitive cells. These cells express very low levels of MDR1 mRNA [25], and only a slight increase in DOX cytotoxicity and DOX uptake was observed after VA exposure.

In conclusion, these data better clarify the key substructural fragments related to MDR reversal activity and cytotoxicity in a series of VAs, and provide information on the interactions of these compounds with P-gp. The methoxy groups in the phenyl rings appear to be crucial in preventing cytotoxic effects when VAs are used alone, whereas the motif of R_5 and R_6 seems to be important for the reversal activity of the VAs and their interaction with P-gp. Most VAs exhibiting high reversal potency also showed high cytotoxic effects. In the planning of new VAs, it is hoped that cytotoxic effects can be minimized while achieving overall optimisation of their MDR reversal activity. Among the VAs investigated, racemic gallopamil showed the lowest cytotoxicity and the highest reversal potency. Unfortunately, racemic gallopamil has a high calcium antagonist activity, which prevents its use in the clinical setting. R-VER and nor-VER, which have a lower calcium antagonist activity, showed high reversal potency and low cytotoxicity, and therefore seem to be the best reversers for clinical use.

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