Biochemical Pharmacology, Vol. 44, No. 9, pp. 1707–1715, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

EFFECTS OF A NEW TRIAZINOAMINOPIPERIDINE DERIVATIVE ON ADRIAMYCIN ACCUMULATION AND RETENTION IN CELLS DISPLAYING P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE

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(*Received* 31 *March* 1992; *accepted* 18 *August* 1992)

Abstract—A new triazinoaminopiperidine derivative, Servier 9788 (S9788), was investigated for its ability to increase Adriamycin® (ADR) accumulation and retention in two rodent (P388/ADR and DC-3F/AD) and three human (KB-A1, K562/R and COLO 320DM) cell lines displaying the P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) phenotype. Depending on the cell line S9788 was shown to be two to five times more active and five to 15 times more potent than Verapamil (VRP) in increasing ADR accumulation in resistant cells. ADR retention in KB-A1 cells maintained in a concentration of 10 μM S9788 was twice that in VRP-treated cells, and similar to that measured in the untreated sensitive KB-3-1 cells. Although 5 μM S9788 and 50 μM VRP gave the same values of ADR uptake in KB-A1 cells, S9788 was shown to induce a greater ADR retention following cell wash and post-incubation in resistance modifier- and ADR-free medium. Taking into account that S9788 had no effects on ADR accumulation and retention in sensitive KB-3-1 cells, it can be suggested that S9788 inhibits specifically the P-gp dependent ADR efflux, and in a manner less reversible than that observed with VRP. Moreover, [^{3}H]azidopine photolabeling of P-gp, in P388/ADR plasma membranes, was completely inhibited by 100 μM S9788. Although S9788, as VRP, had no effect on the cell cycle of P388 cells, 5 μM S9788 increased 700-fold the efficacy of ADR to block P388/ADR cells in the G2+M phase of the cell cycle. Together, these results show that the sensitization, by S9788, of cell lines resistant to ADR is mainly due to an increase in ADR accumulation and retention, leading to an increase in the number of resistant cells blocked in the G2+M phase.

Multidrug resistance (MDR‡) represents a major cause of failure in cancer chemotherapy. The main characteristics of tumor cells displaying the MDR phenotype are (i) cross-resistance to structurally unrelated cytotoxic drugs having different mechanisms of action [1, 2] and (ii) overexpression of a membrane-associated glycoprotein of 170 kDa [3–5] which, by an ATP-dependent efflux pump function, decreases the intracellular drug accumulation [6].

MDR has been shown to be circumvented *in vitro* by a large number of compounds, including calcium channel blockers, calmodulin antagonists [7–9] and immunosuppressants [10]. Most of these compounds act by increasing the intracellular concentration of cytotoxic drugs [9, 11–13] probably through direct interaction with the P-glycoprotein (P-gp). Unfortunately, when used at efficient doses to reverse the MDR phenotype, these drugs are limited in the clinic by toxic effects due to their primary pharmacological activities [14, 15]. A major approach to reverse MDR is therefore to use novel compounds more potent and not limited by pharmacological activities other than reversal properties at the administered doses.

Servier 9788 (S9788) is a new triazinoaminopiperidine derivative which does not belong to any class of compounds described as resistance modifiers and which has been shown to reverse MDR *in vitro* and *in vivo* [16, 17]. S9788 was far more potent than Verapamil (VRP) in sensitizing 7 MDR cell lines *in vitro*. This compound significantly increased the antitumor activity of ADR in P388/ADR-bearing mice and completely restored the sensitivity to Vincristine (VCR) of P388/VCR-bearing mice [16, 17]. The mechanism of action of S9788 is unknown, but, as for VRP [18], interaction at the molecular level with the P-gp is highly suspected.

In order to investigate the mechanism of MDR circumvention by S9788, we studied the effect of this compound, in comparison with VRP, on ADR uptake and retention in two rodent (P388/ADR and DC-3F/AD) and three human (KB-A1, K562/R and COLO 320DM) resistant cell lines overexpressing the P-gp. Exploiting the fluorescence of ADR [19], its cellular accumulation and retention were evaluated by flow cytometry as described [11, 13, 20, 21]. Furthermore, we analysed the cell cycle perturbations induced by ADR in the presence of S9788 or VRP in order to verify that the mechanism of action of ADR on sensitive cells is the same as on resistant cells exposed to such

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‡ Abbreviations: S9788, Servier 9788; ADR, Adriamycin; MDR, multidrug resistance; VRP, Verapamil; P-gp, P-glycoprotein; VCR, Vincristine; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

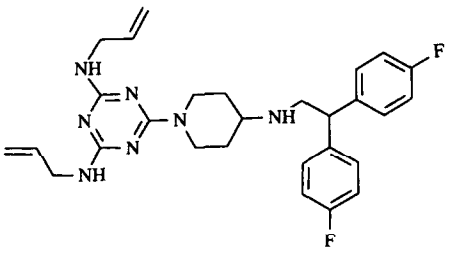

Fig. 1. Chemical structure of S9788.

resistance modifiers. We also verified the ability of S9788 to inhibit [^{3}H]azidopine photolabeling of P-gp in P388/ADR plasma membranes.

MATERIALS AND METHODS

Drugs. VRP and Actinomycin D were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.); ADR was provided by Roger Bellon (Neuilly, France). S9788 [17] was synthesized in our institute (Fig. 1). S9788 was solubilized at 10^{-2} M in dimethyl sulfoxide and diluted directly into culture medium.

Cell lines. The Chinese hamster lung cell line DC-3F and its counterpart 20,600-fold resistant to Actinomycin D, DC-3F/AD, were kindly provided by Dr A. Jacquemin-Sablon (Villejuif, France). The murine leukemia P388/ADR was established from P388/ADR-1 (kindly provided by Dr S. Cros, Toulouse, France) by subsequently growing in culture medium containing 10 μM ADR and was 480-fold resistant to ADR. The KB-3-1 cell line (human epidermoid carcinoma) and its counterpart 340-fold resistant to ADR, KB-A1, were kindly provided by Dr M. Gottesman (Bethesda, MD, U.S.A.) via Dr J. Y. Charcosset (Toulouse, France). The K562/S cell line (human chronic myelogenous leukemia) and its counterpart 56-fold resistant to ADR, K562/R, were kindly provided by Dr H. Tapiero (Villejuif, France). COLO 320DM (human colon adenocarcinoma) was from the American Type Culture Collection (Rockville Pike, MD, U.S.A.), the degree of resistance of this cell line to cytotoxic agents cannot be rigorously determined since there is no parental sensitive line, but it can be estimated as about 10-fold by comparison with all the other sensitive lines. All the cell lines were grown in RPMI medium 1640 (except KB-A1 and KB-3-1 grown in DMEM) supplemented with 10% fetal calf serum, 2 mM *l*-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM Hepes buffer, pH 7.4. The resistant cell lines were cultured in the presence of either 10 μM Actinomycin D (DC-3F/AD), 1 μM ADR (K562/R), 2 μM ADR (KB-A1) or 10 μM ADR (P388/ADR) until one week prior to the beginning of the experiments. The cytotoxicity was measured by the microculture tetrazolium assay after a continuous exposure (four doubling times), and the fold resistance was defined as the ratio of IC_{50} in resistant line/IC_{50} in sensitive line, for a given cytotoxic.

P-gp labeling. Methanol fixed cells were washed twice in phosphate-buffered saline (PBS) and reacted with 5 μg/mL of anti P-gp antibody fluorescein isothiocyanate conjugated (C219-FITC, Centocor Inc., Malvern, PA, U.S.A.) in PBS containing 0.3% Tween 20, 0.5% bovine serum albumin. After 1 hr of incubation at 4°, samples were washed twice in the same buffer and resuspended in PBS prior to flow cytometric analysis.

Results are expressed as relative P-gp over-expression: mean of C219-FITC fluorescence of resistant cells/mean of C219-FITC fluorescence of corresponding sensitive cells. For each cell line, controls done with an FITC-conjugated isotypic antibody showed no P-gp-related fluorescence.

ADR uptake and retention studies. To study the dose-dependent effect of the modulators, resistant cells (P388/ADR, DC-3F/AD, K562/R, KB-A1, COLO 320DM) (5×10^5/mL) were incubated with 50 μM ADR at 37° for 5 hr with 0.5–10 μM S9788 or VRP. Previous experiments have shown that: (i) 50 μM ADR is optimal to discriminate between sensitive and resistant cells, (ii) this high concentration is not toxic (with respect to cell integrity) when used for short periods of treatment and (iii) the accumulation of 50 μM ADR reaches a plateau after 5 hr of incubation at 37°.

The mean ADR fluorescence was measured at 4° by flow cytometry and results are expressed as the increase in the mean ADR fluorescence of treated cells compared with the mean ADR fluorescence of untreated cells.

For the kinetic studies of ADR uptake, KB-A1 and KB-3-1 cells were incubated at 37° with 50 μM ADR for 1 hr, then S9788 (5–10 μM) or VRP (10–50 μM) were added, and mean ADR fluorescence was measured at the indicated times. The measurements of ADR accumulation in KB-A1 and KB-3-1 cells were performed in separate experiments. For ADR retention studies, samples were prepared as described above but cells were washed twice after 4 hr of incubation and then incubated at 37° in ADR-free medium with or without resistance modifiers. ADR fluorescence was measured at the indicated times, and % ADR retention is given at the plateau. Results are given for a single experiment, similar results were obtained in two repeat experiments.

Preparation of plasma membranes and photoaffinity labeling of P-gp. P388 and P388/ADR cells were washed and resuspended in 0.01 M Tris–HCl (pH: 7.5), 0.25 M sucrose, 0.2 mM $CaCl_2$, for 30 min at 4°. Cells were then disrupted with a Dounce homogenizer and centrifuged at 1000 *g* for 10 min. Supernatants were overlaid on 0.01 M Tris–HCl (pH 7.5), 35% sucrose, 1 mM EDTA and centrifuged at 16,000 *g* for 30 min and the membrane fraction, at the interface, was then collected and centrifuged at 100,000 *g* for 75 min. Pellets of plasma membranes were resuspended in 0.01 M Tris–HCl (pH 7.5), 0.25 M sucrose and stored at −80°. Proteins (50 μg) were incubated with various concentrations of S9788 or VRP (1–100 μM) in 0.01 M Tris–HCl (pH 7.5), 0.25 M sucrose, containing 0.5 μM [^{3}H]azidopine (final volume: 150 μL). After 30 min samples were

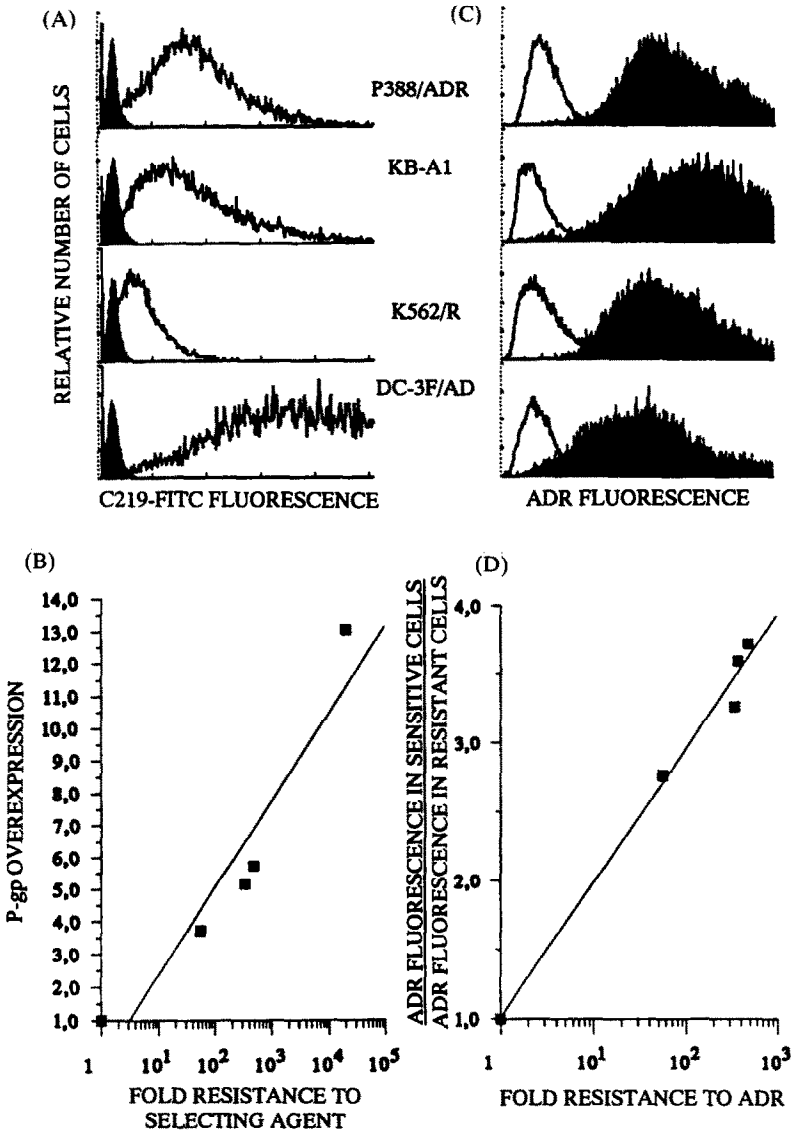

Fig. 2. (A) Flow cytometric analysis of P-gp expression in resistant and sensitive cells. Fixed cells were reacted with 5 μg/mL of C219-FITC antibody and analysed by flow cytometry. Hatched histograms:sensitive cells; open histograms:resistant cells. (B) Correlation between the P-gp overexpression (mean fluorescence of C219-FITC in resistant cells/mean fluorescence of C219-FITC in sensitive cells) and the factor of resistance to the inducing agent. (C) Histograms of ADR fluorescence in resistant and sensitive cells. Cells were incubated with 50 μM ADR for 5 hr and ADR fluorescence was measured by flow cytometry. (D) Correlation between ADR uptake and the factor of resistance to ADR.

irradiated (360 nm) for 30 min, boiled for 1 min and subjected to SDS–PAGE using 4–20% gradient gels. Proteins were stained by a Coomassie blue solution and fixed. Gels were then treated with an amplifying solution (Amersham, France), dried and exposed for 7 days using Hyperfilm-MP film (Amersham).

Cell cycle analysis. P388 and P388/ADR cells (5×10^5/mL) were incubated for two doubling times (21 and 30 hr, respectively) with various concentrations of ADR with or without 5 μM S9788, or 5 and 20 μM (maximum non-toxic dose) VRP. Cells were then fixed by 70% ethanol (v/v), washed twice with PBS and incubated in PBS containing 100 μg/mL RNAse (Sigma), 25 μg/mL propidium iodide for 30 min at 20° before flow cytometric analysis. Results are expressed as the percentage of cells accumulated in the G2+M phase of the cell cycle.

Flow cytometry. All analyses were performed on an ATC3000 flow cytometer (Bruker, Wissembourg, France) using an argon laser (Spectra-Physics, Les Ulis, France) emitting 600 mW at 488 nm. Propidium

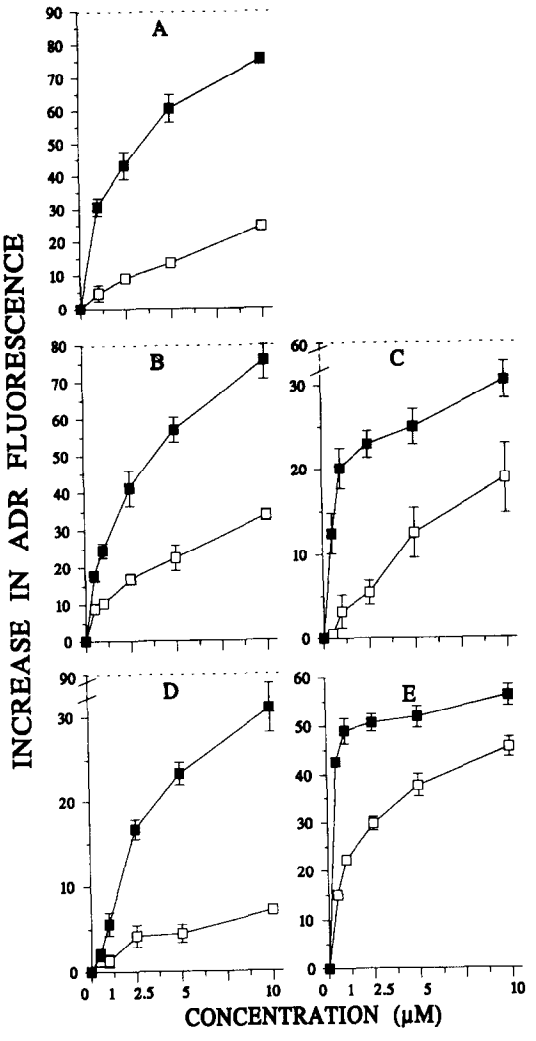

Fig. 3. Effect of S9788 and VRP on ADR accumulation. Resistant cells were incubated with ADR in the presence of S9788 (■) or VRP (□) for 5 hr, and ADR fluorescence was measured by flow cytometry. Results are expressed as the increase in the mean fluorescence of treated cells compared with the mean fluorescence of untreated cells. Bars represent SEM of at least three independent experiments. (A) P388/ADR, (B) KB-A1, (C) K562/R, (D) DC-3F/AD, (E) COLO 320DM. Dashed lines correspond to the difference in ADR fluorescence between sensitive and resistant cells.

iodide fluorescence was collected through a 615 nm long-pass filter; C219-FITC and ADR fluorescence were collected through 520 and 580 nm bandpass filters, respectively. Forward and right-angle light scatters were used to identify and analysed cell populations. For each sample, 10,000 cells were analysed and data were displayed as linear histograms.

RESULTS

P-gp labeling and ADR uptake in resistant and sensitive cells

Figure 2A shows the fluorescence histograms of cells labeled with the anti-P-gp antibody (C219-FITC). Compared to the parental sensitive cell lines, the strong fluorescence of P388/ADR, DC-3F/AD, KB-A1 and K562/R demonstrates an overexpression of P-gp correlating well ($r = 0.90$) with the factor of resistance of each cell line (Fig. 2B). Compared to the control done with the isotypic antibody, COLO 320DM cells also showed an overexpression of P-gp. Furthermore, the intracellular accumulation of ADR was 2.0 (K562/R)–3.7 (P388/ADR)-fold lower (in terms of fluorescence) in the resistant cells than in their sensitive counterparts (Fig. 2C). This ratio was correlated well ($r = 0.99$) with the factor of resistance to ADR (Fig. 2D) suggesting strongly that the cytotoxicity of ADR on these cell lines is directly dependent on its cellular accumulation.

Effect of S9788 and VRP on ADR accumulation by the five MDR cell lines

Resistant cells (P388/ADR, K562/R, DC-3F/AD, KB-A1, COLO 320DM) were incubated for 5 hr with ADR in the presence of increasing concentrations of S9788 or VRP (0.5–10 μM). These concentrations were chosen in the light of previous experiments which showed that 5 μM S9788 was the optimal, non-cytotoxic concentration which completely or partially (depending on the cell line and the cytotoxic drug) reverses MDR after a continuous exposure [16, 17]. Under our experimental conditions (short periods of treatments), 10 μM resistance modifier was not cytotoxic. Figure 3 shows the increase in ADR fluorescence for each cell line as a function of modulator concentration. Both compounds induced, in all resistant cell lines, a dose-dependent increase in ADR accumulation, which for KB-A1 cells treated with 10 μM S9788 reached a level close to that for the sensitive counterpart cell line. Depending on the cell line, S9788 was two to five times more active (at equimolar concentration) and five to 15 times more potent (for similar activity) than VRP.

Effect of S9788 and VRP on the kinetics of ADR accumulation by KB-A1 and KB-3-1 cells

KB-A1 and KB-3-1 cells were incubated with 50 μM ADR for 1 hr, followed by addition of 10 μM S9788 or VRP. The mean ADR fluorescence was determined at the times indicated in Fig. 4. A rapid and high ADR accumulation was observed in KB-3-1 cells with a plateau being reached after 5 hr of incubation. No significant effects of S9788 or VRP were detected on this sensitive cell line. In KB-A1 cells, the slow rate of ADR accumulation was increased from the first few minutes of incubation with the resistance modifiers. Compared to 10 μM VRP, the same concentration of S9788 was three times as efficient in increasing ADR uptake. After 4 hr of incubation, the effect of S9788 was maximum, showing a mean ADR fluorescence closer to the mean measured for the sensitive KB-3-1 cells treated with ADR alone.

Effect of S9788 and VRP on ADR retention by KB-A1 and KB-3-1 cells

Cells were incubated with 50 μM ADR and 10 μM resistance modifier for 4 hr as described above,

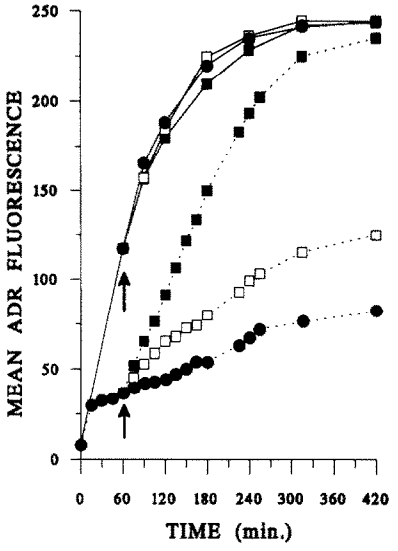

Fig. 4. Effect of S9788 and VRP on the kinetics of ADR uptake by KB-A1 and KB-31 cells. KB-A1 (---) and KB-3-1 (——) cells were incubated with 50 μM ADR (●), with addition of 10 μM S9788 (■), or 10 μM VRP (□). The arrows indicate the time point of addition of resistance modifiers.

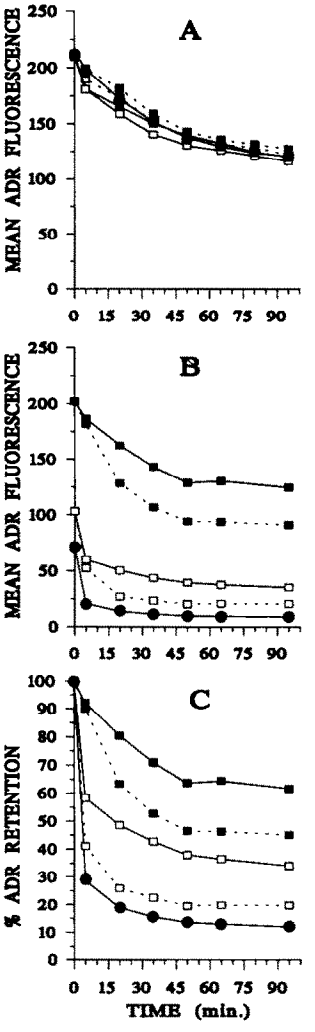

Fig. 5. Effect of S9788 and VRP on ADR retention in KB-A1 and KB-3-1 cells. KB-3-1 (A) and KB-A1 (B, C) cells were incubated as specified in the legend to Fig. 4, washed twice after 4 hr of ADR uptake and incubated in ADR-free medium with (——) or without (----) resistance modifier. Control (●), 10 μM S9788 (■), 10 μM VRP (□) Results are expressed as mean ADR fluorescence (A, B), and as % ADR retention (C).

washed twice and incubated in ADR-free medium with or without 10 μM S9788 or VRP. Mean ADR fluorescence was measured at the times indicated in Fig. 5 (time 0 corresponds to the time 240 min of ADR uptake). Neither S9788 nor VRP significantly modified the efflux of ADR in sensitive KB-3-1 cells (Fig. 5A). In untreated KB-A1 cells (Fig. 5B), a rapid decrease in ADR fluorescence was observed which plateaued 35 min after ADR removal. A lower rate of efflux was observed with resistant cells exposed continuously to VRP or S9788, with 95 min after ADR removal an ADR retention of approximately 36% with VRP and 63% with S9788 (Fig. 5C). The ADR retention in resistant cells treated with S9788 was similar to that in untreated sensitive cells (Fig. 5A, B). When resistant cells were incubated in modulator-free medium, a better ADR retention was observed (Fig. 5C) in cells pre-treated with S9788 (46%) than in cells pre-treated with VRP (20%). These experiments were carried out with the same concentration of resistance modifiers yielding different values of ADR accumulation (Fig. 4). To better compare the reversibility of the effects of the two compounds, cells were incubated with increasing concentrations of resistance modifiers in order to determine two equally active concentrations, i.e. those inducing similar values of ADR uptake. It appears (Fig. 6) that 50 μM VRP is necessary to increase the ADR uptake in KB-A1 cells to a level similar to that observed with 5 μM S9788. A further ADR retention study was thus performed as described above but with 50 μM VRP or 5 μM S9788. The kinetics of ADR efflux, expressed as percentage ADR retention, are shown in Fig. 7. At time 0 (time 240 min of ADR uptake), mean relative ADR fluorescence was about 125 for cells treated with S9788 and VRP, and 60 for untreated KB-A1 cells. When KB-A1 cells were exposed continuously to 5 μM S9788 or 50 μM VRP we observed, as expected, an equal ADR retention of approximately 46% after 95 min of incubation (Fig. 7). However, when cells were incubated in modulator-free medium, ADR retention at the plateau was significantly higher in cells pre-treated with 5 μM S9788 (34%) than in cells pre-treated with 50 μM VRP (21%).

Inhibition of [^{3}H]*azidopine photolabeling of P-gp*

Figure 8 shows the specific labeling of P-gp by [^{3}H]azidopine in the plasma membranes of P388/

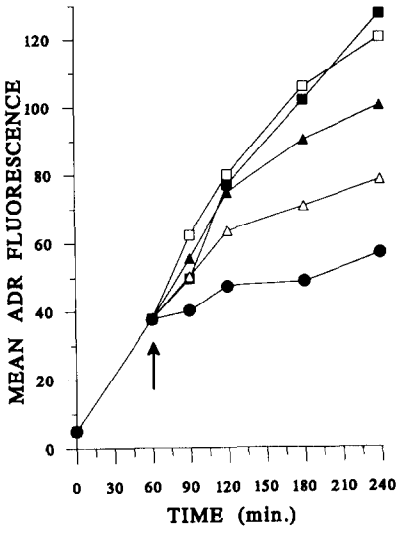

Fig. 6. Effect of S9788 and VRP on ADR uptake by KB-A1 cells. Cells were incubated with 50 μM ADR (●), with addition to 5 μM S9788 (■), or 10 μM VRP (△), 20 μM VRP (▲), 50 μM VRP (□). Arrow indicates the time point of addition of resistance modifiers; gain settings were different from those used for Fig. 4.

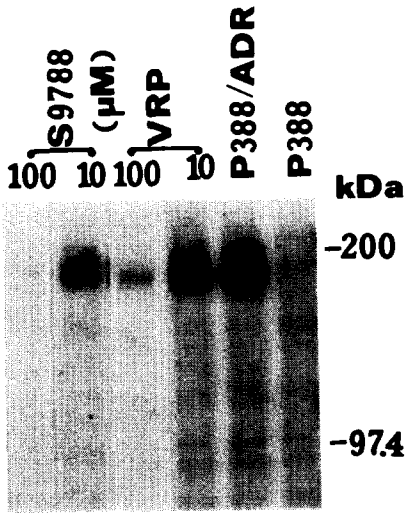

Fig. 8. Inhibition of [^{3}H]azidopine photolabeling of P-gp. Protein (50 μg) of P388/ADR plasma membranes was incubated with or without 1–100 μM S9788 or VRP in the presence of 0.5 μM [^{3}H]azidopine, irradiated at 360 nm and subjected to SDS–PAGE. Proteins were stained by a Coomassie blue solution.

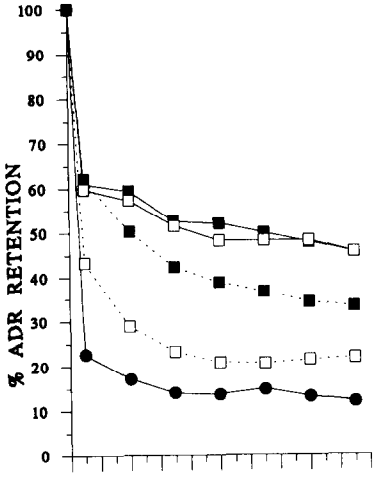

Fig. 7. Effect of S9788 and VRP on ADR retention in KB-A1 cells. Cells were incubated as specified in legend to Fig. 6, washed twice after 4 hr of ADR uptake and incubated in ADR-free medium with (——) or without (----) resistance modifier. Control (●), 5 μM S9788 (■), 50 μM VRP (□).

ADR cells. While 100 μM VRP partially inhibited the photolabeling of P-gp, 100 μM S9788 induced a complete inhibition (96%) of the photolabeling.

Effects of S9788 and VRP on the cell cycle perturbations induced by ADR

Figure 9 shows the percentages of P388 and P388/ADR cells accumulated in the G2+M phase as a function of ADR concentration, with or without resistance modifiers. Under our experimental conditions, no perturbations of the cell cycle and no cytotoxic effects were observed on cells treated with S9788 or VRP alone (not shown). ADR at 200 nM induced a massive accumulation (80%) of sensitive cells in the G2+M phase of the cell cycle, but a similar accumulation was measured when these cells were treated with 50 nM ADR in the presence of 5 μM modulator. The same type of perturbation was observed on P388/ADR cells treated with 100 μM ADR, but the accumulation in G2+M was lower (65%). When resistant cells were treated with ADR in the presence of resistance modifiers, a massive accumulation in G2+M was observed but at different ADR concentrations depending on the modulator concentration: 200 nM ADR with 5 μM S9788, 2 μM ADR with 5 μM VRP, 500 nM ADR with 20 μM VRP. Thus, under our experimental conditions, 5 μM S9788 and 20 μM VRP increased 700 and 300 times, respectively, the ADR efficacy to accumulate P388/ADR cells in the G2+M phase, with however only S9788 completely restoring the activity and potency of ADR on resistant cells.

DISCUSSION

S9788 is a novel triazinoaminopiperidine derivative which has been shown recently to reverse MDR in rodent and human cell lines *in vitro*. Its activity has been confirmed by *in vivo* experiments on P388/VCR-bearing mice where non-toxic doses of S9788 fully restored the sensitivity of this resistant tumor to VCR [16, 17].

To investigate the mechanism of action of S9788, we used flow cytometry to study its ability to modify ADR uptake and retention in resistant cells, properties already described for VRP [13]. The different resistant cell lines used (P388/ADR, DC-3F/AD, K562/R, KB-A1, COLO 320DM) displayed a P-gp-mediated MDR phenotype, as has been defined [1–6]. In addition, the P-gp overexpression correlated well with the factor of resistance of each cell line to the inducing agent and with the ratio of intracellular ADR accumulation in sensitive to resistant cells. These results suggest that the major mechanism of the ADR resistance of these cells is the consequence of P-gp overexpression which lowers the intracellular accumulation of ADR. However, the involvement of other mechanisms of resistance cannot be excluded, since cell lines selected for their resistance to ADR have also displayed changes in topoisomerase II activity [22, 23].

In this report, we show that S9788 increases ADR accumulation in resistant cells, as is the case for other known resistance modifiers [9, 13]. This effect was dose-dependent in all the cell lines studied, and 10 μM S9788 increased ADR accumulation in KB-A1 cells to a level similar to that measured in the parental sensitive KB-3-1 cells. Compared to VRP, S9788 was, at equimolar concentration, two to five times more active and, five to 15 times more potent, depending on the cell line, in increasing ADR uptake. It is important to note that S9788 is significantly more potent that VRP in the case of COLO 320DM, a cell line displaying a level of resistance representative to the resistance to chemotherapy observed in the clinic. Used at an equimolar concentration (10 μM), S9788 and VRP induced a rapid increase in ADR accumulation in KB-A1 cells detected 5 min after the addition of the resistance modifiers, suggesting that these compounds diffuse rapidly across the plasma membrane. This property has been described for VRP, for which cellular accumulation reached a plateau in less than 10 min [24, 25]. Furthermore, 10 μM S9788 induced an increase in ADR fluorescence in KB-A1 cells not significantly different from that measured in the sensitive KB-3-1 cells. Thus, we can suppose that the ADR concentration in sensitive and resistant cells exposed to 10 μM S9788 is the same. However, data are presented in relative fluorescence intensities and not in absolute units of intracellular drug. The ADR accumulation measured by flow cytometry is a global detection [21] and consequently it is difficult to assess the various intracellular compartments of ADR, more precisely the ADR molecules involved in the cytotoxicity (the DNA-intercalated ADR [26]), the fluorescence of which is significantly quenched [19]. Nevertheless, the differences in intracellular ADR fluorescence measured by flow cytometry have been shown to correlate with cell survival [21]. Furthermore, it has been shown [12] that a similar level of anthracycline fluorescence (measured by flow cytometry) in sensitive and resistant cells treated with resistance modifiers corresponds to identical intracellular drug concentrations determined by HPLC.

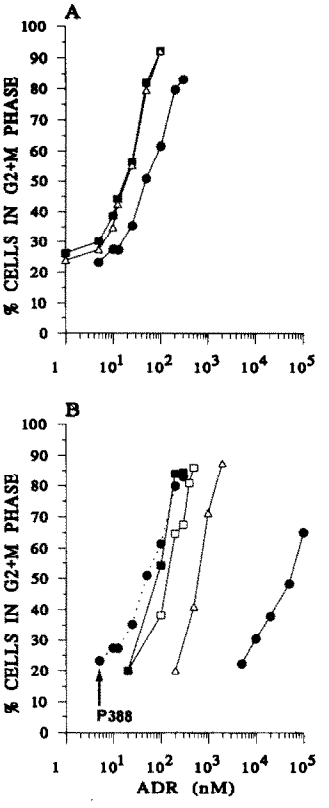

Fig. 9. Effect of ADR on the G2+M phase cell accumulation. P388 (A) and P388/ADR (B) cells were treated for two doubling times with ADR at indicated concentrations in the presence of 5 μM S9788 (■), 5 μM VRP (△), 20 μM VRP (□) or without resistance modifier (●). Percentages of cells in the G2+M phase were determined by flow cytometry.

Under the experimental conditions, the two resistance modifiers did not increase ADR accumulation in sensitive KB-3-1 cells (the same results were obtained by using lower ADR concentrations, data not shown). We can therefore conclude that the effects of S9788 and VRP on KB-A1 cells are independent of an alteration in the membrane permeability. It is thus possible that S9788, because of its high hydrophobicity, accumulates in the membrane of resistant cells and impairs, directly or indirectly, the P-gp function. However, expression of the P-gp was not affected by a 24 hr exposure to 5 μM S9788 (data not shown).

In KB-A1 cells, the rate of ADR efflux was very fast and plateaued after 35 min; a rapid efflux rate was also observed in resistant cells treated with 10 μM VRP, but with a higher ADR retention at the plateau. For KB-A1 cells treated with 10 μM S9788, the ADR efflux rate was slower and plateaued after 50 min. When cells were incubated in modulator-free medium during the ADR efflux, the percentage retention (with respect to initial ADR uptake values) measured with S9788 was twice that measured with VRP. At the plateau, the ADR

retention in KB-A1 cells exposed continuously to S9788 and in untreated KB-3-1 cells was similar, showing that 10 μM S9788 completely inhibits the efflux of ADR. Furthermore, as no significant effects of resistance modifiers were detected on KB-3-1 cells, and taking into account that 100 μM S9788 completely inhibited [^{3}H]azidopine photolabeling of P-gp, a specific inhibition of the P-gp function by S9788 is suggested.

In order to better compare the reversibility of the effect of the two compounds, experiments of ADR accumulation were performed with modulator concentrations inducing similar intensities of ADR fluorescence (5 μM S9788; 50 μM VRP). When cells were incubated in modulator-free medium during the ADR efflux, S9788 induced, at the plateau, a greater ADR retention than VRP with 34% (S9788) and 21% (VRP) of the initial fluorescence. This result shows that even after being removed from the medium, S9788, used at an initial extracellular concentration 10 times lower, induced an inhibition of ADR efflux less reversible than VRP. A possibility is that S9788 remains in cells at a higher concentration than VRP. Measurements of S9788 uptake and retention in resistant and sensitive cells are currently under investigation in our laboratory to verify this hypothesis.

No perturbations of the cell cycle and no cytotoxic effects were detected on P388 or P388/ADR cells treated with resistance modifiers alone. In this experiment, ADR induced the same type of perturbation on resistant and sensitive cells, i.e. an accumulation of the cells in the G2+M phase but at ADR concentrations 1000-fold higher on P388/ADR than on P388 cells. On the resistant line, the ADR efficacy was increased approximately 700 and 300 times by 5 μM S9788 and 20 μM VRP, respectively. These results show a sensitization of the resistant cells, in that (i) the perturbation of the cell cycle by ADR on sensitive cells is the same as that on resistant cells exposed to S9788 and (ii) the increase in ADR accumulation in modulator-treated cells leads to an increase in ADR activity (in terms of G2+M phase accumulation) suggesting that the mechanism of action of ADR on P388/ADR cells exposed to such resistance modifiers is the same as on parental sensitive cells. Furthermore, we have demonstrated that S9788 and VRP increase the ability of ADR to accumulate the sensitive cells in G2+M; this observation may be explained at least in part by a low but significant expression of P-gp in sensitive P388 cells, not observed in KB-3-1 cells.

These results show that the sensitization, by S9788, of cell lines resistant to ADR is due mainly to an increase in ADR accumulation and retention, leading to an enhancement of ADR-induced G2+M blockage in resistant cells. Compared to VRP, the superior effect of S9788 may be explained, in part, by its higher affinity for P-gp, as suggested by its stronger ability to inhibit [^{3}H]azidopine photolabeling. Previous studies concerning the *in vivo* activity of S9788 and the results presented in this report show the interest of this compound as an effective MDR reversal agent.

Acknowledgements—We thank Dr J. A. Boutin for helpful discussions, and M. Burbridge for critical reading of the manuscript. We are indebted to S. Marouillat for her technical assistance.

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