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SHORT COMMUNICATION

S9788 Modulation of P-Glycoprotein- and Multidrug-related Protein-mediated Multidrug Resistance by Servier 9788 in Doxorubicin-resistant MCF7 Cells

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ABSTRACT. Inherent or acquired resistance to multiple natural drugs, termed multidrug resistance (MDR), represents a major obstacle to chemotherapy. Expression of P-glycoprotein (P-gp) in MCF7mdr and MCF7R resistant cells was detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. MCF7R, but not the MDR1 gene-transfected MCF7mdr cells, expressed multidrug-related protein (MRP) concomitantly. Efficacy of an MDR modulator, designated as Servier 9788 (S9788), was estimated by doxorubicin (Dox) sensitization, Dox incorporation, and functional rhodamine 123 assay on MCF7 cell lines. Results showed that S9788 modulates the P-gp-associated MDR of MCF7mdr cells as well as the Multidrug-related protein-associated MDR of MCF7R cells. BIOCHEM PHARMACOL **56**;4:497–502, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. doxorubicin resistance; MDR; MRP; MDR-sensitization; MCF7

Resistance of tumor cells to multiple chemotherapeutic agents, a mechanism termed MDR[‖], is a major obstacle to the success of cancer chemotherapy and has been closely associated with treatment failure. One of the most studied mechanisms of drug resistance is that characterized by reduced drug accumulation and increased drug efflux resulting from overexpression of the plasma membrane protein, P-gp [1]. This protein is a carrier responsible for the energy-dependent efflux of a number of structurally unrelated natural antitumoral agents that include Dox, vinblastine, etoposide and taxol. In addition to P-gp, a second gene is now known to cause MDR *in vitro*. This gene encodes a 190-kDa membrane protein referred to as MRP and, like P-gp, is a member of the ABC superfamily of transporters. MRP, originally isolated from a lung cell line selected with DDX, was subsequently shown to confer drug resistance to a large panel of natural product drugs by cDNA transfection studies [2]. Overexpression of these membrane proteins allows an active efflux of drugs from resistant cells, thereby reducing the intracellular drug concentrations that otherwise would be toxic. A number of noncytotoxic agents (modulators) have been found to sensitize P-gp-associated MDR cells [3, 4]. A novel generation of P-gp and MRP modulators must be identified if resistance is to be circumvented in some malignancies.

S9788 is a triazinoaminopiperidine derivative which was identified through *in vitro* screening for its P-gp-associated MDR reversing activity [5] and its drug accumulation property [6]. Its ability to overcome both intrinsic and acquired P-gp-associated MDR *in vitro* was up to 7 times more potent than that observed with verapamil [6]. S9788 did not affect drug resistance due to an altered topoisomerase II activity [7]. The sensitization of resistant cells to vincristine, vinblastine and Dox is mediated by the interaction of S9788 with P-gp [7], its effect being less reversible than that observed with verapamil.

In order to investigate the efficacy of P-gp- and MRP-associated MDR circumvention by S9788, we studied its effect on two resistant cell lines derived from MCF7. In this study, we confirmed the MDR phenotype of the resistant cell lines by RT-PCR and Western blot methods. S9788 efficacy on P-gp- and MRP-associated MDR cells was estimated by Dox sensitization, incorporation and functional rhodamine 123 assay.

MATERIALS AND METHODS

Cell Lines and Their Characterization

Sensitive (MCF7S) and multidrug-resistant (MCF7mdr and MCF7R) human breast cancer cell lines were obtained from Dr. F. Calvo (Saint-Louis Hospital, Paris, France).

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‖ *Abbreviations:* ABC, ATP binding cassette; AUC, area under the curve; Dox, doxorubicin; LRP, lung-related protein; MDR, multidrug resistance; MTA, microculture tetrazolium assay; MRP, multidrug-related protein; PI, propidium iodide; P-gp, P-glycoprotein; R123, rhodamine 123, RT-PCR, reverse transcription-polymerase chain reaction.

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The sensitive MCF7S cell line was transfected by the human MDR1 gene to obtain a low multidrug-resistant (MCF7mdr) cell line. The high multidrug-resistant cell line (MCF7R) was selected from the parental cell line MCF7S by culture with increasing Dox concentrations. Cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies), 2 mM glutamine (Life Technologies), 100 units/mL of penicillin-streptomycin (Life Technologies), 2.5 μg/mL of amphotericin B (Life Technologies), and incubated at 37° in a 5% CO_2–95% air humidified incubator. The MCF7mdr and MCF7R cell lines were treated with 0.1 μM and 5 μM Dox respectively, until one week prior to the beginning of experiments, when they were cultured in a Dox-free medium.

The MDR1 and MRP gene expression was determined by RT-PCR as described by Bichat *et al.* [8] and Legrand *et al.* [9], respectively with β-actin as internal control [8]. Presence of P-gp and MRP proteins was detected by Western blot analysis. Cell surface membranes were extracted as described by Ferry *et al.* [10], and 50 μg of membrane proteins were subjected to Western blot analysis using either the monoclonal antibody anti-P-gp C494 or the monoclonal antibody anti-MRP MRPm6. P-gp and MRP proteins recognized by their respective antibodies were visualized using a horseradish peroxidase assay kit (Bio-rad) or an ECL detection system (Amersham), respectively.

Treatment Schedule

Dox was purchased from Farmitalia Carlo Erba (Rueil, France) and S9788 was a generous gift from Institut Servier (Courbevoie, France). To measure the Dox influx, cells were preincubated for 1 hr with free medium or with S9788 5 μM (schedules 1 and 2). Then, cells were also postincubated for 3 hr with free medium or with S9788 (schedules 3, 4 and 5) to estimate the Dox efflux (Table 2).

Cytotoxic Assay

Dox and S9788 cytotoxicities were measured by the microculture tetrazolium assay [11]. Cells were exposed to ranged concentrations of cytotoxic drugs for 6 hr, washed with PBS, incubated for 96 hr in free medium, and finally treated for 2 hr at 37° by incubation with microculture tetrazolium assay (at 0.5 mg/mL). Medium was removed and tetrazolium salt was resuspended with DMSO. The absorbance was measured with a Titersoft program (Flow Laboratory, Cergy, France) at 550 nm.

Dox and DNA Quantification

Cells were cultivated in 225 cm^2 plastic culture flasks and were treated as schedules described below. Cell suspensions were aliquoted and frozen to quantitate total Dox and DNA. The remaining material was incubated for 15 min at 37° in hypotonic buffer (4 mL of KCl 0.075 M) and centrifuged for 10 min at 2,500 *g*. Aliquots of supernatant (cytoplasm and membrane) and the pellets were frozen and stored at −20° until cytoplasmic and nuclear Dox quantification. Dox concentrations were determined by HPLC as described by Robert *et al.* [12] using a standard curve calibration obtained after linear regression. Final values were expressed as nanogram of associated Dox per microgram of DNA. Dox AUC was calculated by the trapezoidal rules. Results were expressed as AUC in nanograms of Dox/microgram of DNA × hr. DNA content was determined as described by Fisher-Szafarz *et al.* [13]. DNA concentrations were obtained from a standard curve calibration established after linear regression.

Flow Cytometric Analysis of R123 Accumulation and Efflux

Cells were treated with 5 μM S9788 for 1 hr followed by addition of R123 (0.2 μg/mL) for 1 hr. Thereafter, cells were washed twice with PBS and were either detached for flow cytometric analysis or postincubated for 1 hr with free medium or 5 μM S9788 to determine the R123 efflux. Then, cells were trypsinized, washed with cold phenol-red free RPMI and resuspended in 1 mL of phenol-red free RPMI, to which propidium iodide (PI) (10 μg/mL) (Sigma) was added. PI made it possible to exclude dead cells in order to analyze viable cells only. Samples were analyzed using an EPICS Profile II Coulter flow cytometer equipped with a 15-mA argon day laser, while fluorescence was displayed on a logarithmic scale at 488 nm and emission was recorded at 530 nm. Green fluorescence of R123 was measured on 20,000 cells gated at forward scatter (Fs) and red fluorescence of PI (FL3). Data were stored and analyzed with Phoenix Flow System Multi Plus Software. Results were expressed as histograms with their mean logarithm fluorescence intensity of S9788-treated cells as compared to untreated cells. Experiments were repeated three-fold to improve results.

Statistics

IC_{50} were analyzed by unpaired Student's *t*-test and differences between mean values at $P < 0.05$ were considered to be significant. Statistical comparisons for Dox incorporations (noted in Table 2) were done by ANOVA.

RESULTS AND DISCUSSION

Expression of P-gp and MRP in the MCF7 cell lines was determined by RT-PCR and Western blot analysis. The MCF7R cell line expressed MDR1 and MRP mRNA concomitantly, whereas MCF7mdr only expressed MDR1 to a lesser extent and MCF7S did not express MDR1 and MRP mRNA (Fig. 1A). These results were confirmed by Western blot analysis, 170- and 190-kDa bands being representative of P-gp and MRP proteins, respectively (Fig.

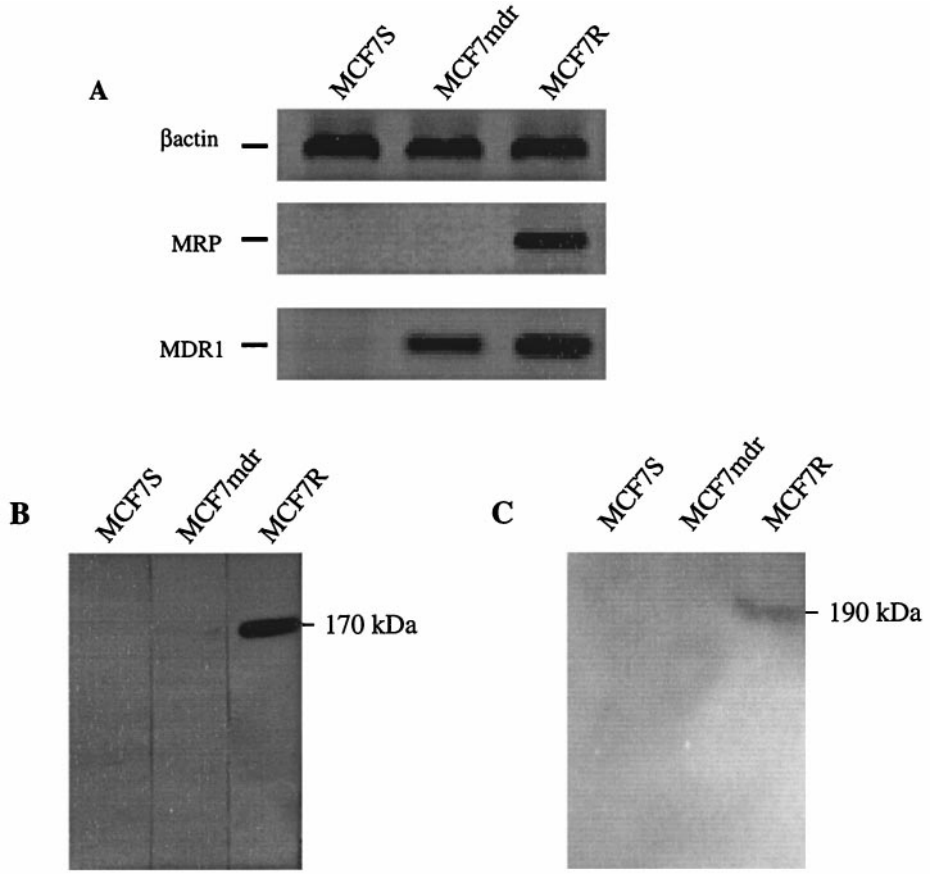

FIG. 1 MDR characterization of MCF7 cell lines. (A) RT-PCR amplifications of MDR1, MRP and βactin mRNA from the parental sensitive MCF7S, the MDR1 gene transfected MCF7mdr and the resistant MCF7R cell lines. (B) Western blot analysis of P-gp in membrane fractions prepared from MCF7S, MCF7mdr and MCF7R cells. P-gp was detected with the monoclonal antibody C494. (C) Western blot analysis of MRP in membrane fractions prepared from MCF7S, MCF7mdr and MCF7R cells. MRP was detected with the monoclonal antibody MRPm6.

1B and 1C). The P-gp level in MCF7R membranes was higher than in MCF7mdr, and the MRP protein was only present in MCF7R membranes.

IC_{50} values deduced from dose-response curves after a 6 hr exposure are summarized in Table 1. The resistance factor of MCF7mdr and MCF7R cells was 27 and 95, respectively. At 5 μM S9788, the cell survival was 90%. The addition of 5 μM S9788 did not modify the Dox IC_{50} value of the MCF7S cell line, but did decrease the Dox IC_{50} value of resistant cell lines, thus defining a reversal factor of 5.3 and 3.6 for the MCF7mdr and MCF7R cell lines, respectively (Table 1).

Dox was quantitated by HPLC in the total, nuclear and cytoplasmic compartments. Ninety percent of cellular Dox was incorporated into the MCF7 cell-line nucleus with or without S9788 incubation. MCF7mdr and MCF7R cells incorporated Dox in amounts of 28 and 12% of the MCF7S cells, respectively (schedule 1 in Table 2). Schedule 3 allowed us to estimate Dox efflux when it was compared to Dox incorporation in schedule 1. The Dox AUC of MCF7S cells was 22% lower with RPMI postincubation; Dox AUC decreased by 53 and 60% for MCF7mdr and MCF7R cells, respectively. Nevertheless, Dox was incorporated to a greater extent in MCF7mdr than in MCF7R cells without S9788 incubation (Table 2). Dox distribution was not altered between the nuclear and cytoplasmic compartments

TABLE 1. Cytotoxicity of Dox after preincubation for 1 hr with 5 μM S9788

	IC_{50} (μM) (mean ± SEM)		
Drugs/Cell lines	**MCF7S**	**MCF7mdr**	**MCF7R**
S9788 (μM)	20.8 ± 6.5	12.0 ± 0.8^{NS}	30.3 ± 8.8^{NS}
Dox (μM)	0.13 ± 0.03	3.4 ± 0.4*	12.4 ± 2.8*
Resistance factor	—	27	95
5 μM of S9788 + Dox (μM)	0.10 ± 0.06^{NS}	0.6 ± 0.4†	3.4 ± 2.1†
Reversal factor	—	5.3	3.6

IC_{50}s (in μM) were determined by MTT assay as described in Materials and Methods. The resistance factor was calculated as the IC_{50} of resistant cells (MCF7mdr or MCF7R)/the IC_{50} of the parental sensitive MCF7S cells. IC_{50}s were also measured after 1 hr of 5 μM S9788 preincubation followed by different concentrations of Dox. Reversal factor for a resistant cell line was calculated as the IC_{50} of Dox alone/the IC_{50} Dox preincubated by S9788. NS: Not significant.

*Statistical significance ($P < 0.05$) of the differences between MCF7S IC_{50} values and those of MCF7mdr or MCF7R.

†Statistical significance ($P < 0.05$) of the differences between IC_{50}s of a cell line preincubated or not by S9788.

TABLE 2. AUC values determined for each schedule of treatment with each cell line

	Dox AUC (ng of Dox/μg of DNA × hr) (mean ± SEM)				
Schedules	**1**	**2**	**3**	**4**	**5**
Preincubation (1 hr)	—	S9788	—	S9788	S9788
	Dox	Dox	Dox	Dox	Dox
Postincubation (3 hr)	—	—	RPMI	RPMI	S9788
MCF7S	211 ± 66	158 ± 41^{NS}	164 ± 29	145 ± 9^{NS}	180 ± 26^{NS}
MCF7mdr transfected	59 ± 30^{NS}	176 ± 18*,^{NS}	28 ± 20‡	77 ± 11‡,^{NS}	136 ± 5†,^{NS}
MCF7R	25 ± 5‡	108 ± 17*,^{NS}	10 ± 1‡	33 ± 4†,‡	83 ± 10†,‡

Before Dox treatment, cells were preincubated for 1 hr with RPMI medium containing or not 5 μM S9788 (schedules 1 and 2) and Dox uptake was measured. In addition to preincubation as for schedules 1 and 3, cells were postincubated for 3 hr with RPMI medium containing or not 5 μM S9788 (schedules 3, 4 and 5) to estimate Dox efflux. AUC were calculated from the mean of triplicate measurements of each drug-exposure time. NS: Not significant.

*Statistical significance ($P < 0.05$) of the differences between the AUC of schedules 1 and 2 (uptake) for the same cell line.

†Statistical significance ($P < 0.05$) of the differences between the AUC of schedule 3 and the AUC of schedule 4 or 5 (efflux) for the same cell line.

‡Statistical significance ($P < 0.05$) of the differences between MCF7S AUC and MCF7mdr or MCF7R AUC for the same schedule.

of S9788-treated cells. S9788 pre-incubation (schedule 2) changed the Dox uptake in comparison with schedule 1: it was slightly decreased in MCF7S and increased by 198 and 332% in MCF7mdr and MCF7R, respectively. Dox uptake in the presence of S9788 increased to a greater extent in MCF7R than in MCF7mdr, although its incorporation remained less than in the latter cell line (176 versus 108 ng of Dox/μg of DNA × hr). The reversal activity of S9788 was estimated by Dox uptake in comparison with schedules 1 and 2.

After preincubation with S9788, Dox efflux was measured by postincubation with (schedule 5) or without S9788 (schedule 4). MCF7S cells did not efflux Dox after postincubation by RPMI, but Dox AUC decreased by 128 and 227% in the MCF7mdr and MCF7R cell lines, respectively. A greater amount of Dox remained incorporated in the MCF7mdr than in MCF7R cell lines (77 vs 33 ng of Dox/μg of DNA × hr). No efflux of Dox was observed in MCF7S cells after postincubation with S9788 (schedule 5). Dox AUC decreased by 29 and 30% in both resistant cells after postincubation by S9788. Nevertheless, this drop in AUC was lower than those obtained after RPMI postincubation (136 vs 77 and 83 vs 33 ng of Dox/μg of DNA × hr for MCF7mdr and MCF7R cells, respectively).

To investigate the effect of S9788 on R123 accumulation and efflux, cells were incubated with R123 and S9788 and analyzed by flow cytometry (Table 3). Without S9788 incubation, the MLFI of the dye was always higher with the sensitive cells than with both resistant cells. R123 efflux from cells after 1 hr postincubation in RPMI was very low (2%) in MCF7S cells, whereas it was active in MCF7mdr and MCF7R cells, with a drop in R123 content of 77% and 16%, respectively. The sensitive cell line accumulated 29% less R123 when treated by 5 μM S9788, whereas the R123 content in the MCF7mdr and MCF7R cell lines increased by 171% and 213%, respectively. S9788 partially restored the R123 uptake to a similar level as that observed with MCF7S cells. No R123 efflux after postincubation in RPMI was observed in MCF7S, but an efflux of 4% and 46% of R123 was measured in MCF7mdr and MCF7R, respectively. This difference contrasted with the observation of a more efficient efflux of R123 in MCF7mdr cells in the absence of S9788, one more spontaneous than in MCF7R cells. Postincubation with S9788 maintained the large amount of preaccumulated R123 in both resistant lines (Table 3).

P-gp and MRP expression was correlated with the resistance factor of each cell line to Dox agent and with the ratio of intracellular Dox accumulation in sensitive and resistant cells. These data suggested that one of the major mechanisms of the Dox resistance of these cells was the reduction of intracellular accumulation of Dox by membrane proteins. The involvement of other mechanisms of Dox resistance in MCF7R is not excluded, e.g. LRP expression [14] and changes in the gluthation-S-transferase π level [15]. In the resistant cell lines, we showed that the cellular content of Dox was increased when S9788 was administered at least 1 hr before Dox. In addition, the Dox efflux from resistant cells was reduced when S9788 was present during the release phase. Thus, the reversal resistance after a short-term Dox exposure resulted not only in an enhanced cellular accumulation of Dox during exposure, but also in an increased retention after cell transfer into fresh free RPMI. These results confirmed that the optimal efficacy of Dox incorporation was achieved by a pre- and postadministration of S9788. Similar results have been found for S9788 [5] and verapamil [16]. Moreover, a previous observation that amounts of intracellular Dox correlate with cell survival [17] was corroborated in our experiments: a relationship was noted between Dox time exposure of cells, Dox incorporation and cytotoxic effects [18].

Different reports studying immediate intracellular anthracyclin distribution showed that in parental cells, Dox was predominantly nuclear, whereas in resistant cells it was distributed into the cytoplasm in distinct punctate regions [19]. In our case, we studied the resultant Dox incorporation after 1-hr treatment. The resistant cells incorporated 90% of Dox into the nucleus, thus confirming that the drugs reached their nuclear targets. Previous studies showed that Dox was accumulated in the nuclei of drug-resistant cells and then redistributed into the cytoplasm [20]. They

TABLE 3. Mean logarithm fluorescence intensity values from R123 uptake and efflux obtained with MCF7S, MCF7mdr and MCF7R

Mean Logarithm Fluorescence Intensity ± SEM			
		R123 efflux with postincubations	
Incubation	R123 Influx	Free medium	5 μM S9788-containing medium
MCF7S			
Free medium	188 ± 22	184 ± 15 (−2)*	ND
S9788 5 μM -containing medium	145 ± 17 (−29)†	152 ± 15 (+5)‡	169 ± 16 (+17)‡
MCF7mdr			
Free medium	52 ± 7	29 ± 2 (−76.9)*	ND
S9788 5 μM -containing medium	141 ± 12 (+171)†	135 ± 11 (−4)*	149 ± 19 (+6)‡
MCF7R			
Free medium	44 ± 4	38 ± 3 (−15.9)*	ND
S9788 5 μM -containing medium	138 ± 9 (+213)†	75 ± 6 (−46)‡	133 ± 10 (−4)‡

Cells were incubated in free medium or in S9788-containing medium to measure the R123 uptake. They were postincubated in free medium or S9788-containing medium to estimate the R123 efflux (see Materials and Methods for details). Data are means ± SEM of three independent experiments. Numbers in parentheses represent the percentage increase (or decrease) in mean logarithm fluorescence intensity. ND: not determined.

*Between cells incubated in free medium followed by a postincubation in free medium and cells incubated in free medium.

†Between cells incubated in S9788-containing medium and cells incubated in free medium.

‡Between cells incubated in S9788-containing medium followed by a postincubation in S9788-containing medium and cells incubated in S9788-containing medium.

also demonstrated that Dox redistribution from the nucleus to the cytoplasm was blocked by verapamil; S9788 might do the same because it was shown to be more incorporated into resistant cells than verapamil [21]. Moreover, enhanced resistance was described as associated with a relocalization of P-gp from a plasma membrane to the cytoplasm [22], which, in turn, was coupled with a redistribution of anthracyclins away from the nucleus, allowing the extrusion of drugs.

The functional rhodamine assay confirmed these observations. Differences in R123 incorporation in both resistant cell lines were the reflection of their different levels of P-gp and the additive resistance mechanisms in MCF7R due to MRP. These results confirmed the choice of the optimal schedule corresponding to the higher efficacy of S9788.

In conclusion, these results form a basis for the design of phase I-II trials by using a combination of a loading dose of S9788 given before the cytostatic administration, followed by a maintenance infusion of the modulator administered as a continuous infusion. This schedule of modulator administration must be tested to sensitize resistant cells expressing P-gp and MRP proteins.

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