Cancer Chemother Pharmacol (1995) 36: 195–203

ORIGINAL ARTICLE

Jacques Soudon · Maryse Berlion · Catherine Lucas
Patrick Haddad · Jean-Pierre Bizzari · Fabien Calvo

In vitro activity of S 9788 on a multidrug-resistant leukemic cell line and on normal hematopoietic cells–reversal of multidrug resistance by sera from phase I-treated patients

Received: 20 June 1994/Accepted: 10 November 1994

Abstract The triazinoaminopiperidine derivative S 9788 is a new multidrug-resistance modulator that is currently being evaluated in phase I clinical trials. In this study, the reversal effect of S 9788 in comparison with verapamil was shown in vitro in human T-leukemic CCRF-CEM/VLB cells expressing the multidrug-resistance (MDR) phenotype. S 9788 increased in a dose-dependent manner the cytotoxic activity of doxorubicin or vinblastine, with complete reversal of resistance occurring at 2 μ*M* for a concomitant continuous exposure (96 h) to the cytotoxic drugs. At respective concentrations equivalent to the IC_{10} value (the concentration inhibiting 10% of cell growth), S 9788 was 44 times more potent than verapamil in CCRF-CEM/VLB cells. S 9788 at 2 μ*M* did not enhance the in vitro toxicity of doxorubicin or vinblastine in the human normal bone-marrow erythroid (BFU-E) and myeloid (CFU-GM) progenitors. The effect of exposure duration and concentrations on the synergistic action of modulator and cytotoxic agent closely depended on the cytotoxic agent studied. Post-incubations with S 9788 alone after a 1-h coadministration with vinblastine and S 9788 dramatically increased the reversal effect (4–41 times) in proportion to both the duration of postincubation and the concentration of S 9788. In contrast, for doxorubicin resistance, post-incubation with S 9788 alone induced a maximal 2-fold increase in the reversal effect that was not proportional to the postincubation duration. In patients treated with S 9788 as a 30-min intravenous infusion during phase I trials, a good correlation was found between the serum levels of S 9788 and the ability to reverse MDR in CCRF-CEM/VLB cells. The reversal effect was dose-dependent and was effective beginning at a plasma concentration of 0.25 μ*M*. These data form a basis for the design of phase II trials using a combination of a loading dose of S 9788 given before vinblastine or doxorubicin administration followed by a maintenance infusion of S 9788 alone for a period of 2–24 h.

Keywords S 9788 · Multidrug resistance
Vinblastine · Doxorubicin · Leukemia
Bone marrow

Introduction

Multidrug resistance (MDR) represents a major obstacle to the successful therapy of neoplastic diseases. MDR is defined as the ability of cells exposed to a single drug to develop resistance to a broad range of structurally and functionally unrelated drugs due to enhanced outward transport of drugs mediated by a membrane glycoprotein called P-glycoprotein (Pgp) [3, 26]. This Pgp appears to be clinically relevant in some malignancies [5, 7, 13, 38, 39].

A wide variety of pharmacological agents have been shown to reverse MDR using in vitro or in vivo experimental models [19, 41]. However, to date the clinical experience with chemosensitizers aimed at the modification of acquired or intrinsic drug resistance has been limited. A few compounds, especially verapamil (VRP) and cyclosporin A, have been evaluated in the clinic [12, 40].

The triazinoaminopiperidine derivative S 9788 is a new modulator of MDR. Its in vitro reversing activity has been demonstrated on a large panel of MDR tumor cell lines with acquired or intrinsic resistance. In MDR-selected lines, S 9788 was, at equimolar concentrations, more potent than VRP [24, 35]. S 9788 was shown to be effective in intrinsically MDR human

J. Soudon · P. Haddad · F. Calvo
Institut de Génétique Moléculaire, Hôpital Saint Louis,
27 rue Juliette Dodu, F-75010 Paris, France

M. Berlion · C. Lucas (✉) · J.-P. Bizzari
Institut de Recherches Internationales SERVIER,
Division Thérapeutique Cancérologie, 6 place des Pléïades,
F-92415 Courbevoie Cédex, France

carcinoma cell lines (kidney, colon, neuroblastoma) [16, 23]. In drug-resistant tumor cell lines expressing atypical MDR, no resistance modulation relative to parental cells was observed [22, 23]. Active in vitro concentrations varied from 0.5 to 5 μ*M*, depending on the cell line and the cytotoxic agent studied. Mechanistic studies suggest that S 9788 modulates MDR through Pgp [14, 30]. The reversal activity of S 9788 was confirmed in vivo in murine P388 leukemia lines resistant to doxorubicin or vincristine [11, 35] and in human xenograft models [36]. S 9788 is currently being evaluated in phase I clinical trials, and plasma peak concentrations of up to 3 μ*M* are achieved in patients treated with a 30-min infusion [27].

Our studies were undertaken with the aim of optimizing future phase I–II clinical trials. To date the clinical use of chemosensitizers has been limited by significant toxicity due either to the pharmacological properties of the modulator or to the potentiation of the antitumor drug's cytotoxicity on normal tissues expressing Pgp. The concentrations of unbound modulator necessary to reverse MDR in vitro cannot usually be achieved in human plasma without producing major side effects.

In the present study, the in vitro reversal activity of S 9788 in combination with doxorubicin (DOX) and vinblastine (VLB) was confirmed in the human MDR T-leukemic CCRF-CEM/VLB cell line. The effect of S 9788 on DOX and VLB cytotoxicity was also evaluated in normal human bone marrow cells. For optimization of phase II dosing schedules, the influence of exposure time and concentration of S 9788 on MDR modulation was investigated. Finally, considering that the high degree of serum protein binding could decrease the reversal activity of modulators [4, 29], the reversing activity of the serum of patients treated with S 9788 during phase I trials was assessed in CCRF-CEM/VLB cells.

Materials and methods

Chemicals

DOX, VLB, and VRP were obtained from Sigma Chemical Co. (France). Cytarabine (Ara-C) was purchased from Upjohn Laboratories (France) and was used as a negative control. S 9788 or 6-{4-[2,2-di(4-fluorophenyl)-ethylamino]-1-piperidinyl}-*N*,*N*′, di-2-propenyl-1,3,5-triazine-2,4-diamine, bismethane sulfonate was synthesized at the Servier Research Institute [15]. All compounds were initially dissolved in water.

Cells

The sensitive parental cell line CCRF-CEM/WT was originally derived from a patient with T-cell lymphoblastic leukemia [18]. The resistant variant CCRF-CEM/VLB, which was established by subsequent growing in culture medium containing increasing concentrations of VLB [2], expresses the MDR phenotype; *mdr*1 gene expression was confirmed by Northern-blot analysis (data not shown). These cell lines were kindly provided by Dr. J.P. Marie (Paris, France). Cells were grown in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum and a 4 m*M* L-glutamine in a humidified chamber containing 5% CO_2. The resistant cell line was cultured in the presence of 0.5 μg VLB/ml until 3 days before the start of the experiments. In these culture conditions, the doubling times of CCRF-CEM/WT and CCRF-CEM/VLB cells were 18 and 24 h, respectively. Bone marrow cells were obtained from posterior iliac crests of healthy donors for bone marrow transplantation with their informed consent. Mononuclear cells were separated by centrifugation with lymphocyte-separation medium (Eurobio, d = 1.077). Cells were washed twice, resuspended in RPMI 1640 medium, and counted. Cytotoxicity assays were performed on erythroid burst-forming units (BFU-E) and granulocyte macrophage colony-forming units (CFU-GM).

Experimental conditions

Modulation of MDR

CCRF-CEM/WT and CCRF-CEM/VLB cells in the logarithmic phase of growth were plated at a final concentration of 2.5×10^4 cells/ml into 96-well microtiter plates on day 0. Medium containing drug or control solvent was added on day 1 for continuous exposure. Each compound was first evaluated alone (six graded concentrations in triplicate) on each cell line. Then, the activity of DOX, VLB, or Ara-C was studied in combination with S 9788 at 0.5, 1, 2, or 4 μ*M*, corresponding respectively to the $IC_{10}/2$, IC_{10}, IC_{15}, and IC_{30} values (i.e., the concentrations required to inhibit cell growth by 10%, 15%, and 30% respectively), or with VRP at 0.4 or 2 μ*M*, corresponding to the IC_{10} and IC_{30} values. Thus, S 9788 and VRP were compared at equitoxic (IC_{10} or IC_{30}) and equimolar concentrations (2 μ*M*). Moreover, these concentrations were within the range of human plasma levels obtained during clinical trials with S 9788 [27] or VRP [37].

After four doubling times, the number and viability of cells were estimated using a colorimetric assay that measures the activity of the endogenous enzyme hexosaminidase [28]. The absorbance was measured at 405 nm. Three separate experiments were performed in triplicate. The results are expressed as IC_{50} values defined as the drug concentration inhibiting by 50% the absorbance of treated cells relative to untreated cells. The resistance of CCRF-CEM/VLB cells relative to the sensitive cells was expressed as the *n*-fold resistance (F. res = IC_{50} of the cytotoxic agent in the resistant cell line/IC_{50} of the cytotoxic agent in the sensitive cell line). The activity of S 9788 or VRP was expressed as *n*-fold reversal (F. rev = IC_{50} of the cytotoxic agent alone/IC_{50} of the cytotoxic agent and modulator) as evaluated for each concentration of modulator.

Hematotoxicity

Bone marrow mononuclear cells were plated at a final concentration of 10^5 cells/ml in petri dishes containing a semisolid Iscove medium (Terry Fox Laboratory) supplemented with 0.8% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} *M* mercaptoethanol, 2 m*M* glutamine, 3 U erythropoietin/ml, and 4.5% phytohemagglutinin in lymphocyte-conditioned medium. DOX and VLB were added with or without 2 μ*M* S 9788 for continuous exposure. BFU-E and CFU-GM colonies were counted on day 14 [10]. The cytotoxicity of S 9788 alone was also evaluated. Two independent experiments were performed in duplicate. The results are expressed as IC_{50} values, defined as the drug concentration inhibiting by 50% the number of colonies formed by treated cells relative to untreated cells.

Time-schedule modulation of MDR

CCRF-CEM/WT and CCRF-CEM/VLB cells were incubated for 1 h in the presence of the cytotoxic agent (DOX or VLB at six graded concentrations) with or without (control) S 9788. At the end of the incubation, cells were centrifuged at 1,500 rpm for 10 min, washed twice in RPMI 1640, and resuspended in complete medium with or without S 9788 for 6, 12, or 24 h (postincubation phase). Cells were then centrifuged again, washed twice in RPMI 1640, and resuspended in complete medium for four doubling times. The number and viability of cells were evaluated using the hexosaminidase assay and IC_{50} values were determined. Concentrations of S 9788 and periods of incubation were determined according to previous experiments.

Different incubation schedules were evaluated as follows:

1. For DOX resistance, cells were coincubated for 1 h with DOX and S 9788 at 50, 100, or 500 n*M* and postincubated for 6, 12, or 24 h with S 9788 alone at 50, 100, or 500 n*M*.
2. For VLB resistance, cells were coincubated for 1 h with VLB and S 9788 at 500 n*M* and postincubated for 12 or 24 h with S 9788 alone at 125, 250, or 500 n*M*.

Two independent experiments were performed in triplicate.

Reversal activity of serum containing S 9788

Blood samples were collected from eight patients in a phase I study who were suffering from tumors expressing intrinsic or acquired resistance [27]. They received S 9788 alone (without cytotoxic drug, which was given 1 week later in combination with S 9788) as a 30-min infusion at doses ranging from 88 to 104 mg/m^2 (last steps of the dose increments). Samples were collected before the infusion, at 15 and 30 min during the 30-min infusion, and at 1, 2 or 24 h (for some samples). S 9788 in serum was quantified by a high-performance liquid chromatography (HPLC) procedure with UV detection following solid-phase extraction as described by Bakes et al. [1]. Sera were decomplemented (30 min at 56° C) and filtered (0.45 μm) with no modification of the concentrations of S 9788 (verified by HPLC before and after decomplementation). CCRF-CEM cells were counted, centrifuged, and then incubated at a final concentration of 2.5×10^4 cells/ml with each serum in the presence or absence of DOX at 10 μ*M*. After a 3-h incubation (at 37° C), cells were washed twice and resuspended in complete culture medium for four doubling times. The number and viability of cells were evaluated using the hexosaminidase colorimetric assay. Two experiments were performed in triplicate. For the calibration curve, the same protocol was used, with decomplemented human serum being spiked in vitro with different concentrations of S 9788 (0.125–4 μ*M*) in the presence of absence of 10 μ*M* DOX.

Results

Modulation of DOX and VLB resistance by S 9788 and VRP during continuous exposure

The results, expressed as IC_{50} values, *n*-fold resistance and *n*-fold reversal, are presented in Table 1. S 9788 alone was not cytotoxic to CCRF-CEM/WT and CCRF-CEM/VLB cells at concentrations lower than 2 μ*M*. Beyond this concentration, cytotoxicity increased rapidly with IC_{10} values of 8 μ*M* being recorded in CCRF-CEM/WT cells and 6 μ*M* in CCRF-CEM/VLB cells. The cytotoxicity of VRP appeared at lower concentrations in CCRF-CEM/VLB cells (IC_{10}, 0.4 μ*M*), but the IC_{50} value was 20 μ*M* (data not shown). CCRF-CEM/VLB cells were highly resistant to VLB (526.3-fold resistance) and DOX (67.5-fold resistance) but remained sensitive to Ara-C, as expected. The addition of S 9788 at concentrations ranging from 0.5 to 4 μ*M* induced a dose-dependent reversal of DOX and VLB resistance in

Table 1 Modulation of DOX and VLB resistance achieved by continuous exposure to S 9788 or VRP in CCRF-CEM cells

Cytotoxic agent	Modulator (μ*M*)		IC_{50} (n*M*)		F. res[a]	F. rev[b] CEM/VLB cells
			CEM/WT cells	CEM/VLB cells		
Doxorubicin		0	65	4,000	61.5	–
	S 9788	0.5	55	220	4.0	18.2
		1.0	42	67	1.6	59.7
		2.0	50	50	1.0	80.0
		4.0	52	43	0.8	93.0
	VRP	0.4	70	2,800	40.0	1.4
		2.0	50	900	18.0	4.4
Vinblastine		0	3.8	2,000	526.3	–
	S 9788	0.5	4.0	38	9.5	52.6
		1.0	4.0	30	7.5	66.7
		2.0	4.0	6	1.5	333.3
		4.0	3.8	4.2	1.1	476.2
	VRP	0.4	3.8	1,300	342.1	1.5
		2.0	4.0	500	125.0	4.0
Cytarabine		0	7.2	5.2	0.7	–
	S 9788	2.0	17	10	0.6	0.5
	VRP	0.4	10	7	0.7	0.7

[a] *n*-Fold resistance = IC_{50} cytotoxic agent in resistant line/IC_{50} cytotoxic agent in sensitive line
[b] *n*-Fold reversal = IC_{50} cytotoxic agent alone/IC_{50} cytotoxic agent and modulator

CCRF-CEM/VLB cells. Reversal of DOX and VLB resistance was complete at S 9788 concentrations of 2 and 4 μ*M*, respectively. In comparison, VRP at 0.4 μ*M* had no effect on CCRF-CEM/VLB cells and exhibited only weak activity at 2 μ*M* (IC_{30}). At equitoxic concentrations, S 9788 was approximately 44 times more potent than VRP in the reversal of DOX and VLB resistance. S 9788 and VRP neither modified the cytotoxicity of Ara-C to sensitive and resistant cell lines nor influenced VLB and DOX cytotoxicity toward parental cells.

Table 2 Cytotoxic effect of DOX and VLB alone or in combination with S 9788 on BFU-E and CFU-GM bone marrow cells

Treatment	IC_{50} (n*M*)	
	BFU-E	CFU-GM
S 9788	9,400	13,000
DOX	13	30
DOX + S 9788 (2 μ*M*)	12	23
VLB	4	4.2
VLB + S 9788 (2 μ*M*)	3	3.2

Effect of S 9788 on DOX and VLB hematotoxicity

The effect of treatment with DOX or VLB in the presence or absence of S 9788 was evaluated in BFU-E and CFU-GM normal hematopoietic precursors. As shown in Table 2, S 9788 alone had a minimal effect on BFU-E and CFU-GM. DOX and VLB alone were very cytotoxic to both BFU-E and CFU-GM (IC_{50} values were comparable with those obtained in the malignant CCRF-CEM/WT cells). S 9788 at 2μ*M*, which corresponded to an active concentration in CCRF-CEM/VLB cells, did not increase the cytotoxicity of VLB or of DOX toward either BFU-E or CFU-GM.

Postincubation effect of S 9788 alone on MDR modulation

The effect of various postincubation times (6, 12, or 24 h) with S 9788 alone following a 1-h incubation with both S 9788 and the cytotoxic agent was evaluated in CCRF-CEM/VLB cells. The *n*-fold resistance values obtained for DOX alone varied from 11 to 24, depending

Table 3 Effect of various periods of postincubation with S 9788 alone at 50, 100, or 500 n*M* on the reversal of DOX resistance in CCRF-CEM/VLB cells

	Incubation for 1 h[a]	Postincubation without S 9788[b]			Postincubation with S 9788			R[e]
		IC_{50} (μ*M*)	F. res[c]	F. rev[d]	IC_{50} (μ*M*)	F. res[c]	F. rev[d]	
Post incubation 6 h:								
with S 97888 (50 n*M*)	DOX alone	17	14.2	–	–	–	–	–
	DOX + S 9788 (50 n*M*)	8	6.7	2.1	7.0	5.8	2.4	1.1
with S 9788 (100 n*M*)	DOX alone	17	14.2	–	–	–	–	–
	DOX + S 9788 (100 n*M*)	6	5.0	2.8	3.4	2.8	5.0	1.8
with S 9788 (500 n*M*)	DOX alone	22	24.0	–	–	–	–	–
	DOX + S 9788 (500 n*M*)	4	4.4	5.5	2.5	2.8	8.8	1.6
Post incubation 12 h:								
with S 9788 (50 n*M*)	DOX alone	15	12.5	–	–	–	–	–
	DOX + S 9788 (50 n*M*)	8	6.7	1.9	7.0	5.8	2.1	1.1
with S 9788 (100 n*M*)	DOX alone	20	16.7	–	–	–	–	–
	DOX + S 9788 (100 n*M*)	6.8	5.7	2.9	3.2	2.7	6.3	2.2
Postincubation 24 h:								
with S 9788 (50 n*M*)	DOX alone	20	18.2	–	–	–	–	–
	DOX + S 9788 (50 n*M*)	8	7.3	2.5	7.0	6.4	2.9	1.2
with S 9788 (100 n*M*)	DOX alone	21	19.1	–	–	–	–	–
	DOX + S 9788 (100 n*M*)	6.5	5.9	3.2	4.0	3.6	5.3	1.7
with S 9788 (500 n*M*)	DOX alone	10	11.1	–	–	–	–	–
	DOX + S 9788 (500 n*M*)	4	4.4	2.5	2.0	2.2	5.0	2.0

[a] DOX alone was also evaluated in CCRF-CEM/WT cells and IC_{50} values were 1.2 (6 and 12 h postincubation with culture medium) and 1.1 μ*M* (24 h postincubation)
[b] Culture medium was added instead of S 9788
[c] F.res = IC_{50} DOX in resistant line/IC_{50} DOX in sensitive line
[d] F.rev = IC_{50} DOX alone/IC_{50} (DOX + S 9788)
[e] R = F. rev with postincubation/F. rev without postincubation

on the experimental conditions. S 9788 at 50, 100, and 500 n*M* associated with DOX for 1 h (without postincubation) induced a partial reversal of resistance, with *n*-fold reversal values ranging from 2 to 5.5. Postincubation with 50 n*M* S 9788 did not improve this reversing activity, whatever the exposure times (6, 12, or 24 h), the *n*-fold reversal values remaining close to 2. Conversely, 100 and 500 n*M* S 9788 led to an increase in the *n*-fold reversal values (1.8- and 1.6-fold, respectively) as early as at 6 h of incubation. Longer postincubation phases did not increase this effect (Table 3).

The resistance values obtained for VLB alone varied from 600- to 1667-fold depending on the experimental conditions. S 9788 at 500 n*M* increased the cytotoxic activity of VLB (without post-incubation), with *n*-fold reversal values ranging from 1.5 to 2.9. Long postincubation phases (12 or 24 h) led to a major increase (4–41 times) in the reversal effect (Table 4). This effect was directly proportional to both the incubation duration and the concentration of S 9788 during this postincubation period. The maximal effect was obtained with the highest concentration of S 9788 (500 n*M*) and the longest postincubation period (24 h), with the reversal value being 119-fold.

Modulation of MDR by the sera of patients treated with S 9788

A total of 58 serum samples were collected, and the concentrations of S 9788 measured at 15 min, 30 min, 1 h, 2 h, or 24 h after the beginning of the infusion varied from 0.16 to 3 μ*M*. As shown in Fig. 1, DOX alone at 10 μ*M* (added in serum samples without S 9788) induced a weak inhibition of cellular growth. The percentages of surviving cells varied from 60% to 80%. Sera containing S 9788 alone had no effect on the

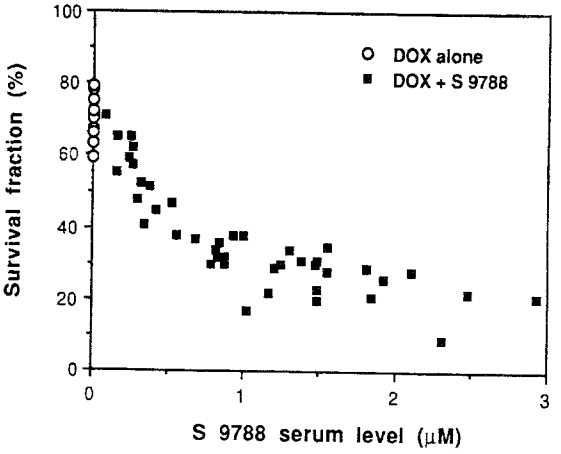

Fig. 1 Modulation of DOX resistance by the sera of patients treated by S 9788 infusion on CCRF-CEM/VLB cells. A total of 58 blood samples were collected from 8 patients before and at 15 min, 30 min, 1 h, 2 h, and 24 h after the beginning of the S 9788 infusion. After HPLC quantification of S 9788, CCRF-CEM/VLB cells were incubated for 3 h with each serum in the presence or absence of DOX at 10 μ*M*. Cytotoxicity was evaluated by the hexosaminidase assay after 4 days of culture. The percentages of surviving cells were calculated as follows:

$$\frac{\text{OD values with cells incubated in sera containing DOX} \pm \text{S 9788}}{\text{OD values with cells incubated in sera collected before the S 9788 infusion, without addition of DOX}} \times 100$$

Table 4 Effect of various periods of postincubation and concentrations of S 9788 alone on the reversal of VLB resistance in CCRF-CEM/VLB cells

	Incubation for 1 h[a]	Postincubation without S 9788[b]			Postincubation with S 9788			R[e]
		IC_{50} (μ*M*)	F. res[c]	F. rev[d]	IC_{50} (μ*M*)	F. res[c]	F. rev[d]	
Post incubation 12 h:								
with S 9788 (125 n*M*)	VLB alone	80	800	–	–	–	–	–
	VLB + S 9788 (500 n*M*)	54	540	1.5	14	140	5.7	3.8
with S 9788 (250 n*M*)	VLB alone	100	1,000	–	–	–	–	–
	VLB + S 9788 (500 n*M*)	80	800	1.3	8	80	12.5	9.6
with S 9788 (500 n*M*)	VLB alone	60	600	–	–	–	–	–
	VLB + S 9788 (500 n*M*)	40	400	1.5	2	20	30.0	20.0
Postincubation 24 h:								
with S 9788 (125 n*M*)	VLB alone	50	1,667	–	–	–	–	–
	VLB + S 9788 (500 n*M*)	17	567	2.9	2	67	25.0	8.6
with S 9788 (250 n*M*)	VLB alone	50	1,667	–	–	–	–	–
	VLB + S 9788 (500 n*M*)	17	567	2.9	0.7	23	71.4	24.6
with S 9788 (500 n*M*)	VLB alone	50	1,667	–	–	–	–	–
	VLB + S 9788 (500 n*M*)	17	567	2.9	0.4	14	119.0	41.0

[a] VLB alone was also evaluated in CCRF-CEM/WT cells and IC_{50} values were 0.1 (12 h postincubation with culture medium) and 0.03 μ*M* (24 h postincubation)
[b] Culture medium was added instead of S 9788
[c] F.res = IC_{50} VLB in resistant line/IC_{50} VLB in sensitive line
[d] F.rev = IC_{50} VLB alone/IC_{50} (VLB + S 9788)
[e] R = F. rev with postincubation/F. rev without postincubation

Table 5 Modulation of DOX resistance by S 9788 added in vitro in human serum on CCRF-CEM/VLB cells

S 9788 (μ*M*)	% Surviving cells	
	Experiment 1	Experiment 2
0	72	67
0.125	66	55
0.250	51	47
0.500	34	40
1	25	29
2	20	21
4	12	16

survival of CCRF-CEM/VLB cells, with the percentages of surviving cells always being higher than 85% (data not shown).

The cytotoxic activity of DOX increased with the concentration of S 9788 used, and the percentage of growth inhibition reached 80% at the highest concentrations tested (between 1.75 and 3 μ*M*). Sera of patients treated with S 9788 were capable of reversing the MDR of CCRF-CEM/VLB cells in a dose-dependent manner (Fig. 1). As shown in Table 5, S 9788 added in vitro at equivalent concentrations in human serum (from 0.125 to 4 μ*M*) induced the same reversing effect in the presence of 10 μ*M* DOX as in the sera of treated patients. The percentages of surviving cells were comparable.

Discussion

Numerous data that have emerged from studies on the clinical expression of P-glycoprotein (Pgp) and its relevance to drug resistance suggest that chemosensitizers could play an important role in cancer chemotherapy. Promising results have recently been obtained using VRP or cyclosporin A in combination with standard drugs for the treatment of refractory lymphoma, acute lymphoblastic leukemia, and multiple myeloma [12, 32, 40]. However, the occurrence of toxic side effects due to either the pharmacological properties of the modulator or to the potentiation of the antitumor drug's cytotoxicity to normal tissues have limited their clinical use.

In the current study, we demonstrated that the new MDR modulator S 9788 exhibited a potent in vitro reversal activity in human T-leukemic CCRF-CEM/VLB cells expressing the MDR phenotype. S 9788 enhanced in a dose-dependent manner the cytotoxic activity of DOX or VLB in CCRF-CEM/VLB cells, with complete reversal of resistance occurring at a concentration of 2 μ*M* when concomitant continuous exposure (96 h) was used. This compound was 44 times more effective than VRP at equitoxic concentrations. Similar results have been described for S 9788 used in combination with cytotoxic drugs in different murine and human MDR cell lines by Pierré et al. [35]. In CCRF-CEM/VLB cells 6,000-fold resistant to VLB, Oum'Hamed et al. [33] showed that 5 μ*M* VRP was 1,000 times less active than 5 μ*M* S 9788 for a 48-h exposure. On the other hand, Hill et al. found that the resistance modulation achieved by VRP and by S 9788 was comparable at equitoxic concentrations (equivalent to the IC_{10} value) in CCRF-CEM/VLB 1000 cells 200-fold resistant to VLB [22, 23]. These results are not directly comparable with our findings due to various differences in both the cell-line characteristics (i.e., origin, level of resistance to VLB and of sensitivity to VRP) and the protocols (incubation time and cytotoxicity tests). The activity of S 9788 was specific for the MDR phenotype since it did not potentiate the cytotoxic effect of Ara-C and had no effect on the parental CCRF-CEM/WT cells.

Hematological toxicity is one of the most important side effects of DOX and VLB in humans. Therefore, we evaluated in vitro the influence of S 9788 on the hematotoxicity of these cytotoxic agents. S 9788 at 2 μ*M*, an active concentration against CCRF-CEM/VLB cells, did not enhance the cytotoxicity of DOX and VLB toward the human bone marrow erythroid (BFU-E) and myeloid (CFU-GM) progenitors. Expression of the *mdr1* gene has been described to be low or negative in normal bone marrow cells [12, 17]. However, recent publications have shown that normal hematopoietic stem cells (CD34 antigen-positive or negative) significantly express the MDR phenotype [9, 21, 31], suggesting a possible function of Pgp in stem-cell protection. Chao et al. [8] described an increase in etoposide cytotoxicity induced in vitro by VRP or cyclosporine in CFU-GM cells.

The importance of exposure sequence and duration on the synergistic action of modulator and cytotoxic agent has previously been demonstrated by Cass et al. [6]. The long coincubation periods usually studied (greater than 48 h) are not relevant to the clinical situation. Therefore, we evaluated the effect of a short duration of exposure (1 h) to both the cytotoxic agent (DOX or VLB) and S 9788 followed by a period of incubation with low concentrations of S 9788 alone for various exposure times (6–24 h). Our results showed that the effect of exposure duration and sequence closely depended on the cytotoxic agent used. After a short-term exposure period (1 h) in the absence of S 9788, CCRF-CEM/VLB cells were about 16- and 1230-fold resistant to DOX and VLB, respectively, relative to the corresponding drug-sensitive cells. However, when resistance was assessed during a long-term period of exposure (96 h), CCRF-CEM/VLB cells were 61.5- and 526-fold resistant to DOX and VLB, respectively. Thus, in contrast to DOX, the degree of resistance to VLB was greater for a short period of exposure than for a long one.

S 9788 was applied concomitantly with the cytotoxic agent for a 1 h period at concentrations that induce

a partial reversal of MDR: 50, 100, and 500 n*M* for DOX (2- to 3-fold reversal) and 500 n*M* for VLB (2-fold reversal). Lower concentrations of S 9788 were used with DOX since CCRF-CEM/VLB cells are clearly less resistant to DOX than to VLB. Post-incubation with low concentration of S 9788 alone after a 1-h coadministration of VLB and S 9788 increased VLB cytotoxicity in CCRF-CEM/VLB cells. This effect was proportional both to the duration of the postincubation period and to the concentration of S 9788. The maximal increase in reversal effect (41 times) was observed after a 24-h postincubation period with 500 n*M* S 9788. Pérez et al. [34] showed that 24 h of postincubation with S 9788 alone at a 10-times higher concentration (5 μ*M*) markedly increased the reversal of vincristine resistance in S1/tMDR cells as compared with a single 4-h period of coincubation. Similar results were obtained by Cass et al. [6] using VRP combined with vincristine in CCRF-CEM/VLB cells; they showed that maximal efficacy could be achieved when VRP was applied both during and after the cytotoxic agent.

On the other hand, postincubation with S 9788 alone weakly improved DOX cytotoxicity with a maximal 2-fold increase in the reversal effect. Prolongation of exposure from 6 to 24 h did not modify the reversal ratio, in contrast to what was observed with VLB. Léonce et al. [30] showed that after a short exposure of KB-A_1 cells to both DOX and S 9788, the administration of S 9788 alone (in DOX-free medium) increased the intracellular retention of DOX. Pérez et al. [34] demonstrated the same phenomenon in S1/tMDR-transfected cells treated with vincristine and S 9788. This increase in retention of the cytotoxic agent could explain the improved reversal effect obtained with a period of postincubation with S 9788 alone.

The differences observed between the two cytotoxic agents could be explained by (a) the CCRF-CEM/VLB cells used, which appeared much more resistant to VLB than to DOX: (b) a better retention of VLB as compared with DOX in the presence of S 9788 in the CCRF-CEM/VLB cells: or (c) the differing mechanisms of actions of these cytotoxic drugs—VLB is active during the shortest G_2M phase of the cell cycle, whereas DOX is active during the longer S phase. Julia et al. [25] showed in the MCF_7/DOX cell line which is highly resistant to DOX (950-fold resistance), that postincubation with S 9788 (1 μ*M*) alone increased the reversal of DOX resistance by a factor ranging from 5 (for a 6-h exposure period) to 9 (for a 24-h period of exposure). Thus, the improvement of reversal activity by post-incubation with S 9788 alone varies with the cytotoxic agent and the level of resistance of the cell line.

Pharmacokinetic analysis during phase I clinical trials showed that plasma concentrations of greater than 2 μ*M* could be reached at nontoxic doses. Linearity between the delivered dose and the area under the concentration versus time curve was also demonstrated [20]. Despite a strong degree of binding to plasma proteins (higher than 98.5% in human plasma in vitro: unpublished data), good correlation was observed between the S 9788 serum levels and the ability to reverse MDR in CCRF-CEM/VLB cells. At S 9788 concentrations ranging from 0.16 to 3 μ*M*, the cytotoxicity of DOX varied from 35% to 80%. The reversal effect of S 9788 in patients' sera was dependent on the concentration of the drug and was effective beginning at a plasma concentration of 0.25 μ*M*. Sera collected 2 h after the beginning of the infusion were inactive (S 9788 concentrations inferior to 0.25 μ*M*). The maximal effect was observed at concentrations higher than 1.75 μ*M*, which were obtained in sera collected at 15–60 min after the beginning of the 30-min infusion at the highest doses of S 9788 tested (96 and 104 mg/m^2, which are the maximal tolerated doses).

In conclusion, these results suggest that reduced doses of S 9788 given as a prolonged intravenous infusion could improve its reversal effect. Moreover, the maximal reversal effect of S 9788 in sera of treated patients was generally obtained at the end of (or immediately after) the infusion. These data form a basis for the design of phase I–II trials using a combination of either a loading dose of S 9788 given over 30 min before VLB or DOX administration followed by a maintenance infusion of S 9788 alone given from 2 (for DOX) to 24 h (for VLB) or a continuous infusion given over 6 h.

Acknowledgements The authors thank Marie-José Calatayud for secretarial assistance, Marie-Gwenaëlle Poullain for critical reading of the manuscript, and T.A. Dunn for participation in the setting of the project.

References

1. Bakes DM, Turner ND, Gordon BM, Hiley MP, Walther B, Lucas C (1993) Method for the analysis of S 9788, a drug to reverse resistance to anticancer agents, in animal plasma and human plasma and serum by high-performance liquid chromatography with ultraviolet detection. J Chromatogr 615:117–126
2. Beck WT, Mueller TJ, Tanzer LR (1979) Altered surface membrane glycoproteins in vinca alkaloid-resistant human leukemia lymphoblasts. Cancer Res 39:2070–2076
3. Bradley G, Juranka PF, Ling V (1988) Mechanism of multidrug resistance. Biochim Biophys Acta 948:87–128
4. Broxterman HJ, Kuiper CM, Schuurhuis GJ, Vander Hoeven JJM, Pinedo HM, Lankelma J (1987) Daunomycin accumulation in resistant tumor cells as a screening model for resistance modifying drugs: role in protein binding. Cancer Lett 35:87–95
5. Campos L, Guyotat, D. Archimbaud E, Calmard OP, Tsuruo T, Troncy J, Treille D, Fiere D (1992) Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. Blood 79:473–476
6. Cass CE, Janoska-Wieczarek A, Lynch MA, Sheinin H, Hindenburg AA, Beck WT (1989) Effect of duration of exposure to verapamil on vincristine activity against multidrug resistant human leukemia cell lines. Cancer Res 49:5738–5804

7. Chan HS, Haddad G, Thorner PS, De Boer G, Lin YP, Ondrusek N, Yeger H, Ling V (1991) P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. N Engl J Med 325:1608–1614
8. Chao NJ, Aihara M, Blume KG, Sikic BI (1990) Modulation of etoposide cytotoxicity by verapamil or cyclosporin in multidrug-resistant human leukemic cell lines and normal bone marrow. Exp Hematol 18:1193–1198
9. Chaudhary PM, Roninson IB (1991) Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell 66:85–94
10. Coulombel L, Eaves AC, Eaves CJ (1983) Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hematopoietic progenitors in the adherent layer. Blood 62:291–297
11. Cros S, Guilbaud N, Berlion M, Dunn TA, Régnier G, Dhainaut A, Atassi G, Bizzari JP (1992) In vivo evidence of complete circumvention of vincristine resistance by a new triazinoaminopiperidine derivative, S 9788, in P388/VCR leukemia model. Cancer Chemother Pharmacol 30:491–494
12. Dalton WS, Grogan TM, Meltzer PS, Sheper RJ, Durie BGM, Taylor CW, Miller TP, Salmon SE (1988) Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J Clin Oncol 7:415–424
13. Dalton WS, Grogan TM, Rybski JA, Scheper RJ, Richter L, Kailey J, Broxterman HJ, Pinedo HM, Salmon SE (1989) Immunohistochemical detection and quantitation of P-glycoprotein in multiple drug-resistant human myeloma cells: association with level of drug resistance and drug accumulation. Blood 73:747–752
14. Demmer A, Dunn TA, Hoof T, Kubesh P, Tümmler B (1992) Study of the ability of S 9788 to competitively inhibit the photoaffinity labelling of P-glycoprotein by a radioiodinated photoaffinity of daunomycin. Clin Exp Metastasis 10 [Suppl 1]:106
15. Dhainaut A, Régnier G, Atassi G, Pierré A, Léonce S, Kraus-Berthier L, Prost JF (1992) New triazine derivatives as potent modulators of multidrug resistance. J Med Chem 35:2481–2496
16. Efferth T, Dunn TA, Berlion M, Langenbahn H, Pommerenke EW, Volm M (1993) Reversal of inherent multidrug resistance in primary human renal cell carcinoma cell cultures by S 9788. Anticancer Res 13:905–908
17. Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman HM, Pastan I (1987) Expression of a multidrug resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 84:265–269
18. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE (1965) Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 18:522–529
19. Ford JM, Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev 42:155–199
20. Gordon B, Briggs M, Khayat D, Clavel M, Foy M, Ardiet C, Bastian G, Benhammouda A, Lucas C (1992) Pharmacokinetically based phase I trials of S 9788 given alone and in combination with vincristine or adriamycin. Ann Oncol 3 [Suppl 5]:51
21. Hegewisch-Becker S, Fliegner M, Tsuruo T, Zander A, Zeller W, Hossfeld DK (1993) P-glycoprotein expression in normal and reactive bone marrows. Br J Cancer 67:430–435
22. Hill BT, Hosking LK (1994) Differential effectiveness of a range of novel drug-resistance modulators, relative to verapamil, in influencing vinblastine or teniposide cytotoxicity in human lymphoblastoid CCRF-CEM sublines expressing classic or atypical multidrug resistance. Cancer Chemother Pharmacol 33:317–324
23. Hill BT, Van Der Graaf WTA, Hosking LK, De Vries EGE, Mulder NH, Whelan DH (1993) Evaluation of S 9788 as a potential modulator of drug resistance against human tumor sublines expressing differing resistance mechanisms in vitro. Int J Cancer 55:330–337
24. Huet S, Chapey C, Robert J (1993) Reversal of multidrug resistance by a new lipophilic cationic molecule, S 9788. Comparison with 11 other MDR-modulating agents in a model of doxorubicin-resistant rat glioblastoma cells. Eur J Cancer 29A:1377–1383
25. Julia AM, Roche H, Berlion M, Lucas C, Milano G, Robert J, Bizzari JP, Canal P (1994) Multidrug resistance circumvention by a new triazinoaminopiperidine derivative S 9788 in vitro: definition of the optimal schedule and comparison with verapamil. Br J Cancer 69:868–874
26. Kartner N, Riordan JR, Ling V (1983) Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221:1285–1288
27. Khayat D, Weil M, Benhamouda A, Villemin E, Bastian G, Antoine E, Rixe O, Auclerc G, Lucas C, Sarkany M, Bizzari JP (1993) Phase I clinical study of a new multidrug resistance modulating agent, S 9788, in combination with vincristine. Proc Am Assoc Cancer Res 34:230
28. Landegren U (1984) Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. J Immunol Methods 67:379–388
29. Lehnert M, Emerson S, Dalton WS, Salmon SE (1991) Identification of potentially useful chemosensitizers to reverse multidrug resistance (MDR1). Eur J Cancer 27 [Suppl 2]:5210
30. Léonce S, Pierré A, Anstett M, Pérez V, Genton A, Bizzari JP, Atassi G (1992) Effects of a new triazinoaminopiperidine derivative on adriamycin accumulation and retention in cells displaying P-glycoprotein mediated multidrug resistance. Biochem Pharmacol 44:1707–1715
31. Marie JP, Brophy NA, Ehsan MN, Aihara Y, Mohamed NA, Cornbleet J, Chao NJ, Sikic BI (1992) Expression of multidrug resistance gene *mdr*1 mRNA in a subset of normal bone marrow cells. Br J Haematol 81:145–152
32. Miller TP, Grogan TM, Dalton WS, Spier CM, Scheper RJ, Salmon SE (1991) P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high dose verapamil. J Clin Oncol 9:17–24
33. Oum'hamed Z, Berlion M, Millot-Broglio C, Dufer J, Joly P, Bizzari JP, Desplaces A (1994) Quantitative cytological study of the activity of a new resistance modulator, S 9788, on human leukemic cells using multiparametric image analysis. Bull Cancer 81:203–211
34. Pérez V, Pierré A, Léonce S, Anstett M, Atassi G (1993) Effect of duration of exposure to S 9788, cyclosporin A or verapamil on sensitivity of multidrug resistant cells to vincristine or doxorubicin. Anticancer Res 13:985–990
35. Pierré A, Dunn TA, Kraus-Berthier L, Léonce S, Saint-Dizier D, Régnier G, Dhainaut A, Berlion M, Bizzari JP, Atassi G (1992) In vitro and in vivo circumvention of multidrug resistance by Servier 9788, a novel triazinoaminopiperidine derivative. Invest New Drugs 10:137–148
36. Poupon MF, Berlion M, Atassi G, Dunn TA, Bizzari JP (1992) S 9788, a new resistance modulator, enhances the antitumoral activity of vepeside/cis-platinum treatment on a P-glycoprotein positive small cell lung cancer xenograft. Proc Am Assoc Cancer Res 33:468
37. Raderer M, Scheithauer W (1993) Clinical trials of agents that reverse multidrug resistance—a literature review. Cancer 72:3553–3563
38. Salmon SE, Grogan TM, Miller T, Scheper R, Dalton WS (1989) Prediction of doxorubicin resistance in vitro in myeloma, lymphoma, and breast cancer by P-glycoprotein staining. J Natl Cancer Inst 81:696–701
39. Sato H, Preisler H, Day R, Raza A, Larson R, Browman G, Goldberg J, Vogler R, Grunwald H, Gottlieb A, Bennett J, Gottesman M, Pastan I (1990) MDR1 transcript levels as an indication of resistant disease in acute myelogenous leukaemia. Br J Haematol 75:340–345

40. Sonneveld P, Durie BG, Lokhorst HM, Marie JP, Solbu G, Suciu S, Zittoun R, Lowenberg B, Nooter K (1992) Modulation of multidrug resistant multiple myeloma by cyclosporin. The leukemia group of the EORTC and the HOVON. Lancet 340:255–259

41. Tsuruo T, Kawabata H, Nagumo N, Iida H, Kitatami Y, Tsukagoshi S, Sagurai Y (1985) Potentiation of antitumor agents by calcium channel blockers with special reference to cross-resistance patterns. Cancer Chemother Pharmacol 15:16–19