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Reversal of Multidrug Resistance by a New Lipophilic Cationic Molecule, S9788. Comparison with 11 other MDR-modulating Agents in a Model of Doxorubicin-resistant Rat Glioblastoma Cells

Sylvie Huet, Christiane Chapey and Jacques Robert

We have compared the properties of the novel multidrug resistance modulator, S9788, to a panel of 11 well-known modulators in a model of rat glioblastoma cells resistant to doxorubicin and displaying a P-glycoprotein-mediated multidrug-resistance phenotype complemented by a mechanism of intracellular drug tolerance not yet identified (*Br J Cancer* 1992, 65, 538–544). S9788, like most modulators, was able to completely restore drug accumulation in the resistant line to the level obtained in the sensitive cells. This was obtained with 10 μmol/l of modulator, which is slightly higher than required for cyclosporine A (3 μmol/l) verapamil and nicardipine (6 μmol/l), but lower than for amiodarone, trifluoperazine and dipyridamole (20 μmol/l), tamoxifen and diltiazem (40 μmol/l), quinine, quinidine and nifedipine (> 100 μmol/l). Complete restoration of drug cytotoxicity was, however, obtained only with amiodarone, and a residual resistance factor of 4 could not be overcome by cyclosporine A or S9788, while other modulators gave residual resistance factors of 5–20 (trifluoperazine, tamoxifen, verapamil, quinine, nicardipine, dipyridamole) or even higher (diltiazem, quinidine, nifedipine). When studying doxorubicin accumulation obtained for an exposure to the IC_{50} of this drug, it appeared that some modulators were able to decrease this "intracellular IC_{50}" independently of their efficiency in resistance reversal (cyclosporine A, S9788, amiodarone, trifluoperazine, quinine, tamoxifen), thus reversing intracellular drug tolerance, whereas other modulators could not reduce this parameter (verapamil, nicardipine, dipyridamole, diltiazem, quinidine). It is suggested that drugs of the first group could be able to segregate doxorubicin in subcellular compartments from which it could not reach its nuclear targets.
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INTRODUCTION

SINCE THE original discovery by Tsuruo *et al.* [1] that verapamil and other calcium channel blockers were able to reverse multidrug resistance (MDR), a great variety of compounds have been shown to possess this property (see for review [2]). Some of them are effectively calcium inhibitors or calmodulin antagonists belonging to several chemical families (verapamil, dihydropyridines, phenothiazines) and it was thought that the mechanism of MDR was closely related to Ca^{2+} availability. The direct role of calcium in the process of multidrug resistance reversal was, however, excluded [3, 4]. It was shown that the compounds able to reverse MDR shared some physico-chemical properties, especially the fact that they were lipophilic cations [5, 6]. The structural requirements are not very strict, and it can be estimated that about 1% of organic compounds display this property (Lavelle, personal communication).

It has been shown that several of these agents were able to compete with the "natural substrates" of P-glycoprotein and were inhibiting its photoaffinity labelling by azidopine or vinblastine analogues [7, 8]. This is especially the case for verapamil, and it has been thought that all MDR reversing agents directly interfere with P-glycoprotein [6]. The restoration of intracellular drug accumulation to the level obtained in sensitive cells has been observed for most modulators and is associated with a restoration of cytotoxicity [9, 10]. However, it is not clear at the present time if all the agents able to reverse MDR can act by the same mechanism. Using photoaffinity labelling with azidopine, and its deplacement with various compounds, it has been shown that several independent sites may exist on P-glycoprotein and that other proteins could be involved [11]. Other evidence for the existence of several mechanisms of multidrug resistance reversal is given by the synergy which is sometimes observed when two reversing agents are used simultaneously: verapamil and cyclosporine A [12], verapamil and dipyridamole [13].

Clinical trials on myelomas and B-lymphomas associating cytotoxic drugs and verapamil have shown a possible chemosensitisation of the tumoral cells [14]; this is, however, at the expense of an important cardiac toxicity mainly due to the vasoactive properties of the drug. There is at the present time a challenge toward the identification of MDR reversing agents devoid of pharmacological effects other than their action on P-glycoprotein, in order to avoid any supplementary toxicity. There have been no clear leads in the resolution of this problem, which is mainly a mass screening of hundreds of molecules. The problem is that the models are quite variable, as well as the techniques of exploring the MDR reversing effect. It is conse-

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quently difficult to compare the results obtained in the numerous studies of the literature, the differences noticed being due either to differences in the properties of the molecules tested, or to differences in the methodology used. In the course of the preclinical development of a new MDR modulating agent, S9788, a triazineaminopiperidine derivative, originating from the Institut de Recherche International Servier [15], we have compared its reversing properties to those of 11 other compounds already known to reverse MDR and belonging to various classes of MDR modulators. This study was performed on a single model of sensitive and multidrug resistant cells that we had already studied from different points of view [16, 17]. The C6 0.5 line, which is 400-fold resistant to doxorubicin, obviously displays a multifactorial resistance, associating a classical MDR phenotype and a non-MDR phenotype which is presently under investigation.

MATERIALS AND METHODS

Drugs and products

Doxorubicin was a generous gift from Laboratoire Roger Bellon. Verapamil and amiodarone were clinical formulation [respectively, Isoptine® (Laboratoires Biosedra, Malakoff, France) and Cordarone® (Laboratoires Sanofi, Paris, France)]; tamoxifen, nifedipine, nicardipine, trifluoperazine, quinine, quinidine and dipyridamole were purchased from Sigma (Missouri, U.S.A.); cyclosporine A was obtained from Sandoz (Rueil Malmaison, France), and diltiazem from Synthelabo (Paris, France). S9788 (6-{4-[2,2-di(4-fluorophenyl)ethylamino] piperidin 1-yl}*N*,*N*'-dipropen-2-yl 1,3,5-triazine 2,4-diamine, bimethanesulphonate), structural formula Fig. 1, was obtained from Laboratoires Servier (Courbevoie, France). The following drugs had to be first dissolved in absolute ethanol for the preparation of stock solutions at 10 mmol/l: cyclosporine A, dipyridamole, tamoxifen, nifedipin, nicardipin, and trifluoperazine. We have verified that the amount of ethanol thus introduced during cell incubations had no effect on cell growth and doxorubicin cytotoxicity.

Cell culture

The C6 rat glioblastoma clone [18] and its doxorubicin-resistant counterpart C6 0.5 [19] were routinely cultivated in Petri dishes (Nunc) with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Seromed) and antibiotic mixture, at 37°C, in a humidified atmosphere containing 5% CO_2. The cultures were replicated each week and the medium was changed each 2 or 3 days, depending on cell density. A selection pressure of 0.5 μg/ml of doxorubicin was constantly maintained in the routine culture medium of the C6 0.5 cells, but was omitted 1 week before any experiment.

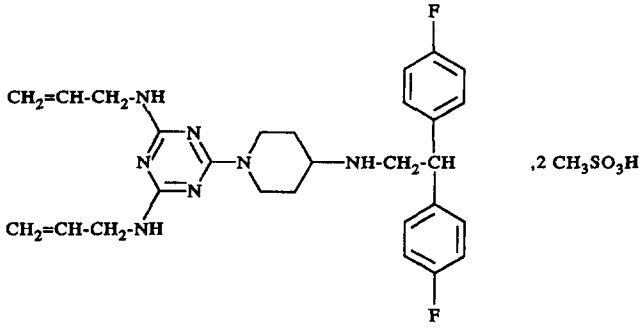

Fig. 1. Structural formula of S9788.

Doxorubicin accumulation

8×10^4 C6 cells were seeded in 10 cm^2 Petri dishes with 3 ml medium or 50×10^4 C6 0.5 cells were seeded in 20 cm^2 Petri dishes with 5 ml of medium without drug. The effect of the various MDR reversing agents was then tested on doxorubicin accumulation at several concentrations of the modulator between 0.1 and 100 μmol/l. The concentration of doxorubicin was set at 17.2 μmol/l in a first series of experiments, then it was equalled to the IC_{50} of the drug in the presence of various concentrations of the modulator. All incubations were performed for 2 h in the presence of both doxorubicin and the modulator. At the end of this incubation, the monolayers were washed twice with 0.15 mol/l NaCl, harvested after gentle stirring, and pelleted at 3000 rpm for 5 min. This was done rapidly in order to avoid any significant drug efflux during these steps. Water (0.5 ml) and 40% trichloroacetic acid (0.5 ml) were successively added and the sample were kept at 4°C overnight, then centrifuged for 30 min at 3000 rpm. The acid-soluble fraction was used to evaluate the intracellular concentrations of non-covalently bound drug by fluorometry with a Kontron spectrofluorometer, model SFM 25.

The acid-insoluble pellet was solubilised with 1 mol/l NaOH and used to evaluate the protein content [21]. All incubations were performed in triplicate and at least two independent experiments were performed for each drug.

Doxorubicin cytotoxicity

A colorimetric assay using the tetrazolium salt MTT [22] was used to assess cytotoxicity of a 2-h exposure to doxorubicin in the presence or absence of the various multidrug resistance modulators. 5×10^2 C6 cells or 20×10^2 C6 0.5 cells were plated in 96-well plates (Nunc) in a volume of 200 μl of culture medium. After 24 h for C6 cells or 48 h for C6 0.5 cells, the cells were exposed for 2 h at the appropriate concentrations of doxorubicin and the modulator. Culture medium was then removed, the cell layers were rinsed twice; fresh tissue culture medium was added, and the cells allowed to grow for a further 4 days (C6 cells) or 5 days (C6 0.5 cells). These conditions have been established, after a careful study of the growth curves of the cells, in order to keep the cells in the exponential phase of growth over the 5 or 7 days of culture. At the end of the incubation, 300 μl of MTT-containing medium were added and maintained for 4 h; after elimination of the medium, 200 μl of dimethylsulphoxide were added and the plate shaken for 5 min. Absorbance was then immediately measured on a two-wavelength microplate photometer set at 570 and 630 nm, respectively. Triplicate determinations were always performed, and the results presented are the means of at least three experiments. Cytotoxicity was expressed as IC_{50}, i.e. the concentration of doxorubicin causing 50% decrease of absorbance as compared to controls incubated simultaneously. Additional blank controls without cells were substracted from the absorbance values. It was possible to study the cytotoxicity of the modulator itself by comparing the cell survivals obtained with the various concentrations of the modulator in absence of doxorubicin. The results presented below have been restricted to the non-cytotoxic concentrations of the modulators.

RESULTS

The C6 0.5 line has been already characterised in our laboratory [16, 17, 19]. The resistance factor to doxorubicin is around 400 (IC_{50} = 62 vs. 0.155 μmol/l in the sensitive line). Doxorubicin accumulation for a 2-h exposure at 17.2 μmol/l is reduced

by a factor of 8 in this resistant line (0.205 nmol/10^6cells vs. 2.95 in the sensitive line). This line is, however, able to accumulate nearly 20 times more doxorubicin than the sensitive line when exposed at doses giving the same cytotoxicity (intracellular IC_{50}: 0.495 nmol/10^6cells vs. 0.029 in the sensitive line).

Several compounds tested in this study presented some cytotoxicity at the concentration used for multidrug resistance modulation. This is the case for nicardipine (IC_{50} = 26 and 23 μmol/l for C6 and C6 0.5 cells, respectively); for trifluoperazine (IC_{50} = 18 and 31 μmol/l); for tamoxifen (IC_{50} = 19 and > 30 μmol/l); and for S9788 (IC_{50} = 26 and 50 μmol/l). All other compounds were not cytotoxic themselves at the highest concentration tested (generally 50 or 100 μmol/l). There was no collateral sensitivity of the resistant C6 0.5 line to verapamil or other modulators; there was even rather a slight cross-resistance of this line to the cytotoxic effect of S9788, tamoxifen or trifluoperazine, as evident from the IC_{50} indicated above.

The effect of the modulators on doxorubicin accumulation after a 2-h exposure at the dose of 17.2 μmol/l is presented Fig. 2 on a series of charts. The resistant cells accumulated 10–12-fold less doxorubicin than the sensitive cells in these conditions. Only some compounds (cyclosporine A, S9788 and quinine) had a significant effect on doxorubicin accumulation in the sensitive line; most of them were able to restore, in the resistant cells, the intracellular doxorubicin concentration measured in the sensitive cells, but with very different efficiencies. Complete restoration occurred with 2 μmol/l of cyclosporine A, 6 μmol/l of verapamil and nicardipine, 10 μmol/l of S9788, 20 μmol/l for amiodarone, trifluoperazine and dipyridamole, 40 μmol/l of diltiazem and tamoxifen; complete restoration did not occur at the tested concentrations (up to 100 μmol/l) for nifedipine, quinine and quinidine.

The effect of the multidrug resistance modulators on doxorubicin cytotoxiciy is presented in Fig. 3 on a series of charts. For each compound, we have plotted the doxorubicin IC_{50} of the C6 0.5 line as a function of the concentration of modulator. Control values were obtained by growth without doxorubicin, but in the presence of the modulator, so as to eliminate the cytotoxic effect of the modulator. We have calculated the ratio of the IC_{50} obtained with modulator in the C6 0.5 line to the IC_{50} of the sensitive C6 line; this "residual resistance factor" is presented in Table 1 for each modulator at all the concentrations tested. Only one compound, amiodarone, was able to completely reverse the doxorubicin IC_{50} value of the resistant C6 0.5 line to that observed in the sensitive line. This occurred at a concentration of 20 μmol/l, but a residual resistance factor of only 4 was observed at 3 μmol/l. None of the other compounds were able to completely reverse doxorubicin resistance. For cyclosporine A and S9788, a residual resistance factor of 4 could not be overcome, even at high concentrations, but a residual resistance factor of only 6.5 was observed at 1 μmol/l for cyclosporine. The compounds which completely restored doxorubicin accumulation did not completely reverse resistance to the drug, and a residual resistance factor of 10–20 was never overcome for verapamil, nicardipine, dipyridamole, diltiazem, trifluoperazine and tamoxifen. When considering the dose–effect relationship, it appeared that this maximal effect was obtained at lower doses for verapamil and nicardipine (around 1–3 μmol/l) than for trifluoperazine and tamoxifen (around 10–30 μmol/l) and was reached only at higher dose (30–50 μmol/l) for dipyridamole and diltiazem. The three compounds not able to completely restore drug incorporation in the resistant line were, however, able to reverse doxorubicin resistance in the same proportions as others (about 20-fold), and only at high concentrations (40–60 μmol/l). From the curves presented in Figs 2 and 3, it is possible to evaluate by interpolation the concentration of modulator required for a 50% reduction of doxorubicin IC_{50}, and to compare it to the doxorubicin incorporation obtained at this concentration of modulator with 17. 2 μmol/l of doxorubicin. It appears that for some modulators, the 50% reduction of IC_{50} occurred without modification of doxorubicin incorporation; these modulators were amiodarone, cyclosporine A, trifluoperazine, quinine, tamoxifen and quinidine. In contrast, the 50% reduction of doxorubicin IC_{50} occurred with modification of doxorubicin incorporation for verapamil, S9788, nicardipine, dipyridamole, diltiazem and nifedipine.

We have also measured doxorubicin accumulation in C6 0.5 cells for exposures corresponding to the IC_{50} values obtained at each concentration of the modulator ('intracellular IC_{50}', Fig. 3). Amiodarone was the only modulator able to decrease efficiently this parameter low enough to reach the values observed in sensitive C6 cells. Some compounds were able to decrease this intracellular IC_{50} in important proportions: cyclosporine A, S9788, trifluoperazine, tamoxifen, quinine and nifedipine. In marked contrast, verapamil and nicardipine, although very efficient in drug resistance reversal, were unable to decrease significantly the amount of drug incorporated in C6 0.5 cells for an IC_{50} exposure; this was also the case for dipyridamole, diltiazem and quinidine.

DISCUSSION

We have studied in this paper several aspects of multidrug resistance reversal: the reversal of the IC_{50} of the drug; the reversal of drug accumulation to its level in sensitive cells and the reversal of the intracellular IC_{50} (i.e. the intracellular drug concentration providing a 50% growth inhibition). We have shown that the various drugs tested on this model differ qualitatively and quantitatively in their ability to reverse MDR and that the phenomena of reversal of resistance and restoration of drug accumulation are dissociated. This may have consequences in the choice of the best candidate for clinical studies and provides guidelines for the screening of MDR reversing agents. We have especially tested a new compound S9788, a triazine derivative which does not belong to a family already known to reverse MDR, and which has been proven to be active *in vitro* and *in vivo* [15, 23, 24].

The reversal of resistance itself does not appear to directly depend on the level of accumulation, in contrast to the observations of other authors working on other cell systems [9, 10]. First, the reversal is complete in only one case (amiodarone); all the other compounds can restore drug accumulation but never allow the resistant line to display an IC_{50} of doxorubicin as low as the sensitive line. This clearly indicates that drug extrusion cannot explain the doxorubicin resistance phenotype of the C6 0.5 line, as we have already pointed out [16]. Secondly, a compound like quinine is able to restore drug sensitivity to an important level (residual resistance factor = 14 at 10 μmol/l) without restoring intracellular doxorubicin amounds to levels as high as those found with other compounds. Third, several compounds are able to potentiate 2-fold the cytotoxicity of doxorubicin without any change in doxorubicin accumulation; cyclosporine A, amiodarone, trifluoperazine, tamoxifen, quinine and quinidine. These observations clearly dissociate doxorubicin activity from its intracellular accumulation.

The modulators tested can be divided in two categories: those able to decrease the intracellular IC_{50} (cyclosporine A, S9788,

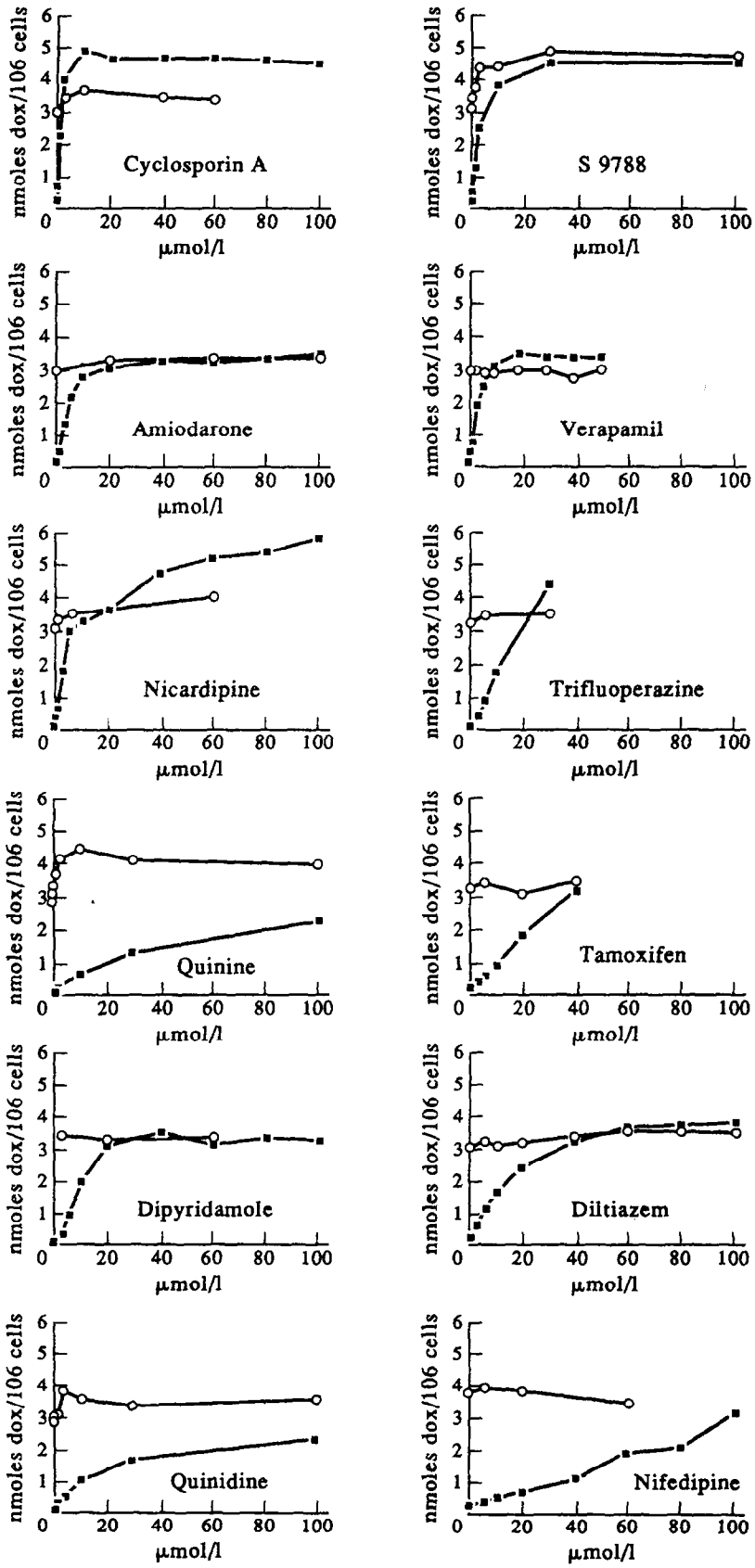

Fig. 2. Incorporation of doxorubicin in C6 sensitive cells (—○—) and in C6 0.5 cells (—■—) in the presence of various concentrations of 12 different modulators. Doxorubicin was maintained at 17.2 µmol/l for 2 h at the contact of the cells; cells were harvested immediately after exposure and doxorubicin was extracted and assayed as described in Materials and Methods. At least two independent experiments were performed in triplicate for each determination. The S.D. obtained were always within ± 10% of the means.

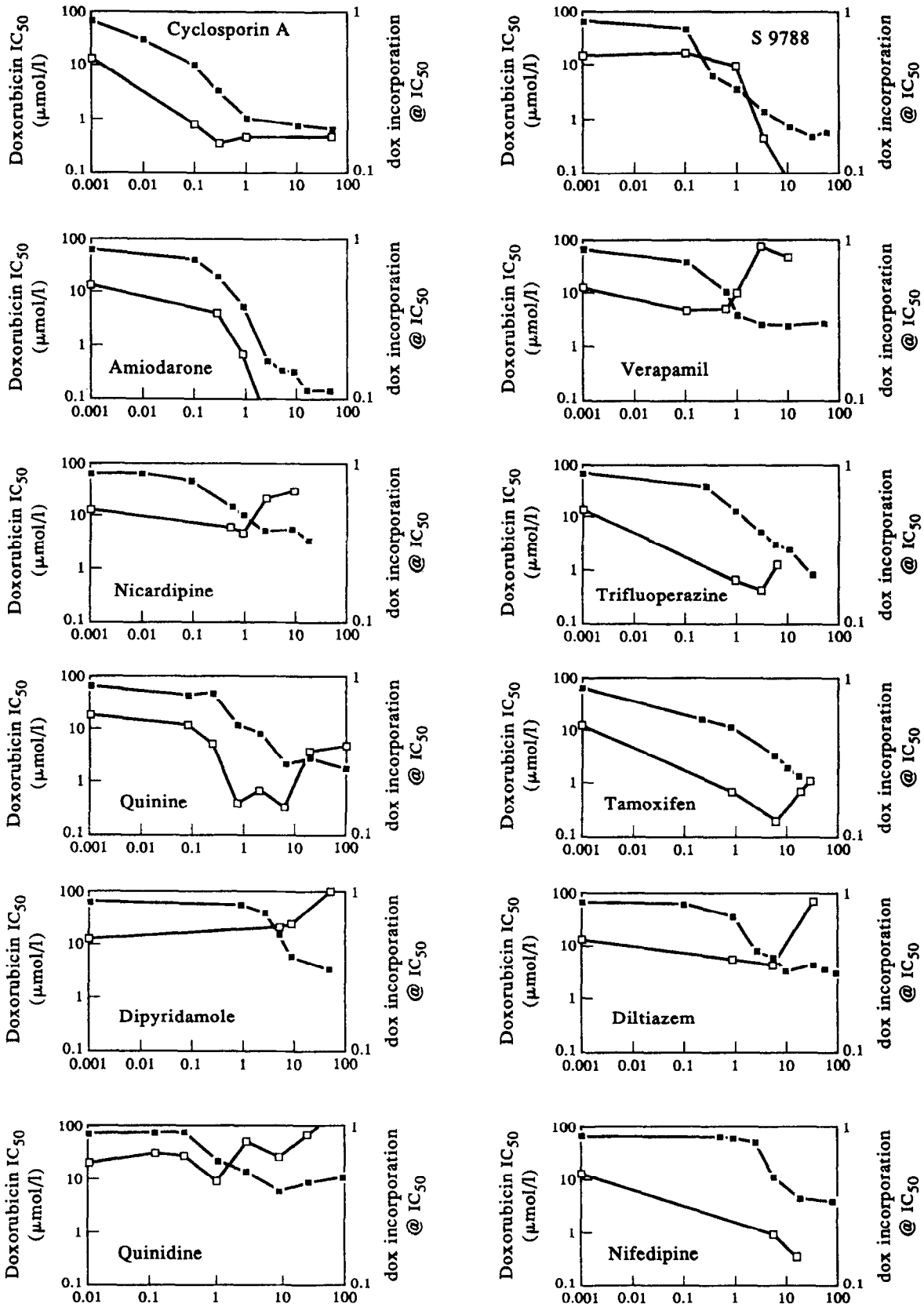

Fig. 3. Doxorubicin IC_{50} (—■—) and doxorubicin accumulation at IC_{50} exposure (—□—) in C6 0.5 cells for various concentrations of 12 different modulators. IC_{50} were evaluated in the presence of various concentrations of doxorubicin, from 0.1 to 100 μmol/l, maintained for 2 h in the presence of the cells, simultaneously with the modulator. The cell layer was then rinsed and allowed to grow for appropriate times until MTT evaluation of cell survival. Doxorubicin accumulations at IC_{50} were evaluated after 2-h exposures of the cells to the IC_{50} determined previously as indicated before. Doxorubicin was extracted and measured as described in Materials and Methods. In each case, at least 3 independent experiments were performed in triplicate. The S.D. obtained were within ± 25% of the means for doxorubicin IC_{50} and within ± 10% of the means for doxorubicin accumulation at IC_{50}.

Table 1. Residual resistance factors obtained for the various modulators at the various concentrations tested

	Concentration of the modulator (μmol/l)									
	0.01	0.1	0.3	0.6	1	3	6	10	30	50
Cyclosporine A	187	65	22	—	6.5	—	—	4.7	—	4
S9788	—	322	42	—	30	10	—	5.5	3.4	3.9
Amiodarone	—	265	142	—	39	3.9	—	2.6	1.0	—
Verapamil	—	258	—	71	26	19	—	17	—	19
Nicardipine	—	303	—	97	65	34	—	36	22	19
Trifluoperazine	—	—	245	—	84	33	21	19	16	—
Quinine	—	—	290	—	74	51	—	14	18	—
Tamoxifen	—	—	110	—	77	22	—	13	7.1	—
Dipyridamole	—	—	—	—	355	245	97	40	—	24
Diltiazem	—	380	—	—	213	52	37	—	29	—
Quinidine	—	—	400	—	140	92	—	39	59	—
Nifedipine	—	—	—	400	387	322	77	—	33	27

Residual resistance factors were obtained by dividing the IC_{50} of doxorubicin evaluated in the C6 0.5 line in the presence of the modulator by the IC_{50} of doxorubicin in the C6 sensitive line.

amiodarone, trifluoperazine, tamoxifen, quinine and nifedipine) and those which do not decrease this parameter, or even increase it when used at higher concentrations (verapamil, nicardipine, dipyridamole, diltiazem and quinidine). This property of decreasing intracellular IC_{50} appears independently of the efficacy of the modulator to reverse doxorubicin resistance on a molar basis. We had hypothesised [16] that a mechanism of doxorubicin tolerance to high intracellular concentrations was operating in our C6 0.5 cells in addition to the classical MDR P-glycoprotein-mediated drug extrusion. It appears that those modulators able to lower the intracellular IC_{50} can reverse the phenomenon of tolerance, whereas the other compounds cannot do so, and only reverse classical MDR resistance. This interpretation would suggest that these are intracellular targets only to the first class of modulators, those of the second class being either unable to reach them or devoid of activity on these targets. It is tempting to assign these intracellular targets to transport proteins analogous or identical to P-glycoprotein, located in subcellular membrane systems and devoted to the segregation of the drug in compartments from where it cannot reach its usual nuclear targets. It should be mentioned also that the intracellular effect of these modulators could be mediated simply by their physico-chemical interactions with membrane systems; Hindenburg *et al.* [25] have shown especially that verapamil was able to modify the organic phase-water distribution of anthracyclines *in vitro*. Other works are in favour of alterations of the disposition of anthracyclines at the intracellular level in resistant cells: it had been shown by Sehested *et al.* [26] that multidrug resistant cells presented alterations in membrane traffic processes suggesting endosomal drug trapping; it was more recently shown by fluorescence microscopy that doxorubicin could be trapped in vesicles in MDR cells, a phenomenon which does not occur in sensitive cells [27].

From a clinical point of view, it appears difficult to choose the better MDR modulator only from *in vitro* studies, since the problem of the other pharmacological properties of the drugs are not taken into account, especially the vasoactive properties shared by numerous modulators. Several clinical studies have been undertaken, mostly in haematological malignancies with verapamil [14], quinine [28] and cyclosporine A [29], but definitive results showing a clinical reversal of resistance of solid tumours are still lacking. In view of the possible existence of multiple types of targets of these modulators, it could be interesting to try to associate the best compounds acting on drug accumulation (verapamil type) to the best compounds acting on intracellular tolerance (quinine type). This could allow the use of smaller doses of each instead of the optimal dose of compounds acting on both targets. This would maintain their activity on tumour cells whilst reducing their unwanted effects. Another approach would be to develop selectively compounds showing no toxicity at the doses required for resistance reversal. This is presently the case for non-immunosuppressive cyclosporins or for the compound studied here, S9788, which is presently in phase I trial.

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Ploidy and Proliferative Index in Medulloblastoma: Useful Prognostic Factors?

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Paraffin sections from 32 patients with primary medulloblastoma were analysed by flow cytometry for DNA ploidy and proliferative index to assess the value of these measurements in determining prognosis. Twenty-seven samples were informative. Of these 27 patients, 8 had had a total resection. The tumours were diploid in 13 patients and aneuploid in 14. Neither ploidy nor S-phase fraction were prognostic factors for survival, even when considered in conjunction with the type of surgery performed. This is in contrast to other published data, emphasising the need for large multicentre studies of biological prognostic factors in this rare tumour.
Eur J Cancer, Vol. 29A, No. 10, pp. 1383–1387, 1993.

INTRODUCTION

ALTHOUGH SINGLE centre studies report 5-year survival rates in the order of 70–80%, multicentre trials suggest that the overall survival for medulloblastoma is less satisfactory and more in the region of 50–55% [1–3]. In addition, the late toxicity associated with conventional treatment of surgery plus postoperative cranio-spinal irradiation, with a radiation boost to the posterior fossa, is not insignificant and may have important consequences on the quality of life of survivors.

The aim of future therapeutic manoeuvres is 2-fold; to improve survival and, at the same time, decrease the toxic effects of treatment. This can only be achieved if reliable prognostic