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Competitive inhibition of photoaffinity labelling of P-glycoprotein by anticancer drugs and modulators including S9788

Annette Demmer [a,*], Theresa Dunn [b], Torsten Hoof [a], Peter Kubesch [a], Burkhard Tümmler [a]

[a] *Klinische Forschergruppe Molekulare Pathologie der Mukoviszidose im Zentrum Biochemie der Medizinischen Hochschule Hannover, OE 4350, D-30623 Hannover, Germany*

[b] *Abteilung Haematologie der Medizinischen Hochschule Hannover, OE 6860, D-30623 Hannover Germany*

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Abstract

The affinity of the multidrug resistance modulator S9788 to interact with P-glycoprotein was characterized by its ability to inhibit the photoaffinity labelling of plasma membranes of multidrug resistant chinese hamster ovary B30 cells by iodomycin. This iodinated analogue of daunomycin specifically photolabels P-glycoprotein in membrane vesicles as well as in intact cells. The multidrug resistance reversing agents verapamil and cyclosporin and the cytotoxic drugs vinblastine and daunomycin which are known to be recognized by P-glycoprotein competed with iodomycin for its binding site on P-glycoprotein. Vinblastine and cyclosporin bound with high affinity, S9788 and verapamil with medium affinity to P-glycoprotein.

Keywords: Chemotherapy; Modulator; Multidrug resistance; P-glycoprotein

1. Introduction

Major problems of the chemotherapy of cancer are the toxicity of drugs to normal tissues and the outgrowth of drug-resistant tumor cell populations. The latter phenomenon is frequently associated with the development of multidrug resistance, which is characterized by resistance to several unrelated cytotoxic compounds and to reduced cellular accumulation of the drugs (Riordan and Ling, 1985; Gerlach et al., 1986). A broad variety of compounds have meanwhile been shown to reverse the multidrug resistance phenotype in vitro, including Ca^{2+} channel antagonists, antiarrhythmics, and immunosuppressants (Ford and Hait, 1990). Verapamil is the only collaterally sensitizing drug which has been extensively studied in clinical trials on its multidrug resistance reversing ability, however, side effects from the cardiovascular system were observed at the doses required to reverse multidrug resistance in vitro (Presant et al., 1986; Dalton et al., 1989; Miller et al., 1991; Pennock et al., 1991).

The most thoroughly investigated multidrug resistance phenomenon in vitro that appears to be of clinical significance in vivo is the overexpression of a 170 kDa plasma membrane protein, called P-glycoprotein (Kartner et al., 1983). P-glycoproteins play a causative role for both multidrug resistance and collateral sensitivity. Equilibrium binding and photoaffinity labelling studies with radiolabeled *Vinca* alkaloids and anthracyclines and photoreactive azido- or Bolton-Hunter derivatives of vinblastine, verapamil, daunomycin, and nifedipine revealed that human and rodent P-glycoproteins bind the drugs to which an multidrug resistance cell is resistant or collaterally sensitive (Cornwell et al., 1986a,b,1987; Safa et al., 1986, 1987; Busche et al., 1989a,b).

S9788 is a triazinoaminopiperidine derivative (Fig. 1B) which was identified through in vitro screening for its multidrug resistance reversing activity (Regnier et al., 1992; Merlin et al., 1994; Julia et al., 1994). Transport studies with adriamycin in multidrug resistant cells indicate that S9788 may act by increasing drug accumulation (Pierré et al., 1992). S9788 induces a dose dependent inverse in doxorubicin accumulation. Its ability to overcome multidrug resistance both in vitro in intrinsic and acquired resistant cell lines was up to seven times more potent than

* Corresponding author. Tel.: (49-511) 532-2920; Fax: (49-511) 532-5966.

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that of verapamil (Pierré et al., 1992). In vivo S9788 was able to overcome multidrug resistance in murine P388 leukemic cells resistant to vincristine (Cros et al., 1992) and also in human P-glycoprotein positive tumor xenograft after treatment with vepeside and cis-platinum (Poupon et al., 1992). Resistance modulation by S9788 was only detected in cell lines which exhibited significant P-glycoprotein expression. S9788 effected in contrast no change in drug resistance induced by altered topoisomerase II activity (Hill et al., 1993). The authors proposed that the resensitization to vincristine, vinblastine or adriamycin is mediated by interaction of S9788 to P-glycoprotein.

In order to evaluate this interaction, we investigated the ability of S9788 to competitively inhibit the photoaffinity labelling of plasma membranes from multidrug-resistant hamster cells by a radioiodinated analogue of daunomycin, termed iodomycin (Busche et al., 1989a).

2. Materials and methods

S9788 was provided by the Servier Research Institute, Suresnes, France (batch no. EB865). S9788 is 6-[4-[2,2-di-(4-fluorophenyl)ethylamino]-1-piperidinyl]-N,N′-di-2-propenyl-1,3,5-triazine-2,4-diamine bis methane sulfonate (Fig. 1B). Daunomycin was purchased from ICN Biomedicals (Costa Mesea, CA, USA), vinblastine from Lilly (Indianapolis, IN, USA), cyclosporin from Sandoz AG (Nürnberg, Germany) and verapamil from Knoll AG (Ludwigshafen, Germany). All other chemicals were of at least analytical grade. The colchicine-resistant mutant Chinese hamster ovary (CHO) cell line CHO B30 (Kartner et al., 1985) was selected from the glycine-, adenosine- and thymidine-requiring auxotroph AuxBl (McBurney and Whitmore, 1974). CHO cells were cultured in α-MEM (alpha minimum essential medium) supplemented with glutamine, nucleosides and 10 vol% fetal calf serum. Colchicine was added to the medium at a concentration of 30 μg/ml. Cultures were split every 4–5 days.

For the isolation of plasma membranes, the α-MEM medium was substituted by 20 mM Hepes buffer, pH 7.3. Cells from 50 tissues culture plates (diameter 9 cm) were scraped off with a rubber policeman and disrupted by ultrasonication (MSE desintegrator) until 80% of cells were lysed as checked by light microscopy. After removal of cell debris by centrifugation ($4000 \times g$; 10 min), the membraneous fraction of the supernatant was collected by centrifugation at $40\,000 \times g$ for 1 h. The pellet was subsequently centrifuged for 90 min ($40\,000 \times g$) through a 35% sucrose cushion. After resuspension in 5 mM Tris buffer, pH 7.5, the plasma membrane fraction was collected by a spin of $100\,000 \times g$ for 1 h. The yield varied between 1–2 mg plasma membrane protein per 50 tissues culture dishes.

[^{125}I]Iodomycin was prepared from daunomycin by reaction with ^{125}I-labelled Bolton-Hunter reagent (ICN Biomedicals, Costa Mesa, CA, USA) and subsequently purified as described previously (Busche et al., 1989a,b). The absence of contaminations with unreacted Bolton-Hunter reagent and daunomycin in the purified product was controlled by thin-layer chromatography (Busche et al., 1989a).

For photoaffinity labelling, 50 μg plasma membrane protein from CHO B30 cells, 0.05 pmol ^{125}I-labelled iodomycin, and variable concentrations of cytotoxic drugs or modulators were suspended in 150 μl in a quartz cuvette and were illuminated for 10 min with visible light emitted from a 250 W Xenon lamp that had been passed through two 3 cm filters of water (to absorb most infrared light) and of a saturated aqueous $CuSO_4$ solution (to absorb most of the UV light). By this procedure [^{125}I]iodomycin is specifically covalently linked to P-glycoprotein. Protein was collected by centrifugation in an airfuge, and the resuspended pellet was applied to separation by polyacrylamide gel electrophoresis (Fairbanks gels) (Busche et al., 1989a). The gel was dried and exposed to X-ray films. The autoradiograms were evaluated by one-dimensional laser densitometry. The signals were integrated on-line via a homebuilt analog-digital converter on an IBM PC.

3. Results

Iodomycin is an iodinated analogue of daunomycin (Fig. 1A) that specifically photolabels P-glycoprotein in membrane vesicles (Fig. 2A, left outermost lane) as well as in intact cells (Busche et al., 1989a). Photoaffinity labelling of P-glycoprotein with [^{125}I]-labelled iodomycin was performed in the presence of 0 nM, 3 nM, 30 nM, 300 nM, 3 μM, 30 μM, or 300 μM of compounds to which multidrug resistant cells are either cross-resistant or collaterally sensitive (Fig. 2 and Fig. 3). To compare the affinity

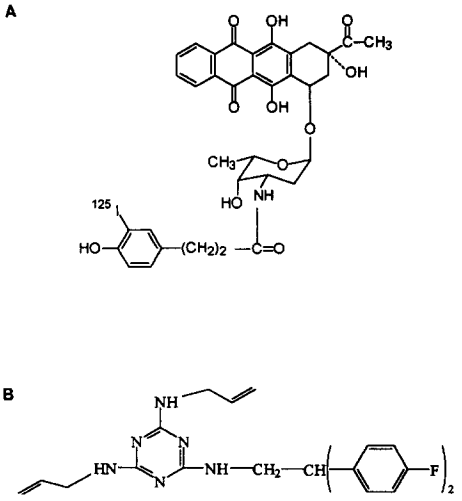

Fig. 1. Structures of [^{125}I]iodomycin (A) and S9788 (B).

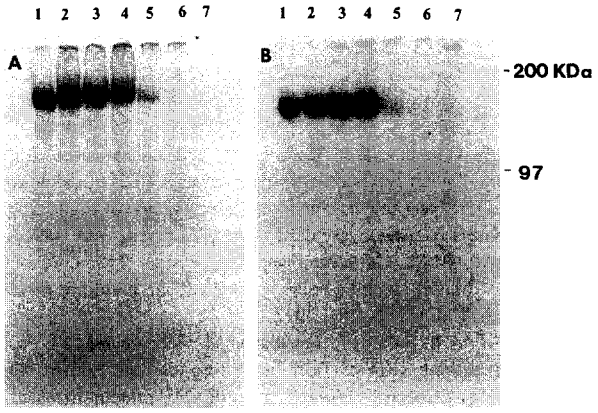

Fig. 2. Autoradiogram of gel-separated plasma membrane proteins of CHO B30 cells that had been photolabelled with ^{125}Iiodomycin in the presence of (from left to right) 0, 3 nM, 30 nM, 300 nM, 3 μM, 30 μM, 300 μM of S9788 (A) and verapamil (B).

of the novel sensitizer S9788 to P-glycoprotein with typical representatives of these two categories, vinblastine and daunomycin were chosen as examples of the former category, and verapamil and cyclosporin were selected as the multidrug resistance modulators. All titrations were done at least in duplicate with separate membrane preparations. The concentration dependence of inhibition of photoaffinity labelling was evaluated by densitometry of the autoradiograms (Fig. 3). By comparing the individual data for the separate experiments, the corresponding points of the titrations differed by 30% or less from each other; i.e., the average relative error was about 15%.

S9788 inhibited like verapamil the photobinding of [^{125}I]iodomycin in a dose-dependent manner (Fig. 2). Whereas 3 nM and 30 nM of S9788 had no significant influence on photoincorporation of radioactive anthracycline relative to controls, the reduction in photobinding was 35%, 74%, 85%, and > 97% for 0.3 μM, 3 μM, 30 μM, and 300 μM of S9788, respectively (Fig. 2A). These data demonstrate that S9788 efficiently competes with

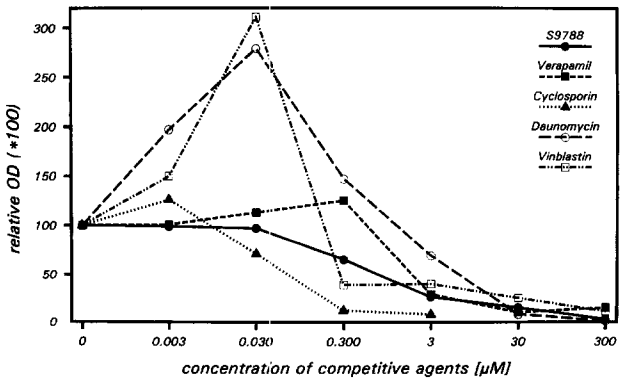

Fig. 3. Competitive inhibition of ^{125}Iiodomycin photolabelling of P-glycoprotein by daunomycin, vinblastine, cyclosporin A, verapamil and S9788. The same concentrations were applied as in Fig. 2.

iodomycin for its binding sites on P-glycoprotein, and hence we conclude that S9788 is recognized by P-glycoprotein. In our study it could be shown that verapamil and S9788 interacted with medium affinity with P-glycoprotein, but they show striking difference in the titration curve. Fig. 3 displays the densitometric analysis of the autoradiograms of the titrations with daunomycin, vinblastin, cyclosporin, and verapamil. All compounds were able to displace iodomycin from its sites on P-glycoprotein, but in quantitative terms each drug had a different effect on photobinding of P-glycoprotein with [^{125}I]iodomycin. For most compounds, the lowest concentrations of 3 nM and 30 nM increased the yield of radioactive photoproduct relative to controls, and photobinding was inhibited by all drugs if concentrations of 3 μM or more had been chosen.

4. Discussion

In this study it could be shown that S9788 was able to compete with iodomycin for its binding site on P-glycoprotein. In contrast to all other tested compounds S9788 was not able to increase the yield of photoproduct by non-saturating drug concentrations. This bimodal dependence of the yield of photoproduct on the concentration of competing drug can be interpreted in terms of the relative affinities of drug for the iodomycin-binding sites on P-glycoprotein (Busche et al., 1989a).

The binding constant K_I for the non-covalent complex of P-glycoprotein and iodomycin was determined by fluorescence titrations to be $K_I = (3–5) \cdot 10^7$ M^{-1} (Busche et al., 1989a). For photolabelling, the iodomycin concentration was chosen to be 0.3 nM; i.e. two orders of magnitude below the apparent dissociation constant of the iodomycin-P-glycoprotein complex. Since P-glycoprotein amounted in the CHO B30 membrane preparation to 15–20% (w/w) of total protein, the P-glycoprotein concentration in the photolabelling assays was about 0.2 μM. The approximately thousandfold molar excess of P-glycoprotein over radioiodinated indicator drug allowed a semiquantitative estimate of the affinity of competing drug: The irreversible photoreaction ($t_{1/2} \approx 1–2$ min) is slower than the rapid non-covalent equilibration between iodomycin and its binding sites residing on P-glycoprotein(Busche et al., 1989a). Thus, as long as the binding sites are not saturated and provided that the competing drug does not prevent the access of iodomycin to its binding pocket on P-glycoprotein, one expects an increase of photoincorporated product up to an concentration of about K^{-1} competitor. In other words, the relative yield of covalent photoproduct as a function of the concentration of competing drug provides information about the affinity of the competing drug for binding to P-glycoprotein and about the mode of inhibition of the binding of the daunomycin analogue to P-glycoprotein.

The data in Fig. 3 show that substoichiometric concentrations of daunomycin or vinblastine (3 nM <, 30 nM < concentration of P-glycoprotein, respectively) increased the yield of covalent photoproduct. Hence, both drugs compete with iodomycin for the same high-affinity site whereby the binding constant decreases in the order vinblastine > daunomycin. Cyclosporin turned out to be the most potent inhibitor of iodomycin photolabelling among the five tested drugs (Twentyman, 1992). Photolabelling was already suppressed by 30 nM cyclosporin when only a minority of P-glycoprotein binding sites can be occupied by drug. Verapamil and S9788 were somewhat less effective inhibitors of photoaffinity labelling of P-glycoprotein. At non-saturating drug concentrations, S9788 did not influence the yield of photoproduct. However, S9788 efficiently competed with iodomycin when the concentration of S9788 exceeded that of drug binding sites, and the binding of iodomycin could be completely inhibited by 300 μM of S9788. In conclusion, with regard to its ability to compete for the iodomycin binding sites on P-glycoprotein, S9788 may be classified into the same category as verapamil. Both drugs interact with medium affinity with P-glycoprotein.

Léonce et al. (1992) examined the ability of S9788 to compete with [^{3}H]azidopine, a photoreactive Ca^{2+} channel antagonist and substrate of P-glycoprotein, for its binding site on this protein. In contrast to our study S9788 was more effective than verapamil to inhibit [^{3}H]azidopine photolabelling which could reflect differential affinity and/or localization of the binding sites of P-glycoprotein for azidopine and iodomycin. Our study demonstrates that S9788 binds with medium affinity to the iodomycin binding site on P-glycoprotein which is recognized with high affinity by vinblastin and cyclosporin A, a potent modulator of P-glycoprotein. Interestingly, the cyclic dodecapeptide cyclosporin A shows no obvious structural similarity with anthracyclins indicating that chemical structure alone is not sufficient to predict drug binding affinity to P-glycoprotein.

The possibility that mechanisms of action other than binding of the substrates to P-glycoprotein contribute to the modulation of multidrug resistance cannot be excluded. The main pharmacological effects of S9788 noted during the clinical phase I studies were Q-T prolongations on the electrocardiogram associated with some cases of ventricular arrythmia. These findings, explored and confirmed by in vitro and in vivo preclinical experiments, were in favor of a class Ia antiarrythmic effect (quinidine like) rather than a Ca^{2+} antagonist effect as with verapamil (data not published).

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