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Interaction of cytostatics and chemosensitizers with the dexniguldipine binding site on P-glycoprotein

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Abstract

The interaction of cytostatics and chemosensitizers with the dexniguldipine binding site of P-glycoprotein was investigated in photoaffinity labeling experiments. A tritiated azidoderivative of the chemosensitizer dexniguldipine with dihydropyridine structure, [^{3}H]B9209-005, was used to irreversibly label P-glycoprotein. The apparent affinity of cytostatics and chemosensitizers to this binding site was estimated from labeling experiments in the presence of increasing concentrations of compounds. From the cytostatics tested, the vinca alkaloids and taxol showed the highest affinity, anthracyclins possessed moderate affinity while methotrexate, ara C and camptothecin, cytostatics not involved in P-glycoprotein-mediated multidrug resistance, were almost inactive. The chemosensitizers GF 120918, cyclosporin A and SDZ PSC-833 inhibited photoincorporation with the highest potency. Steep dose-inhibition curves were obtained with the cyclic peptides and S9788, indicating that these compounds may bind allosterically to a separate binding site. Compounds with dihydropyridine structure with or without chemosensitizing potency were also tested and some structure-activity relationships could be derived from the data. Our data show that inhibition of photoaffinity labeling by [^{3}H]B9209-005 is a valuable and reliable system for measuring the interaction with and potency of chemosensitizing compounds at P-glycoprotein. Furthermore, data obtained in this test system are well suited to investigate structure-activity relationships for chemosensitizers at P-glycoprotein. In addition cytostatics underlying P-glycoprotein-mediated multidrug resistance can be identified.

Keywords: P-Glycoprotein; Photoaffinity labeling; Chemosensitizer; Multidrug resistance

1. Introduction

Multidrug resistance is a serious problem in the treatment of human cancers. Initially responsive tumors often develop drug resistance during multiple cycles of chemotherapy, while some tumors are intrinsically resistant to chemotherapy (Nooter and Herweijer, 1991). One important mechanism leading to drug resistance is the expression of P-glycoprotein in the cell membranes of resistant tumor cells. P-glycoprotein is an ATP-driven drug efflux pump, which transports a variety of different cytostatic drugs out of the cells, thereby reducing the intracellular concentrations below the effective threshold (Gottesman and Pastan, 1993; Endicott and Ling, 1989). The substrate specificity of P-glycoprotein is rather broad and includes cytostatics, such as vinca alkaloids, anthracyclins taxanes and colchicin. In addition to cytostatics, the protein transports various cationic dyes such as rhodamine-123 (Kessel, 1989) or acridine orange (Neyfakh, 1988), cardiac glycosides (De Lannoy and Silverman, 1992) and some steroid hormones (Ueda et al., 1992). A variety of compounds with very different structures, termed chemosensitizers, have been identified as inhibitors of P-glycoprotein's pumping function. Consequently, inhibition of P-glycoprotein's transport function by chemosensitizers is tested clinically to overcome drug resistance and some compounds including verapamil, cyclosporin A, SDZ PSC-833, S9788, dexniguldipine and others have already entered clinical trials.

The biochemical mechanisms of drug transport and inhibition of transport by chemosensitizers are still

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unclear. Competitive and noncompetive inhibition of transport has been described (Yusa and Tsuruo, 1989; Horio et al., 1991; Tamai and Safa, 1990; Tamai and Safa, 1991). Some chemosensitizers such as cyclosporin A and verapamil are substrates by themselves (Saeki et al., 1992, 1993; Cano-Gauci and Riordan, 1987), while others are not transported by P-glycoprotein. In radioligand binding experiments with [^{3}H]vinblastine as a ligand for the vinca alkaloid binding site, allosterically coupled binding sites for dihydropyridines and taxanes were identified (Ferry et al., 1992; Malkhandi et al., 1994). Up to now no radioligand for monitoring reversible binding to the dihydropyridine binding site is available. [^{3}H]Azidopine was used as a photoligand to irreversibly label P-glycoprotein (Safa et al., 1987; Yang et al., 1988) and some experiments to pharmacologically characterize the azidopine binding site were performed with this dihydropyridine compound (Safa et al., 1987; Friche et al., 1993). Azidopine is only a weak chemosensitizer in cellular assays in vitro (Borchers et al., 1995) and might therefore not be very well suited for this purpose. In the present work we used an azido derivative of the potent chemosensitizer dexniguldipine, [^{3}H]B9209-005, as a photoligand to label the dihydropyridine binding site of P-glycoprotein. It has been previously shown that [^{3}H]B9209-005 specifically photoincorporates into P-glycoprotein of multidrug-resistant and P-glycoprotein-overexpressing human T lymphoblastoid CCRF ADR-5000 cell membranes (Borchers et al., 1995).

B9209-005 shows high chemosensitizing potency in cellular assays, and higher affinity to P-glycoprotein than azidopine (Borchers et al., 1995). The interaction of cytostatics and chemosensitzers with the dihydropyridine binding site of P-glycoprotein was investigated in photolabeling competition experiments. Although photoaffinity competition experiments represent irreversible binding under nonequilibrium conditions, valuable information concerning the relative potency of different chemosensitizers and structure-activity relationships can be obtained by this procedure.

2. Material and methods

2.1. Materials

Dexniguldipine, B9109-012 (3-[3-(4,4-diphenyl-1-methyl-1-piperidinio)propyl]-5-methyl-4(*R*)-(3-nitrophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate-chloride), S9788 (6-[4-[2,2bis(4-fluorophenyl)ethylamino]piperidine-1-yl]-2,4-bis(allylamino)-1,3,5-triazine dimethanesulfonate), GF 120918 (*N*-[4-[2-(1,2,3,4,-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide), azidopine and [^{3}H]B9209-005 (3-[3-(4-[^{3}H]phenyl-4-phenyl-1-piperidinyl)propyl]-5-methyl-4(*R*)-(3-azidophenyl)-1,4-dihydro-2,6-dimethyl-pyridine-3,5-dicarboxylate) were synthesized at the chemical department of Byk Gulden. For measurements with cyclosporin A, the Sandimmun preparation, (Sandoz, Basel, Switzerland) was used. SDZ PSC-833 was a kind gift of Sandoz Pharma. Verapamil, rhodamine-123, methotrexate, colchicine, actinomycin-D, taxol, camptothecin, and daunomycin were from Sigma (Deisenhofen, Germany). Doxorubicin was from Farmitalia (Freiburg, Germany) and vincristine was from Aldrich (Milwaukee, WI, USA). Vinblastine (Velbe) was obtained from Lilly (Giessen, Germany) and ara C (Alexan) was from Mack (Illertissen, Germany).

2.2. Cell lines and cell culture

The human T-lymphoblastoid cell line CCRF-CEM was obtained from American Type Culture Collection (Rockville, MD, USA). The selection of the multidrug-resistant CCRF ADR-5000 subline has been reported elsewhere (Kimmig et al., 1990). Cells were propagated at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium containing 10% fetal calf serum, 2 mM glutamine and 50 μg/ml gentamicin. Stock cultures were grown in the presence of 5000 ng/ml adriamycin.

2.3. Preparation of plasma membranes

All steps for membrane preparation were carried out at 4°C. Cells were harvested by centrifugation and homogenized with a Teflon/glass homogenizer in lysis buffer containing 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at $3000 \times g$ for 10 min, and at $40\,000 \times g$ for 30 min. The resulting high-speed pellets were resuspended in 50 mM Tris/HCl pH 7.4, 0.1 mM PMSF buffer. Protein content was determined by a bicinchroninic acid assay (BCA; Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

2.4. Photoaffinity labeling and fluorography

Membranes (0.6 mg/ml) were incubated in phosphate-buffered saline pH 7.4 for 1 h at room temperature in the dark in the presence of 0.01–1.3 μM [^{3}H]B9209-005 in a total volume of 50 μl. Irradiation was performed with an UV lamp (Camag, Berlin, Germany) at 366 nm for 20 min at 4°C at a distance of 10 cm. For photoincorporation inhibition experiments a [^{3}H]B9209-005 concentration of approximately 1 μM was used and experiments were performed in the presence of increasing concentrations of compounds. For separation of photolabeled proteins by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a 40 μl aliquot of the irradiation mixture was diluted 1:1 in sample buffer (0.125 M Tris/HCl, pH 6.8, 5% mercaptoethanol, 2% SDS, 0.01% bromphenolblue, 50% glycerol) and 60 μl were applied onto 8.5% polyacrylamide-SDS gels. For liquid scintillation counting gels were cut into 1 mm slices and radioactivity was eluted by 3 h incubation in Biolute S (Zinsser, Frankfurt, Germany) at 50°C and radioactivity was quantified by liquid scintillation counting. When fluorography was employed for measurements of photoincorporation, whole gels were incubated with Amplify (Amersham, Braunschweig, Germany) for 30 min and dried on filter paper. Dried gels were applied onto a Hyperfilm (Amersham, Braunschweig, Germany) for 8 days at −80°C. Radioactivity was quantified by densitometric tracing of the fluorograms, using the CS-1 video imaging system (Cybertech, Berlin, Germany).

2.5. Data analysis

Data were analyzed with the Prism program (GraphPad, San Diego, CA, USA). For radioligand saturation experiments a one-site binding hyperbola was fitted to the data. Photoincorporation inhibition data were analyzed by nonlinear least-square fitting. A sigmoid curve was fitted to the data and the IC_{50} value yielding half-maximal inhibition was calculated. IC_{50} values were corrected for the presence of radiolabeled ligand according to the following formula of Cheng and Prusoff (1973):

$$K_i = IC_{50}/1 + (L/K_d)$$

where K_i is the corrected affinity constant, L is the concentration of radioligand and K_d is the affinity of the radioligand to its binding site (obtained from saturation experiments as shown in Fig. 1).

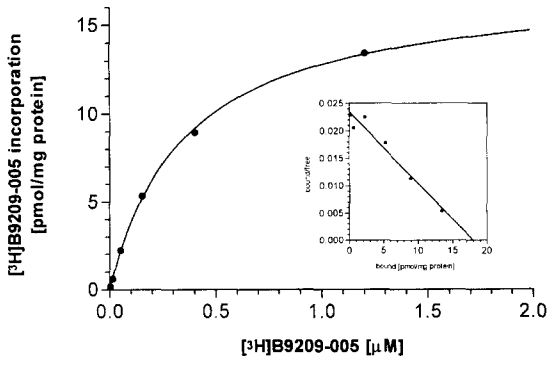

Fig. 1. Dose-dependent photoincorporation of [^{3}H]B9209-005 into P-glycoprotein after photoaffinity labeling of CCRF ADR-5000 membranes and separation of the membrane proteins by SDS-PAGE. Photoincorporation into the 170 kDa protein (native P-glycoprotein) was determined by liquid scintillation counting. The inset shows the incorporation data after transformation according to Scatchard. An apparent affinity constant of 0.3 μM was obtained from the above experiment.

3. Results

In photoaffinity labeling experiments with membranes from CCRF ADR-5000 cells [^{3}H]B9209-005 specifically labeled two proteins with molecular masses of 170 and 95 kDa, while two other proteins with molecular masses of 55 and 38 kDa were nonspecifically labeled. The 170 and 95 kDa proteins were identified immunologically as intact P-glycoprotein and the *N*-terminal proteolytic fragment thereof (Borchers et al., 1995). In a first set of radioligand saturation experiments increasing concentrations of [^{3}H]B9209-005 were used to evaluate the binding characteristics of the compound. For these experiments photolabeled membranes were separated on 8% polyacryamide-SDS gels. After slicing of the gels, photoincorporation into the 170 kDa protein was determined by liquid scintillation counting. [^{3}H]B9209-005 binding and photoincorporation into native P-glycoprotein was saturable and of high affinity. Half-maximal photolabeling is achieved at a ligand concentration of 0.35 μmol/l. Transformation of the data according to Scatchard resulted in a straight line, indicating an interaction with and incorporation into a homogeneous population of binding sites (Fig. 1).

When photoaffinity labeling experiments with [^{3}H]B9209-005 were performed in the presence of increasing concentrations of unlabeled B9209-005, photoincorporation of [^{3}H]B9209-005 was inhibited dose-dependently. After slicing of the gels and determination of radioactivity by liquid scintillation counting an IC_{50} value (concentration resulting in half-maximal inhibition) of 1 μmol/l was obtained. Correction for the presence of the radioligand concentration was performed according to Cheng and Prusoff (1973), resulting in an affinity constant of 0.34 μmol/l, which is in excellent agreement with data from saturation experiments. Affinity constants do not represent true K_I values as defined by the theory of mass action law and should therefore be considered as apparent affinity constants.

Fig. 2 shows the inhibition of [^{3}H]B9209-005 photoincorporation by increasing concentrations of unlabeled B9209-005. When photolabeling-inhibition experiments with B9209-005 were analyzed by fluorography an affinity constant of 0.77 μmol/l was obtained, which is also in good agreement with the data obtained by liquid scintillation counting. The slopes of the inhibition curves were slightly above unity with values of 1.5 in the case of liquid scintillation counting and 1.3 employing fluorographic detection.

The interaction of cytostatics and chemosensitizers

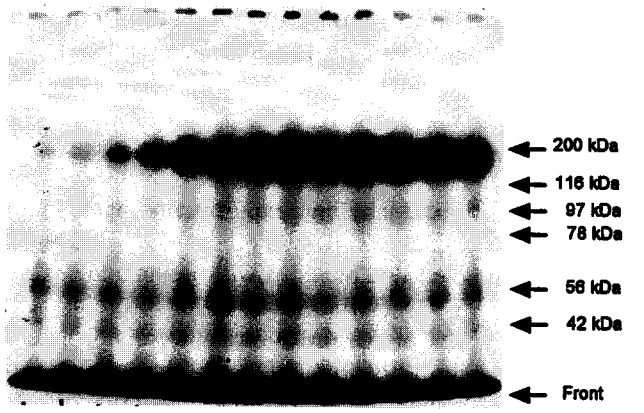

Fig. 2. Inhibition of photoincorporation of [^{3}H]B9209-005 (1.3 μM) by unlabeled B9209-005. The following concentrations in μM of unlabeled B9209-005 were employed from left to right: 50, 22, 10, 5, 2.2, 1, 0.5, 0.22. 0.1, 0.05, 0.02, 0.01 and 0 (solvent control). After photoincorporation, membrane proteins from CCRF ADR-5000 membranes were separated by SDS-PAGE. Gels were dried and fluorography was performed as described in the Material and methods section. Photoincorporation into the 170 kDa band of P-glycoprotein was determined by densitometric scanning of the fluorogram. From the above experiment an IC_{50} value of 3 μM was obtained, resulting in an apparent affinity constant of 0.66 μM.

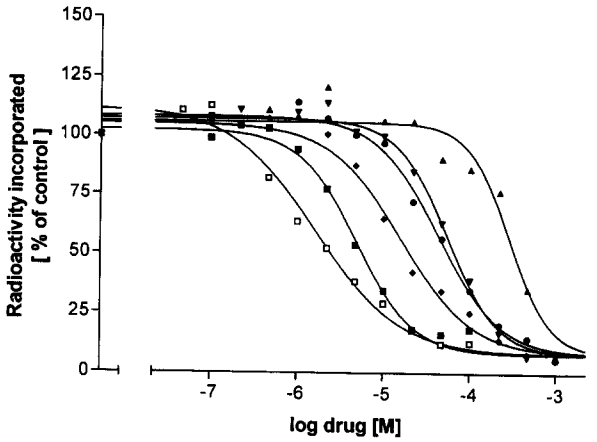

Fig. 3. Inhibition of [^{3}H]B9209-005 photoincorporation into P-glycoprotein from CCRF ADR-5000 cells by (□) vinblastine, (■) taxol, (♦) vincristine, (●) daunomycin, (▼) rhodamine-123, and (▲) colchicine.

with the [^{3}H]B9209-005 binding site was investigated in photolabeling inhibition experiments as described above for B9209-005. Fluorography was used for quantitation of the radioactivity incorporated into the 170 kDa protein. Photoincorporation of [^{3}H]B9209-005 was inhibited dose-dependently by all chemosensitizers and most cytostatics. Potent inhibition of photoincorporation is seen with taxol and vinblastine, resulting in apparent affinity constants of 0.63 and 0.46 μmol/l, respectively. Vincristine, daunomycin, and actinomycin-D showed intermediate affinity with constants between 3 and 10 μmol/l. Colchicine gave a value of 30 μmol/l, while methotrexate, ara C and camptothecin did not interact with this binding site up to concentrations of 300 μmol/l. Rhodamine-123, a fluorescent dye often used to monitor drug transport by P-glycoprotein, interacted with relatively low affinity (26 μmol/l) with the dihydropyridine binding site. The slopes of the inhibition curves obtained with taxol, doxorubicin, actinomycin-D and rhodamine-123 are slightly above unity with values up to 1.3. Fig. 3 shows the photolabeling inhibition curves obtained with taxol, vinblastine, vincristine, daunomycin, colchicine and rhodamine-123. In Table 1 the log IC_{50} values (pIC_{50}), the apparent affinity constants and the slopes of the inhibition curves for cytostatics are given.

From the chemosensitizers tested the cyclic peptides cyclosporin A and SDZ PSC-833 and the acridone carboxamide derivative GF 120918 showed the highest affinity with apparent constants around 0.1 μmol/l. The dihydropyridines dexniguldipine and nicardipine were of intermediate affinity, while nitrendipine displayed somewhat weaker activity. The permanently charged quaternary dexniguldipine derivative B9109-012 was almost 10-fold more active than the parent compound dexniguldipine, and devoid of a permanent charge in the side chain. For azidopine, an often used dihydropyridine photoaffinity ligand for P-glycoprotein, an apparent affinity constant of 1 μmol/l was obtained. The triazinoaminopiperidine derivative S9788, another potent chemosensitiser, gave an apparent affinity constant of 11 μmol/l. Verapamil was of rela-

Table 1
Inhibition of [^{3}H]B9209-005 photolabeling by cytostatics

Cytostatics	IC_{50} value (log mol/l ± S.D.)	Affinity constant (log mol/l ± S.D. and μmol/l) [a]	Slope ± S.D.
Taxol	5.50 ± 0.19	6.19 ± 0.23 (0.65 μM)	1.31 ± 0.67
Vinblastine	5.65 ± 0.28	6.34 ± 0.27 (0.46 μM)	0.96 ± 0.06
Vincristine	4.81 ± 0.01	5.52 ± 0.04 (3.02 μM)	1.18 ± 0.13
Daunomycin	4.28 ± 0.08	5.00 ± 0.13 (10.0 μM)	1.01 ± 0.22
Doxorubicin	3.74 ± 0.28	4.44 ± 0.31 (36.3 μM)	1.25 ± 0.22
Actinomycin-D	4.50 ± 0.45	5.20 ± 0.45 (6.31 μM)	1.29 ± 0.12
Colchicine	3.86 ± 0.28	4.51 ± 0.29 (30.9 μM)	1.29 ± 0.20
Methotrexate	< 3.0	< 3.7 [b] (< 200 μM)	
Ara-C	< 3.0	< 3.7 [b] (< 200 μM)	
Camptothecin	< 3.0	< 3.7 [b] (< 200 μM)	
Rhodamine-123	4.41 ± 0.18	4.98 ± 0.25 (10.5 μM)	1.38 ± 0.18

[a] For convenience the affinity constants are also given in nonlogarithmic form in μmol/l. [b] Estimated.

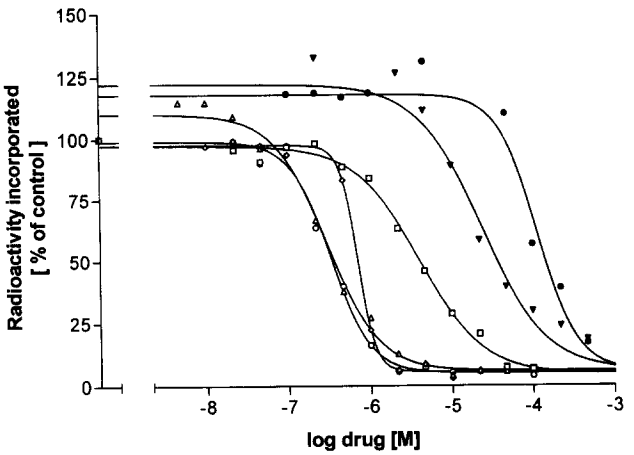

Fig. 4. Inhibition of [^{3}H]B9209-005 photoincorporation into P-glycoprotein from CCRF ADR-5000 cells by (□) dexniguldipine, (○) B9109-012, (△) GF 120918, (◇) SDZ PSC-833, (▼) verapamil, and (●) S 9788.

tively low affinity (apparent affinity constant = 3.5 μmol/l). Fig. 4 shows the dose-inhibition curves for the chemosensitizers SDZ PSC-833, GF 120918, S 9788, verapamil, dexniguldipine and B9109-012. In Table 2 the pIC_{50} values, the apparent affinity constants and the slopes obtained with these chemosensitizers are listed. The slopes for cyclosporin A, SDZ PSC-833 and for S 9788 are clearly above unity and vary between 3 and 4. In two out of eight experiments with the cyclic peptides the slopes were extremely high, exceeding a value of 10. These values were not included in the calculation of mean values given in Table 2. To exclude the possibility that these high values are caused by problems in the fluorometric detection of incorporated radioactivity, dose-response curves with cyclosporin A and SDZ PSC-833 were also analyzed by liquid scintillation counting after slicing of the gels. Similar steep dose-response curves were obtained by both methods of detection.

For some chemosensitizers, especially verapamil and S9788, a slight stimulation of photoincorporation (up to 30%) was seen before the expected inhibition due to displacement of [^{3}H]B9209-005 occurred (Fig. 4).

Table 2
Inhibition of [^{3}H]B9209-005 photolabeling by chemosensitizers

Chemosensitizer	IC_{50} value (log mol/l ± S.D.)	Affinity constant (log mol/l ± S.D. and μmol/l) [a]	Slope ± S.D.
Cyclosporin A	6.30 ± 0.10	6.96 ± 0.15 (0.11 μM)	3.65 ± 1.21
SDZ PSC-833	6.22 ± 0.07	6.87 ± 0.10 (0.13 μM)	3.95 ± 1.85
S9788	4.23 ± 0.20	4.95 ± 0.19 (11.2 μM)	3.65 ± 0.46
GF 120918	6.53 + 0.15	7.02 + 0.15 (0.095 μM)	1.47 + 0.31
Dexniguldipine	5.36 ± 0.07	6.10 ± 0.07 (0.79 μM)	1.19 ± 0.07
B9109-012	6.34 ± 0.16	7.00 ± 0.06 (0.1 μM)	1.41 ± 0.13
B9209-005	5.43 [b]	6.11 [b] (0.78 μM)	1.33
Azidopine	5.29 ± 0.18	5.99 ± 0.15 (1.02 μM)	1.42 ± 0.13
Nicardipine	5.76 [b]	6.26 [b] (0.55 μM)	0.99
Nitrendipine	5.05 [b]	5.62 [b] (2.4 μM)	1.43
Verapamil	4.75 ± 0.01	5.45 ± 0.11 (3.55 μM)	1.24 ± 0.31

[a] For convenience the affinity constants are also given in nonlogarithmic form in μmol/l. [b] Mean from two independent experiments not differing more than 0.25 from each other.

4. Discussion

The purpose of the present work was to evaluate the interaction of cytostatics and chemosensitizers with the dexniguldipine binding site on P-glycoprotein. Furthermore, we compared the potency of different chemosensitizers for their interaction with P-glycoprotein, i.e. the dexniguldipine binding site on P-glycoprotein. We have chosen the human multidrug-resistant lymphoblastoid cell line CCRF ADR-5000 which was selected from the drug-sensitive parental CCRF-CEM cell line by sequential exposure to adriamycin (Kimmig et al., 1990). This cell line shows high expression of P-glycoprotein as demonstrated immunologically (Kimmig et al., 1990). High expression of P-glycoprotein is an important prerequisite for a reasonable signal-to-noise ratio in photoaffinity labeling studies.

The dihydropyridine derivative dexniguldipine is a potent chemosensitizer in vitro (Hofmann et al., 1992; Boer et al., 1994) and has entered clinical trials as a chemosensitizer in phase I/II studies. The chemosensitizing potency of dexniguldipine is due to a direct interaction with P-glycoprotein and inhibition of drug transport by P-glycoprotein (Boer et al., 1994; Borchers et al., 1995). The most suitable technique to measure interaction of compounds with a receptor or binding site is the determination of affinity constants in reversible radioligand binding assays. Unfortunately, up to now no suitable dihydropyridine radioligand for this approach is available. [^{3}H]Vinblastine has been successfully used to measure interaction with the vinca alkaloid binding site (Ferry et al., 1992; Malkhandi et al., 1994). From these data it is clear that the dihydropyridines bind to a different site which is allosterically coupled to the vinca alkaloid binding site. Therefore, the use of [^{3}H]vinblastine may be not optimal for the characterization of the dihydropyridine binding site. The relatively low affinity of [^{3}H]azidopine, another dihydropyridine photoligand for P-glycoprotein,

limits the use of this compound in reversible binding experiments. In addition azidopine is only a weak chemosensitizer, while dexniguldipine and the azido-derivative B9209-005 possess potent chemosensitizing activity in cellular assays (Borchers et al., 1995). Therefore, a radiolabeled azido derivative of dexniguldipine, [^{3}H]B9209-005, was used to monitor the interaction of compounds with its binding site. [^{3}H]B9209-005 has been shown to specifically label P-glycoprotein and the N-terminal 95 kDa fragment thereof (Borchers et al., 1995).

Inhibition of photoincorporation into the native 170 kDa protein was used to assess the potency of compounds to interact with P-glycoprotein. Although photoaffinity labeling represents irreversible binding, reversible binding precedes the photoactivation and incorporation of the radioligand and the results are assumed to mirror the equilibrium situation obtained during the incubation period before photoactivation. Photoactivation was routinely performed for 20 min at 0°C, where dissociation of bound ligands should be minimized. Nevertheless, some dissociation may occur during that time period. Therefore, for some compounds the photoactivation time was reduced to 2 min, resulting in very similar IC_{50} values (data not shown). This supports the assumption that dissociation of ligands is not critical for the outcome of the experiments.

[^{3}H]B9209-005 binds and photoincorporates into P-glycoprotein in a saturable and monophasic manner and half-maximal photoincorporation is seen at a concentration of 0.35 μmol/l. Transformation of the data according to Scatchard results in a straight line, indicating binding to and incorporation of photoligand into a homogeneous population of binding sites. Again, as stated above Michaelis-Menten kinetics and Scatchard plots are normally used to describe reversible equilibrium binding. Nevertheless, the data are accurately fitted by these equations. The affinity constants calculated from the above data should therefore be regarded not as true affinity constants but as experimental constants describing the affinity of a compound for the two coupled processes of binding and photoincorporation. The same holds true for the conversion of IC_{50} values into affinity constants according to Cheng and Prusoff (1973). When photoaffinity labeling experiments were performed in the presence of increasing concentrations of the nonradioactive ligand an affinity constant of 0.77 μmol/l was obtained after correction according to Cheng and Prusoff (1973). This value is in good agreement with the value of 0.35 μmol/l obtained from saturation experiments. The 2-fold higher value is probably due to absorption of lipophilic B9209-005 to glass and plastics, which can be controlled and compensated for in saturation studies with only the radioactive compound, but cannot be measured easily in competition studies, where unlabeled B9209-005 is used as competitor. The good agreement between saturation studies and photoaffinity competition studies demonstrates that photolabeling inhibition studies can be applied reliably to measure apparent affinity constants of nonradioactive compounds for P-glycoprotein.

To further characterize the dexniguldipine binding site pharmacologically, and to address the question whether the interaction of cytostatics with P-glycoprotein can be measured quantitatively, photolabeling inhibition experiments with various cytostatics were performed. From the cytostatics tested, taxol and the vinca alkaloids showed the highest affinity, followed by actinomycin-D and the anthracyclins, daunomycin and doxorubicin. Very low affinities for methotrexate, ara C and camptothecin, cytostatics not transported by P-glycoprotein, were found. This rank order of potency roughly mirrors the picture seen in P-glycoprotein-mediated multidrug resistance in vitro. Vinblastine was a 7 times more potent inhibitor than vincristine, which is in accordance with the data of others (Ferry et al., 1992; Malkhandi et al., 1994), who reported a 30- to 60-fold higher affinity for vinblastine in radioligand binding studies with [^{3}H]vinblastine as a radioligand for P-glycoprotein. Furthermore the rank order of potency for cytostatics in their [^{3}H]vinblastine binding assay is almost the same as the rank order obtained in our experiments. Rhodamine-123, a substrate often used for monitoring P-glycoprotein-mediated drug transport, showed intermediate affinity for the dexniguldipine binding site. The slopes of the competition curves obtained with the cytostatics are generally near unity, indicating a competitive interaction with the dihydropyridine binding site. Slope values slightly above unity are difficult to interpret, especially in a nonequilibrium system, and may also be caused by substance depletion due to adsorption to glass and plastics.

Among the chemosensitizers the cyclic peptides cyclosporin A and SDZ PSC-833 showed the highest affinity with apparent affinity constants of 0.1 μmol/l. No significant difference in the potency of both compounds is seen. This is in agreement with the data of others (Jonsson et al., 1992) who reported identical chemosensitizing potency of both compounds in cellular proliferation tests versus different cytostatics. Similarly, [^{3}H]vincristine or daunomycin uptake in EHR2/DNR + cells is modulated with almost equal potency by both compounds (Friche et al., 1992). In contrast to these data an approximately 10-fold higher chemosensitization towards different cytostatics was reported by others (Boesch et al., 1991; Twentyman and Bleehen, 1991). Again, in agreement with our data high affinity of cyclosporin A to P-glycoprotein is also found in reversible radioligand binding assays with [^{3}H]vinblastine using membrane preparations from the same cell line (Malkhandi et al., 1994). Two new and potent

modulators of multidrug resistance, GF 120918 and S9788, also interact with P-glycoprotein, GF 120918 with high affinity and S9788 with considerably lower affinity. High potency of GF 120918 as a chemosensitizing agent has been reported by others (Hyafil et al., 1993). The relatively low affinity of S9788 is unexpected, as this compound is an efficient chemosensitizer in cellular assays in vitro (Leonce et al., 1992). One possible explanation for this would be that the compound might interact with a different binding site on P-glycoprotein with weak allosteric coupling to the dihydropyridine binding site, or that the modulating potency is at least in part due to other qualities of the compound. For instance, changes in membrane fluidity have been reported to be relevant for the chemosensitizing potency of tamoxifen and detergents such as cremophor EL (Woodcock et al., 1992; Wadkins and Houghton, 1993; Wiseman, 1994). Furthermore, the stimulation of photoincorporation by S9788 which is seen at concentrations below those necessary for inhibition of photoincorporation also point to a noncompetitive mechanism of interaction with P-glycoprotein by S9788.

B9109-012, a permanently charged derivative of dexniguldipine, carrying a positive charge in the side chain of the molecule, shows an approximately 10-fold higher affinity than dexniguldipine, pointing to the importance of a positive charge for the interaction of this compound with P-glycoprotein. These results are in line with data from others (Zamora et al., 1988; Hait and Aftab, 1992) who identified a positive charge as one important structural element in the interaction of compounds with P-glycoprotein. In contrast to the high affinity of B9109-012 for inhibition of photoaffinity labeling (8-fold more potent than dexniguldipine), the compound is approximataly 5- to 10-fold less active than dexniguldipine in the augmentation of drug accumulation in various multidrug-resistant cell lines (data not shown; manuscript in preparation). Furthermore, the compound is only a weak chemosensitizer in cellular proliferation tests with various cytostatics (V. Gekeler; personal communication). These data may indicate an intracellular localization of the dexniguldipine binding site and the inability of permanently charged compounds to cross the cell membrane in cellular assays of multidrug resistance.

Taken together, our data show that the above system can be used to measure the interaction of cytostatics and chemosensitizers with P-glycoprotein. Cytostatics subject to P-glycoprotein-mediated multidrug resistance such as vinca alkaloids, anthracyclins and taxanes show high to intermediate affinity, but cytostatics not involved in P-glycoprotein-mediated multidrug resistance do not inhibit photoaffinity labeling and show very low, if any, affinity to P-glycoprotein. Other substrates for P-glycoprotein such as rhodamine-123 also inhibit photoaffinity labeling. The system is therefore useful to identify cytostatics and other experimental compounds which underly P-glycoprotein-mediated drug transport and are subject to P-glycoprotein-mediated multidrug resistance. In addition to this, the interaction of chemosensitizers with P-glycoprotein can be investigated which may lead to information about the molecular mechanisms of chemosensitization and the involvement of P-glycoprotein in chemosensitization. Furthermore, as demonstrated for dexniguldipine and the quarternary dexniguldipine analogue, the system is well suited to investigate structure-activity relationships of chemosensitizing compounds and can be used as a convenient screening system for chemosensitizers and may therefore be valuable in the design of new and more potent compounds.

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