0026-895X/95/010021-09$3.00/0

MOLECULAR PHARMACOLOGY, 48:21–29 (1995).

B9209–005, an Azido Derivative of the Chemosensitizer Dexniguldipine-HCl, Photolabels P-Glycoprotein

CHRISTOPH BORCHERS, WOLF-RÜDIGER ULRICH, KURT KLEMM, WOLFGANG ISE, VOLKER GEKELER, SABINE HAAS, ANGELIKA SCHÖDL, JÜRGEN CONRAD, MICHAEL PRZYBYLSKI, and RAINER BOER

Fakultät für Chemie, Universität Konstanz, D-78434 Konstanz, Germany (C.B., J.C., M.P.), and Byk Gulden Lomberg GmbH, D-78467 Konstanz, Germany (W.-R.U., K.K., W.I., V.G., S.H., A.S., R.B.)

Received November 30, 1994; Accepted April 3, 1995

SUMMARY

P-glycoprotein is an energy-dependent drug extrusion pump for a variety of anticancer drugs and is involved in the development of multidrug resistance in cancer. Dexniguldipine-HCl is a potent chemosensitizer for P-glycoprotein-mediated multidrug resistance *in vitro*, and clinical phase I/II trials are underway. To investigate the mechanisms of chemosensitization and to identify the binding sites for dexniguldipine-HCl on target proteins involved in chemosensitization, [^{3}H]B9209–005, an azido derivative of dexniguldipine-HCl, was synthesized and used as a photoaffinity ligand. In two models of multidrug resistance reversal, i.e., sensitization to vincristine and modulation of rhodamine-123 uptake, B9209–005 and dexniguldipine-HCl showed identical biological activities. Photoaffinity labeling experiments with [^{3}H]B9209–005 in cell membranes from multidrug-resistant CCRF ADR-5000 cells, in comparison with labeling experiments with [^{3}H]azidopine (an established photoaffinity ligand for P-glycoprotein), showed that [^{3}H]B9209–005 labeled two proteins, with apparent molecular masses of 170 and 95 kDa. The pharmacological specificity of labeling was demonstrated by inhibition of photoincorporation by several cytostatic drugs transported by P-glycoprotein, as well as by chemosensitizers. Immunoprecipitation of the labeled proteins with the P-glycoprotein-specific monoclonal antibody C 219 and with a site-directed polyclonal antibody to the amino-terminal sequence of P-glycoprotein (amino acids 389–406) identified these proteins as intact P-glycoprotein and the amino-terminal fragment thereof. No specific labeling was obtained in the drug-sensitive parent cell line CCRF-CEM, which is devoid of significant P-glycoprotein expression. Maximal labeling of 17 pmol of the 170-kDa protein/mg of crude membrane protein was obtained. The affinity of [^{3}H]B9209–005 for binding to and photoincorporation into P-glycoprotein was 5-fold greater than that of [^{3}H]azidopine, and photoincorporation of [^{3}H]B9209–005 showed a different photoincorporation pattern, compared with [^{3}H]azidopine, in that the latter compound was incorporated specifically into the carboxyl-terminal 55-kDa fragment of P-glycoprotein. In contrast to [^{3}H]azidopine, no specific labeling of this fragment was obtained with [^{3}H]B9209–005, indicating different binding sites for or different photoincorporation of the two dihydropyridine ligands. Because B9209–005 carries the photoreactive azido group in the dihydropyridine moiety, whereas the azido group of azidopine is located in the side chain, these results suggest that the dihydropyridine moiety of the two compounds probably interacts with the amino-terminal part of P-glycoprotein, whereas the side chains react preferentially with the carboxyl-terminal 55-kDa fragment. The data clearly show that the chemosensitizing potency of dexniguldipine-HCl is due to direct interaction of dexniguldipine-HCl with and inhibition of P-glycoprotein. Furthermore, [^{3}H]B9209–005 is a valuable ligand for the identification, at the molecular level, of the dexniguldipine-HCl binding sites on P-glycoprotein.

Drug resistance is a major problem in the treatment of cancer. Initially sensitive tumors often develop drug resistance during multiple cycles of chemotherapy, whereas some tumors are intrinsically resistant to chemotherapy. A variety of mechanisms can lead to resistance, such as reduced cellular drug accumulation, increased detoxification, intracellular vesicularization of drugs, altered enzymatic activities, up- or down-regulation of targets, and enhanced DNA repair (1–3). One important mechanism leading to cross-resistance to chemically unrelated cytostatic drugs is the expression of P-glycoprotein in the membranes of tumor cells (4, 5). P-glycoprotein is an ATP-dependent drug efflux pump that transports a variety of different cytostatic drugs out of the cells, resulting in low and ineffective intracellular drug concentrations and in multidrug resistance (6).

As deduced from the cDNA sequence, human P-glycoprotein consists of 1280 amino acids (7) and is composed of two homologous halves with hydrophobic and hydrophilic domains. Structural features derived from hydropathy plots suggest a membrane topology of 12 transmembrane seg-

ABBREVIATIONS: BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ara-C, 1-[β-D-arabinofuranosyl]cytosine.

ments (8, 9), although other structures have also been proposed (10, 11). The amino-terminal half of the protein is composed of transmembrane segments 1–6, whereas the carboxyl-terminal half includes transmembrane segments 7–12. Each homologous half of the protein contains an ATP-binding cassette. Hydrolysis of ATP provides the energy source for transport of cytostatic drugs and other compounds such as rhodamine-123 and various lipophilic dyes (12, 13). The substrate specificity of P-glycoprotein is rather broad and includes *Vinca* alkaloids, anthracyclines, colchicine, and the taxanes.

Reversal of multidrug resistance is of major clinical concern, and chemosensitizers, which block P-glycoprotein activity, are being intensively investigated for clinical use (14) (for review, see Ref. 15). A number of chemically diverse compounds were shown to reverse multidrug resistance *in vitro* by blocking the P-glycoprotein pumping function. The hitherto best characterized compound is verapamil, with which a first clinical trial was reported in 1984 (16). Other compounds with promising *in vitro* activity are cyclosporin A (reviewed in Ref. 15), SDZ PSC 833 (17), S9788 (18), and the dihydropyridine derivative dexniguldipine-HCl (19).

Photoaffinity labeling is a valuable technique for the evaluation of drug binding sites and the elucidation of the function of P-glycoprotein. An often used photoaffinity labeling agent for P-glycoprotein has been [^{3}H]azidopine (20), another dihydropyridine derivative, which is an effective label for L-type Ca^{2+} channels and has shown some affinity for P-glycoprotein. Azidopine binding sites have been associated with two distinct regions of P-glycoprotein, with one site being within the amino-terminal half of the protein, between amino acids 198 and 440, and the other site being in the carboxyl-terminal half of the protein (21). An azidopine binding site on murine P-glycoprotein, encoded by the mouse *mdr-1b* gene, was identified near transmembrane segments 11/12 (22, 23).

To investigate the mechanism of multidrug resistance reversal by the new chemosensitizer dexniguldipine-HCl, and to identify its binding sites on P-glycoprotein, a photoaffinity derivative of dexniguldipine-HCl, B9209–005, was synthesized (Fig. 1). In this derivative, the nitro group in the phenyldihydropyridine moiety was replaced by the photolabile azido group. In contrast to azidopine, in which the photoreactive azido group is located in the side chain of the dihydropyridine moiety, B9209–005 carries this group at position 3 of the phenyl ring in the 4-phenyldihydropyridine moiety. Therefore, comparative photoaffinity labeling with [^{3}H]azidopine and [^{3}H]B9209–005, two dihydropyridine derivatives carrying the photoreactive group in two different pharmacophores (the dihydropyridine moiety and the side chain), is expected to provide valuable information for evaluation of the dihydropyridine binding site on P-glycoprotein.

Materials and Methods

Chemicals and supplies. [^{3}H]Azidopine was from Amersham (Braunschweig, Germany). The monoclonal antibody C 219 was purchased from Centocor (Malvern, PA). Fetal calf serum and Triton X-100 were from Boehringer (Mannheim, Germany). Adriamycin was purchased from Farmitalia (Freiburg, Germany). Anti-mouse IgG-agarose, RPMI 1640 medium, gentamicin, BSA, daunomycin, vincristine, vinblastine, methotrexate, campthotecin, taxol, and verapamil were from Sigma (Deisenhofen, Germany). Ara-C (Alexan)

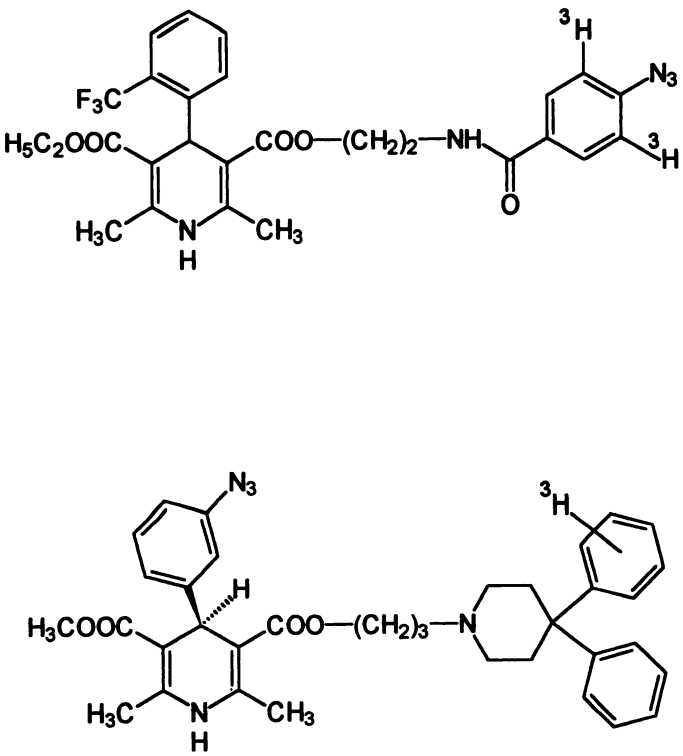

Fig. 1. Chemical structures of [^{3}H]azidopine (*top*) and [^{3}H]B9209–005 (*bottom*). Dexniguldipine-HCl carries a nitro group instead of the azido group in position 3 of the phenyl ring.

was purchased from Mack (Illertissen, Germany). All other chemicals were from commercial suppliers and were of the highest available purity. The structure of B9209–005 [3-[3-(4-[^{3}H]phenyl-4-phenyl-1-piperidinyl)propyl]-5-methyl-4(*R*)-(3-azidophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate] is shown in Fig. 1, together with the structure of [^{3}H]azidopine. [^{3}H]B9209–005 was prepared by tritium exchange in the precursor compound 4,4-diphenylpiperidine. A specific radioactivity of 414 GBq/mmol (11.2 Ci/mmol) was obtained.

Cell lines and cell culture. The human T lymphoblastoid cell line CCRF-CEM was obtained from the American Type Culture Collection (Rockville, MD). The selection of the multidrug-resistant CCRF-CEM sublines CCRF VCR-1000 and CCRF ADR-5000 has been reported elsewhere (24). Cells were propagated at 37° 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 (CCRF ADR-5000) or 1000 ng/ml vincristine (CCRF VCR-1000).

Preparation of plasma membranes. All steps for membrane preparation were carried out at 4°. Cells were homogenized with a Teflon/glass homogenizer in lysis buffer containing 10 mM Tris·HCl, pH 7.4, 10 mM NaCl, and 1.5 mM $MgCl_2$, in the presence or absence of 0.1 mM PMSF. After centrifugation at 300 × *g* for 10 min, the supernatant was centrifuged twice at 4000 × *g* for 20 min and at 35,000 × *g* for 1 hr. The resulting pellets were resuspended in lysis buffer containing 50% glycerol, and protein content was determined by a bicinchoninic acid assay (Pierce, Rockford, IL), using BSA as a standard.

Cellular proliferation. For monitoring of cellular proliferation, a modification of the original MTT colorimetric assay was used (25), by plating 40,000 cells in 80 μl of medium in microtiter plates. Chemosensitizers were dissolved in DMSO at a concentration of 10 mM and diluted with medium. Ten microliters of cytostatic agents and 10 μl of chemosensitizers were then added to achieve the desired concentrations in the test solution. All concentrations were assayed

in triplicate. After a 3-day incubation in a humidified 5% CO_2 atmosphere at 37°, the plates were analyzed by the MTT test. Ten microliters of tetrazolium dye (final concentration, 0.5 mg/ml) were added to each well and incubated for 4 hr. After centrifugation for 5 min at 200 × *g*, 60 μl of the supernatant were discarded and DMSO was added to the cells to achieve a final volume of 200 μl. After 1 hr of incubation, the formation of formazan was quantified spectrophotometrically at a wavelength of 540 nm.

Rhodamine-123 accumulation. Cells were centrifuged at 100 × *g* for 10 min and resuspended in serum-free RPMI 1640 medium in the presence of 10 mM HEPES/NaOH, pH 7.3. Cells (200,000 cells/sample) were incubated for 30 min at 37°, in a total volume of 1 ml, in the presence of chemosensitizers or solvent (DMSO). Glass tubes were used for all experiments, to avoid underestimation of the potency of the lipophilic dihydropyridines (26). Rhodamine-123 was added to achieve a total concentration of 0.8 mg/liter, and incubation was continued for 60 min. Cells were analyzed on an Epics Profile II fluorescence-activated cell sorter (Coulter, Krefeld, Germany). The excitation wavelength was 488 nm, and cell-associated rhodamine-123 fluorescence was measured at 520 nm. Concentration-response curves were constructed for each chemosensitizer. The concentration leading to a half-maximal increase of cell-associated rhodamine-123 (EC_{50}) was calculated by fitting a sigmoidal curve to the data.

Photoaffinity labeling and fluorography. Membranes (0.6 mg/ml) were incubated in phosphate-buffered saline, pH 7.4, for 1 hr at room temperature in the dark, in the presence of [^{3}H]B9209–005 or [^{3}H]azidopine. Irradiation was performed with a UV lamp (Camag, Berlin, Germany) at 366 nm for 20 min at 4°, at a distance of 10 cm. For separation of photolabeled proteins by SDS-polyacrylamide gel electrophoresis (27), 40-μl aliquots of the irradiated mixture were diluted 1/1 in sample buffer (0.125 M Tris·HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 0.01% bromphenol blue, 50% glycerol) and applied to 8% SDS-polyacrylamide gels. Gels were cut into 1-mm slices, and radioactivity was eluted by a 3-hr incubation in Biolute S (Zinsser, Frankfurt, Germany) at 50°. Radioactivity was quantified by liquid scintillation counting. When fluorography was used for measurement of photoincorporation, whole gels were incubated with Amplify (Amersham) for 30 min and dried on filter paper. Dried gels were applied to Hyperfilm (Amersham) for 10 days at −80°. Radioactivity was quantified by densitometric tracing of the fluorograms.

Production of polyclonal antisera. A peptide corresponding to amino acids 389–406 of human P-glycoprotein was synthesized by solid-phase synthesis. After purification by high performance liquid chromatography, the peptide was characterized by electrospray and plasma desorption mass spectrometry. The peptide was coupled to BSA with glutaraldehyde. Rabbits were immunized with 100 nmol of peptide in complete Freund's adjuvant and were boosted every 3 weeks. Antibody titers versus solid phase-bound peptides were tested by enzyme-linked immunosorbent assay. After high titers were obtained, sera were collected and stored. Specificity of the antibodies was tested in Western blots with membranes of CCRF ADR-5000 cells.

Immunoprecipitation. Fifty microliters of photolabeled membranes (30 μg) were solubilized with 10 μl of 2% SDS, heated for 3 min at 95°, and incubated for 30 min at room temperature. The mixture was diluted 20-fold with buffer (0.1% Triton X-100, 25 mM Tris·HCl, pH 7.8, 150 mM NaCl, 0.5 mg/ml globulin-free BSA), incubated for 30 min at 4°, and centrifuged at 15,000 × *g* for 30 min at 4°. Solubilized membranes were incubated for 2 hr at room temperature with immobilized antibodies bound to Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) (for antibody AB 389) or anti-mouse IgG-agarose beads (for antibody C 219). Beads were washed twice with the buffer described above and then three times with BSA-free buffer. Bound proteins were eluted from the beads by incubation in electrophoresis sample buffer and were separated by SDS-polyacrylamide gel electrophoresis.

Results

Activity of B9209–005 as a chemosensitizer of multidrug resistance. Dexniguldipine-HCl has been shown to possess strong multidrug resistance-reverting potency in various *in vitro* models of multidrug resistance. To obtain the photoreactive derivative [^{3}H]B9209–005, the nitro group in position 3 of the phenyl ring in the 4-phenyldihydropyridine moiety was replaced by an azido group, as shown in Fig. 1.

Before this compound could be used as a photolabel, it was necessary to establish that the biological activity was unaffected by the structure modification. The chemosensitizing potencies of B9209–005 and dexniguldipine-HCl to sensitize multidrug-resistant CCRF ADR-5000 cells to vincristine (as an established substrate for P-glycoprotein) were determined and compared with that of azidopine. Cellular proliferation was measured by using a 3-day colorimetric MTT test. For CCRF ADR-5000 cells and CCRF-CEM cells, dose-response curves for vincristine were generated in the absence or presence of chemosensitizers. In the absence of chemosensitizers, IC_{50} values of 0.7 ng/ml and 4.4 μg/ml were determined for vincristine in CCRF-CEM cells and CCRF ADR-5000 cells, respectively, corresponding to a 6285-fold vincristine resistance in CCRF ADR-5000 cells. Vincristine dose-response curves in CCRF ADR-5000 cells were then determined in the presence of B9209–005, dexniguldipine-HCl, and azidopine. All compounds sensitized CCRF ADR-5000 cells to the cytostatic effect of vincristine, with a concomitant decrease of vincristine IC_{50} values. The chemosensitization was dose dependent and showed complete reversal of vincristine resistance in the presence of high concentrations of chemosensitizers. Fig. 2 shows the reversal of vincristine resistance in CCRF ADR-5000 cells by B9209–005, dexniguldipine-HCl, and azidopine. EC_{50} values of 1.95, 1.55, and 6.31 μM were calculated for B9209–005, dexniguldipine-HCl, and azidopine, respectively. Thus, B9209–005 and dexniguldipine-HCl showed almost identical activities for multidrug resistance reversal, whereas azidopine was 3–4-fold less effective.

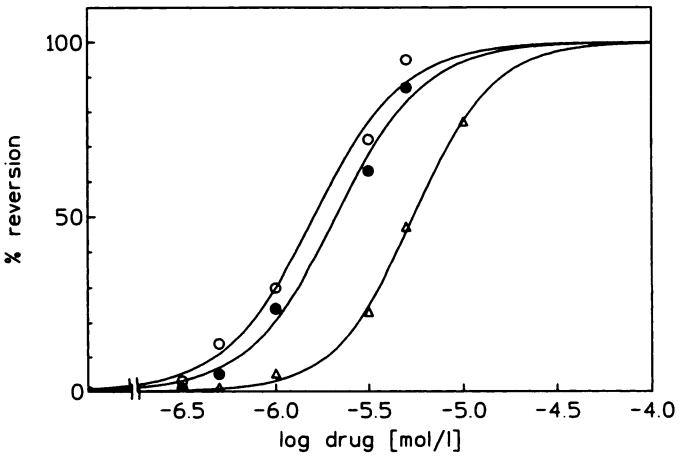

Fig. 2. Reversal of vincristine resistance in CCRF ADR-5000 cells by B9209–005 (●), dexniguldipine-HCl (○), and azidopine (△). Reversal of resistance was calculated as follows. The difference between vincristine log IC_{50} values in CCRF ADR-5000 cells and in CCRF-CEM cells equals 100% resistance. The modulator-mediated shifts of the log IC_{50} values at different chemosensitizer concentrations in the resistant cell line, toward the values for the sensitive cell line, were taken into relation to the 100% resistance value and give the percentage of inhibition at the particular concentration of chemosensitizer.

The increase in rhodamine-123 accumulation in P-glycoprotein-overexpressing and multidrug-resistant cells was determined as a further sensitive test system for evaluating the potency of chemosensitizing compounds. B9209–005, dexniguldipine-HCl, and azidopine elevated the cellular rhodamine-123 content in resistant and P-glycoprotein-positive CCRF VCR-1000 cells in a dose-dependent manner, as shown in Fig. 3 by the corresponding dose-response curves. Identical EC_{50} values of 80 nM were obtained for B9209–005 and dexniguldipine-HCl, whereas azidopine yielded a 13-fold higher EC_{50} value (1 μM). These results demonstrated identical biological activities for B9209–005 and the parent compound dexniguldipine-HCl, thus suggesting similar binding sites. Azidopine was only a weak chemosensitizer in both test systems.

Photoaffinity labeling of P-glycoprotein. Fig. 4 shows the photoaffinity labeling pattern of crude membranes derived from multidrug-resistant and P-glycoprotein-positive CCRF ADR-5000 cells with [^{3}H]B9209–005 and [^{3}H]azidopine. After photoaffinity labeling, membrane proteins were separated by SDS-polyacrylamide gel electrophoresis. [^{3}H]B9209–005 predominantly labeled four proteins, with apparent molecular masses of 170 kDa, 95 kDa, 55 kDa, and 38 kDa (12 experiments) (Fig. 4a). Photoincorporation into these proteins was quantified by calculating the area under the peak after subtraction of the background labeling. When photoaffinity labeling experiments were carried out in the presence of 10 μM dexniguldipine-HCl, labeling of the 170-kDa and 95-kDa proteins was reduced to 17 ± 9% (three experiments), whereas labeling of the 55-kDa proteins remained almost unchanged. A slight reduction of photolabeling to 66 ± 14% (three experiments) was seen for the 38-kDa protein in the presence of 10 μM dexniguldipine-HCl. Dexniguldipine-HCl at 100 μM completely inhibited photoincorporation into the 170- and 95-kDa proteins and reduced photolabeling of the 38-kDa protein to 51%. Vinblastine at concentrations of up to 100 μM did not inhibit photaffinity

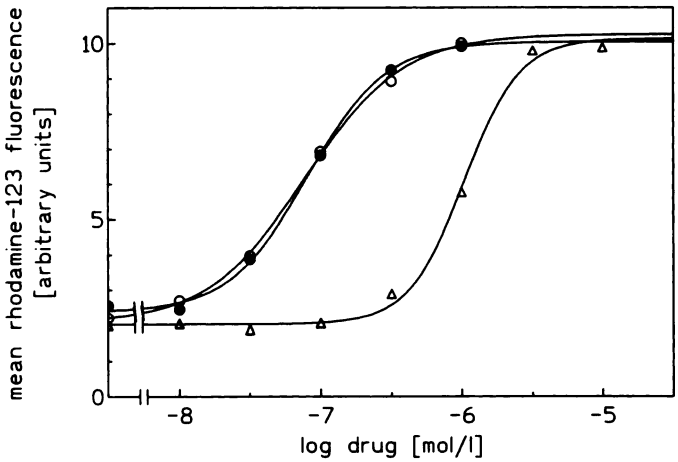

Fig. 3. Modulation of rhodamine-123 accumulation in multidrug-resistant CCRF VCR-1000 cells by B9209–005, dexniguldipine-HCl, and azidopine. CCRF VCR-1000 cells were incubated with rhodamine-123 in the presence of increasing concentrations of B9209–005 (●), dexniguldipine-HCl (○), or azidopine (△). Cellular rhodamine-123 content was determined at the single-cell level by fluorescence-activated cell sorting, as described in Materials and Methods. EC_{50} values of 80 nM were obtained for B9209–005 and dexniguldipine-HCl. For azidopine an EC_{50} value of 1 μM was obtained.

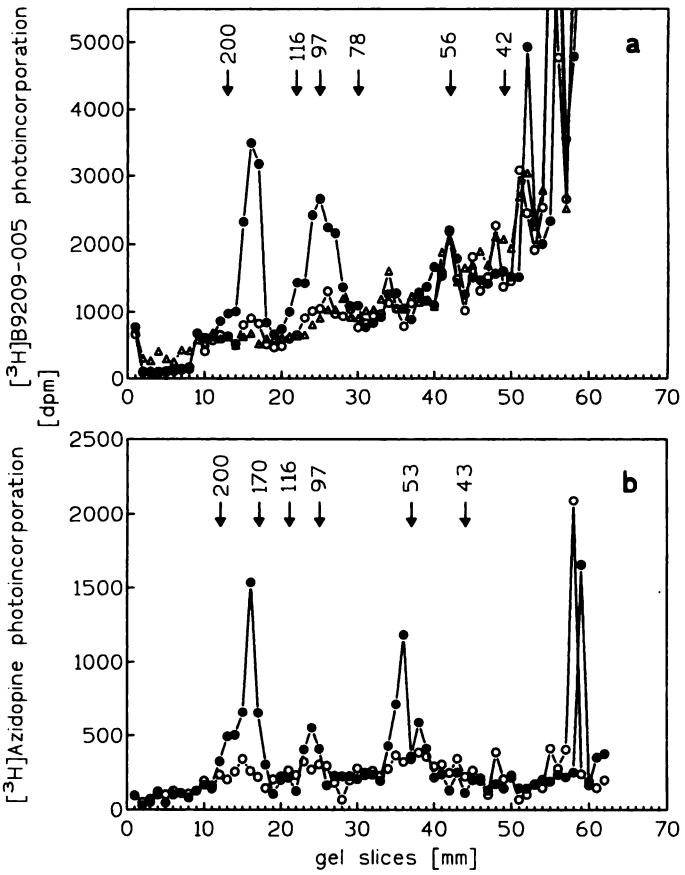

Fig. 4. Photoaffinity labeling of crude plasma membranes from CCRF ADR-5000 and CCRF-CEM cells. Plasma membranes (24 μg) from CCRF ADR-5000 (○, ●) and CCRF-CEM (△) cells were photolabeled with 1 μM [^{3}H]B9209–005 (a) or 0.3 μM [^{3}H]azidopine (b), in the absence (●) or presence of 10 μM (a, ○) or 30 μM (b, ○) unlabeled dexniguldipine-HCl. Plasma membrane proteins were separated on 8% polyacrylamide-SDS gels. Gels were cut into 1-mm slices and radioactivity was quantified by liquid scintillation counting. *Numbers at the top*, molecular mass standards (in kDa).

labeling of the 55- and 38-kDa proteins (data not shown). A ratio of approximately 1:1 for photoincorporation into the 170-kDa and 95-kDa proteins was found. The presence of PMSF during membrane preparation and photoaffinity labeling by [^{3}H]B9209–005 increased photoincorporation into the 170-kDa protein by approximately 42 ± 8%, with a concomitant 34 ± 7% (three experiments) decrease in the labeling of the 95-kDa protein, whereas labeling of the 55-kDa protein remained unchanged.

Identical experiments performed with [^{3}H]azidopine yielded labeling of three proteins, with apparent molecular masses of 170, 95, and 55 kDa (eight experiments) (Fig. 4b), whereas the presence of 30 μM dexniguldipine-HCl completely suppressed photoincorporation into these proteins. A ratio of 1:0.1:0.4 for photoincorporation into the 170-, 95-, and 55-kDa proteins was found. Greater background photolabeling was consistantly observed with [^{3}H]B9209–005 in the lower molecular mass proteins, in comparison with [^{3}H]-azidopine. The reason for this is not clear but may be related to the high lipophilicity of B9209–005, resulting in greater nonspecific photoincorporation. Membranes prepared from drug-sensitive CCRF-CEM cells were also labeled with [^{3}H]B9209–005. Only two proteins, with apparent molecular

masses of 55 kDa and 38 kDa, were labeled, whereas no photoincorporation into the 170- and 95-kDa proteins was found (Fig. 4a). These results suggest specific photolabeling of the 170- and 95-kDa proteins by [^{3}H]B9209–005.

To further characterize the photoaffinity labeling by [^{3}H]B9209–005 and [^{3}H]azidopine, experiments were performed with increasing concentrations of the two photoaffinity ligands. For [^{3}H]B9209–005 this was performed with higher concentrations of the radioactive photoligand, whereas in the case of [^{3}H]azidopine isotope dilution with unlabeled azidopine was used to achieve saturating concentrations. Fig. 5 shows the saturation isotherms for photoincorporation of both compounds into the 170-kDa protein. Fig. 5, *inset*, represents the results after Scatchard transformation. The linear Scatchard plots indicate homogeneity of the target proteins with regard to binding and photoincorporation. Half-maximal photolabeling with [^{3}H]B9209–005 was obtained at 0.35 μM, with maximal photoincorporation (I_{max}) of 17.3 pmol/mg of protein. The "affinity" of [^{3}H]azidopine for the process of photoaffinity labeling was 5-fold lower, with half-maximal labeling at 1.62 μM. The I_{max} value for [^{3}H]-azidopine was 22 pmol/mg of protein, which is in good agreement with the value obtained for [^{3}H]B9209–005.

The pharmacological specificity of [^{3}H]B9209–005 photolabeling was assessed with experiments in the presence of 10 μM vinblastine, vincristine, taxol, daunomycin, and doxorubicin (cytostatic agents known to be transported by P-glycoprotein). Methotrexate, ara-C, and camptothecin (cytostatic agents not related to multidrug resistance) were also tested. Furthermore, dexniguldipine-HCl, the photoaffinity ligands B9209–005 and azidopine, and verapamil were evaluated in these experiments. A concentration of 10 μM was chosen to facilitate a comparison of the potencies of these compounds. Higher concentrations (100 μM) led to almost complete inhibition of photoincorporation for several compounds, rendering a comparison of the potencies of these compounds more difficult. Most cytostatic agents and all chemosensitizing compounds significantly inhibited the photoaffinity labeling of the 170- and 95-kDa proteins (Table 1). Of the cytostatic agents tested, taxol and vinblastine were most active (74% and 71% inhibition, respectively), whereas doxorubicin and daunomycin showed only little inhibition (3% and 11%). Ara-C, methotrexate, and camptothecin (cytostatic agents unrelated to P-glycoprotein-mediated multidrug resistance) did not inhibit photoincorporation. Dexniguldipine-HCl and B9209–005 at 10 μM inhibited [^{3}H]B9209–005 photoincorporation by 77 and 80%, respectively, whereas verapamil yielded only 36% inhibition. Azidopine showed intermediate potency, with 51% inhibition. Complete dose-inhibition curves for photolabeling were determined for dexniguldipine-HCl and B9209–005, yielding similar IC_{50} values of 4.37 and 3.8 μM (mean of two experiments), respectively. These results were in agreement with the aforementioned data on the biological activities of both compounds, confirming that the replacement of the nitro group by an azido group does not modify the potency for multidrug resistance reversal. Fig. 6 shows the fluorographic determination of dexniguldipine-HCl inhibition of photoaffinity labeling. Concentrations varied from 100 μM to 22 nM. Parallel decreases in photoincorporation of the radioligand into the 170- and 95-kDa proteins were found with increasing concentrations of dexniguldipine-HCl. In contrast, no change in photoincorporation into the 55-kDa and 38-kDa proteins was found in the presence of dexniguldipine-HCl, pointing to a nonspecific labeling of the 55-kDa and 38-kDa proteins by [^{3}H]B9209–005.

To assess the identity of the photolabeled proteins, immunoprecipitations with the P-glycoprotein-specific monoclonal antibody C 219 and the polyclonal antibody AB 389 (raised against amino acids 389–406 of human P-glycoprotein) were performed. Immunoprecipitated proteins were separated on SDS gels, which were sliced and assayed for radioactivity. Control experiments were performed by eluting the solubilized proteins from Protein A-Sepharose or anti-mouse IgG-agarose without antibody. Only two labeled proteins, with molecular masses of 170 and 95 kDa, were detected after photolabeling with [^{3}H]B9209–005 and immunoprecipitation with C 219 and AB 389 (Fig. 7). When labeling experiments were performed with [^{3}H]azidopine instead of [^{3}H]B9209–

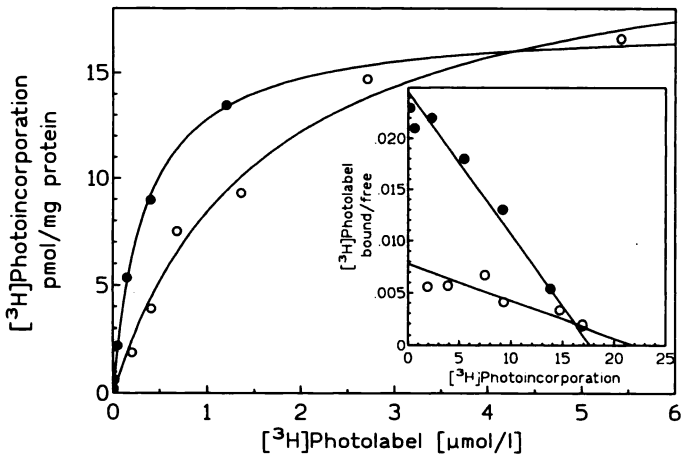

Fig. 5. Saturation isotherms for [^{3}H]B9209–005 (●) and [^{3}H]azidopine (○) photoincorporation. Plasma membranes (24 μg) from CCRF ADR-5000 cells were photolabeled with increasing concentrations of radioligands. After UV irradiation and separation of the labeled proteins on 8% polyacrylamide-SDS gels, incorporation of radioligands into the 170-kDa band was calculated. A one-binding site saturation isotherm was fitted to the data, and the concentration leading to half-maximal photoincorporation (K_d) was calculated. K_d values of 0.34 μM and 1.6 μM were obtained for [^{3}H]B9209–005 and [^{3}H]azidopine, respectively. The maximal incorporation values (I_{max}) were 17 pmol/mg of protein and 22 pmol/mg of protein for [^{3}H]B9209–005 and [^{3}H]azidopine, respectively. *Inset*, transformation of the data according to the method of Scatchard.

TABLE 1

Inhibition of photoaffinity labeling with [^{3}H]B9209–005 by cytostatic agents and chemosensitizers

All compounds were tested at a concentration of 10 μM. Photoincorporation was determined by fluorography and densitometric scanning of the fluorograms.

Compound	Inhibition
	%
Vinblastine	71
Vincristine	42
Daunomycin	11
Doxorubicin	3
Taxol	74
Methotrexate	0
Ara-C	0
Camptothecin	0
Verapamil	36
Dexniguldipine-HCl	77
B9209–005	80
Azidopine	51

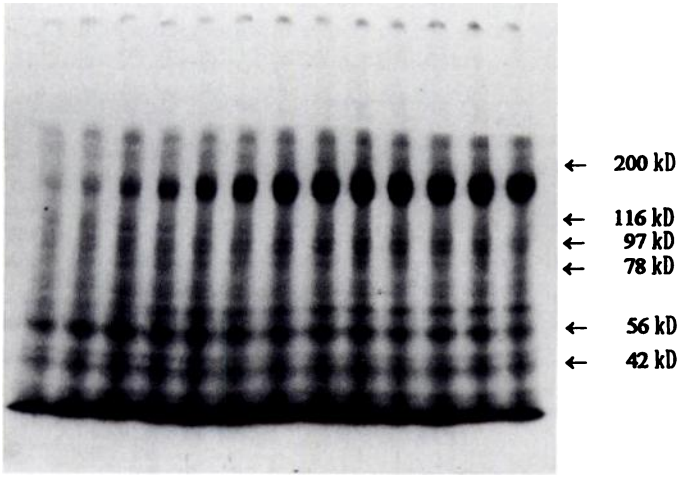

Fig. 6. Fluorography of a [^{3}H]B9209–005 photolabeling dose-inhibition experiment with dexniguldipine-HCl. Plasma membranes (18 μg) from CCRF ADR-5000 cells (prepared in the presence of PMSF) were photolabeled with 1.7 μM [^{3}H]B9209–005 in the absence (*right lane*) or presence of increasing concentrations of dexniguldipine-HCl. Dexniguldipine-HCl concentrations were (from *left* to *right*) 100, 47, 22, 10, 4.7, 2.2, 1, 0.47, 0.22, 0.1, 0.047, and 0.022 μM, plus control in the presence of solvent only. Radiolabeled proteins were separated by 8% polyacrylamide-SDS gel electrophoresis, and fluorography was performed as described in Materials and Methods. For the experiment shown, an IC_{50} value of 4.2 μM was determined. The concentration (L) of [^{3}H]B9209–005 was 1.7 μM. Calculation of an affinity constant (K_i) for dexniguldipine-HCl was performed according to the formula of Cheng and Prusoff, $K_i = IC_{50}/(1 + L/K_d)$. A value of 0.35 μM was taken as the K_d value (see Results). A K_i value of 0.7 μM was calculated.

005, an additional 55-kDa fragment was immunoprecipitated by antibody C 219 (data not shown). These results are in accord with the identity of the 170-kDa protein as intact P-glycoprotein and that of the 95-kDa band as the amino-terminal proteolytic fragment of P-glycoprotein. Immunoprecipitations using C 219 and AB 389 with [^{3}H]B9209–005-photolabeled membranes prepared in the presence of PMSF demonstrated approximately 40% greater photoincorporation into the 170-kDa protein and a reduction in the labeling of the 95-kDa protein by approximately 30%.

Discussion

The present study was performed to characterize the biochemical mechanism of multidrug resistance reversal by dexniguldipine-HCl and to identify binding sites on putative target proteins. Dexniguldipine-HCl is a potent new chemosensitizer for P-glycoprotein-mediated multidrug resistance (28, 29). It was previously shown to reverse resistance in a number of P-glycoprotein-positive and multidrug-resistant cell lines (30) and to increase accumulation of rhodamine-123, a well known substrate for P-glycoprotein, in these cell lines (31, 32). From these data it was deduced that dexniguldipine-HCl exerts its effect by an interaction with P-glycoprotein. Nevertheless, definite proof of a direct interaction with P-glycoprotein was still lacking. Although it seems clear that P-glycoprotein is involved in the action of dexniguldipine-HCl, mechanisms other than direct interaction with and inhibition of P-glycoprotein are possible. For instance, for tamoxifen, another multidrug resistance modulator, a decrease in membrane fluidity was reported and considered to be relevant for multidrug resistance reversal (33). Similar

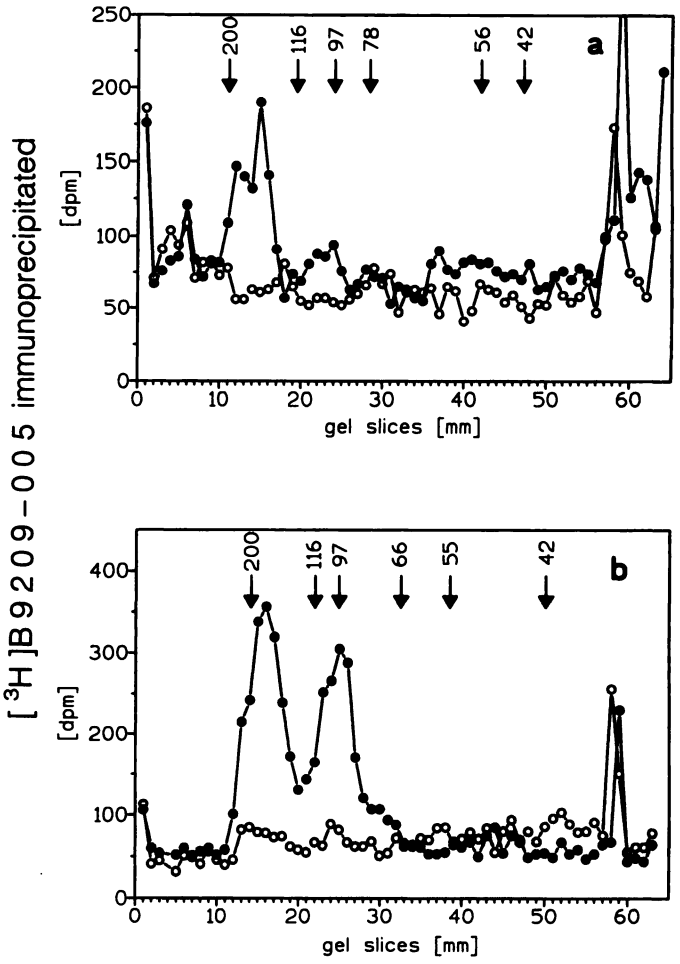

Fig. 7. Immunoprecipitation of membrane proteins from CCRF ADR-5000 cells labeled with [^{3}H]B9209–005, with monoclonal antibody C 219 (a, ●) or polyclonal antibody 389 (b, ●). Control precipitations of Protein A-Sepharose- or anti-mouse IgG-agarose-bound proteins in the absence of specific antibodies (○) are also shown. Plasma membranes (30 μg) were photolabeled with 0.6 μM [^{3}H]B9209–005 and solubilized with SDS/Triton X-100. Precipitated proteins were solubilized in sample buffer and separated on 8% polyacrylamide-SDS gels. Gels were sliced and counted as described for Fig. 4.

situations were found for Cremophor EL and detergents like Tween 80, whose chemosensitizing property may be attributed to membrane perturbations (34).

Therefore, it seemed necessary to address the question of the molecular mechanism of multidrug resistance reversal by dexniguldipine-HCl. The most suitable way seemed to be the development of a photoreactive dexniguldipine-HCl derivative and the use of this tool for the identification of target proteins. The use of photoaffinity ligands bears the problem that parent compounds must be modified by the addition of photolabile azido, benzophenone, or diazirine groups. This often results in a concomitant decrease in biological activity, which makes the resulting compounds less suitable for this purpose. However, the biological activity of B9209–005 with respect to multidrug resistance reversal remained almost unchanged. In a 3-day colorimetric MTT test monitoring cellular proliferation and in a short-term rhodamine-123 accumulation test measuring P-glycoprotein transport function, B9209–005 had activity similar to that of dexniguldipine-HCl. In addition, the two compounds showed almost

identical potency in competing with [^{3}H]B9209–005 photolabeling in CCRF ADR-5000 membranes, suggesting that a direct interaction of compounds with P-glycoprotein is relevant for multidrug resistance reversal. These results render [^{3}H]B9209–005 a highly suitable ligand for the investigation of the target protein for multidrug resistance reversal.

[^{3}H]B9209–005 specifically labeled two proteins, with apparent molecular masses of 170 kDa and 95 kDa, in P-glycoprotein-positive CCRF ADR-5000 cell membranes. These proteins were not labeled in the drug-sensitive control cell line CCRF-CEM. The specificity of this labeling pattern was examined in labeling experiments performed in the presence of cytostatic agents known to be transported by P-glycoprotein. *Vinca* alkaloids, anthracyclines, and taxanes inhibited the incorporation of the photoligand into the 170- and 95-kDa proteins. The rank order of potency for vinblastine, vincristine, and doxorubicin to inhibit photoincorporation was the same as that found in radioligand binding studies with P-glycoprotein, using [^{3}H]vinblastine as radioligand (35). In addition, the chemosensitizers dexniguldipine-HCl and verapamil also inhibited photoincorporation. The low potency of verapamil is in agreement with the weak activity of this compound in the rhodamine-123 accumulation assay in CCRF VCR-1000 cells (32) and with the low potency of verapamil in inhibiting [^{3}H]azidopine photolabeling of P-glycoprotein (20, 21). Furthermore, azidopine inhibition of photoincorporation suggests that B9209–005 and azidopine interact with the same, or at least overlapping, binding sites. These pharmacological data strongly suggest that the target protein of B9209–005 is indeed P-glycoprotein.

The most definitive evidence that the labeled proteins correspond to P-glycoprotein comes from our immunoprecipitation data with the monoclonal antibody C 219 and the site-directed polyclonal antibody AB 389. C 219 is an established antibody against P-glycoprotein and recognizes amino- and carboxyl-terminal intracellular epitopes (amino acids 568–574 and amino acids 1213–1219) near both ATP-binding cassettes (36). Immunoprecipitation with this monoclonal antibody yielded two radiolabeled proteins, of 170 and 95 kDa. The 95-kDa protein was identified as the amino-terminal fragment of P-glycoprotein by immunoprecipitation with AB 389, which is directed against amino acids 389–406 of P-glycoprotein. A protein with the same molecular mass (95 kDa) was observed previously and was identified as the amino-terminal tryptic fragment of P-glycoprotein by the same technique (22, 37).

In addition to the specifically labeled 170- and 95-kDa proteins, [^{3}H]B9209–005 labeled two other proteins, with apparent molecular masses of 55 and 38 kDa. The 55-kDa protein labeling is clearly nonspecific, because high concentrations of vinblastine and dexniguldipine-HCl did not suppress photoincorporation of the labeled ligand. Furthermore, in the drug-sensitive cell line CCRF-CEM (without significant P-glycoprotein expression), a protein with the same molecular mass (55 kDa) was labeled by [^{3}H]B9209–005, indicating that this protein is nonspecifically labeled and is not involved in the P-glycoprotein-mediated multidrug resistance phenotype. For the 38-kDa protein the situation is less clear. Photoincorporation was reduced by 34% (compared with 83% reduction for the 170-kDa protein) in the presence of 10 μM dexniguldipine. However, no inhibition of photoincorporation into this protein was observed with up to 100 μM vinblastine. This finding and the appearance of a similar, although somewhat smaller, peak in the sensitive cell line are more compatible with nonspecific labeling. Furthermore, when low concentrations (30–200 μM) of [^{3}H]B9209–005 were used for photoincorporation, this protein was not labeled, whereas the 170-, 95-, and 55-kDa proteins showed prominent photoincorporation (data not shown).

The nonspecific nature of [^{3}H]B9209–005 incorporation into the 55-kDa protein contrasts with reported results obtained with [^{3}H]azidopine (22, 37). It was reported that, after tryptic cleavage, specific [^{3}H]azidopine incorporation into two proteins, with apparent molecular masses of 95 and 55 kDa, was observed. These proteins were identified, by using site-directed antibodies against P-glycoprotein, as the amino-terminal and carboxyl-terminal halves, respectively, of P-glycoprotein (37). Therefore, we decided to perform additional photolabeling experiments with [^{3}H]azidopine and to compare the labeling patterns of these two dihydropyridine photoligands. [^{3}H]Azidopine specifically labeled three proteins, with apparent molecular masses of 170, 95, and 55 kDa, which were identified as P-glycoprotein and the amino-terminal and carboxyl-terminal proteolytic fragments thereof, respectively. The appearance of the 95-kDa and 55-kDa fragments of P-glycoprotein indicates the presence of some proteolytic activity in our preparation in the absence of protease inhibitors. Indeed, when high concentrations of PMSF were included during membrane preparation, the amount of photoincorporation into the 170-kDa protein was increased, with a concomitant decrease in the labeling of the 95-kDa and 55-kDa fragments. Taken together, these data indicate that [^{3}H]azidopine labels two different sites of P-glycoprotein, one in the amino-terminal 95-kDa fragment and the other in the carboxyl-terminal 55-kDa fragment of P-glycoprotein. These data are in agreement with the literature (22, 37). In contrast, [^{3}H]B9209–005 is specifically photoincorporated only into the 170-kDa and 95-kDa proteins, indicating a somewhat different reactivity of this compound with the molecular structures of P-glycoprotein.

The apparent affinities of [^{3}H]B9209–005 and [^{3}H]azidopine for P-glycoprotein were determined in experiments where P-glycoprotein was photolabeled in the presence of increasing concentrations of both photoligands. The concentration of [^{3}H]B9209–005 necessary to achieve half-maximal photoincorporation (K_d value) was 0.35 μM. A 5-fold higher concentration was necessary with [^{3}H]azidopine, clearly indicating the lower affinity of this compound for binding and photoincorporation. These data are in accord with the 5–10-fold lower biological activity of azidopine in the two biological models of multidrug resistance reversal, i.e., potentiation of vincristine cytotoxicity in CCRF ADR-5000 cells and modulation of rhodamine-123 uptake in CCRF VCR-1000 cells. Transformation of the data according to the method of Scatchard resulted in straight lines, showing homogeneity of the target proteins with respect to the binding and photoincorporation process. Because Scatchard transformation is normally used for reversible binding under equilibrium conditions, data should not be interpreted as true K_d values but as apparent K_d values. Nevertheless, these data cleary document the greater efficacy of [^{3}H]B9209–005, compared with azidopine. The maximal incorporation (I_{max}) values were similar for the two compounds, and I_{max} reached an extrapolated value of 17 pmol/mg of crude membrane protein for

[^{3}H]B9209–005. This value is in excellent agreement with a B_{max} value of 17 pmol/mg of membrane protein obtained in the same cell line with [^{3}H]vinblastine as radioligand for monitoring reversible binding to P-glycoprotein (38). Our K_d value of 1.6 μM for [^{3}H]azidopine is similar to data from Yoshimura *et al.* (37), who reported half-maximal [^{3}H]azidopine photoincorporation at a concentration of 0.8 μM, but contrasts with data from others, who obtained an affinity constant of approximately 0.1 μM for [^{3}H]azidopine in Chinese hamster lung cells selected for resistance to vincristine (20). The latter difference may be due to the use of cell lines derived from different organs (lung versus hematopoietic cells) or species (hamster versus human). Also, interlaboratory differences in the UV irradiation procedures may be responsible.

Our photoaffinity labeling experiments with [^{3}H]azidopine are in accord with data obtained by others. Yoshimura *et al.* (37) reported predominant incorporation of [^{3}H]azidopine into the carboxyl-terminal 55-kDa fragment of P-glycoprotein. Similar data were reported by Bruggemann *et al.* (21), who showed that the compound is incorporated into both halves of P-glycoprotein. In their work, photoincorporation into the amino- and carboxyl-terminal halves was inhibited equally by vinblastine. From those data, those authors concluded that the two halves come together to form a common binding site for [^{3}H]azidopine and that the azido group of azidopine, bound to its binding site, is able to react with both halves of the protein. Furthermore, those authors speculated that the amino- and carboxyl-terminal sites labeled by [^{3}H]-azidopine are confined to transmembrane domains 5/6 and 11/12. Our data obtained with [^{3}H]azidopine are in accord with their results. In contrast to azidopine, which carries the photoreactive moiety in the side chain, B9209–005 contains the reactive species in the dihydropyridine moiety and is not incorporated into the carboxyl-terminal half of P-glycoprotein, i.e., the 55-kDa fragment. From these data, a refined model of the dihydropyridine binding site can be deduced, in which the dihydropyridine moiety interacts with the amino-terminal half of P-glycoprotein, i.e., transmembrane segments 1–6, and the side chain extends to the carboxyl-terminal half, i.e., transmembrane segments 7–12. Depending on the position of the photolabile group, different labeling patterns are observed for [^{3}H]azidopine and [^{3}H]B9209–005. The flexibility of the side chain, which carries the azido group in azidopine, may be responsible for the labeling of both the 95-kDa and 55-kDa fragments when this compound is used as a photoligand. It may well be that transmembrane domains 5/6 in the amino-terminal half and transmembrane domains 11/12 in the carboxyl-terminal half of P-glycoprotein are involved in the formation of a common binding site for chemosensitizers. This assumption is also supported by the data of Greenberger (39), who showed that [^{125}I]iodoarylazidoprazosin is incorporated into an amino-terminal 5-kDa fragment and a carboxyl-terminal 4-kDa fragment of murine P-glycoprotein. The amino-terminal fragment is probably derived from a region near transmembrane domain 6, whereas the carboxyl-terminal fragment most probably extends from amino acid 998 to amino acid 1044 and is located carboxyl-terminally to transmembrane domain 12.

In summary, our data show that the new photoaffinity ligand [^{3}H]B9209–005 possesses high specificity and high affinity for P-glycoprotein. In comparison with azidopine, B9209–005 seems to be a more suitable ligand for the evaluation of the binding sites for chemosensitizers with dihydropyridine structures, because it possesses a greater chemosensitizing potential for multidrug resistance reversal than does azidopine. Furthermore, we demonstrated that this compound is a valuable tool for photoaffinity labeling of P-glycoprotein and will be useful for the biochemical and molecular evaluation of the dihydropyridine binding site on P-glycoprotein.

Acknowledgments

The authors thank Prof. H. Glossmann and Dr. J. Striessnig for the synthesis of peptide 389 and the production of polyclonal antisera and antibodies.

References

1. Judson, I. R. Understanding anticancer drug resistance: opportunities for modulation and impact on new drug design. *Eur. J. Cancer* **28**:285–289 (1992).
2. Beck, W. T. Mechanisms of multidrug resistance in human tumor cells: the role of P-glycoprotein, DNA topoisomerase II, and other factors. *Cancer Treat. Rev.* **17**:11–20 (1990).
3. Hayes, J. D., and C. R. Wolf. Molecular mechanisms of drug resistance. *Biochem. J.* **272**:281–295 (1990).
4. Juliano, R. L., and V. Ling. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* **455**:152–162 (1976).
5. Gottesman, M. M., and I. Pastan. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**:385–427 (1993).
6. Gottesman, M. M., and I. Pastan. Resistance to multiple chemotherapeutic agents in human cancer cells. *Trends Pharmacol. Sci.* **9**:54–58 (1988).
7. Chen, C.-J., J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesmann, and I. B. Roninson. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47**:381–389 (1986).
8. Gros, P., J. Croop, and D. E. Housman. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell* **47**:371–380 (1986).
9. Gerlach, J. H., J. A. Endicott, P. F. Juranka, G. Henderson, F. Sarangi, K. L. Deuchars, and V. Ling. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature (Lond.)* **324**:485–489 (1986).
10. Zhang, J. T., M. Duthie, and V. Ling. Membrane topology of the N-terminal half of the hamster P-glycoprotein molecule. *J. Biol. Chem.* **268**:15101–15110 (1993).
11. Skach, W. R., M. C. Calayag, and V. R. Lingappa. Evidence for an alternate model of human P-glycoprotein structure and biogenesis. *J. Biol. Chem.* **268**:6903–6908 (1993).
12. Doige, C. A., and F. S. Sharom. Transport properties of P-glycoprotein in plasma membrane vesicles from multidrug-resistant Chinese hamster ovary cells. *Biochim. Biophys. Acta* **1109**:161–171 (1992).
13. Shapiro, A. B., and V. Ling. ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. *J. Biol. Chem.* **269**:3745–3754 (1994).
14. Sikic, B. I. Modulation of multidrug reistance: at the threshold. *J. Clin. Oncol.* **11**:1629–1635 (1993).
15. Raderer, M., and W. Scheithauer. Clinical trials of agents that reverse multidrug resistance. *Cancer (Phila.)* **72**:3553–3563 (1993).
16. Presant, C. A., P. Kennedy, C. Wiseman, K. Gala, and M. Wyres. Verapamil plus Adriamycin: a phase I-II clinical study. *Proc. Am. Soc. Clin. Oncol.* **3**:32 (1984).
17. Fisher, G. A., J. Halsey, J. Hausdorff, B. L. Lum, N. A. Brophy, H. Collins, D. Nix, and B. I. Sikic. A phase I study of paclitaxel (taxol®) (T) in combination with SDZ PSC 833, a potent modulator of multidrug resistance (MDR). *Anti-Cancer Drugs* **5**(Suppl. 1):43 (1994).
18. Mazier, B., M. Berlion, C. Lucas, M. Paraire, J. Berille, B. Giroux, B. Gerard, M. Sarkani, and J. P. Bizarri. S9788: multidrug resistance modulator. *Drugs Future* **18**:711–713 (1993).
19. Reiter, W. W., C. Ludescher, B. Wörmann, V. Nüßler, C. Gattringer, C. Reiber, C. Weimar, M. R. Nowrousian, and J. Thaler. A phase II trial with dexniguldipine, a new multidrug resistance modulator in combination with VAD or VECD in patients with refractory myeloma. *Ann. Oncol.* **5**(suppl.):136 (1994).
20. Safa, A. R., C. J. Glover, J. L. Sewell, M. B. Meyers, J. L. Biedler, and R. L. Felsted. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J. Biol. Chem.* **262**:7884–7888 (1987).
21. Bruggemann, E. P., S. J. Currier, M. M. Gottesman, and I. Pastan. Char-

acterization of the azidopine and vinblastine binding site of P-glycoprotein. *J. Biol. Chem.* **267**:21020–21026 (1992).
22. Greenberger, L. M., C. J. Lisanti, J. T. Silva, and S. B. Horwitz. Domain mapping of the photoaffinity drug-binding sites in P-glycoprotein encoded by mouse *mdr1b*. *J. Biol. Chem.* **266**:20744–20751 (1991).
23. Greenberger, L. M., C. P. H. Yang, E. Gindin, and S. B. Horwitz. Photoaffinity probes for the α_1-adrenergic receptor and the calcium channel bind to a common domain in P-glycoprotein. *J. Biol. Chem.* **265**:4394–4401 (1990).
24. Kimmig, A., V. Gekeler, M. Neumann, G. Frese, R. Handgretinger, G. Kardos, H. Diddens, and D. Niethammer. Susceptibility of multidrug resistant human leukemia cell lines to human interleukin 2-activated killer cells. *Cancer Res.* **50**:6793–6799 (1990).
25. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63 (1983).
26. Boer, R., A. Grassegger, C. Schudt, and H. Glossmann. (+)-Niguldipine binds with very high affinity to Ca^{2+} channels and to a subtype of α_1-adrenoceptors. *Eur. J. Pharmacol.* **172**:131–146 (1989).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
28. Hill, B. T., and L. K. Hosking. 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 (1994).
29. Reymann, A., G. Looft, C. Woermann, M. Dietel, and R. Erttmann. Reversal of multidrug resistance in Friend leukemia cells by dexniguldipine-HCl. *Cancer Chemother. Pharmacol.* **32**:25–30 (1993).
30. Ise, W., V. Gekeler, and K. H. Sanders. Use of dexniguldipine-HCl (B8509–035) to circumvent multidrug resistance (MDR) mediated by P-170 glycoprotein. *Clin. Exp. Metastasis* **10** (Suppl. 1):104 (1992).
31. Hofmann, J., A. Wolf, M. Spitaler, G. Böck, J. Drach, C. Ludescher, and H. Grunicke. Reversal of multidrug resistance by B859–35, a metabolite of B859–35, niguldipine, verapamil and nitrendipine. *J. Cancer Res. Clin. Oncol.* **118**:361–366 (1992).
32. Boer, R., S. Haas, and A. Schödl. Influence of dexniguldipine-HCl on rhodamine-123 accumulation in a multidrug-resistant leukaemia cell line: comparison with other chemosensitisers. *Eur. J. Cancer* **30A**:1117–1123 (1994).
33. Wiseman, H. Tamoxifen: new membrane-mediated mechanisms of action and therapeutic advances. *Trends Pharmacol. Sci.* **15**:83–89 (1994).
34. Woodcock, D. M., M. E. Linsenmeyer, G. Chojnowski, A. B. Kriegler, V. Nink, L. K. Webster, and W. H. Sawyer. Reversal of multidrug resistance by surfactants. *Br. J. Cancer* **66**:62–68 (1992).
35. Ferry, D. R., M. A. Russel, and M. H. Cullen. P-glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is allosterically coupled to a *Vinca* alkaloid-selective binding site. *Biochem. Biophys. Res. Commun.* **188**:440–445 (1992).
36. Georges, E., G. Bradley, J. Gariepy, and V. Ling. Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **87**:152–156 (1990).
37. Yoshimura, A., Y. Kuwazuru, T. Sumizawa, M. Ichikawa, S. Ikeda, T. Uda, and S. Akiyama. Cytoplasmic orientation and two-domain structure of the multidrug transporter, P-glycoprotein, demonstrated with sequence-specific antibodies. *J. Biol. Chem.* **264**:16282–16289 (1989).
38. Malkhandi, J., D. R. Ferry, R. Boer, V. Gekeler, W. Ise, and D. J. Kerr. Dexniguldipine-HCl is a potent allosteric inhibitor of [^{3}H]vinblastine binding to P-glycoprotein of CCRF ADR-5000 cells. *Eur. J. Pharmacol.* **288**: 105–113 (1994).
39. Greenberger, L. Major photoaffinity drug labeling sites for iodoaryl azidoprazosin are within, or immediately C-terminal to, transmembrane domains 6 and 12. *J. Biol. Chem.* **268**:11417–11425 (1993).

Send reprint requests to: Rainer Boer, Byk Gulden Lomberg GmbH, Byk Gulden-Str. 2, D-78467 Konstanz, Germany.