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The α_1-ADRENERGIC PHOTOAFFINITY PROBE [^{125}I]ARYLAZIDOPRAZOSIN BINDS TO A SPECIFIC PEPTIDE OF P-GLYCOPROTEIN IN MULTIDRUG-RESISTANT CELLS

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SUMMARY: Much evidence suggests that P-glycoprotein (P-gp) confers multidrug-resistance (MDR) in tumor cells by energy-dependent efflux of hydrophobic cytotoxic agents. In this study, we have used the α_1-adrenergic photoaffinity probe, [^{125}I]arylazidoprazosin ([^{125}I]AAP), and identified P-gp as a specific acceptor for prazosin. Drugs to which MDR cells are resistant, including vincristine, vinblastine, doxorubicin, actinomycin D and colchicine as well as agents reversing MDR, including verapamil, nicardipine, prenylamine, diltiazem, trifluoperazine, dibucaine, reserpine, monensin, and progesterone, differentially reduced [^{125}I]AAP photolabeling of P-gp. We also analyzed the influence of α_2-adrenergic drugs and dopaminergic drugs on [^{125}I]AAP photolabeling of P-gp. Limited proteolysis of [^{125}I]AAP photolabeled P-gp with Staphylococcus aureus V8 protease revealed that prazosin binds to a single 8 kDa fragment of P-gp.

Tumor cells selected in vitro for resistance to natural product anticancer drugs such as vinblastine or doxorubicin are cross-resistant to a variety of structurally and functionally unrelated cytotoxic agents (1, 2). The hallmark of this multidrug resistance (MDR) is the overexpression of a transmembrane glycoprotein called P-glycoprotein (P-gp) (2). Evidence that P-gp is the mediator of MDR comes from DNA transfection experiments. Transfection of sensitive cells with cDNA encoding P-gp results in the MDR phenotype (3). The protein binds to drugs (4, 5) and ATP (6), and in pure form displays Mg^{2+}-dependent ATPase activity (7). These data suggest that P-gp functions as an efflux pump, extruding drugs out of MDR cells by an active energy-dependent process (8).

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We have recently demonstrated that P-gp binds specifically to photoactive analogs of vinblastine (4), colchicine (5), calcium channel blockers (9, 10) and many agents which reverse MDR (11). In this study, we have identified P-gp as a specific acceptor for an α_1-adrenergic receptor antagonist, prazosin, by photoaffinity labeling experiments using [^{125}I]arylazidoprazosin ([^{125}I]AAP) (12). Our data indicate the presence of a distinct, specific prazosin-binding peptide on P-gp which is cross-reactive with several classes of MDR cross-resistant agents and compounds reversing MDR.

MATERIALS AND METHODS

MATERIALS. The α_1-adrenergic photoaffinity probe, [^{125}I]AAP (2200 Ci/mmol), was purchased from New England Nuclear. [^{14}C]-Labeled-methylated molecular weight standards (carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21.5 kDa; cytochrome C, 12.5 kDa and aprotinin, 6.5 kDa) were purchased from Amersham. Vinblastine and vincristine were obtained from Eli Lilly & Co. Actinomycin D, doxorubicin, colchicine, methotrexate, (±)verapamil, (±)nicardipine, diltiazem, prenylamine, trifluoperazine, dibucaine, reserpine, progesterone, (±)epinephrine HCl, (±)norepinephrine HCl, methoxamine, clonidine HCl, yohimbine HCl, dopamine (3-hydroxytyramine), haloperidol, and sulpiride were purchased from Sigma. Monensin was obtained from Calbiochem. Monoclonal antibody C219 specific for P-gp was purchased from Centacor. Staphylococcus aureus V8 protease was purchased from ICN Biochemicals.

CELL CULTURE AND CYTOTOXICITY ASSAYS. DC-3F Chinese hamster lung cells and MDR variant DC-3F/VCRd-5L cells (2750-fold resistant to vincristine) and cross-resistance to doxorubicin (220-fold), vinblastine (1000-fold), actinomycin D (1000-fold) and colchicine (1000-fold) were cultured as previously described (13, 14). The effect of prazosin on the growth of DC-3F and DC-3F/VCRd-5L cells was determined as previously described (15).

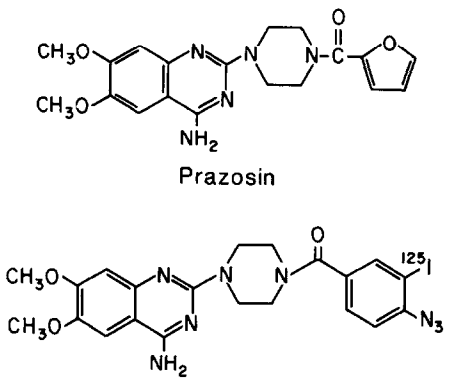
ACCUMULATION AND OUTWARD TRANSPORT STUDIES. [^{125}I]AAP accumulation and outward transport studies were carried out by previously published procedures (8, 16). Radioactivity associated with the cells was measured in a Packard gamma counter.

PHOTOAFFINITY LABELLING. Exponentially growing cells (5 x 10^5 cells/assay) or plasma membranes prepared from DC-3F and DC-3F/VCRd-5L cells (10) were photoaffinity labeled with 7.5 nM of [^{125}I]AAP (2200 Ci/mmol) in the absence or presence of competing ligands (4,5,9). Immunoprecipitations of [^{125}I]AAP photolabeled membrane vesicles with anti-P-gp monoclonal antibody C219 was carried out as previously described (4). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography (4,5). The percent inhibition of labeling was determined by cutting bands from the gels and measuring their radioactivity.

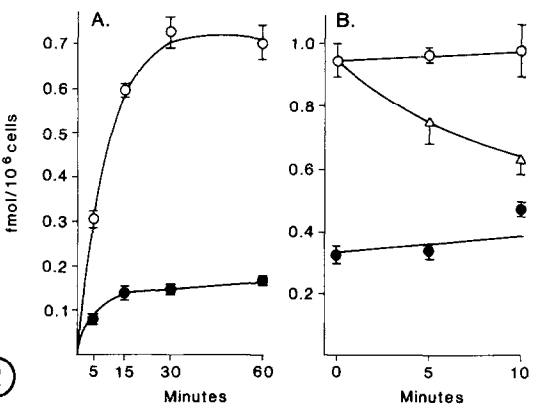
PEPTIDE MAPPING. The method of Cleveland et al. (17) using S. aureus V8 protease was used for peptide mapping of the [^{125}I]AAP photolabeled P-gp. The drug-bound peptides were resolved by a 10-20% SDS-urea gel (4,17).

RESULTS AND DISCUSSION

The chemical structures of prazosin, an α_1-adrenergic receptor antagonist, and its photoaffinity analog [^{125}I]AAP are presented in Fig. 1. To determine whether [^{125}I]AAP is a substrate for the outward drug transport system of the MDR cells, we measured its cellular uptake into sensitive DC-3F and resistant variant DC-3F/VCRd-5L cells (Fig. 2A). The internal concentration of [^{125}I]AAP reached steady-state after 30 min incubation of cells with 8 pM [^{125}I]AAP. There was a 4-fold decrease in the intracellular accumulation of [^{125}I]AAP in the resistant cells. This defect in intracellular accumulation of [^{125}I]AAP accounts for the presence of an active efflux of the drug across the plasma membrane in the MDR cells. Fig. 2B shows that [^{125}I]AAP was actively effluxed from the resistant cells. The [^{125}I]AAP accumulation in the resistant cells increased to the level of sensitive cells when 10 mM sodium azide, an inhibitor of oxidative phosphorylation (8, 16), was added to the cells in Hank's Balanced Salt Solution (HBSS) without glucose (8, 16). At steady-state, [^{125}I]AAP accumulation (30 min), 1 g/l glucose was added and outward transport of [^{125}I]AAP was measured



(1)



(2)

Fig. 1 Structure of prazosin and its photoaffinity analog, [^{125}I]arylazidoprazosin.

Fig. 2 A. [^{125}I]AAP uptake into DC-3F (O) and DC-3F/VCRd-5L (●) cells. Cells were incubated with 8 pM [^{125}I]AAP (2200 Ci/mmol) in the growth medium at 37°C for various periods and the intracellular drug level was determined at the indicated times.
B. DC-3F/VCRd-5L cells were incubated at 37°C in normal HBSS (●) and in glucose-free HBSS containing 10 mM NaN_3 (O) (8) with 8 pM [^{125}I]AAP for 30 min. After washing with cold glucose-free HBSS, the cells were exposed to HBSS containing 1 g/l glucose at 37°C for 10 min (Δ). After 5 and 10 min incubation, radioactivities associated with cells were determined.

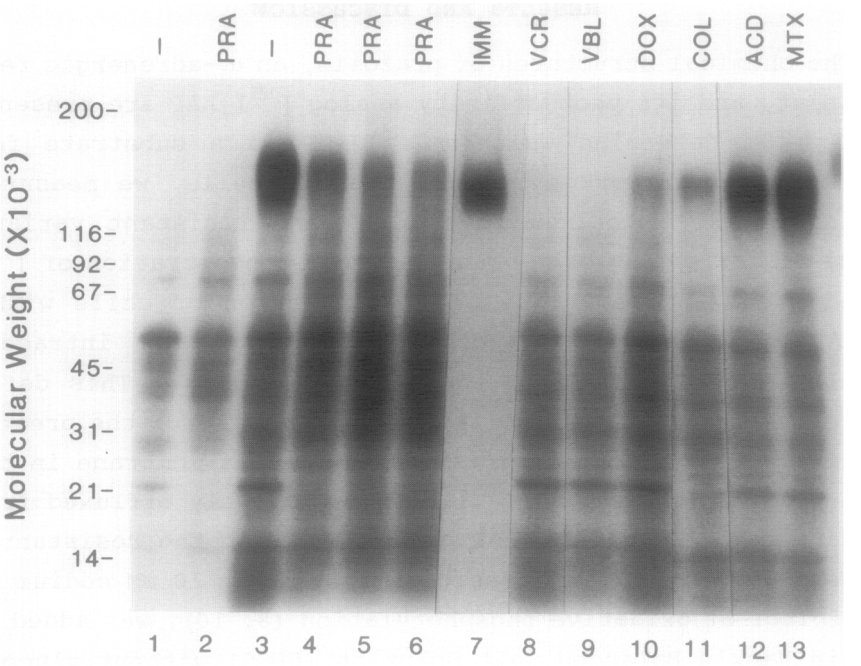


Fig. 3 SDS-PAGE autoradiography of [^{125}I]AAP photolabeled DC-3F cells (lanes 1 and 2) and DC-3F/VCRd-5L cells (lanes 3-13) in the absence (lanes 1 and 3) or presence of 100, 200 or 500 μM (lanes 4-6) non-radioactive prazosin (PRA), or 100 μM vincristine (VCR), vinblastine (VBL), doxorubicin (DOX), colchicine (COL), actinomycin D (ACD) and methotrexate (MTX) (lanes 8-13), respectively. For immunoprecipitation (IMM, lane 7), 180 μg [^{125}I]AAP photolabeled and deoxycholate solubilized (4) membrane vesicles from the resistant cells and monoclonal antibody C219 specific for P-gp were used.

for 10 min. Glucose induced a pronounced efflux of [^{125}I]AAP as measured by determining radioactivity in the intact cells.

The cytotoxic effect of prazosin revealed that the concentration that inhibited cell growth by 50% was 12 μM for DC-3F and 100 μM for DC-3F/VCRd-5L cells, respectively. This indicates that the resistant variant is only 8.3-fold resistant to prazosin. Furthermore, prazosin did not reverse vincristine resistance significantly when it was used in combination with vincristine.

In order to identify the specific prazosin acceptors in the resistant cells, intact cells were photoaffinity labeled with 8 nM of [^{125}I]AAP and examined by SDS-PAGE. Autoradiography (Fig. 3) showed that [^{125}I]AAP prominently binds to a 150-180 kDa polypeptide in DC-3F/VCRd-5L cells that was not detectable in the drug-sensitive DC-3F parental cells. Photolabeling specificity was determined by performing the experiments in the presence of excess

non-radioactive prazosin (Fig. 3, lanes 2 and 4-6). In these experiments 53.2, 63.9, and 71.5% inhibition of photolabeling of the 150-180 kDa polypeptide was seen in the presence of 100, 200 and 500 μM prazosin, respectively. Another polypeptide (22 kDa) was also found to bind specifically to [^{125}I]AAP and the extent of labeling of this protein in the resistant cells was approximately 2-fold more than that in the sensitive cells. At 100 μM, prazosin totally inhibited labeling of this protein (Fig. 3, lane 4) while labeling of other protein bands was unaffected even at 500 μM prazosin (Fig. 3, lane 6), indicating non-specific binding of [^{125}I]AAP to several proteins. The [^{125}I]AAP photolabeled membrane vesicles from the drug resistant cells were detergent solubilized (4) and immunoprecipitated with the P-gp specific monoclonal antibody C219 (10). Only the radiolabeled 150-180 kDa polypeptide was immunoprecipitated with this monoclonal antibody indicating that this protein is P-gp (Fig. 3, lane 7). At 100 μM vinblastine, vincristine, doxorubicin, colchicine and actinomycin D, the drugs to which the resistant cells display cross-resistance, [^{125}I]AAP photolabeling was inhibited by 100, 96.3, 82, 81.7 and 46.7%, respectively (Fig. 3, lanes 8-12). Methotrexate, an antitumor agent to which DC-3F/VCRd-5L cells are resistant (14), had no effect on the [^{125}I]AAP labeling of P-gp (Fig. 3, lane 13).

It is well documented that certain lipophilic agents enhance antitumor drug cytotoxicity in MDR cells presumably by increasing cellular accumulation and retention of the antitumor agents through interaction with P-gp (9-11). We screened a series of agents known to reverse MDR for their ability to inhibit [^{125}I]AAP labeling of P-gp in intact DC-3F/VCRd-5L cells. The [^{125}I]AAP labeling of P-gp in the presence of 100 μM nicardipine, diltiazem, prenylamine, and verapamil representatives of four classes of calcium channel blockers were reduced by 100, 83.2, 71, and 46%, respectively (Fig. 4A, lanes 3-6). At 100 μM, reserpine, trifluoperazine, monensin, progesterone and dibucaine, decreased the [^{125}I]AAP photoaffinity labeling of P-gp by 96, 85.8, 85, 62, and 57.5% respectively (Fig. 4A, lanes 8-12). These results demonstrate that most agents that can reverse MDR also inhibit [^{125}I]AAP photolabeling of P-gp. Previously, we have shown that some of these agents interact with P-gp and inhibit the binding of vinblastine, verapamil and azidopine to P-gp (4,5,11). While prazosin specifically interacts with P-gp, DC-3F/VCRd-5L cells are only 8.3-fold resistant to this agent. Furthermore, prazosin does not reverse vincristine

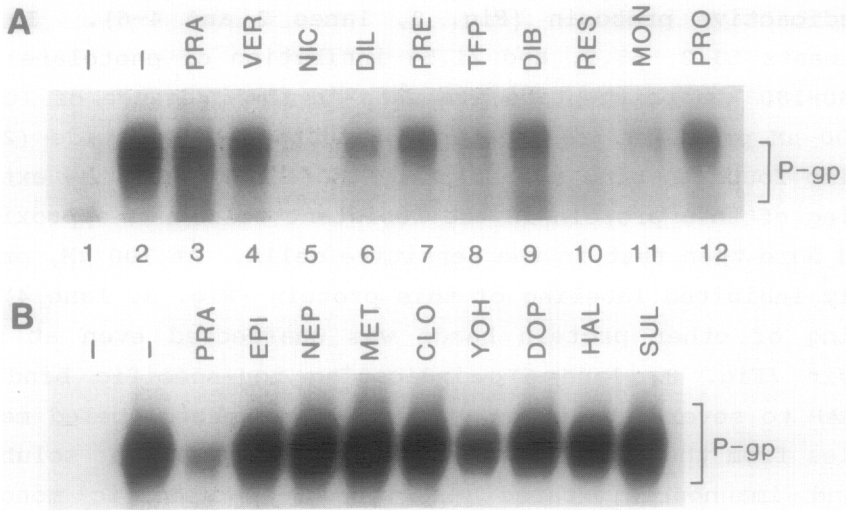


Fig. 4 A. SDS-PAGE autoradiography of [^{125}I]AAP photolabeled DC-3F cells (lane 1) and DC-3F/VCRd-5L cells (lanes 2-12) in the absence (lane 2) or presence of 100 μM prazosin (PRA), verapamil (VER), nicardipine (NIC), diltiazem (DIL), prenylamine (PRE), trifluoperazine (TFP), dibucaine (DIB), reserpine (RES), monensin (MON) and progesterone (PRO), respectively.
B. Photolabeling of DC-3F (lane 1) and DC-3F/VCRd-5L cells (lanes 2-11) in the absence (lane 2) or presence of 100 μM prazosin (PRA), epinephrine (EPI), norepinephrine (NEP), methoxamine (MET), clonidine (CLO), yohimbine (YOH), dopamine (DOP), haloperidol (HAL) and sulpiride (SUL).

resistance in these cells. These results and our previous data (4, 5, 9-11) collectively imply that various hydrophobic agents may have overlapping binding sits on P-gp. Identification of the amino acid sequence of the drug binding domain(s) of P-gp will be essential for characterizing the precise mechanism of drug interaction with this protein and for the development of effective MDR modulating agents.

We further analyzed the ability of α_2-adrenergic drugs (epinephrine, norepinephrine, methoxamine, clonidine and yohimbine) and dopaminergic drugs (dopamine, haloperidol and sulpiride) to inhibit the [^{125}I]AAP labeling of P-gp (Fig. 4B). At 500 μM, epinephrine, haloperidol, dopamine, and yohimbine inhibited [^{125}I]AAP photolabeling of P-gp by 8.5, 19.5 and 51.9%, respectively. While norepinephrine and sulpiride had no significant effect on the [^{125}I]AAP photolabeling of P-gp, clonidine and methoxamine enhanced the binding of this probe to P-gp by 20.1 and 41.5%, respectively. These latter two drugs may have a positive al-

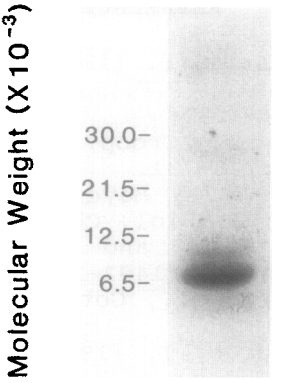


Fig. 5 Peptide mapping of the [^{125}I]AAP photolabeled P-gp. The photolabeled P-gp was subjected to peptide mapping by limited proteolysis with S. aureus V8 protease as described by Cleveland et al. (17).

losteric effect on [^{125}I]AAP labeling by binding to a separate site on P-gp.

To identify the specific [^{125}I]AAP binding domain of P-gp, we performed limited proteolysis of the photolabeled P-gp by digestion with S. aureus V8 protease using the Cleveland method (17). The results in Fig.5 show that [^{125}I]AAP predominantly binds to a single 8 kDa peptide. Our data strongly suggest that there is one major specific binding site for prazosin on P-gp. However, it is also possible that P-gp may have two identical binding sites for prazosin that are located on the two homologous halves (18-20) of the protein, and limited proteolysis by S. aureus V8 protein yields 8 kDa prazosin binding fragments from each half of P-gp molecule.

While the relationship, if any, between P-gp and the α_1-adrenergic receptors is not known, our data indicate that prazosin, an α_1-adrenergic antagonist, binds to P-gp and that this binding is influenced by other agents shown to interact with this protein. Our ability to photoaffinity label P-gp with [^{125}I]AAP should be of great value for further characterization of the structure and function of P-gp.

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