Binding of an Optically Pure Photoaffinity Analogue of Verapamil, LU-49888, to P-Glycoprotein from Multidrug-resistant Human Leukemic Cell Lines

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ABSTRACT

Verapamil enhances anticancer drug cytotoxicity in multidrug resistant (MDR) cells, apparently by competing with these agents for binding to P-glycoprotein (Pgp). In this study, we provide direct evidence for this competition. We studied the binding of an optically pure photoaffinity analogue of verapamil, (S)-5-[3-azidophenethyl]-1-[N-methyl-3H]-methylamino-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile (LU-49888), to Pgp from MDR cell lines. LU-49888 specifically labelled a single M, 170,000 protein that was identified as Pgp on Western blots and also by specific immunoprecipitation with monoclonal antibody C-219. A 200-fold molar excess of vinblastine or vincristine specifically inhibited this binding by >98%. LU-49888 labeling of Pgp was also inhibited by actinomycin D (45%), podophyllotoxin (47%), and amascrine (82%), marginally by doxorubicin (25%), colchicine (22%), daunorubicin (18%), and etoposide (14%), but not by teniposide. Modulators of Pgp-MDR also compete with LU-49888 for binding to Pgp; verapamil (82%), diltiazem (73%), quinidine (91%), reserpine (91%), rescinamine (88%), and trimethoxybenzoylyohimbine (89%). Chloroquine was moderately inhibitory (25%), whereas chlorpromazine and yohimbine, which are not modulators in our MDR cell lines, did not inhibit the binding of LU-49888 to Pgp. LU-49888 labeling of Pgp was also completely inhibited by (R)-, (S)-, and racemic desmethoxyverapamil, all with the same efficiency. Our results demonstrate that the verapamil analogue LU-49888 specifically binds to Pgp and suggest that verapamil and some MDR modulators exert their effects by interacting with Pgp.

INTRODUCTION

MDR cells are cross-resistant to a wide variety of structurally and functionally unrelated cytotoxic agents (1–3). The multidrug resistance phenotype is often associated with overexpression of Pgp (4, 5) and is characterized by decreased steady state drug resistance (9–11), one of the most effective being verapamil in MDR cells, apparently by competing with these agents for binding to Pgp-MDR. For it has less cardiovascular activity. Since LU-49888 used in this study is a pure S-enantiomer of a verapamil analogue, we also explored the effect of stereochemistry of verapamil on its binding to Pgp. A preliminary report of this work has been presented (24).

MATERIALS AND METHODS

Chemicals and Supplies. LU-49888 (25), the structure of which shown in Fig. 1, was kindly provided by Knoll Pharmaceutical AG (Ludwigshafen, West Germany), trimethoxybenzoylyohimbine, VLB, and VCR were provided by Dr. Homer Pierce, Eli Lilly and Co. (Indianapolis, IN). ADR and DNR were from Adria Laboratories (Columbus, OH), and VM-26 and VP-16 were from Bristol Laboratories (Wallingford, CT). CLC, POD, reserpine, rescinamine, diltiazem, quinidine, chloroquine, chlorpromazine, yohimbine, actinomycin D, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). R- and S-enantiomers of desmethoxyverapamil, also shown in Fig. 1, were generously provided by Dr. Jack Yalowich (University of Pittsburgh, Pittsburgh, PA), and m-AMSA was provided by the National Cancer Institute (Bethesda, MD). Eagle’s minimal essential medium was purchased from Whittaker M. A. Bioproducts (Walkersville, MD), and fetal bovine serum was obtained from Hyclone Laboratories, Inc. (Logan, UT). All other chemicals and supplies were obtained from standard commercial sources unless otherwise indicated.

Cells and Culture Conditions. CEM human leukemic lymphoblasts and their drug-resistant variants were grown as described earlier (5). Drug-resistant CEM/VLB sublines were selected by growth in the continuous presence of sublethal concentrations of drug and were cloned from the original by the limiting dilution method (26). Preparation of Plasma Membrane Fractions and Photoaffinity Labeling. Membrane fractions were prepared by a nitrogenvillation method, as described previously (19). Membranes prepared from drug-sensitive and -resistant cell lines were diluted to 4 mg protein/ml in 10 mM Tris-

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3 The abbreviations used are: MDR, multidrug-resistant; Pgp, P-glycoprotein; CLC, colchicine; DNR, daunorubicin; ADR, adriamycin; POD, podophyllotoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VCR, vincristine sulfate; VLB, vinblastine sulfate; VM-26 (teniposide), 4’-demethyl-epipodophyllotoxin 9,4,6-O-2-thienylidine-β-D-glucopyranoside; VP-16 (etoposide), 4’-demethyleneepipodophyllotoxin 9,4,6-O-2-thiocarbamoyl-β-D-glucopyranoside; m-AMSA, (amascrine, 4’,4’-19-azidinylationo-methanesulphonyl-m-aminoiodophenyl-3H)-methylamino-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; NABAV, N1-(azido)3,5-1Hbenzoylaminomethylverapamil; CEM, CCRF-CEM.
HCl buffer, pH 7.4, and can be stored for up to 2 months at -70°C. Membrane proteins (200 µg) were incubated in buffer containing 250 mM sucrose and 10 mM Tris-HCl, pH 7.4, at 25°C for 20 min with 100 nM LU-49888, in the absence or presence of various competitors, in a total volume of 150 µl. The incubation mixture was then irradiated with a germicidal UV light (GE Germicidal Lights, G30T8, 30 Watts), commonly used in laminar flow hoods, for 20 min at a distance of 10 cm.

Immunoprecipitation. Immunoprecipitation was performed according to the methods of Safa (20) and Cornell et al. (17), with minor modification. LU-49888-labeled membrane proteins (200 µg) were solubilized in 200 µl of deoxycholate buffer (20 mM Tris-HCl, pH 8.0; 140 mM NaCl; 0.5% deoxycholate) for 30 min at 4°C. Solubilized samples were then incubated overnight with 50 µg of either nonspecific mouse myeloma IgG1 or C219 monoclonal antibody specific for Pgp. Protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) was then added to the tubes and incubated for 30 min at 4°C, after which the immune complexes were washed with deoxycholate buffer 5 times. The final pellets were resuspended in Laemmli sample buffer (27) and the immune complexes were washed with deoxycholate buffer 5 times. The final pellets were resuspended in Laemmli sample buffer (27) and the Sepharose beads were removed by centrifugation. Supernatants were then electrophoresed.

SDS-PAGE and Fluorography. Photolabeled proteins or immune complexes were separated by one-dimensional 5-15% SDS-PAGE under reducing conditions, using the discontinuous buffer system of Laemmli (27). After staining with Coomassie blue and destaining, the gels were soaked in Amplify (Amersham Corp., Arlington Heights, IL) for 30 min and dried under vacuum at 75°C. The dried gels were exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for 2-3 days at -70°C and developed. Radioactively labeled bands were scanned in their centers with a densitometer (model E-C 910; EC Apparatus Corp., St. Petersburg, FL) and quantitated by integration with a chromatography recorder (Chromatopac C-R6A; Shimadzu Corp., Kyoto, Japan). The quantitation of radiolabeled bands was also done by cutting out the gel slices, digesting them in hydrogen peroxide, and counting them for radioactivity according to a standard procedure (28).

Western Blot. Membrane proteins were transferred to nitrocellulose using a Polyblot apparatus (American Biionetics, Hayward, CA) according to the directions of the manufacturer. The remainder of the procedure was essentially that of Towbin et al. (29). Nonspecific binding sites on the nitrocellulose filter papers were blocked with bovine serum albumin by incubation at 40°C for 30 min in buffer A (3% bovine serum albumin, 10 mM Tris-HCl, 0.9% NaCl, 0.02% sodium azide, pH 7.4). The papers were then incubated with monoclonal antibody C219 (Cen
tocor, Inc., Malvern, PA) in buffer A at a concentration of 0.2 µg/ml, at 4°C for 16 hr. The filters were washed 3 times with buffer B (10 mM Tris-HCl, 0.9% NaCl, pH 7.4) and incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA) in buffer A (1:200 dilution) at room temperature for 3 h. After 3 washes with buffer B, the filters were developed in 0.5% 5-bromo-4-chloro-3-indoly phosphate.

RESULTS AND DISCUSSION

Specific Binding of LU-49888 to Pgp. We labeled the membrane proteins from a highly MDR cell line, CEM/VELbK, with a photoaffinity analogue of verapamil, LU-49888. Fluorography of SDS-polyacrylamide gels (Fig. 2) showed that LU-49888 specifically binds to a major single protein with a molecular weight of 170,000. The specificity of the labeling of this protein by LU-49888 was determined by photoaffinity labeling experiments in the presence of increasing amounts of verapamil or VLB. The binding of LU-49888 to this M, 170,000 protein was completely inhibited with 20 µM VLB or 200 µM verapamil, indicating that the LU-49888 binding sites on this M, 170,000 protein are saturable. The results of this experiment also suggest that the M, 170,000 protein has a higher binding affinity for VLB than for verapamil. These results are consistent with our earlier data showing that 10 µM verapamil was required in our Pgp-MDR cell line CEM/VELbK0 to reverse its resistance to 400 nM VLB (15).

The labeling of membrane proteins by LU-49888 was also studied by using our VLB-selected series of Pgp-MDR cell lines that exhibit increasing resistance to VLB, ranging from 18- to 1400-fold (Table 1). It is seen in this table that 10 µM verapamil increases VLB cytotoxicity in all of the cell lines, including a modest effect on the drug-sensitive CEM cells. As demonstrated in Fig. 3, the labeling of the M, 170,000 protein increased in rough proportion to the degree of VLB resistance of the cells.
Table 1  Drug resistance of CCRF-CEM-derived MDR cell lines in the absence or presence of verapamil

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VLB IC₅₀ (nm)</th>
<th>-Verapamil</th>
<th>+Verapamil (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>3.4 (1.0)</td>
<td>0.93 (3.7)</td>
<td></td>
</tr>
<tr>
<td>CEM/VLB₁₀⁻²</td>
<td>60.0 (17.6)</td>
<td>2.45 (24.6)</td>
<td></td>
</tr>
<tr>
<td>CEM/VLB₁₀⁻¹</td>
<td>230 (67.6)</td>
<td>4.0 (57.5)</td>
<td></td>
</tr>
<tr>
<td>CEM/VLB₅⁻²</td>
<td>415 (122.0)</td>
<td>19.2 (21.5)</td>
<td></td>
</tr>
<tr>
<td>CEM/VLB₁₀⁻¹</td>
<td>2250 (661.0)</td>
<td>29.0 (77.6)</td>
<td></td>
</tr>
<tr>
<td>CEM/VLB₅⁻¹</td>
<td>2900 (852.0)</td>
<td>40.0 (72.5)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parenthesis are fold-resistance, relative to CEM cells.

† Numbers in parenthesis indicate the fold-decrease in VLB IC₅₀ in the presence of verapamil.

Fig. 3. Photoaffinity labeling of plasma membranes from CCRF-CEM cell lines of increasing VLB resistance. Cell membrane fractions were prepared from six CEM-derived cell lines according to methods previously described (19). See Table 1 for the fold-resistance of the cell lines used in this experiment. Membrane proteins photoaffinity labeled by 100 nM LU-49888 were separated by SDS-PAGE and detected by fluorography. See “Materials and Methods” for experimental details.

Expression of Pgp also correlated with the resistance of the cell lines (Fig. 4). To confirm the identity of the LU-49888-labeled protein as Pgp, we reacted labeled membrane preparations with either C219 monoclonal antibody or an isotype-specific “irrelevant” monoclonal antibody. After collecting and washing the complexes and subsequent electrophoresis and fluorography of the gels, we found that C219, which identifies a highly conserved epitope on Pgp, specifically precipitated a radioactively labeled band, as shown in Fig. 5. The irrelevant antibody did not precipitate this complex. Also shown in the figure, for comparison, is a nonprecipitated membrane preparation labeled with LU-49888. Based on the results in Figs. 2–5, it is clear that the Mₗ 170,000 protein labeled by LU-49888 is Pgp.

Effect of Cytotoxic Drugs on the Binding of LU-49888 to Pgp. The CEM/VLB₅⁻¹ cell line is cross-resistant to a variety of cytotoxic drugs commonly used in cancer chemotherapy (Table

2). However, while verapamil has a strong effect in reversing the resistance of VLB and VCR in these cells, it was much less effective in reversing the cross-resistance of other drugs, such
or presence of 20 μM cytotoxic drug, as indicated. The labeled proteins were photoaffinity labeled by 100 nM LU-49888 in the absence or presence of 20 μM concentrations of modulators of multidrug resistance as indicated. The labeled proteins were separated by SDS-PAGE and detected by fluorography. The inhibitory effects of the anticancer drugs on Pgp labeling were determined by scanning the film with a densitometer. See “Materials and Methods” for experimental details.
binding to Pgp (31) or bind to different sites on the protein. Our results suggest a complex relationship between the ability of a compound to modulate MDR and its ability to bind to Pgp.

Effect of Stereoisomers of Desmethoxyverapamil on the Binding of LU-49888 to Pgp. Verapamil as used in the clinic is chemically synthesized as a racemic mixture. Since the R- and S-enantiomers of verapamil exhibit substantial differences in cardiac activity and side effects (13), we studied the effects of stereoisomers of a verapamil analogue, desmethoxyverapamil (Fig. 1), on the binding of LU-49888 to Pgp. The results, shown in Fig. 8, indicate that both the S- and R-isomers of desmethoxyverapamil, as well as the racemic desmethoxyverapamil, are equally effective in inhibiting the binding of LU-49888 to Pgp. These results are in agreement with the data from the study by Cornwell et al. (17), in which it was shown that S- and R-enantiomers of desmethoxyverapamil are equally efficient in inhibiting the reversible binding of verapamil to Pgp. This observation suggests that the pure R-enantiomer of verapamil, which has less cardiac activity, may be useful in the treatment of clinical MDR associated with overexpression of Pgp.

Summary and Conclusions. Our results provide evidence to support the hypothesis that verapamil reverses Pgp-MDR by blocking the binding of cytotoxic drugs to Pgp, thereby blocking the drug efflux function of Pgp. The labeling of Pgp by LU-49888 is in general proportional to the amount of this protein in the CEM/VLB cell lines; similarly, the amount of Pgp present appears to reflect the resistance of these lines. The inhibition of LU-49888 labeling of Pgp by various cytotoxic drugs is in good agreement with the cross-resistance pattern of our CEM/VLB cells lines. The inhibition of LU-49888 labeling of Pgp by modulators of MDR also correlates with their effects on anticancer drug cytotoxicity. These results complement our efforts to define the multidrug resistance "pharmacophore" (32) and will be useful in the design of effective agents to circumvent Pgp-associated multidrug resistance.

LU-49888 was designed to identify verapamil-inhibitable, voltage-gated calcium channels in excitable tissues by photoaffinity methods (25). Since MDR cell lines, including ours, do not express these calcium channels (16, 33), there must be a site on Pgp that is similar to the LU-49888 binding site on calcium channels in excitable cells. Support for this idea comes from recent results showing that azidopine specifically labels Pgp (34, 35). Further support comes from the recent cloning of bovine brain adenyl cyclase, which was found to have a remarkable topographical similarity to Pgp (36). Desmethoxyverapamil has actions similar to those of verapamil (37) and can compete with verapamil for binding to calcium channel proteins (25) and Pgp-containing membranes from multidrug resistant cells (17), suggesting that this compound also recognizes similar structures on the two proteins. Whether these apparently similar sites on the two proteins subserve similar functions is not known.

In two recent studies with another photoactive analogue of verapamil (20, 21), the nitrile group was substituted by a rather bulky benzyolaminomethyl group and the photoactive azido group was not directly attached on the verapamil moiety per se. Despite these structural differences between NABAV and LU-49888, our results are in good agreement with those with NABAV (20, 21) and indicate that either the benzyolaminomethyl group does not interfere with NABAV binding to Pgp or Pgp recognizes a fairly broad range of structures. Indeed, our data demonstrate clearly the promiscuous characteristics of Pgp in recognizing substrates with apparently diverse molecular structures. This property is in agreement with the hypothesized efflux function of Pgp that can extrude a wide range of structurally unrelated natural products. We note, however, that these molecules may all be similar in three dimensions and share a common volume element (9). Indeed, our work (32) and that of others (38, 39) suggest that there is specificity in the structures recognized by Pgp. Studies characterizing the verapamil and VLB binding sites on Pgp are presently in progress.

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REFERENCES

LABELING OF P-GLYCOPROTEIN BY VERAPAMIL


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