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-azidophenylethyl)-N-methyl]-H-methylamino-2,3,4,5-tetramethoxyphenyl-2-isopropylvaleronitrile (LU-49888), to Pgp from MDR cell lines. LU-49888 specifically labeled 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%), reserpin (91%), rescinnamine (88%), and trimethoxybenzoylhydrophosphate (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 intracellular drug levels (6-8). Identification of Pgp as the protein that mediates MDR in human cells, therefore, has provided a target for current efforts aimed at overcoming clinical MDR neoplasms.

Several compounds have been found that can reverse multidrug resistance (9-11), one of the most effective being verapamil (12), a calcium channel inhibitor used in cardiology to treat angina (13). Although verapamil can inhibit drug efflux from MDR cells and increase the accumulation of VCR or ADR in drug-resistant cell lines (14, 15), its precise mechanism of action is not clear, since Pgp-MDR cells do not have voltage-gated calcium channels (16). It was demonstrated in other studies, however, that verapamil can competitively inhibit the binding of VLB (17) or its photoaffinity analogue (18, 19) to Pgp, suggesting that it exerts its action by interacting with Pgp and inhibiting its drug efflux function. Here we provide direct evidence of binding of an optically pure verapamil analogue, LU-49888, to Pgp in human leukemic MDR cell lines established in this laboratory. During the course of the present study, two reports appeared showing that another photoactive analogue of verapamil, NABAV, could also covalently bind to Pgp (20, 21).

In one clinical trial, verapamil was used to treat patients with refractory ovarian cancer (22). Unfortunately, when used at the doses required to reverse drug resistance in vitro, it caused unacceptable cardiotoxicity, and the trial was terminated. Even in a more successful study in patients with multiple myeloma, verapamil at the doses administered produced cardiac toxicity (23). The verapamil used clinically is a racemic mixture, and the R-isomer has 5 to 10 times less cardiovascular activity than the S-isomer (13). If both isomers are equally effective in reversing multidrug resistance, it would be clinically advantageous to use the R-isomer of verapamil or its analogue to reverse 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). Trimethoxybenzoylhydrophosphate, 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). PLC, P0D, reserpine, rescinnamine, 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 Vaiowich (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 originals by the limiting dilution method (26). Preparation of Plasma Membrane Fractions and Photoaffinity Labeling. Membrane fractions were prepared by a nitrogen cavitation 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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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-β-D-glucopyranoside); VP-16 (etoposide), 4'-demethylleptopodophyllotoxin 9-(4,6-O-β-ethylidine-β-β-D-glucopyranoside); m-AMSA, amascrine; 4'-[9-α-acidinyl]aminomethanesulfon-m-amine; ICso concentration of drug required to inhibit 48-h cell growth by 50% relative to untreated controls; LU-49888, (S)-5-[(3-azidophenethyl)-N-methyl]-H-methylamino-2,3,4,5-tetramethoxyphenyl-2-isopropylvaleronitrile; NABAV, N-[p-azido]-3,5-H[benzoylaminoethyl]verapamil; CEM, CCRF-CEM.
commonly used in laminar flow hoods, for 20 min at a distance of 10 cm. The incubation mixture was then irradiated with a germicidal UV light (GE Germicidal Lights, G3OT8, 30 Watts), total volume of 150 nm. The incubation mixture was then irradiated with a germicidal UV light (GE Germicidal Lights, G3OT8, 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 Cornwell et al. (17), with minor modification. LU-49888-labeled membrane proteins (200 ng) 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 IgGl 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 using a Polyblot apparatus (American Bio-Technologies, 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 (Centor, 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-indolyl phosphate.

RESULTS AND DISCUSSION

Specific Binding of LU-49888 to Pgp. We labeled the membrane proteins from a highly MDR cell line, CEM/VLB5K, 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/VLB100 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.

Immunological Quantitation and Identification of Labeled Protein as Pgp. Western blots probed with the Pgp-specific monoclonal antibody C-219 (30) showed that the increased expres-
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>
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<tbody>
<tr>
<td>CEM</td>
<td>3.4 (1.0)</td>
</tr>
<tr>
<td>CEM/VLB₁₀⁻²</td>
<td>60.0 (17.6)</td>
</tr>
<tr>
<td>CEM/VLB₂₅⁻⁸</td>
<td>230 (67.6)</td>
</tr>
<tr>
<td>CEM/VLB₁₀₀</td>
<td>415 (122.0)</td>
</tr>
<tr>
<td>CEM/VLB₅K</td>
<td>2250 (661.0)</td>
</tr>
<tr>
<td>CEM/VLB₅K</td>
<td>2900 (852.0)</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.

Fig. 4. Immunoblot analysis of Pgp present in membrane fractions of CEM and CEM/VLB cell lines. Membrane fractions (200 μg protein) prepared from CEM and CEM/VLB cell lines with increasing resistance to VLB were separated by SDS-PAGE, transferred to nitrocellulose, and detected by monoclonal antibody C-219, as detailed in “Materials and Methods.”

Fig. 5. Immunoprecipitation of Pgp from membrane fractions of CEM/VLB₅K cells photoaffinity labeled with LU-49888. Membrane proteins (200 μg) prepared from CEM/VLB₅K cells were photoaffinity labeled with 100 nM LU-49888 and solubilized in deoxycholate buffer. The solubilized samples were run without antibody treatment as a control (Lane 1) or were incubated with 50 μg of either nonspecific mouse myeloma IgG1 (Lane 2) or C219 monoclonal antibody (Lane 3). Immunoprecipitation was with Protein A-Sepharose CL-4B. Proteins were separated by SDS-PAGE and detected by fluorography. See “Materials and Methods” for experimental details.

Labeling of P-glucoprotein by verapamil

The CEM/VLB₅K 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 as doxorubicin (Dox) and adriamycin (ADM).
as CLC, ADR, and DNR (Table 2). To gain a better understanding of this phenomenon, we chose several drugs to which these cells express cross-resistance and examined their effects on the binding of LU-49888 to Pgp. As shown in Fig. 6, many cytotoxic drugs were able to inhibit this binding when present in a 200-fold molar excess. Inhibition of LU-49888 binding was quantitated by scanning the fluorographic bands with a densitometer, and those values are given in this figure. Among the cytotoxic drugs studied, VLB, VCR (both >98%), and m-AMSA (82%) exerted the greatest inhibition of LU-49888 binding, while actinomycin D (45%) and POD (47%) produced an intermediate inhibition. Little or no inhibition was seen with the antitumor drugs doxorubicin (DNR), ADR, the epipodophyllotoxins (VM-26, VP-16), and CLC.

Comparing these results with those in Table 2, it is clear that the drugs whose cytotoxicity was strongly enhanced by verapamil (VLB, VCR) are those that can best compete with LU-49888 for binding to Pgp. Of considerable interest, drugs such as CLC, ADR, and VM-26 do not compete effectively with LU-49888 for binding to Pgp, suggesting that they have a low binding affinity for the protein at the same or different sites. Studies titrating the binding to Pgp of substrate and inhibitor will be required to resolve this matter. Since the cells were only marginally cross-resistant to m-AMSA, its cytotoxicity was not greatly enhanced, despite its strong inhibition of LU-49888 binding. At this time, we have no explanation for this apparent discrepancy between cytotoxicity and binding inhibition, but it may complicate efforts to screen potential modulators.

Effect of Modulators of Multidrug Resistance on the Binding of LU-49888 to Pgp. Several classes of chemicals are able to enhance the cytotoxic actions of anticancer drugs in Pgp-MDR cells (9, 10). However, the mechanism by which these agents exert this effect is unclear. Earlier studies from this laboratory indicated that many of these agents shared physical and chemical features (9), and many, but not all, were able to compete with a photoaffinity analogue of VLB for binding to Pgp (19). In the present study, we asked whether these modulators interact with Pgp as does verapamil by photoaffinity labeling this protein with LU-49888 in the presence of 200-fold molar excesses of these compounds. The inhibitory effects of these agents on the binding of LU-49888 to Pgp, as demonstrated by fluorography (Fig. 7), were: verapamil, 82%; reserpine, 91%; rescinnamine, 88%; trimethoxybenzoyloxyhimbine, 89%; quinidine, 91%; dil-tiazem, 73%; and chloroquine, 25%. Chlorpromazine and yohimbine, which have marginal effects on VLB cytotoxicity in our cell lines (9), have no effect on the labeling of Pgp by LU-49888. These results suggest that modulators such as reserpine, diltiazem, quinidine, rescinnamine, and trimethoxybenzoyloxyhimbine may exert their effects through interaction with Pgp to interfere the binding of the cytotoxic drug to Pgp. The present results also indicate that these modulators probably interact at the same site on Pgp to which verapamil binds. By the same reasoning, it is possible that chloroquine and chlorpromazine act by a mechanism other than competition for

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ (nm)</th>
<th>Fold-resistance</th>
<th>IC₅₀ (nm)</th>
<th>Fold-decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLB</td>
<td>2,900</td>
<td>2.636</td>
<td>40</td>
<td>72.0</td>
</tr>
<tr>
<td>VCR</td>
<td>31,000</td>
<td>8.378</td>
<td>470</td>
<td>62.0</td>
</tr>
<tr>
<td>ADR</td>
<td>5,200</td>
<td>21.7</td>
<td>750</td>
<td>6.9</td>
</tr>
<tr>
<td>DNR</td>
<td>8,600</td>
<td>183</td>
<td>3,690</td>
<td>2.4</td>
</tr>
<tr>
<td>VM-26</td>
<td>9,000</td>
<td>100</td>
<td>8,200</td>
<td>1.1</td>
</tr>
<tr>
<td>VP-16</td>
<td>2,600</td>
<td>81</td>
<td>440</td>
<td>5.9</td>
</tr>
<tr>
<td>CLC</td>
<td>4,300</td>
<td>53</td>
<td>1,400</td>
<td>3.1</td>
</tr>
<tr>
<td>POD</td>
<td>52</td>
<td>1.1</td>
<td>18</td>
<td>2.9</td>
</tr>
<tr>
<td>m-AMSA</td>
<td>320</td>
<td>2.6</td>
<td>70</td>
<td>4.6</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>140</td>
<td>3.8</td>
<td>52</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* The decrease in drug IC₅₀ of the verapamil-treated CEM/VLB₅K cells relative to the parent CEM cell line.
* The resistance of the CEM/VLB₅K cells relative to the parent CEM cell line.

Fig. 6. Inhibitory effect of anticancer drugs on photoaffinity labeling of Pgp by LU-49888. Membrane fractions (200 µg protein) prepared from CEM or CEM/VLB₅K cells were photoaffinity labeled by 100 nm LU-49888 in the absence or presence of 20 µM cytotoxic drug, 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 details ACT-D, actinomycin D.

Fig. 7. Inhibitory effect of modulators of multidrug resistance on photoaffinity labeling of Pgp by LU-49888. Membrane fractions (200 µg protein) prepared from CEM or CEM/VLB₅K cells 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 inhibition of Pgp labeling was quantitated by scanning the film with a densitometer. See "Materials and Methods" for experimental details. VRP, verapamil; DILT, diltiazem; QND, quinidine; CLQ, chloroquine; CPZ, chlorpromazine; RES, reserpine; RESCIN, rescinnamine; YOH, yohimbine; TMBY, trimethoxybenzoyloxyhimbine.
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/VELB 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/VELB cell 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 adenylly 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 benzoylaminomethyl 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 benzoylaminomethyl 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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