# N-(p-Azido-3-[125I]iodophenethyl)spiperone Binds to Specific Regions of P-glycoprotein and Another Multidrug Binding Protein, Spiperophilin, in Human Neuroblastoma Cells†

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ABSTRACT: P-glycoprotein (P-gp) is an energy-dependent drug extrusion pump with broad specificity for diverse hydrophobic anticancer agents and compounds known to reverse multidrug resistance (MDR). Among MDR reversing agents, phenothiazines (PTZs) and related compounds may sensitize MDR by interacting with a specific binding site(s) on P-gp and by other mechanisms. In order (1) to identify a binding site for PTZs and related compounds on P-gp, (2) to examine whether these compounds and other MDR modulators bind to the same domains of P-gp, and (3) to identify proteins with high specificity for these neuroleptic agents and other MDR modulators, we used a butyrophenone $D_2$-dopamine receptor photoaffinity probe, N-(p-azido-3-[125I]iodophenethyl)spiperone ([125I]NAPS). [125I]NAPS was actively effluxed from vincristine (VCR)-resistant SH-SY5Y/VCR human neuroblastoma cells, and nonradioactive I-NAPS was a potent chemosensitizing agent. After photolabeling, the probe bound specifically and with high efficiency to P-gp and to another multidrug binding 17-kDa membrane-bound protein, spiperophilin, in these cells. The efficiency of [125I]NAPS binding to P-gp was 5–6-fold more than [3H]azidopine and [125I]arylazidoprazosin ([125I]AAP), known photoaffinity analogs for P-gp. [125I]NAPS photolabeling of P-gp was preferentially competed by MDR-related drugs, with vinblastine > VCR > colchicine > doxorubicin > actinomycin D. Many drugs that are known to reverse MDR were potent inhibitors of [125I]NAPS binding to P-gp. While PTZs and related compounds were potent inhibitors of [125I]NAPS binding to P-gp, most of them enhanced the binding of [125I]AAP significantly. *cis*-Flupentixol increased the binding of [125I]AAP to P-gp 9-fold more than did *trans*-flupentixol, but both were potent inhibitors of [125I]NAPS binding, suggesting their stereoselective effect on the [125I]AAP binding site. Proteolysis of [125I]NAPS-bound P-gp with *Staphylococcus aureus* V8 protease revealed that this probe binds to two major peptides, 6 and 8 kDa, and a number of minor ones, while [125I]AAP binds to only an 8-kDa peptide. These results suggest that modulators of MDR may interact with separate or overlapping domains. Furthermore, most MDR modulators, dopaminergic drugs, and β-adrenergic antagonists used also inhibited binding of [125I]-NAPS to spiperophilin, suggesting that this protein may be a target for these drugs.

Multidrug resistance (MDR),[1] a phenomenon whereby tumor cells acquire resistance to a wide variety of structurally and functionally unrelated natural product cytotoxic agents (Endicott & Ling, 1989), is caused by overexpression of a 150–180-kDa transmembrane phosphoglycoprotein termed P-glycoprotein (P-gp) (Juliano & Ling, 1976). Sensitive cells transfected with a human or mouse P-gp gene acquire MDR (Gros et al., 1986; Ueda et al., 1987). P-gp is believed to act as an energy-dependent drug efflux pump with broad substrate specificities (Danø, 1973; Skovsgaard, 1978; Tamai & Safa, 1990, 1991). The predicted amino acid sequences of mammalian P-gp indicate an open reading frame of approximately 1280 amino acids which form homologous N-terminal and C-terminal halves (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986). Each half of P-gp resembles bacterial transport proteins, particularly hemolysin B (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986). P-gp consists of 12 putative membrane-spanning domains, 6 in each half, and are thought to form a channel for extruding its substrates (Gros et al., 1986; Azzaria et al., 1989; Safa et al., 1990a). Each half of P-gp has a nucleotide binding site consensus domain facing the cytosol which can hydrolyze ATP independently and produce the energy for active transport (Shimabuku et al., 1992). It is known that P-gp binds to ATP (Cornwell et al., 1987; Azzaria et al., 1989) and its purified form has ATPase activity (Hamada & Tsuruo, 1988).

Drugs involved in the MDR phenotype bind specifically to P-gp (Safa, 1993). Although these compounds tend to be chemically diverse, it has been postulated that their similar overall tertiary structure allows binding to drug acceptor site(s) on the P-gp cytoplasmic and transmembrane domains and subsequent transport out of the cells (Safa et al., 1990b; Gros et al., 1991). In general, these drugs are highly lipophilic natural products with a planar ring structure (Zamora et al., 1988). The most intriguing question is the mechanism of the

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[1] Abbreviations: [125I]AAP, [125I]arylazidoprazosin; HBSS, Hank's balanced salt solution; [125I]NAPS, N-(p-azido-3-[125I]iodophenethyl)spiperone; $Me_2SO$, dimethyl sulfoxide; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PTZs, phenothiazines; P-gp, P-glycoprotein; RIPA, radioimmune precipitating buffer; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VCR, vincristine.

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broad chemical specificity of P-gp.

Recently, there has been major interest in developing strategies to circumvent MDR in experimental systems and ultimately in the resistant cell population in patients. It is known that lipophilic agents such as certain calcium channel blockers and calmodulin inhibitors, reserpine and its analogs, lysosomatropic agents, indole alkaloids, quinidine, local anesthetics, cyclosporins, progesterone, and megestrol acetate enhance drug cytotoxicity in MDR cells, possibly through interaction with P-gp (Tamai & Safa, 1991; Safa, 1993; Fleming et al., 1992).

We have demonstrated that P-gp binds specifically to photoaffinity analogs of vinblastine (Safa et al., 1986), colchicine (Safa et al., 1989), dihydropyridines (Safa et al., 1987a), verapamil (Safa, 1988), and the $\alpha_1$-adrenergic receptor antagonist prazosin (Safa et al., 1990b). Furthermore, other investigators have shown that photoaffinity analogs of daunorubicin (Busche et al., 1989; Beck & Qian, 1992), a synthetic isoprenoid (Akiyama et al., 1989), cyclosporin A (Foxwell et al., 1989), and forskolin (Morris et al., 1991) likewise bind specifically to P-gp. Progesterone can also covalently bind to P-gp when activated by UV light (Qian & Beck, 1990).

We recently showed that many phenothiazines (PTZs) and compounds structurally related to PTZs known to reverse MDR (Ford et al., 1989) inhibit the binding of photoactive analogs of vinblastine, colchicine, verapamil, and azidopine to P-gp from DC-3F/VCRd-5L Chinese hamster lung cells (Fleming et al., 1990), suggesting that they reverse MDR at least in part through interaction with the cytotoxic drug binding site(s) of this protein. However, because these compounds may affect many cellular functions including several cellular enzymes (Pang & Brigg, 1976; Ruben & Rasmussen, 1981) and block the function of critical cellular receptors, such as those for dopamine (Creese & Sibley, 1981), it is possible that these agents may exert their effects and reverse MDR through more than one mechanism (Ford et al., 1990). In this study, we used a butyrophenone $D_2$-dopamine receptor photoaffinity probe, *N*-(*p*-azido-3-iodophenethyl)-spiperone (I-NAPS) (Amlaiky & Caron, 1986) (1) to determine whether it modulates MDR, (2) to identify the cellular components in MDR cells with the highest affinity and specificity for [$^{125}$I]NAPS and the other PTZs and related compounds, (3) to examine whether [$^{125}$I]NAPS binds to domains of P-gp different from that of the photoaffinity analog arylazidoprazosin, which is known to modulate MDR, and (4) to determine whether other MDR modulators differentially affect the binding of these two photoaffinity probes to P-gp.

## MATERIALS AND METHODS

*Materials.* The $D_2$-dopamine photoaffinity probe *N*-(*p*-azido-3-[$^{125}$I]iodophenethyl)spiperone ([$^{125}$I]NAPS, 2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and was also synthesized as previously described (Amlaiky & Caron, 1986). The nonradioactive iodoazido-NAPS (I-NAPS) was prepared as described previously (Amlaiky & Caron, 1986). [$^{125}$I]Arylazidoprazosin ([$^{125}$I]-AAP, 2200 Ci/mmol) and $^{125}$I-Bolton–Hunter-labeled calmodulin (74.4 $\mu$Ci/$\mu$g) were purchased from New England Nuclear. [$^3$H]Vincristine (10 Ci/mmol) and [$^3$H]azidopine (40 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Protein A–Sepharose and protein G–Sepharose were purchased from Pharmacia LKB (Piscataway, NJ). Vinblastine and vincristine (VCR) were obtained from Eli Lilly and Co. (Indianapolis, IN). Actinomycin D, doxorubicin, colchicine, methotrexate, (±)-verapamil, (±)-nicardipine, diltiazem, prenylamine, trifluoperazine, chlorpromazine hydrochloride, *cis*-thiothixine, dibucaine, reserpine, progesterone, (±)-epinephrine hydrochloride, (±)-norepinephrine hydrochloride, methoxamine, clonidine hydrochloride, yohimbine hydrochloride, dopamine hydrochloride, and haloperidol were purchased from Sigma (St. Louis, MO). Monensin was obtained from Calbiochem (La Jolla, CA). Cyclosporin A was supplied by Sandoz (Basel, Switzerland). *cis*- and *trans*-flupentixol hydrochloride were kindly supplied by H. Lundbeck (Copenhagen, Denmark). Spiperone hydrochloride, (*R*)-(L)-propylnorapomorphine hydrochloride-(*S*)-(−)- and (*R*)-(+)-sulpiride, pimozide, fluspirilene, 5-hydroxytryptamine hydrochloride, (−)- and (+)-propranolol hydrochloride, fluphenazine hydrochloride, fluphenazine N-mustard hydrochloride, thioridazine hydrochloride, and W-7 HCl [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride] were purchased from Research Biochemical, Inc. (Natick, MA). Monoclonal antibody C219 specific for P-gp was purchased from Centocor (Malvern, PA). The anti-calmodulin monoclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). All other chemicals were obtained commercially and were of reagent grade.

*Cell Culture.* The SH-SY5Y human neuroblastoma cell line and its VCR-resistant derivative SH-SY5Y/VCR (Meyers et al., 1985) expressing 600-fold resistance to VCR were maintained as described (Meyers et al., 1985). SH-SY5Y/VCR cells were maintained in 5 $\mu$g/mL VCR. The KB-3-1 human epidermoid carcinoma cell line and its vinblastine-resistant derivative KB-V1 expressing 12 800-fold resistance to VCR were maintained as described (Fojo et al., 1985). The KB-V1 cells were maintained in 1 $\mu$g/mL vinblastine. VCR was removed from the cultures 4 weeks before experiments.

*Drug Accumulation and Efflux Studies.* For [$^{125}$I]NAPS accumulation studies, SH-SY5Y and SH-SY5Y/VCR cells were scraped from monolayer cultures and suspended in Hank's balanced salt solution (HBSS). Cells at a density of $2 \times 10^5$ per experiment were treated with 3.12 pM [$^{125}$I]NAPS (2200 Ci/mmol). After 5-, 15-, or 30-min incubation at room temperature in HBSS containing 10 mM glucose, cells were rapidly washed with ice-cold HBSS, and the radioactivity associated with the cells was determined by Packard Cobra 1 $\gamma$ counter.

The time course of accumulation of 50 nM [$^3$H]VCR (2.3 Ci/mmol) in SH-SY5Y and SH-SY5Y/VCR cells and the effect of nonradioactive I-NAPS on [$^3$H]VCR accumulation were determined as previously described (Tamai & Safa, 1990). Briefly, after 5-, 15-, 30-, or 60-min incubation at 37 °C with [$^3$H]VCR in HBSS containing 1.5 g/L D-glucose in the absence or presence of 5 $\mu$M I-NAPS, cells were rapidly washed twice with HBSS and trypsinized, and the radioactivity associated with the cells was determined.

*Growth Inhibition Measurements.* Growth inhibition assays were performed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as we recently reported (Fleming et al., 1992). Briefly, cells were plated in 50-$\mu$L aliquots of growth medium into 96-well microtiter plates at 7500 cells/well and incubated at 37 °C overnight. Drugs were dissolved in dimethyl sulfoxide ($Me_2SO$) before being diluted in medium; conditions were adjusted to keep the final concentration of $Me_2SO$ to less than 0.1%, concentrations at which $Me_2SO$ had no effect on cell survival and growth. Drugs diluted in medium were added to achieve a final volume of 100 $\mu$L; cells were treated with I-NAPS (at 3 and 5 $\mu$M) for 30–60 min before various concentrations of VCR were added.

Concentrations of VCR which reduced cell growth by 50% after 72 h treatment ($IC_{50}$) in the absence or presence of I-NAPS were determined from growth inhibition plots.

*Membrane Preparations.* Cell membrane vesicles were prepared by the nitrogen cavitation and sucrose differential centrifugation procedure previously described (Safa et al., 1989; Lever, 1977). High-speed (160000*g*) supernatant and pellet fractions were prepared as previously reported (Safa et al., 1987b) using a Beckman Airfuge. Protein concentrations were determined by the method of Bradford (1976) using the BioRad protein assay kit (BioRad; Richmond, CA).

*Photoaffinity Labeling.* Exponentially growing cells were harvested by a cell scraper. Trypan blue viable (>90%) cell suspensions ($5 \times 10^5$ cells/assay) in $Ca^{2+}$–$Mg^{2+}$-free Dulbecco's phosphate-buffered saline, or membrane vesicles (100 μg of protein) in 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 10 mM NaCl, 1.5 mM $MgCl_2$, and 3 mM ATP containing 4% $Me_2SO$ and 2 nM [$^{125}$I]NAPS (2200 Ci/mmol) or [$^{125}$I]-AAP (2200 Ci/mmol) in a final volume of 50 μL, were photolabeled after preincubation for 30 min at 25 °C in the absence or presence of nonradioactive competing ligands. Samples were then photolabeled as previously described (Safa et al., 1986). To determine whether the 17-kDa [$^{125}$I]NAPS binding protein spiperophilin is an integral membrane protein or whether it peripherally interacts with cellular membranes, $1 \times 10^7$ cells were photolabeled with 1 μCi of [$^{125}$I]NAPS. The high-speed (160000*g*) pellet fraction was washed 3 times with 0.6 M NaCl (Safa & Felsted, 1987) and recentrifuged at 160000*g*. The photolabeled pellet fraction, membrane vesicles, and cells were solubilized in SDS sample buffer (Safa & Felsted, 1987) and subjected to SDS–PAGE. Quantitation of [$^{125}$I]NAPS-labeled P-gp was carried out by densitometry tracings of autoradiograms.

*Immunoprecipitation.* For immunoprecipitation with anti-P-gp monoclonal antibody C219 (Kartner et al., 1985) or the anti-calmodulin monoclonal antibody. SH-SY5Y/VCR cells were photolabeled with 1 μCi of [$^{125}$I]NAPS in the absence or presence of 50 μCi of nonradioactive I-NAPS. Cells were solubilized in radioimmune precipitating buffer (RIPA) for 1 h as previously described (Safa et al., 1986). The mixtures were then incubated for 1 h at 4 °C with 10 μg each of anti-P-gp monoclonal antibody C219, the anti-calmodulin monoclonal antibody, or nonimmune mouse serum. Immune complexes were obtained by incubating the mixture with protein A–Sepharose (for C219 monoclonal antibody) and protein G–Sepharose (for the anti-calmodulin monoclonal antibody) for 1 h at 4 °C and recovering the beads after washing once with 1.5 mL of 50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (buffer A) containing 0.1% SDS and 2 times with buffer A. The bound proteins were then solubilized by resuspending the immune complexes in equal volumes of SDS sample buffer, and the supernatant fraction was analyzed by SDS–PAGE. Aliquots of $^{125}$I-Bolton–Hunter-labeled calmodulin (50 ng/immunoprecipitation) in either buffer A or RIPA buffer were used as positive controls in immunoprecipitations with the anti-calmodulin monoclonal antibody. Immunoprecipitation experiments were repeated in two independent experiments.

*Peptide Mapping.* For peptide mapping, $5 \times 10^6$ SH-SY5Y/VCR cells were photolabeled with 2 nM [$^{125}$I]NAPS or [$^{125}$I]AAP. The photolabeled P-gp from $8 \times 10^6$ cells was immunoprecipitated with anti-P-gp monoclonal antibody C219. Immunoprecipitated P-gp was then subjected to *Staphylococcus aureus* V8 protease (60 μg) in 100 mM Tris-HCl, pH 7.5, for 90 min at room temperature and applied to 10–20% SDS–PAGE gradient gels.

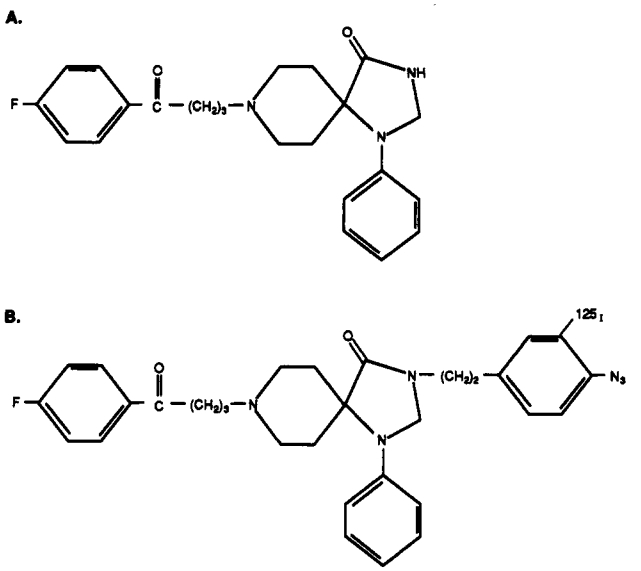


FIGURE 1: Structure of spiperone and its photoaffinity analog *N*-(*p*-azido-3-[$^{125}$I]iodophenethyl)spiperone ([$^{125}$I]NAPS).

*Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE).* Photolabeled samples were electrophoresed on SDS–PAGE 5–15% gradient gels containing 4.5 M urea and processed for fluorography or autoradiography as previously described (Safa et al., 1986).

## RESULTS

*[$^{125}$I]NAPS Accumulation and Efflux.* The chemical structure of [$^{125}$I]NAPS, a $D_2$-dopamine receptor photoaffinity probe, is presented in Figure 1. To examine whether [$^{125}$I]-NAPS is a substrate for P-gp and is actively extruded from the MDR cells, we measured its cellular uptake and efflux by sensitive parental SH-SY5Y human neuroblastoma cells and MDR variant SH-SY5Y/VCR cells. The time course of [$^{125}$I]-NAPS accumulation by SH-SY5Y and SH-SY5Y/VCR cells was measured in the presence (1.5 g/L D-glucose) or absence (10 mM sodium azide) of metabolic energy supply and is shown in Figure 2. The accumulation of [$^{125}$I]NAPS in both cell lines reached steady-state after 30-min incubation in 3.12 pM [$^{125}$I]NAPS. There is a 3-fold decrease in the intracellular accumulation of [$^{125}$I]NAPS in resistant cells compared to the sensitive parental cells. This decreased intracellular accumulation of [$^{125}$I]NAPS accounts for the presence of an active extrusion pump in MDR cells. Such active efflux of MDR-related drugs and some MDR modulators has been previously described (Danø, 1973; Skovsgaard, 1978; Tamai & Safa, 1990, 1991). In the drug-sensitive SH-SY5Y cells, there was no significant difference in [$^{125}$I]NAPS accumulation in the presence or absence of a metabolic energy supply. However, SH-SY5Y/VCR cells in the absence of energy accumulated an amount of [$^{125}$I]NAPS comparable to that accumulated by SH-SY5Y cells, suggesting that an energy-dependent efflux pump regulates [$^{125}$I]NAPS accumulation in the resistant cells. In the presence of 10 mM sodium azide, when [$^{125}$I]NAPS accumulation reached steady-state, the efflux of [$^{125}$I]NAPS was measured by determining the radioactivity associated with the cells (Figure 2). The efflux was dependent on the presence of metabolic energy. In the presence of 1.5 g/L D-glucose, efflux was rapid, and about 75% of the drug was extruded within 5 min and reached steady-state thereafter. More than 90% of the radioactivity remained

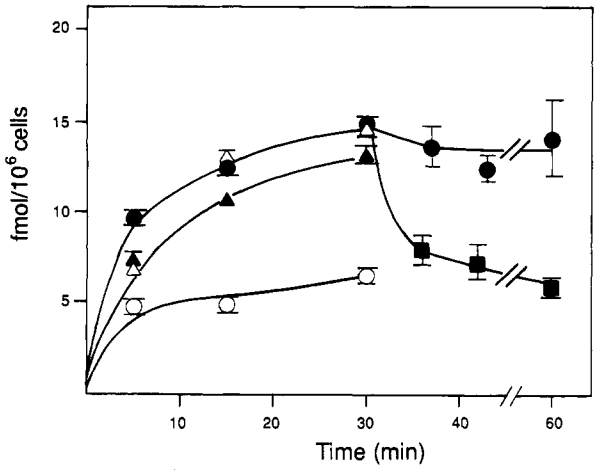


FIGURE 2: Time course of [$^{125}$I]NAPS uptake and efflux by SH-SY5Y and SH-SY5Y/VCR cells. Uptake of 3.2 pM [$^{125}$I]NAPS was measured in HBSS in the presence of 1.5 g/L D-glucose (▲, ○) or 10 mM sodium azide (△, ●) at room temperature for 30 min: SH-SY5Y cells (▲, △); SH-SY5Y/VCR cells (○, ●). After 30-min accumulation of [$^{125}$I]NAPS in the presence of 10 mM sodium azide by SH-SY5Y/VCR cells, the cells were washed with ice-cold HBSS, and efflux medium was added. Efflux was measured in HBSS containing either 10 mM sodium azide (●) or 1.5 g/L D-glucose (■). Each point is the mean value of triplicate experiments.

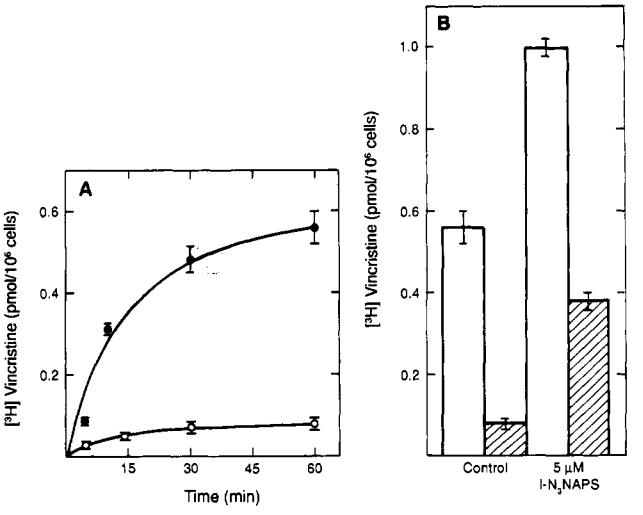


FIGURE 3: (A) Time course of accumulation of [$^3$H]vincristine in SH-SY5Y (●) and SH-SY5Y/VCR cells (○). Cells ($2 \times 10^5$/well) in multiwell plates were used, and the accumulation of 50 nM [$^3$H]-vincristine was measured in HBSS containing 1.5 g/L D-glucose. (B) [$^3$H]Vincristine accumulation in SH-SY5Y (open bars) and SH-SY5Y/VCR (hatched bars) cells in the absence or presence of a 5 μM aliquot of the nonradioactive photoactive analog I-NAPS.

with the cells even after 30 min in the presence of sodium azide, suggesting that more than 90% of [$^{125}$I]NAPS efflux is energy-dependent. These results and our photoaffinity labeling data discussed below indicate that [$^{125}$I]NAPS is an excellent substrate for P-gp and is actively effluxed from drug-resistant cells. When 5 μM [$^{125}$I]NAPS was used in accumulation experiments, the ratio of drug accumulation in SH-SY5Y/VCR cells to that in the SH-SY5Y cells approached unity. This finding suggests that the probe might itself be acting as a blocker of P-gp. Indeed, as shown in Figure 3, I-NAPS was found to enhance the accumulation of [$^3$H]VCR in these cells.

*Effect of I-NAPS on [$^3$H]VCR Accumulation.* We further determined the effect of I-NAPS on [$^3$H]VCR accumulation in the parental SH-SY5Y and MDR variant SH-SY5Y/VCR cell lines (Figure 3). The accumulation of [$^3$H]VCR in both cell lines reached steady-state after 60-min incubation of cells with 50 nM [$^3$H]VCR. After 60-min incubation with 50 nM [$^3$H]VCR, SH-SY5Y/VCR cells accumulated 6.8-fold less drug than SH-SY5Y cells (Figure 3). The addition of 5 μM I-NAPS increased [$^3$H]VCR accumulation by 1.8-fold in SH-SY5Y cells and by 4.75-fold in SH-SY5Y/VCR cells (Figure 3).

Table 1: Effect of [I-NAPS] on Cell Survival and VCR Sensitization

| [I-NAPS] (μM) | cell survival[a] (% control) SH-SY5Y | cell survival[a] (% control) SH-SY5Y/VCR | VCR $IC_{50}$[b] (nM) SH-SY5Y | VCR $IC_{50}$[b] (nM) SH-SY5Y/VCR |
|---|---|---|---|---|
| none | 100 | 100 | 5.2 | 3200 |
| 3 | 98 | 100 | 0.45 | 620 |
| 5 | 99 | 100 | 0.25 | 105 |

[a] Cell survival in the absence or presence of I-NAPS was determined by MTT assay. Values are the average of three independent experiments and duplicate determinants in each experiment. [b] Concentration of VCR which reduced cell survival by 50% in the absence or presence of I-NAPS after 72-h treatment. Values are the average of three independent experiments and duplicate determinants in each experiment.

*Effect of I-NAPS on Cell Survival and VCR Sensitization.* The cytotoxic effect of I-NAPS alone and in combination with VCR on parental SH-SY5Y human neuroblastoma cells and MDR variant SH-SY5Y/VCR cells was evaluated by MTT assay, and the results are presented in Table 1. The assay is based on the principal that living cells can convert soluble MTT to an insoluble formazen precipitate. The purple formazen crystals are then dissolved in lysing buffer, and the optical density of the solution is measured on a multiplate spectrophotometer. The VCR concentration that inhibited cell growth by 50% ($IC_{50}$) was 5.2 nM in SH-SY5Y and 3200 nM in SH-SY5Y/VCR cells, indicating that the resistant cells are 615-fold-resistant to VCR (Table I). At 3 and 5 μM, I-NAPS alone had no significant effect on the survival of sensitive or resistant cells (Table 1). The addition of 3 and 5 μM I-NAPS to sensitive SH-SY5Y cells in the presence of increasing concentrations of VCR reduced the $IC_{50}$ to 0.45 and 0.25 nM, respectively, indicating that I-NAPS at these concentrations increases VCR sensitivity 11.5- and 20.8-fold, respectively (Table 1). At 3 and 5 μM, I-NAPS decreased the VCR $IC_{50}$ of SH-SY5Y/VCR cells to 620 and 105 nM, respectively, indicating that at these concentrations I-NAPS sensitizes the resistant cells 5.16- and 30.4-fold, respectively (Table 1). To further determine the modulating effect of I-NAPS on VCR cytotoxicity in sensitive cells, we used the human epidermoid carcinoma cell line KB-3-1, known to lack P-gp (Noonan et al., 1990). At 3 and 5 μM, I-NAPS decreased the VCR $IC_{50}$ of KB-3-1 cells from 0.25 nM to 0.1 and 0.018 nM, respectively, indicating that at these concentrations I-NAPS sensitizes these cells 2.5- and 15.5-fold, respectively. These results suggest that a mechanism(s) independent of P-gp is (are) involved in sensitizing KB-3-1 cells to I-NAPS.

*Identification of Specific [$^{125}$I]NAPS Binding Proteins by Photoaffinity Labeling.* In order to identify the [$^{125}$I]NAPS binding proteins in SH-SY5Y and SH-SY5Y/VCR cells, the intact cells, membrane vesicles, or high-speed supernatant was photoaffinity labeled with a 2 nM aliquot of the photoaffinity probe and examined by SDS–PAGE and autoradiography. Photoaffinity labeling and autoradiography showed that [$^{125}$I]NAPS prominently and specifically binds to two proteins: a 150–180-kDa protein in SH-SY5Y/VCR cells which is also detectable in the drug-sensitive SH-SY5Y parental cells, and a 17-kDa protein which we termed spiperophilin. The specificity of binding was determined by

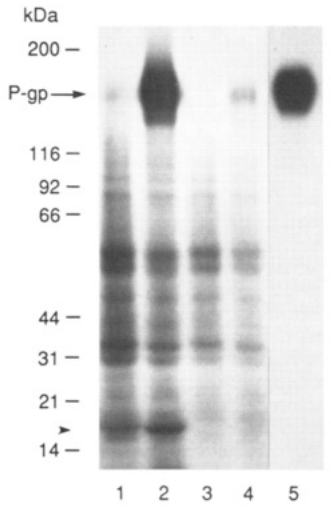


FIGURE 4: SDS–PAGE autoradiography of [$^{125}$I]NAPS-photolabeled SH-SY5Y (lanes 1 and 3) and SH-SY5Y/VCR (lanes 2 and 4) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of a 10 $\mu$M aliquot of the nonradioactive probe I-NAPS. Lane 5 shows the 150–180-kDa [$^{125}$I]NAPS-photolabeled protein from $1 \times 10^7$ SH-SY5Y/VCR cells immunoprecipitated with the anti-P-gp monoclonal antibody C219.

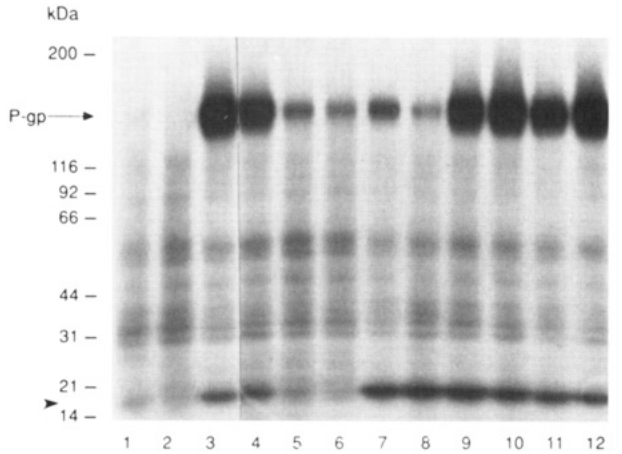


FIGURE 5: SDS–PAGE autoradiography of [$^{125}$I]NAPS-photolabeled SH-SY5Y (lanes 1 and 2) and SH-SY5Y/VCR cells (lanes 3–12). Photolabeling was performed in the absence (lanes 1 and 3) or presence of 1, 10, and 100 $\mu$M spiperone (lanes 4–6) or 100 $\mu$M vincristine, vinblastine, doxorubicin, actinomycin D, colchicine, or methotrexate (lanes 7–12), respectively. The arrow and the arrowhead indicate the positions of the photolabeled P-gp and spiperophilin, respectively. The positions of molecular mass standards in kilodaltons are indicated on the left.

performing experiments in the presence of excess I-NAPS (Figure 4) or the nonradioactive parent compound spiperone (Figure 5). At 10 $\mu$M, I-NAPS inhibited the photolabeling of the 150–180- and 17-kDa proteins by 90 and 100%, respectively (Figure 4). At 10, 100, and 500 $\mu$M, spiperone inhibited the [$^{125}$I]NAPS photolabeling of P-gp by 61, 93, and 94%, respectively (Figure 5). When the [$^{125}$I]NAPS-photolabeled SH-SY5Y/VCR cells were solubilized and immunoprecipitated with monoclonal antibody C219 specific for P-gp, only the radiolabeled 150–180-kDa protein was precipitated (Figure 4). This indicates that P-gp is a specific acceptor for [$^{125}$I]NAPS. As shown in Table 2, the photolabeling efficiency of P-gp by [$^{125}$I]NAPS was 9.41%. [$^{125}$I]-AAP and [$^3$H]azidopine, two known photoactive MDR modulators, incorporated into P-gp with efficiencies of 1.58 and 1.76%, respectively (Table 2), demonstrating that [$^{125}$I]-NAPS bound to P-gp with much higher efficiency. At 100 $\mu$M, vinblastine, VCR, colchicine, doxorubicin, or actinomycin

Table 2: Efficiency of Photoaffinity Labeling of P-gp by [$^{125}$I]NAPS, [$^{125}$I]APP, and [$^3$H]Azidopine

| photoaffinity drug | efficiency[a] (%) |
|---|---|
| [$^{125}$I]NAPS | 9.41 |
| [$^{125}$I]AAP | 1.58 |
| [$^3$H]azidopine | 1.76 |

[a] Efficiency is defined as the perdent of the initial concentration of the photoactive drug bound to P-gp. Photoaffinity labeling was carried out using 2 nM [$^{125}$I]NAPS, 2 nM [$^{125}$I]AAP, or 50 nM [$^3$H]azidopine and $5 \times 10^5$ SH-SY5Y/VCR cells as described under Materials and Methods. Values are the average of three independent experiments. Radioactivity associated with P-gp was determined by cutting bands corresponding to this protein from autoradiograms or fluorograms.

Table 3: Effect of MDR-Related Drugs and MDR Modulators on [$^{125}$I]NAPS and [$^{125}$I]AAP Photoaffinity Labeling of P-gp and Spiperophilin

| drugs | [$^{125}$I]NAPS[a] (% control) | | [$^{125}$I]AAP (% control) |
|---|---|---|---|
| | P-gp | spiperophilin | P-gp |
| control | 100 | 100 | 100 |
| vinblastine | 3 | 282 | 11 |
| vincristine | 13 | 280 | 44 |
| colchicine | 56 | 102 | 50 |
| doxorubicin | 64 | 217 | 82 |
| actinomycin D | 87 | 271 | 62 |
| nicardipine | 0 | 7 | 12 |
| prenylamine | 0 | 24 | 320 |
| verapamil | 3 | 25 | 114 |
| diltiazem | 26 | 101 | 40 |
| cyclosporin A | 0 | 324 | 0 |
| reserpine | 3 | 64 | 0 |
| dibucaine | 15 | 60 | 25 |
| monensin | 12 | 150 | 8 |
| progesterone | 100 | 7 | 27 |

[a] Photoaffinity labeling was carried out using 2 nM [$^{125}$I]NAPS or [$^{125}$I]AAP in the absence or presence of 100 $\mu$M drugs as described under Materials and Methods. Quantitation was carried out by densitometric analysis of SDS–PAGE autoradiograms.

D, the drugs to which SH-SY5Y/VCR cells display cross-resistance, differentially inhibited [$^{125}$I]NAPS photolabeling of P-gp (Figure 5 and Table 3). The MDR-unrelated antitumor agent methotrexate had no effect on the [$^{125}$I]NAPS labeling of P-gp (Figure 5 and Table 3).

The extent of spiperophilin labeling in SH-SY5Y/VCR cells was 3-fold more than that in the sensitive parental line SH-SY5Y (Figure 4). [$^{125}$I]NAPS binding to this protein represented 2.07% of the initial radioactivity. Densitometry tracings of Coomassie brilliant blue-stained cells after SDS–PAGE revealed no significant differences between the amounts of this 17-kDa protein in the sensitive and resistant cells. Photoaffinity labeling of membrane and cytosolic fractions of SH-SY5Y/VCR cells revealed that spiperophilin is membrane-bound. Spiperophilin was associated with the membrane fraction even after repeated washings with a high salt concentration (0.6 M NaCl), and detergent was required for its solubilization, indicating that it is an integral membrane protein. Spiperophilin was also detected in KB-3-1 human epidermoid carcinoma cells lacking P-gp, revealing that this protein is not a proteolytic fragment of P-gp. Furthermore, the extent of spiperophilin photolabeling in the MDR variant KB-V1 cells was similar to that in KB-3-1 cells (data not shown). While spiperone at 100 $\mu$M completely inhibited the binding of [$^{125}$I]NAPS to this protein, actinomycin D, doxorubicin, vinblastine, and VCR enhanced its binding as compared with the control (Figure 5 and Table 3).

Spiperophilin has the same molecular weight as calmodulin, a ubiquitous calcium binding protein that mediates many

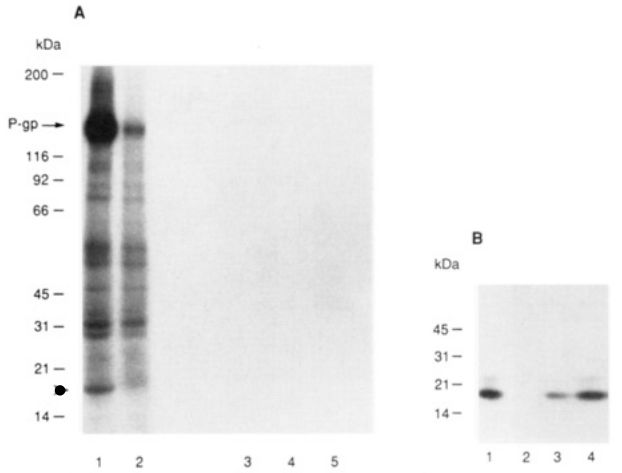


FIGURE 6: (A) [$^{125}$I]NAPS photolabeling of SH-SY5Y/VCR cells and immunoprecipitation with anti-calmodulin monoclonal antibody. (B) Immunoprecipitation of $^{125}$I-Bolton–Hunter-labeled calmodulin with anti-calmodulin monoclonal antibody. (A) SH-SY5Y/VCR cells ($5 \times 10^5$ cells/lane) were photolabeled with 2 nM [$^{125}$I]NAPS in the absence (lane 1) or presence of 50 $\mu$M nonradioactive I-NAPS (lane 2). Lanes 3–5 show [$^{125}$I]NAPS-photolabeled detergent-solubilized SH-SY5Y/VCR cells ($9.5 \times 10^7$ cells/lane) in the absence (lane 3) or presence (lane 4) of 50 $\mu$M I-NAPS immunoprecipitated with the anti-calmodulin monoclonal antibody (lanes 3 and 4) or normal mouse serum (lane 5). (B) Fifty nanograms of $^{125}$I-Bolton–Hunter-labeled calmodulin (lane 1) was used as a control, and immunoprecipitated under identical conditions as for panel A using normal serum (lane 2) or the anti-calmodulin monoclonal antibody (lanes 3 and 4) before (lane 3) and after addition of RIPA buffer for 1 h as described under Materials and Methods. The arrow and the arrowhead indicate the positions of the photolabeled P-gp and spiperophilin, respectively. The positions of molecular mass standards in kilodaltons are indicated on the left.

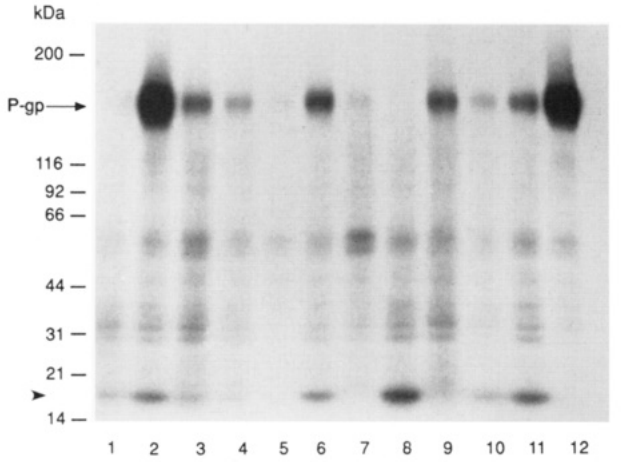


FIGURE 7: SDS–PAGE autoradiography of [$^{125}$I]NAPS-photolabeled SH-SY5Y (lane 1) and SH-SY5Y/VCR cells (lanes 2–12). Photolabeling was carried out in the absence (lanes 1 and 2) or presence of 100 $\mu$M spiperone (lane 3) or the MDR modulators verapamil, nicardipine, diltiazem, prenylamine, cyclosporin A, dibucaine, reserpine, monensin, or progesterone (lanes 4–12), respectively. The arrow and the arrowhead indicate the positions of the photolabeled P-gp and spiperophilin, respectively. The positions of molecular mass standards in kilodaltons are indicated on the left.

cellular functions, and like calmodulin (Hait & Lazo, 1986) this protein interacts with a diverse group of drugs as shown below. Therefore, to determine whether this protein is different from calmodulin, the RIPA buffer-solubilized [$^{125}$I]NAPS-photolabeled SH-SY5Y/VCR cells were immunoprecipitated with anti-calmodulin antibody (Figure 6A). As shown in Figure 6A, no radiolabeled protein was immunoprecipitated with this antibody. Moreover, in control experiments, the anti-calmodulin antibody immunoprecipitated $^{125}$I-labeled calmodulin in the presence or absence of RIPA buffer (Figure 6B). These results indicate that the spiperophilin is different from calmodulin.

It is known that certain structurally unrelated lipophilic agents may reverse MDR by interacting with the cytotoxic drug binding sites of P-gp and increasing the cellular accumulation and retention of antitumor agents (Safa, 1993). To determine whether MDR modulators inhibit the binding of [$^{125}$I]NAPS to P-gp and spiperophilin, we screened a series of agents known to reverse MDR in photoaffinity labeling experiments. [$^{125}$I]NAPS photolabeling of P-gp in the presence of 100 $\mu$M each of nicardipine, prenylamine, verapamil, and diltiazem, representatives of four classes of calcium channel blockers (dihydropyridines, diphenylalkylamines, phenylalkylamines, and benzodiazepines), was significantly reduced (Figure 7 and Table 3). At 100 $\mu$M, other MDR modulators including cyclosporin A, reserpine, monensin, and dibucaine were potent inhibitors of [$^{125}$I]NAPS photolabeling of P-gp (Figure 7 and Table 3). The steroid hormone progesterone, known to interact with P-gp, did not affect [$^{125}$I]NAPS photolabeling of P-gp (Figure 7 and Table 3).

At 100 $\mu$M, nicardipine, progesterone, prenylamine, verapamil, dibucaine, and reserpine reduced [$^{125}$I]NAPS photolabeling of spiperophilin (Figure 7 and Table 3) while monensin and cyclosporin A increased the radiolabeling of P-gp, and diltiazem had no effect (Figure 7 and Table 3).

Several PTZs and structurally related compounds sensitize MDR cells to chemotherapeutic agents, possibly by interacting with P-gp (Ford et al., 1989; Fleming et al., 1990). We tested the ability of these agents to interact with the [$^{125}$I]NAPS binding site of P-gp and inhibit its binding to this protein. At 100 $\mu$M, trifluoperazine, thioridazine, fluphenazine N-mustard, *trans*-flupentixol, *cis*-flupentixol, chlorpromazine, and fluphenazine inhibited [$^{125}$I]NAPS photolabeling of P-gp by more than 95% (Figure 8A). W-7 inhibited P-gp photolabeling by 70%. At 100 $\mu$M, pimozide, fluspirilene, and *cis*-thiothixine inhibited P-gp photolabeling by 100, 100, and 80%, respectively (Figure 8A).

At 100 $\mu$M, trifluoperazine, chlorpromazine, thioridazine, fluphenazine N-mustard, W-7, pimozide, and fluspirilene totally inhibited [$^{125}$I]NAPS binding to spiperophilin (Figures 8A and 9A). Furthermore, *cis*- and *trans*-flupentixol, fluphenazine, and *cis*-thiothixine inhibited the photolabeling of this protein by 80% (Figure 8A).

We also analyzed the ability of a $\beta$-adrenergic antagonist, (–)-propranolol, and its inactive enantiomer, (+)-propranolol, dopaminergic drugs [dopamine, haloperidol, (*S*)-(–)- and (*R*)-(+)-sulpiride, and (*R*)-(–)-propylnorapomorphine], the neurotransmitter serotonin (5-hydroxytryptamine), and $\alpha_2$-adrenergic drugs (epinephrine, norepinephrine, methoxamine, clonidine, and yohimbine) to inhibit [$^{125}$I]NAPS photolabeling of P-gp (Figures 9A and 10). At 100 $\mu$M, (*R*)-(–)-propylnorapomorphine, (+)-propranolol, (–)-propranolol, serotonin, haloperidol, yohimbine and methoxamine partially inhibited photolabeling (Figures 9A and 10 and Table 4). (*S*)-(–)- and (*R*)-(+)-sulpiride, clonidine, norepinephrine, epinephrine, and dopamine had no significant effect on [$^{125}$I]-NAPS photolabeling of P-gp (Figures 9A and 10 and Table 4).

At 100 $\mu$M, while (*R*)-(–)-propylnorapomorphine, (+)-propranolol, (–)-propranolol and serotonin also inhibited [$^{125}$I]-NAPS photolabeling of spiperophilin, (*S*)-(–)- and (*R*)-(+)-sulpiride did not inhibit photolabeling of this protein (Figure 9A). Furthermore, haloperidol, dopamine, clonidine, and

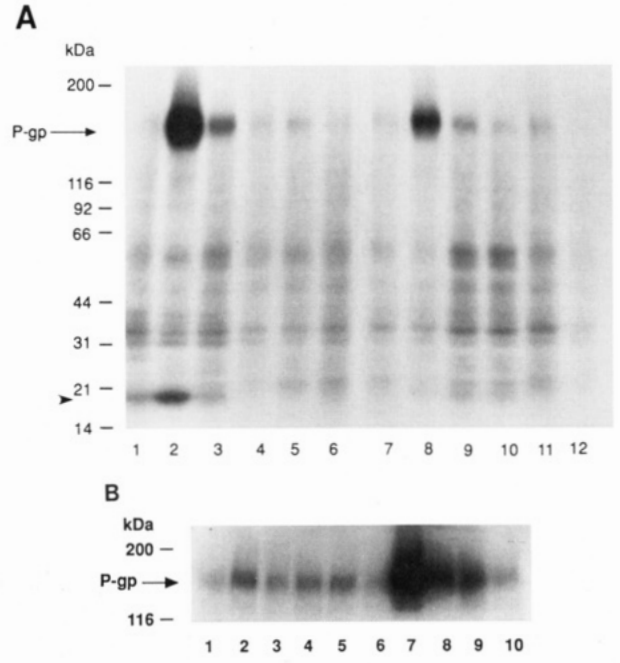


FIGURE 8: (A) SDS–PAGE autoradiography of [$^{125}$I]NAPS-photolabeled SH-SY5Y (lane 1) and SH-SY5Y/VCR cells (lanes 2–12) in the absence (lanes 1 and 2) or presence of 100 $\mu$M spiperone (lane 3) or phenothiazines and the related agents trifluoperazine, chlorpromazine, thioridazine, perphenazine. W-7, *cis*-flupentixol, *trans*-flupentixol, fluphenazine, or fluphenazine N-mustard (lanes 4–12), respectively. The arrow and the arrowhead indicate the positions of the photolabeled P-gp and spiperophilin, respectively. (B) Photoaffinity labeling of P-gp from SH-SY5Y/VCR cells with [$^{125}$I]AAP (lane 1) and effects of trifluoperazine, chlorpromazine, thioridazine, perphenazine. W-7, *cis*-flupentixol, *trans*-flupentixol, fluphenazine, and fluphenazine mustard (lanes 2–10), respectively. The positions of molecular mass standards in kilodaltons are indicated on the left.

yohimbine reduced photolabeling of this protein (Table 4). Norepinephrine, epinephrine, and methoxamine enhanced [$^{125}$I]NAPS photolabeling of spiperophilin (Figure 10 and Table 4).

To examine whether [$^{125}$I]NAPS interacts with P-gp in a manner similar to that of arylazidoprazosin, a known photoaffinity analog of P-gp and an MDR modulator, we used two approaches: (1) comparing patterns of inhibition of [$^{125}$I]-NAPS and [$^{125}$I]AAP photoaffinity labeling of P-gp by MDR modulators and related agents; (2) identifying sites of interaction of these two photoaffinity probes with P-gp by comparing the drug-bound fragments after proteolysis. [$^{125}$I]-AAP bound specifically to P-gp, and at 100 $\mu$M, the parent compound, prazosin, inhibited binding by 65%. However, spiperophilin did not bind to [$^{125}$I]AAP. The results presented in Table III show that photoaffinity labeling of P-gp with [$^{125}$I]AAP was preferentially inhibited by vinblastine > VCR > colchicine > actinomycin D > doxorubicin. Among the calcium channel blockers, while verapamil and nicardipine totally inhibited [$^{125}$I]NAPS binding to P-gp, they increased the binding of [$^{125}$I]AAP (Table 3). In contrast, while progesterone had no effect on the binding of [$^{125}$I]NAPS, it inhibited the binding of [$^{125}$I]AAP to P-gp by 73% (Table 3). RTZs and the related compounds tested, including W-7, chlorpromazine, fluphenazine N-mustard, thioridazine, perphenazine, and triflupentixol, increased the binding of [$^{125}$I]-AAP to P-gp by 124, 314, 326, 521, 641, and 943%, respectively (Figure 8B). Moreover, *trans*-flupentixol, fluphenazine, and *cis*-flupentixol increased the binding of [$^{125}$I]AAP to P-gp by 12-, 22.6-, and 107.2-fold, respectively (Figure 8B). Identical results were obtained when KB-V1 cells were used and [$^{125}$I]-

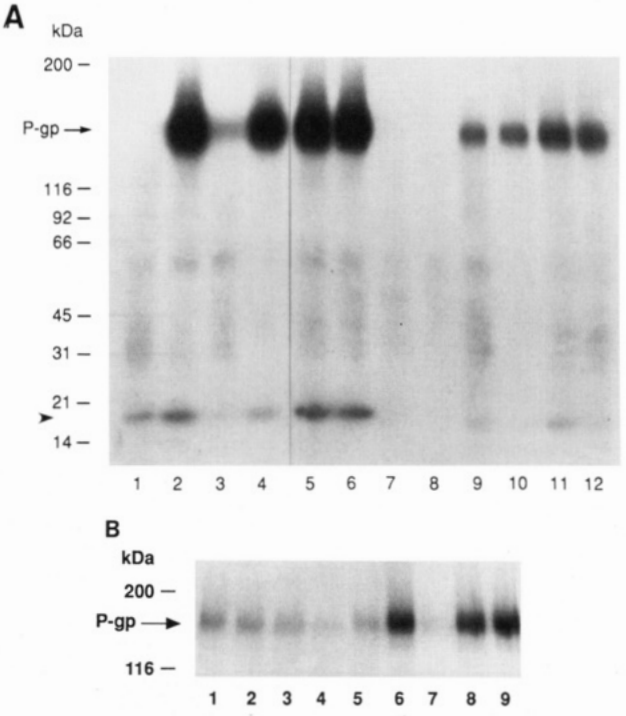


FIGURE 9: (A) SDS–PAGE autoradiography of [$^{125}$I]NAPS-photolabeled SH-SY5Y (lane 1) and SH-SY5Y/VCR cells (lanes 2–12) in the absence (lanes 1 and 3) or presence of 100 $\mu$M each of spiperone, serotonin. (*S*)-(–)- and (*R*)-(+)-sulpiride, pimozide, fluspirilene, *cis*-thiothixene, (*S*)-(+)-propylnorapomorphine, (–)-propranolol, and (+)-propranolol (lanes 4–12), respectively. The arrow and the arrowhead indicate the positions of the photolabeled P-gp and spiperophilin, respectively. (B) Photoaffinity labeling of P-gp from SH-SY5Y/VCR cells with [$^{125}$I]AAP (lane 1) and effects of serotonin, (±)-sulpiride, pimozide, fluspirilene, *cis*-thiothiexene, (*S*)-(+)-propylnorapomorphine, (–)-propanolol, and (+)-propanolol (lanes 2–9), respectively. The positions of molecular mass standards in kilodaltons are indicated on the left.

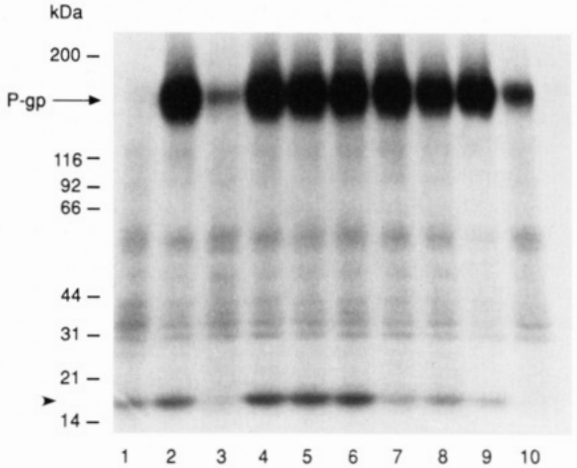


FIGURE 10: SDS–PAGE autoradiography of [$^{125}$I]NAPS-photolabeled SH-SY5Y (lane 1) and SH-SY5Y/VCR cells (lanes 2–10). Photolabeling was performed in the absence (lanes 1 and 2) or presence of 100 $\mu$M spiperone, epinephrine, norepinephrine, methoxamine, clonidine, yohimbine, dopamine, or haloperidol (lanes 3–10). The arrow and the arrowhead indicate the positions of the photolabeled P-gp and spiperophilin, respectively. The positions of molecular mass standards in kilodaltons are indicated on the left.

AAP photolabeling was performed in the presence of PTZs and related compounds (data not shown). Among the PTZs and related compounds used, only fluspirilene and pimozide inhibited [$^{125}$I]AAP binding to P-gp by 23 and 100%, respectively (Figure 9B). Dopamine, norepinephrine, clonidine, methoxamine, haloperidol, and (–)- and (+)-propanolol

Table 4: Effect of Dopaminergic and $\alpha_2$-Adrenergic Drugs on [$^{125}$I]NAPS and [$^{125}$I]AAP Photoaffinity Labeling of P-gp and Spiperophilin

| drugs | [$^{125}$I]NAPS[a] (% control) | | [$^{125}$I]AAP (% control) |
|---|---|---|---|
| | P-gp | spiperophilin | P-gp |
| control | 100 | 100 | 100 |
| epinephrine | 112 | 150 | 84 |
| norepinephrine | 100 | 136 | 137 |
| methoxamine | 86 | 150 | 169 |
| clonidine | 100 | 36 | 148 |
| yohimbine | 84 | 47 | 81 |
| (−)-propanolol | 25 | 30 | 645 |
| (+)-propanolol | 30 | 15 | 738 |
| dopamine | 98 | 24 | 128 |
| haloperidol | 30 | 0 | 454 |
| (*R*)-(−)-propylnorapomorphine | 20 | 10 | 0 |
| serotonin | 75 | 70 | 95 |

[a] Photoaffinity labeling was carried out using 2 nM [$^{125}$I]NAPS or [$^{125}$I]AAP in the absence or presence of 100 $\mu$M drugs as described under Materials and Methods. Quantitation was carried out by densitometric analysis of SDS–PAGE autoradiograms.

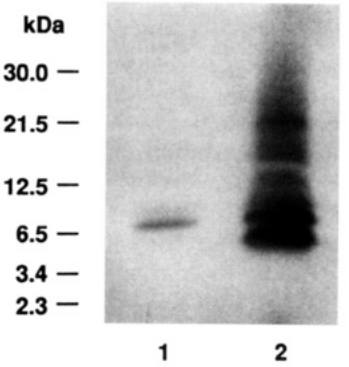


FIGURE 11: *S. aureus* V8 protease-digested P-gp photoaffinity-labeled with [$^{125}$I]AAP (lane 1) or [$^{125}$I]NAPS (lane 2). SH-SY5Y/VCR cells ($10^8$ cells per experiment) were photoaffinity-labeled with 2 nM [$^{125}$I]AAP or [$^{125}$I]NAPS, solubilized with RIPA buffer, immunoprecipitated with the anti-P-gp monoclonal antibody C219, and subjected to V8 protease digestion as described under Materials and Methods.

increased the binding of [$^{125}$I]AAP to P-gp (Figure 9B and Table 4). (*R*)-(−)-Propylnorapomorphine totally inhibited [$^{125}$I]AAP binding to P-gp.

These results suggest that [$^{125}$I]NAPS and [$^{125}$I]AAP may interact with separate domains of P-gp. Identification of the [$^{125}$I]NAPS binding domain(s) of P-gp will help to further understand the molecular mechanism of substrate recognition and transport by P-gp and how the MDR modulators and diverse lipophilic agents interact with this protein. To identify the [$^{125}$I]NAPS and [$^{125}$I]AAP binding domain(s), radiolabeled P-gp was immunoprecipitated from detergent-solubilized membrane vesicles of SH-SY5Y/VCR cells using the anti-P-gp monoclonal antibody C219 and then digested with *S. aureus* V8 protease (Figure 11). The results in this figure show that when the [$^{125}$I]NAPS-photolabeled P-gp from SH-SY5Y/VCR cells was digested with 60 $\mu$g of V8 protease, two major peptides, 6 and 8 kDa, and a number of minor ones were labeled (Figure 11). Identical peptide mapping experiments were performed to determine whether [$^{125}$I]AAP binds to the same domain(s) of P-gp in SH-SY5Y/VCR human neuroblastoma cells. Interestingly, [$^{125}$I]AAP binds to only one fragment of P-gp, an 8-kDa peptide, suggesting that P-gp may possess more than one drug binding site and that modulators of MDR may interact with separate binding sites.

## DISCUSSION

A variety of cationic, lipophilic agents can reverse MDR by directly and specifically binding to the cytotoxic drug binding sites of P-gp (Endicott & Ling, 1989; Tamai & Safa, 1990; Safa, 1993). We have previously shown that the modes of interaction of P-gp binding substrates are competitive or noncompetitive (Tamai & Safa, 1990, 1991). In this report, we used [$^{125}$I]NAPS, a $D_2$-dopamine receptor photoaffinity probe (Amlaiky & Caron, 1986), and identified P-gp and a 17-kDa protein, spiperophilin, as its specific acceptors in SH-SY5Y/VCR cells. [$^{125}$I]NAPS has been shown to bind specifically to a $D_2$-dopamine receptor with a molecular mass of 92 kDa (Amlaiky & Caron, 1986) and to the serotonin ($5\text{-HT}_{1A}$) receptor with a molecular mass of 64 kDa (Raymond et al., 1989).

Our results demonstrate that the steady-state accumulation of [$^{125}$I]NAPS in drug-resistant SH-SY5Y/VCR cells was significantly less than that of the sensitive parental cells. Furthermore, the efflux experiments proved that [$^{125}$I]NAPS is transported out of resistant cells by an energy-dependent process. Our data also show that the nonradioactive probe I-NAPS increases VCR accumulation and is a highly effective modulator of VCR resistance in SH-SY5Y/VCR cells when it is used with increasing concentrations of VCR. Furthermore, while the effects of 5 $\mu$M I-NAPS are much greater in SH-SY5Y/VCR cells, sensitization in the parental SH-SY5Y cells is also evident. This is expected since SH-SY5Y cells express P-gp (Meyers et al., 1989) and this cell line contains an amount of MDR1 mRNA similar to that in KB-8-5 cells (Bates et al., 1989). Indeed, [$^{125}$I]NAPS photolabeled P-gp in SH-SY5Y cells (Figure 1A) and was clearly seen in autoradiograms exposed for longer times (data not shown). Interestingly, I-NAPS alone had no significant effect on the growth of sensitive SH-SY5Y cells and resistant SH-SY5Y/VCR cells at the concentrations examined. Therefore, modulation of VCR resistance occurred at dose levels of I-NAPS which are not in themselves growth-inhibitory.

These data suggest that I-NAPS may increase VCR sensitivity at least in part by interacting with P-gp and increasing VCR accumulation. However, the weak correlation between the magnitude of the effect of I-NAPS on VCR accumulation and cell survival particularly in SH-SY5Y cells suggests that this compound may increase VCR sensitivity by other mechanisms, possibly by interaction with spiperophilin. Interestingly, 5 $\mu$M I-NAPS increased the VCR sensitivity of sensitive KB-3-1 human epidermoid carcinoma cells lacking P-gp by 15.5-fold. Moreover, [$^{125}$I]NAPS specifically bound to spiperophilin in this cell line, suggesting that this protein may mediate VCR chemosensitization by I-NAPS. Furthermore, while I-NAPS at 5 $\mu$M increased the VCR sensitivity of SH-SY5Y and SH-SY5Y/VCR cells by 20.8- and 30.4-fold, respectively, there was a much greater increase in drug accumulation in SH-SY5Y/VCR cells. This suggests that other resistance mechanisms that are insensitive to I-NAPS and independent of VCR accumulation may be present in SH-SY5Y/VCR cells.

The photoaffinity labeling of P-gp by [$^{125}$I]NAPS was 5–6-fold more efficient than when [$^{125}$I]AAP or [$^3$H]azidopine was used, and was preferentially inhibited by MDR-related drugs, vinblastine > VCR > colchicine > doxorubicin > actinomycin D. Consistent with these results, we (Safa, 1988; Safa et al., 1987, 1989, 1990) have previously shown that *Vinca* alkaloids are the most effective inhibitors of the photoaffinity drug analogs that bind to P-gp. Moreover, many drugs that are known to interact with P-gp and reverse MDR

(Endicott & Ling, 1989; Safa, 1993) inhibited the binding of [$^{125}$I]NAPS to this protein. Interestingly, while progesterone has been shown to bind to P-gp and reverse MDR (Yang et al., 1989; Qian & Beck, 1990; Fleming et al., 1992), it did not inhibit the binding of [$^{125}$I]NAPS to this protein, suggesting that progesterone may bind to a separate binding site on P-gp.

To identify the specific [$^{125}$I]NAPS binding domain(s) of P-gp and to determine if the photolabeled site(s) for this probe is (are) identical to the [$^{125}$I]AAP binding domain(s) of P-gp, we performed peptide mapping by *S. aureus* V8 protease digestion of this protein. Our results revealed that [$^{125}$I]NAPS binds to two major and a number of minor fragments of P-gp from SH-SY5Y/VCR cells. The major [$^{125}$I]NAPS-bound fragments were 6 and 8 kDa while [$^{125}$I]AAP bound primarily to an 8-kDa fragment of P-gp. The latter result confirms our previous work showing that [$^{125}$I]AAP binds to a single 8-kDa fragment of P-gp from DC-3F/VCRd5L Chinese hamster cells (Safa et al., 1990a). Further, V8 proteolysis of [$^{3}$H]-azidopine-photolabeled P-gp encoded by mouse *mdr*1a and *mdr*1b has also resulted in a 6-kDa peptide (Greenberger et al., 1990), suggesting that arylazidoprazosin and azidopine bind to the same domain of P-gp. While both [$^{125}$I]NAPS and [$^{125}$I]AAP bind to a common 8-kDa fragment of P-gp, the former binds to the other domains as well. These results suggest several possibilities: (1) P-gp may have several binding sites, and [$^{125}$I]NAPS binds to a number of these sites; (2) the drug-bound fragments may form a single drug binding site for [$^{125}$I]NAPS which partially overlaps with the binding site domain of [$^{125}$I]AAP; or (3) both photoaffinity analogs bind to adjacent amino acid residues of a common domain, while [$^{125}$I]NAPS also binds to other drug binding sites. Further support that [$^{125}$I]NAPS binds to separate sites from [$^{125}$I]AAP was provided by competition experiments with other MDR modulators. For instance, while prenylamine, methoxamine, clonidine, haloperidol, dopamine, (−)- and (+)-propanolol, and many PTZs and related compounds inhibited the binding of [$^{125}$I]NAPS, they significantly increased the binding of [$^{125}$I]AAP to P-gp. It is possible that these compounds competitively inhibit the binding of [$^{125}$I]NAPS to P-gp while allosterically affecting the [$^{125}$I]AAP binding site and increasing the binding of the latter photoaffinity prove to P-gp. Indeed, we have previously shown by detailed kinetic analysis the presence of a single class of binding sites on P-gp for [$^{3}$H]azidopine which is distinct from that of *Vinca* alkaloids and cyclosporin A (Tamai & Safa, 1991). It has been shown that *trans*-flupentixol reverses MDR more effectively than *cis*-flupentixol, but both compounds equally inhibited [$^{3}$H]-azidopine binding to P-gp (Ford et al., 1990). While our results also showed that these stereoisomers equally inhibited the binding of [$^{125}$I]NAPS to P-gp, *cis*-flupentixol increased the binding of [$^{125}$I]AAP to P-gp 9–10-fold more than did *trans*-flupentixol. This is the first indication that P-gp may interact with its substrates stereoselectively.

The specific amino acid residues involved in drug recognition by P-gp have not been identified. Nevertheless, analysis of P-gp mutations which express altered patterns of drug resistance relative to the normal protein has permitted identification of particular functional domains of P-gp which may be involved in drug recognition and transport. We have previously shown that the Gly-185→Val-185 substitution in a predicted cytoplasmic loop linking transmembrane domain (TMD) 2 and TMD 3 changes the relative efficiency of dissociation of MDR-related drugs from P-gp and their release outside the cells, rather than altering initial drug binding (Safa et al., 1990a). In mouse *mdr*1 and *mdr*3, a single Ser-939→Phe-939 or Ser-941→Phe-941 substitution, respectively, altered the pattern of drug resistance and reduced azidopine and arylazidoprazosin binding to the mutant proteins (Kajiji et al., 1993). Furthermore, the double substitution of Gly-338→Ala-338 and the adjacent Ala-339→Pro-339 in TMD 6 of the Chinese hamster *Pgp*1 gene alters the pattern of cross-resistance to MDR-related drugs (Devine et al., 1992). Mutations of either Phe-335→Ala-335 in TMD 6 or Phe-978→Ala-978 in TMD 12 drastically altered the drug resistance profile conferred by the mutant P-gp in transfected cells (Loo & Clarke, 1993). These studies strongly suggest that TMDs 6, 11, and 12 are involved in drug recognition and efflux. Amino acid sequencing of [$^{125}$I]NAPS-bound peptides is essential for identifying the exact location of its binding sites on P-gp and for characterizing the molecular mechanisms of MDR reversal.

Photoaffinity labeling with [$^{125}$I]NAPS revealed for the first time that in addition to P-gp, another membrane protein, spiperophilin, with a molecular mass of 17 kDa specifically bound [$^{125}$I]NAPS 2-fold more in SH-SY5Y/VCR cells than in the parental SH-SY5Y cell line. Our results revealed that spiperophilin in MDR cells binds more efficiently to [$^{125}$I]-NAPS than in sensitive cells. Moreover, most of the compounds known to reverse MDR including calcium channel blockers, PTZs and related compounds, and progesterone were potent inhibitors of binding of [$^{125}$I]NAPS to this protein. Vinblastine, VCR, actinomycin D, and doxorubicin, the MDR-related agents, increased the binding of [$^{125}$I]NAPS to spiperophilin. It is possible that these agents may bind to separate binding site(s) on this protein and enhance [$^{125}$I]-NAPS binding allosterically. Our results indicate that spiperophilin is a multidrug acceptor in both sensitive and MDR cells.

Immunoprecipitation results using the anti-calmodulin monoclonal antibody revealed that spiperophilin is different from calmodulin. Previously, increased expression of a calcium binding soluble cytoplasmic protein (sorcin) with a molecular mass of 19–22 kDa was reported in several multidrug resistant cells, including SH-SY5Y/VCR cells (Hamada et al., 1988; Meyers et al., 1988). However, evidence suggests that spiperophilin is not sorcin: (1) the molecular mass of sorcin is larger than 17 kDa; (2) spiperophilin is an integral membrane protein; and (3) in contrast to this protein, which we showed interacts with many drugs such as calmodulin inhibitors and calcium channel blockers, sorcin has been shown not to interact with these drugs (Hamada et al., 1988). It is known that [$^{125}$I]NAPS binds to the $D_2$-dopamine receptor (Amlaiky & Caron, 1986). Furthermore, while dopamine significantly inhibited the binding of this photoprobe to spiperophilin, two lines of evidence suggest that this protein is not the known $D_2$-dopamine receptor: (1) the molecular mass of the $D_2$-dopamine receptor is 92 kDa (Amlaiky & Caron, 1986); (2) the labeling specificity of spiperophilin is not dopaminergic in character (Amlaiky & Caron, 1986; Raymond et al., 1989). For instance, neither the active dopamine antagonist (*S*)-(−)-sulpiride nor the inactive (*R*)-(+) isomer inhibited [$^{125}$I]-NAPS binding to spiperophilin, while only the active isomer inhibits the binding of the photoprobe to the $D_2$-dopamine receptor (Niznik et al., 1986). These results suggest that the mechanism of MDR reversal by I-NAPS appears to be separate from its specific dopamine receptor binding activity. A similar conclusion was also reached by Ford et al. (1989) that thioxanthenes may reverse MDR independent of their specific dopamine receptor binding activity.

Thus, the data presented in this report for the first time identify P-gp and spiperophilin as acceptors of [$^{125}$I]NAPS in MDR cells, indicate that [$^{125}$I]NAPS binds to binding site(s) on P-gp distinct from [$^{125}$I]AAP, and reveal that this probe can be used for investigating the molecular mechanism of MDR and its reversal by chemosensitizers. [$^{125}$I]NAPS will be particularly useful for identification of drug-bound amino acid residues of P-gp since it incorporates into this protein with high efficiency compared to [$^{3}$H]azidopine and [$^{125}$I]-AAP. Furthermore, while we identified spiperophilin in both sensitive and resistant cells and found that many chemosensitizers, particularly PTZs and related compounds, are able to interact with this protein, its functional role in cancer cells remains to be found.

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