Synthetic and Natural Opiates Interact with P-glycoprotein in Multidrug-resistant Cells*

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The ability of P-glycoprotein (P-gp) to transport naturally occurring and synthetic opiate analogues was investigated. Multidrug-resistant Chinese hamster ovary cells (B30) were found to accumulate significantly lower amounts of morphine than their drug-sensitive counterparts (B1). This decreased accumulation was reversed upon depletion of cellular stores of ATP. In addition, morphine bound to plasma membranes from B30 cells in a specific, saturable fashion. Verapamil and vinblastine, compounds transported by P-gp, were able to increase accumulation and displace the binding of morphine in B30 cells. In turn, the synthetic opiates meperidine, pentazocine, and methadone were able to increase the accumulation of vinblastine in resistant cells. These compounds were also able to displace the specific binding of vinblastine and the photoaffinity labeling of P-gp in plasma membranes by the radioiodinated anthracycline, idomycin. The implications of these findings in relation to the distribution, tolerance, and gastrointestinal side effects of opiates are discussed.

Multidrug resistance represents a serious hurdle to the successful chemotherapy of cancer. Hence, cultured cells selected for resistance to chemotherapeutic agents have been extensively investigated (1). Most drug-resistant cells accumulate substantially lower amounts of cytotoxic drugs and overexpress a 180-180-kDa integral membrane protein, P-glycoprotein (P-gp) (2). P-gp is responsible for the decreased cellular accumulation of chemotherapeutic agents by extruding a wide range of compounds in an ATP-dependent fashion (3). Some of the evidence implicating P-gp as a drug transporter is that chemotherapeutic agents bind specifically to P-gp within plasma membranes from resistant cells (4). In addition, photoactive analogues of these agents label a 180-180-kDa plasma membrane protein (5). Substrates for P-gp bear few structural or functional similarities and include vinca alkaloids, anthracycline antibiotics, phenothiazines, and calcium channel blockers (6-8). However, several physical properties are shared by substrates for P-gp, including aromaticity and hydrophobicity; a nitrogen cationic group is also characteristic of many compounds recognized by P-gp (9).

In addition to drug-resistant cells, P-gp has been localized to several sites throughout the body (10). A precise physiological role for P-gp has not been rigorously established at these sites. P-gp has been postulated to modulate steroid hormone transport in the adrenal gland and the uterus as well as transport of toxic metabolites by the liver. Recently, hydrophobic extracts from rat urine and human serum have been demonstrated to reverse multidrug resistance (11, 12). In this paper we demonstrate that cells overexpressing P-gp accumulate significantly less of the opiate morphine. In addition, several synthetic opiate agonists increase the cellular accumulation of vinblastine in drug-resistant CHO cells.

EXPERIMENTAL PROCEDURES

Materials—[3H]Vinblastine sulfate (23 Ci/mmol) was purchased from Amersham Life Sciences (Ontario, Canada). [3H]Daunomycin (2.1 Ci/mmol) and [3H]morphine (80 Ci/mmol) were purchased from Du Pont Pharmaceuticals, and pentazocine lactate from Winthrop Sterling Drug Ltd. Tissue culture products were purchased from Gibco BRL (Burlington, Ontario). All other reagents and chemicals were obtained commercially and were of at least reagent grade.

Cell Culture—Drug-sensitive Chinese hamster ovary cells (AuxB1) and the drug-resistant line (B30) were grown in -minimum essential medium containing 10% heat-inactivated bovine serum, streptomycin (100 ng/ml), and penicillin (100 ng/ml) (13). The resistant cells were maintained in medium containing colchicine (30 μg/ml).

Drug Accumulation—Accumulation of [3H]vinblastine was assayed by using previously published methods (13). Briefly, cells were grown as monolayers in 60 x 15-mm tissue culture plates to a density of approximately 2.5 x 10⁶ cells/plate. Monolayers were then washed with phosphate-buffered saline and incubated in transport buffer made of up 107 mM NaCl, 10 mM Tris, 26 mM NaHCO3, 5.3 mM KCl, 1.9 mM CaCl2, 1 mM MgCl2, and 7 mM dextrose. [3H]Vinblastine (0.6 Ci) was added to each plate in addition to unlabeled drug bringing the vinblastine concentration to 0.25 μM. Competing drug, in the 15-75 μM concentration range, was also added to each plate. Incubation was carried out at 37 °C. Monolayers were then washed twice with 2 ml of ice-cold phosphate-buffered saline. Cells were harvested with 0.4 ml of 0.4 M NaOH and neutralized with 0.8 ml of 0.25 M ammonium acetate (pH 6.6). The cell extract was then added to 10 ml of aqueous scintillant for liquid scintillation counting. For ATP depletion studies transport buffer was essentially as described above with the omission of dextrose and the addition of 10 mM NaN3. Cells were incubated in this buffer for 10 min prior to the addition of labeled and unlabeled drug.

Preparation of Plasma Membranes from B1 and B30 Cells—A
plasma membrane fraction was prepared from drug-sensitive and -resistant cells by using nitrogen cavitation (400 p.s.i., 15 min) as previously described (4). Protein concentration was determined by a micro-Lowry method. Fractions were stored in 0.01 M Tris-HCl, pH 7.4, 0.25 M sucrose at -70 °C prior to use.

Displacement of Drug Binding to Plasma Membranes—Displacement of \(^{3}H\)-drug binding to plasma membranes was determined by rapid filtration. Membranes containing 25 \(\mu\)g of protein were incubated with \(^{3}H\)-drug (0.16 \(\mu\)M) and competing drug (0-100 \(\mu\)M) in 0.01 M Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM MgCl\(_2\) buffer. Total incubation volume was 100 \(\mu\)l, and incubation time was 60 min at 25 °C. Following incubation membranes were collected on cellulose nitrate filters (Whatman, 0.45 \(\mu\)M) and washed twice with 3 ml of buffer. The filters were previously soaked for 3 h in 3% bovine serum albumin. Radioactivity associated with each filter was determined by liquid scintillation counting in Cytoscan ES (ICN Chemicals). Specific binding of \(^{3}H\)-drug was determined by subtracting binding in the presence of a 1000-fold concentration of unlabeled drug. Specific binding of [\(^{3}H\)]vinblastine accounted for 80-85% of total binding, whereas specific binding of [\(^{3}H\)]morphine was 55-60%.

Photoaffinity Labeling of Plasma Membranes—Photoaffinity labeling of plasma membranes containing P-gp by the anthracycline analogue [\(^{125}\)I]iodomycin was based on previously published methods (15). Briefly, membranes (25 \(\mu\)g protein) were incubated with [\(^{125}\)I]iodomycin (3.5 nM) in the absence or presence of competing drug (50-100 \(\mu\)M) followed by irradiation for 15 min. Membranes were pelleted by an Aruffle (Beckman) and rebalubilized in sodium dodecyl sulfate buffer and 20 \(\mu\)g of protein run on 6% polyacrylamide gels.

RESULTS

\[^{3}H\]Morpheine Accumulation—Fig. 1, upper panel, shows the uptake and accumulation of morphine by sensitive (B1) and resistant (B30) CHO cells. There was a progressive accumulation of the drug by the wild type cells that reached an apparent steady state by 20 min. Uptake of multidrug-resistant cells was barely discernible with the amount accumulated after 60 min more than 3-fold less than in sensitive cells. Depletion of cellular ATP by incubation with 10 mM sodium azide significantly increased the accumulation of morphine by B30 cells so that it approached the level observed in sensitive cells (Fig. 1, lower panel). There was only a minor effect of the energy depletion on uptake by the drug-sensitive B1 cells. These observations are generally similar to those obtained with several different anti-neoplastic drugs (16).

Specific Binding of \[^{3}H\]Morphine to Plasma Membranes—The specific binding of morphine and the anti-neoplastic agent daunomycin to plasma membranes from B1 and B30 cells was compared. There was no specific binding of either compound to membranes from drug-sensitive B1 cells (data not shown). The amount (fmol/mg protein) of drug bound to B30 membranes was plotted as a function of concentration. The binding parameters \(K_d\) (dissociation constant) and \(B_{max}\) (maximal binding capacity) determined by non-linear least squares regression (Sigma Plot) are shown in Table I. These binding constants indicated that morphine bound to B30 plasma membranes with higher affinity than daunomycin. However, the amount of the opiate bound specifically to membranes was 2.5-fold less than daunomycin.

Effect of Verapamil, Vinblastine, and Daunomycin on \[^{3}H\]Morpheine Accumulation—The calcium channel blocker verapamil and the anti-neoplastic agents vinblastine and daunomycin were examined for their effects on morphine accumulation by B1 and B30 cells (Fig. 2). Verapamil, an agent known to reverse drug resistance, significantly increased the accumulation of morphine in B30 cells. Vinblastine had a similar competitive effect of somewhat lesser magnitude. Daunomycin at concentrations up to 75 \(\mu\)M did not cause any increase in morphine uptake. This presumably reflects the lower affinity of daunomycin binding to P-gp. There was a slight decrease (<10%) in morphine accumulation in response to these drugs in sensitive B1 cells.

Displacement of Specific Binding of Morpheine to B30 Cell Membranes—The specific binding of morphine to plasma membranes was displaced by verapamil, vinblastine, daunomycin, unlabeled morphine, and an opiate analgesic with similar structure to morphine, hydromorphone (Fig. 3). Unlabeled morphine was able to efficiently displace most of the binding of labeled compound from B30 plasma membranes. This indicates that bound radioactivity probably represented the parent compound rather than a metabolite or merely label. Verapamil exhibited moderate potency in displacing approximately 50% of the binding of labeled morphine to B30 membranes at a concentration of 100 \(\mu\)M. This parallels its potency in increasing the accumulation of morphine in drug-resistant cells. The opiate hydromorphone and the anti-neoplastic vinblastine and daunomycin were all less effective, decreasing the binding by only about 30%. The similar ability of daunomycin and vinblastine to displace the binding of morphine is surprising given the inability of daunomycin to increase the cellular accumulation of morphine. Possibly the interaction between vinblastine and morphine in cells is non-competitive as apparently is the case between vinblastine and morphine (18).

The Effect of Synthetic and Natural Opiates on the Accumulation of Vinblastine—The ability of natural and synthetic opiates to increase the accumulation of vinblastine in drug-sensitive and -resistant cells was also investigated (Fig. 4). There was no significant effect of these drugs on sensitive B1 cells. Neither the natural opiates morphine and hydromorphone nor the opiate antagonist naloxone was able to significantly alter vinblastine accumulation in drug-resistant cells. However, the other synthetic opiate analgesics meperidine, pentazocine, and methadone significantly increased the accumulation of vinblastine by the P-gp-expressing B30 cells. The effect was dose-dependent for opiate concentrations in the range 15-75 \(\mu\)M as shown in Fig. 5. The order of potency was meperidine > pentazocine > methadone, which is identical to their analgesic potency (17).

Displacement of \[^{3}H\]Vinblastine Binding to Plasma Membranes by Natural and Synthetic Opiates—To determine whether opiates increase accumulation of vinblastine by competing for binding sites on P-gp binding, displacement assays were performed. The results are shown in Fig. 6. As expected from their inability to alter accumulation of vinblastine, morphine and hydromorphone also did not displace its binding to B30 membranes. This was presumably due to a lower affinity of binding of opiates compared with vinca alkaloids. In contrast, the synthetic opiates are able to partially displace the binding of vinblastine to plasma membranes from B30 cells. The potency of displacement was similar to their ability to increase cellular accumulation of vinblastine. However, these compounds were not as potent displacers as verapamil.

Displacement of [\(^{125}\)I]Iodomycin Photolabeling of P-gp in B30 Plasma Membranes—The ability of several opiates to displace the photoaffinity labeling of the anthracycline derivative iodomycin to P-gp in plasma membranes is shown in Fig. 7. [\(^{125}\)I]Iodomycin bound to a 180-kDa membrane protein band from drug-resistant cell membranes that has been confirmed as P-gp (2, 15). The calcium channel blocker verapamil was the most potent compound in displacing the binding of iodomycin. The order of potency of natural and synthetic opiates to displace the photoaffinity labeling of iodomycin was pentazocine > meperidine > naloxone > morphine. These effects are in good agreement with the competition for the equilibrium binding of vinblastine to membranes. Therefore
Transport of Opiates by P-glycoprotein

Fig. 1. Upper panel, time course of accumulation of [3H]morphine in drug-sensitive (B1, ●) and drug-resistant (B30, ○) CHO cells. Lower panel, the effect of ATP depletion on the time course of [3H]morphine accumulation in CHO cells. Each point represents the mean ± S.E. of at least three experiments.

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_d$ (μM)</th>
<th>$B_{max}$ (fmol/mg protein)</th>
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<tr>
<td>Morphine</td>
<td>6.9 ± 0.8</td>
<td>910 ± 200</td>
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<tr>
<td>Daunomycin</td>
<td>16.6 ± 2.1</td>
<td>2410 ± 340</td>
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we have shown that opiate compounds are able to displace the binding of vinca alkaloids and anthracyclines to multidrug-resistant cell membranes and that the site of this displacement is P-gp.

Discussion

Natural and synthetic opiates are potent analgesics that bind to receptors for endogenous opiates in the brain (17). The structural formulas of natural and synthetic opiates used in this study are shown in Fig. 8. Naturally occurring opiates share a planar 3-ringed aromatic structure with a substituted piperidine ring. On this basis, they are reasonable candidates for the general class of substrates for P-gp (9). Morphine is the most potent natural opiate in terms of analgesia and affinity for the opiate receptor (17). We have demonstrated that cells selected for resistance to colchicine accumulate significantly less morphine than their parental counterparts. The difference in accumulation of morphine was reversed by depletion of cellular ATP consistent with P-gp-mediated active drug transport. The maintenance of decreased cellular
levels of drugs such as colchicine, vinblastine, daunomycin, and azidopine in P-gp-expressing cells also depends on energy in the form of ATP (6, 18). In fact, partially purified P-gp has been shown to exhibit ATPase activity (19). The diminished accumulation of morphine by multidrug-resistant cells relative to drug-sensitive cells (~3-fold) was less pronounced than that of vinblastine (12-fold). This may reflect differing affinities of morphine and vinblastine for P-gp. In fact, preferential resistance of multidrug-resistant cells to specific classes of cytotoxic drugs has been previously demonstrated, and the vinca alkaloids are known to be among the most strongly bound to P-gp (20, 21).

Morphine also bound specifically to plasma membranes from drug-resistant but not -sensitive cells. The dissociation constant for morphine was significantly higher than that observed for high affinity substrates such as azidopine (18, 22) and vinblastine (4). However, it was in the range observed for daunomycin, an anthracycline antibiotic with intermediate binding affinity for P-gp. Verapamil and vinblastine were able to significantly impair the active extrusion of morphine from resistant cells. Furthermore, these two compounds displaced the specific binding of morphine to B30 plasma membranes. Taken together, these results suggest that morphine competes for binding and transport with vinca alkaloids and calcium channel blockers. There is, however, evidence that multiple binding sites exist on P-gp (18). In addition, substrates may interact noncompetitively to alter accumulation or binding to P-gp (18).

Neither morphine nor hydromorphone was able to increase accumulation of vinblastine or displace its binding to membranes from resistant cells. This may be explained by the significantly lower binding affinity of morphine (and presumably hydromorphone) compared with vinblastine or the calcium channel blockers. Only the opiate antagonist naloxone was able to partially displace vinblastine binding to plasma membranes. In addition, naloxone and to a lesser extent morphine were able to displace the photoaffinity labeling of B30 cell membranes by iodomycin that has an intermediate binding affinity to P-gp (15). Naloxone has a similar structure to morphine differing by a propenyl substitution on the nitrogen group (23). A polar substitution on the nitrogen group has been proposed to result in greater affinity for the putative binding site(s) on P-gp (24). The synthetic opiates were, however, able to inhibit the extrusion of vinblastine and displace its binding to plasma membranes in B30 cells. Meperidine and pentazocine were also able to displace the photoaffinity labeling of B30 cell membranes by iodomycin that has an intermediate binding affinity to P-gp (15). Naloxone has a similar structure to morphine differing by a propenyl substitution on the nitrogen group, indicating that binding displacement occurs at P-gp. The synthetic opiates were, however, able to inhibit the extrusion of vinblastine and displace its binding to plasma membranes in B30 cells. Meperidine and pentazocine were also able to displace the photoaffinity labeling of B30 cell membranes by the anthracycline analogue iodomycin, indicating that binding displacement occurs at P-gp. The structures of synthetic opiates differ from that of natural opiates, but they still retain potent narcotic agonist/antagonist activity. Their ability to alter accumulation and binding of the vinca alkaloid is not as potent as...
Inhibition of the specific binding of \([\text{SH}]\)vinblastine to plasma membranes from B30 cells. Membranes (20 μg of protein) were incubated with 0.16 μM \([\text{3H}]\)vinblastine and competing drug for 60 min at room temperature. Data are expressed as the percent of the specific binding of vinblastine measured in the absence of competing drug. Each point represents the mean ± S.E. of three experiments.

Displacement of the photoaffinity labeling of plasma membranes from B30 cells by \([\text{125I}]\)iodomycin. Plasma membranes (25 μg) were incubated with 3.5 nM \([\text{125I}]\)iodomycin in the presence or absence of competing drug as described under "Experimental Procedures." Following solubilization, 20 μg of each sample was run on 6% SDS-polyacrylamide gel electrophoresis gels and subsequently autoradiographed. Lane assignments are: 1, control, no drug; 2, 50 μM verapamil; 3, 100 μM meperidine; 4, 100 μM pentazocine; 5, 100 μM morphine; and 6, 100 μM naloxone.

In summary we have demonstrated that morphine and cellular responses to opiates, and distribution of these drugs have been proposed (26). Indeed, P-gp has been localized at the blood-brain barrier (10) and could therefore possibly contribute to the regulation of the amount of exogenously administered opiate reaching the brain. Cross-tolerance to several classes of opiates has been observed, consistent with the involvement of a broad substrate specificity system such as P-gp. Whether the amount of P-gp expressed at the blood-brain barrier can be influenced by exposure to drugs has not been determined.

A commonly encountered side effect of opiate analgesics is constipation (17). In fact, opiates are administered clinically in certain cases of diarrhea. P-gp has also been localized in the small and large intestine (27) and has recently been demonstrated to influence volume-regulated chloride channel activity (28). Volume regulation of epithelial cells in the colon is stimulated by activation of Cl⁻ and K⁺ channels (29). Conceivably, the chloride channel activity associated with P-gp expression may have a role in salt and H₂O flux across the intestinal epithelium. P-gp is localized apically in epithelial cells (27). In addition, compounds that are transported by P-gp have also recently been shown to inhibit the associated chloride channel activity (30). A significant amount of administered morphine is excreted in the feces as a glucuronide conjugate (31). B-Glucuronidase activity in the bowel is responsible for releasing morphine from its conjugate, thus facilitating its reabsorption in the colon (14). Hence, significant levels of morphine are present in the bowel to perhaps interact with P-gp and alter the Cl⁻/H₂O flux across the colonic epithelium.
related narcotic analogs interact with the multidrug-resistant transport protein P-gp. P-gp may be involved in determining the distribution and side effects of these compounds.

REFERENCES