Interaction of tamoxifen with the multidrug resistance P-glycoprotein

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Summary Tamoxifen is an anti-oestrogen which is currently being assessed as a prophylactic for women at high risk of breast cancer. Taxotrex has also been shown to reverse multidrug resistance in P-glycoprotein (P-gp)-expressing cells, although the mechanism of action is unknown. In this study we demonstrate that tamoxifen interacts directly with P-gp. Plasma membranes from P-gp-expressing cells bound [3H]tamoxifen in a specific and saturable fashion. A 180 kDa membrane protein in these membranes, labelled by the affinity analogue tamoxifen aziridine and azidopine, was shown to be P-gp. Tamoxifen reduced the binding of vinblastine and azidopine to P-gp, and tamoxifen increased [3H]vinblastine accumulation in P-gp-expressing cells to levels approaching those in non-P-gp-expressing cells. However, the cellular accumulation of [3H]tamoxifen itself was not influenced by the presence of P-gp. Thus, tamoxifen appears to reverse multidrug resistance by binding to P-gp and inhibiting the transport of cytotoxic drugs, but does not itself appear to be transported by the protein.

Keywords: P-glycoprotein; tamoxifen; drug transport; multidrug resistance

Tamoxifen is an anti-oestrogen which displays tumourstatic properties (Lerner and Jordan, 1990). The use of tamoxifen in the clinic has progressed from palliation in advanced breast cancer (Jordan, 1990, 1992; Lerner and Jordan, 1990) to efficacious treatment of all stages of oestrogen receptor-positive breast cancer. Furthermore, tamoxifen is currently being assessed as a prophylactic agent for women at high risk of developing breast cancer (Jordan, 1990). Tamoxifen acts by binding to the cytosolic oestrogen receptor (Katzenellenbogen et al., 1983; Berthois et al., 1986) and inhibiting the binding of oestrogens (Jordan and Prestwich, 1977; Jordan and Naylor, 1979). Tamoxifen is tolerated at high doses and has few reported side-effects owing to its high target specificity (Jordan, 1992).

Tamoxifen has also been demonstrated to reverse the drug resistance phenotype of several P-glycoprotein (P-gp)-expressing cell lines (Ramus et al., 1984; DeGregorio et al., 1989; Kirk et al., 1993a,b). However, it is not known whether this is an indirect effect or the result of the direct interaction of tamoxifen with P-gp. P-gp is frequently associated with the phenomenon of multidrug resistance (MDR) (Kartner et al., 1983), acting as an ATP-dependent drug efflux pump to reduce the intracellular accumulation of antineoplastic drugs (Inaba et al., 1979; Cornwell et al., 1986a; for reviews see also Riordon and Ling 1985; Horio et al., 1988; Gottesman and Pastan, 1993). A number of compounds are known to antagonise the drug efflux activity of P-gp, such as verapamil and other calcium channel blockers, immunosuppressants (e.g. cyclosporin A), antiarrhythmics and antiinhibitamines (for review see Gottesman and Pastan, 1993). Many of these compounds bind to P-gp and are believed to reverse drug resistance by competing with drug binding to and/or drug transport (Wigler and Patterson, 1993). Thus, it seemed possible that tamoxifen might also interact directly with P-gp. In this study we demonstrate that tamoxifen does indeed bind to P-gp and inhibits the transport of cytotoxic drugs, although it does not appear to be a substrate for transport. This identifies a second target for tamoxifen in certain tissues and has implications for the therapeutic use of tamoxifen.

Materials and methods

Chemicals

[3H]Vinblastine sulphate (11 Ci mmol-1), [3H]azidopine (52 Ci mmol-1), [3H]tamoxifen (84 Ci mmol-1) and [3H]tamoxifen aziridine (24 Ci mmol-1) were purchased from Amer sham Life Sciences (Amerham, U.K). Vinblastine sulphate, tamoxifen and protein A-Sepharose were obtained from Sigma Chemicals. The monoclonal antibodies against P-gp (C219) was purchased from Centocor Diagnostics. Tissue culture reagents were provided by the ICRF Clare Hall Laboratory.

Cell culture

The human epidermal carcinoma cell line KBV-1 and its drug-resistant derivative KBV-1 were provided by Dr M Gottesman (Shen et al., 1986). Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and supplemented with penicillin and streptomycin. The KBV-1 cell line was maintained in medium which also contained 1 μg ml-1 vinblastine. The level of resistance displayed by KBV-1 cells to vinblastine, colchicine and adriamycin was 213-, 171- and 422-fold respectively (Shen et al., 1986).

Plasma membrane isolation

Plasma membrane fractions were isolated according to previously published methods (Cornwell et al., 1986b). Disruption of cells (5 × 10⁶) was achieved by nitrogen cavitation (1500 p.s.i., 20 min). All buffers contained the following protease inhibitors: phenylmethyl sulphonyl fluoride (PMSF) 1 mM, benzamidine 1 mM, aproton 1 μg ml⁻¹ and EDTA 1 mM. Membranes were snap frozen in liquid nitrogen and stored in 0.01 M Tris–HCl pH 7.4, 0.25 M sucrose, at −70°C. The protein concentration of each sample was determined by a micro-Lowry assay using bovine serum albumin as a standard.

Affinity labelling of plasma membranes

Photoaffinity labelling of membranes with [3H]azidopine was done according to previously published methods (Safa et al., 1987). Briefly, membranes (40 μg) were incubated in labelling buffer (0.01 M Tris–HCl pH 7.4, 0.25 M sucrose, 5 mM magnesium chloride) containing 45 nm [3H]azidopine. Tamoxifen (1, 10, 50 or 100 μM), verapamil (50 μM) or vinblastine (50 μM) was used to compete with [3H]azidopine for binding to P-gp, as indicated. The total reaction volumes were 50 μl. Membranes and drug were allowed to reach equilibrium binding for 20 min in the dark and then irradiated with UV light (265 nm) for 25 min on a transilluminator (LKB Instruments). Membranes were labelled with [3H]tamoxifen azidopine by incubation at 25°C with 0.84 μM labelled drug.

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The reactions were stopped by the addition of 2 ml of ice-
cold labelling buffer and membranes were pelleted by cen-
trifugation at 120,000 g in a Beckman TL-100 ultracentrifuge
for 15 min at 4°C. Pellets were solubilised in Laemml sample
buffer and proteins separated on a 6% sodium dodecylsul-
phate (SDS)–polyacrylamide gel. The gels were treated with
Ampilify (Amersham, UK), dried onto filter paper, and
labelled proteins visualised by autoradiography.

**Drug binding to plasma membranes**

Drug binding to plasma membranes was assayed using a
rapid filtration assay as previously described (Callaghan and
Riordan, 1993). Briefly, membranes (40 μg of total protein)
were incubated with either [3H]tamoxifen (50 nM) or
[3H]vinblastine (55 nM), and any appropriate unlabelled com-
peting drug, in a buffer composed of 0.01 M Tris pH 7.4,
0.25 M sucrose, 5 mM magnesium chloride. After 60 min
incubation at 25°C samples were filtered by light suction
through 0.25 μm nitrocellulose filters and washed with ice-
cold buffer (4 ml) in a Millipore multichannel filtration
manifold. Filters were added to Ready Protein (Amersham,
UK) scintillation fluid and counted for radioactivity. Non-
specific binding to plasma membranes was defined as the
binding detected in the presence of a 2000-fold excess of un-
labelled drug. Binding to nitrocellulose filters did not exceed
5–10% of total radioactivity added.

**Drug accumulation assay**

Accumulation of [3H]tamoxifen in cell monolayers was
assayed using previously published methods (Cano-Gauci
and Riordan, 1987). Briefly, cells were grown as monolayers
on 60 x 15 mm tissue culture plates to a density of approx-
imately 2.5 x 10^6 cells per plate. To determine the time course
of drug accumulation, [3H]tamoxifen (0.15 μCi) was mixed
with unlabelled tamoxifen to a final concentration of 20 μM
and added to each plate. Cells were harvested after the
appropriate time points and the amount of radioactivity
accumulated determined by scintillation counting.

For [3H]vinblastine accumulation, labelled drug (0.6 μCi)
was added to each plate together with unlabelled drug to a
dinal vinblastine concentration of 21 nM. Tamoxifen was
added as a competing agent in the concentration range
0–60 μM. Cells were harvested and treated as above.

**Results**

[3H]Tamoxifen displayed specific and saturable binding to
plasma membranes isolated from P-gp-expressing (KBV-1)
and non-expressing (KB3-1) cell lines (Figure 1). Of the total
amount of [3H]tamoxifen added, about 85% was associated
with the membranes of either cell type. Specific binding was
defined as the binding sensitive to the addition of a 2000-fold
excess of unlabelled tamoxifen. For P-gp-expressing cells
38.7 ± 2.4% of total binding was specific, while for the non
P-gp-expressing cells specific binding accounted for only
5–10% of the total. Thus, a significant amount of the
tamoxifen appears to associate non-specifically with the lipid
phase. Non-linear, least-squares regression of the binding
isothersms of [3H]tamoxifen to KBV-1 and KB3-1 membranes
gave binding capacities of 35.4 ± 8.5 μmol mg⁻¹ (n = 5) and
11.1 ± 1.5 μmol mg⁻¹ (n = 2) respectively. Thus P-gp-contain-
ing membranes have a 3.2-fold greater binding capacity for
[3H]tamoxifen than non-P-gp-containing membranes. Tamox-
ifen aziridine binds to several proteins other than P-gp (see
Figures 4 and 5 below) in KBV-1 and KB3-1 cells, suggesting
multiple plasma membrane targets for tamoxifen. Nonethe-
less, the significant difference in binding capacity between
KBV-1 and KB3-1 suggests that the drug binds to P-gp. The
dissociation constant for specific [3H]tamoxifen binding to
KBV-1 membranes was 17.3 ± 1.9 μM (n = 5), which is
higher than values reported for vinblastine (Yusa and Tsuro,
1989) and azidopine (Tamai and Safa, 1991), but similar to
those reported for daunomycin and morphine (Callaghan and
Riordan, 1993).

Tamoxifen inhibits the binding of vinblastine to P-gp
expressing membranes

Vinblastine is a cytotoxic drug which binds to P-gp and is a
transported substrate. The ability of tamoxifen to displace
the specific binding of [3H]vinblastine to KBV-1 membranes
is shown in Figure 2. About 20.1 ± 0.9% of the total [3H]
vinblastine added to the membranes was bound. For P-gp-
expressing cells, 81 ± 2% of this binding was specific, as
defined by its sensitivity to a 2000-fold excess of unlabelled
vinblastine. In contrast, no specific binding could be detected
for membranes from non-P-gp-expressing KB3-1 cells (data
not shown). Tamoxifen concentrations of 10 μM were suffi-
cient to displace approximately 50% of the specific binding
of vinblastine to P-gp-containing membranes. This implies
that tamoxifen and vinblastine bind to a common site in
these membranes. The ability of tamoxifen to displace vin-
blastine binding compares favourably with that reported for
verapamil and other calcium channel blockers.

**Site of [3H]tamoxifen binding to KBV-1 plasma membranes**

To identify the site of specific tamoxifen binding to KBV-1
membranes it was necessary to use affinity labelling tech-
niques. Tamoxifen aziridine, an electrophilic analogue of
tamoxifen, has previously been used to demonstrate the
ability of tamoxifen to bind the oestrogen receptor (Katze-
lenbogen et al., 1983). We therefore examined the abilities
of [3H]tamoxifen aziridine and [3H]azidopine to label plasma
membranes from P-gp-expressing and non-expressing cells
(Figure 3). [3H]Azidopine has previously been demonstrated
to label P-gp (Safa et al., 1987) and, thus, served as a positive
control. [3H]Azidopine strongly labelled a protein of approx-
imately 180 kDa in membranes from P-gp-expressing cells
which was absent from membranes of non-P-gp-expressing
cells (Figure 3a). Immunoprecipitation with the monoclonal
antibody C219 showed that the 180 kDa peptide was P-gp
(data not shown). The 180 kDa protein present only in mem-
branes from P-gp expressing cells was also labelled by [3H]
tamoxifen aziridine. Thus, a significant proportion of the
increased binding capacity of KBV-1 membranes to tamox-
ifen (Figure 1) appears to be due to P-gp. Labelling with the

![Figure 1](image-url)

**Figure 1** The specific binding of [3H]tamoxifen to plasma mem-
branes isolated from KBV-1 (●) and KB3-1 (○) cells. [3H]-
Tamoxifen binding was measured after 60 min at room temper-
ature in 10 mM Tris- HCL 0.25 M sucrose and 5 mM magnesium
chloride. Specific binding was determined by subtracting the
amount of tamoxifen bound in the presence of a 2000-fold excess
of unlabelled tamoxifen. Each point represents the mean ± s.e.m.
of at least six independent experiments.
tamoxifen analogue was not as sensitive as with azidopine and may reflect a lower affinity of this compound for its binding site in the membranes.

Two smaller (50 and 100 kDa) polypeptides were also labelled by [3H]azidopine. These two polypeptides are probably proteolytic fragments of P-gp since they were detected by the anti-Pgp antibody C219 (data not shown). Several proteins not specific to P-gp-expressing cells were also weakly labelled by both [3H]azidopine and [3H]tamoxifen azidirine. This was not unexpected since the targets for [3H]azidopine and [3H]tamoxifen azidirine are reactive functional groups on proteins such as methionines and cysteines (Peters and Richards, 1977).

Tamoxifen inhibits the binding of other compounds to P-gp

Affinity labelling techniques were used to demonstrate that tamoxifen interacts with the same binding site(s) on P-gp as vinca alkaloids on calcium channel blockers. The ability of vinblastine (0.1–100 μM) and tamoxifen (100 μM) to displace the binding of [3H]tamoxifen azidirine to KBV-1 membranes is shown in Figure 4. The displacement of tamoxifen azidirine from P-gp by vinblastine was dose dependent and almost complete at 100 μM. In addition, we studied the ability of tamoxifen to displace the specific photoaffinity labelling of P-gp by [3H]azidopine (Figure 5). Azidopine is a high-affinity substrate for P-gp with a Kd of 1.5 μM (Tamai and Saka, 1991). Labelling of Pgp by azidopine was significantly reduced by 10 μM tamoxifen and completely inhibited by 50 μM tamoxifen. Tamoxifen had a similar IC50 for displacing both [3H]vinblastine binding and [3H]azidopine labelling of P-gp. An equimolar concentration of vinblastine displayed similar potency in displacing azidopine binding. Thus, tamoxifen appears to interact at the same binding site on P-gp as vinblastine and the calcium channel blocker azidopine. In contrast, a similar concentration of verapamil, an MDR reversing agent, did not appreciably reduce photolabelling by azidopine.

Effects of tamoxifen on [3H]vinblastine accumulation in KB cells

Tamoxifen reverses drug resistance in P-gp-expressing cells. As tamoxifen binds to P-gp, it seemed likely that it reverses drug resistance by inhibiting drug transport. To address this point we studied the effects of tamoxifen on the accumulation of [3H]vinblastine (21 nM) in P-gp-expressing (KBV-1) and non-expressing (KB3-1) cell lines (Figure 6). The results expressed as the increase in vinblastine accumulation observed in the presence of tamoxifen above the levels observed in the absence of tamoxifen. In the absence of tamoxifen, non-Pgp-expressing KB3-1 cells accumulated approximately 6-fold
Tamoxifen was also found to have a significant effect on the accumulation of $[^{3}H]$vinblastine in KBV-1 cells. For example, when KBV-1 cells were incubated with $[^{3}H]$vinblastine and tamoxifen, the amount of $[^{3}H]$vinblastine accumulated in KBV-1 cells was approximately 50% of the level in drug sensitive KB3-1 cells. These data show that tamoxifen impairs the P-gp-dependent transport of vinblastine.

**Tamoxifen is not itself transported by P-gp**

The accumulation of $[^{3}H]$tamoxifen by P-gp-expressing and non-expressing cells is shown in Figure 7. In both cell lines there was a rapid initial accumulation of tamoxifen which plateaued by approximately 30 min. There was no significant difference in the steady-state accumulation of labelled tamoxifen between the two cell lines. This is in contrast to $[^{3}H]$vinblastine, which as reported in the preceding section, has a 6-fold lower accumulation in KBV-1 cells than in KB3-1 cells. Tamoxifen is highly lipophilic, and approximately 80% of the total counts added were associated with the cells. The data above, which show that a similar proportion of $[^{3}H]$tamoxifen associates with isolated plasma membranes, indicate that a significant proportion of the tamoxifen is non-specifically bound to the cell membrane rather than being accumulated intracellularly. In plasma, tamoxifen is extensively bound to proteins which alter its distribution between cells and serum (Chaterjee and Harris, 1990). The addition of albumin (0–2%, w/v) to the transport medium caused a fall (30% at a concentration of 1.0%, w/v) in the amount of tamoxifen bound by both cell lines (data not shown). However, there was still no significant difference in accumulation between the two cell lines under these conditions. Thus, tamoxifen does not appear to be transported by P-gp despite its ability to bind to the protein and displace the binding of transported substrates.

**Discussion**

Tamoxifen exerts tumorstatic effects through its interaction with the oestrogen receptor. Tamoxifen has generally been assumed to be relatively specific in its interactions with cellular proteins. In this study we identify a new cellular target for tamoxifen, the multidrug resistance P-glycoprotein. Not only does tamoxifen bind to P-gp, it inhibits P-gp-mediated drug transport. This defines the mechanism whereby tamoxifen can reverse multidrug resistance. As tamoxifen is well tolerated at high doses in vivo (Stuart et al., 1992), it may prove to be a valuable tool in overcoming drug resistance in neoplastic disorders.

Tamoxifen has been reported to reverse P-glycoprotein-mediated multidrug resistance in vitro (Ramu et al., 1984; DeGregorio et al., 1989; Kirk et al., 1993a). The mechanism by which this is achieved has not been established, although a recent study suggests an interaction between P-gp and tamoxifen in oestrogen receptor-positive MCF7 cells (Leonessa et al., 1994). Many agents which reverse MDR bind specifically to P-gp and to compete with cytotoxic drugs for active transport (Cornwell et al., 1987; Safa et al., 1987; Ryffel et al., 1991). We have shown that tamoxifen also interacts directly with P-gp. Furthermore, tamoxifen appears to inhibit drug transport since it dramatically increased vinblastine accumulation in P-gp-expressing cells. Thus, it appears that the reversal of multidrug resistance by tamoxifen is due to the interaction of tamoxifen with P-gp and the consequent inhibition of P-gp-dependent drug transport.

Tamoxifen was shown to displace the specific binding of azidopine and vinblastine to P-gp. This suggests that tamoxifen interacts directly with the drug binding site on P-gp. The precise nature of this site is unclear. Two regions on P-gp are labelled by azidopine (Bruggemann et al., 1989, 1992; Yoshimura et al., 1989), one in each half of the protein. However, it is believed that these two regions form a single drug binding site (Bruggemann et al., 1992). It has, however, been suggested on the basis of binding and competition studies, that P-gp may have distinct binding sites for vinca alkaloids and azidopine (Tamai and Safa, 1991). Verapamil is more efficacious at inhibiting binding to the vinca alkaloid site. However, the steroid hormone progesterone competes equally well with either vinca alkaloids or azidopine for binding to P-gp (Yang et al., 1989). As tamoxifen competes with both vinblastine and azidopine for binding to P-gp, if P-gp is able to bind or handle vinca alkaloids and azidopine differentially, then tamoxifen (like progesterone) must interfere with both sites/mechanisms.

Compounds which reverse multidrug resistance may prevent drug transport simply by occupying the drug binding site on P-gp, or by competing directly with cytotoxic drugs for transport. Many MDR reversing agents, such as verapamil (Cano-Gauci and Riordan, 1987; Yusa and Tsuro, 1989),
azidopine (Tamai and Safa, 1991), cyclosporin and FK506 (Ueda et al., 1992), have been reported to be transported by P-gp. However, we could detect no difference in the abilities of drug-resistant and drug-sensitive cells to accumulate tamoxifen. This suggests that tamoxifen is not transported by P-gp. A previous report has also suggested that tamoxifen is not a substrate for ATP-sensitive drug efflux in adriamycin-resistant P388 murine leukemia cells (Kessel, 1986). The interaction of tamoxifen with P-gp may be similar to that of the steroid hormone progesterone. Like tamoxifen, progesterone inhibits azidopine photolabelling of P-gp (Yang et al., 1989) and vinca alkaloid binding to P-gp-containing plasma membranes (Yang et al., 1990) and increases the cellular levels of vinblastine (Yang et al., 1989), yet does not appear to be a substrate for transport by P-gp (Yang et al., 1989; Saecki et al., 1993). Thus, there appear to be two classes of reversers of P-gp-mediated drug resistance. The first class consists of compounds which compete with cytotoxic drugs for binding and transport by P-gp. The second class of reversers, which includes tamoxifen and progesterone, compete for drug binding, thereby blocking transport of chemotherapeutic agents, but are not themselves transported.

In addition to its role in drug resistance, expression of P-gp is also associated with a cell volume-regulated chloride channel (Gill et al., 1992; Valverde et al., 1992). Tamoxifen is also a high-affinity inhibitor of this chloride channel, although it is not known whether or not this inhibitory effect is mediated through the interaction of tamoxifen with P-gp (Zhang et al., 1994). The blockage of chloride channels by tamoxifen in the lens of the eye leads to opacity, suggesting a molecular mechanism by which tamoxifen might lead to visual impairment (Zhang et al., 1994). Together with the present finding that tamoxifen binds to P-gp, these data suggest that tamoxifen may interact with a number of functionally important targets, with consequent implications for the therapeutic use of this anti-cancer drug.

Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; PMSF, phenylmethyloxysulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

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