Tamoxifen inhibits nitrobenzylthioinosine-sensitive equilibrative uridine transport in human MCF-7 breast cancer cells

Jun CAI and Chee Wee LEE*  
Department of Physiology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260

INTRODUCTION

Tamoxifen is an anti-cancer drug widely used in the treatment of breast cancer. The anti-tumour effect of tamoxifen is not entirely understood but is believed to result from competitive interaction with the oestrogen receptor as a frank oestrogen, as an agonist or as an antagonist [1-3], depending on the species studied, the target organ assessed and the amount of tamoxifen used. Nevertheless, not all effects of tamoxifen can be explained by mediation through the oestrogen receptor. Some studies have shown that tamoxifen inhibition of cell proliferation in vitro cannot be prevented by oestriadiol [4]. Furthermore tamoxifen at micromolar concentrations had profound inhibitory effects on the growth of oestrogen-receptor-negative cancer cell lines such as ral Nb2 lymphoma [5,6], human BT47 breast carcinoma [7], human A549 lung adenocarcinoma [8] and human fetal fibroblast strains from lung and pituitary [9]. In addition, approx. 14-30% of oestrogen-receptor-negative human breast cancer patients have been reported to respond to tamoxifen therapy [10,11]. A variety of potentially important actions of tamoxifen have been reported. For example, tamoxifen has been shown to induce cells surrounding the cancer cells to secrete the transforming growth factor β (TGF-β) [9,12]. Owing to its ability to regulate TGF-β production, tamoxifen might have additional applications in the prevention and treatment of both oestrogen-receptor-positive and oestrogen-receptor-negative breast cancer [9]. Tamoxifen also acts as a membrane fluidity modulator and this is thought to be the basis of its anti-oxidant, anti-cancer, anti-viral, anti-multidrug-resistance and cardioprotective actions [13-15]. In pancreatic cancer, the anti-cancer effect of tamoxifen is mediated by inhibiting the action of protein kinase C [16].

At a molecular level, tamoxifen is assumed to be specific in its interactions with the oestrogen receptor. However, there are other cellular proteins that might be directly affected by tamoxifen. Among them are high-affinity membrane-associated binding proteins called anti-oestrogen binding sites (AEBS) [4,17]. These proteins, found in a variety of tissues, have a ubiquitous distribution that does not follow the pattern of oestrogen receptor distribution. Although the AEBS have been widely studied, the physiological function of these proteins remains unknown. Tamoxifen has also been shown to inhibit the binding of lactogen to the lactogen-binding protein, which is also a member of the AEBS family [18,19]. In addition, muscarinic receptor [20], histamine-like receptors [21], P-glycoprotein [22], protein kinase C [23] and calmodulin [24,25] are all affected by tamoxifen.

In this study we have identified a possible additional cellular target for tamoxifen, the nitrobenzylthioinosine (NBMPR)-sensitive equilibrative nucleoside transporter protein. The importance of nucleoside transport proteins in modulating the anti-tumour properties of nucleoside drugs has previously been demonstrated in vitro. For example, dipyridamole (a nucleoside transport inhibitor) is able to enhance the cytotoxicity of 5-fluorouracil by prolonging the retention of active metabolites of 5-fluorouracil such as FdUMP [26]. In practice, however, combinations with dipyridamole were no more effective than 5-fluorouracil alone [27]. One of the possible reasons for the ineffectiveness observed in patients may be an insufficient level of free dipyridamole, as dipyridamole itself is bound in the plasma and can thus be rendered inactive. It is difficult to achieve, without severe side effects, the effective concentration of dipyridamole in vivo as is required in vivo for this modulation. In contrast, tamoxifen is well tolerated at high doses in vivo and has few complications [28]. Thus tamoxifen might be an alternative to dipyridamole as a nucleoside transport inhibitor in modulating the bioactivity of nucleoside drugs in cancer therapy.

EXPERIMENTAL

Cell culture

Human MCF-7 breast carcinoma, human HL-60 promyelocytic leukaemia, rat Morris 7777 hepatoma and murine EL4 lympho-
oma cells were maintained in continuous culture in RPMI 1640 medium (Gibco, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) heat-inactivated (50 °C for 30 min) fetal bovine serum (Gibco) plus 50 i.u./ml penicillin and 50 µg/ml of streptomycin (Gibco).

**[3H]Uridine transport assay**

Cells were harvested just before reaching confluency or plateau growth phase and prepared for transport studies as follows. Monolayer cells were detached by trypsinization (0.05% trypsin plus 0.02% EDTA in PBS). The cell suspension was centrifuged at 1000 g for 5 min in a Sorvall GL-C 4 centrifuge. The cell pellet was washed once with 50 ml of Na+-free (choline replacement) Hanks balanced salt solution (HBSS) plus 5.5 mM d-glucose and 4 mM Heps buffer, pH 7.4. The final cell pellet was resuspended with an appropriate volume of Na+-free HBSS. Cell numbers were determined with a Coulter particle counter. The equilibrative uptake of [3H]uridine (final concentration 50 µM) was initiated by mixing 70 µl of cell suspension (treated with or without tamoxifen) with 140 µl of radioactive substrate (in the presence or absence of Na+) in a 1.5 ml Microfuge tube at 22–24 °C. The detailed composition of the final transport medium is given in each figure legend. After an appropriate interval the reactions were terminated by transferring a 60 µl aliquot of the cell mixture into an ‘oil-stop’ tube consisting of a 400 µl Microfuge tube 125 µl of silicon oil (density 1.04 g/ml) layered over 25 µl of 15% (w/v) trichloroacetic acid. Cells were separated from the transport medium by pelleting into the trichloroacetic acid layer via centrifugation in a Beckman model E microcentrifuge. Microfuge tubes were then cut through the oil layer and both the supernatant and cell pellet were placed in separate plastic mini-vials for scintillation counting. To each sample, 3.5 ml of scintillation fluid (Amersham, Arlington Heights, IL, U.S.A.) was added and vortex-mixed vigorously. Radioactivity was determined with a liquid-scintillation counter. Approximate initial rates of uridine uptake were determined by using an uptake interval of 15 s. NBMPR-sensitive equilibrative transport was defined as the difference between transport determined in the presence and in the absence of 100 nM NBMPR. NBMPR-insensitive equilibrative transport was defined as the transport in the presence of 100 nM NBMPR after subtracting the linear component determined in the presence of 30 µM dipyridamole. ‘Time-zero’ values for transport, attributed to radioactivity trapped in the cell pellet, were determined by centrifuging a cell suspension (20 µl) through a layer of [3H]uridine (40 µl) layered over the oil in an ‘oil-stop’ tube. These values were subtracted from measurements of uptake by cells. The intracellular cell volume was calculated in all experiments by using [3H]Sucrose as a measurement of extracellular space.

**[3H]NBMPR-binding assay**

Equilibrium [3H]NBMPR-binding assays were conducted as follows. Cells (20 µl) suspended in HBSS were added to either 1 nM [3H]NBMPR in a total volume of 200 µl at 22–24 °C in the presence or absence of 20 nM nitrobenzylthioguanosine (NBTRG). NBTRG is a competing non-radioactive ligand. After 30 min of incubation, reactions were terminated by the ‘oil-stop’ method as described above. Specific binding was defined as the difference in membrane content of [3H]NBMPR in the presence and in the absence of NBTRG.

**Data analysis**

Kinetic constants for equilibrative uridine transport (apparent $K_m$ and $V_{max}$) and for NBMPR binding (apparent $K_d$ and $B_{max}$) were assessed by the nonlinear squares fit of the Michaelis–Menten equation with the ENZFIT program [29].

**Chemicals**

[5-3H]Uridine (25 Ci/mmol), [3H]NBMPR (25 Ci/mmol) and 3H2O (18 µCi/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). [3C]Sucrose (475 mCi/ml) was purchased from Amersham. Tamoxifen, oestradiol, NBMPR, NBTRG and dipyridamole were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

**RESULTS**

**Effect of tamoxifen on the equilibrium binding of [3H]NBMPR in MCF-7, HL-60 and EL4 cells**

One approach to investigating the properties of an equilibrative nucleoside transporter is to use NBMPR, a potent nucleoside transport inhibitor, as a ligand probe for the protein [30,31]. As shown in Figure 1, MCF-7 cells were incubated with 1 nM [3H]NBMPR in the presence of various concentrations of tamoxifen ranging from 0 to 30 µM for 30 min. Results show that tamoxifen decreases the specific binding of [3H]NBMPR in MCF-7 cells with an IC50 of 8 µM without affecting the non-specific binding (results not shown).

It is possible that the inhibitory effect of tamoxifen on NBMPR binding is unique to MCF-7 cells and not to other cell types, as the MCF-7 cell is known to be responsive to various actions of tamoxifen. To resolve this issue we examined the effect of tamoxifen on the binding of NBMPR in other cell lines. Figure 1 shows that the binding of [3H]NBMPR in HL-60 and EL4 cells is also sensitive to tamoxifen inhibition with IC50 values of 5 and 20 µM respectively.

**Figure 1** Effect of tamoxifen on equilibrium binding of [3H]NBMPR in MCF-7, HL-60 and EL4 cells

MCF-7 (●), HL-60 (○) and EL4 (●) cells were incubated with 1 nM [3H]NBMPR in the presence of various concentrations of tamoxifen (0–30 µM) for 30 min at room temperature. Specific binding was defined as the difference in membrane content of [3H]NBMPR in the presence and in the absence of NBTRG. Results are expressed as a percentage of untreated cells. The values are the means ± S.E.M. for triplicate determinations, and are from a representative of four separate experiments.
Tamoxifen inhibits nucleoside transport

Effect of tamoxifen on equilibrative transport of \([^{3}H]\)uridine

MCF-7 (●), HL-60 (○), Morris 7777 (■) and EL4 (□) cells suspended in Na\(^+\)-free (choline replacement) HBSS were pretreated with various concentrations of tamoxifen ranging from 0 to 30 µM for 20 min before the addition of \([^{3}H]\)uridine (50 µM final concentration). NBMPR-sensitive transport (A) was defined as the difference between transport determined in the presence and in the absence of 100 nM NBMPR. NBMPR-insensitive transport (B) was defined as transport in the presence of 100 nM after subtracting the linear component determined in the presence of 30 µM dipyridamole. The results are shown as a percentage of the control influx rate (measured at 15 s uptake intervals) against tamoxifen concentration. The values are the means ± S.E.M. for triplicate analyses, and are representative of four separate experiments.

Effect of tamoxifen on NBMPR-sensitive and NBMPR-insensitive equilibrative transport of \([^{3}H]\)uridine in MCF-7, HL-60, EL4 and Morris 7777 cells

Figure 2(A) shows that the NBMPR-sensitive equilibrative transport of \([^{3}H]\)uridine (50 µM) in MCF-7 cells was inhibited by tamoxifen with IC\(_{50}\) values of 7–10 µM. However, the NBMPR-insensitive transport activity was not significantly affected by tamoxifen at concentrations below 30 µM (Figure 2B).

The same experiments were repeated with HL-60, Morris 7777 and EL4 cells. Like MCF-7 cells, HL-60 cells possess both the NBMPR-sensitive and NBMPR-insensitive transport systems [32,33]. EL4 cells, however, possess only the NBMPR-sensitive transport system, and Morris 7777 cells possess mostly the NBMPR-insensitive transport system [34]. The unique characteristic of the EL4 and Morris 7777 cell lines allowed us to investigate the effects of tamoxifen on each subtype of equilibrative nucleoside transport systems accurately without the interference of the other transport system. Figure 2(A) shows that NBMPR-sensitive transport of \([^{3}H]\)uridine in HL-60 and EL4 cells was also inhibited by tamoxifen with IC\(_{50}\) values of 7–10 µM. In contrast, the NBMPR-insensitive transport system in HL-60 and Morris 7777 cells was not inhibited by tamoxifen at concentrations as high as 30 µM (Figure 2B). These results suggest that tamoxifen is specifically targeted at the NBMPR-sensitive equilibrative nucleoside transport system.

Effect of tamoxifen on 

Effect of tamoxifen on the kinetics of equilibrium binding of \([^{3}H]NBMPR\) in MCF-7 cells

Figure 3 shows that the \(K\(_m\)\) values for the binding of \([^{3}H]NBMPR\) to the NBMPR-sensitive equilibrative nucleoside transporter molecules was increased significantly in response to tamoxifen treatment. The apparent \(K\(_m\)\) values were 0.63 ± 0.12, 1.79 ± 0.29 and 4.75 ± 0.58 nM (\(n = 3\)) for cells treated with 0, 10 and 30 µM tamoxifen respectively. In contrast, the \(B\(_{max}\)\) values of 311 000 ± 76 000, 245 000 ± 95 000 and 263 000 ± 46 000 sites per cell (\(n = 3\)) for cells treated with 0, 10 and 30 µM tamoxifen respectively were not significantly different. The result indicates that the inhibition of NBMPR binding by tamoxifen is a competitive mechanism.

Effect of tamoxifen on the kinetics of NBMPR-sensitive and NBMPR-insensitive transport of \([^{3}H]\)uridine in MCF-7 cells

Lineweaver–Burk plots of the concentration dependence of NBMPR-sensitive and NBMPR-insensitive \([^{3}H]\)uridine influx are shown in Figure 4. The apparent \(K\(_m\)\) values of uridine influx for the NBMPR-sensitive (Figure 4A) and NBMPR-insensitive transport systems (Figure 4B) were 390 ± 30 and 430 ± 120 µM, with \(V\(_{max}\)\) values of 12.0 ± 0.1 and 3.7 ± 1.6 µM/s (\(n = 3\)) respectively. After treatment with 15 µM tamoxifen for 20 min there was a 4-fold increase in the \(K\(_m\)\) of uridine influx for NBMPR-sensitive component (apparent \(K\(_m\)\) 1500 ± 250 µM/s; \(n = 5\)) with little change in \(V\(_{max}\)\) (11.3 ± 4.3 µM/s, \(n = 5\)) (Figure 4A). These results suggest a competitive inhibition of NBMPR-sensitive uridine transport by tamoxifen. As expected, the transport kinetics for NBMPR-insensitive transport of uridine was not affected by 15 µM tamoxifen (apparent \(K\(_m\)\) 340 ± 150 µM; \(V\(_{max}\)\) 4.4 ± 1.3 µM/s, \(n = 5\)) (Figure 4B).

Effect of tamoxifen on Na\(^+\)-dependent \([^{3}H]\)uridine uptake by murine splenocytes

Previous studies have demonstrated that uridine is actively
concentrated in murine splenocytes by an Na⁺-dependent nucleoside transport system [35,36]. This transport system is different from the equilibrative nucleoside transport system in that it is concentrative and insensitive to equilibrative nucleoside transport inhibitors such as NBMPR and dipyridamole. Figure 5 shows that the Na⁺-dependent transport of [³H]uridine (5 µM) in murine splenocytes was not inhibited by tamoxifen at concentrations as high as 30 µM. Figure 6 shows that in splenocytes, owing to the presence of the Na⁺-dependent nucleoside transport system, the uptake of [³H]uridine in the presence of Na⁺ was at least 2–3-fold higher than the uptake in the absence of Na⁺ (choline replacement) after an uptake interval of 5 min. The uptake of uridine in the absence of Na⁺ was significantly inhibited by 30 µM tamoxifen. In contrast, there was a slow increase in intracellular accumulation of radioactivity in cells treated with tamoxifen in the presence of Na⁺. The level of intracellular radioactivity in these cells eventually exceeded the level of those control cells suspended in the absence of Na⁺. These results suggest that tamoxifen is able to function like NBMPR and dipyridamole, which under physiological conditions are able to enhance the intracellular accumulation of uridine in murine splenocytes by inhibiting the efflux of uridine via the NBMPR-sensitive equilibrative transport system.

**DISCUSSION**

Mammalian nucleoside transporters have been studied widely. These transporters are categorized into two main systems, the equilibrative transport system [30,33–37] and the Na⁺-dependent concentrative transport system [35,36,38,39]. The equilibrative transport system is further divided into the NBMPR-sensitive and the NBMPR-insensitive equilibrative transport systems.

In this paper we have demonstrated that tamoxifen, an anti-oestrogen, has an inhibitory effect on the NBMPR-sensitive equilibrative nucleoside transport system. Tamoxifen, however, has little or no effect on either the NBMPR-insensitive equilibrative system or the Na⁺-dependent nucleoside transport system.
These findings were obtained from collective studies with various cell lines such as HL-60, EL4, Morris 7777 cells and murine splenocytes. The decrease in the NBMPR-sensitive equilibrative nucleoside transport activity in MCF-7 cells in the presence of tamoxifen was due mainly to a profound decrease in the apparent transport affinity of this transport system. These changes are not the result of non-specific effects of the tamoxifen on the plasma membranes or on the transporter protein itself because no decrease in the rate of NBMPR-insensitive equilibrative (Figure 4B) and Na+-dependent (Figure 5) uridine transport was observed with tamoxifen at concentrations as high as 30 &mu;M. Furthermore solvents such as ethanol and DMSO at concentrations as high as 2.5 %, a concentration known to perturb the structural integrity of the cell membrane, showed no effect on NBMPR-sensitive equilibrative nucleoside transport system in MCF-7 cells. Steroid hormones such as oestradiol, at a cytotoxic concentration of 30 &mu;M, also had little effect on this transport system (results not shown).

In our studies the IC_{50} values for tamoxifen’s inhibiting the NBMPR-sensitive equilibrative uridine transport system in MCF-7, HL-60 and EL4 cells were at concentrations of 7–20 &mu;M. The amount of tamoxifen used to exert the inhibitory effect on this transport system is rather high if it is compared with &mu;Molar concentrations of tamoxifen were required. For example, molecules in MCF-7 cells (results not shown). There are numerous studies showing the non-estrogen-receptor-dependent effects of tamoxifen on various cellular systems, where micromolar concentrations of tamoxifen were required. For example, IC_{50} values for the inhibition of calmodulin actions [25], protein kinase C activation [23] and breakdown of phosphomonoesterases [40] by tamoxifen were 9, 5 and 9 &mu;M respectively. Those studies also showed that low micromolar concentrations of tamoxifen had little or no apparent adverse effect on cell membrane integrity.

Thus changes in the NBMPR-sensitive equilibrative uridine transport activity associated with tamoxifen might be due to a direct interaction of tamoxifen with the transporter, hence affecting transport activity. The mechanism might be similar to that of NBMPR, i.e. it binds to a site that is either on or near the nucleoside-transporting site, thus decreasing the affinity of the transporter for binding or transporting the substrate.

There are reports that the salvage pathway of DNA synthesis is important for the proliferation of tumour cells, and that purine and pyrimidine nucleosides protect tumour cells from inhibitors of the de novo pathway of DNA synthesis [41,42]. It is possible for tamoxifen to have some anti-proliferative effects merely by inhibiting the DNA salvage pathway. However, one can argue that tamoxifen is a much weaker inhibitor of equilibrative nucleoside transport than other better characterized equilibrative nucleoside transport inhibitors, including NBMPR, dilazep and dipyridamole. This makes the physiological significance of tamoxifen in inhibiting the uptake of nucleoside questionable. Nevertheless our parallel studies had demonstrated that prolonged exposure of MCF-7 cells to tamoxifen also causes down-regulation of the NBMPR-sensitive equilibrative nucleoside transporters on the cell membranes [43]. These studies, together with the previous findings that tamoxifen is well tolerated at high concentrations in vivo and has multiple modes of action, support a new clinical role for tamoxifen in development of anti-cancer regimens.

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REFERENCES