

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

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Tamoxifen inhibits the binding of [³H]nitrobenzylthioinosine ([³H]NBMPR) to human MCF-7 breast cancer cells with an IC₅₀ of 8 μM. Tamoxifen at 30 μM changed the apparent K_d for [³H]NBMPR binding from 0.63 ± 0.12 to 4.75 ± 0.58 nM, with little effect on the B_{max} (311 000 ± 76 000 and 263 000 ± 46 000 sites per cell for untreated and tamoxifen-treated cells respectively). Corresponding to this decrease in binding of [³H]NBMPR in the presence of tamoxifen was an inhibition of NBMPR-sensitive equilibrative transport of 50 μM [³H]uridine (IC₅₀ 7–10 μM). In the presence of 15 μM tamoxifen, the apparent K_m for [³H]uridine transport was increased from 390 ± 30 to 1500 ± 250 μM, with no change in V_{max} (12.0 ± 0.1 and 11.3 ± 4.3 μM/s for untreated and

tamoxifen-treated cells respectively). The inhibitory effect of tamoxifen on NBMPR-sensitive equilibrative uridine transport was specific, as similar results were also observed in HL-60 leukaemia and EL4 lymphoma cells. Furthermore a similar concentration of tamoxifen had no effect on the NBMPR-insensitive equilibrative transport of uridine in MCF-7, HL-60 and Morris 7777 hepatoma cells, and on the Na⁺-dependent transport of uridine in murine splenocytes. In this paper we demonstrate that tamoxifen by itself might have some anti-proliferative effects through inhibition of DNA synthesis by blocking the nucleoside salvage pathway.

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 oestradiol [4]. Furthermore tamoxifen at micromolar concentrations had profound inhibitory effects on the growth of oestrogen-receptor-negative cancer cell lines such as rat Nb₂ lymphoma [5,6], human BT₂₀ 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 [12]. Tamoxifen also acts as a membrane fluidity modulator and this is thought to be the basis of its anti-oxidant, anti-cancer, anti-viral, antimultidrug-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 vitro* 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 lymph-

Abbreviations used: AEBS, anti-oestrogen binding sites; HBSS, Hanks balanced salt solution; NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine.

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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 GLC-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 Hepes 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 containing 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 dipyrindamole. '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 ³H₂O to determine total water space, and by using [¹⁴C]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 µM nitrobenzylthioguanosine (NBTGR). NBTGR 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 NBTGR.

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.). [¹⁴C]sucrose (475 mCi/mmol) was purchased from Amersham. Tamoxifen, oestradiol, NBMPR, NBTGR and dipyrindamole 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.

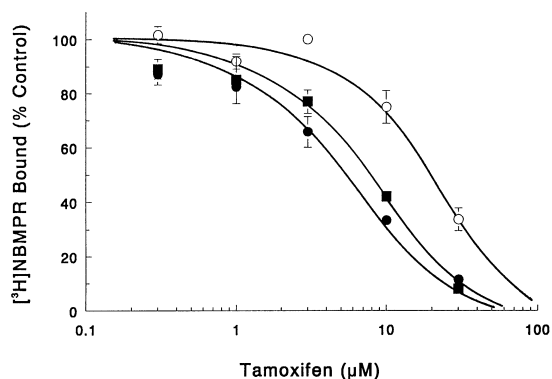


Figure 1 Effect of tamoxifen on equilibrium binding of [³H]NBMPR

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 20 µM NBTGR. 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.

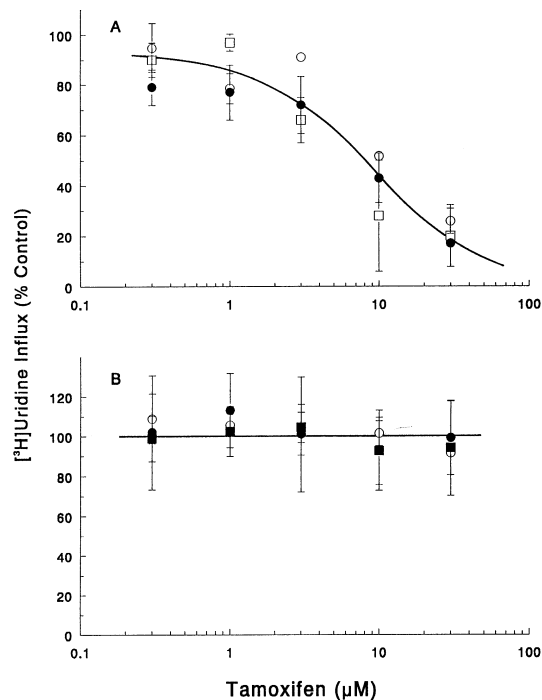


Figure 2 Effect of tamoxifen on the equilibrative transport of [³H]uridine

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 [³H]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 dipyrindamole. 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 [³H]uridine in MCF-7, HL-60, EL4 and Morris 7777 cells

Figure 2(A) shows that the NBMPR-sensitive equilibrative transport of [³H]uridine (50 µM) in MCF-7 cells was inhibited by tamoxifen with IC₅₀ 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 [³H]uridine in HL-60 and EL4 cells was also inhibited by tamoxifen with IC₅₀ 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.

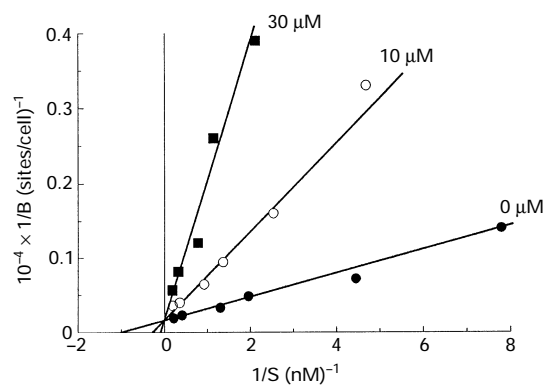


Figure 3 Effects of tamoxifen on kinetics of [³H]NBMPR equilibrium binding

MCF-7 cells were pretreated with 0 (●), 10 (○), or 30 (■) µM tamoxifen for 1 min before they were incubated with various concentrations of [³H]NBMPR (0.2–6 nM final concentration) for an additional 30 min. Lineweaver–Burk plots of the results are presented. The values are the means ± S.E.M. for triplicate analyses, and are representative of three separate experiments. For the results shown, the apparent K_d values were 0.90 ± 0.10 , 1.80 ± 0.30 and 5.77 ± 1.99 nM, with B_{max} values of $490\,000 \pm 25\,000$, $390\,000 \pm 28\,000$ and $370\,000 \pm 78\,000$ sites per cell for cells treated with 0, 10 and 30 µM tamoxifen respectively.

Effect of tamoxifen on the kinetics of equilibrium binding of [³H]NBMPR in MCF-7 cells

Figure 3 shows that the K_d values for the binding of [³H]NBMPR to the NBMPR-sensitive equilibrative nucleoside transporter molecules was increased significantly in response to tamoxifen treatment. The apparent K_d 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 $311\,000 \pm 76\,000$, $245\,000 \pm 95\,000$ and $263\,000 \pm 46\,000$ 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 equilibrative transport of [³H]uridine in MCF-7 cells

Lineweaver–Burk plots of the concentration dependence of NBMPR-sensitive and NBMPR-insensitive equilibrative [³H]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 [³H]uridine uptake by murine splenocytes

Previous studies have demonstrated that uridine is actively

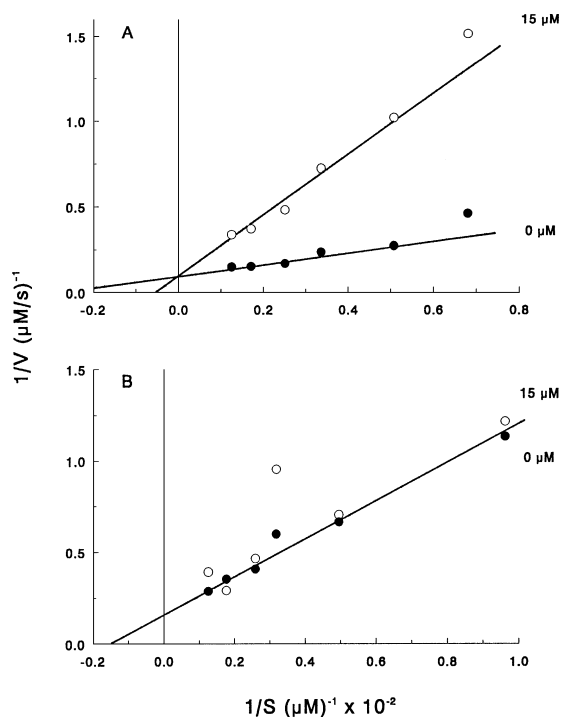


Figure 4 Effect of tamoxifen on the kinetics of NBMPR-sensitive and NBMPR-insensitive equilibrative transport of [³H]uridine in MCF-7 cells

MCF-7 cells were treated with (○) or without (●) 15 μM tamoxifen for 15 min before uridine transport was measured. Approximate initial rates of uridine transport were determined by using an uptake interval of 15 s with [³H]uridine concentrations of 50–800 μM. Lineweaver–Burk plots of the results are presented. 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 NBMPR after subtracting the linear component determined in the presence of 30 μM dipyridamole. The values are the means ± S.E.M. for triplicate analyses, and for each panel are representative of at least three separate experiments. For the results shown, the apparent K_m values were 360 ± 80 and 580 ± 120 μM, with V_{max} values of 5.88 ± 0.82 and 5.91 ± 0.73 μM/s for NBMPR-sensitive and NBMPR-insensitive transport systems respectively. After treatment with 15 μM tamoxifen, the apparent K_m values were 1800 ± 670 and 580 ± 530 μM, with V_{max} values of 6.53 ± 1.87 and 5.18 ± 2.58 μM/s for NBMPR-sensitive and NBMPR-insensitive transport systems respectively.

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

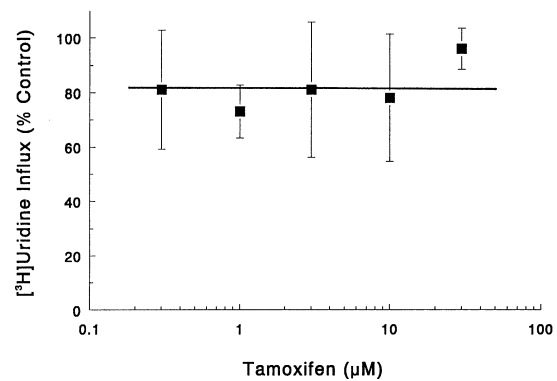


Figure 5 Effect of tamoxifen on Na⁺-dependent transport of [³H]uridine in murine splenocytes

Murine splenocytes treated with 30 μM NBMPR were incubated with various concentrations of tamoxifen (0–30 μM) for 5 min before the addition of 5 μM [³H]uridine (final concentration). The results are shown as a percentage of control influx rate (measured at 15 s uptake intervals) against tamoxifen concentration. The values are the means ± S.E.M. for triplicate analysis, and are representative of three separate experiments and were corrected for the uptake in the absence of Na⁺ (choline replacement).

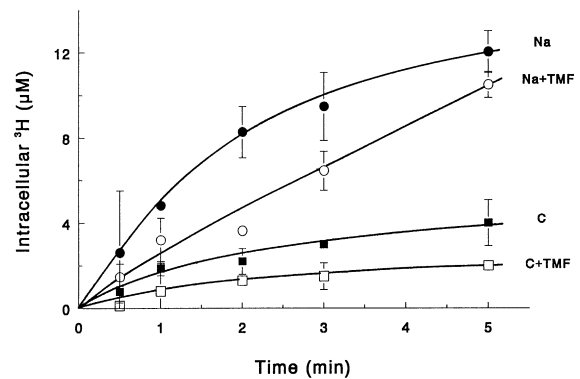


Figure 6 Effects of tamoxifen on intracellular accumulation of [³H]uridine in murine splenocytes

Cells were incubated without (●,■) or with 30 μM tamoxifen (○,□) for 20 min before exposure to [³H]uridine (final concentration 5 μM) in the presence (●,○) or absence (■,□) of Na⁺ (choline replacement). At appropriate intervals, uptake was terminated by the 'oil-stop' method. The values are the means ± S.E.M. for triplicate analysis, and are representative of four separate experiments. Abbreviations: Na, sodium; C, choline; TMF, tamoxifen.

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 μ 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 μ M, also had little effect on this transport system (results not shown).

In our studies the IC₅₀ 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 μ M. The amount of tamoxifen used to exert the inhibitory effect on this transport system is rather high if it is compared with the binding affinity of tamoxifen (K_d 4.8 nM) for the oestrogen receptor [1]. However, we believe that the inhibitory effect of tamoxifen on NBMPR-sensitive equilibrative transport system is not an oestrogen-receptor-dependent mechanism. Oestradiol at 30 μ M was unable to reverse the inhibitory effect of 30 μ M tamoxifen on the binding of [³H]NBMPR to the transporter molecules in MCF-7 cells (results not shown). There are numerous studies showing the non-oestrogen-receptor-dependent effects of tamoxifen on various cellular systems, where micromolar concentrations of tamoxifen were required. For example, IC₅₀ values for the inhibition of calmodulin actions [25], protein kinase C activation [23] and breakdown of phosphoinositides [40] by tamoxifen were 9, 5 and 9 μ 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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