Reduction of equilibrative nitrobenzylthioinosine-sensitive nucleoside transporter in tamoxifen-treated MCF-7 cells: an oestrogen-reversible phenomenon

Lay-Beng GOH and Chee-Wee LEE¹

Department of Physiology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260

MCF-7 cells display both nitrobenzylthioinosine (NBMPR)-sensitive (*es*) and NBMPR-insensitive (*ei*) equilibrative, but not the Na⁺-dependent, nucleoside transport. Transport of uridine by *es* is more sensitive to inhibition by purine nucleosides, whereas the *ei* component is more sensitive to nucleosides without an amino side group, such as inosine and thymidine. When exposed to 10 μ M tamoxifen for 5 days, MCF-7 cells displayed a 44% decrease in the total number of NBMPR-binding sites [$B_{\max}$ from 245000 ± 18000 to 136000 ± 25000 sites per cell (mean $\pm$ S.E.M.; $n = 5$; $P < 0.05$)], and a 57% decrease in cell growth with no significant change in binding affinities [K_d from 0.37 ± 0.05 to 0.45 ± 0.08 nM ($n = 5$; $P > 0.05$)]. Kinetic studies

of [³H]uridine transport showed a decrease in the $V_{\max}$ of the *es* component from 21.7 ± 0.3 ($n = 8$) to 8.4 ± 2.2 μ M/s ($n = 4$; $P < 0.05$), whereas the $V_{\max}$ of the *ei* component [from 4.7 ± 0.5 ($n = 8$) to 5.8 ± 1.6 μ M/s ($n = 4$; $P > 0.05$)] and K_m values for both components [*es* from 460 ± 80 to 630 ± 280 μ M ($n \geq 3$; $P > 0.05$) and *ei* from 355 ± 115 to 440 ± 220 μ M ($n \geq 4$; $P > 0.05$)] did not change significantly. Oestradiol at 100 nM reversed almost completely the NBMPR-binding site decrease and growth retardation in tamoxifen-treated cells. Thus tamoxifen is shown to cause an oestrogen-reversible decrease of *es* nucleoside transporters in MCF-7 cells.

INTRODUCTION

Nucleosides traverse a mammalian cell membrane primarily via the equilibrative and/or Na⁺-dependent concentrative transport processes. The former can be subdivided into nitrobenzylthioinosine (NBMPR)-sensitive (*es*) and NBMPR-insensitive (*ei*) components, where NBMPR is a specific inhibitor of equilibrative nucleoside transport. Equilibrative nucleoside transport is present in both normal and neoplastic cells, but the activity is higher in neoplastic cells, with the *es* component predominating [1]. In contrast, Na⁺-dependent concentrative nucleoside transport is usually present in normal cells, but either absent or functioning at a very low level in neoplastic cells [1]. Combined with a higher metabolism in neoplastic cells, this might explain why normal cells are able to accumulate intracellular uridine pools that are absent from neoplastic cells [2].

On differentiation *in vitro*, equilibrative nucleoside transport tends to be down-regulated, whereas the Na⁺-dependent concentrative nucleoside transport is usually up-regulated. Our previous studies show that when human HL-60 promyelocytic leukaemia cells are induced into monocytic or granulocytic differentiation with PMA or DMSO respectively, a rapid 80–94% decrease in growth occurs [3–5], together with a decrease in the number of *es* transporter proteins ($B_{\max}$ from 198 000 to 12 000 sites per cell [3] and from 280 000 to 9790 sites per cell [5] for monocytic and granulocytic differentiation respectively). At the same time, Na⁺-dependent concentrative transport affinity for uridine increases (K_m from 44 μ M to approx. 1 μ M) [4,5]. Further stimulation of DMSO-differentiated HL-60 cells by a chemotactic peptide, *N*-formylmethionyl-leucylphenylalanine, results in an instantaneous 3-fold increase in $V_{\max}$ of the Na⁺-dependent concentrative transport of uridine [6]. Even in growth-retarded tamoxifen-treated MCF-7 cells, an upsurge in the Na⁺-dependent concen-

trative transport of uridine has been reported [7], where dome formation in cultures points to tamoxifen's role as a differentiation inducer for MCF-7 cells.

In the present study we have found that prolonged exposure of MCF-7 cells to tamoxifen resulted in a decrease in the number of *es* nucleoside transporters on the cell membrane. A preliminary account of some of the work presented here has been published in abstract form [8].

EXPERIMENTAL

Materials

[5-³H]Uridine (39.7 Ci/mmol), [³H]NBMPR (19.5 Ci/mmol) and ³H₂O (32.1 μ Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). [¹⁴C]Sucrose (667 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). 17 β -Oestradiol, tamoxifen, NBMPR, nitrobenzylthioguanosine and dipyrindamole were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). All other reagents were of A. R. grade. Tamoxifen was dissolved in DMSO and 17 β -oestradiol was dissolved in 100% ethanol. The final concentrations of DMSO and ethanol in the culture medium were always at 0.03% and 0.1% (both v/v) respectively.

Cell culture

MCF-7 human breast cancer cells were maintained as a continuous culture in RPMI 1640 medium (Gibco, Gaithersburg, MD, U.S.A.) containing Phenol Red and supplemented with 10% (v/v) heat-inactivated (50 °C for 30 min) fetal bovine serum (FBS) (Gibco) plus 50 i.u./ml penicillin and 50 μ g/ml strep-

Abbreviations used: *es*, NBMPR-sensitive equilibrative nucleoside transporter; *ei*, NBMPR-insensitive equilibrative nucleoside transporter; FBS, fetal bovine serum; HBSS, Hanks balanced salt solution; NBMPR, nitrobenzylthioinosine.

¹ To whom correspondence should be addressed.

tomycin (Sigma). Cells were cultured to near confluency in 50 ml of culture medium in 75 cm² flasks unless stated otherwise in the legends. The culture medium was completely aspirated before cells were harvested via 3–4 min of trypsin treatment [0.05 % trypsin/0.02 % EDTA in 150 mM NaCl/5 mM KCl/0.3 mM potassium phosphate/5.6 mM D-glucose/4 mM Hepes (pH 7.4)]. Next, cells were pelleted, suspended in 50 ml of Hanks balanced salt solution (HBSS) and left for 5 min to allow any membrane-bound tamoxifen to dissociate. This was followed by centrifugation before the final pellet was resuspended in respective buffers. For experiments involving tamoxifen and/or 17 β -oestradiol, appropriate amounts of these two compounds were added 2 days after subculture. Ethanol up to a concentration of 0.5 % (v/v) has no apparent effect on the proliferation of MCF-7 cells (results not shown). Complete removal of trypsin was not necessary for cell reattachment.

Determination of cell numbers and viability

Cells were counted after trypsin treatment with the help of an electronic Coulter counter (Coulter Electronics, Luton, Beds., U.K.). The percentage of viable cells was determined by the Trypan Blue exclusion assay.

Measurement of [³H]uridine transport

Cells were harvested via trypsin treatment as mentioned above and finally suspended in Na⁺-free (choline replacement) HBSS. The uptake of [³H]uridine (final concentration of 50 μ M, at 22–24 °C) was initiated by incubating 70 μ l of cell suspension [(5–6) $\times 10^5$ cells] with 140 μ l of [³H]uridine for 15 s, and terminated in triplicates of 60 μ l each by the ‘oil-stop’ method [3]. The 15 s time point fell just outside the initial linearity of the transport curve (see Figure 1). However, it was chosen to give a reasonable signal-to-noise ratio with little interference from cellular metabolism, as approx. 90 % of intracellular metabolites were [³H]uridine as analysed by HPLC (results not shown). In inhibition studies, cells were preincubated with NBMPR, dipyrindamole or dilazep for 15 min before assay. Detailed compositions of the final incubation medium is provided in each Figure legend. ‘Zero time’ values for transport, attributed to radioactivity trapped in the cell pellet, were determined by centrifuging 20 μ l of cell suspension through a layer of 40 μ l [³H]uridine over the oil in an ‘oil-stop’ tube. Intracellular volume was estimated by using ³H₂O to determine total water space, and [¹⁴C]sucrose as a measurement of extracellular space. NBMPR-sensitive equilibrative transport of uridine (*es*) is defined as the difference between the intracellular concentrations of [³H]uridine in cells suspended in Na⁺-free (choline replacement) HBSS treated with or without 100 nM NBMPR. Similarly, NBMPR-insensitive equilibrative transport of uridine (*ei*) is defined as the difference between those treated with 100 nM NBMPR and those treated with 30 μ M dipyrindamole.

[³H]NBMPR-binding assays

Equilibrium [³H]NBMPR-binding assays were initiated by incubating 110 μ l of cell suspension [(5–6) $\times 10^5$ cells] in HBSS with graded concentrations of [³H]NBMPR (0.1–12 nM) for 60 min in a total volume of 660 μ l at 22–24 °C. The reaction was terminated by filtering the incubated mixture as three different aliquots of 200 μ l each, via the membrane vacuum filtration method [9], washing each filter once with 5 ml of ice-cold Na⁺-buffer [150 mM NaCl/5 mM Tris/HCl (pH 7.4)]. Filters with trapped cells were dried and soaked overnight in scintillants before being counted for radioactivity. Specific binding is defined

as the difference in [³H]NBMPR content in the absence and the presence of 20 μ M nitrobenzylthioguanosine, a competing non-radioactive ligand.

Data and statistical analysis

Kinetic constants for the equilibrative transport of [³H]uridine (apparent K_m and V_{max}) and the specific binding of [³H]-NBMPR (apparent K_d and B_{max}) were assessed by the use of non-linear squares fitting of the Michaelis–Menten equation with the ENZFIT computer program [10]. Data were analysed with paired Student's *t* tests where appropriate. *P* < 0.05 implies that results are significantly different from the untreated cells.

RESULTS

Time course of uridine uptake by MCF-7 cells

The MCF-7 cells tested did not possess the active type of uridine transport requiring the presence of Na⁺ (Figure 1). There was no difference in the uptake of 50 μ M [³H]uridine for up to 5 min, regardless of whether Na⁺-containing or Na⁺-free (choline replacement) HBSS was used as the transport medium. The only uptake component measurable was almost completely abolished by 30 μ M dipyrindamole, a potent inhibitor of equilibrative transport, in both the presence and the absence of Na⁺. Although the initial linearity of the transport curve lasted for slightly less than 15 s, all subsequent transport kinetic studies were conducted with a 15 s uptake interval. This was to obtain reasonable signal-to-noise ratio. Furthermore the amount of intracellular uridine metabolized at this uptake interval was only about 10 %, as analysed by HPLC (results not shown). This equilibrative nucleoside transport system was next ascertained for its sensitivity towards NBMPR and other inhibitors of nucleoside transport.

Inhibition of equilibrative uridine transport by NBMPR, dipyrindamole and dilazep in MCF-7 cells

The concentration responses of MCF-7 cells to the three equilibrative nucleoside transport inhibitors were investigated in the absence of Na⁺ ions. All three transport-inhibition curves were

Figure 1 Time course of uridine uptake by MCF-7 cells

MCF-7 cells suspended in Na⁺-containing (●, ■) or Na⁺-free [choline (Cho) replacement] HBSS (○, □) were preincubated with (■, □) or without (●, ○) 30 μ M dipyrindamole (Dp) for 15 min before the addition of [³H]uridine (final concentration 50 μ M). Data presented are representative of two separate experiments. Values are means $\pm$ S.E.M. for triplicate determinations.

Figure 2 Inhibition of equilibrative uridine transport by nucleoside transport inhibitors

MCF-7 cells in Na^+ -free (choline replacement) HBSS were preincubated with various concentrations of NBMPR (●), dipyrindamole (○) or dilazep (■) for 15 min before studying the uptake (15 s) of [^3H]uridine (50 μM). Results were expressed as the percentage of control against the inhibitor concentration. Data are representative of at least three separate experiments. Values are means $\pm$ S.E.M. for triplicate determinations.

Table 1 Substrate specificity of equilibrative uridine transport systems in MCF-7 cells

MCF-7 cells suspended in Na^+ -free (choline replacement) HBSS were preincubated with or without 100 nM NBMPR for 15 min and exposed simultaneously to both [^3H]uridine (50 μM) and different concentrations of adenosine, cytidine, guanosine, inosine or thymidine (ranging from 0.03 to 30 mM) for 15 s. K_i values for both *es* and *ei* components were calculated from the IC_{50} values for the inhibition of uridine influx by using the equation $K_i = \text{IC}_{50}/(1 + L/K_m)$, where L is the [^3H]uridine concentration at 50 μM and K_m values are taken as 430 and 355 μM for *es* and *ei* respectively. Values reported are means $\pm$ S.E.M. for the number of experiments indicated in parentheses. Abbreviation: n.i., no inhibition at 30 mM inhibitor concentration.

Inhibitor	K_i (μM)	
	<i>es</i>	<i>ei</i>
Adenosine	80 $\pm$ 10 (3)	2610 $\pm$ 1900 (3)
Guanosine	255 $\pm$ 60 (3)	715 $\pm$ 120 (3)
Inosine	345 $\pm$ 60 (3)	75 $\pm$ 15 (3)
Thymidine	495 $\pm$ 55 (3)	60 $\pm$ 1 (2)
Cytidine	660 $\pm$ 120 (4)	n.i. (4)

biphasic (Figure 2). Approx. 70% of the carrier-mediated [^3H]uridine (50 μM) transport activity was inhibited by NBMPR, dipyrindamole and dilazep, with IC_{50} values of 0.1, 1 and 0.1 nM respectively. There was no further inhibition until the concentrations of NBMPR, dipyrindamole and dilazep exceeded 0.1, 3 and 0.1 μM respectively. Hence MCF-7 cells were found to possess both the *es* and *ei* components of equilibrative uridine transport. These two equilibrative nucleoside transport components were also distinguishable with dipyrindamole and dilazep.

Substrate specificity of equilibrative uridine transporter proteins

The substrate specificity of the *es* and *ei* components in MCF-7 cells was studied by investigating the effect of uridine uptake inhibition by physiological nucleosides such as adenosine, guanosine, inosine, thymidine and cytidine (Table 1). Details of the reactions are given in the Table legend.

Figure 3 Concentration dependence of effects of tamoxifen on cell growth and NBMPR binding

MCF-7 cells were seeded at an initial density of 2.5×10^4 cells/ml for 2 days before they were exposed to different concentrations of tamoxifen (0.1–10 μM) for a further 5 days. Thereafter cell density (A) and the number of specific NBMPR-binding sites (B) were determined as described in the Experimental section. NBMPR-binding sites were determined with a single concentration of [^3H]NBMPR (5 nM). Control cells without tamoxifen were treated with an equal volume of the vehicle (DMSO). Data are representative of at least three separate experiments. Values are means $\pm$ S.E.M. for triplicate determinations; where absent, the S.E.M. is smaller than the symbol.

The *es* and *ei* components of nucleoside transport in MCF-7 cells apparently exhibited different substrate specificities. In the *es* component, purine nucleosides were more effective inhibitors of uridine uptake than the pyrimidine nucleosides. The calculated mean K_i values were 80 $\pm$ 10 ($n = 3$), 255 $\pm$ 60 ($n = 3$) and 345 $\pm$ 60 μM ($n = 3$), for adenosine, guanosine and inosine respectively. For pyrimidine nucleosides such as thymidine and cytidine, the mean K_i values were 495 $\pm$ 55 ($n = 3$) and 660 $\pm$ 120 μM ($n = 4$) respectively. The *ei* component, however, seemed to prefer inosine ($K_i = 75 \pm 15 \mu\text{M}$; $n = 3$) and thymidine ($K_i = 60 \pm 1 \mu\text{M}$; $n = 2$) as a substrate. Adenosine ($K_i = 2610 \pm 1900 \mu\text{M}$; $n = 3$), cytidine ($K_i > 10 \text{ mM}$; $n = 4$) and guanosine ($K_i = 715 \pm 120 \mu\text{M}$; $n = 3$) were poorly accepted by this component.

Concentration dependence and temporal effects of tamoxifen on MCF-7 cell growth and NBMPR binding

MCF-7 cells were exposed to various concentrations of tamoxifen for 5 days. The maximum effect of tamoxifen was at 10 μM , which caused a 63% decrease in cell density (Figure 3A), and a 48% decrease in the number of NBMPR binding sites (from 190000 $\pm$ 2300 to 100000 $\pm$ 20000 sites per cell) measured with 5 nM [^3H]NBMPR (Figure 3B). Note that the culture medium used in this experiment contained Phenol Red, which is mildly oestrogenic [11]. The FBS supplement also contains oestrogen at approx. 10 nM. With this huge amount of oestrogen present,

Figure 4 Temporal effects of tamoxifen on cell growth and NBMPR binding

MCF-7 cells were subcultured at an initial density of 2.5×10^4 cells/ml for 2 days before exposure to 10 μ M tamoxifen (TMF). The number of specific NBMPR-binding sites (**A**) and cell density (**B**) were determined as described in the Experimental section. The numbers of NBMPR-binding sites were determined with a single concentration of [3 H]NBMPR (5 nM). Control cells without tamoxifen were treated with an equal volume of the vehicle (DMSO). Data are representative of at least three separate experiments. Values are means $\pm$ S.E.M. for triplicate determinations; where absent, the S.E.M. is smaller than the symbol.

tamoxifen at 10 μ M was non-cytotoxic to MCF-7 cells for at least 5 days (cell viability more than 90%). This is in contrast with an earlier report, using cell culture medium free of oestrogens, which cited this concentration of tamoxifen to be cytotoxic [12]. Thus, in subsequent studies, 10 μ M tamoxifen was used as it exerted the greatest effect on both growth and NBMPR-binding sites without compromising cell viability at the same time.

Next, MCF-7 cells were exposed to 10 μ M tamoxifen for up to 6 days. By day 6 of incubation, the number of NBMPR-binding sites determined with 5 nM [3 H]NBMPR had decreased by 47%, from 220000 ± 15000 sites per untreated cell to 117000 ± 2500 sites per tamoxifen-treated cell (Figure 4A). The cell density was decreased by 57% (Figure 4B). More than 90% of the cells were viable throughout the 6 days of incubation. However, to coincide with the exponential phase of growth in control cells, so as to minimize complications arising from cell arrest effects, a 5-day exposure to tamoxifen (10 μ M) was employed in subsequent studies.

Effects of tamoxifen on the kinetics of NBMPR equilibrium binding

To determine whether tamoxifen's effect on NBMPR binding was a result of a change in K_d or B_{max} , MCF-7 cells were treated with or without 10 μ M tamoxifen for 5 days before being harvested for the high affinity [3 H]NBMPR-binding assay (Figure 5). Tamoxifen induced a 44% decrease in B_{max} from 245000 ± 18000 to 136000 ± 25000 sites per cell ($n = 5$; $P < 0.05$). K_d

Figure 5 Effect of tamoxifen on the kinetics of NBMPR binding

MCF-7 cells seeded at an initial density of 2.5×10^4 cells/ml were cultured for 2 days before treatment with (●) or without (○) 10 μ M tamoxifen (TMF) for a further 5 days. For the determination of K_d and B_{max} values, high-affinity [3 H]NBMPR-binding assays were performed as described in the Experimental section. The kinetic constants for the data presented are K_d values of 0.29 ± 0.08 and 0.33 ± 0.08 nM, with B_{max} values of 154000 ± 9000 and 255000 ± 15000 sites per cell for tamoxifen-treated and untreated cells respectively. Values are means $\pm$ S.E.M. for triplicate determinations; where absent, the S.E.M. is smaller than the symbol. Data shown were corrected for non-specific binding determined in the presence of 20 μ M nitrobenzylthioguanosine and are representative of three separate experiments. The inset shows the Scatchard plot of the results.

values, however, remained relatively unchanged at 0.37 ± 0.05 and 0.45 ± 0.08 nM, for untreated and tamoxifen-treated cells respectively ($n = 5$; $P > 0.05$).

Effects of tamoxifen on the kinetics of *es* and *ei* transport of uridine

The effect of tamoxifen on the *es* component was investigated via high-affinity NBMPR binding. However, the effect on the *ei* component could not be determined similarly owing to the absence of a suitable ligand for the latter. However, the effect of tamoxifen on the *ei* component can be directly studied through tamoxifen's influence on the kinetics of *ei* uridine transport. Thus MCF-7 cells were treated with or without 10 μ M tamoxifen for 5 days and both the *es* (Figure 6A) and *ei* (Figure 6B) transport of various concentrations of [3 H]uridine (15 s) were determined. Treatment with tamoxifen led to a 61% decrease in V_{max} of the *es* component from 21.7 ± 3.0 μ M/s ($n = 8$) to 8.4 ± 2.2 μ M/s ($n = 4$; $P < 0.05$). The change in V_{max} for the *ei* component was, however, insignificant [from 4.7 ± 0.5 μ M/s ($n = 8$) to 5.8 ± 1.6 μ M/s ($n = 4$; $P > 0.05$)]. Exposure to tamoxifen did not change the apparent K_m values of either the *es* [K_m from 430 ± 80 to 630 ± 280 μ M ($n \geq 4$)] or the *ei* [K_m from 355 ± 115 to 440 ± 220 μ M ($n \geq 4$)] component. Thus it is shown that tamoxifen could decrease the maximum transport velocity of the *es* component without any substantial change in its transport affinity for uridine. From the previous section we know that this is a result of fewer *es* nucleoside transporters and not a decrease in the efficiency of nucleoside translocation, as indicated by the unchanged affinity of the NBMPR-binding sites. The *ei* component was, in contrast, not susceptible to the down-regulatory effect of tamoxifen. This implied that tamoxifen's effect on the *es* component is not a non-specific phenomenon.

Figure 6 Effect of tamoxifen on the kinetics of *es* and *ei* transport of uridine

MCF-7 cells seeded at an initial density of 2.5×10^4 cells/ml were cultured for 2 days before treatment with (●) or without (○) 10 μ M tamoxifen (TMF) for a further 5 days. Cells in Na⁺-free (choline replacement) HBSS were preincubated with or without 100 nM NBMPR or 30 μ M dipyrindamole for 15 min. The transport of different concentrations of [³H]uridine (50–1000 μ M) was measured with an uptake interval of 15 s. The *es* (A) and *ei* (B) components were derived as described in the Experimental section. Kinetic constants for the *es* component shown are K_m values of 510 ± 15 and 370 ± 70 μ M, with V_{max} values of 7.5 ± 0.1 and 19.4 ± 1.5 μ M/s for tamoxifen-treated and untreated cells respectively. For the *ei* component shown, K_m values are 340 ± 15 and 260 ± 6 μ M, with V_{max} values of 5.13 ± 0.08 and 4.39 ± 0.03 μ M/s for tamoxifen-treated and untreated cells respectively. Values are means $\pm$ S.E.M. for triplicate determinations. Data are representative of three separate experiments. The inset shows the Eadie–Hofstee plot of the results.

Reversal of tamoxifen's effect on growth and NBMPR binding by oestradiol

MCF-7 cell growth increased with increasing concentrations of oestradiol from 0 to 3000 nM in the presence of 10 μ M tamoxifen (Table 2). It is evident that oestradiol concentrations between 10 and 3000 nM were capable of reversing the anti-proliferative effect of tamoxifen. Cell density was increased in the presence of 100 nM oestradiol, to that of the tamoxifen-untreated control. A similar profile was observed with changes in the number of NBMPR-binding sites in the presence of various concentrations of oestradiol. Apparently, 100 nM oestradiol was sufficient to reverse the decrease in NBMPR-binding sites resulting from treatment with tamoxifen, almost to that of the tamoxifen-untreated control. Tamoxifen-untreated MCF-7 cells did not respond in terms of growth and NBMPR binding to the same concentration range of oestradiol (results not shown).

Table 2 Reversal of effect of tamoxifen on growth and NBMPR binding by oestradiol

MCF-7 cells were cultured in six-well plates at an initial density of 4×10^3 cells/ml for 2 days, after which 10 μ M tamoxifen and graded concentrations of oestradiol were added. After 5 days of incubation, the cell density and the number of specific NBMPR-binding sites were measured as described in the Experimental section. The numbers of NBMPR-binding sites were determined with a single concentration of [³H]NBMPR (5 nM). Control cells were treated with neither tamoxifen nor oestradiol. Values are means $\pm$ S.E.M. for six independent determinations. ** $P < 0.01$; *** $P < 0.005$ with respect to the tamoxifen-treated control; where not indicated, the difference between the test value and the tamoxifen-treated control was statistically insignificant ($P > 0.05$).

Tamoxifen	Oestradiol (nM)	$10^{-3} \times$ Growth (cells/ml)	$10^{-3} \times$ NBMPR binding (sites/ml)
Present	0	48 ± 7	134 ± 27
	1	88 ± 24	153 ± 35
	10	$130 \pm 23^{***}$	$197 \pm 54^{***}$
	100	$133 \pm 20^{***}$	$261 \pm 51^{***}$
	1000	$110 \pm 22^{***}$	$246 \pm 40^{***}$
	3000	$112 \pm 23^{***}$	$279 \pm 90^{***}$
Absent	0	$134 \pm 50^{**}$	$245 \pm 16^{***}$

DISCUSSION

In the present study, nucleoside transport activities in MCF-7 cells have been characterized. These cells do not possess a functional Na⁺-dependent uridine transport system. The only carrier-mediated uridine transport detectable seemed to be an equilibrative system. This system was resolvable into two unique components distinguishable by their sensitivity towards nucleoside transport inhibitors such as NBMPR, dipyrindamole and dilazep. Approx. 70 % of the total uridine transport activity was inhibited by NBMPR, dipyrindamole and dilazep, with IC_{50} values of 0.1, 1 and 0.1 nM respectively. This proportion of transport activity is categorized as *es* transport. No further inhibition was observed until inhibitor concentrations exceeded 0.1, 3 and 0.1 μ M for NBMPR, dipyrindamole and dilazep respectively. This next portion of transport activity is categorized as *ei* transport. Similar biphasic inhibition curves of uridine transport for these inhibitors have also previously been observed in human HL-60 leukaemia cells [4], murine Sp2/0-Ag14 myeloma [13], murine L1210 leukaemia cells [14] and guinea-pig synaptosomes [15]. However, the inhibition curves for dipyrindamole in others such as the rat synaptosomes [9], Chinese hamster ovary cells, murine P388 leukaemia cells and a few other mammalian cell lines [16] were monophasic, even though the inhibitions by NBMPR were biphasic. These results suggest that the two forms of equilibrative nucleoside transporters distinguishable with NBMPR are probably not distinguishable with dipyrindamole in some cells and tissues in certain species.

The substrate specificities of the *es* and *ei* components in MCF-7 cells were distinctly different. Apparently the *es* component preferred purine nucleosides (K_i from 80 to 345 μ M) as substrates more than pyrimidine nucleosides (K_i from 495 to 660 μ M). The K_i values obtained were very similar to those reported for HL-60 cells [4], where K_i values of purine nucleosides in inhibiting uridine uptake ranged from 170 to 700 μ M and those for pyrimidine nucleosides from 510 to 1430 μ M. In murine Sp2/0-Ag14 myeloma cells [13], however, there was no distinct preference of the *es* component for either purine or pyrimidine nucleosides. In contrast, the *ei* component in MCF-7 cells seemed more sensitive to nucleosides without an amine side group, such as inosine and thymidine. This is not the case in guinea-pig

synaptosomes [15], which showed that the transport affinities for most physiological nucleosides were generally higher for the *es* than for the *ei* component. Nevertheless, the present observation is similar to that in HL-60 cells [4] and murine Sp2/0-Ag14 myeloma cells [13], except that adenosine is a good substrate for the *ei* component in HL-60 and Sp2/0-Ag14 cells.

The presence of the *es* component in MCF-7 cells enabled us to monitor and compare its changes in these cells with the high-affinity [³H]NBMPR-binding assay. During prolonged exposure to tamoxifen, an anti-proliferative effect was observed in MCF-7 cells as expected. The approx. 60% decrease in MCF-7 cell growth observed is an improvement over those previously reported, where a 40–55% decrease in growth was observed with a similar concentration of tamoxifen [12,17–19], albeit under slightly different cell culture conditions [5% (v/v) dextran-charcoal-treated FBS instead of the 10% (v/v) native FBS used in this study]. Concurrent with retardation in cell growth, the total number of NBMPR-binding sites decreased by 44%. The $V_{\max}$ of *es* transport of uridine in these cells also decreased by 61% with relatively unaltered uridine transport affinity and NBMPR-binding affinity. Tamoxifen has been shown to decrease membrane fluidity in MCF-7 cells. However, the effect on *es* transporter was not a non-specific membrane phenomenon, because the *ei* component of uridine transport remained unaffected by tamoxifen. Moreover, Trypan Blue exclusion assays of tamoxifen-treated cells consistently revealed a cell viability above 90%. Control experiments also showed that the vehicle (0.03% DMSO) did not contribute any apparent effects to cell growth. In our recent paper, tamoxifen was found to inhibit uridine uptake and NBMPR binding [20]. However, this does not pose as a problem in this study. Our control experiments have shown that the cell-washing method adopted and described in the Experimental section was effective. Hence there was no significant amount of tamoxifen left from the culture medium to interfere with NBMPR binding or uridine uptake.

An almost completely reversed condition of sustained growth was observed in tamoxifen-treated MCF-7 cells exposed to 100 nM oestradiol. Cell growth was increased from 48000 to 133000 cells/ml, close to the control value of 134000 cells/ml. Other oestradiol-reversible effects of tamoxifen have been documented earlier in tamoxifen's inhibition of cell growth [12,19,21], although some of the reversals were partial, in contrast with this study. By using an oestrogen receptor enzymic immunoassay, we have found that incubation of MCF-7 cells with 10 μ M tamoxifen for 5 days led to a 1.3-fold increase in the number of oestrogen receptors per cell compared with untreated control cells (results not shown). Hence the cell model that we were working with has increased the number of available oestrogen receptors in the presence of tamoxifen. This might perhaps be an attempt to

compensate for the inhibitory effects of tamoxifen. With oestradiol counteracting tamoxifen's anti-proliferative effect, the number of NBMPR-binding sites also reverted from 134000 to 261000 sites per cell, close to the control level of 245000 sites per cell. This positive correlation of NBMPR-sensitive nucleoside transporter proteins with proliferation has also been observed in HL-60 cells induced to differentiate [3–5]. Further supportive evidence of this positive correlation can be found in our recent studies where, *in vivo*, the numbers of NBMPR binding sites were found to be significantly higher in freshly harvested human breast, colorectal, liver and stomach tumour tissues than in their normal counterparts from the same patient [22]. In conclusion, the decrease in *es* nucleoside transporter proteins in tamoxifen-treated MCF-7 cells was found to occur via a mechanism that can be interfered with by oestradiol.

This work was supported by the National Medical Research Council research grant RP950392/N.

REFERENCES

- 1 Belt, J. A. (1983) *Mol. Pharmacol.* **24**, 479–484
- 2 Darnowski, J. W. and Handschumacher, R. E. (1986) *Cancer Res.* **46**, 3490–3494.
- 3 Lee, C. W. (1994) *Biochem. J.* **300**, 407–412
- 4 Lee, C. W., Sokoloski, J. A., Sartorelli, A. C. and Handschumacher, R. E. (1991) *Biochem. J.* **274**, 85–90
- 5 Lee, C. W., Sokoloski, J. A., Sartorelli, A. C. and Handschumacher, R. E. (1994) *In Vivo* **8**, 795–802
- 6 Goh, L. B., Sokoloski, J. A., Sartorelli, A. C. and Lee, C. W. (1993) *Biochem. J.* **294**, 693–697
- 7 Liu, M. P. and Handschumacher, R. E. (1995) *Cancer J.* **1**, 210–214
- 8 Goh, L. B. and Lee, C. W. (1996) *Proc. Am. Assoc. Cancer Res.* **37**, 498
- 9 Lee, C. W. and Jarvis, S. M. (1988) *Biochem. J.* **249**, 557–564
- 10 Leatherbarrow, R. J. (1987) *ENZFIT*, Elsevier Biosoft, Cambridge
- 11 Berthois, Y., Katzenellenbogen, J. A. and Katzenellenbogen, B. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2496–2500
- 12 Reddel, R. R., Murphy, L. C. and Sutherland, R. L. (1983) *Cancer Res.* **43**, 4618–4624
- 13 Lee, C. W., Goh, L. B. and Tu, Y. (1995) *Biochim. Biophys. Acta* **1268**, 2200–2208
- 14 Belt, J. A. (1983) *Biochem. Biophys. Res. Commun.* **110**, 417–423
- 15 Lee, C. W. and Jarvis, S. M. (1988) *Neurochem. Int.* **12**, 483–492
- 16 Plagemann, P. G. W. and Wohlhueter, R. M. (1984) *Biochim. Biophys. Acta* **773**, 39–52
- 17 Lippman, M., Bolan, G. and Huff, K. (1976) *Cancer Res.* **36**, 4595–4601
- 18 Pasqualini, J. R., Sumida, C. and Giambiagi, N. (1988) *J. Steroid Biochem.* **31**, 613–643
- 19 Coezy, E., Borgna, J. L. and Rochefort, H. (1982) *Cancer Res.* **42**, 317–323
- 20 Cai, J. and Lee, C. W. (1996) *Biochem. J.* **320**, 991–995
- 21 Vignon, F., Bouton, M. M. and Rochefort, H. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1502–1508
- 22 Goh, L. B., Mack, P. and Lee, C. W. (1995) *Anticancer Res.* **15**, 2575–2580