Interactions of ATP, oestradiol, genistein and the anti-oestrogens, faslodex (ICI 182780) and tamoxifen, with the human erythrocyte glucose transporter, GLUT1

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INTRODUCTION

A number of phytooestrogens inhibit glucose transport; in particular the isoflavone genistein, which is present in large amounts in diets containing soya-bean extracts [1]. Genistein inhibits glucose transport directly by interacting with GLUT1, the glucose transporter protein found abundantly in human erythrocytes. It competitively antagonizes oestradiol, genistein and cytochalasin B (CB)-dependent inhibitions of glucose exit, \( K_{i(ATP/ED)} = 2.5 \pm 0.23 \text{ mM}, K_{i(ATP/GEN)} = 0.99 \pm 0.17 \text{ mM} \) and \( K_{i(ATP/CB)} = 0.76 \pm 0.08 \text{ mM} \). Tamoxifen and faslodex reverse oestradiol-dependent inhibition of glucose exit with ATP > 1 mM \( K_{i(ED/TAM)} = 130 \pm 5 \text{ nM} \) and \( K_{i(ED/FAS)} = 2.7 \pm 0.9 \mu\text{M} \). The cytoplasmic surface of the glucose transporter (GLUT1) contains four sequences with close homologies to sequences in the ligand-binding domain of human oestrogen receptor \( \beta \) (hers-2). One homology is adjacent to the Walker ATP-binding motif II (GLUT1, residues 225–229) in the large cytoplasmic segment linking transmembrane helices 6 and 7; another GLUT (residues 421–423) contains the Walker ATP-binding motif III. Mapping of these regions on to a three-dimensional template of GLUT indicates that a possible oestrooestrogen-binding site lies between His\(^{357}\), Arg\(^{349}\) and Glu\(^{449}\) at the cytoplasmic entrance to the hydrophobic pore spanning GLUT, which have a similar topology to His\(^{357}\), Glu\(^{365}\) and Arg\(^{368}\) in hers-2 that anchor the head and tail hydroxy groups of oestradiol and genistein, and thus are suitably placed to provide an ATP-sensitive oestrogen binding site that could modulate glucose export.

Key words: ATP, faslodex, GLUT1 structure, oestradiol, tamoxifen.
stimulation of DNA synthesis. High concentrations of genistein inhibited DNA synthesis [18]. Genistein also inhibits the growth of human prostatic carcinoma cells LNCaP and PC-3 cells, but leaves tyrosine kinase activity unaffected [19].

Because phytoestrogens and selective oestrogen receptor modulators (SERMs) may affect tumour growth and metabolism at genomic and non-genomic levels, it is important to isolate the non-genomic effects on cell transport systems. The effects of these drugs on human erythrocyte glucose transport are a simple and unambiguous means of assessing some of their non-genomic interactions on transport. For this reason the effects of two SERMs, e.g. 4-0H-tamoxifen and faslodex on inhibition of glucose transport by the oestradiol, genistein, and also ATP and cytochalasin B in erythrocytes have been examined in the present study.

There have been no previous reports of direct non-genomic effects of the pure anti-oestrogen faslodex [20] or tamoxifen on glucose transport, although there is some suggestion that these drugs may affect glucose metabolism in mammary adenoma cells [21]. The effects of the steroids and steroid inhibitors (see Figure 1) on net glucose exit from human erythrocytes at 25 °C were monitored using a modified form of the light-scattering method developed by Sen and Widdas [22].

EXPERIMENTAL

Solutions

The suspension medium was isotonic PBS, adjusted to pH 7.4 (Sigma, Poole, Dorset, U.K.). D-Glucose, genistein, phloretin, 4-0H-tamoxifen and cytochalasin B were obtained from Sigma. Faslodex was kindly donated by Dr Frances Sutcliffe (R and D Division, AstraZeneca, Alderley Park, Alderley Edge, Cheshire, U.K.).

Cells

Fresh human erythrocytes were obtained by venipuncture, and washed three times in isotonic saline by repeated centrifugation and resuspension. The cells were then suspended in solutions containing sugars at the preloading concentration, usually 100 mM, final haematocrit 10%. The cells were incubated for at least 2 h, in the case of D-glucose. The cells were then re-centrifuged to obtain a thick suspension, approx. 95% haematocrit. This was kept at 4 °C until required. Aliquots of pre-warmed cells suspension (7.5 μl) were added to a 1 cm³ fluorescence cuvette containing 3 ml of saline solution, which had been pre-warmed to the required temperature (25 °C). The cell suspensions were mixed vigorously and photometric monitoring was started within 5 s of mixing. The final glucose concentration in nominally glucose-free solution with this regime was maximally 0.25 mM, which was at least 10-fold less than the lowest Km of D-glucose measured here: contamination of the external solution with glucose had a negligible effect on D-glucose exit.

Ghosts

Resealed ghosts were prepared according to the method described by Bjerrum [23] with a few modifications. Briefly, freshly drawn human blood was washed three times in isotonic saline, and cells were lysed in ice-cold haemolysing medium (pH 3.6). Prior to rescaling, the ghost suspension was loaded with the appropriate reconstitution medium (pH 10.5) by adding 1 ml of reconstitution medium per 1 ml of ghost suspension and was left at 0 °C for 10 min. The reconstitution medium consisted of KCl, ATP (concentrations as required) and 25 mM Tris. The ghosts were then rescaled by incubating at 38 °C for a period of 45 min. Finally, the rescaled ghosts were washed twice in the appropriate wash medium (pH 7.4) containing equimolar concentrations of ATP and NaCl, as the reconstitution medium, and also 100 mM glucose.

Photometric monitoring: glucose exit

This method is adapted from that introduced by Sen and Widdas [22], as described previously [24,25]. The exit rates of D-glucose from cells were monitored photometrically, using a Hitachi 2000-F fluorescence spectrometer with a temperature-controlled and monitored cuvette; Em = Eem = 650 nm. The output was recorded and stored directly using a MacLab 2e (AD Instruments). Data were collected at a rate of 0.33–5 points s⁻¹, depending on the time course of exit; each run consisted of 200–2000 data points. The light scattering response was found to be approximately linear for osmotic perturbations ±50 mM NaCl above or below the normal isotonic saline solution. With glucose present in the cytosol, after the initial 2–3 s period of cell swelling due to osmotic equilibration, the rate of shrinkage was a linear function of the rate of glucose loss from the cells. When glucose transport was abolished with high concentrations of transport inhibitors, e.g. cytochalasin B or phloretin, no change in light scattering after the initial osmotic equilibration occurred. The initial amplitude of the light scattering signal is proportionately reduced by raising [glucose] in the external solution, or reducing [glucose] in the loading solution. In the absence of a glucose concentration gradient no light scattering change occurs.

The time courses of D-glucose exit were fitted to monoeponential curves of the form:

\[ y_i = A[1 - B \cdot \exp(C \cdot t)] \]

using Kaleidagraph 3.51 (Synergy Software). The voltage, y, is recorded at time, t; the coefficient, A, is a scaling factor that fits the curves to the voltage signal, B and C are the exponential coefficients and t is the time in s at which the observed data y were obtained. These fits gave correlation coefficients, r > 0.98, and S.E.M. of the rate coefficients.

Statistics

All the probabilities were estimated from two-tailed Student’s t values for unpaired means, even although the data were always matched.

The Ki values for direct inhibitors of glucose exit were obtained by non-linear regression of the change in the exponential exit rate of glucose exit, C against the inhibitor concentration [I] using the following equation:

\[ y = V_{\text{max}} \cdot K_i/(K_i + [I]) \]

where K is the inhibitor concentration giving 50% decrease of the rate of exit obtained in the absence of inhibitor. The regression coefficient is expressed as a mean ± S.E.M. Each Ki plot was obtained from the means of glucose exit rates at least 3–4 inhibitor concentrations, i.e. typically 16–20 glucose exit rates were determined per estimate of each Ki. Each Ki estimate was repeated 3–4 times.
The $K_i$ values for indirect inhibition, e.g. effect of faslodex on oestradiol, are obtained by linear regression of the apparent $K_i$ values against the inhibitor concentration $[I]$:

$$K_{app} = K_i(I + [I]/K_{mi})$$

It follows that $K_{mi}$ is the concentration of modulator, e.g. faslodex (FAS), required to raise the $K_i$ of the primary inhibitor, e.g. $K_i$ [oestradiol (ED)] by a factor of 2. Thus $K_{mi}$ is equivalent to $K_{i(ED/FAS)}$ for the modulator (faslodex) antagonizing the primary inhibition of oestradiol. This is obtained from (intercept/slope) $\pm$ S.E.M. of the linear regression line of $K_{app}$ versus $[I]$. Each secondary $K_i$ is estimated from replicates of 80–100 glucose exits. Where activation occurs, e.g. with faslodex modulation of cytochalasin B in the presence of ATP, the $K_{mi}$ is obtained in the same way as the $K_i$, but $K_{app}$ is the $[I]$ causing a decrease in $K_i$ by 50%.

The rates of glucose exit were examined without pre-incubation of the cells or ghosts with the inhibitors. These were present only in the incubation solutions during zero-trans glucose exit.

**RESULTS**

**Effects of genistein, oestradiol, cytochalasin B, faslodex and tamoxifen on glucose exit from human erythrocytes at 25 °C**

Both oestradiol and genistein inhibited the rate of glucose exit into glucose-free solutions with similar $K_i$s: oestradiol $K_{i(ED)} = 4.71 \pm 0.70 \mu M$ and genistein $K_{i(GEN)} = 4.24 \pm 0.83 \mu M$ (Figure 1, Table 1). This inhibitory effect due to the action of a single modulator is termed a direct effect here. Neither genistein nor oestradiol had any significant effect on the glucose or phloretin affinity for the external site (results not shown).

The effects of the SERMs, tamoxifen, faslodex and the specific inhibitor of glucose transport, cytochalasin B on net glucose exit into glucose-free solutions are shown in (Figures 2A and 1 respectively, and Tables 1 and 2). The pure anti-oestrogen, faslodex, had no direct effect on net glucose exit, whereas tamoxifen had no direct effect on net glucose exit, whereas tamoxifen directly inhibited the rate of glucose exit with a $K_{i(TAM)} = 0.3 \pm 0.1 \mu M$.

Tamoxifen, unlike faslodex, also had a small effect on the affinity of glucose at the external site (Figures 2A and 2B). Cytochalasin B reduced the rate of glucose exit from the cells with a $K_{i(CB)} = 0.46 \pm 0.09 \mu M$ (Table 2), and had no effect on the affinity of glucose for the external site (results not shown).

**Effects of faslodex and tamoxifen on the inhibition of glucose exit by cytochalasin B or phloretin**

Faslodex antagonized the inhibitory effect of cytochalasin B with a $K_{i(CB/FAS)} = 5.8 \pm 1.4 \mu M$ in cells, whereas it had no significant effect on phloretin action (Figures 3A and 3B, Table 2). Thus, faslodex had a very asymmetric action, acting exclusively at the inner face of the transporter (see the Discussion section).

Tamoxifen differs from faslodex in that, in whole cells or ghosts, it had no competitive effect on either cytochalasin B or phloretin-dependent inhibition of glucose exit (Table 2).

**Indirect effects of faslodex and tamoxifen of the inhibition of glucose exit by oestradiol and genistein**

Tamoxifen and faslodex each competitively inhibited oestradiol and genistein-dependent inhibition of glucose exit, as was evident from the linear increases in the apparent $K_i$s of oestradiol, $K_{i(ED)}$, and genistein-dependent inhibition $K_{i(GEN)}$ of glucose exit with increasing concentrations of the anti-oestrogens (results not shown). The $K_{i(GEN/FAS)}$ of faslodex against oestradiol was $2.84 \pm 0.16 \mu M$ and against genistein was $K_{i(GEN/FAS)} = 1.9 \pm 0.3 \mu M$ (Table 1), whereas the $K_i$ of tamoxifen against genistein was $K_{i(GEN/TAM)} = 0.35 \pm 0.02 \mu M$ and against oestradiol $K_{i(ED/TAM)} = 0.100 \pm 0.002 \mu M$ (Table 1).

**Effects of varying intracellular [ATP] on cytochalasin B and phloretin-dependent inhibition of glucose exit in human erythrocytes ghosts**

Glucose transport in human erythrocyte ghosts, prepared as described in the Experimental section, differed only slightly from whole-cell glucose transport. The photometric method worked equally well with ghosts as with whole cells. However, ATP raises the $V_{max}$ for zero-trans net glucose exit in ghosts [9]. This necessitated using a lower temperature, 20 °C, rather than 25 °C with cells.

Raising intracellular [ATP] in ghosts from 0–2 mM increased the apparent $K_i$ of cytochalasin B inhibition of glucose exit from $K_{i(CB)} = 0.18 \pm 0.04 \mu M$ to 1.12 $\pm 0.07 \mu M$. There was no significant alteration in the $V_{max}$ of glucose exit. A replot of the apparent $K_{i(CB)}$ of cytochalasin B inhibition of glucose exit gave a $K_{i(CB/ATP)} = 0.76 \pm 0.08 \mu M$ for the ATP-dependent reversal of cytochalasin B inhibition of glucose exit (Figures 4A and 4B, Table 2).

Increasing intracellular [ATP] in ghosts also had an effect on phloretin-dependent inhibition of glucose exit. The $K_i$ of phloretin increased from $K_{i(PH)} = 0.13 Your content is now accurately converted into a plain text representation. The image content is not necessary for the text as it contains only graphical data.
Table 1  Effects of the oestrogen 17β-oestradiol, the isoflavone genistein and the anti-oestrogens tamoxifen and faslodex on glucose exit from erythrocytes or on glucose exit from erythrocyte ghosts with or without ATP

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Modulator</th>
<th>Effect on erythrocytes</th>
<th>Comment on cell fluxes</th>
<th>Effect on erythrocyte ghosts (ATP &lt; 0.2 mM)</th>
<th>Effect on erythrocyte ghosts (ATP = 1–4 mM, $K_i$(ATP))</th>
<th>Comment on erythrocyte ghost fluxes</th>
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<tr>
<td>Oestradiol</td>
<td>—</td>
<td>$K_i$(ED) = 4.71 ± 0.70 μM</td>
<td>Oestradiol is a competitive inhibitor of glucose exit</td>
<td>$K_i$(ED) = 6.6 ± 0.85 μM</td>
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<td>Oestradiol</td>
<td>Tamoxifen</td>
<td>$K_i$(ED/TAM) = 0.100 ± 0.002 μM</td>
<td>Tamoxifen is a competitive antagonist of oestradiol inhibition of glucose exit</td>
<td>ATP = 0 mM, tamoxifen has no effect on oestradiol inhibition of glucose exit from ghosts</td>
<td>$K_i$(ED/TAM) = 0.130 ± 0.005 μM</td>
<td>Tamoxifen is a competitive antagonist of oestradiol inhibition of glucose exit only with [ATP] &gt; 0.1 mM</td>
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<td>Oestradiol</td>
<td>Faslodex</td>
<td>$K_i$(ED/FAS) = 2.64 ± 0.16 μM</td>
<td>Faslodex is a competitive antagonist of oestradiol inhibition of glucose exit</td>
<td>0 mM ATP, faslodex has no effect on oestradiol inhibition of glucose exit from ghosts</td>
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<td>Oestradiol</td>
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<td>$K_i$(ED/TAM) = 2.5 ± 0.23 mM</td>
<td>ATP is a competitive antagonist of oestradiol inhibition of glucose exit only with [ATP] = 2 mM</td>
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<td>Genistein</td>
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<td>$K_i$(GEN) = 4.2 ± 0.83 μM</td>
<td>Genistein is a competitive inhibitor of glucose exit</td>
<td>ATP = 0 mM, no effect of genistein</td>
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<td>Genistein</td>
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<td>$K_i$(GEN) = 3.2 ± 0.19 μM</td>
<td>Genistein only inhibits with ATP present</td>
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<td>Genistein</td>
<td>Faslodex</td>
<td>$K_i$(GEN/FAS) = 1.9 ± 0.3 μM</td>
<td>Faslodex is a competitive antagonist of genistein inhibition of glucose exit from cells</td>
<td>ATP = 0 mM, no effect of faslodex</td>
<td>ATP = 2–4 mM, $K_i$(GEN/FAS) = 14.9 ± 2.7 μM</td>
<td>Genistein interaction with the site of glucose export (note difference between cells and ghosts)</td>
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<td>Genistein</td>
<td>Tamoxifen</td>
<td>$K_i$(GEN/TAM) = 0.35 ± 0.02 μM</td>
<td>Tamoxifen competitively antagonizes genistein inhibition of glucose exit</td>
<td>No effect</td>
<td>ATP = 2 mM, no effect of tamoxifen</td>
<td>Tamoxifen does not alter genistein inhibition of glucose exit from ghosts in contrast with cells</td>
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<td>Tamoxifen</td>
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<td>$K_i$(TAM) = 0.3 ± 0.14 μM</td>
<td>Tamoxifen inhibits glucose exit</td>
<td>$K_i$(TAM) = 0.08 ± 0.01 μM</td>
<td>ATP = 2 mM, $K_i$(TAM) = 0.10 ± 0.01 μM</td>
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<td>No effect</td>
<td>Faslodex has no direct effect on glucose exit</td>
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Anti-oestrogenic actions of faslodex and tamoxifen on glucose transport

Table 2

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Inhibitor: Cytochalasin B

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Figure 2

Comparison of direct inhibitions of glucose exit from erythrocytes by (A) tamoxifen and faslodex, and (B) phloretin

(A) The rates of glucose exit were estimated by exponential fitting and then the inhibitions were fitted to the equation

\[ y = V_{\text{max}} \left( \frac{I}{K_i + I} \right) \]

The \( K_i(\text{TAM}) \) of tamoxifen is \( 0.3 \pm 0.14 \mu M \), and \( K_i(\text{FAS}) \) of faslodex is not significant. \( K_i(\text{PHL/ATP}) \) of ATP-dependent antagonism of the oestradiol effect is \( 2.5 \pm 0.23 \mu M \) (Table 1). Varying \[ \text{[ATP]} \] from 0–4 mM altered the \( K_i(\text{GEN}) \) of genistein on zero-trans exit of glucose from human erythrocyte ghosts at 20°C. With \[ \text{[ATP]} \] = 0 mM, the \( K_i(\text{GEN}) \) is \( 3.30 \pm 0.44 \mu M \), and with \[ \text{[ATP]} \] = 4 mM, the \( K_i(\text{GEN}) \) is \( 15.3 \pm 4.9 \mu M \). The \( K_i(\text{GEN/ATP}) \) of [ATP] for counteracting genistein inhibition with glucose transport is \( 0.99 \pm 0.17 \mu M \) (Figures 5A and 5B).
Effects of faslodex on oestradiol-dependent inhibition of glucose exit from human erythrocyte ghosts

As in whole erythrocytes, faslodex in the range 0–5 μM, had no direct effect on zero-trans exit of glucose from human erythrocyte ghosts (Figure 6). In the absence of ATP, faslodex had zero effect on oestradiol-dependent inhibition. However, when ATP was raised to 2 mM, faslodex raised the apparent $K_i$ of oestradiol for inhibition of glucose exit. This effect of faslodex on oestradiol-dependent inhibition in ghosts was identical to the effects of faslodex on oestradiol-dependent inhibition in whole erythrocytes, $(K_i^{(RED/FAS)} = 2.7 ± 0.9 \mu M$ of faslodex with ATP = 2 mM, Table 1).

Effects of faslodex on cytochalasin B and genistein-dependent inhibition of glucose exit from human erythrocyte ghosts at 20 °C

In the presence of 2 mM ATP in ghosts, faslodex reduced the $K_i^{(CB)}$ of cytochalasin B inhibition, i.e. increased the affinity of cytochalasin B, of glucose from $K_i^{(CB)} = 1.13 ± 0.46 \mu M$ with faslodex = 0 μM, to $K_i^{(CB)} = 0.60 ± 0.12 \mu M$ with faslodex = 5 μM (Table 2). With lower concentrations of ATP, the faslodex induced proportionally smaller effects on the $K_i^{(CB)}$ and in the absence of ATP, faslodex had a negligible effect on $K_i^{(CB)}$ (Table 2).

Faslodex also enhanced the inhibitory effects of genistein on glucose exit from ghosts in the presence 2 mM ATP, $K_i^{(GEN)} = 8.8 ± 0.75 \mu M$, faslodex = 0 μM, to $K_i^{(GEN)} = 3.1 ± 0.62 \mu M$ with faslodex = 10 μM (Figure 7A). However, in the absence of ATP, faslodex had a negligible effect on the $K_i^{(GEN)}$ (Figure 7B). These results confirm those in Table 2 showing that there is, in ghosts, an ATP-dependent increase in the apparent affinity of cytochalasin B-dependent inhibition induced by faslodex.
Anti-oestrogenic actions of faslodex and tamoxifen on glucose transport

Figure 5 Effects of ATP on genistein-dependent inhibition of glucose exit from human erythrocyte ghosts

(A) The effects of various concentration of genistein on ATP = 0 mM, $K_{i(GEN)} = 3.30 \pm 0.45$ μM; $K_{i(GEN)} = 5.83 \pm 0.29$ μM, ATP = 1 mM; $K_{i(GEN)} = 8.4 \pm 1.71$ μM, ATP = 2 mM; $K_{i(GEN)} = 15.32 \pm 4.93$ μM, ATP = 2 mM. (B) Replot of effects of ATP on $K_{i(GEN)} K_{i(GEN/ATP)} = 0.99 \pm 0.17$ mM.

Effects of tamoxifen on oestradiol-dependent inhibition of glucose exit from erythrocyte ghosts in the absence or presence of ATP (2 mM)

In the absence of ATP, tamoxifen is without effect on oestradiol-dependent inhibition of glucose exit from erythrocyte ghosts (Table 1). However, with ATP = 2 mM, there was a concentration-dependent increase in the $K_{i(ED)}$ of oestradiol with increasing tamoxifen concentrations. Thus, like the actions of faslodex, tamoxifen’s action was dependent on the presence of ATP; however, tamoxifen, unlike faslodex, is ineffective in inhibiting cytochalasin B, or genistein-dependent action on glucose exit in erythrocyte ghosts.

Absence of effect of tamoxifen on genistein-dependent inhibition of glucose exit from erythrocyte ghosts

Tamoxifen in the range 0.5–1 μM had no significant effect on the inhibition of glucose exit by genistein from erythrocyte ghosts (Table 1) in the presence (2 mM) or absence of ATP. This result contrasts with its strong effect on genistein-dependent inhibition of glucose exit from whole cells, and suggests that some component is lost or changed during erythrocyte-ghost preparation. This component enables tamoxifen to inhibit genistein interactions with GLUT1 in whole cells.

DISCUSSION

Polarized action of oestrogens, phytoestrogens and anti-oestrogens

Because membrane transporters have two sides at which ligands can bind, they can have more complex kinetics than an enzyme that is exposed to a uniform distribution of ligands. Cytochalasin B behaves as a competitive inhibitor to glucose exit from cells and as a non-competitive inhibitor of glucose uptake; it does not alter the affinity of glucose or ligands at the external site, although it inhibits the maximal rate of glucose entry [6–8,26,27].
genistein and faslodex with ATP

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These results indicate that tamoxifen and faslodex may have different modes or sites of action at the internal site of the glucose transporter (see below).

Faslodex competitively antagonizes oestradiol, genistein and cytochalasin B inhibition of glucose exit from cells, indicating that it is a competitive inhibitor of steroid binding at the internal site of the glucose transporter. Corroboration of this view that faslodex and tamoxifen act exclusively on the inside of GLUT is that no significant antagonism occurs between either faslodex or tamoxifen and phloretin at the external surface.

This view is further supported by the finding that intracellular ATP modulates the effects of faslodex on cytochalasin B-, oestradiol- and genistein-dependent inhibition of glucose exit from ghosts (see below). Similarly, it is evident that tamoxifen acts mainly at the internal site of GLUT, since it competitively antagonizes oestradiol- and genistein-dependent inhibition of glucose exit from cells and from ghosts, when ATP > 1 mM is present (Tables 1 and 2).

Effects of ATP on modulator action on glucose transport

Metabolic depletion of intracellular erythrocyte ATP has been reported to slow net glucose exit [9,13] from human erythrocytes and to increase the affinity of cytochalasin B [14]. Raised intracellular ATP in human erythrocytes ghosts has been shown to increase the amount of 3-O-methyl-d-glucoside bound to GLUT1. This ATP-dependent ‘occlusion’ of 3-O-methyl-d-glucoside binding suggests that ATP binding alters the conformation of the internal binding site or some modifier site of GLUT1 [13]. Tyrosine-kinase inhibitors, e.g. genistein, that inhibit ATP activation of tyrosine kinase, also inhibit glucose transport across GLUT1 in a number of different cell types, including human erythrocytes, and also inhibit cytochalasin B binding to GLUT1 [5].

The results of the present study indicate that binding of the steroid ligands at the internal site of GLUT is modified by ATP. Increasing [ATP] from 0–4 mM reduces the affinity of oestradiol, genistein and cytochalasin B (Tables 1 and 2). The ATP-dependent decrease in oestradiol affinity is synergized by faslodex, whereas faslodex antagonizes the ATP-dependent decreases in affinities of cytochalasin B or genistein. In the absence of ATP, there is no effect of faslodex on steroid (oestradiol or genistein) or cytochalasin B-dependent inhibition of glucose exit. Also, in the absence of ATP, tamoxifen is without effect on oestradiol-dependent inhibition of glucose exit. However, tamoxifen on its own still acts as an inhibitor of glucose exit and is unaffected by an [ATP] of 0–4 mM. Tamoxifen is ineffective against cytochalasin B in either whole erythrocytes or ghosts; in ghosts, it is also ineffective against genistein-dependent inhibition of glucose exit in the absence or presence (2 mM) of ATP.

Differences between modulator effects in whole erythrocytes and ghosts

Although raising [ATP] seems to act qualitatively in the same way in both whole cells and ghosts in that it reduces the affinity of cytochalasin B [14], there are important quantitative differences. The $K_s$ of genistein, oestradiol and cytochalasin B are similar in ghosts (with ATP = 0 mM) and in fresh whole

Phloretin, an inhibitor that binds competitively to the external site of the glucose transporter [28], reduces the apparent affinity of glucose and other ligand binding to the external site of the transporter, without affecting the maximal rate of glucose uptake. It also reduces the maximal rate of glucose exit without affecting the affinity of ligands at the inside site.

Since cytochalasin B binds to the inside and phloretin to the outside of the glucose transporter, exploring the effects of oestrogen antagonists, such as faslodex or tamoxifen, on the inhibitor effects of cytochalasin B and phloretin on glucose transport may give some insight on the site and mode of action of these anti-oestrogens on glucose transport.

The evidence from the Results section shows that both faslodex and tamoxifen act exclusively on the inside of GLUT. However, faslodex is a much more specific antagonist of oestradiol-dependent inhibition than tamoxifen. Faslodex on its own does not inhibit glucose transport, whereas tamoxifen does. However, tamoxifen has a 20–30-fold higher potency on oestradiol-dependent inhibition of glucose exit than faslodex (Table 1).
cells, where the ATP 1–2 mM [14]. However, raising ATP in ghosts to 2–4 mM increases the $K_s$ of genistein-, oestradiol- or cytochalasin-dependent inhibition of glucose exit by a factor of 3–4-fold (Table 1). Furthermore, faslodex or tamoxifen act differently on glucose transport in whole cells from ghosts (with ATP present). In ghosts with ATP, faslodex increases the affinity of genistein and cytochalasin B; whereas in fresh whole cells, faslodex reduces the affinity of these ligands. Similarly, tamoxifen reduces the affinity of genistein in cells, but does not alter the affinity of either cytochalasin B or genistein in ghosts in the absence or presence of ATP.

A number of ATP-binding proteins providing linkages between GLUT and the cytoskeleton are disrupted during ghosting [29–31]. This alteration in the local environment of GLUT may provide the basis of the differences between the effects of faslodex and ATP on cytochalasin B, and genistein interactions with glucose transport, in ghosts and whole cells. These results also draw attention to the need for caution in interpreting ligand interactions on transporters or other proteins when studied in isolation from their natural environment.

**Kinetic schemes consistent with faslodex, ATP interactions with cytochalasin B or genistein inhibition of glucose exit from whole erythrocytes or ghosts**

The kinetic schemes and their simulations (results not shown) indicate that in ghosts (Scheme 1B) faslodex stabilizes steroid binding to the internal site of GLUT when ATP is bound; whereas, in whole cells (Scheme 1A) faslodex binding occludes the steroid-binding site, thereby preventing steroid or cytochalasin B inhibition of glucose exit (Schemes 1A and 1B). Faslodex may act by prevention of cytochalasin B binding, but without itself blocking the pore (Scheme 1C). In so doing, it also inhibits oestradiol binding and also, in whole cells at least, it competes with genistein. This scheme is consistent with the findings of May and Beecham [32] who suggest that cytochalasin B blocks entry of glucose to the hydrophilic pore at the cytoplasmic surface of the transporter.

Simulations of these kinetic schemes were used to obtain accurate matches to the observed data with ATP, faslodex and cytochalasin interactions with glucose export in both whole and ghost erythrocytes. These simulations were obtained using a fast modelling package Berkeley Madonna version 8.0.1 (http://www.berkeleymadonna.com/).

**Deductions on the structure–function relationships of ATP and oestrogen binding to GLUT1**

Several sequences in GLUT1 have close homology with an ATP-binding region in adenylate cyclase [11,12]. Three Walker ATP-binding domains that are found in a number of adenine-nucleotide-binding proteins, including glycogen phosphorylase [33,34], have been identified in GLUT1 [29]. Two of these GLUT1 Walker domains are likely to be the sites of high affinity-binding site for ATP, as they are subject to photo-phosphorylation with 8-azido-[gamma-32P]ATP [12].

Since ATP has been shown here to reduce the affinity of oestrogens and cytochalasin B at the glucose export site, we investigated whether there were any further homologies in GLUT, which might explain these interactions. Accordingly, we searched to see if there are any homologies within the ligand-binding domains (LBD) of esrs and GLUTs.

Using retrieval software at the HGMP (Hinxton Hall, Cambridge, U.K.), searches in SwissProt identified some

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**Scheme 1 How glucose, cytochalasin B, ATP and faslodex interact kinetically at the inside site of GLUT1**

(A) Kinetic scheme showing interactions between glucose, ATP, cytochalasin B and faslodex (ICI) and the internal binding site C_i of the glucose transporter of whole human erythrocytes. The carrier forms which permit glucose transport from inside to outside $C_{i}$ are $C_iGATP.C_i, GATP.C_i$ and $G.C_i$. ATP competitively inhibits cytochalasin B (CB), cytochalasin B binding and faslodex stabilizes the ATP-ligated forms. This Scheme illustrates the competitive interaction of faslodex with cytochalasin B. (B) Kinetic scheme showing interactions between glucose, ATP, faslodex and cytochalasin B at the internal site of erythrocyte ghosts. In this Scheme only the glucose and ATP-ligated forms of the transporter permit glucose transport. Neither cytochalasin B nor faslodex liganded-forms permit glucose transport. However, the carrier permits both faslodex and cytochalasin B to bind simultaneously, but ATP competes with both of these ligands. This Scheme accounts for the faslodex-dependent increase in cytochalasin B affinity in contrast with (A), which pertains in whole cells. (C) Simplified Scheme showing how faslodex (Fac) competes with oestradiol (ED) or genistein or cytochalasin B binding, and thereby permits glucose (Glc) transport, whereas oestradiol binding blocks the passage to glucose.
interesting homologies between sequences on the inside surface of GLUT1, including regions adjacent to Walker domains 2 and 3 in GLUT1, with sequences within the LBD of esr-β contiguous with the ligand, as identified from the following crystallographic structures. Bonding interactions of the ligands, oestriadiol, genistein, tamoxifen and raloxifene with the LBD of oestrogen receptors (α and β) were obtained from searches in the X-ray crystallography literature [35–37]. The documented three-dimensional peptide maps of the LBDs within a radius of 6 Å (1Å = 0.1 nm) to the head and tail groups of the ligand (A and D rings of oestradiol) were selected using Swiss-Pdb-Viewer (http://www.expasy.ch/spdbv/text/server.html) (SWISSPROT: GTR1_HUMAN Accession No P11166 Description GLUCOSE TRANSPORTER TYPE 1, ERYTHROCYTE/BRAIN, SWISSPROT: ESR2_HUMAN Accession No Q92731 Description OESTROGEN RECEPTOR BETA (ER-BETA)).

Other oestrogen receptors were also examined for homologies, but these searches are not discussed here. Sequence homologies between these selected sequences close to the oestrogen ligand of esr-2 were sought and obtained in GLUT1 as follows.

The 492 amino acids of GLUT1 were split into overlapping 20-mers, with an overlap of 10 sequence symbols. The program FASTA [38] was used to identify the partial matches between each of the 20-mers, and sequences in esr-β that were identified as being adjacent to the ligand binding cleft (Table 3). Matches between esr-2 and GLUT1 were evaluated by FASTA. Many matches were found, but the searches were restricted to those matching the inside facing regions of GLUT1, as predicted by the hydrophathy plots [39]. This constraint was observed as the kinetic interactions between genistein, oestriadiol, ATP and cytochalasin B all show that these ligands bind exclusively to the glucose export site on the inside of GLUT 1 (Figures 1–7 and Scheme 1). The sequence matches between esr-β and GLUT1 are shown in Table 3.

These homologous regions in human esr-β are amino acid residues 290–296 (blue), 301–310 (lemon), 339–346 (‘chlorine’, a term used by Microsoft for greenish yellow) and 472–480 (violet), where the residue numbering comes from human esr-2 (Figures 8A and 8B). These contain the consensus H-bonding groups, His87, Glu89, and Arg416, that H-bond to the hydroxy residues of oestriadiol and genistein (Table 3).

The matches were applied to the three-dimensional template structures of GLUT3 [40] and GLUT1 [41] and viewed with Swiss-Prot viewer. The Protein DataBank file of the coordinates of GLUT3 was kindly made available to us by Dr D. S. Dywer (Departments of Psychiatry and Pharmacology, LSU Health Sciences Center, Shreveport, LA, U.S.A.) (results not shown). The coordinates and structure factors of the three-dimensional model of GLUT1 (accession number 1JAS) are not shown in the Protein DataBank [Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ, U.S.A. (http://www.rcsb.org)]. There is > 70% correspondence between the amino acid sequences of GLUT3 and GLUT1, particularly in the regions adjacent to the inside facing regions of the transporters, so the three-dimensional structures of these two transport proteins are similar enough to map the GLUT1–esr-β homologies on to the three-dimensional templates of GLUT1 (Figure 9) and GLUT3 (results not shown).

A number of important features of GLUT1 and GLUT3 simplify matching of potential binding sites at the inner face of GLUT1 to the LBD of the esr. (1) There is only one conserved histidine group in GLUT1 (His337) and GLUT3 (His345). A histidine group is required to hydrogen-bond to the hydroxy group in ring A of the oestrogens or isoflavone ligands, like genistein, consequently this single group has a key role in anchoring the oestrogen ligand on GLUT1 (Figures 8C and 8D). Another histidine group is present in the cytoplasmic linker region between TM6 and TM7 in GLUT1, His338. This is close enough to His327 to form contiguous anchoring sites for oestriadiol (Table 4). (2) Five glutamic acid–arginine pairs are close enough to His327, < 20 Å, to form a second anchor with the oestrogen ligand and close enough to each other to hydrogen-bond donor–acceptor grouping required to link with the 17-hydroxy group of oestriadiol. Similarly, there are five glutamic acid–arginine pairs close enough to His319 to form possible second anchors for oestriadiol. Glu350 and Arg320 can form anchors with either His337 or His339 (Figures 8E and 8F). (3) The most likely site for oestrogen–isoflavone interaction with GLUT1 is between His337, Arg416 and Glu454. The separation between the arginine and glutamic acid residues is 3.9 Å, between His317 and Arg419 is 14.3 Å, and between His and Glu454 is 14.1 Å. The comparable distances in the LBD of esr-β are 4.5 Å, 16.1 Å and 15.1 Å respectively. Σδ2 of the differences between these distances in esr-β and GLUT3 is 0.35. The nearest alternative groupings are His–Arg157–Glu189, Σδ2 = 0.62, or Arg205–Glu198, Σδ2 = 0.35 (Table 4). (4) The sequences in GLUT corresponding to the Walker ATP-binding domains 2 and 3, namely GLUT1 residues 225–229 (KSVLK) and 332–338 (GRTRHR) [33,34] (coloured red in Figure 8), lie adjacent or within the homologous regions to the LBD of esr-β. (5) The best-fit binding site for oestrogen within GLUT1

Table 3 H-bonding amino acids in the LBD of the esr isoforms to the polar head and tail groups of the steroid ligands as determined by X-ray crystallography

<table>
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<tr>
<th>SwissProt number</th>
<th>Ligand</th>
<th>Protein</th>
<th>Arg 394</th>
<th>Glu 353</th>
<th>His 524</th>
<th>Asp 351</th>
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<tr>
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<td>Stilboestrol</td>
<td>esr-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1TERE</td>
<td>Oestradiol</td>
<td>esr-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1IAS2</td>
<td>Oestradiol</td>
<td>esr-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1TERR</td>
<td>Raloxifene</td>
<td>esr-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>305</td>
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<tr>
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<td>OH-Tamoxifen</td>
<td>esr-α</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8 Two-dimensional and putative three-dimensional locations in GLUT1 of the homologous sequences to the LBD of esr-2

(A) The positions of the homologous sequences between GLUT1 and esr-β marked in four colours are shown on the two-dimensional model of GLUT1 [40] with 12 transmembrane helices the numbers at the top and bottom represent the beginning and end amino acids in the transmembrane helix within the primary sequence of GLUT1. (B) The red lines on the two-dimensional model show the positions of the Walker ATP-binding domains. (C–F) show the three-dimensional structures of the interactions. (C) The LBD of esr-β with genistein (red) in the binding pocket. The homologous sequences to GLUT1 are shown in the same colours as in (A) and (B). (D) A close view of the H-bonding interactions of genistein with His and Arg GLUT. The colours match the homologous sequence in GLUT1. The H-bonding amino acids in esr-β to genistein as obtained from SwissProt Protein DataBank (accession number 1XDM) and Shiaw et al. [35]. (E) A three-dimensional reconstruction of GLUT1 [41], SwissProt accession number 1IAS2, showing the positions of the homologous sequences to those in the ligand binding domain of esr-β, see (A–D). The red colours show the positions of the Walker domains as in (B). (F) A close view of the cytoplasmic surface of GLUT1 centred on His317. The diagram is the sum of all the amino acids within a 20 Å radius of His317 and His337. All the possible matching anchoring sites for oestrogen (Arg blue and Glu red) are shown. The white-labelled amino acid behind His329 is the C-terminal Val412.
Figure 8 For legend see facing page.
or GLUT3 lies within the cytoplasmic entrance to the hydrophilic pore (Figure 9). The hydrophilic central core is evident from Dwyer’s three-dimensional structure of GLUT3 [40], and therefore is an ideal site for placement of a competitive inhibitor of glucose exit. These similarities between the sequences of esr-β and the putative oestradiol-binding domain of GLUT1 on the inside surface (Tables 3 and 4, and Figure 8A) support the view that there is a potential oestrogen-binding site at the inner surface of GLUT, which interferes with glucose transport and responds rapidly to direct interaction with either the hormone or its analogues or competitors.

The best-fit binding site for oestrogen within GLUT1/GLUT3 lies within the cytoplasmic entrance to the hydrophilic pore (Figure 9). A hydrophilic central core is considered likely in both recent three-dimensional reconstructions of GLUT1 and GLUT3 [40,41]. There is also evidence from studies of cysteine mutagenesis of GLUT1 in support for a central hydrophilic core within GLUT1 [42]. Therefore, the site, at the rim of the entrance to the hydrophilic pore region, proposed here as a potential locus for oestradiol or genistein binding, is well placed to act as a competitive inhibitor of glucose exit. This site could therefore be a non-genomic site of oestrogen action.

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