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Title page

Title:

Involvement of Estrone-3-Sulfate Transporters in Proliferation of Hormone-Dependent Breast Cancer Cells

Authors:

TAKASHI NOZAWA, MASATO SUZUKI, KOICHI TAKAHASHI, HIKARU YABUUCHI, TOMOJI MAEDA, AKIRA TSUJI, and IKUMI TAMAI

Department of Molecular Biopharmaceutics, Faculty of Pharmaceutical Sciences, Tokyo University of Science (T.N., M.S., K.T., H.Y., T.M., I.T.), Noda, Japan, Department of Pharmaceutical Biology, Faculty of Pharmaceutical Sciences, Kanazawa University (T.N., A.T.), Kanazawa, Japan, and GenoMembrane, Inc. (H.Y.), Tsurumi, Kanagawa, Japan.

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Running Title Page

Running title:

Estrone-3-sulfate transport in breast cancer

Correspondence:

Ikumi Tamai, Ph.D.,

Department of Molecular Biopharmaceutics,

Faculty of Pharmaceutical Sciences,

Tokyo University of Science,

2641 Yamasaki,

Noda, Chiba 278-8510, Japan.

Phone/Fax: +81-4-7121-3615

e-mail: tamai@rs.noda.tus.ac.jp

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Abbreviations:

DCC, dextran-coated charcoal; DMSO, dimethylsulfoxide; FCS, fetal calf serum ; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; DHEAS, dehydroepiandrosterone sulfate.

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Abstract

Although circulating estrone-3-sulfate is a major precursor of biologically active estrogen, permeation across the plasma membrane is unlikely to occur by diffusion because of the high hydrophilicity of the molecule. The object of this study was to clarify the involvement of specific transporter(s) in the supply of estrone-3-sulfate to human breast cancer-derived T-47D cells, which grow in an estrogen-dependent manner. The proliferation of T-47D cells was increased by the addition of estrone-3-sulfate, or estradiol, to the cultivation medium. The initial uptake rate of estrone-3-sulfate kinetically exhibited a single saturable component, with Km and Vmax values of 7.6 μM and 172 pmol/mg protein/min, respectively. The replacement of extracellular Na^+ with Li^+, K^+ or N-methylglucamine$^+$ had no effect on the uptake of [^{3}H]estrone-3-sulfate. The uptake was strongly inhibited by sulfate conjugates of steroid hormones, but not by estradiol-17ß-glucuronide. Taurocholate and sulfobromophthalein inhibited the uptake, while other tested anionic and cationic compounds did not. The expression of organic anion transporting polypeptides, OATP-D and OATP-E, which are candidate transporters of estrone-3-sulfate, was detected by RT-PCR analysis, though their actual involvement in the uptake of estrogen remains to be clarified.

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In conclusion, the uptake of estrone-3-sulfate by T-47D cells was mediated by a carrier-mediated transport mechanism, suggesting that the estrogen precursor is actively imported by estrogen-dependent breast cancer cells.

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Introduction

Breast cancer is one of the major types of cancers, and two-thirds of breast tumors are estrogen-dependent (Henderson et al., 1980), i.e., their cell growth is regulated by estrogens. The biologically active form of estrogen is estradiol, which is synthesized through two main pathways (Tilson-Mallett et al., 1983; Santner et al., 1986). One is the aromatase pathway, in which aromatase converts androgens to estrogens, and the other is the sulfatase pathway, in which steroid sulfatase converts estrone-3-sulfate to estrone. Since sulfatase activity is 50-200 times higher than aromatase activity in pre- and post-menopausal patients with breast cancer, the sulfatase pathway is the major source of biologically active estrogen derived from estrone-3-sulfate (Santen et al., 1986; Pasqualini et al., 1996, 1997). Moreover, the circulating plasma concentration of estrone-3-sulfate is about 5-10 times higher than that of unconjugated estrogens and the half-life of estrone-3-sulfate is longer than that of estradiol (Purdy et al., 1961; Ruder et al., 1972; Pasqualini et al., 1996). Therefore, estrone-3-sulfate is thought to play an important role in the progression of breast cancer as a reservoir of active estrogen, even though estrone-3-sulfate itself is not biologically very active.

The stimulation of proliferation of breast cancer cells by estrone-3-sulfate involves the following sequence of processes: uptake of estrone-3-sulfate into the cells,

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desulfation by estrogen sulfatase, conversion of estrone to estradiol by 17ß-hydroxysteroid dehydrogenase type I, binding to nuclear estrogen receptor, and the regulation of gene transcription (MacIndoe 1988; Kuiper et al., 1997; Puranen et al., 1997; Suzuki et al., 2002). Although the enzymes and the receptor involved in the response of breast cancer cells to estrone-3-sulfate have been well investigated, the transport mechanism of ligands such as estrone-3-sulfate across the plasma membrane remains to be clarified. Since the logP value of estrone-3-sulfate is reported as 1.4, which is significantly lower than that of estrone (4.4) and estradiol (3.9), it would not be easy to cross the plasma membrane by diffusion, though estrone and estradiol are diffusible (Verheugen et al., 1984; Tan et al., 1999). Estrone-3-sulfate was detected in intact form in the cytosol fraction of MCF-7 cells after incubation with estrone-3-sulfate (Vignon et al., 1980), and exhibited estrogenic effects (Santen et al., 1986; Pasqualini et al., 1996; Billich et al., 2000). Accordingly, a specific transporter may be involved in the supply of estrone-3-sulfate to breast cancer cells which show hormone-dependent growth.

Several members of the organic anion transporter (OAT, SLC22A) (Sekine et al., 2000) and organic anion transporting polypeptide (OATP, SLC21A, SLCO) (Hagenbuch and Meier, 2002, 2003) families are already known to transport estrone-3-sulfate. They are expressed mainly in liver, kidney, brain, intestine, and elsewhere, but

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their expression in breast cancer cells has not been reported. Their Km values for estrone-3-sulfate are in the range from 0.05 μM to 59 μM. Since plasma levels of estrone-3-sulfate fluctuate in the range of 1 to 10 nM in females (Honjo et al., 1987), these or other transporters may play an role in the uptake of estrone-3-sulfate into cancer cells as the first step of hormone-dependent proliferation.

Recently, Pizzagalli et al. suggested the expression of a steroid sulfate transporter, OATP-B, in the human mammary gland (Pizzagalli et al., 2003). However, OATP-B mRNA was not detected in estrogen-dependent MCF-7 and T-47D cells, while OATP-D and OATP-E mRNAs were found in these cells. Since OATP-D and OATP-E exhibited transport activity for estrone-3-sulfate when their genes were transiently expressed in HEK293 cells (Tamai et al., 2001), they may be involved in the transport of estrone-3-sulfate in cancer cells. However, the transporter-mediated uptake of estrone-3-sulfate in estrogen-dependent cells has not previously been examined, while it has been reported in other tissues such as liver, placenta, and brain (Tan et al., 1999; Kitazawa et al., 2000; St-Pierre et al., 2002). To fully understand the mechanism of proliferation of breast cancer cells, the mechanism of supply of estrogen to the cells must be clarified. Thus, the purpose of this study is to characterize the transport mechanism of estrone-3-sulfate in estrogen-dependent cells as the first step to identify the transporter molecule in hormone-dependent breast cancer cells.

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Materials and methods

Materials

[^{3}H]Estrone-3-sulfate, ammonium salt (1702.0 GBq/mmol) was purchased from PerkinElmer Life Science Products, Inc. (Boston, MA, USA). T-47D cells were kindly provided by Prof. Takuma Sasaki, Kanazawa University Cancer Research Institute. Fetal calf serum (FCS) was obtained from Invitrogen Corp. (Carlsbad, CA, USA). All other reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan).

T-47D cell proliferation assay

T-47D cells were routinely grown in RPMI 1640 medium (Sigma Chemicals) containing phenol red and 10% untreated FCS in a humidified incubator at 37°C and 5% CO_2. For the proliferation assay, FCS was incubated with 0.5% dextran-coated charcoal (DCC) at 4°C overnight, then the medium was decanted and DCC was filtered off (0.2 μm) to remove steroid hormones. This procedure was repeated three times. Then T-47D cells were seeded into 96-well plates at a density of 8,000 cells/well in phenol red-free RPMI 1640 medium containing 2.5% DCC-treated FCS. At 1 day after seeding, estradiol or estrone-3-sulfate was added at graded concentrations from stock solutions in water or dimethylsulfoxide (DMSO, 0.1%). The negative control was

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solvent only. At designated days after seeding, the cells were treated with trypsin, and cell numbers were counted.

Transport Experiments

Transport experiments were performed as described previously (Tamai et al., 2001). Briefly, after cultivation of T-47D cells in 15 cm dishes, the cells were harvested and suspended in the transport medium containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM Hepes, adjusted to pH 7.4. The cell suspension and a solution containing a radiolabeled test compound in the transport medium were separately incubated at 37°C for 20 min, then transport was initiated by mixing them. At appropriate times, 150 µL aliquots of the mixture were withdrawn and the cells were separated from the transport medium by centrifugal filtration through a layer of a mixture of silicone oil (SH550, Toray Dow Corning Co., Tokyo, Japan) and liquid paraffin (Wako Pure Chemical Industries) with a density of 1.03. Each cell pellet was solubilized in 3 N KOH, and neutralized with HCl. Then, the associated radioactivity was measured by means of a liquid scintillation counter using Clearsol-1 as a liquid scintillation fluid (Nacalai tesque, Kyoto, Japan).

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Reverse Transcription PCR Method

Expression of OATP and OAT transporters in T-47D cells was examined by the RT-PCR method. Single-stranded cDNA was constructed using an oligo(dT) primer (Invitrogen Corp., CA, USA). The following specific primers were originally designed as follows and used for PCR; OATP-A: 5'-AAACAAGCTGCCCACATAGG-3' for forward and 5'-AGACAGTCGAACAGAACGAC-3' for reverse, OATP-B: 5'-CCTGCCGCTCTTCTTTATCGG-3' and 5'-GTTGCACGTCGGTAGACCA-3', OATP-C: 5'-CACTTGGAGGCACCTCACA-3' and 5'-TTCCCAGATGAACCCGAACA-3', OATP-D: 5'-CAGGCCATGCTCTCCGAAA-3' and 5'-CCTCTAACGTCACCACCGA-3', OATP-E: 5'-CCCTGGGAATCCAGTGGATTG-3' and 5'-GAGAAGAAACGGTATCGGACGA-3', OATP-F: 5'-GGAAATTCCTCAGGCATAGTGG-3' and 5'-CTCAAGAACGTCCTTAGGGTC-3', OATP8: 5'-GGGAATCATAACCATTCCTACGG-3' and 5'-CAGATCGTCCTACGTTTAGGAG-3', OAT1: 5'-CTGATGGCTTCTCACAACAC-3' and 5'-GACCAAGAAGTAACTCAGCC-3', OAT2: 5'-GCTGGTTTTACCATCATCGT-3' and 5'-GAGGATAATGCCGGACTCAG-3', OAT3: 5'-AAGTGACCTGTTCCGGATAC-3' and 5'-GTAGTCCGTTTGTCCATACC-3', and OAT4: 5'-GGCGTTATCTCCATTGCTTC-3'

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and 5'-TCTCTGACCCAAGGTTAGAG-3'. The reaction was performed 35 cycles of 94°C for 30 sec, 58°C (OATPs) or 56°C (OATs) for 30 sec, 72°C for 30 sec and final elongation at 72°C for 10 min in the presence of deoxynucleotides and *Tag* polymerase (Takara Bio Inc., Shiga, Japan). PCR products were analyzed by means of 1% agarose gel (w/v) electrophoresis and the gel was stained with ethidium bromide to visualize bands. For positive control, PCR reaction was performed using pcDNA3 vector, including cDNA of OATP-B, -C, -D, -E or 8 as previously described (Tamai et al., 2000; Nozawa et al., 2004), human brain Marathon-Ready cDNA for OATP-A, and F, or human kidney Marathon-Ready cDNA for OAT1, 2, 3, and 4 (Clontech, Palo Alto, CA) as templates.

Analytical Methods

Cellular protein content was determined according to the method of Bradford by using a BioRad protein assay kit (Hercules, CA, USA) with bovine serum albumin as the standard (Bradford, 1976). Initial uptake rate of estrone-3-sulfate uptake by T-47D cells was evaluated by the uptake at 1 min after subtraction of the uptake in the presence of excess concentration of unlabeled estrone-3-sulfate (1 mM). To estimate kinetic parameters for saturable transport, the initial uptake rate (v) was fitted to the following

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equations by means of nonlinear least-squares regression analysis using KaleidaGraph™ (Synergy, PA, USA).

$v=Vmax*s/(Km+s) + kd*s$

where v and s are the uptake rate and concentration of substrate, respectively, and Km,Vmax, and kd represent the half-saturation concentration (Michaelis constant), the maximum transport rate, and the apparent nonsaturable first-order rate constant, respectively. All data were expressed as means ± S.E.M., and statistical analysis was performed by the use of Student's *t* test with $P < 0.05$ as the criterion of significance. Cell-to-medium ratio was obtained by dividing the cellular uptake amount by the concentration of test compound in the uptake medium.

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Results

Effect of estradiol and estrone-3-sulfate on the growth of T-47D cells

To elucidate the effect of estrone-3-sulfate on the growth of T-47D cells, the cells were treated with estradiol and estrone-3-sulfate. In line with a previous study using estrogen-dependent MCF-7 cells (Billich et al., 2000), the proliferation of T-47D cells was stimulated by 1 pM estradiol or 100 nM estrone-3-sulfate compared with the cells treated with solvent alone as control (Fig. 1A). When evaluated at 6 days after seeding, the estrogen-dependent stimulation was increased in a concentration-dependent manner (Fig. 1B). The values of median effective concentration (EC_{50}) of estradiol and estrone-3-sulfate were calculated as approximately 22.5 fM and 17.1 nM, respectively, by nonlinear regression analysis.

Uptake of estrone-3-sulfate by T-47D cells

Figure 2 shows the time course of the uptake of [^{3}H]estrone-3-sulfate (6.1 nM) by T-47D cells in the presence or absence of unlabeled 1 mM estrone-3-sulfate. The uptake of [^{3}H]estrone-3-sulfate increased linearly over 3 min. In the presence of unlabeled 1 mM estrone-3-sulfate, no significant increase in the uptake of [^{3}H]estrone-3-sulfate were observed over 30 min (Fig. 2), suggesting that estrone-3-sulfate was hardly taken up into T-47D cells by simple diffusion. Accordingly, in the following

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experiments, uptake of estrone-3-sulfate at 1 min by T-47D cells was kinetically analyzed after correction by the uptake in the presence of excess concentration of unlabeled estrone-3-sulfate (1 mM).

To characterize the transport mechanism of estrone-3-sulfate, the effect of replacement of Na^+ with various cations on estrone-3-sulfate uptake was examined (Fig. 3). When Na^+ was replaced with K^+, Li^+, or *N*-methylglucamine$^+$, the uptake of [^{3}H]estrone-3-sulfate was not significantly different from that in the presence of Na^+, suggesting a Na^+-independent transport mechanism.

To obtain the kinetic parameters of the uptake of estrone-3-sulfate by T-47D cells, the concentration dependence of estrone-3-sulfate uptake was examined. The uptake of estrone-3-sulfate after subtraction of the value in the presence of 1 mM estrone-3-sulfate was saturable, as shown in Fig. 4. In Eadie-Hofstee plot analysis after correction of non-saturable uptake, which was estimated from the first-order rate constant (4.69 ± 0.38 μL/mg protein/min) obtained by the nonlinear least-squares regression analysis, a single straight line was obtained with the kinetic parameters, Km, and Vmax values (mean ± S.E.) of 7.6 ± 1.2 μM, and 172 ± 21.0 pmol/mg protein/min, respectively. These results suggested that a single saturable mechanism is involved for the uptake of estrone-3-sulfate by T-47D cells.

To characterize the substrate specificity of the transport system, the inhibitory

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effects of hormones, their conjugated metabolites, and various organic compounds on [^{3}H]estrone-3-sulfate uptake by T-47D cells were examined (Table 1). Unlabeled estrone-3-sulfate itself exhibited the strongest inhibitory effect (to 34.7% and 6.6% of the control at 5 μM and 50 μM, respectively). The inhibitory effect of DHEAS was significant (to 44.8% of the control at 50 μM), but weaker than that of estrone-3-sulfate. Glucuronide conjugate, estradiol-17ß-glucuronide, had no effect at 50 μM. Unconjugated steroid hormones all significantly inhibited the uptake of [^{3}H]estrone-3-sulfate. However, their affinities were lower than that of sulfated conjugates, estrone-3-sulfate and DHEAS. Among other organic compounds examined, sulfobromophthalein and taurocholic acid were inhibitory at 100 μM (to 18.9 and 69.3% of the control, respectively). Other organic compounds, salicylate, *p*-aminohippuric acid, tetraethylammonium, cyclosporin A, and digoxin had no effect, while probenecid and benzylpenicillin showed slight inhibitory effects.

To identify the transporters which mediate the uptake of estrone-3-sulfate in T-47D cells, we investigated the expression of OATP and OAT transporters, which could potentially transport estrone-3-sulfate, by RT-PCR analysis. Figures 5A and 5B show the positive control for the specific primers for OATP and OAT transporters, respectively (upper panels) and the signals of expression of OATP-D and OATP-E in T-

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47D cells (lower panels). Thus, OATP-D and OATP-E are candidates to mediate the uptake of estrone-3-sulfate in estrogen-dependent breast cancer cells.

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Discussion

Estrone-3-sulfate is a major circulating estrogen, and participates in the progression of estrogen-dependent breast cancer (Pasqualini et al., 1989). It is subjected to desulfation by steroid sulfatase, followed by conversion to biologically active estrogen in tumor tissues. Since steroid sulfatase was detected in the cytoplasm of breast carcinoma cells (Saeki et al., 1999), estrone-3-sulfate must be transported into the cells across the plasma membrane prior to desulfation. In view of the hydrophilicity of estrone-3-sulfate, the internalization of estrone-3-sulfate is likely to be mediated by a specific transport mechanism, whereas hydrophobic unconjugated steroid hormones seem simply to diffuse into the cells (Verheugen et al., 1984). So, we examined in the present study whether there is a specific transport system which mediates the uptake of estrone-3-sulfate into T-47D breast cancer cells, which are regarded as model cells that show estrogen-dependent growth.

First of all, we confirmed the estrogen dependence of the cell growth. Treatment with estrone-3-sulfate, or estradiol, increased T-47D cell proliferation with an EC_{50} value of estrone-3-sulfate of 17.1 nM, which is close to the physiological plasma concentration of estrone-3-sulfate. Since estrone-3-sulfate itself induces a low direct biological response at the estrogen receptor (Kuiper et al., 1997), the results suggested the internalization of estrone-3-sulfate across the plasma membrane followed

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by conversion to active unconjugated estrogen in the cells. Accordingly, this study was particularly focused on the uptake of the sulfate conjugate of estrogen by T-47D cells, as the first step in the manifestation of estrogen activity.

The time course of estrone-3-sulfate uptake by T-47D cells suggested that the uptake was mediated by a specific transporter(s), since the uptake in the presence of a high concentration of estrone-3-sulfate (1 mM) was significantly decreased (Fig. 2). The specific uptake of estrone-3-sulfate was not affected by replacement of Na^+ with Li^+, K^+ or *N*-methylglucamine$^+$ (Fig. 3). Members of the OATP and OAT families are candidates for Na^+-independent estrone-3-sulfate uptake transporters. The Km value for estrone-3-sulfate uptake by T-47D cells was calculated to be 7.6 ± 1.2 μM (Fig. 4), which is consistent with the known range of Km values of OATPs and OATs of 0.05 μM to 59 μM (Kusuhara et al., 1999; Cha et al., 2000; Tamai et al., 2001). Since the plasma concentrations of estrone-3-sulfate is approximately 1-10 nM (Honjo et al., 1987), breast cancer cells may effectively take it up without saturation.

To further characterize the transport mechanism and to identify the transporter involved, an inhibition study on the uptake of estrone-3-sulfate by T-47D cells was performed. The inhibitory effects of sulfate conjugates of steroid hormones such as estrone-3-sulfate and DHEAS were stronger than those of unconjugated steroid hormones, while a glucuronide conjugate was not inhibitory. Thus, a sulfate moiety

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might confer high affinity for the transporter, while a glucuronide moiety might be hardly recognized. Since sulfate conjugates have lower ionization constant (pKa) values compared with unconjugated steroids or glucuronide conjugates, these results suggested that an anionic moiety is essential for substrate recognition by the transporter. Because the plasma concentration of sulfate conjugates of steroid hormones is higher than that of unconjugated hormones or their glucuronide conjugates, the transporter should act effectively in the supply of estrogens to breast tumors. In the case of postmenopausal women, androgens and their sulfated conjugates also play an important role in the progression of breast cancer as a source of active estrogen. Since DHEAS is present at high concentration (about 1 μM) in most postmenopausal patients, it is a major precursor of estrogen (Worgul et al., 1982). Since DHEAS strongly inhibited the uptake of estrone-3-sulfate, DHEAS may be preferentially taken up into breast cancer cells as well as estrone-3-sulfate, while its affinity for the transporter which transports estrone-3-sulfate may be lower than that of estrone-3-sulfate.

Some members of OATPs, OAT3 and OAT4 can transport sulfate conjugates of steroid hormones (Kusuhara et al., 1999; Saeki et al., 1999; Hagenbuch and Meier, 2002, 2003). Among them, OATP-B and OAT4 recognize estrone-3-sulfate with Km values of 9.04 μM and 1.01 μM, respectively, while they have no affinity for glucuronide conjugates of steroid hormones (Cha et al., 2000; Tamai et al., 2001).

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Since the uptake of [^{3}H]estrone-3-sulfate was not inhibited by glucuronide conjugates in T-47D cells, the functional characteristics of OATP-B or OAT4 are consistent with the uptake mechanism in T-47D cells as to the substrate recognition of steroid conjugates. On the other hand, since OATP-A, OATP-C, OATP8, and OAT3 transport both sulfate and glucuronide conjugates, these transporters are not likely to be involved in the transport of estrone-3-sulfate in T-47D cells (Kusuhara et al., 1999; Hagenbuch and Meier, 2002, 2003). The typical substrates for OATP, taurocholate and sulfobromophathalein strongly inhibited the uptake of estrone-3-sulfate by T-47D cells, while digoxin and cyclosporinA, which interact with OATP, had no effect (Hagenbuch and Meier, 2002, 2003; Nozawa et al., 2003). Anionic compounds, salicylate and *p*-aminohippuric acid, and cationic compound, tetraethylammonium, which are substrates of OAT and OCT, respectively, had negligible effect. These results suggested the characteristics of the transporter for estrone-3-sulfate in T-47D cells is partly similar to those of OATP or OAT transporters. To obtain insight into the nature of the key transporter, we examined the expression of various human OATPs and OATs by RT-PCR analysis (Fig. 5). We detected the expression of only OATP-D and OATP-E in T-47D cells, in agreement with a previous report (Pizzagalli et al., 2003). Since OATP-D and OATP-E exhibited transport activity for estrone-3-sulfate when they were transiently expressed in HEK293 cells (Tamai et al., 2000), they may take part in the

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uptake of estrone-3-sulfate across the plasma membrane in T-47D cells. However, their functional characteristics have not been well clarified. On the other hand, the expression of OATP-B and OAT4 which can transport estrone-3-sulfate with the similar characteristics as observed in the present study were not expressed in T-47D cells. Therefore, the key transporter molecule involved in the uptake of estrone-3-sulfate in T-47D cells could not be identified in the present study. It has also been reported that OSTalpha-OSTbeta can accept estrone-3-sulfate as a substrate (Seward et al., 2003). Therefore, transporters other than OATPs and OATs might also be involved in the uptake of estrone-3-sulfate in T-47D cells, and further studies remain necessary.

In conclusion, in the present study, it was clearly demonstrated that estrone-3-sulfate is taken up by estrogen-dependent T-47D cells via a specific transport mechanism. This is the first demonstration that the major circulating estrogen, estrone-3-sulfate, is supplied to estrogen-dependent breast cancer cells via a specific transporter. Although further study is required to identify the transporter in T-47D cells, such a transporter molecule would be a novel molecular target for therapeutic intervention in estrogen-dependent breast cancer.

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References

Billich A, Nussbaumer P, and Lehr P (2000) Stimulation of MCF-7 breast cancer cell proliferation by estrone sulfate and dehydroepiandrosterone sulfate: inhibition by novel non-steroidal steroid sulfatase inhibitors. *J Steroid Biochem Mol Biol* **73:** 225-235.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248-254.

Cha SH, Sekine T, Kusuhara H, Yu E, Kim JY, Kim DK, Sugiyama Y, Kanai Y, and Endou H (2000) Molecular cloning and characterization of multispecific organic anion transporter 4 expressed in the placenta. *J Biol Chem* **275:** 4507-4512.

Hagenbuch B, and Meier PJ (2002) The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* **1609:** 1-18.

Hagenbuch B, and Meier PJ (2003) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* **447:** 653-665.

Henderson IC, and Canellos GP (1980) Cancer of the breast: the past decade (second of two parts). *N Engl J Med* **302:** 78-90.

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JPET Fast Forward. Published on August 24, 2004 as DOI: 10.1124/jpet.104.071522
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Honjo H, Kitawaki J, Itoh M, Yasuda J, Iwasaku K, Urabe M, Naitoh K, Yamamoto T, Okada H, Ohkubo T, and Nambara T (1987) Serum and urinary estrone sulfate during the menstrual cycle, measured by a direct radioimmunoassay, and fate of exogenously injected estrone sulfate. *Hormone Res* **27:** 61-68.

Kitazawa T, Hosoya K, Takahashi T, Sugiyama Y, and Terasaki T (2000) In-vivo and in-vitro evidence of a carrier-mediated efflux transport system for oestrone-3-sulphate across the blood-cerebrospinal fluid barrier. *J Pharm Pharmacol* **52:** 281-288.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, and Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138:** 863-870.

Kusuhara H, Sekine T, Utsunomiya-Tate N, Tsuda M, Kojima R, Cha SH, Sugiyama Y, Kanai Y, and Endou H (1999) Molecular cloning and characterization of a new multispecific organic anion transporter from rat brain. *J Biol Chem* **274:** 13675-13680.

MacIndoe JH (1988) The hydrolysis of estrone sulfate and dehydroepiandrosterone sulfate by MCF-7 human breast cancer cells. *Endocrinology* **123:** 1281-1287.

Nozawa T, Tamai I, Sai Y, Nezu J, and Tsuji A (2003) Contribution of organic anion transporting polypeptide OATP-C to hepatic elimination of the opioid

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JPET Fast Forward. Published on August 24, 2004 as DOI: 10.1124/jpet.104.071522
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pentapeptide analogue [D-Ala2, D-Leu5]-enkephalin. *J Pharm Pharmacol* **55:** 1013-1020.

Nozawa T, Sugiura S, Nakajima M, Goto A, Yokoi T, Nezu J, Tsuji A, and Tamai I (2004) Involvement of organic anion transporting polypeptides in the transport of troglitazone sulfate: implications for understanding troglitazone hepatotoxicity. *Drug Metab Dispos* **32:** 291-294.

Pasqualini JR, Chetrite G, Blacker C, Feinstein MC, Delalonde L, Talbi M, and Maloche C (1996) Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. *J Clin Endocrinol Metab* **81:** 1460-1464.

Pasqualini JR, Cortes-Prieto J, Chetrite G, Talbi M, and Ruiz A (1997) Concentrations of estrone, estradiol and their sulfates, and evaluation of sulfatase and aromatase activities in patients with breast fibroadenoma. *Int J Cancer* **70:** 639-643.

Pasqualini JR, Gelly C, Nguyen BL, and Vella C (1989) Importance of estrogen sulfates in breast cancer. *J Steroid Biochem* **34:** 155-163.

Pizzagalli F, Varga Z, Huber RD, Folkers G, Meier PJ, and St-Pierre MV (2003) Identification of steroid sulfate transport processes in the human mammary gland. *J Clin Endocrinol Metab* **88:** 3902-3912.

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JPET Fast Forward. Published on August 24, 2004 as DOI: 10.1124/jpet.104.071522
This article has not been copyedited and formatted. The final version may differ from this version.

Puranen T, Poutanen M, Ghosh D, Vihko P, and Vihko R (1997) Characterization of structural and functional properties of human 17 beta-hydroxysteroid dehydrogenase type 1 using recombinant enzymes and site-directed mutagenesis. *Mol Endocrinol* **11:** 77-86.

Purdy RH, Engel LL, and Oncley JL (1961) The characterization of estrone sulfate from human plasma. *J Biol Chem* **236:** 1043-1050.

Ruder HJ, Loriaux L, and Lipsett MB (1972) Estrone sulfate: production rate and metabolism in man. *J Clin Invest* **51:** 1020-1033.

Saeki T, Takashima S, Sasaki H, Hanai N, and Salomon DS (1999) Localization of estrone sulfatase in human breast carcinomas. *Breast Cancer* **6:** 331-337.

Santen RJ, Leszczynski D, Tilson-Mallet N, Feil PD, Wright C, Manni A, and Santner SJ (1986) Enzymatic control of estrogen production in human breast cancer: relative significance of aromatase versus sulfatase pathways. *Ann N Y Acad Sci* **464:** 126-137.

Santner SJ, Leszczynski D, Wright C, Manni A, Feil PD, and Santen RJ (1986) Estrone sulfate: a potential source of estradiol in human breast cancer tissues. *Breast Cancer Res Treat* **7:** 35-44.

Sekine T, Cha SH, and Endou H (2000) The multispecific organic anion transporter (OAT) family. *Pflugers Arch* **440:** 337-350.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 20, 2024

JPET Fast Forward. Published on August 24, 2004 as DOI: 10.1124/jpet.104.071522
This article has not been copyedited and formatted. The final version may differ from this version.

Seward DJ, Koh AS, Boyer JL, and Ballatori N (2003) Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta. *J Biol Chem* **278:** 27473-27482.

St-Pierre MV, Hagenbuch B, Ugele B, Meier PJ, and Stallmach T (2001) Characterization of an organic anion-transporting polypeptide (OATP-B) in human placenta. *J Clin Endocrinol Metab*. **87:** 1856-1863.

Suzuki T, Moriya T, Ishida T, Kimura M, Ohuchi N, and Sasano H (2002) In situ production of estrogens in human breast carcinoma. *Breast Cancer* **9:** 296-302.

Tan E, Tirona RG, and Pang KS (1999) Lack of zonal uptake of estrone sulfate in enriched periportal and perivenous isolated rat hepatocytes. *Drug Metab Dispos* **27:** 336-341.

Tamai I, Nezu J, Uchino H, Sai Y, Oku A, Shimane M, and Tsuji A (2000) Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem Biophys Res Commun* **273:** 251-260.

Tamai I, Nozawa T, Koshida M, Nezu J, Sai Y, and Tsuji A (2001) Functional characterization of human organic anion transporting polypeptide B (OATP-B) in comparison with liver-specific OATP-C. *Pharm Res* **18:** 1262-1269.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 20, 2024

JPET Fast Forward. Published on August 24, 2004 as DOI: 10.1124/jpet.104.071522
This article has not been copyedited and formatted. The final version may differ from this version.

Tilson-Mallett N, Santner SJ, Feil PD, and Santen RJ (1983) Biological significance of aromatase activity in human breast tumors. *J Clin Endocrinol Metab* **57:** 1125-1128.

Verheugen C, Pardridge WM, Judd HL, and Chaudhuri G (1984) Differential permeability of uterine and liver vascular beds to estrogens and estrogen conjugates. *J Clin Endocrinol Metab* **59:** 1128-1132.

Vignon F, Terqui M, Westley B, Derocq D, and Rochefort H (1980) Effects of plasma estrogen sulfates in mammary cancer cells. *Endocrinology* **106:** 1079-1086.

Worgul TJ, Santen RJ, Samojlik E, and Wells SA (1982) How effective is surgical adrenalectomy in lowering steroid hormone concentrations? *J Clin Endocrinol Metab* **54:** 22-26.

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JPET Fast Forward. Published on August 24, 2004 as DOI: 10.1124/jpet.104.071522
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Footnotes

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Send reprint requests to: Prof. Ikumi Tamai, Department of Molecular Biopharmaceutics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamasaki, Noda, Chiba, 278-8510, Japan.

e-mail: tamai@rs.noda.tus.ac.jp

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Figure Legends

Figure 1

Time course (a) and concentration dependence (b) of stimulation of proliferation of T-47D breast cancer cells by estrogens

(a) T-47D cells were seeded at a density of 8,000 cells/well and cultured. Twenty-four hours after seeding (day 1), estradiol (1 pM, closed triangles), estrone-3-sulfate (100 nM, closed circles), DMSO (0.1 %, open triangle) or water (open circles) was added. After the designated number of days, T-47D cells were trypsinized and counted. (b) T-47D cells were grown in the presence of various concentrations of estradiol (closed triangles) or estrone-3-sulfate (closed circles), ranging from 10^{-16} to 10^{-11} M, or from 10^{-11} to 10^{-6} M, respectively. The cell number was counted at day 6 after seeding. Each value represents the mean ± S.E.M. (n = 3).

Figure 2

Time course of [^{3}H]estrone-3-sulfate uptake by T-47D cells

Cultured T-47D cells were incubated at 37°C over 30 min in medium containing [^{3}H]estrone-3-sulfate (6.1 nM) with (opened circles) or without (closed circles) 1 mM unlabeled estrone-3-sulfate. Each value represents the mean ± S.E.M. (n = 4). When the error bars are not shown, they are smaller than the symbols.

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Figure 3

Effect of extracellular cations on [^{3}H]estrone-3-sulfate uptake by T-47D cells

The uptake rate of [^{3}H]estrone-3-sulfate (6.1 nM) for 1 min was measured in the presence or absence of extracellular Na^+. Extracellular Na^+ was replaced with K^+, Li^+, or *N*-methylglucamine$^+$ (NMG^+). Each value represents the mean ± S.E.M. (n = 4).

Figure 4

Concentration dependence of uptake of estrone-3-sulfate by T-47D cells

Uptake of estrone-3-sulfate at various concentrations, ranging from 6.1 nM to 20 μM, was measured at 37°C for 1 min. The saturable uptake was obtained after subtraction of the uptake in the presence of excess of unlabeled estrone-3-sulfate (1 mM) and used for the evaluation of kinetic constants by nonlinear least-squares analysis. The inset shows an Eadie-Hofstee plot of the saturable uptake of estrone-3-sulfate. Each value represents the mean ± S.E.M. (n = 3-4). When error bars are not shown, they are smaller than the symbols.

Figure 5

Expression of OATP and OAT transporters in T-47D cells

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RT-PCR analysis was performed using mRNA obtained from T-47D cells. The reaction using specific primers for OATP (A) or OAT (B) were performed as described in Materials and Methods. Positive controls are shown in upper panels using the pcDNA3 vector including cDNA of each OATP (OATP-B, C, D, E, and 8), human brain (OATP-A, and F) or human kidney (OAT1, 2, 3, and 4) Marathon-Ready cDNA (Clontech, Palo Alto, CA) as templates. Lower panels show the results of the reaction using cDNA of T-47D cells. The arrows show the specific bands of OATP-D and OATP-E.

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TABLE 1

Inhibitory Effects of Various Compounds on [^{3}H]Estrone-3-sulfate Uptake by T-47D Cells

Inhibitor	Concentration (μM)	% of control
Control		100.0 ± 4.7
Estrone-3-sulfate	5	34.7 ± 4.3*
	50	6.6 ± 0.9*
DHEAS	5	66.4 ± 5.7*
	50	27.0 ± 7.6*
Estradiol-17ß-glucuronide	5	73.8 ± 8.7
	50	90.4 ± 11.5
Estrone	5	78.8 ± 3.3*
	50	64.8 ± 7.5*
Dehydroepiandrosterone	5	75.6 ± 3.8*
	50	44.8 ± 8.0*
Estradiol	5	76.9 ± 4.8*
	50	45.7 ± 5.3*
Sulfobromophthalein	100	18.9 ± 2.8*
	1000	14.2 ± 1.2*
Taurocholic acid	100	69.3 ± 2.7*
	1000	24.1 ± 2.3*
Probenecid	100	103.2 ± 10.2

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	1000	79.1 ± 3.8*
Benzylpenicillin	100	83.2 ± 2.0*
	1000	75.9 ± 10.5
Salicylate	100	94.9 ± 6.0
	1000	82.7 ± 7.3
p-Aminohippuric acid	100	87.8 ± 1.5
	1000	112.7 ± 18.6
Tetraethylammonium	100	87.4 ± 1.5*
	1000	112.7 ± 18.6
Control (DMSO 1%)		100.0 ± 9.7
Cyclosporin A (DMSO 1%)	10	112.2 ± 8.3
	100	90.3 ± 3.7
Digoxin (DMSO 1%)	100	90.3 ± 3.5
	1000	94.8 ± 4.4

Cultured T-47D cells were incubated at 37°C for 10 min in medium containing [^{3}H]estrone-3-sulfate (7.1 nM) with or without (control) an inhibitor at the indicated final concentration. Transporter-mediated uptake was calculated by subtracting the uptake in the presence of excess estrone-3-sulfate (1 mM) from the total uptake. Uptake was expressed as percent of the control uptake. Each value represents the mean ± S.E.M. (n = 4). *P < 0.05, compared with control (Student's *t* test).

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Fig. 1

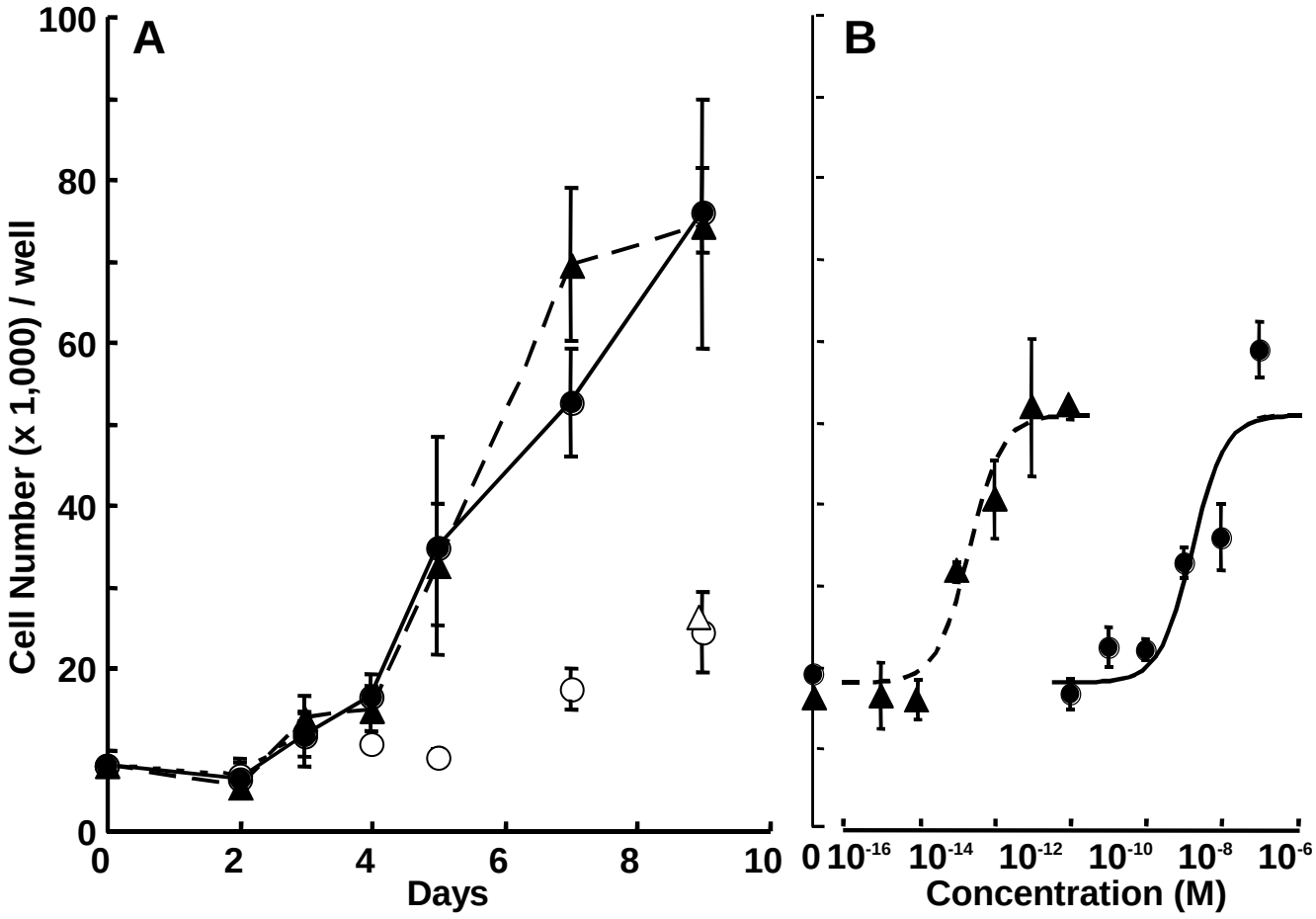

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Fig. 2

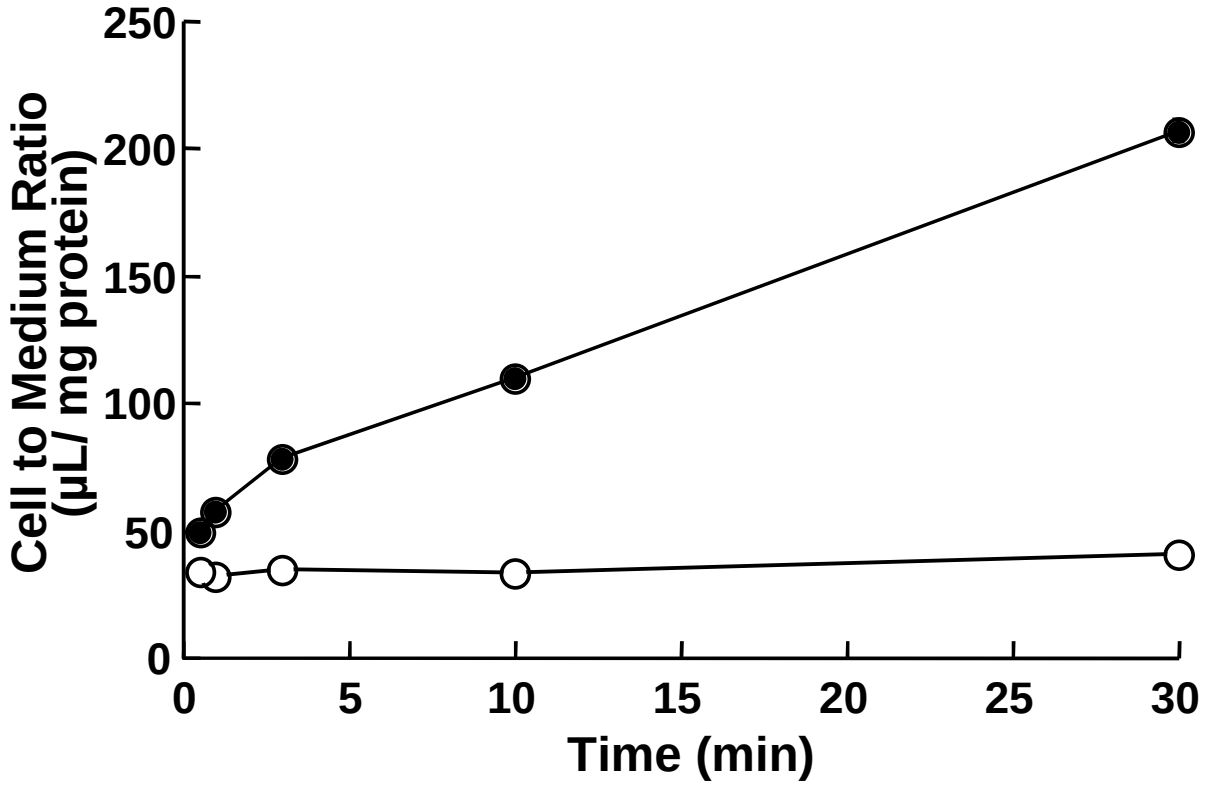

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Fig. 3

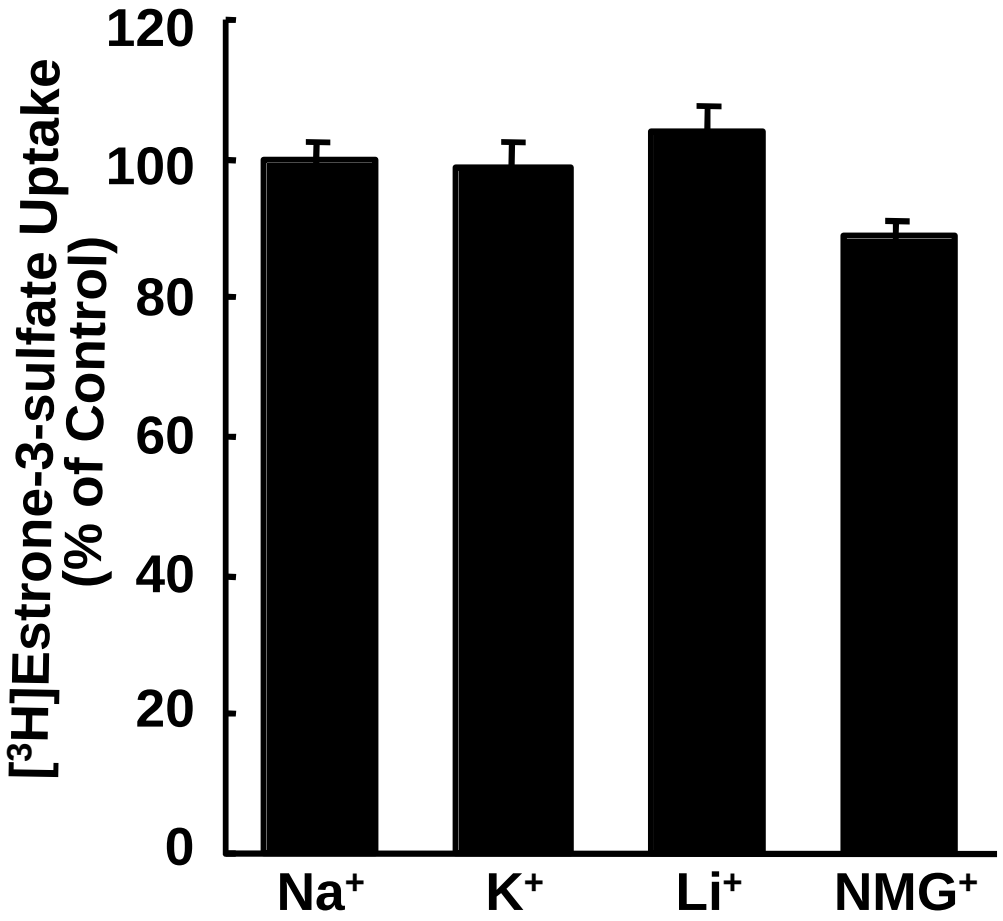

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Fig. 4

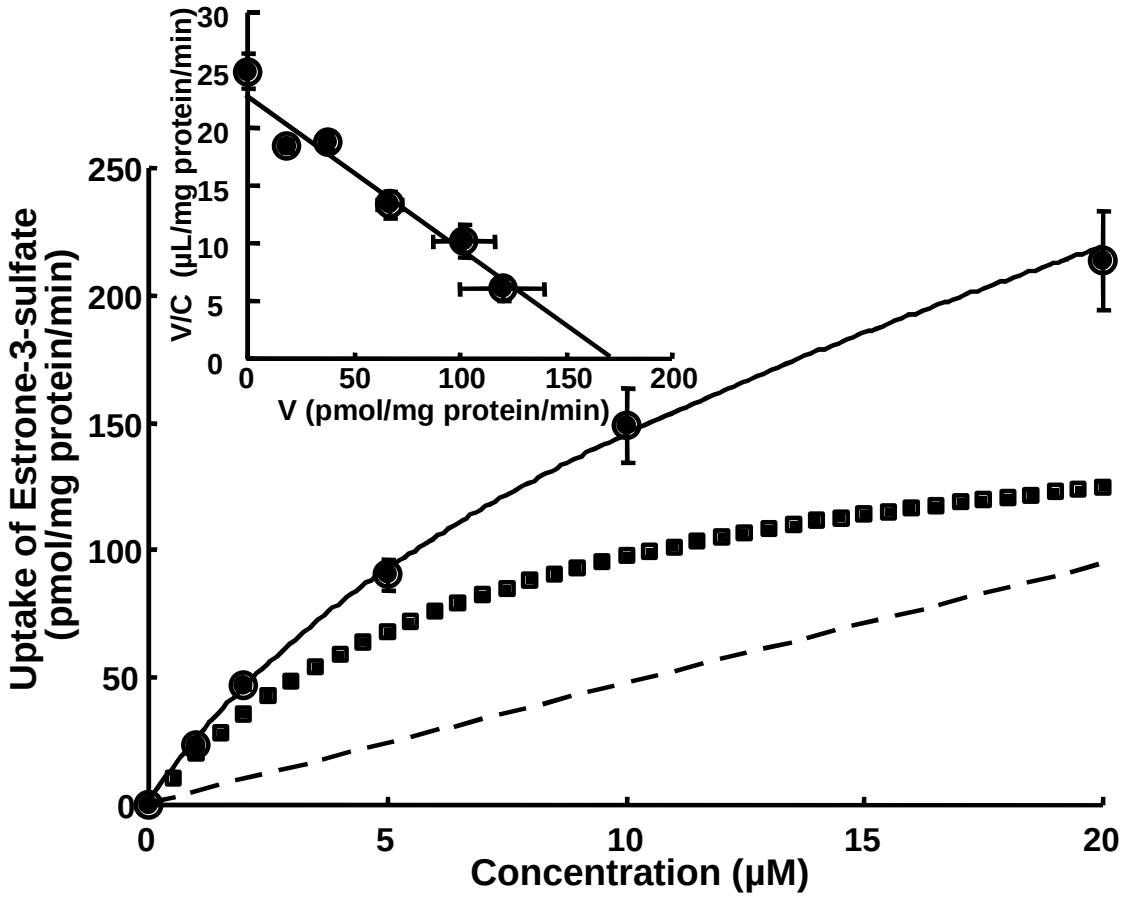

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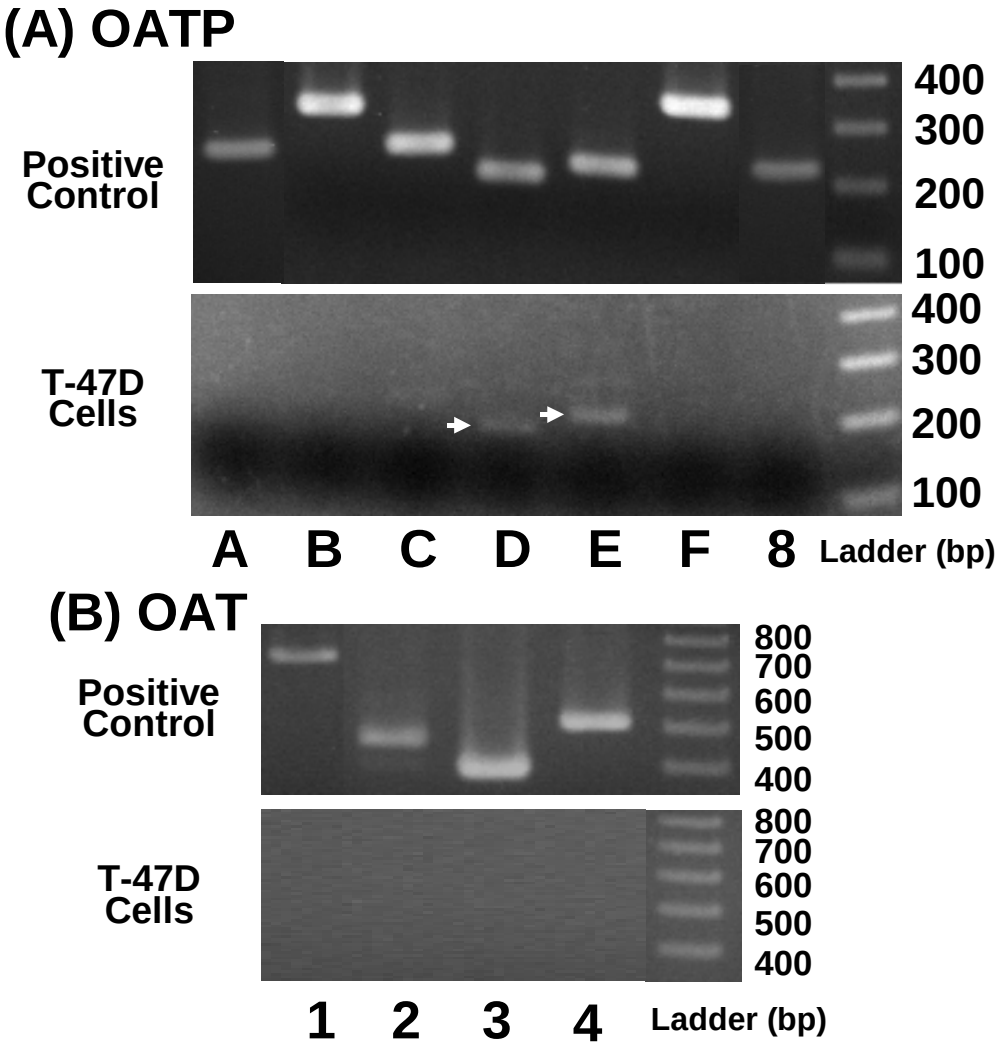
Fig. 5



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