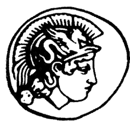

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Development of (*p*-*O*-sulfamoyl)-*N*-alkanoyl-phenylalkyl amines as non-steroidal estrone sulfatase inhibitors

Aparna Kolli[a], Guo-Hua Chu[b], Michael E. Rhodes[a], Kengo Inoue[c], Kyle W. Selcer[a], Pui-Kai Li[b,*]

[a]*Department of Biology, Bayer School of Natural and Environmental Sciences, Duquesne University, Pittsburgh, PA 15282, USA*
[b]*Department of Medicinal Chemistry and Pharmaceutics, Mylan School of Pharmacy, Duquesne University, Pittsburgh, PA 15282, USA*
[c]*Pharmaceutical Research & Development Centre, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan*

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Abstract

Estrogen levels in breast tumors of postmenopausal women are as much as 10 times higher than estrogen levels in plasma, presumably due to in situ formation of estrogen. The major source of estrogen in breast cancer cells may be conversion of estrone sulfate to estrone by the enzyme estrone sulfatase. Thus, inhibitors of estrone sulfatase are potential agents for treatment of estrogen-dependent breast cancer. Several steroidal compounds have been developed that are potent estrone sulfatase inhibitors, most notably estrone-3-*O*-sulfamate. However, these compounds and their metabolites may have undesired effects, including estrogenicity. To avoid the problems associated with a potentially active steroid nucleus, we designed and synthesized a series of nonsteroidal estrone sulfatase inhibitors, the (*p*-*O*-sulfamoyl)-*N*-alkanoyl phenylalkyl amines. The compounds synthesized vary in the length of their alkanoyl chain and in the number of carbons separating the phenyl ring and the carbonyl carbon. The ability of these compounds to inhibit estrone sulfatase activity was tested using human placental microsomes and intact cultured human breast cancer cells. Estrogenicity was also evaluated, using growth of estrogen-dependent human breast cancer cells. All of the test compounds inhibited estrone sulfatase activity of human placental microsomes to some extent, with the most effective compound having an IC_{50} value of 72 nM. In general, compounds with longer alkanoyl chains (12–14 carbons) were more effective than those with shorter chains. The test compounds also inhibited estrone sulfatase activity in intact cultures of MDA-MB-231 human breast cancer cells. Again, the longer chain compounds were more effective. In both the placental and breast cancer cell sulfatase assays, the optimal distance between the phenyl ring and the carbonyl carbon was 1–2 carbons. The MCF-7 cell proliferation assay revealed that estrone and estrone-3-*O*-sulfamate were both estrogenic, but the (*p*-*O*-sulfamoyl)-*N*-alkanoyl phenylalkyl amines were not. Our data indicate the utility of (*p*-*O*-sulfamoyl)-*N*-alkanoyl phenyl alkylamines for inhibition of estrone sulfatase activity. Furthermore, our data support the concept that nonsteroidal estrone sulfatase inhibitors may be useful as therapeutic agents for estrogen-dependent breast cancers. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Estrogen levels in breast tumors of postmenopausal women are at least ten times higher than estrogen levels in plasma [1,2]. The high levels of estrogen in these tumors are presumably due to in situ formation of estrogen, possibly through conversion of estrone sulfate to estrone by the enzyme estrone sulfatase [3,4]. Therefore, inhibitors of estrone sulfatase are potential agents for the treatment of estrogen-dependent breast cancers. Of the estrone sulfatase inhibitors reported in the literature, estrone 3-*O*-sulfamate (EMATE, Fig. 1) is the most potent. It inhibited over 99% of estrone sulfatase activity in intact MCF-7 cells at 0.1 μM. In addition, it exhibited time- and concentration-dependent inactivation of estrone sulfatase and is classified as an active-site directed irreversible inhibitor [5–7]. However, a recent report by Elger et al. demonstrated

* Corresponding author. Tel.: +1-412-396-5661; fax: +1-412-396-5130; e-mail: li@duq.edu

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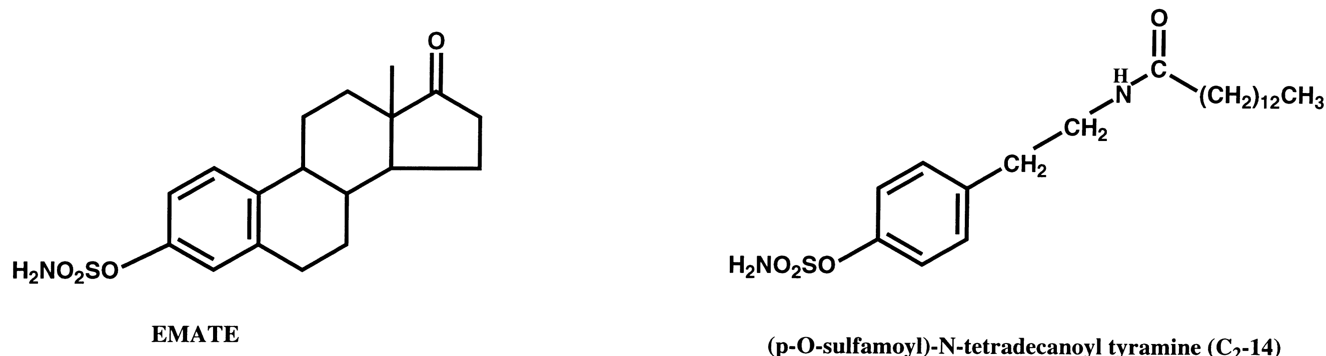
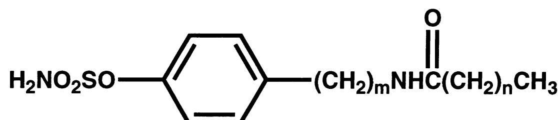




Inhibitor #	m	n
1 (a-d)	0	6,8,10,12
2 (a-d)	1	6,8,10,12
3 (a-e)	3	6,8,10,12,14

Proposed inhibitors

Fig. 1. Structures of EMATE, (*p*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine (C_2-14) and proposed inhibitors 1(a–d), 2(a–d) and 3(a–e).

that EMATE is a potent estrogen [8]. Therefore, there is a need for a potent nonsteroidal sulfatase inhibitor that is metabolically st , more selective and devoid of estrogenic activity. Since estrone sulfatase is a membrane-bound protein, we have reported the synthesis and sulfatase inhibitory activities of (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramines, as nonsteroidal estrone sulfatase inhibitors [9]. The phenyl sulfamoyl group of the inhibitors mimic the A ring of EMATE, and the long alkanoyl chain is proposed to provide additional binding through insertion into the membrane. One of the most potent inhibitors in this series is (*p*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine (C_2-14) (Fig. 1) with an IC_{50} value of 55.8 nM in human placental microsomal assay [9]. The structure-activity-relationships of the amide functionality in C_2-14 was investigated. Replacement of the amide functionality in C_2-14 with an ethylene moiety resulted in complete loss of sulfatase inhibitory activity (IC_{50} of 61.3 nM vs. >20 μM). The keto, hydroxy and ester analogs have 8 to 15 times lower in affinity for the sulfatase than inhibitor 1. These results suggest that the amide functionality is favorable for sulfatase inhibitory activity and there may be a hydrogen-bonding component to the enzyme interaction in this region [10]. Hence, three additional series of inhibitors [1 (a–d), 2 (a–d) and 3 (a–e)] were designed by varying the chain length between the phenyl ring and the amide functionality and the length of the N-alkanoyl group to examine the optimum chain length for maximum inhibitory activity (Fig. 1). Estrone sulfatase inhibition of each compound was tested in two distinct assay systems, human placental microsomes, and intact cultures of human breast cancer cells (MDA-MB-231). Additionally, selected compounds were tested for their abilities to stimulate growth of estrogen-dependent MCF-7 human breast cancer cells, a marker of estrogenicity.

2. Experimental

2.1. Chemicals and reagents

Chemicals and silica gel were purchased from Aldrich Chemical Company (Milwaukee, WI). Chemicals were checked for purity by thin layer chro-

matography and NMR. All radioinert steroids and tissue culture reagents were obtained from Sigma Chemical Company (St. Louis, MO). [6,7-^{3}H] Estrone sulfate and [C4, ^{14}C] estrone were purchased from Dupont Company (Boston, MA). Melting points were determined on a Thomas Hoover capillary melting point apparatus and were uncorrected. IR spectral data were obtained with a Perkin-Elmer 1430 ratio recording spectrophotometer. Proton NMR spectra were obtained with a Bruker WH-300 (300 MHz) spectrophotometer. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Centrifugation was performed on a Damon CRU-5000 Centrifuge and ultracentrifugation on a Beckman type B Ultracentrifuge. Radioactive samples were analyzed with a Packard Tri-Carb 4530 Liquid scintillation Counter. Liquid scintillation cocktail was Ultima Gold from Packard (Meriden, CT).

2.2. Chemistry

2.2.1. Synthesis of p-methoxy-N-octanoyl-benzylamine

To the solution of the *p*-methoxy-benzylamine (2.0 g, 14.6 mmol), triethylamine (4.1 ml, 29.2 mmol) and 4-dimethylaminopyridine-DMAP (178 mg, 1.46 mmol) in dry methylene chloride-CH_2Cl_2 (50 ml) was added dropwise 1-octanoyl chloride (3.32 ml, 29.2 mmol) at 0°C. The reaction mixture was stirred at room temperature overnight and then washed with sat. sodium bicarbonate ($NaHCO_3$) solution and water, dried (Na_2SO_4) and concentrated. The residue was purified by chromatography on silica gel (ethyl acetate (EtOAc)/CH_2Cl_2, 1:10), yielding 3.6 g (94%) of p-methoxy-*N*-octanoyl-benzylamine. m.p.; 95–96°C; ^{1}H NMR (300 MHz,$CDCl_3$) δ 0.81 (t, 3H, $J=6.6$ Hz, CH_3), 1.20 (m, 8H, 4 x CH_2), 1.57 (m, 2H, CH_2), 2.12 (t, 2H, $J=7.5$ Hz, CH_2CO), 3.72 (s, 3H, OCH_3), 4.28 (d, 2H, $J=5.7$ Hz, $ArCH_2$), 5.80 (brs, 1H, NH), 6.78 (d, 2H, $J=8.4$ Hz, ArH), 7.13 (d, 2H, $J=8.4$ Hz, ArH). Analysis calculated for $C_{16}H_{25}NO_2$: C, 72.96; H, 9.57; N, 5.32. Found C, 72.88; H, 9.62; N, 5.47.

2.2.2. Synthesis of p-hydroxy-N-octanoyl-benzylamine

A 1 M solution of boron tribromide (BBr_3) in CH_2Cl_2 (19.8 ml, 19.8 mmol) was added dropwise to a solution of *p*-methoxy-*N*-octanoyl-benzylamine (2.37 g, 8 mmol) in 80 ml of CH_2Cl_2 at 0°C. After stirring at 0°C for 2.5 h, the reaction mixture was quenched by adding H_2O and 10% HCl. The mixture was then extracted with CH_2Cl_2. The organic extracts were washed with brine and dried (Na_2SO_4). After concentration, the residue was purified by silica gel chromatography (CH_2Cl_2/EtOAc, 5:1), giving the product N-octanoyl-p-hydroxy-benzylamine (2.1 g, 93.8%). m.p: 83–85°C; ^{1}H NMR (300 MHz, DMSO-d6) δ 0.86 (t, 3H, $J=6.6$ Hz,CH_3), 1.23 (m, 8H, 4 x CH_2), 1.50 (m, 2H, CH_2), 2.09 (t, 2H, $J=7.5$ Hz, CH_2CO), 4.12 (d, 2H, $J=5.7$ Hz, $ArCH_2$), 6.68 (d, 2H, $J=8.4$ Hz, ArH), 7.03 (d, 2H, $J=8.4$ Hz, ArH), 8.18 (t, 1H, $J=5.7$ Hz, NH), 9.28 (s, 1H, OH). Analysis calculated for $C_{15}H_{23}NO_2$: C, 72.25; H, 9.30; N, 5.62. Found C, 72.12; H, 9.38; N, 5.56.

2.2.3. Synthesis of (p-O-sulfamoyl)-N-octanoyl-benzylamine (2a)

Sodium hydride-NaH (111 mg, 4.4 mmol) was added to p-hydroxy-*N*-octanoyl-benzylamine (500 mg, 2 mmol) in anhydrous dimethylformamide-DMF (20 ml) at 0°C under nitrogen. The solution was stirred for 30 min and chlorosulfonamide-$ClSO_2NH_2$ (350 mg, 6 mmol) was slowly added in one portion. The solution was then stirred at room temperature for 24 h. The mixture was poured into a cold saturated sodium bicarbonate solution and the resulting solution was extracted with CH_2Cl_2 (3×30 ml). The organic layer was separated, dried (Na_2SO_4) and concentrated under reduced pressure to give a light yellow solid. The product was purified by chromatography on a silica gel column eluted with CH_2Cl_2/EtOAc (25:1) (410 mg, 62.3% yield). m.p.: 118.5–119°C; ^{1}H NMR (300 MHz, DMSO-d6) δ 0.83 (t, 3H, $J=6.6$ Hz, CH_3), 1.21 (m, 8H, $4\times CH_2$), 1.48 (m, 2H, CH_2), 2.09 (t, 2H, $J=7.5$ Hz, CH_2CO), 4.22 (d, 2H, $J=5.6$ Hz, $ArCH_2$), 7.18 (d, 2H, $J=8.4$ Hz, ArH), 7.27 (d, 2H, $J=8.4$ Hz, ArH), 7.94 (s, 2H, NH_2), 8.31 (t, 1H, $J=5.6$ Hz, NH). Analysis calculated for $C_{15}H_{24}N_2O_4S$: C, 54.86; H, 7.37; N, 8.53. Found C, 54.98; H, 7.23; N,8.49.

2.2.4. Enzyme preparation

Microsomal membranes were obtained by differential centrifugation of the homogenates of human placentas from normal term pregnancies and were stored at −80°C until assayed [11]. Stock solution of estrone sulfate was prepared in 0.02 M Tris–HCl buffer, pH 7.2. All the inhibitors were dissolved in ethanol prior to the experiments. Estrone sulfatase activity was assayed radiometrically using [6,7-^{3}H] estrone sulfate. Conversion of [^{3}H] E_1S to [^{3}H] unconjugated steroids (E_1 and E_2) was used as a measure of estrone sulfatase activity.

2.2.5. Sulfatase assay procedure

The final volume of the enzyme assay was 1 ml. An inhibitor at various concentrations in ethanol was added to a 5 ml test tube. The ethanol was removed with a stream of nitrogen. Estrone sulfate (20 μM/tube; 300,000 dpm/tube) and Tris–HCl buffer (0.02 M, pH 7.2, 0.2 ml) were then added to each tube. The assay tubes containing the estrone sulfate, Tris–HCl buffer and inhibitor were preincubated for 5 min at 37°C in a water bath shaker. The assay began by the addition of placental microsomes (150 μg) diluted with

0.02 M Tris–HCl buffer, pH 7.2 (0.8 ml). After 20 min of incubation at 37°C, 4 ml of toluene was added to quench the assay. [^{14}C] Estrone (10,000 dpm/tube) was added concurrently with the toluene as the internal standard for the determination of extraction efficiency. Control samples with no inhibitor were incubated simultaneously. Blank samples were obtained by incubating boiled microsomes. The quenched samples were vortexed for 45 s and centrifuged (2000 rpm for 10 min). One milliliter of toluene was obtained from each quenched sample to determined the amount of product formation. All the samples were run twice in triplicate with variation of less than 7%. The IC_{50} value in nanomolar (nM) or micromolar (μM) concentration, represents the concentration of the inhibitor needed to achieve 50% inhibition of estrone sulfatase activity in the homogenate when compared to the control with no inhibitor.

2.3. Cell culture methods

The human breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely grown in a CO_2 incubator at 5% CO_2 and 37° C. Cells were grown 100 mm^2 tissue culture dishes in RPMI-1640 medium containing 0.2% (v/v) sodium bicarbonate, 5% heat-inactivated fetal calf serum, 10 mg/ml gentamycin and 1% (v/v) antibiotic/antimycotic. Estrogen-free medium consisted of RPMI-1640 phenol-red-free medium, 5% dextran-coated-charcoal-stripped fetal calf serum, 0.2% (v/v) sodium bicarbonate, 10 mg/ml gentamycin, and 1% (v/v) antibiotic/antimycotic.

2.3.1. In vitro estrone sulfatase assay

Intact monolayers of MDA-MB-231 (estrogen-independent) cells were used to determine the ability of compounds to inhibit estrone sulfatase activity. Confluent cells were washed twice in phosphate buffered saline (PBS), detached with trypsin-EDTA, and viable cells were counted in the presence of Trypan-blue dye using a hemocytometer. Cells were then seeded in 6-well plates at 500,000 cells/well and incubated overnight in whole media to allow for attachment. ^{3}H-E_1S (20,000 dpm/ml) was added in the presence or absence of experimental compounds (1 μM). In all experiments, cells were incubated for 18 h. Plates were removed and cooled at room temperature for 30 min and then 0.5 ml of media was removed, in duplicate, into test tubes. Unconjugated steroids were extracted from the samples using 3 ml of toluene. Samples were vortexed for 1 min followed by centrifugation at 1000×g for 3 min to separate the aqueous and organic phases. One ml aliquots of the organic phase were removed and placed into a scintillation vials and 4 ml of scintillation cocktail was added. Radioactivity was counted in a Packard Tri-carb scintillation counter. All treatments were run in triplicate and each experiment was repeated five times. Wells containing media, but no cells, were included in each experiment to control for spontaneous hydrolysis of the ^{3}H-E_1S. Conversion of [^{3}H] E_1S to [^{3}H] unconjugated steroids (E_1 and E_2) was used as a measure of estrone sulfatase activity.

The in vitro -conversion assay was also used to determine dose-dependent inhibition of estrone sulfatase activity. Experiments were run as above except that test compounds were added at concentrations ranging from 10 μM to 0.01 nM. From these data, IC_{50} values were calculated. IC_{50} was defined as the concentration of a compound that inhibited estrone sulfatase activity to 50% of its maximal activity in the absence of inhibitor.

2.4. Cell proliferation assay for estrogenicity

Growth of intact monolayers of MCF-7 (estrogen-dependent) cells was used to assess experimental compounds for estrogenicity. Confluent cells were washed twice in PBS, detached using trypsin-EDTA and viable cells were counted in the presence of Trypan-blue dye using a hemocytometer. Cells were seeded in 96-well plates at 5000 cells/well and incubated in whole medium for two days, in order to allow the cells to adhere. The whole medium was then aspirated off and 200 μl of estrogen-free medium (E-free medium) was added to each well. The cells were incubated for five days in the E-free medium, with the medium being replaced once during this period. After five days, compounds were added at a 1 μM concentration in E-free medium. The cells were once again incubated for five days, replacing the medium once during this period. Cells were then counted using the MTT assay.

2.5. MTT assay

An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) assay was used to determine cell concentration for all experiments. MTT was made as a stock solution (1 mg/ml) in charcoal-stripped estrogen-free RPMI 1640 medium and stored at −20°C. MTT was added in a 1:10 ratio to the medium volume of the wells, and cells were incubated for 3 h. The MTT reaction was terminated by removing the medium and adding 1 ml of acidic isopropanol to the cells to solubilize the product. Absorbance was read at a wavelength of 595 nm and a background wavelength of 655 nm, using a BioRad model 3550 microtiter plate reader.

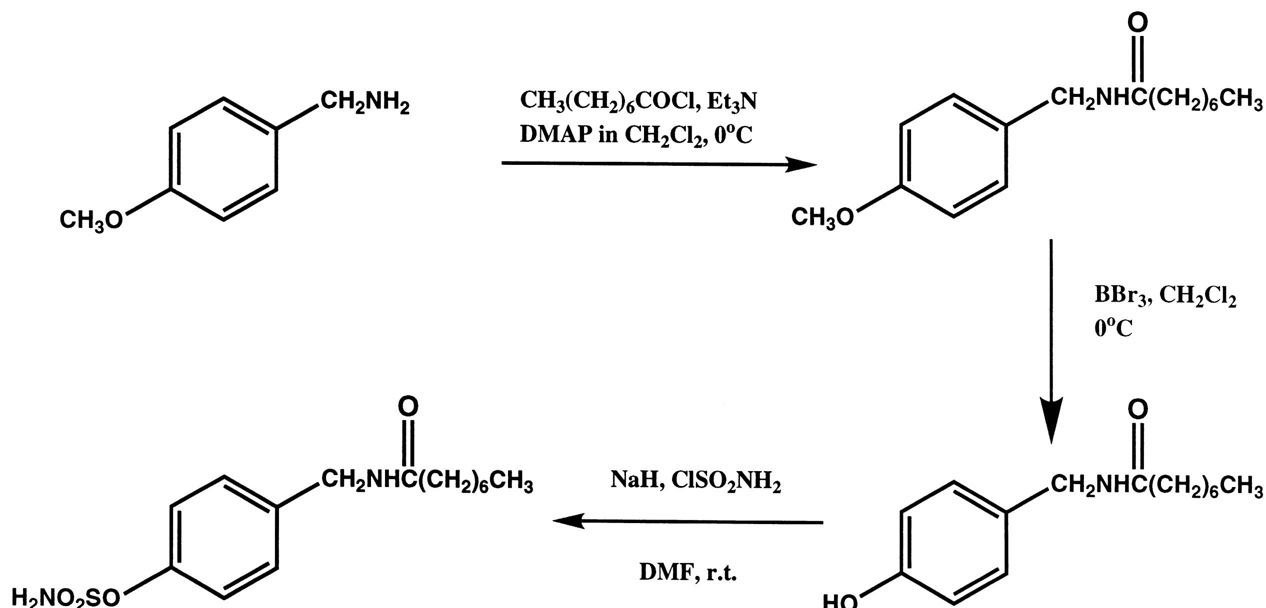


Fig. 2. Synthesis of inhibitor 2a.

2.6. Statistical analyses

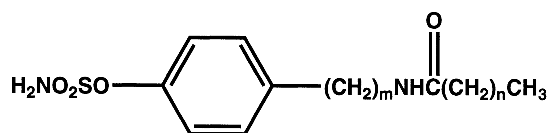
One-way analysis of variance (ANOVA) was used to determine differences between treatment means. A Newman–Keuls test was used for a posteriori comparisons of means. A nonlinear regression analysis was performed on the dose-response curves (plotted as estrone sulfatase activity vs. $\log_{10}$ inhibitor concentration) using the computer program Prism (GraphPad Software, Inc., San Diego, CA). The resulting equation was used to determine the IC_{50} value for each test compound. For all statistical analyses, probabilities of $P < 0.05$ were considered significant.

3. Results

3.1. Chemical results

Inhibitors 1 (a–d), 2 (a–d) and 3 (a–e) were syn-

Table 1
Inhibitory effects of nonsteroidal sulfatase inhibitors to estrone sulfatase from human placental microsome, utilizing estrone sulfate (20 μM) as the substrate. Each value represents the mean of two determinations in triplicate



Compound #	*m*	*n*	IC5 (nM)
la	0	6	1140 ± 85
lb	0	8	684 ± 48
lc	0	10	171 ± 22
Id	0	12	737 ± 42
2a	1	6	1800 ± 132
2b	1	8	300 ± 21
2c	1	10	190 ± 11
2d	1	12	72 ± 6
3a	3	6	> 20,000
3b	3	8	> 20,000
3c	3	10	5100 ± 230
3d	3	12	1230 ± 65
3e	3	14	760 ± 51

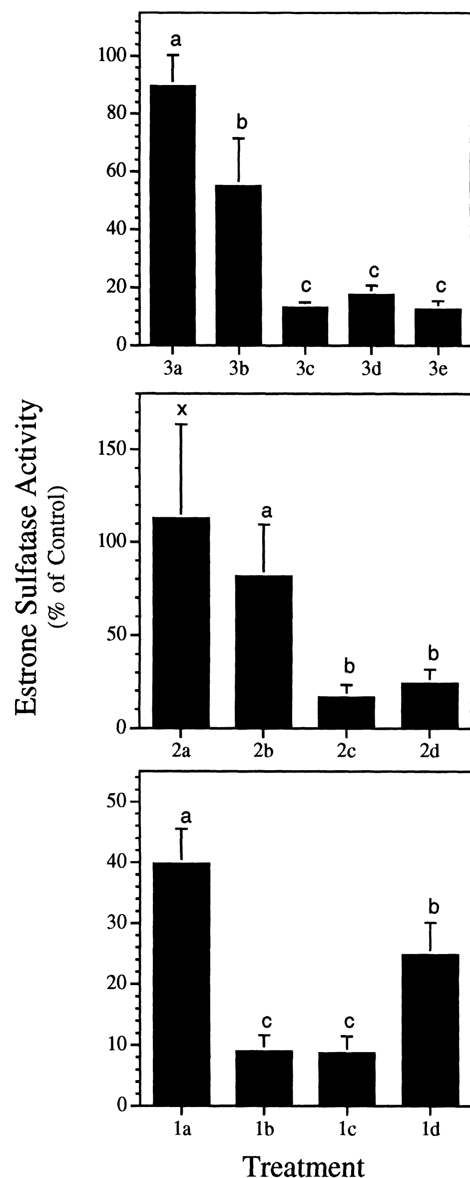


Fig. 3. Comparison of estrone sulfatase inhibition, in MDA-MB-231 cells, by inhibitors 1(a–d), 2(a–d) and 3(a–e). Cells were seeded at 500,000 cells/well in 6-well plates and incubated overnight in whole media. Tritiated estrone sulfate was added (average dpm: 20,000; 0.35 nM) in the presence or absence of inhibitors (1 μM). Cells were incubated for 18 h. After termination of the reaction, ^{3}H-unconjugated steroids were extracted with toluene. Radioactivity was determined by scintillation spectroscopy. Data (cpm) are expressed as percent of control, which was the level of conversion in the absence of inhibitors. Each data set represents triplicate determinations of five separate experiments. Bars represent means±1 SEM. Different letters indicate significant differences by a Student Newman–Keuls test ($p<0.05$).

thesized with similar procedures and the synthesis of inhibitor 2a is shown in Fig. 2. The first step involved adding 1-octanoyl chloride to a solution of p-methoxybenzylamine, triethylamine, 4-dimethylaminopyridine (DMAP) in methylene chloride at 0°C to form p-methoxy-*N*-octanoyl-benzylamine. The *p*-methoxy-*N*-octanoyl-benzylamine was then treated with boron tribromide to form *p*-hydroxy-*N*-octanoyl-benzyllamine. Treatment of *p*-hydroxy-*N*-octanoyl-benzyllamine with sodium hydride in DMF at 0°C followed by chlorosulfonamide yielded inhibitor 2a. The melting points and elemental analyses of the inhibitors are shown in Table 3.

3.2. Biochemical results

3.2.1. Determination of IC_{50} values of inhibitors using estrone sulfatase isolated from human placenta

The sulfatase activity in the presence of increasing amounts of inhibitor concentration were determined to evaluate the relative potency of the inhibitor. For these experiments, a microsomal protein concentration of 150 μg/ml was used. The microsomes were incubated with various concentrations of inhibitor and a fixed concentration of the substrate estrone sulfate (20 μM). The concentration of the inhibitor needed to achieve 50% inhibition of estrone sulfatase activity in the microsome when compared to the control with no inhibitor was given an IC_{50} value in nanomolar or micromolar concentration. The IC_{50} values of the inhibitors are shown in Table 1. The IC_{50} values ranged from 72 nM to >20 μM.

3.2.2. In vitro conversion assay

3.2.2.1. Single-dose comparisons. Inhibition of estrone sulfatase activity was tested using the intact MDA-MB-231 cell estrone sulfatase assay at a concentration of 1 μM. Inhibition was tested separately for each series of inhibitors [1(a–d), 2(a–d) and 3(a–e)]. Fig. 3 shows a comparison of estrone sulfatase inhibition for the three series of compounds. For inhibitors 1(a–d), ANOVA revealed significant differences among treatment groups ($F=11.60$; 3,16 df; $p<0.001$). Inhibitor 1a was the weakest inhibitor and 1b and 1c were the best. There was no statistical difference between these two compounds. Inhibitor 1d showed intermediate inhibition of estrone sulfatase. The calculated percent of control values for 1a, 1b, 1c and 1d were 39.7, 9.0, 8.6 and 24.8%, respectively.

For inhibitors 2(a–d), an initial ANOVA revealed no statistical differences between any treatments ($F=2.796$; 3,16 df 16; $p>0.05$). However, evaluation of the data indicated that highly variable values for inhibitor 2a were interfering with the statistical analysis. A subsequent ANOVA, excluding the 2a data, showed significant differences among treatment means ($F=4.324$; 2,12 df; $p<0.05$). In general, the longer alkanoyl chain inhibitors showed greater inhibitory action than did the shorter chain compounds. The inhibitory values of 2a were similar to those of controls. The longest alkanoyl chain inhibitors (2c and 2d) were

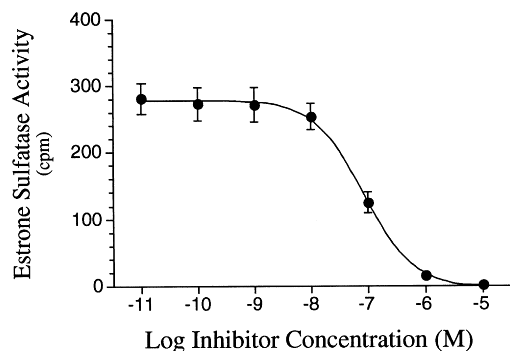


Fig. 4. Dose-response for inhibitor 1d. Dose-responsive inhibition of estrone sulfatase activity by inhibitor 1d. Cells were plated at 500,000 cells/well in 6-well plates and incubated overnight. Tritiated estrone sulfate was added (average dpm: 20,000; 0.35 nM) in the presence of varying concentrations of 1d (10 μM–0.01 nM). After an 18 h incubation period, the reaction was terminated. ^{3}H-unconjugated steroids extracted with toluene and radioactivity was determined by scintillation spectroscopy. Each data point represents triplicate determinations of five separate experiments.

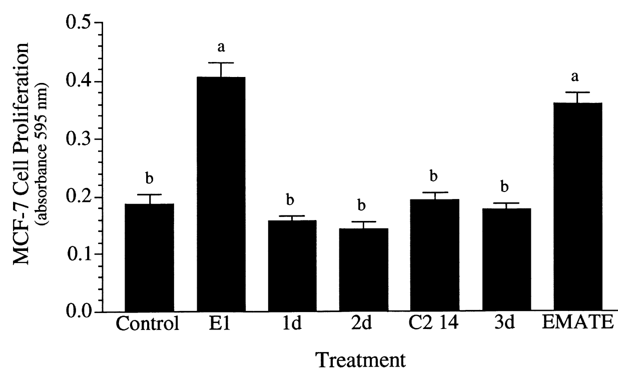


Fig. 5. Estrogenicity of various compounds as determined by MCF-7 cell proliferation. Cells were plated at 5000 cells/well in a 96-well plate and incubated two days in whole media. On day three, the whole media was removed. 200 μl of estrogen-free media was added and the cells were incubated for five days (media was changed once during this interval). On day eight, the serum free media was removed and replaced with 200 μl of serum free media with and without compounds (1 μM). Cells were again incubated for five days (media was changed once during this interval) and on day thirteen the reaction was terminated by adding MTT to each well (1:10). The insoluble formazan product was solubilized with acidic isopropanol and cell number was determined spectrophotometrically. Each data set is an average of 16 wells except Control, E_1, 1d (average of 14 wells) and 2d (average of 22 wells). Bars represent means±SEM. Different letter indicate significant differences by a Student Newman–Keuls test ($p < 0.05$).

significantly different from the control and 2b ($p < 0.05$) but not from each other ($p > 0.05$).

For inhibitors 3(a–e), ANOVA revealed significant differences among treatment means ($F = 14.02$; 4,20 df; $p < 0.0001$). Inhibitors with longer alkanoyl chains, 3c–e, showed the greatest inhibition and were indistinguishable from one another. Inhibitor 3b was statistically different from all other treatment groups ($p < 0.05$). The inhibitor with the shortest alkanoyl chain in this series, 3a, was the least potent. There was no significant difference between 3a and the control ($p > 0.05$).

3.2.2.2. Dose-response and IC_{50} estimates. Estrone sulfatase inhibition of inhibitors 1c, 2d and 3e were tested at concentrations ranging from 10 μM–0.01 nM. All three compounds showed dose-dependent inhibition of estrone sulfatase activity. For example, inhibitor 1c showed percent of control values of 2.0% at 10 μM, 14.1% at 1 μM, 129.8% at 0.1 μM and 254.0% at 0.01 μM (Fig. 4). Using nonlinear regression analyses, the IC_{50} values of the three inhibitors ranged from 31 to 121 nM (Table 2).

3.3. Estrogenicity

Estrogenicity of compounds was examined using the estrogen-dependent MCF-7 cell line. An MTT assay was used to quantify cell proliferation. The experimental compounds, 1d, 2d, 3e, (*p*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine and EMATE, were tested at a 1 μM concentration. Additionally, a negative control (absence of inhibitors) and a positive control (100 nM estrone) were run simultaneously. Fig. 5 shows the cell proliferation in the presence of the various treatment groups. ANOVA showed significant differences among treatment groups ($F = 43.87$, 6, 153 df, $p < 0.0001$). There were no statistical differences among the inhibitors 1d, 2d, 3e, (p)sulfamoyl)-*N*-tetradecanoyl tyramine ($p > 0.05$) and they showed comparable cell proliferation values to that of the negative control ($p > 0.05$). In contrast, cells incubated in the presence of 100 nM estrone (E_1) and EMATE showed cell proliferation that was statistically different from the negative control ($p < 0.001$).

Table 2
IC_{50} values for compounds 1c, 2d and 3d

Inhibitor	IC50 (nM)	(standard error)
lc	84.88	46.29–155.6
2d	31.72	15.11–66.63
3d	121.2	51.44 -795 56

4. Discussion

Postmenopausal women have limited ovarian activity and correspondingly low levels of circulating plasma estrogen; however, they show a higher incidence of estrogen-dependent breast cancer than do premenopausal women [12]. One explanation for this apparent paradox is that breast tissues themselves are the source of the estrogens supporting tumor growth

Table 3
Melting points and elemental analyses of inhibitors 1(a–d), 2(b–d) and 3(a–e)

Compound #	*m*	*n*	m.p. (°C)	Calculated (%)			Found (%)		
				C	H	N	C	H	N
la	0	6	108.5–109.5	53.48	7.05	8.91	53.42	7.09	8.90
lb	0	8	110–111.5	56.12	7.65	8.18	56.38	7.33	8.04
lc	0	10	114–115	58.35	8.16	7.56	58.12	8.23	7.68
1d	0	12	116.5–118	60.27	8.60	7.03	60.14	8.69	7.12
2b	1	8	126.5–127.5	57.28	7.92	7.86	57.49	7.91	7.89
2c	1	10	128.5–129.5	59.35	8.39	7.28	59.32	8.61	7.42
2d	1	12	133–134	61.13	8.79	6.79	61.02	8.90	6.78
3a	3	6	124.5–125.5	57.28	7.92	7.86	57.22	7.90	7.67
3b	3	8	128.5–129.5	59.35	8.39	7.28	59.22	8.30	7.45
3c	3	10	133–133.5	61.13	8.79	6.79	61.01	8.97	6.80
3d	3	12	134–134.5	62.69	9.15	6.36	62.56	9.29	6.48
3e	3	14	135–136	64.07	9.46	5.98	64.21	9.55	5.67

in postmenopausal women. In support of this assertion, postmenopausal women with breast cancer demonstrate a ten-fold greater level of estrogens in their breast tissue than in their plasma [13,14]. There are two potential pathways for estrogen formation in breast tissues, the aromatase pathway and the sulfatase pathway. Of these, the sulfatase pathway appears to be quantitatively the more important source of breast tissue estrogens [3,4,15]. This pathway involves conversion of plasma estrone sulfate to estrone via the enzyme estrone sulfatase (sterol sulfatase, E.C. 3.1.6.2). Thus, the development of compounds that inhibit estrone sulfatase could have therapeutic potential for treatment of estrogen-dependent breast cancers.

Recently, a number of estrone sulfatase inhibitors have been developed and tested. Many of these are structural modifications of the steroid substrate estrone sulfate. These steroidal inhibitors include estrone-3-methythiophosphonates [16,17], estrone-3-amino and estrone-3-thiol derivatives [18,19], and estrone-3-sulfamates [5–7]. These various steroidal compounds have been shown to be effective estrone sulfatase inhibitors, with the most potent being estrone-3-*O*-sulfamate. However, because they are based on a steroid backbone, these compounds and their metabolites can have unwanted activities that make them undesirable for treatment of estrogen-dependent breast cancers. Indeed, estrone-3-*O*-sulfamate has been found to be estrogenic [8].

To avoid the potential problems associated with a steroid nucleus, nonsteroidal compounds have been developed as estrone sulfatase inhibitors. A series of coumarin sulfamates have been shown to effectively inhibit estrone sulfatase [20,21] and to be devoid of estrogenic activity. However, they were not nearly as potent as estrone-3-*O*-sulfamate. Our lab recently designed and synthesized the (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramines as potential nonsteroidal estrone sulfatase inhibitors [9]. Based on an estrone sulfamate backbone, but lacking the B, C and D rings of the steroid nucleus, these compounds were demonstrated to be potent inhibitors of estrone sulfatase in both human placental microsomes [9] and in human breast cancer cells [22]. These compounds were found to be irreversible inhibitors of estrone sulfatase activity in both systems.

In the present study, we investigated the properties of three additional series of inhibitors that are structurally related to the (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramines. These compounds also have a phenyl sulfamoyl and a long alkanoyl chain attached to an amide group, but they have varying chain lengths between the phenyl ring and the amide group. This was done to investigate the optimal distance between the phenyl ring and the carbonyl carbon for interaction with the enzyme. Additionally, the length of the alkanoyl chain, which presumably serves as membrane insertion region, was varied.

Initially, the (*p*-*O*-sulfamoyl)-*N*-alkanoyl phenylalkyl amines [inhibitors 1(a–d), 2(a–d) and 3(a–e)] were screened using a human placental microsome assay. There were considerable differences in the abilities of the various compounds to inhibit placental estrone sulfatase activity. IC_{50} values ranged from greater than 20 μM for the least effective compounds (3a and b) to 72 nM for the most effective (2d). The differences between compounds largely resulted from differences in the length of the alkanoyl chains. Within each of the three series, there was a clear trend towards increased estrone sulfatase inhibition with increasing length of the alkanoyl chain. Similar results were found for the related (*p*-*O*-sulfamoyl)-*N*-alkanoyl tyramine series using the same assay [9].

Some of the differences between the inhibitors' abilities to inhibit estrone sulfatase resulted from differ-

ences in the distance between the phenyl ring and the amide group. For a given length of the alkanoyl chain, series 2 [inhibitors 2(a–d)] was the most effective, series 1 [inhibitors 1(a–d)] was intermediate, and series 3 [inhibitors 3(a–e)] was the least effective estrone sulfatase inhibitor. This indicates that 1 methylene group separating the phenyl ring and the amide group is more effective than 0 or 3 methylene groups. Comparing these results with those for the related *p-O*-sulfamoyl)-*N*-alkanoyl tyramines, which have 2 methylene groups, the tyramines are somewhat more effective than series 2. For example, compound 2d showed an IC_{50} value of 72 nM, compared with 55 nM for (*p-O*-sulfamoyl)-*N*-tetradecanoyl tyramine, which has the same number of carbons in the alkanoyl chain. These data suggest that 2 carbons provide the optimal distance between the phenyl ring and the amide ring for estrone sulfatase inhibition.

We next employed an in vitro -conversion assay using intact monolayers of hormone-independent breast cancer cells (MDA-MB-231). This cell line was chosen because of its greater level of estrone sulfatase activity as compared to the hormone-dependent MCF-7 cell line [22,23]. All three of the series tested showed significant inhibition of the estrone sulfatase enzyme in the in vitro -conversion assay. Again, compounds with longer alkanoyl chains tended to be the more effective inhibitors at the 1 μM concentration used for this assay. This trend was previously seen for the related (*p-O*-sulfamoyl)-*N*-alkanoyl tyramine series [22], using the same assay. The results of the in vitro conversion assay corroborated those from the placental microsome assay. Thus, it appears that 12–14 carbons in the alkanoyl chain provides the greatest inhibition of estrone sulfatase activity, based on data from two distinct assays for the four different series. The improved estrone sulfatase inhibition of the longer-chain compounds may be due to greater hydrophobic interaction with the lipid bilayer.

Selected compounds from each series were subsequently assayed in dose-response experiments. Using the in vitro conversion assay, the compounds were tested in concentrations ranging from 10 μM to 0.01 nM. The representative compounds from the three series were similar in their effectiveness as inhibitors, with IC_{50} values ranging from 32 nM for compound 2d to 121 nM for compound 3d. There was a high degree of concordance when the IC_{50} values determined in the in vitro assay are compared to the IC_{50} values obtained using a human placental microsome screen. Indeed, compounds 1c and 2d showed almost identical IC_{50} values to those determined in the placental assay. However, compound 3d showed quite different results between the two assays. In the human placental system, 3d was largely ineffective; whereas, in the intact cell assay this compound exhibited more effective levels of enzyme inhibition. It is unclear why these assays differ with regard to this series.

For clinical utility, estrone sulfatase inhibitors must not be estrogen agonists; therefore these compounds were also assessed for estrogenic properties using an in vitro cell proliferation assay. We have previously used this assay to test for estrogenicity of steroidal estrone sulfatase inhibitors [24] As expected, cells incubated in 100 nM estrone showed a high degree of cell growth. Estrone-3-*O*-sulfamate also stimulated cell proliferation above control levels. This finding is consistent with previous reports documenting the estrogenic properties of estrone-3-*O*-sulfamate [9,24]. None of the three series of compounds showed any indication of stimulation of cell proliferation. We also included *p-O*-sulfamoyl)-*N*-tetradecanoyl tyramine in this assay, because it had proven to be the most effective of all three series. This compound was not estrogenic in the MCF-7 cell proliferation assay.

The compounds tested, the (*p-O*-sulfamoyl)-*N*-alkanoyl phenylalkyl amines, represent a new generation of nonsteroidal estrone sulfatase inhibitors. These compounds are potent estrone sulfatase inhibitors and they are not estrogenic. Thus, the (*p-O*-sulfamoyl)-*N*-alkanoyl phenylalkyl amines may prove useful for the treatment of estrogen-dependent breast cancer.

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