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Stimulation of MCF-7 breast cancer cell proliferation by estrone sulfate and dehydroepiandrosterone sulfate: inhibition by novel non-steroidal steroid sulfatase inhibitors

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Abstract

Steroid sulfatase (STS) regulates the formation of active steroids from systemic precursors, such as estrone sulfate and dehydroepiandrosterone sulfate (DHEAS). In breast tissues, this pathway is a source for local production of estrogens, which support the growth of endocrine-dependent tumours. Therefore, inhibitors of STS could have therapeutic potential. In this study, we report on substituted chromenone sulfamates as a novel class of non-steroidal irreversible inhibitors of STS. The compounds are substantially more potent (6- to 80-fold) than previously described types of non-steroidal inhibitors when tested against purified STS. In MCF-7 breast cancer cells, they inhibit STS activity with IC_{50} below 100 pM. Importantly, the compounds also potently block estrone sulfate-stimulated growth of MCF-7 cells, again with IC_{50} below 100 pM. For one compound, we also observed a lack of any estrogenic effect at high concentrations (1 μM). We also demonstrate for the first time that STS inhibitors can block the DHEAS-stimulated growth of MCF-7 cells. Interestingly, this cannot be achieved with specific inhibitors of the aromatase, suggesting that stimulation of MCF-7 cell growth by DHEAS follows an aromatase-independent pathway. This gives further justification to consider steroid sulfatase inhibitors as potential drugs in the therapy of breast cancer.

Keywords: Steroid sulfatase; Breast cancer; Estrone sulfate; Dehydropiandroesterone sulfate; Enzyme inhibition

1. Introduction

Estrogens support the growth of hormone-dependent tumours of the breast and endometrium. Enzymes involved in estrogen synthesis in women, such as the aromatase and the 17β-hydroxysteroid dehydrogenase type I, therefore, are obvious targets for therapeutic intervention. An enzyme, which has more recently been proposed as a potential point of attack for anti-tumour therapy, is steroid sulfatase (STS) [1,2]. The rationale for an important role of STS and its substrates estrone sulfate and dehydroepiandrosterone sulfate (DHEAS) in breast cancer is based on the following arguments:

1. Estradiol concentrations in breast tumours of postmenopausal women are 10–40 times higher than serum levels [3–7]. Furthermore, tissue levels of estradiol in breast tumours of post-menopausal women are nearly equivalent to those in premenopausal patients [7], even though plasma estradiol levels are 5- to 60-fold lower after menopause [8]. These observations are best explained by the local formation of the estrogens by enzymes in the tumour [9,10]. Biosynthesis of estrogens in tumour tissues may occur via two different routes, either from androstenedione (aromatase pathway) [11,12] or from estrone sulfate (sulfatase pathway) [13,14]. Direct evidence has been obtained that in situ formation of estrone in breast tumour tissue via the sulfatase pathway is at least 10-fold higher than by the aromatase route [9,10,15]. In another study, the activity of STS in both pre- and postmenopausal patients was found to be 50–200 times higher than that of aromatase [16].

Abbreviations: ADIOL, androst-5-ene-3β,17β-diol; DCC, dextran-coated charcoal; DHEA(S), dehydroepiandrosterone (sulfate); ER, estrogen receptor; STS, steroid sulfatase.

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2. Estrone sulfate, featuring higher concentration [17] and half-life [18] than estradiol in plasma and high concentration in breast tissue [1], appears to represent a reservoir for the formation of biologically active estrogens in the breast tissue [2].
3. In addition to the active estrogens estrone and estradiol, androst-5-ene-3β,17β-diol (ADIOL) as another ligand of the estrogen receptor may be important for growth of breast cancer [19–22]. About 90% of ADIOL are derived from DHEAS in a pathway involving the sulfatase [23]. DHEAS is found in significant amounts in breast cancer tissue [24].
4. Recent epidemiological studies identified STS expression as an independent predictor of recurrence in human breast cancer [25] and revealed an increased risk of postmenopausal breast cancer in women with elevated prediagnostic serum levels of estrone sulfate and DHEAS [26].

Both the formation of active estrogens from estrone sulfate and the formation of ADIOL from DHEAS may be blocked by inhibitors of STS. Such compounds could provide an addition to aromatase blockers, which are in clinical use for the treatment of mammary carcinoma, in order to complete the estrogen blockade. To this end, a considerable number of STS inhibitors have been synthesized (see Refs. [27–32] for some recent examples). In this study, we describe a novel chemotype, which is a more potent blocker of estrone sulfate-stimulated proliferation of MCF-7 breast cancer cells as compared to previously published reference compounds. Importantly, for one selected non-estrogenic derivative, we also demonstrate inhibition of DHEAS-stimulated MCF-7 cell growth, which is not blocked by specific aromatase inhibitors.

2. Materials and methods

2.1. Chemicals and reagents

2,5-Dihydroxyacetophenone, pivaloyl, benzoyl and decanoyl chloride were purchased from the Aldrich Chemical Co. Amidochlorosulfonic acid was prepared according to the method of Appel and Berger [33].

Biochemicals were obtained from Sigma (St. Louis, MO). Materials for protein chromatography were bought from Pharmacia, Uppsala, Sweden. Steroids were checked for purity by HPLC. Commercial batches of estrone sulfate or DHEAS (sodium salts) contained up to 3% of estrone or DHEA, respectively. Therefore, we extracted solutions of the steroid sulfates in phosphate-buffered saline (PBS) with toluene or, alternatively, with methylene chloride/diethylether 4:1. This procedure reduced the content of unconjugated steroid in the aqueous solution below the levels of 0.01% that was detectable by HPLC. The purified PBS solutions of estrone sulfate and DHEAS were prepared fresh on the same day prior to supplementing cell cultures, filtered (0.2 μm) and diluted with culture medium as required.

Tritiated estrone sulfate (estrone sulfate, ammonium salt [6,7-^{3}H(N)] −; 53 Ci/mmol) and ^{14}C-labeled reference steroids were bought from DuPont/New England Nuclear (Boston, MA). Liquid scintillation cocktail was from Packard (Meriden, CT).

2.2. Chemistry

Melting points were determined on a Reichert Thermovar microscope and are not corrected. The temperature is given in Celsius units. Thin-layer chromatography was performed using silica gel F_{254} plates (Merck) detection by UV light or potassium permanganate. Column chromatography was performed using silica gel 60 (0.040–0.063 mm; Merck), pressure 3–5 bar. NMR spectra were recorded at 250 MHz (Bruker WM 250 spectrometer) with $(CH_3)_4Si$ as internal standard. Chemical shifts are given in δ units. Elemental analyses were performed by Novartis Services AG, Basle, Switzerland.

The reference inhibitors estrone-3-*O*-sulfamate (**1**), 4-methylumbelliferyl sulfamate (**2**), and (*p*-*O*-sulfamoyl)-*N*-tetradecanoyltyramine (**3**) were synthesized according to published procedures [28,34,35]. Letrozole was obtained from Novartis Basel. Lentaron, nafoxidine and aminoglutethimide were bought from Sigma.

2.2.1. 2,5-Di-t-butylcarbonyloxyacetophenone (7)

A solution of 2,5-dihydroxyacetophenone (**6**) (5 g, 32.8 mmol) in dry pyridine (35 ml) was treated with pivaloyl chloride (10 g, 83 mmol) under cooling with an ice-bath to keep the reaction temperature at ≈20°C. After stirring for 18 h at room temperature, the solvent was partially distilled off in vacuo. The residue was poured onto ice and hydrochloric acid (32%, 30 ml) and extracted with diethylether. The combined organic layers were washed with aqueous sodium carbonate solution and water, dried ($MgSO_4$) and evaporated to give crude **7** (10.6 g, 100%), which was used in the next step without further purification, as yellow crystals, mp 35°C; ^{1}H NMR ($CDCl_3$) δ 7.45 (d, J = 2.8 Hz, 1H), 7.23 (dd, J = 2.8 + 8.7 Hz, 1H), 7.04 (d, J = 8.7 Hz, 1H), 2.54 (s, 3H), 1.38 (s, 9H), 1.36 (s, 9H).

2.2.2. 2,2-Dimethylpropionic acid 3-(4,4-dimethyl-3-oxopentanoyl)-4-hydroxyphenyl ester (8)

Crude **7** (10.5 g, 32.8 mmol) was dissolved in dry dimethylformamide (30 ml) and added slowly at 0°C under argon to a suspension of sodium hydride (95% pure, 870 mg, 34.4 mmol) in dry dimethylformamide (30 ml). After stirring for an additional 2 h at 0–5°C,

the mixture was cautiously treated with acetic acid (2.5 ml), then poured into water (300 ml) and extracted with ethyl acetate (3 × 70 ml). The combined organic layers were washed with saturated aqueous sodium chloride solution, dried ($MgSO_4$) and evaporated to yield crude **8** (11 g, ≈100%), which was used in the next step without further purification, as orange semi-crystalline mass.

*2.2.3. 2-t-Butyl-6-hydroxy-4H-chromen-4-one (**9**)*

Crude **8** (10.4 g, 30.2 mmol) was heated in formic acid (60 ml) to 100°C for 1 h. Then hydrochloric acid (32%, 10 ml) and water (5 ml) were added and the mixture was stirred for an additional hour at 100°C. The cooled mixture was poured onto ice/water (500 ml) and extracted with ethyl acetate. The combined organic layers were cautiously washed with saturated aqueous sodium bicarbonate solution (3 × 100 ml), dried ($MgSO_4$) and concentrated in vacuo. The dark green residue was chromatographed on silica gel (cyclohexane/ethyl acetate = 2/1) to give the hydroxychromenone **9** (4.9 g, 74%) as colourless crystals, mp 170°C; ^{1}H NMR ($CDCl_3$) δ 8.10 (br s, 1H), 7.88 (d, J = 3 Hz, 1H), 7.39 (d, J = 9 Hz, 1H), 7.28 (dd, J = 3 + 9 Hz, 1H), 6.30 (s, 1H), 1.36 (s, 9H).

*2.2.4. Sulfamic acid 2-t-butyl-4-oxo-4H-chromen-6-yl ester (**4**)*

Sodium hydride (95%, 0.6 g, 23.8 mmol) was carefully added to a solution of **9** (4 g, 18.3 mmol) in dry dimethylformamide (40 ml). After stirring for 30 min at room temperature, the mixture was cooled (ice/water) and amidochlorosulfonic acid (6.3 g, 55 mmol) was added in portions. The mixture was stirred for additional 3 h at room temperature and subsequently concentrated in vacuo. The residue was partitioned between water and ethyl acetate and after separation the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and evaporated to give the crude product which was purified either by crystallisation from toluene or by chromatography on silica gel (cyclohexane/ethyl acetate = 2/1) yielding the sulfamate **4** (4.3 g, 79%) as colourless crystals, mp 180°C; ^{1}H NMR (d_6-DMSO) δ 8.10 (br s, 2H), 7.86 (d, J = 2.9 Hz, 1H), 7.79 (d, J = 9 Hz, 1H), 7.66 (dd, J = 2.9 + 9 Hz, 1H), 6.28 (s, 1H), 1.33 (s, 9H); ^{13}C NMR (d_6-DMSO) δ 177.1, 176.6, 154.3, 147.2, 129.2, 123.9, 120.7, 117.8, 106.1, 36.8, 27.9; $C_{13}H_{15}NO_5S$ (mw = 297.33) calc. 52.51% C, 5.08% H, 4.71% N, found: 52.41% C, 5.05% H, 4.77% N.

*2.2.5. 5-Benzoyloxy-2-hydroxyacetophenone (**10**)*

Benzoyl chloride (9.2 g, 65.7 mmol) was added under stirring at 0°C to a solution of **6** (10 g, 65.7 mmol) and triethylamine (13.6 ml, 98.5 mmol) in dry dichloromethane (150 ml). The mixture was stirred overnight at room temperature, then poured into water and extracted with dichloromethane. The combined organic layers were dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica gel (cyclohexane/ethyl acetate = 8/1) yielding **10** (13.95 g, 83%) as colourless crystals, mp 82°C; ^{1}H NMR ($CDCl_3$) δ 12.18 (s, 1H), 8.18-8.23 (m, 2H), 7.62–7.72 (m, 1H), 7.59 (d, J = 2.8 Hz, 1H), 7.48–7.58 (m, 2H), 7.35 (dd, J = 2.8 + 9 Hz, 1H), 7.05 (d, J = 9 Hz, 1H), 2.64 (s, 3H).

2.2.6. 5-Benzoyloxy-2-decanoyloxyacetophenone

Decanoyl chloride (3.8 g, 20 mmol) was added to a solution of **10** (5.1 g, 20 mmol) in dry pyridine (50 ml). The solution was stirred for an additional 3 h at room temperature and then poured into 15% aqueous hydrochloric acid (300 ml). Extraction with ethyl acetate, followed by washing with aqueous sodium carbonate solution, drying ($MgSO_4$) and evaporation yielded the crude product (8.2 g, 100%), which was used in the next step without further purification, as yellowish crystals, mp. 37°C; ^{1}H NMR ($CDCl_3$) δ 8.18–8.23 (m, 2H), 7.62–7.72 (m, 1H), 7.67 (d, J = 2.8 Hz, 1H), 7.48–7.58 (m, 2H), 7.41 (dd, J = 2.8 + 8.7 Hz, 1H), 7.18 (d, J = 8.7 Hz, 1H), 2.64 (t, J = 7.5 Hz, 2H), 2.56 (s, 3H), 1.78 (qui, J = 7.5 Hz, 2H), 1.22–1.42 (m, 12H), 0.89 (t, J = 7.5 Hz, 3H).

*2.2.7. 1-(5-Benzoyloxy-2-hydroxyphenyl)-1,3-dodecanedione (**11**)*

Following the procedure described for the synthesis of **8** the title compound was obtained as crude product from 5-benzoyloxy-2-decanoyloxyacetophenone and used in the next step without further purification.

*2.2.8. 6-Benzoyloxy-2-nonyl-4H-chromen-4-one (**12**)*

Crude **11** (8.2 g, 20 mmol) was heated in formic acid (50 ml) to 100°C for 45 min. After cooling the solvent was distilled off in vacuo and the residue was chromatographed on silica gel (cyclohexane/ethyl acetate = 8/1) to give the protected chromenone **12** (3.95g, 50%) as colourless crystals, mp 80°C; ^{1}H NMR ($CDCl_3$) δ 8.18–8.23 (m, 2H), 8.00 (dd, J = 1 + 2.3 Hz, 1H), 7.62–7.72 (m, 1H), 7.48–7.58 (m, 4H), 6.20 (s, 1H), 2.64 (t, J = 7.5 Hz, 2H), 1.75 (qui, J = 7.5 Hz, 2H), 1.22–1.42 (m, 12H), 0.88 (t, J = 7.5 Hz, 3H).

*2.2.9. 6-Hydroxy-2-nonyl-4H-chromen-4-one (**13**)*

A solution of **12** (3.3 g, 8.4 mmol) in dioxane (100 ml) was treated with 10% aqueous potassium hydroxide solution (35 ml) and stirred for 1 h at room temperature. The mixture was poured into 2 M aqueous pH 7 buffer solution and extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and evaporated. The residue was chromatographed (SiO_2, cyclohexane/ethyl acetate = 1/1) to yield the

hydroxychromenone **13** (2.25 g, 93%) as colourless crystals, mp 104°C; ^{1}H NMR (d_6-DMSO) δ 9.96 (s, 1H), 7.47 (d, J = 9 Hz, 1H), 7.27 (d, J = 3 Hz, 1H), 7.18 (dd, J = 3 + 9 Hz, 1H), 6.15 (s, 1H), 2.61 (t, J = 7.5 Hz, 2H), 1.65 (qui, J = 7.5 Hz, 2H), 1.16–1.38 (m, 12H), 0.84 (t, J = 7.5 Hz, 3H).

2.2.10. Sulfamic acid 2-nonyl-4-oxo-4H-chromen-6-yl ester (5)

Compound **5** was prepared from 6-hydroxy-2-nonyl-4H-chromen-4-one (13, 2 g, 6.9 mmol) following the procedure described for the synthesis of **4** and was obtained after chromatographic purification (silica gel, cyclohexane/ethyl acetate = 3/1) as colourless crystals (1.91 g, 75%), mp 92°C; ^{1}H NMR (d_6-DMSO) δ 8.10 (br s, 2H), 7.86 (d, J = 2.9 Hz, 1H), 7.76 (d, J = 9 Hz, 1H), 7.64 (dd, J = 2.9 + 9 Hz, 1H), 6.30 (s, 1H), 2.68 (t, J = 7.5 Hz, 2H), 1.68 (qui, J = 7.5 Hz, 2H), 1.20–1.38 (m, 12H), 0.85 (t, J = 7.5 Hz, 3H).

$C_{18}H_{25}NO_5S$ (mw = 367.46) calc. 58.84%C, 6.86%H, 3.81%N, found: 58.56%C, 6.60%H, 3.88%N.

2.3. Expression and purification of human STS

STS was produced from a clone of recombinant Chinese Hamster Ovary cells that stably express the human enzyme (A. Billich et al., manuscript in preparation). Briefly, the enzyme contained in the microsomal fraction of the cells was solubilized using Triton X-100. The protein then was carried through a sequence of anion exchange and affinity chromatography (DEAE Sephacel, ConA Sepharose, Blue Sepharose); further purification was achieved on PBE94 chromatofocusing and Sephadex G-100 gel filtration columns. The purity of the final enzyme preparation was estimated to > 95%. The identity of the protein was confirmed by its reaction with anti-STS polyclonal and monoclonal antibodies in ELISA and on Western blots (kindly provided by Dr J-I Kawano, Miyazaki, Japan [36]) and by N-terminal sequencing.

2.4. Assay of purified human STS

Sulfatase activity was assessed using a method originally described by Eto et al. [37] with modifications. The assay was conducted in white 96-well microtiter plates (Packard). To 100 μl 0.75 mM 4-methylumbelliferyl sulfate (Sigma) in buffer (0.1 M Tris–HCl, pH 7.5, 0.1% Triton), were added 0.6 enzyme units contained in 50 μl buffer (we define 1 U as the amount of enzyme producing 1 nmol 4-methylumbelliferone per hour). After incubation for 60 min at 37°C, 100 μl 0.2 M NaOH were added and fluorescence was measured (λ_{ex} = 355 nm, λ_{em} = 448 nm) using a Titertek reader. For inhibition studies, compounds were included at graded concentrations from stock solutions in ethanol; final ethanol content did not exceed 2%. IC_{50} values were calculated using non-linear regression (GraFit, Erithacus Software Ltd.). Since IC_{50} values measured for irreversible inhibitors strongly depend on incubation conditions, we always performed parallel determination of **1** as a reference, which yielded $IC_{50} = 56 \pm 6$ nM.

2.5. Culture of MCF-7 cells

RPMI 1640 medium (with and without Phenol red) and fetal calf-serum (FSC) was bought from Life Technologies. To remove steroid hormones, FSC was incubated with 0.5% dextran-coated charcoal (DCC; Sigma) at 4°C overnight; then the medium was decanted and DCC filtered off (0.2 μm). This procedure was carried out three times.

For maintenance of MCF-7 cells, they were routinely cultivated in RPMI containing 5% FCS and 10 nM estradiol at 37°C under 5% CO_2. Estradiol was included in these long-term cultures in order to prevent loss of estrogen-responsivenes. To deplete cells from estradiol prior to experiments, they were cultured for 10 days in the absence of the hormone in Phenol red-free RPMI containing 5% FCS. Phenol red-free trypsin was used to detach cells from such hormone-depleted cultures.

2.6. Measurement of STS activity in cell cultures

Cells were seeded into culture plates (2.4 cm diameter) in RPMI medium containing 5% FCS and incubated until confluence was reached. Then the medium (2.0 ml) was changed to serum-free RPMI medium and 0.2 μCi [^{3}H]estrone sulfate was added (final concentration: ≈2 nM). Inhibitors were included at graded concentrations in triplicate experimental wells. They were added from stock solutions in ethanol; total solvent concentration did not exceed 0.2%. Positive control wells received solvent only. After incubation for 24 h at 37°C, the culture medium was harvested. A 1.4-ml aliquot of the medium was extracted with 4 ml toluene. The organic layer was removed and radioactivity in a 2-ml aliquot was quantified by liquid scintillation counting. To correct for trace amounts of toluene extractable radioactivity contained in the original estrone sulfate solution or their possible non-enzymatic formation during the incubation period, medium controls consisting of cell-free medium plus radiolabeled estrone sulfate were processed similarly; radioactivity in medium controls was usually 20 times lower than negative controls. IC_{50} values were calculated from the data (radioactivity in experimental wells and in positive control wells, each corrected by the medium control value) using non-linear regression. We found that the inhibition of metabolite formation in the medium was paralleled by inhibition in the cellular compartment and for convenience we chose to analyse the medium only.

To demonstrate the conversion of estrone sulfate to desulfated products, toluene extracts were evaporated to dryness; the residue was redissolved in mobile phase (acetonitrile/10 mM ammonium sulfate, pH 6, 40:60) and analysed by HPLC (Beckman Ultrasphere Octyl 25×4.6 mm column, isocratic elution with mobile phase at 1 ml/min; peak detection by continuous flow scintillation counting). Metabolites were identified by co-migration with respective ^{14}C-labeled steroids.

2.7. MCF-7 cell proliferation assay

Cells were seeded into Costar 24-well plates at a density of 20 000 cells/cm^2 ($\approx$ 40 000 cells/well) in Phenol red-free RPMI medium containing 2.5% DCC-treated FCS. The cells were incubated for 5 days with one medium change on day 3 to allow for estrogen depletion from the cells. On day 5, the medium was changed again and test compounds were added at graded concentrations from stock solutions in ethanol or DMSO (solvent concentrations did not exceed 0.1%); each concentration was tested in triplicate experimental wells. Negative controls (no test compound added) received solvent only. Medium was changed on day 8 with concomitant fresh addition of test compounds; incubation was continued until day 10.

Cell numbers were determined: (i) in parallel plates on day 5 (i.e. before addition of test compounds); and (ii) on day 10, using the sulforhodamine staining method as described by Skehan et al. [38]. Results are reported either as absolute cell numbers per well with S.D. or as '% stimulation of proliferation', i.e.

$$\left[\left(\frac{\text{increase in cell number between day 5 and 10 for experimental wells}}{\text{increase in cell number between day 5 and 10 for negative controls}}\right) \times 100\right] - 100.$$

To assess the effect of STS inhibitors on the estrone sulfate-stimulated proliferation of MCF-7 cells, the same schedule as outlined above was followed, with experimental wells receiving 100 nM estrone-free estrone sulfate on day 5 plus inhibitor at graded concentrations. Two sets of controls were included in each experiment. (i) Negative control wells were the same as experimental wells, except that after day 5, they were continued in estrogen-free medium. These wells represented the number of cells in the absence of estrone sulfate stimulation of cell proliferation. (ii) Positive control wells were the same as experimental wells, except that they lacked STS inhibitors. Estrone sulfate-dependent cell proliferation was determined as the number of cells in the experimental wells minus the number of cells in the negative control wells. Inhibition of cell proliferation by the test compounds was expressed as percentage of the positive control:

$$\left(\frac{\text{cell proliferation in the presence of inhibitors}}{\text{cell proliferation in the absence of inhibitors}}\right) \times 100.$$

Experiments on the DHEAS-stimulated growth of MCF-7 cells were conducted in analogy, with 10 μM DHEAS being added in place of estrone sulfate.

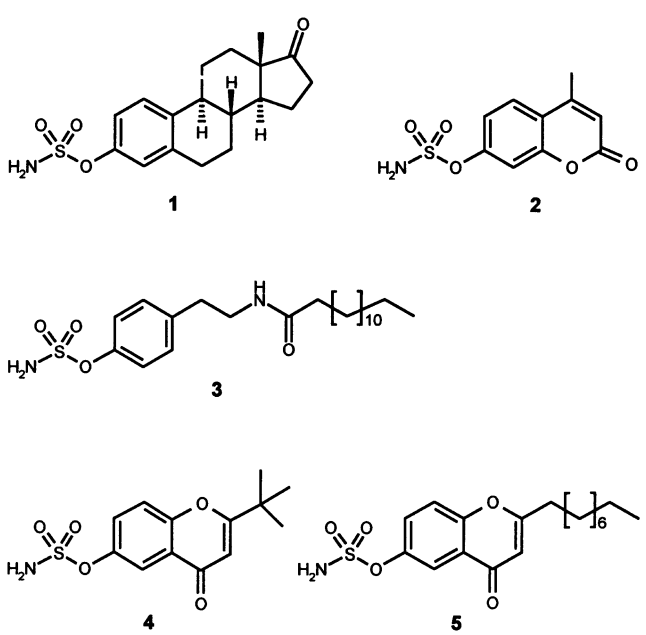


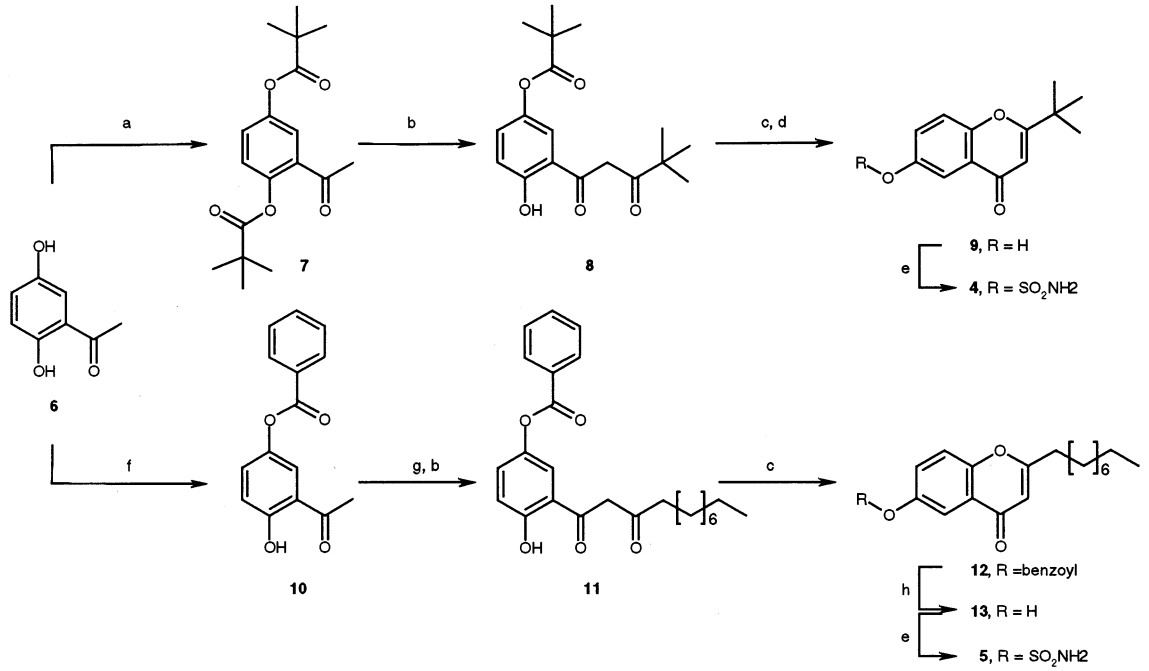
Fig. 1. Chemical structures of STS inhibitors.

3. Results

3.1. Synthesis of non-steroidal STS inhibitors

We designed and synthesized novel non-steroidal aryl sulfamates (compounds **4** and **5**, Fig. 1) featuring a 4-chromenone ring system as steroid sulfatase inhibitors. The bicyclic ring system serves as estrone AB-ring mimicry and the alkyl groups fill the space of the steroidal CD-rings. The sulfamate functionality is attached to the benzene ring in diagonally opposite position relative to the alkyl groups in the heteroaromatic ring, which is important for high inhibitory potency.

The synthesis of **4** and **5** was designed to first generate the 6-hydroxychromen-4-one system and to introduce the sulfamate moiety in the final step (Scheme 1). The 2-substituted 4-chromenones were prepared from a common starting material, i.e. 2,5-dihydroxy-acetophenone (**6**), following the routes depicted in Scheme 1.



Scheme 1. Synthetic routes for the synthesis of **4** and **5**. (a) 2.5 equ. pivaloyl chloride, pyridine, rt, 18 h; (b) NaH, DMF, 0–5°C, 2 h; (c) HCOOH, 100°C, 1 h; (d) HCl, 100°C, 1 h; (e) NaH, DMF, 3 equ. $ClSO_2NH_2$, rt, 3 h; (f) benzoyl chloride, Et_3N, CH_2Cl_2, rt, 16 h; (g) decanoyl chloride, pyridine, rt, 3 h; (h) 10% aqu. KOH, dioxane, rt, 1 h.

For the synthesis of the 2-*t*-butyl derivative, the pivaloyl group was used as both building block and hydroxy protecting group (**7**), whereas for the 2-nonyl derivative the hydroxy group in position 5 of the starting material was selectively protected by benzoylation (**10**). Treatment with sodium hydride effected the O→C migration of the acyl residues within the hydroxyacetophenone moiety without harming the protecting groups in position 5. The resulting crude (3-oxopropio)phenones **8** and **11** were then cyclyzed to the protected 2-alkyl chromenones using formic acid without prior purification. For the 2-*t*-butyl series, we developed a one pot reaction for the cyclization and the deprotection step. Thus, cleavage of the pivaloyl protecting group was accomplished just by adding aqueous hydrochloric acid to the reaction mixture. In the 2-nonyl series the protected chromenone **12** was isolated and subsequently deprotected by treatment with aqueous potassium hydroxide in dioxane to give **13**. Finally, the hydroxychromenones **9** and **13** were transformed into the corresponding sulfamates by reaction of their phenolates, generated by treatment with sodium hydride, with amidochlorosulfonic acid.

3.2. Inhibition of STS

The compounds were tested for inhibition of human STS. In contrast to previous authors (e.g. Refs. [27,28,31]), we used a highly purified enzyme instead of microsomal fractions or cell extracts. In place of radiolabeled estrone sulfate, 4-methylumbelliferyl sulfate as a synthetic substrate was used as a convenient assay. We demonstrated that relative IC_{50} values of compounds measured with both substrates are identical (data not shown). Table 1 summarizes the IC_{50} values obtained under the specified conditions. Compounds **4** and **5** are substantially more potent than the previously described non-steroidal inhibitors 4-methylumbelliferyl sulfamate (**2**) and (*p*-*O*-sulfamoyl)-*N*-tetradecanoyltyramine (**3**) (see Fig. 1 for structures); compound **4** is more active than the steroidal prototype inhibitor estrone sulfamate (**1**). Compounds **4** and **5** were found to be irreversible inhibitors of the STS by using the charcoal-binding method described by Purohit et al. [39] (data not shown).

The activity of STS in MCF-7 breast cancer cells was measured by incubating confluent monolayer cells with radiolabeled estrone sulfate for 24 h; the estrone sulfate concentration was ≈ 2 nM, which is about the physiological concentration in the plasma of postmenopausal women [26]. Subsequently, the culture medium was removed and the desulfated products contained in the medium were extracted with toluene and quantified by liquid scintillation counting. We determined a substrate cleavage rate of 204 ± 32 fmol/24 h/10^6 cells. As seen by HPLC analysis, the toluene-extractable metabolites of estrone sulfate produced by MCF-7 cells were estrone and estradiol in a ratio of $\approx 10{:}1$ after the 24 h incubation period (data not shown). Addition of sulfatase inhibitors during incubation of the cells with the

Table 1
Activity of STS inhibitors in MCF-7 breast cancer cells

No.	Inhibition of STS		Inhibition of estrone sulfate-induced proliferation	Stimulation of proliferation at 1 μM (%)[a,c]
	Isolated enzyme IC_{50} (nM)[a]	Enzyme in MCF-cells IC_{50} (pM)[a]	IC_{50} (pM)[a,b]	
1	56 ± 8	21 ± 4	None	123 ± 20
2	1680 ± 250	23 000 ± 8000	400 000 ± 60 000	0
3	1176 ± 170	593 ± 158	110 000 ± 40 000	20 ± 12
4	21 ± 4	47 ± 5	40 ± 20	100 ± 13
5	203 ± 26	89 ± 11	95 ± 25	0

[a] Mean of three independent experiments, ± S.D.
[b] Inhibition of MCF-7 cell proliferation stimulated by 100 nM estrone sulfate.
[c] Stimulation of MCF-7 cell growth in estrogen-depleted medium.

substrate blocked formation of these metabolites. Also, the ratio of estrone to estradiol was increased in presence of inhibitors, at concentrations near to the IC_{50} to ≈20:1. IC_{50} values are summarised in Table 1. Our determination for estrone sulfamate (**1**) ($IC_{50} = 21$ pM) is in acceptable agreement with a published value of 65 pM obtained in an analogous experimental setting [28]. The new non-steroidal sulfamates (**4**, **5**) were 2- to 4-fold weaker inhibitors than estrone sulfamate, yet with IC_{50} values below 100 pM. In contrast, the reference compounds **2** ($IC_{50} = 23$ nM, published value [28]: 380 nM) and **3** ($IC_{50} = 0.6$ nM) were found to be much less active.

3.3. Inhibition of estrone sulfate-stimulated proliferation of MCF-7 cells

In line with previous reports [40], we observed stimulation of the proliferation of hormone-depleted MCF-7 cells by estrone sulfate (see Fig. 2 for dose–response curve). To test the effect of sulfatase inhibitors, we incubated the cells with a fixed concentration of estrone sulfate (100 nM, producing about half-maximal stimulation) and simultaneously with graded concentrations of the test compounds. Cell proliferation during a 5-day incubation period corrected for cell growth in negative control wells (no inhibitor/no estrone sulfate) was measured as percentage of positive control (estrone sulfate only).

Estrone sulfamate (**1**) (Fig. 3A) did not block estrone sulfate-stimulated proliferation, but rather enhanced cell growth at concentrations ≥1 nM. When MCF-7 cells were incubated with estrone sulfamate alone in estrogen-depleted medium, a >2-fold stimulation of cell growth was observed at a concentration of 1 μM (see Table 1, last column). Compounds **2** and **3** blocked estrone sulfate-stimulated cell proliferation (Fig. 3A), with IC_{50}-values of 400 and 110 nM, respectively, while showing no or only a marginal growth-stimulating effect (Table 1). The value for compound **3** is in acceptable agreement with a published IC_{50} of 39 nM in a similar experimental setting [27].

Compounds **4** and **5** exhibited much lower IC_{50}-values of 40 and 95 pM, respectively (Table 1). In the case of compound **4**, cell proliferation was stimulated at higher concentrations, leading to a U-shaped dose–response curve (Fig. 3B). The growth-stimulating effect of **4** was abolished when including the estrogen-receptor antagonist nafoxidine at 1 μM during the incubation period, indicating that this compound stimulates cell growth via an estrogen-like effect. In contrast, compound **5** inhibited estrone sulfate-induced cell growth completely, also at the high concentrations (Fig. 3B).

3.4. Inhibition of DHEAS-stimulated proliferation of MCF-7 cells

In line with earlier work [21,41–44], we observed stimulation of MCF-7 cell proliferation by ADIOL, DHEA and DHEAS; in our hands, half-maximal stimulation occurring at concentrations of 5×10^{-10}, 1×10^{-8} and 3×10^{-6} M, respectively (Fig. 2). The stimulation induced by 10 μM DHEAS was inhibited by compound **5** with $IC_{50} = 4.8 \pm 3$ nM ($n = 4$).

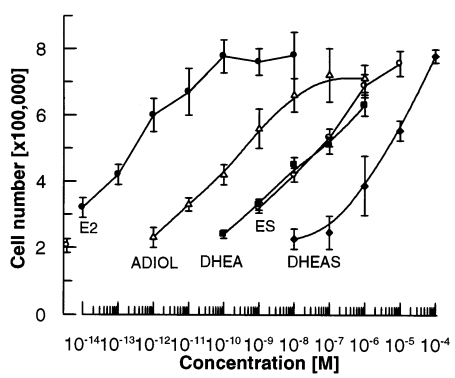


Fig. 2. Stimulation of MCF-7 breast cancer cell proliferation by estradiol (E2, ●); ADIOL (△); DHEA (■); estrone sulfate (ES, ○); and DHEAS (◆).

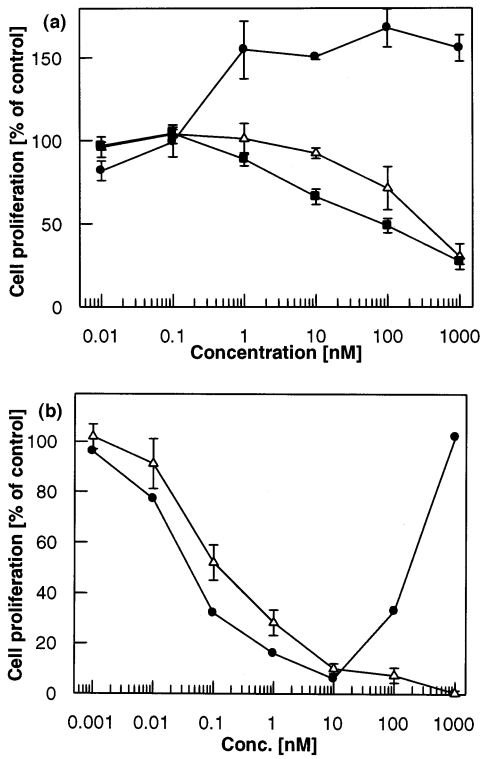


Fig. 3. Effect of STS inhibitors on the proliferation of MCF-7 cells stimulated by estrone sulfate (≈ 2 nM). (A) Compound **1** (●), **2** (△) and **3** (■). (B) Compound **4** (●) and **5** (△).

We asked whether aromatase inhibitors would block stimulation of MCF-7 cell growth by ADIOL, DHEA or DHEAS. Therefore, we incubated cells with the steroids alone or in combination with the aromatase inhibitors lentaron, letrozole and aminoglutethimide, each of the latter being used at a concentration of 1 μM. The results summarised in Table 2 show that lentaron and letrozole did not influence the stimulation of the MCF-7 cells, while aminoglutethimide inhibited the steroid-stimulated growth by 30–50%. The STS inhibitor **5** at 1 μM completely blocked the stimulation exerted by DHEAS, but had no effect on the stimulation effected by the non-sulfated steroids. The estrogen-receptor antagonist nafoxidine at 1 μM completely blocked stimulation by all three steroids (data not shown).

4. Discussion

The most active inhibitors of steroid sulfatase reported so far are aryl sulfamates, featuring as aryl moiety either 3-hydroxy steroids [28–31], 7-hydroxy-coumarins [28,34], or *N*-alkanoyl-phenylalkyl amines [27,32,35]. We have identified a novel non-steroidal chemotype, exemplified by compounds **4** and **5**, which exhibit a chromenone group. The present compounds are irreversible enzyme inhibitors; as such, their IC_{50} values strongly depend on the assay conditions (incubation time, enzyme concentration, K_m and concentration of substrate). Therefore, it is not valid to compare, e.g. the reported IC_{50} for **3** (55.8 nM [35], determined on microsomal fractions with estrone sulfate as substrate) with our values (purified enzyme, synthetic substrate). However, the side-by-side comparisons performed here show that compounds **4** and **5** have inhibitory potency against the isolated enzyme comparable to the steroidal inhibitor **1** (0.4-4-fold IC_{50}), and are 6- to 80-fold more potent than the non-steroidal inhibitors **2** and **3** in our enzyme assay. One may further conclude that inhibitors **4** and **5** are significantly more potent than the previously described series of 7-hydroxy-coumarins [28], and *N*-alkanoyl-phenylalkyl amines [27,32,35].

A similar ranking of the compounds is observed when measuring enzyme activity in intact MCF-7 cells, with compounds **4** and **5** showing IC_{50} values below 100 pM. The only major difference to the situation in the assay using purified STS is that the coumarin derivative **2** is much weaker in the cellular assay. This may be attributed to the at least 20-fold faster hydrolysis of the sulfamic acid ester in **2** as compared to the other compounds (AB, unpublished observations), which becomes more important in the 24-h cell assay than in the 1-h enzyme assay.

When comparing the absolute IC_{50} values between the assays using isolated enzyme and intact cells, one has again to take into account the strong dependence on incubation time and on enzyme concentration. This explains the very low IC_{50} values (pM-range) observed with the MCF-7 cells, where the enzyme activity is low and incubation time is 24 h. Importantly, compounds **4** and **5** not only block the enzyme activity in cultured

Table 2
Influence of aromatase inhibitors on the stimulation of MCF-7 cell growth by ADIOL, DHEA and DHEAS

Stimulating steroid	Concentration	% Stimulation of proliferation in presence of 1 μM of			
		No inhibitor	Lentaron	Letrozole	Aminoglutethimide
ADIOL	1 nM	263 ± 29	286 ± 32 (n.s.)[a]	221 ± 32 (n.s.)	116 ± 13**
DHEA	100 nM	233 ± 20	212 ± 10 (n.s.)	200 ± 35 (n.s.)	118 ± 30**
DHEAS	10 μM	176 ± 12	184 ± 8 (n.s.)	172 ± 3 (n.s.)	123 ± 12**

[a] In parantheses: Student's *t*-test in comparison to 'no inhibitor'. n.s., not significant ($P > 0.05$); ** $P < 0.01$.

MCF-7 cells with IC_{50} values in the range of $\approx$50–100 pM, but also inhibit the estrone sulfate-stimulated proliferation of MCF-7 cells at concentrations comparable to those needed to block the enzyme. This indicates that the antiproliferative effect is due to inhibition of the sulfatase, as expected.

Besides potency against the enzyme, another critical property of STS inhibitors is estrogenicity. All aryl sulfamates are slowly degraded to the corresponding phenols in aqueous solution (AB, unpublished observations) and these phenols may be ligands for the estrogen receptor(s): it is conceivable that the aryl moiety of inhibitors that tightly fit into the active site of STS (which accommodates estrone sulfate) also fits into the binding pocket of the estrogen receptors (ER) α and β (which bind, e.g. estrone). As expected from an earlier report on estrogenicity of **1** in vivo [45], we found a significant growth-stimulating effect of **1** (where the phenol is estrone) towards MCF-7 cells. Published data on the activity of **1** in estrone sulfate-stimulated MCF-7 cells are somewhat conflicting: Selcer et al. [46] found an IC_{50} of 100 nM; Purohit et al. [47] found stimulation of growth at lower concentration (10 and 100 nM), no effect at 1 μM and inhibition at 10 μM. In both cases, the concentrations reported to produce inhibition are higher by at least a factor of 5000 than expected from the activity of the compound in blocking the enzyme activity in the same cells (IC_{50} $\approx$20 pM).

In contrast, compound **4** showed some degree of estrogen-like effect only at high concentrations. This estrogenicity is in principle an unwanted property for compounds that are intended for use in estrogen-suppression therapy. Future experiments in vivo will have to show how large the therapeutic window between sulfatase inhibition and estrogenic effects might be for such a compound. With compound **5**, however, we have identified one inhibitor that is devoid of estrogenic activity on MCF-7 cells. While recently steroidal inhibitors of the sulfatase with reduced estrogenicity have been reported [29,30], the chromenone derivative **5** is the first example of a highly potent non-steroidal sulfatase-blocker, which at the same time is non-estrogenic.

We have confirmed earlier studies [19,42] showing that adrenal steroids (DHEAS, DHEA and androstenediol) are capable of stimulating breast cancer cell growth. In our hands, DHEAS concentrations needed to stimulate MCF-7 cell growth (half-maximal stimulation at $3{\cdot}10^{-6}$ M) are in the physiological range (plasma levels in postmenopausal women: 0.9–5.4 μM [26]). We have now shown for the first time that DHEAS-stimulated growth of these cancer cells can be blocked by a steroid sulfatase inhibitor. The different IC_{50}-values for compound **5** in inhibiting DHEAS- and estrone sulfate-stimulated growth (4.8 and 0.09 nM, respectively) are most readily explained by the very different concentrations of DHEAS (10 μM) and estrone sulfate (100 nM) used in the assays. While this work was in progress, a comparison of the effects of estrone sulfate and DHEAS on the proliferation of MCF-7 and T47D breast cancer cells was published [43]; the authors conclude that DHEAS but not estrone sulfate may be the relevant substrate for the sulfatase pathway. In any case, compounds like derivative **5** exhibit sufficient potency to block utilisation of both steroid sulfates.

Androstenediol, as the product of the subsequent action of STS and 3β-hydroxysteroid dehydrogenase on DHEAS, acts as an estrogen [19,42], also in the presence of aromatase inhibitors [19]. Androstenediol has $\approx$20-fold lower affinity to the ER than estradiol [19] and in our hands stimulated MCF-7 cell growth at $\approx$500-fold higher concentrations than estradiol. However, tissue levels of androstenediol in breast tumours of postmenopausal women ($\approx$2.5 nmol/kg [24]) are higher than the concentration needed for half-maximal stimulation of MCF-7 cells (0.5 nM) determined here.

The specific and potent aromatase inhibitors letrozole and lentaron failed to block DHEA(S)-stimulated growth. We conclude that metabolism of DHEAS to aromatic estrogens, such as estradiol, is not important in these cells. On the other hand, we have confirmed the observation of Le Bail et al. [44] that aminoglutethimide partially inhibits the growth of MCF-7 cells stimulated by DHEA(S) and some other steroids. We assume that the partial inhibition exerted by this drug can be attributed to its known action as an inhibitor of a variety of cytochrome P450-dependent enzymes [48]. In consequence, it appears that at least in MCF-7 cells, stimulation by DHEAS occurs via an aromatase-independent pathway, which is susceptible to STS inhibition. It remains to be studied whether other estrogen-dependent tumour cell lines and finally, tumours in vivo respond similarly.

The existence of an aromatase-independent pathway of stimulation of tumour cells by a sulfated estrogen precursor, namely DHEAS, gives further justification to consider STS inhibitors as potential drugs in the therapy of breast cancer. The new inhibitors **4** and **5** appear as suitable tools to explore this therapeutic modality further, e.g. in tumour models in vivo.

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