Estrogenic Potential of 2-Alkyl-4-(thio)chromenone 6-*O*-Sulfamates: Potent Inhibitors of Human Steroid Sulfatase

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2-Alkylchromen-4-one 6-*O*-sulfamates, a new class of potent steroid sulfatase (STS) inhibitors, were evaluated for their estrogenic potential. Structure−activity relationships for estrogenic activity were identified; however, no correlation with STS inhibition was found. Estrogenicity is favored by bulky side chains and can be effectively abrogated by an (additional) linear substituent. Compound **2g**, which lacks estrogenicity while potently inhibiting STS, has an ideal in vitro profile for the treatment of breast cancer.

Introduction

The enzyme steroid sulfatase (EC 3.1.6.2, STS) has emerged as a highly attractive target for the therapy of a number of disorders. It catalyzes the hydrolysis of the sulfate esters of 3-hydroxy steroids, which are inactive transport or precursor forms of the active 3-hydroxy steroids. Inhibitors of STS are considered as potential new therapeutic agents for the treatment of estrogen- and androgen-dependent disorders.[1] Indications range from cancers of the breast, endometrium, and prostate[2] to androgenic alopecia (male-pattern hair-loss)[3] and acne.[4] In addition, STS inhibitors are proposed to have positive effects on cognitive dysfunction.[5,6]

Estrogenicity of an STS inhibitor at pharmacologically relevant concentrations, as observed with the prototype inhibitor estrone 3-*O*-sulfamate[7] (**1**, Figure 1), is prohibitive for its use in the treatment of estrogen-dependent diseases; in fact, the estrogenic effect of **1** has been documented both in cellular systems[8] and in vivo.[9] So far it has not been elucidated whether this is a direct effect of the sulfamate **1** or of estrone (E1) and its metabolites. E1 may be formed from **1** either by metabolism or by chemical hydrolysis. We and others observed that aryl sulfamate-type STS inhibitors including **1** show slow degradation to the corresponding phenols in solution[9,10] (about 10% hydrolysis of **1** to estrone within 24 h in buffered aqueous solution, pH 7.5; our unpublished observations). In any case, a number of groups have aimed at the synthesis of steroidal and nonsteroidal sulfamate-type STS inhibitors lacking estrogenicity.[11]

Recently, we reported on 2-substituted 4-(thio)-chromenone 6-*O*-sulfamates as highly potent STS inhibitors.[12] For two prototype compounds (**2b** and **2d** in this paper, Table 1) we showed previously that they can block the growth of MCF-7 cells after stimulation by either estrone sulfate or dehydroepiandrosterone sulfate, the latter also serving as a source for estrogenic steroids.[8] From further results of this initial study we concluded that **2b** lacks estrogenic effects even at high concentrations, whereas **2d** showed some degree of
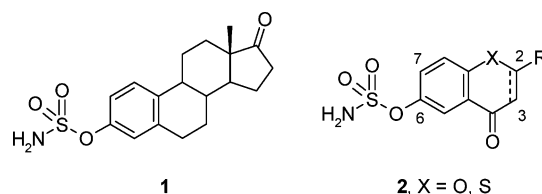

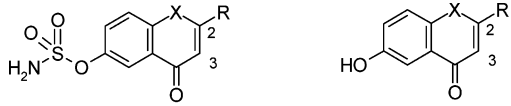**Figure 1.** Structures of estrone sulfamate (**1**) and the chromenone-based sulfamates **2**.

estrogen-like activity at a concentration of 1 μM (to be compared to the IC$_{50}$ values of <100 pM against STS in cellular systems). In the present study, we report on the detailed evaluation of the potential estrogenicity of a selection of chromenone-based STS inhibitors (**2**, Figure 1), as well as their phenolic metabolites, in two different systems and discuss the resulting structure−activity relationships (SARs).

Results and Discussion

The (thio)chromenone-based STS inhibitors **2** do not feature the full steroidal backbone, but structural and biological similarities between steroidal estrogens and hydroxy-substituted flavones (= 2-phenylchromenones) are well documented.[13] To assess the estrogenic potential and possible structure−activity relationships of 2-alkyl-(thio)chromenone 6-*O*-sulfamates, we tested compounds **2a**−**j** for their capacity to stimulate the growth of the estrogen-dependent human breast cancer cell line MCF-7. In addition, binding affinity to purified human estrogen receptors (ER) α and β (ED$_{50}$ derived via competition assays using fluorescent E2) was measured for the sulfamates **2**, as well as for the corresponding phenols **3**. Determination of the estrogenicity of the phenols is important, because they can be formed in cells and in vivo (i) by hydrolysis/metabolism of **2**, and (ii) as postulated products after irreversible inhibition of STS by the sulfamates **2**.[7,14] This set of data should allow a solid judgment on the estrogenic potential of a sulfamate-type STS inhibitor. **1**, E1, and E2 were used as reference compounds, where appropriate.

Table 1 lists the results obtained in these assays in comparison to the inhibitory potencies of sulfamates **1** and **2** against purified STS in the enzymatic assay.

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Table 1. Effect of Selected Chromenone-Based STS Inhibitors on STS Activity, Proliferation of MCF-7 Breast Cancer Cells, and Estrogen Receptor Binding

2a-h, X = O   **3a-h**, X = O
2i,   X = S   **3i**,   X = S
2j,   X = O, $\Delta^{2,3}$ hydrogenated   **3j**,   X = O, $\Delta^{2,3}$ hydrogenated

compd	R	STS IC$_{50}$ [nM]	% stimulation^a of MCF cells at			estrogen receptor, ED$_{50}$ [μM]^b				
						sulfamates		phenols		
			1000 nM	100 nM	10 nM	α	β		α	β
17β-estradiol = E2		-	n.t.^{c}	n.t.	400	-	-	E2^d	0.0073	0.011
1	-	56	123	112	97	2.6	3.7	E1^d	0.051	0.070
2a	2-phenylethyl	190	45	33	16	>100	>100	**3a**	26	>100
2b	*n*-nonyl	403	0	0	0	>50	>100	**3b**	>100	>100
2c	1,1-dimethylnonyl	78	0	0	0	>30	>100	**3c**	>100	>100
2d	*tert*-butyl	22	100	45	12	>100	>100	**3d**	64	69
2e	2,2,3,3-tetramethylcyclopropyl	45	120	85	52	30	47	**3e**	0.9	2.6
2f	cyclododecyl	45	118	72	48	>10	>30	**3f**	0.8	1.5
2g	4-pentylbicyclo[2.2.2]-oct-1-yl	11	0	0	0	>100	>100	**3g**	>100	>100
2h	1-adamantyl	5.6	116	88	71	>30	>30	**3h**	1.5	1.4
2i	1-adamantyl	0.34	120	99	78	4.8	18	**3i**	0.21	0.48
2j	1-adamantyl	140	130	90	79	13	14	**3j**	0.10	0.035

^{a} Data are the mean of four culture wells; SD was in the range of 8 to 22%. ^{b} Data are the mean of two to six independent determinations; SD was in the range of 12 to 73%, except for **2i**, where SD was 122% against the ER β. ^{c} n.t., not tested. ^{d} E1 = estrone, E2 = 17β-estradiol.

The estrogenic STS inhibitor **1** stimulated growth of MCF-7 cells by 97% at a concentration of 10 nM (to be compared with 400% stimulation for the reference compound and estrone metabolite 17β-estradiol (E2)). Sulfamate **1** also displayed weak affinity to the estrogen receptors α and β, whereas the corresponding phenol E1 and its metabolite E2 showed high affinity to the estrogen receptors (ED$_{50}$ values of 51 and 70 nM for E1; 7.3 and 11 nM for E2). We conclude that the estrogenicity of **1** seen in both assay systems is due to its hydrolysis to E1 (and further conversion to E2 in the cellular system), similar to the effect of estrone sulfate which yields estrone by enzymatic hydrolysis and stimulates MCF-7 proliferation by 250% at 10 nM.[8] Furthermore, the high in vivo estrogenicity observed for sulfamate **1**[9] should be due to its conversion to estrone rather than to direct binding to the estrogen receptors.

Most of the chromenone-based STS inhibitors (**2a–d,f–h**), did not show measurable affinity to the purified estrogen receptors. Similar to the situation with **1**, compounds **2e,i,j** showed weak, variable activity (4.8 to 47 μM), most likely due to trace contamination with phenols **3e,i,j**, which themselves exhibited strong binding to both estrogen receptors. These findings are in line with the fact that estrone sulfate does not bind to estrogen receptors;[15] therefore, affinity of the sulfamates to the receptors was not to be expected.

In the cellular assay system some sulfamates (**2b,c,g**) were inactive up to the highest test concentration of 1 μM, whereas others clearly stimulated the growth of the MCF-7 cells even at 10 nM. These results for the sulfamates **2** correlated nicely with the activities for the corresponding phenols **3** in the receptor assays, supporting again the postulate that estrogenic activity of sulfamate-type STS inhibitors is caused by their degradation products, the phenols. Furthermore, the data confirm that our in vitro assays are useful to assess the estrogenic potential of members of this compound class.

According to the test results, the compounds can be classified in three groups: compounds with no estrogenic potential (**2b,c,g**), with weak-to-moderate estrogenic potential (**2a,d**), and with high estrogenic potential (**2e,f,h–j**) similar to **1**. When comparing the profiles within this group of compounds, it becomes obvious that STS inhibitory potency and estrogenic activity do not correlate at all. This conclusion is also supported by the observation that the phenolic counterparts of sulfamate-type STS inhibitors are consistently devoid of STS inhibitory activity (including E1, E2, and representative examples of compounds **3**; data not shown).

In detail, the results can be discussed as follows: Derivative **2a** shows weak stimulatory effect in the MCF-7 assay, whereas its corresponding phenol **3a** does not show significant binding to the ERs. Compounds **2b,c,g** and **3b,c,g** are essentially devoid of any estrogen-like activity in both assay systems. The common feature of these analogues is an extended, linear aliphatic group in the substituent (R) at the chromenone ring. In contrast, compounds with very bulky but shorter side chains (**2e,f,h–j**) show marked growth stimulatory activity in MCF-7 cells even at 10 nM. Analogues **2h–j** featuring the 1-adamantyl residue are almost as effective as **1** in that respect. Phenols **3e,f,h–j** also show binding affinity to the estrogen receptors α and β, without any preference for either subtype, although their activity is substantially lower compared to that of E1. Only the phenolic derivatives **3b,c,g** of nonestrogenic compounds **2b,c,g** do not bind to the estrogen receptors at concentrations up to 100 μM.

The results suggest that, within the series of chromenone-based STS inhibitors, estrogenic activity increases with the bulkiness of the side chain R (**2d** < **2e** < **2h–j**) and that this can be effectively abrogated by introduction of an additional linear substituent (**2c,g**). Compound **2d** with the *tert*-butyl side chain shows moderate estrogenic potential (45% growth stimulation of MCF-7 cells at 100 nM). An increase in steric bulkiness of the side chain, as in the 2,2,3,3,-tetramethylcyclopropyl

analogue **2e**, results in higher estrogenic effects in the cellular assay (52% growth stimulation at 10 nM) and in increased binding to the estrogen receptors. The spherelike adamantyl moiety present in **2h**–**j** is the most bulky residue investigated and gives rise to strong cellular estrogenic activity, comparable to that of **1**, irrespective whether the core element is a chromenone (**2h**), a thiochromenone (**2i**), or a chromanone (**2j**) ring system. When the bulky residue bears an additional linear moiety, as in **2g**, no estrogenic effect is observed. This result demonstrates that the STS inhibitory potency and the estrogenic activity can be separated in the series of 2-substituted 4-chromenone 6-*O*-sulfamates. Compound **2a** shows only a weak estrogenic effect in the cellular assay (45% growth stimulation at 1 μM), which is consistent with the SAR derived thus far. First, the end of the side chain of **2a** is not linear, so that some estrogenic potential has to be expected. Second, the bulky component of the side chain, which would cause estrogenicity, is not as sterically demanding as an adamantyl residue and is connected to the bicyclic core element via a spacer. Results from the analogue for a direct comparison (**2**, R = phenyl) are not available. Nevertheless, it can be concluded that direct attachment of the bulky residue to the chromenone system is essential for strong estrogenic activity.

In summary, we could demonstrate for potent STS inhibitors **2** that estrogenic activity does not correlate at all with the inhibitory potency against STS and that structure–activity relationships for their estrogenic potential exist. In vitro estrogenicity of **2** increases with the bulkiness of the side chain R attached to the chromenone core moiety. Strong estrogenic effects are caused by highly bulky side chains but can be effectively abrogated by introduction of an (additional) linear substituent. Thus, compounds **2b**,**c**,**g** featuring such a linear substituent are completely devoid of any estrogenic activity in the cellular assay. The phenols **3** follow the same SAR regarding potency in the ER binding assay. For example, the phenols **3b**,**c**,**g** of the nonestrogenic sulfamates **2b**,**c**,**g** also show no estrogenic potential. On the basis of our data obtained with isolated human estrogen receptors and an estrogen-responsive human cell line, we clearly identified potent, nonestrogenic STS inhibitors. To address the possibility of any in vivo metabolism to estrogenic derivatives, future studies will have to include in vivo estrogenicity assays.

Compound **2g** is the most interesting inhibitor out of the described series considering both the results of the present study (no in vitro estrogenicity) and the inhibitory potency against STS. Therefore we tested **2g** for inhibition of estrogen-dependent MCF-7 cell growth, as reported previously for analogue **2b**.[8] With an IC$_{50}$ value of about 20 pM in this assay, compound **2g** potently blocks estrone sulfate-stimulated cell proliferation and proved to be superior to **2b** (IC$_{50}$ = 95 pM). Thus, **2g** has an ideal in vitro profile for the development as therapeutic agent in breast cancer.

Experimental Section

Test compounds **1**, **2**, and **3** were all published previously[7,12] and were prepared according to published procedures. Biochemicals were obtained from Sigma (St. Louis, MO). Human STS was expressed and purified as described previously.[12]

1. Assay of Purified Human STS. The compounds were tested for their inhibitory potency in a fluorimetric assay using 4-methylumbelliferone sulfate as substrate.[12] IC$_{50}$ values were calculated using nonlinear regression (GraFit, Erithacus Software Ltd.). Values reported here are the mean of triplicate determinations, which typically lie in the range of $\pm 20\%$.

2. MCF-7 Cell Proliferation Assay. 2.1. Culture of MCF-7 Cells. RPMI 1640 medium (with and without phenol red) and fetal calf-serum (FSC) were 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-responsiveness. To deplete cells of 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.

2.2. MCF-7 Cell Proliferation Assay. Cells were seeded into Costar 24-well plates at a density of 20 000 cells/cm² (∼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.[15] Results are reported as "% stimulation of proliferation", i.e.

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

3. Fluorescence Polarization-Based Ligand Displacement Assay for ER-α and ER-β. Test compounds were prepared as stocks either in DMSO or ethanol. The final solvent concentration in assay buffer at each compound concentration was 0.5% (v/v). Competitor screening kits for estrogen receptor-alpha (ER-α) and estrogen receptor-beta (ER-β) were obtained from PanVera Corporation (Madison, WI). These kits contain the reagents necessary to perform competition assays to assess the affinity of test compounds for the human estrogen receptors.[17] ER-α or ER-β was added to a fluorescent estrogen ligand (Fluormone ES2 = FES2, which is 17β-estradiol conjugated with fluorescein), to form a FES2/ER complex with high fluorescence polarization. The complex was then added to test compounds in black 96-well microtiter plates followed by incubation at room temperature for 2–2.5 h; each concentration was tested in triplicate (i.e., three wells per concentration). The final concentration of FES2 was 1 nM. The final concentration of ER was 13 nM and 10 nM in the ER-α and ER-β assays, respectively. Measurements of fluorescence polarization were made with the microtiter plate reader Fluorolite FPM-2 (DYNEX Technologies, Inc., Chantilly, VA). The shift (i.e., reduction) in polarization in the presence of test compounds was used to determine the relative affinity of test compounds for ER-α or ER-β. Data were transferred into Excel for calculations of means and standard deviations. The dose–response curves for each test compound were fitted via the Excel add-on program XLfit (ID Business Solutions, Guilford, Surrey, UK) to a four-parameter logistic equation. The ED$_{50}$ is defined as the concentration of test substance causing a reduction of the fluorescence polarization to a value midway between the values of the high (+FES2,

+ER) and low (+FES2, -ER) solvent controls. This midway point (i.e., the *x* value thereof) was calculated by XLfit via the fitted logistic equation. Compounds were generally tested up to their solubility limits, but accounting also for an artifactual increase in fluorescence polarization at high concentrations, thus, resulting in lower estimates for inactivity (e.g., >10 or >30 μM). Values reported are the means of at least three (for inactive compounds two) determinations.

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