First Dual Aromatase-Steroid Sulfatase Inhibitors

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Abstract: Aromatase inhibitors in clinical use block the biosynthesis of estrogens. Hydrolysis of estrone 3-sulfate by steroid sulfatase is an important additional source of tumor estrogen, and blockade of both enzymes should provide a more effective endocrine therapy. Sulfamoylated derivatives of the aromatase inhibitor YM511 inhibited sulfatase and aromatase in JEG-3 cells with respective IC_{50} values of 20−227 and 0.82−100 nM (cf. letrozole, 0.89 nM). One dual inhibitor was potent against both enzymes in vivo, validating the concept.

Breast carcinoma is the most common form of female cancer. Each year, there are about 38 000 new cases of breast cancer in the U.K., and the lifetime risk of breast cancer in women is 1 in 9.[1] Many breast tumors are initially hormone-dependent, with estrogens playing a pivotal role in supporting the growth and development of such tumors. The highest incidence of breast cancer occurs in postmenopausal women at a time when their ovaries have become nonfunctional and estrogens are synthesized exclusively in peripheral tissues. Since most hormone-dependent breast carcinomas are expected to regress upon estrogen deprivation,[2] many endocrine agents have been developed to interfere with estrogen action or reduce the level of estrogen.

The most widely used forms of endocrine therapy for postmenopausal women with hormone-dependent breast cancer (HDBC) include selective estrogen receptor modulators, primarily tamoxifen, that block estrogen at the receptor level and, more recently, aromatase inhibitors (AIs) that inhibit the biosynthesis of estrogen.

AIs act through inhibition of the cytochrome P450 enzyme aromatase, which catalyzes the conversion of androgens to estrogens. The first-generation AI, aminoglutethimide, became available in late 1970s.[3] Although this agent is efficacious, its widespread use has been limited by both toxicity and lack of selectivity for the aromatase enzyme.[3] A number of steroidal and nonsteroidal inhibitors have now been developed, and the latest generation of AIs that are achieving clinical prominence include exemestane, formestane, anastrozole, and letrozole.[4] Exemestane and formestane are mechanism-based or suicide inhibitors. They compete with the endogenous ligands androstenedione and testosterone for the active site of the enzyme, where they are metabolized to intermediates that bind irreversibly to the active site. Anastrozole and letrozole are selective reversible inhibitors. They compete with the endogenous ligands, excluding both ligands and oxygen from the enzyme active site, by coordinating strongly but reversibly to the heme iron of aromatase via a heteroatom of the inhibitor. Invariably, all nonsteroidal AIs contain a heteroaryl ring and the pharmacophore for this class of AI is a heme-ligating moiety (e.g., a triazole) supported by a scaffold that interacts favorably with those amino acid residues lining the active site of aromatase.

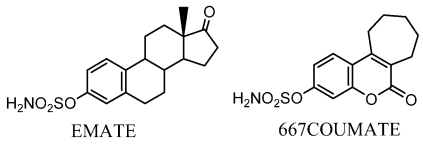

Figure 1. Structures of EMATE and 667COUMATE.

There is now abundant evidence to suggest that the hydrolysis of estrone sulfate (E1S) to estrone (E1) by steroid sulfatase (STS) is the main source of estrogens in tumors.[5−11] In addition, the production of androstenediol (Adiol), and to a lesser extent estradiol, via dehydroepiandrosterone (DHEA) could also significantly contribute to the estrogenic stimulation of hormone-dependent breast tumors.[12−15] Adiol, an androgen that binds to the estrogen receptor and has estrogenic properties, originates from DHEA sulfate (DHEA-S) once it has been hydrolyzed to DHEA by DHEA-sulfatase.[14−18] Therefore, STS inhibitors, when used alone or in concert with an aromatase inhibitor, may enhance the response of hormone-dependent breast tumors to this type of endocrine therapy by reducing not only the formation of E1 from E1S but also the synthesis of other steroids with estrogenic properties, such as Adiol, from DHEA-S via DHEA.

Since the discovery of estrone 3-*O*-sulfamate (EMATE, Figure 1) as a highly potent irreversible inhibitor of STS,[19] considerable progress has been made in developing a number of potent steroidal/nonsteroidal STS inhibitors based on sulfamate esters. These inhibitors are structurally diverse, ranging from derivatives of EMATE,[20] *N*-alkanoylphenylalkylamine sulfamates,[21] bicyclic and tricyclic coumarin sulfamates (e.g., 667COUMATE, Figure 1),[22] and polycyclic fused and nonfused sulfamates[23] to nonfused bicyclic sulfamates.[23a,24] However, all known STS inhibitors share a common pharmacophore, i.e., a phenol sulfamate ester with substituents that exploit favorable hydrophobic interactions with the enzyme active site.[22a] It is thought that such compounds sulfamoylate the enzyme active site in a classical active site-directed fashion.

There is now a strong case to be made to inhibit both the aromatase and STS enzymes in order to achieve a more effective estrogen ablation for treating HDBC. While administering an AI in conjunction with an STS inhibitor as two separate agents might be an obvious choice for a combined endocrine therapy, an attractive alternative approach is to design a dual aromatase and

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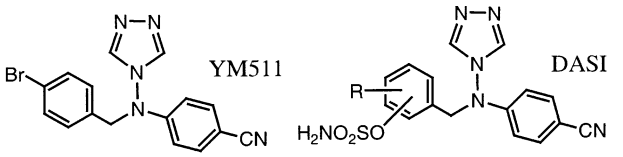


Figure 2. Structure of YM511 and a YM511-based dual aromatase-sulfatase inhibitor (DASI). R is a substituent that enhances inhibition of aromatase and sulfatase.

sulfatase inhibitor (DASI) that will inhibit both enzymes as a single agent. To explore the feasibility of the DASI concept, a design strategy was adopted in which the pharmacophore for STS inhibition is incorporated into a known, highly potent, and selective AI. Such a strategy should introduce STS inhibitory potential to the chosen AI while preserving most of its aromatase inhibitory characteristics.

A new class of nonsteroidal AIs was reported by Okada et al.[25] The best inhibitor in the series, YM511 (Figure 2), which inhibits aromatase potently with a high degree of selectivity, progressed to a phase II clinical trial but was withdrawn for market competitive considerations. Unlike anastrozole and letrozole, which are symmetrical molecules, YM511 is a tertiary amine possessing three different substituents (i.e., a *p*-cyanophenyl group, a *p*-bromobenzyl group, and a 1,2,4-triazol-4-yl group) on a central N atom, thus avoiding the presence of a tetrahedral center and chirality considerations. This latter property allows the implementation of our design strategy for a DASI that requires a group within an AI to be replaced by a moiety containing the pharmacophore for STS inhibition, something not applicable to, for example, letrozole without chirality complications. The *p*-bromobenzyl group of YM511 seemed to be the most attractive group for modification. A general formula for potential DASIs structurally related to YM511 is shown in Figure 2.

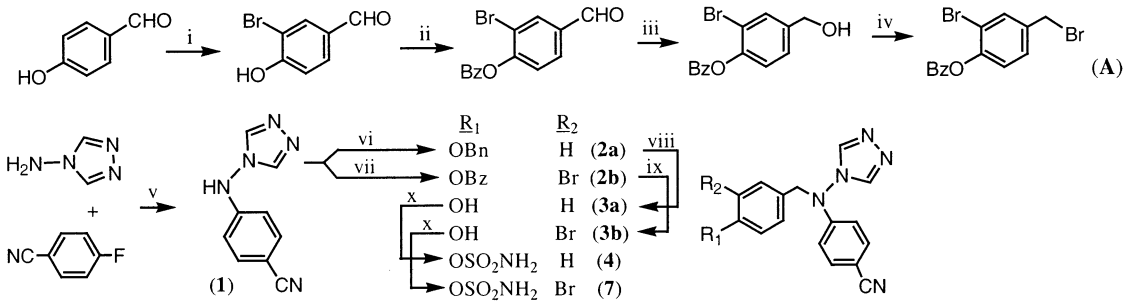
An initial YM511-based DASI candidate (**4**) was synthesized that is structurally different from YM511 only by its *p*-sulfamoyloxybenzyl group. The synthesis of **4** is shown in Scheme 1. 4-[(4-Cyanophenyl)amino]-4*H*-1,2,4-triazole (**1**) was prepared from 4-amino-4*H*-1,2,4-triazole and 4-fluorobenzonitrile essentially according to the method described by Okada et. al.[25] except that **1** crystallized out of the neutralized aqueous workup as small yellow crystals upon standing at room temperature overnight. The triazole **1** was allowed to react with 4-benzyloxybenzyl chloride to give the tertiary amine **2a**. After debenzylation of **2a** by hydrogenation, the resulting phenol **3a** in *N*,*N*-dimethylacetamide (DMA) was sulfamoylated with 2 equiv of sulfamoyl chloride[26] according to the conditions described by Okada et al.[27] to give sulfamic acid 4-{[(4-cyanophenyl)-[1,2,4]triazol-4-ylamino]methyl}phenyl ester (**4**). The biological activities of **4** are shown in Table 1. In JEG-3 cells,[28] the IC_{50} values for **4** to inhibit aromatase and STS were 100 and 227 nM, respectively. Under the same assay conditions, YM511 inhibited aromatase by 50% at 0.5 nM but was not active against STS (Table 1). In comparison, 667COUMATE (Figure 1),[22b] a highly potent nonsteroidal STS inhibitor, inhibited STS by 50% at 3.3 nM. Inhibition with **4** was reversible for aromatase. When JEG-3 cells were pretreated with **4** at 1 μM and cells were then washed to remove unbound inhibitor, the inhibition of aromatase was reduced by 63.5% (from 86.5 ± 1.4% to 23.0 ± 1.0%). Recovery from inhibition of sulfatase was negligible under the same conditions (from 91.1 ± 0.4% to 89.9 ± 0.1%), suggesting that **4**, like other sulfamate-based STS inhibitors, inhibited the enzyme irreversibly. Although **4** is apparently only a relatively weak DASI in vitro, it is nevertheless the first lead compound possessing submicromolar dual aromatase and sulfatase inhibitory properties. It was reasoned that the potency of **4** against both enzymes might be increased by a substituent(s) on its *p*-sulfamoyloxybenzyl moiety that enhances the binding potential of **4** to aromatase and the "sulfamoylating potential" of its sulfamate group.

Okada et. al. have already demonstrated that other para-substituted derivatives of YM511 that contain electron-withdrawing (EW) groups such as halogens are also active against the aromatase enzyme.[25] Our experience from the design of steroidal STS inhibitors has also indicated that substitution at the 2-position of EMATE with halogens increases the STS inhibitory activity (unpublished results). To investigate the effects of halogen as a substituent on the dual aromatase and sulfatase inhibitory activity of **4**, compounds **5**, **6**, and **7** were prepared, which are the *m*-fluoro, *m*-chloro, and *m*-bromo derivatives of **4**, respectively (Scheme 1 for **4** and **7**). In addition, the effect of relocating the sulfamate group of **4** to the meta position of the ring was investigated and **8** was synthesized.

Compounds **5**−**8** were synthesized by alkylating 4-[(4-cyanophenyl)amino]-4*H*-1,2,4-triazole (**1**) with the corresponding protected hydroxyhalobenzyl halide in a manner similar to the synthesis of **4** and **7** as outlined in Scheme 1. The key reagent for preparing **5** is 1-benzyloxy-4-chloromethyl-2-fluorobenzene, which was prepared in three steps from 3-fluoro-4-hydroxybenzaldehyde in an overall yield of 88%. 3-Chloro-4-hydroxybenzoic acid methyl ester, in three steps, was converted to 1-benzyloxy-4-bromomethyl-2-chlorobenzene in an overall yield of 91%, which was used for the preparation of **6**. The key reagent for preparing **7** is 3-bromo-4-benzoyloxybenzyl bromide (**A**, Scheme 1) which was prepared by brominating 4-hydroxybenzaldehyde followed by protection of the phenol as a benzoate. Reduction of the formyl group with sodium borohydride followed by bromination of the resulting primary alcohol with phosphorus tribromide gave 3-bromo-4-benzoyloxybenzyl bromide (**A**). 3-Benzyloxybenzyl bromide is the alkylating agent for the preparation of **8**, which was synthesized by brominating 3-benzyloxybenzyl alcohol using established literature methods. Starting from **1** and the corresponding protected benzyl halide, the overall yields of sulfamates **5**, **6**, and **8** synthesized in three steps were 63%, 11%, and 12%, respectively.

The in vitro inhibitory activities of **4**−**8** against both enzymes are shown in Table 1. It is clear that a halogen substituted at the position ortho to the sulfamate group of **4** significantly increases the potency of the derivatives against both aromatase and STS. For aromatase inhibition, the increase in potency in comparison with **4** is between 8-fold for the fluoro derivative **5** to 120-fold for the bromo derivative **7**. It has been suggested that the EW groups (such as CN and Cl) commonly found in potent nonsteroidal AIs act as hydrogen-bonding acceptors and hence increase the binding affinity of the

Scheme 1. Synthesis of Reagent 3-Bromo-4-benzoyloxybenzyl Bromide (**A**) and DASIs, **4**, and **7**[a]



[a] (i) Br_2/AcOH, 25 °C, 53%; (ii) BzCl/EtOAc/NEt_3, 87%; (iii) $NaBH_4$/THF, room temp, quantitative; (iv) PBr_3/CH_2Cl_2, 0 °C to room temp, 87%; (v) ^{t}BuOK/DMSO, 38%; (vi) NaH/DMF, 0 °C, 4-benzyloxybenzyl chloride, 80−90 °C, 3 h, 94%; (vii) NaH/DMF, 0 °C, **A**, room temp, 18 h, 78%; (viii) Pd/C (10%), THF/EtOH, H_2, 26%; (ix) KOH/MeOH, 77%; (x) 2 equiv of H_2NSO_2Cl/DMA, room temp, **4** (57%) and **7** (44%).

Table 1. In Vitro Inhibition of the Aromatase and STS Activity in a JEG-3 Cells Preparation by YM511 and **4**−**8**[a]

			aromatase	STS
compd	R_1	R_2	IC_{50} (nM)	IC_{50} (nM)
YM511	Br	H	0.5 ± 0.03	
4	OSO_2NH_2	H	100 ± 7.8	227 ± 28
5	OSO_2NH_2	F	12 ± 1.8	40 ± 3.8
6	OSO_2NH_2	Cl	2.3 ± 0.3	20 ± 2.1
7	OSO_2NH_2	Br	0.82 ± 0.3	39 ± 4.2
8	H	OSO_2NH_2	39 ± 10.1	5133 ± 65.4

[a] The extent of in vitro inhibition of aromatase and sulfatase activities was assessed using intact monolayers of JEG-3 cells. Aromatase activity was measured using [1β-^{3}H]androstenedione (30 Ci/mmol) over 3 h. Sulfatase activity was measured using [6,7-^{3}H]E1S (50 Ci/mmol) over 3 h. Each value represents the mean ± SE of triplicate measurements.

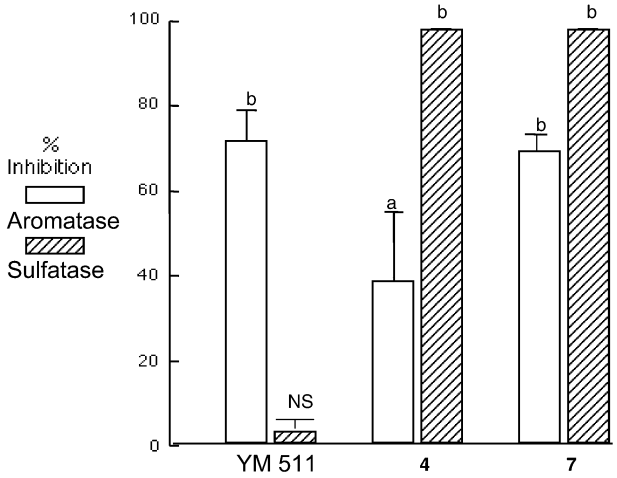


Figure 3. Percentage inhibition of in vivo aromatase and STS activities. Female Wistar rats received a single subcutaneous injection of PMSG (200 IU). Three days later, drugs were administered orally in propylene glycol as a single dose. Blood and liver samples were obtained 3 h after administration. Plasma concentrations of estradiol were measured using a radioimmunoassay kit. Liver STS activity was determined as in reference 20b. Results are expressed as the percentage inhibition of PMSG-stimulated estradiol levels for aromatase or STS activity in untreated animals (mean ± SE, $n = 3$). Where no error bars are shown, SE < 1%. The statistical significance for aromatase and STS activities in control and treated groups was assessed using Student's t test: (a) $p < 0.05$; (b) $p < 0.001$; NS, not significant.

inhibitors for the enzyme active site. Our findings for **5**−**7** do not appear to support a significant involvement of hydrogen bonding in the activities of these compounds because **5**, the 3-fluoro atom of which is the strongest hydrogen-bond acceptor, is the weakest AI in the series. For this reason, other factors such as size and hydrophobicity of the halogens might contribute to the activities of these inhibitors.

While IC_{50} values for the inhibition of STS activity in JEG-3 cells by **5**−**7** are similar, these compounds are about 5- to 11-fold more active than **4**. From our findings in previous work[19,22a] and the inhibitory activities observed for **4** here, **5**−**7** are expected to be irreversible inhibitors. The increase in potency observed for **5**−**7** against STS is in agreement with our findings that 2-halo-substituted derivatives of EMATE are generally more potent STS inhibitors than EMATE. The EW effects of halogens most likely increase the leaving group ability of the corresponding meta-halogenated phenol precursors of sulfamates **5**−**7** and hence their enzyme "sulfamoylation potential" as sulfamates, rendering them better STS inhibitors than **4**. The fact that the potencies of **5**−**7** did not follow the expected EW inductive effect order (i.e., F > Cl > Br) suggests that there are other determining factors for the biological activities of these inhibitors. YM511-sulfamoylated derivatives are anticipated to act as reversible inhibitors of aromatase but irreversible inhibitors of STS. Like YM511, these sulfamoylated derivatives are expected to be selective for CYP19 inhibition.

The inhibitory activities shown by **8** suggest that a relocation of the sulfamate group from the para position of **4** to the meta position of **8** results in a small but significant increase in potency against aromatase. However, such relocation appears to be detrimental to the STS inhibitory property of **8** and the substantial increase in the IC_{50} value suggests that a *p*-sulfamate moiety is required for optimal STS inhibition.

To demonstrate that the DASI concept also works in vivo, the activities of **4** and YM511 were studied in female Wistar rats (weighing 150−200 g). On day zero, rats were given 200 IU/0.1 mL sc of PMSG (pregnant mare's serum gonadotropin). After 3 days, rats were given a single oral dose [10 mg/kg (0.2 mL)] of either YM511 or **4**. The rats were sacrificed 3 h after the drug dosing, and their plasma and liver samples were taken for assessment of estradiol level and sulfatase inhibition, respectively. The results are shown in Figure 3. YM511 reduces the plasma estradiol level by 70% after 3 h of treatment. For **4**, a reduction of plasma estradiol level by 38% is observed after 3 h of treatment. When the rat liver sulfatase activity was assayed,[20b] YM511

showed no significant inhibition, as expected, since it lacks the pharmacophore for STS inhibition. However, DASI **4** showed nearly complete inhibition (≥98%) after 3 h of treatment. These results clearly indicate that the concept of a DASI works in principle and in vivo. We also studied the in vivo activities of **7**, which is the best DASI in vitro. After 3 h of treatment, **7** reduced plasma estradiol level by 68% (Figure 3), a potency similar to that of YM511 (Figure 3). Like **4**, **7** is also highly active against the rat liver sulfatase activity, showing an inhibition of ≥98% after 3 h of treatment (Figure 3). Clearly, structural optimization can lead to highly potent in vivo active dual inhibitors with nanomolar potencies, and further optimization is in progress.

In conclusion, we have validated in this work the novel concept of a dual aromatase and STS inhibitor. For the first time, a small series of DASI candidates have been prepared and found to possess high potency against both enzymes in vitro. In vivo, one example (**4**) shows inhibitory activities against both aromatase and STS. Compound **7**, a *m*-bromo derivative of **4**, shows not only high potency against STS but also a similar potency to YM511 against aromatase, demonstrating that the potency of this class of DASIs can be further optimized. This strategy should be applicable to other AIs and should allow the therapeutic potential of dual inhibition of estrogen formation in breast tumors to be assessed.

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