Discovery of Estrogen Sulfotransferase Inhibitors from a Purine Library Screen

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There is now substantial evidence that sulfated biomolecules (i.e., carbohydrates, proteins, and steroids) contribute to many disease states,[1] including chronic inflammation,[2] HIV-1 infection,[3,4] and hormone-dependent breast tumor growth.[5,6] The sulfate ester is often a key determinant of bioactivity, directing significant attention to the corresponding enzymes, the sulfotransferases, as a new class of therapeutic targets.

Estrogen sulfotransferase (EST) catalyzes the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to estrogen (3,17-*β*-estradiol) and estrogen-like compounds in the cytosol, solubilizing them to maintain hormone homeostasis (Figure 1). The delicate balance of sulfated and nonsulfated estrogens in vivo is dictated by EST and the desulfating enzyme, estrogen sulfatase.[7] Unusually high levels of estrogen sulfate are found in breast tumor cells,[6,8] a discovery which has focused interest on both enzymes as potential therapeutic targets. Moreover, EST has been implicated in the bioactivation of environmental pro-carcinogens and cooked-food mutagens.[9] Selective EST inhibitors would be beneficial to fully investigate the pathogenic characteristics of sulfated estrogens.

Fortunately, EST is one of the best-characterized sulfotransferases. The X-ray crystal structure was solved with bound estrogen and 3′,5′-diphosphoadenosine (PAP, a product of the sulfation reaction, Figure 1).[10] In addition, both the structure of a vanadate complex[11] and a detailed kinetic investigation support an in-line direct sulfate transfer mechanism.[12] However, current reported inhibitors of EST are limited to nonbioavailable PAPS analogues,[13] environmental toxins such as polychlorinated biphenols (PCBs),[14] tertiary amine drugs,[15] dietary flavonoids,[7] and synthetic steroids.[16] While many of these inhibitors have K_i values in the low micromolar range, secondary biological activities are prevalent, such as the disruption of hormone action.[15] Herein we report the discovery of potent and selective EST inhibitors derived from a purine-based library that possess all of the qualities required for cell-based and pharmacological studies. In addition, we report the application of a recently described mass spectrometry (MS) assay[17] for rapid identification of novel inhibitors for this therapeutically interesting sulfotransferase.

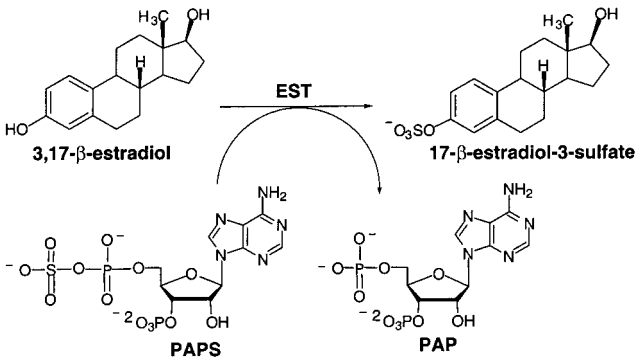

Figure 1. Estrogen sulfotransferase reaction.

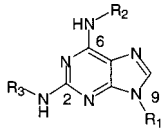

Figure 2. Purine scaffold of the combinatorial library screened against EST, showing three sites of diversity.

The purine library comprised 275 compounds and was generated with structural variations introduced at the 2-, 6-, and 9-positions of the purine ring (Figure 2).[18] Members of this purine-based library have the benefit of drug-like properties, and were originally targeted against the cyclin-dependent protein kinase CDK2. However, the library was also profitable in our search for carbohydrate sulfotransferase inhibitors.[19]

We expressed and purified murine EST bearing an *N*-terminal GST fusion,[20] and screened this purine library using two complementary assays. For detecting inhibition of enzyme activity, we employed a radiolabel transfer assay[21] in which ^{35}S-sulfate is transferred from ^{35}S-PAPS to estrone.[22] The extent of the reaction was determined by TLC separation followed by phosphorimaging analysis. This assay, although useful for obtaining kinetic data, is exceedingly time-consuming and laborious for the screening of large compound libraries. We therefore investigated the potential for complementing this assay with a higher-throughput MS-based binding assay.[17]

In this assay, the enzyme is immobilized on an agarose column by reductive amination and incubated with a compound mixture. An excess amount of enzyme is used compared to the concentration of the compound mixture, to allow both strong and weakly binding compounds equal access to the enzyme. Spectral subtraction of pre- and post-incubation mixtures was used

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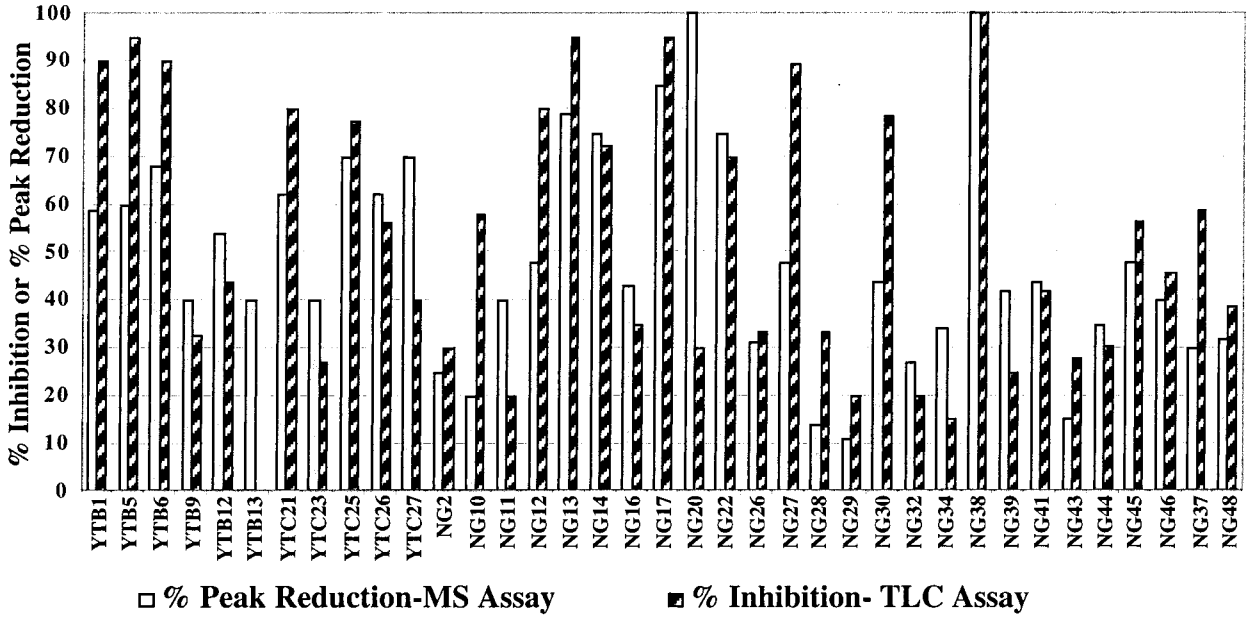
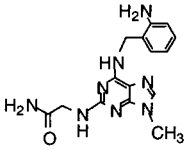
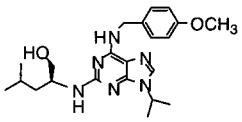
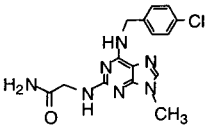
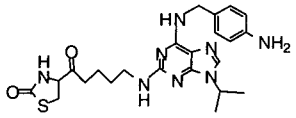
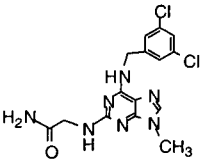
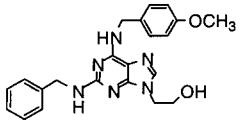
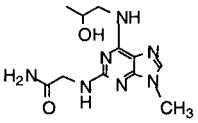
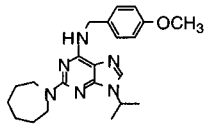
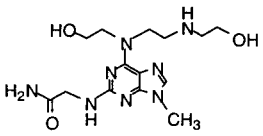
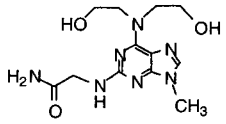
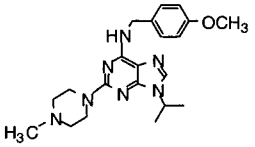
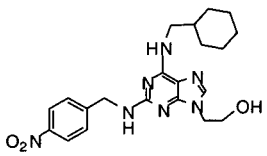


Figure 3. Correlation of inhibition and binding data obtained by the TLC and MS assay, respectively. "Hits" are defined as compounds displaying ≥45% peak intensity reduction by the MS assay and >70% inhibition by the TLC assay (each compound screened at 100 μM).

Table 1. Inhibitors Identified via the MS Assay and the TLC Radiolabel Transfer Assay[a]

Cmpd Label	% Peak Intensity Decrease	IC_{50} (μM)	Structure	Cmpd Label	% Peak Intensity Decrease	IC_{50} (μM)	Structure
YTB1	59	30		NG14	75	60	
YTB5	60	35		NG30	45	72	
YTB6	68	60		NG17	85	30	
YTC21	62	60		NG38	100	0.5	
YTC25	70	100		YTC27	70	>200	
NG13	79	80		NG20	100	>200	

[a] Reported IC_{50} values were obtained using the TLC-based assay

to identify compounds with binding activity. To date, this assay has found success in correctly identifying known inhibitors of two well-studied enzymes, glutathione S-transferase and pepsin.[17] Features of the assay include the discrimination of mass differences as small as 0.001 Da by the FT-ICR instrument utilized. In

addition, the immobilized enzyme is recyclable; and, as opposed to other mass spectrometry methods, chromatography of library components after incubation is not necessary. Due to the potential for rapid inhibitor identification using relatively small amounts of enzyme and compound, we chose to apply the assay to a library screen for inhibitors of EST. However, because the mass spectrometry assay may detect compounds that bind to but do not inhibit the enzyme, initial "hit" identification must be confirmed by screening via the TLC radiolabel transfer assay.

We first performed an initial screen of the 275-compound library using the TLC-based assay, and identified 30 compounds as primary hits. We then selected 40 members of the library at random (including representative hits and nonhits), and screened them using the MS assay. Presently, 30−40 library components can be readily screened at once via the MS assay. We are currently investigating the potential for screening larger library pools. We found that the same compounds were identified as hits by each assay with few exceptions (Figure 3). Only compounds YTC27 and NG20 showed binding activity in the MS assay, yet lacked inhibitory activity in the radiolabel transfer assay, supporting the use of both assays in conjunction. We are currently investigating whether these compounds bind outside of the active site.

Table 1 shows the IC_{50} values for representative hits. Notably, NG38, the most potent compound with an IC_{50} value of 500 nM, was clearly identified using the MS assay (peak intensity reduction = 100%). To determine the selectivity of these hits for EST, we next screened them against three representative members of the carbohydrate sulfotransferase family, a major subclass of enzymes with over 25 members.[23] No inhibitory activity was observed with the bacterial GlcNAc-6-sulfotransferase NodH[24] and the human GlcNAc-6-sulfotransferases HEC-GlcNAc6ST[25,26] and CHST2[27] at a concentration of 200 μM. Thus, these compounds appear to be selective inhibitors of EST, a representative member of the cytosolic sulfotransferase subclass.

In addition, it should be noted that all of the compounds in Table 1 displayed weak to moderate activity against the CDKs (IC_{50} values in the 100−1000 nM range). For instance, NG38 showed an IC_{50} value of 4000 nM against CDK1, in sharp contrast to more potent library members with IC_{50} values in the 20−35 nM range.[18]

In conclusion, we have discovered several purine-based inhibitors, including one with nanomolar potency, for EST using two parallel screening methods. Future studies will involve use of the MS method for rapid lead identification, followed by the TLC method for specific inhibition data. The co-crystal structure of EST with these selected inhibitors is in progress and will provide substantial insight into the mechanism of inhibition and the potential for second-generation, EST-directed inhibitor libraries. The most potent of these compounds may prove useful as chemical tools for elucidating the role of EST in steroid homeostasis and tumor cell proliferation.

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Supporting Information Available: Experimental details for enzyme expression, purification, library screens, and structures of all library components are available free of charge via the Internet at http://pubs.acs.org.

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