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A Potent and Highly Selective Sulfotransferase Inhibitor

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Sulfotransferases (ST) catalyze the transfer of a sulfuryl group from the ubiquitous donor adenosine 3′-phospate 5′-phosphosulfate (PAPS) to either an hydroxy group or an amine to yield the corresponding sulfate and adenosine 3′,5′-diphosphate (PAP). These enzymes are involved in a number of important biological processes including molecular recognition, detoxification, hormone regulation, drug processing, and modulation of receptor binding.[1] They have also been implicated in a number of disease states such as chronic inflammation, cancer metastasis, and HIV and herpes virus entry.[2] For this reason, there is interest in the generation of potent, specific small-molecule inhibitors of various ST. A number of reports on the inhibition of various ST exist,[3] but all of the inhibitors discovered show only modest inhibition and generally lack specificity. In the present study, we have used a highly sensitive fluorescence-based assay for the high throughput screening of a library of approximately 35 000 purine and pyrimidine analogues against β-arylsulfotransferase-IV (β-AST-IV).

β-AST-IV is a detoxification enzyme originally isolated from rat liver. Being a cytosolic ST, β-AST-IV is quite tractable with large quantities of recombinant protein available.[4] This enzyme also accepts a large number of aromatic alcohols and amines, facilitating the development of an assay amenable to high-throughput inhibitor discovery.[5] We have developed a highly sensitive fluorescence-based assay based on the reverse sulfotransferase reaction using 4-methylumbelliferyl sulfate and PAP as the substrates (Figure 1). We followed the reaction by monitoring fluorescence emission at 449 nm in a single-reaction format. Since this assay is uncoupled, uses few reagents, is quite sensitive, and can be followed at an emission range that is outside the range of most of the compounds to be screened, we decided to use this system for our high throughput screening. The assay was easily converted to a 96-well format with reaction volumes of 100 μL and then to a 384-well format with reaction volumes of 50 μL.

The synthesis of the library to be screened has been reported previously.[6] We reasoned that a library of this type should competitively inhibit PAPS binding. Our initial screen was run in 100 mM Tris pH 7.6, 5 mM mercaptoethanol, 25 μM PAP (K_m = 26.4 μM), 1 mM 4-methylumbelliferyl sulfate (K_m = 1.2 mM), and 10 μM inhibitor using 384-well plates in a final volume of 50 μL.[7] Reaction progress was followed using an excitation wavelength of 360 nm and reading emission at 450 nm. From the initial screen we found 13 molecules that showed at least 50% inhibition. We then rescreened these 13 at a final inhibitor concentration of 4 μM. Two of the original compounds screened proved to be false positives. We selected the best of these to resynthesize and scrutinize more carefully.[8] For the rescreening, the concentration

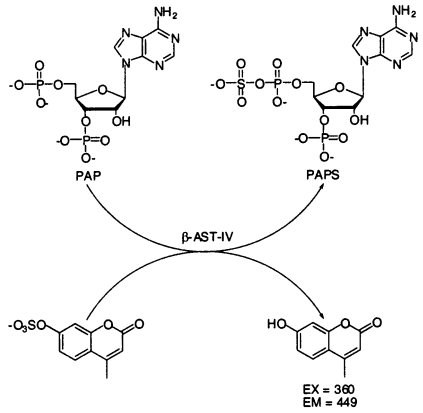

Figure 1. Fluorescence-based assay used for high-throughput screen of purine and pyrimidine library.

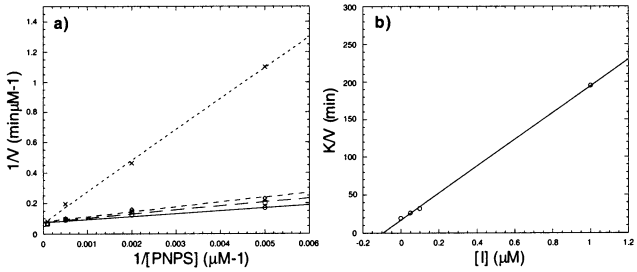

Figure 2. Inhibition of β-AST-IV. (a) Reciprocal rate vs reciprocal *p*-nitrophenolsulfate concentration at 1, 0.1, 0.05, and 0 μM inhibitor. The concentrations of PNPS used were 10, 2, 0.5, and 0.2 mM. (b) Slope replot. The inhibitor data shown here is for the first entry from Table 1.

of PAP or *p*-nitrophenol sulfate (PNPS) was varied at varying concentrations of inhibitor, a double reciprocal plot of 1/V verses 1/[substrate] was constructed, and the slopes of the resulting lines were plotted verses inhibitor concentration. The corresponding K_i values were determined from the *x*-intercept.[9] All of the data from these studies are shown in Table 1. Surprisingly, the inhibitors screened more carefully were noncompetitive versus PAP, but were competitive versus PNPS (Figure 2).

Of the 11 compounds remaining after the second round of screening, compound 1 showed the best inhibition constant with K_i = 96 nM. All of the other compounds showed K_i or IC_{50} values greater than 1 μM. We then studied the specificity of the best inhibitor (compound 1). The molecule was screened against a variety of nucleotide binding proteins. All of the enzymes screened are shown in Table 2. Compound 1 showed no inhibition of all but one of the enzymes at concentrations as high as 100 μM. The enzyme Sult1A1 is a human cytosolic ST involved in hormone regulation with a wide range of substrates. It is not surprising that this enzyme was inhibited, as compound 1 competes for the aryl-

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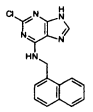
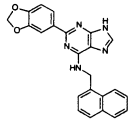
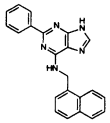
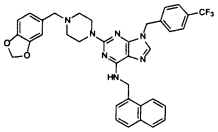
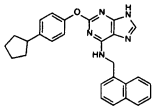
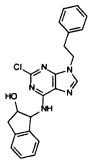
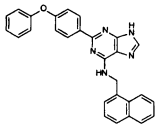
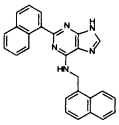
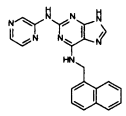
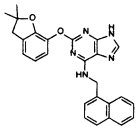
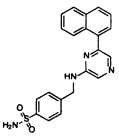
Table 1. β-AST-IV Inhibitors

Compound	#	IC_{50} (nM)[a]	Compound	#	IC_{50} (nM)[a]
	1	96[b]		7	7300
	2	4200		8	1700
	3	3300		9	2000[b]
	4	1800		10	2100
	5	3900[b]		11	9600
	6	3100			

[a] Measured at 20 μM PAP and 1 mM PNPS. [b] Values reported are K_i.

Table 2. Inhibitor Selectivity

enzyme	IC_{50}	enzyme	IC_{50}
Sult 1A1	770 nM	pyruvate kinase	no inhibition[b]
Nod factor ST	no inhibition[a]	lactic dehydrogenase	no inhibition[b]
protein kinase G	no inhibition[a]	fucosyltransferase V	no inhibition[b]
hexokinase	no inhibition[b]		

[a] At 10 μM inhibitor. [b] At 100 μM inhibitor.

binding site. Surprisingly, however, the inhibition was nearly an order of magnitude weaker than the inhibition observed for β-AST-IV.

Given the biological importance of ST, a method of finding specific potent inhibitors is of great importance. In the current study, we have achieved this goal by screening a large library of potential small-molecule inhibitors. The molecule found to be the best inhibitor competed for the aryl binding site and showed high selectivity. Coupled with another ST, this method could be used for high throughput screening of other ST. Recent mechanistic studies suggest that β-AST-IV catalysis proceeds through a sulfotrioxide-like transition state[10] (Figure 3). Further studies are

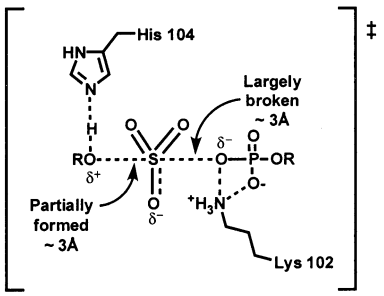


Figure 3. Proposed mechanism indicating the transition-state structure, the nucleophile, and the participating histidine for the forward reaction.

underway to characterize the specifics of binding and to modify this lead compound to make more potent and specific inhibitors, as well as to explore the scope of this assay.

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(7) We also studied the sulfonation of 4-methylumbelliferone, but were unable to use the fluorescence method due to a signal that was outside of the linear range at even K_m concentrations. To determine k_{cat} and K_m we followed the reaction using a UV/vis spectrophotometer at 390 nm ($\epsilon_{390} = 1080$). K_m was determined to be 320 ± 60 μM and k_{cat} was foud to be 11.2 ± 0.7 s^{-1}.

(8) The compounds that were resynthesized were done using the procedure reported in ref 6, except all reactions were carried out in the solution phase.

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