A Potent Inhibitor of the Melatonin Rhythm Enzyme

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Since the structural elucidation of melatonin (5-methoxy-*N*-acetyltryptamine) in 1959,[1] there has been growing interest in this "molecular pacemaker" hormone.[2] The biological roles of melatonin have been widely speculated on and include effects on aging, sleep, mood, immune response, cardiovascular fitness, and cancer.[3] The primary pharmacologic intervention that has been available to elucidate the actions of melatonin has been to administer the hormone to animals or people in large doses. Lacking from the arsenal of neuroendocrinologists has been a specific inhibitor of melatonin biosynthesis. Inhibitors of melatonin biosynthesis could not only help improve our understanding of circadian rhythm but they might also have therapeutic roles in mood and sleep disorders. The key enzyme to be targeted in this regard is serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AANAT), also called the "melatonin rhythm enzyme".[4] Here we report on the first potent and specific inhibitor of AANAT.

AANAT catalyzes the transfer of the acetyl group from acetyl-CoA to the primary amine group of serotonin, affording *N*-acetylserotonin (Figure 1). The enzyme is found primarily in the pineal and regulates the circadian rhythm of melatonin production.[4] Like melatonin production itself, AANAT activity varies with diurnal periodicity with up to 100-fold increases at night. The pineal enzyme hydroxyindole *O*-methyltransferase catalyzes the conversion of *N*-acetylserotonin to melatonin,[5] and this step is primarily regulated by *N*-acetyl-5-hydroxytryptamine availability. AANATs constitute a family of 23 kDa proteins which share 80% amino acid identity and are members of a superfamily of proteins defined by the presence in tandem of two weakly conserved 15 amino acid sequences (motifs A and B).[4] Included in this superfamily are some histone *N*-acetyltransferases important in gene regulation[6] and aminoglycoside *N*-acetyltransferases important in antibiotic resistance.[7]

Recent steady-state kinetic studies on AANAT showed evidence for an ordered "BiBi" ternary complex kinetic mechanism in which acetyl-CoA binds first followed by serotonin (or the alternative substrate tryptamine).[8] In this mechanism, both substrates must bind to the enzyme prior to the release of either product, and the acetyl group is most likely transferred directly from acetyl-CoA to serotonin without the involvement of a covalent acetyl−enzyme intermediate. In this model, a "bisubstrate analog" containing the components of serotonin and acetyl-CoA covalently linked could potentially be a potent AANAT inhibitor.[9] We therefore synthesized compound **1** to evaluate this possibility (Scheme 1).

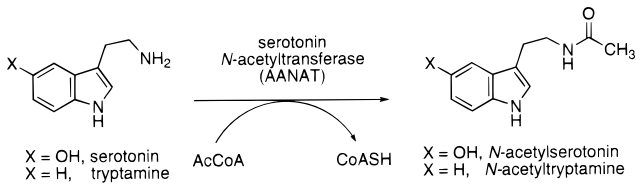

Figure 1. Enzyme reaction catalyzed by AANAT.

Tryptamine **3** was reacted with bromoacetyl bromide to afford the bromoacetamide derivative **2**. CoASH was alkylated with **2** in the presence of weakly basic conditions leading to thioether **1**. Compound **1** was purified by reversed phase HPLC, demonstrated high purity (>98%) based on ^{1}H NMR, ^{31}P NMR, MS, and analytical HPLC, and was reasonably stable for extended periods in aqueous solution.

A screening assay carried out with recombinant sheep AANAT[8] with varying concentrations of compound **1** and fixed substrate concentrations demonstrated that **1** was a very potent inhibitor with $IC_{50} \approx 150$ nM. This value is approximately 1000-fold lower than the substrate K_m values or the IC_{50} of any other reported inhibitor to date.[8] A time course demonstrated that in the presence of inhibitor the activity was still linear with time for at least 3 min, making less likely a "slow-binding" inhibition kinetic scheme. Steady-state kinetic inhibitor analysis of AANAT was carried out, and compound **1** was shown to be a linear competitive inhibitor versus the varied substrate acetyl-CoA ($K_i = 90$ nM) and a noncompetitive inhibitor versus tryptamine (Figure 2). This pattern of inhibition further supports an ordered BiBi model with acetyl-CoA binding before tryptamine (or serotonin).[10]

A useful analysis of bisusbtrate inhibitory potency has been to compare the K_i of the bisubstrate analogue to the product of the individual K_m values of the substrates involved.[9] In this case, the (K_m-acetyl-CoA)(K_m-tryptamine)/$K_i \approx 1$ M. The entropic gain for linking the two substrates is comparable to the more potent examples of reported bisubstrate analogue enzyme inhibitors.[9] The potency of inhibition of AANAT by compound **1** is in a range that could potentially be useful for physiologic studies.

We were next interested in exploring the specificity of compound **1** as an acetyltransferase inhibitor. In particular, we wanted to examine whether **1** could block the enzyme arylamine *N*-acetyltransferase. Arylamine *N*-acetyltransferase catalyzes the transfer of the acetyl group from acetyl-CoA to arylamines as well as arylalkylamines including serotonin.[11] Because it is also present in pineal, it can be a source of interference in assaying AANAT activity in crude extracts.[12] In contrast to AANAT,

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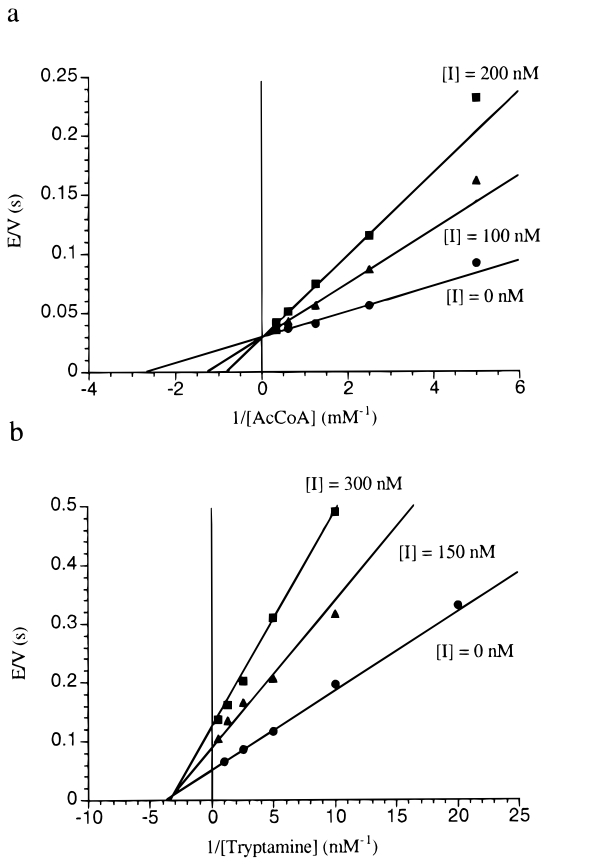
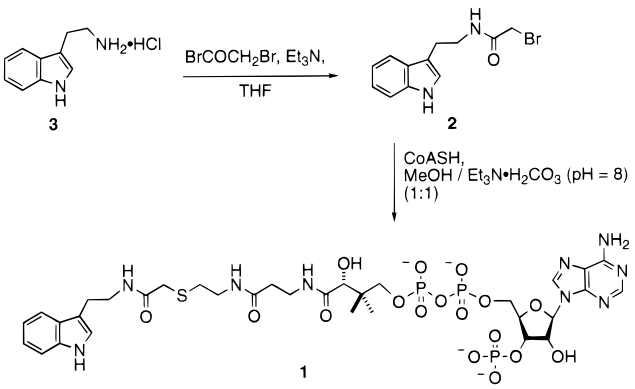


Figure 2. Inhibition of AANAT with bisubstrate analogue **1**. (a) 1/Velocity (*E*/*V*) versus 1/AcCoA at varying concentrations of bisubstrate analogue **1**. Tryptamine concentration was fixed at 1.0 mM. Fit is to linear competitive inhibitor model (Kinetasyst II). The calculated K_{is} is 91 ± 11 nM and the K_m (apparent) for acetyl-CoA is 0.36 ± 0.04 mM. (b) 1/Velocity (*E*/*V*) versus 1/tryptamine at varying concentrations of bisubstrate analogue **1**. AcCoA concentration was fixed at 0.35 mM. Fit is to linear noncompetitve inhibitor model (Kinetasyst II). The calculated K_{is} is 174 ± 38 nM and K_{ii} is 209 ± 25 nM and the K_m (apparent) for tryptamine is 0.26 ± 0.03 mM. Assays were performed using the DTNB kinetic assay according to previously described procedures.[8]

Scheme 1. Synthesis of Bisubstrate Analog **1**



arylamine *N*-acetyltransferase follows a ping-pong mechanism and involves a covalent acetyl−cysteine enzyme intermediate.[11] Therefore, arylamine *N*-acetyltransferases would be predicted to be resistant to bisubstrate analogue inhibitors. Indeed, this turned out to be the case. In the presence of the substrates acetyl-CoA (0.1 mM) and nitroaniline (0.1 mM), bisubstrate analogue **1** was a comparatively weak inhibitor with $IC_{50} \approx 140$ μM.[13] Thus, compound **1** is roughly 1000-fold more potent in inhibiting AANAT activity compared to arylamine *N*-acetyltransferase. This finding augurs well for the development of specific agents for in vivo work.

(13) Assays employed pig liver arylamine *N*-acetyltransferase purchased from Sigma and were carried out as described in the Supporting Information.

Table 1. Inhibition of Serotonin *N*-Acetyltransferase by Fatty Acyl-CoA Derivatives[a]

fatty acyl-CoA analog	IC_{50} (μM)	fatty acyl-CoA analog	IC_{50} (μM)
C_8-SCoA	45	C_{14}-SCoA	2.4
C_9-SCoA	20	C_{16}-SCoA	5
C_{10}-SCoA	11	C_{18}-SCoA	43
C_{12}-SCoA	3.5		

[a] Assays were performed at fixed concentrations of acetyl-CoA (0.3 mM) and tryptamine (0.3 mM) with varying concentrations of the fatty acyl-CoA analogues (typically a 10-fold concentration range varied around the IC_{50}) and Dixon analysis (1/V vs [I]) of the data showed linear inhibition. Fatty acyl-CoA derivatives were purchased from Sigma and the nomenclature C_8-SCoA is an abbreviation for octanoyl-SCoA with the others indicating the number of carbons in the fully saturated, unsubstituted fatty acid. All assays were performed using the DTNB activity assay[8] at least two times and standard errors were ±10%.

Given the general inability of acetyl-CoA and other CoA analogues to penetrate cell plasma membranes,[14] it is likely that compound **1** in its current form would be unable to block melatonin production in vivo. Of concern in future work will be developing analogues of **1** that can enter cells and inhibit AANAT in living organisms.

It seemed plausible that the bisubstrate analogue **1** could be mimicked by compounds that contain a CoA moiety attached to a less specific hydrophobic moiety rather than indole. To test this possibility, a series of commercially available fatty acyl-CoA analogues were screened as potential inhibitors (Table 1). That none of these compounds could be alternative substrates was established by demonstrating the absence of CoASH production in the presence of AANAT plus tryptamine.[8] Interestingly, some members of the series of fatty acyl-CoA compounds were able to potently inhibit AANAT action. Remarkably, the optimal inhibitor (C_{14}-SCoA) contained a nearly identical chain length to the number of carbon and nitrogen atoms of the tryptamine group. This compound with $IC_{50} = 2.4$ μM was only 16-fold less potent than compound **1**. This suggests that a wide array of hydrophobic indole replacements can be screened to optimize inhibitory potency of bisubstrate analogues.

Given these results, it seems reasonable to speculate on the possibility that fatty acyl-CoA levels could affect melatonin levels in in vivo settings. For example, most tissues including rat brain have on the order of 10−100 μM total long chain acyl-CoA levels.[15] Given the IC_{50} of the more potent fatty acyl-CoA compounds shown in Table 1, fatty acyl-CoA inhibition of AANAT in vivo might be predicted. However, caution must be taken with this interpretation since much of the fatty acyl-CoA in cells is bound to specific acyl-CoA binding proteins and the free concentration in cells is not actually known.[16] Nevertheless it is interesting that fasting for 24−48 h in man, which generally leads to a 2-fold elevation in total fatty acyl-CoA levels,[17] causes a significant drop in melatonin production in the pineal.[18] That such an effect on melatonin production is modulated by alterations in intracellular fatty acyl-CoA levels is provocative and could represent a novel regulatory mechanism for circadian rhythm.

Supporting Information Available: Experimental details (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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