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Development of bisubstrate analog inhibitors of aspartate *N*-acetyltransferase, a critical brain enzyme

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Abbreviations

ANAT, aspartate *N*-acetyltransferase; ASPA, aspartoacylase; boc, t-butyloxy carbonyl; CD, Canavan disease; CNS, central nervous system; CoA, coenzyme A; DCM, dichloromethane; DTNB, dithionitrobenzoate; EDCl, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; NAA, *N*-acetyl-L-aspartate; PDB, protein data bank; TLC, thin layer chromatography; THF, tetrahydrofuran.

Abstract

Canavan disease (CD) is a fatal leukodystrophy caused by mutations in the *aspA* gene coding for the enzyme aspartoacylase. Insufficient catalytic activity by this enzyme leads to the accumulation of its substrate, *N*-acetyl-L-aspartate (NAA), and diminished production of acetate in brain oligodendrocytes of CD patients. There is growing evidence that this accumulation of NAA is the cause of many of the developmental defects observed in these patients. NAA is produced in the brain by a transacetylation reaction catalyzed by aspartate *N*-acetyltransferase (ANAT), and this membrane-associated enzyme has recently been purified as a soluble maltose binding protein fusion. Designing selective inhibitors against ANAT has the potential to slow the accumulation of NAA and moderate these developmental defects, and this is the goal of this project. Several bisubstrate analog inhibitors of ANAT have been synthesized that have achieved nanomolar level binding affinities against this enzyme. Truncated versions and fragments of these bisubstrate analog inhibitors have identified the essential structural elements needed for high binding affinity. More drug-like versions of these inhibitors can now be built, based on these essential core structures.

1. INTRODUCTION

Canavan disease (CD) is a neurological disorder caused by inherited genetic defects (Canavan, 1931), leading to brain atrophy and degeneration into spongy tissues with microscopic fluid-filled cystic cavities. CD is classified as a leukodystrophy, since it causes disruptions in the growth and maintenance of the myelin sheath that serves to insulate neuronal connections. Children born with the most severe forms of this disorder lack motor skills and speech, and they also develop conditions such as hypotonia and macrocephaly (Adachi *et al.*, 1973, Adornato *et al.*, 1972). The average life expectancy of CD patients is only about 10 years. The underlying molecular basis for CD is due to multiple mutations in the *aspA* gene (Matalon *et al.*, 1988), which leads to deficiencies in the catalytic activity of the enzyme aspartoacylase (ASPA) (Zano *et al.*, 2013) that is localized in oligodendrocytes in the brain. ASPA is the enzyme responsible for the production of the important CNS metabolite acetate by hydrolysis of *N*-acetyl-L-aspartate (NAA). Acetate is the key starting point for fatty acid and steroid synthesis, compounds which are further used as building blocks in making myelin sheaths (Moffett *et al.*, 2007). Different strategies have been employed in an attempt to identify treatment therapies for CD, including brain-targeted gene therapy against defective *aspA* gene (Janson *et al.*, 2002), metabolic therapy by providing acetate from external sources (Arun *et al.*, 2010), lithium therapy (Janson *et al.*, 2005), and enzyme replacement therapy by intraperitoneal injection of protein surface modified ASPA (Zano *et al.*, 2011). So far, while each of these approaches have shown some promising results in lowering NAA levels, none of these different strategies have identified a clear path to a treatment therapy.

Some recent findings have supported the need for a completely different approach, with the potential to produce a novel treatment for CD. It has been found that a knockout of the *Nat8l* (*N*-acetyltransferase 8-like) gene that codes for aspartate *N*-acetyltransferase (ANAT), the enzyme responsible for the synthesis of NAA, led to normal CNS myelination during the early stages of brain development (Guo *et al.*, 2015, Sohn *et al.*, 2017), thereby eliminating the symptoms of this leukodystrophy in a mouse model of CD. ANAT belongs to the acetyltransferase enzyme superfamily, and catalyzes the acetylation of L-aspartate for conversion into the nervous system specific metabolite NAA. This compound is an important constituent in CNS metabolism, in fact NAA is one of the most abundant amino acids in the brain, constituting about 3-4% of the total brain osmolarity (Baslow, 2000). NAA is synthesized in neurons and is subsequently transported into the cytoplasm of oligodendrocytes (Baslow, 2003), where it is hydrolyzed by the enzyme aspartoacylase to release the acetate moiety which is then utilized as a building block in myelin biosynthesis (Figure 1).

Based on these studies showing the important role that elevated NAA plays in the etiology of CD, our planned approach involves designing and synthesizing selective inhibitors against ANAT. The advantage of this approach is the capability to directly control the elevated levels of NAA found in the brain of CD patients by rebalancing the rates of NAA synthesis and utilization. In previously published work, we have produced and characterized a soluble form of this membrane-associated enzyme (Wang *et al.*, 2016), and have synthesized several initial ANAT inhibitors based on two different core structures (Thangavelu *et al.*, 2017). These compounds are the first synthesized inhibitors against ANAT that have been produced to date, with the best compounds having inhibition constants (K_i values) in the low to sub-micromolar range.

To expand this search for improved inhibitors we have incorporated the structures of the ANAT substrates into the design strategy. Coenzyme A (CoA) functions primarily through the activation of acyl groups *via* formation of a thioester for their subsequent transfer to a variety of different acceptor molecules (Jencks, 1973). There is general agreement that the purpose of the extended coenzyme A structure, composed of adenosine-3′-monophosphate linked by a pyrophosphate to a pantothenate coupled in a peptide linkage to β-mercaptoethylamine, is to provide the specificity and binding affinity to its enzyme partners (Engel & Wierenga, 1996). However, these individual moieties typically have little or no affinity to these enzymes, suggesting a requirement for a coordinated ensemble of interactions to achieve tight binding of this coenzyme. This need for binding cooperativity between multiple functional groups makes the design and synthesis of potent and selective inhibitors against CoA-utilizing enzymes particularly challenging.

As part of a systematic effort to develop inhibitors against an important brain enzyme that catalyzes an acetyl transfer reaction, the affinities of bisubstrate analog inhibitors and various truncated versions have been explored. We now report the synthesis and kinetic evaluation of a series of CoA-based bisubstrate analogs with very high affinity towards ANAT. In addition, this study includes the development and evaluation of a series of truncated derivatives of this bisubstrate analog, compounds that are now available to be further elaborated as a new family of potent inhibitors against ANAT.

2. METHODS AND MATERIALS

2.1 Materials

Solvents and reagents were used as purchased without further purification. The reaction time courses for the newly synthesized enzyme inhibitors were monitored by TLC using an ethyl acetate/hexane solvent system on Silica gel HLF plates (Analtech, Inc., Newark, DE). The final products were purified by silica gel (230-400 mesh) flash column chromatography.

2.2 Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on either a Varian VXRS 400 MHz or a Varian INOVA 600 MHz spectrometer. Electrospray–Atmospheric Pressure ionization (ES-API) mass spectrometry of the final inhibitors were carried out on Agilent 1260 infinityII spectrometer.

2.3 Enzyme production and inhibitor measurements

The recombinant maltose-binding protein-human aspartate *N*-acetyltransferase (MBP-ANAT) fusion enzyme, produced in order to solubilize the membrane-associated enzyme, was expressed and purified as reported previously (Wang *et al.*, 2016). ANAT activity was measured by using a previously described DTNB-based assay (Wright & Viola, 1998) using a SpectraMax 190 spectrophotometer plate reader (Molecular Devices, CA). For inhibition constant (K_i) determinations a two-fold serial dilution of each inhibitor was performed starting from a stock inhibitor solution, with the starting concentration determined according to the potency of the inhibitors. A negative control, in which no inhibitor was added, and a background signal control, in which neither L-aspartate nor inhibitor were added, were included in each set of assays.

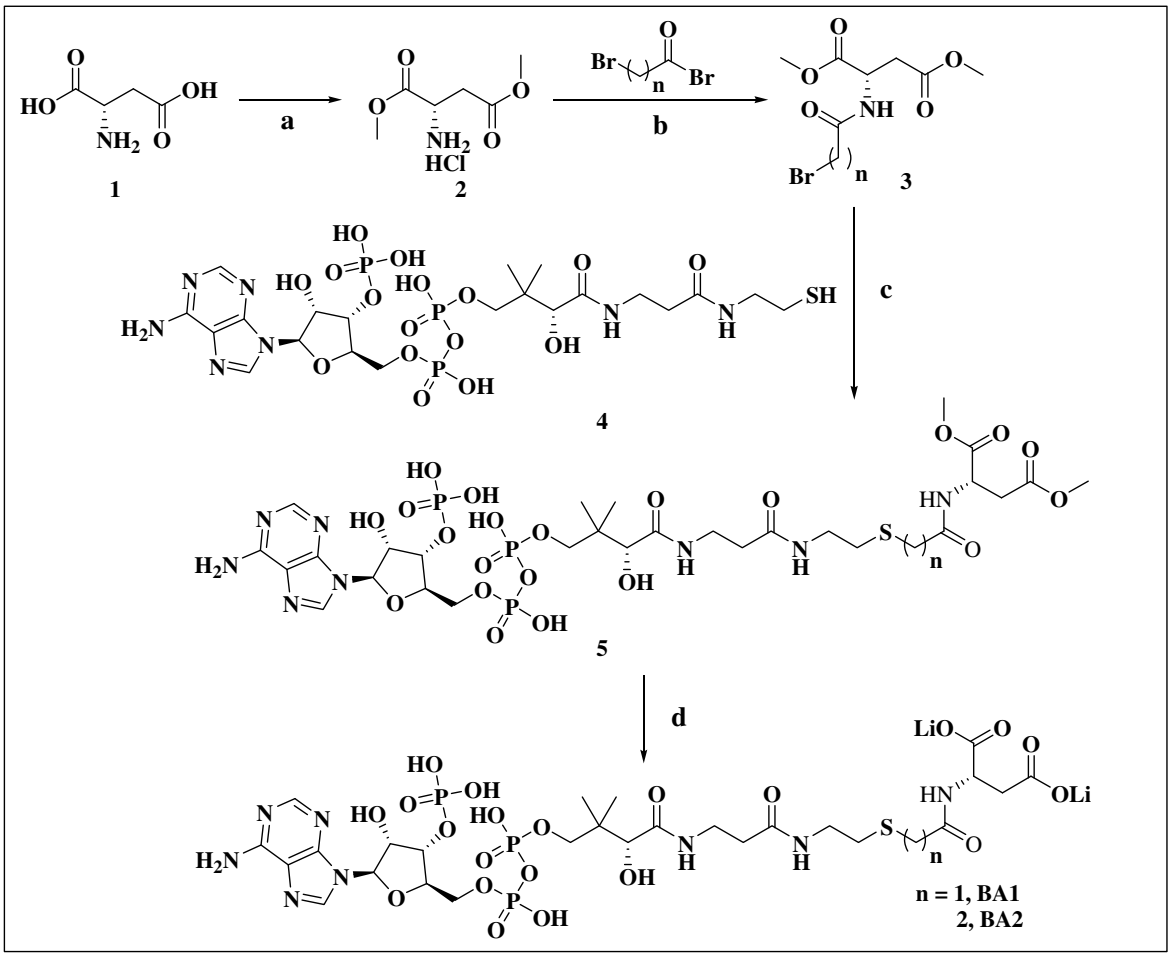
3 RESULTS

3.1 Design of bisubstrate analog inhibitors

The transfer of the acetyl unit from acetyl-CoA to L-aspartate catalyzed by the enzyme ANAT involves the nucleophilic attack of the α-amino group of aspartate on the carbonyl carbon of the acetyl group. This reaction is proposed to go through a transition state in which the newly forming C-N bond will alter the geometry around the carbonyl carbon of the acetyl group as the C-S bond becomes elongated during the departure of coenzyme A (Figure 2). To mimic this transition state a pair of bisubstrate analogs has been produced in which aspartate is covalently coupled to acetyl-CoA, with the distance between the carbonyl carbon and the departing thiol group varied from 1 to 2 bonds (Figure 2).

3.2 Synthesis of full length bisubstrate analogs

These full-length bisubstrate analogs have been synthesized by coupling coenzyme A to activated *N*-acetyl or *N*-propionyl aspartate (Scheme 1). The bisubstrate analog with a single methylene carbon linker (BA1) is a potent inhibitor of ANAT, with a K_i value of 275 nM (Table 1). The importance of the aspartate carboxyl groups in substrate binding is demonstrated by a greater than 60-fold loss of affinity when those functional groups are converted to methyl esters (BA1Me). Increasing the distance between the thiol group and the carbonyl carbon through the incorporation of an additional methylene carbon leads to a further 6-fold improvement in binding affinity, with this bisubstrate analog (BA2) possessing a K_i value of 48 nM as an inhibitor of ANAT (Table 1).



SCHEME 1 Synthesis of full length bisubstrate analogs BA1 and BA2. Reagents and conditions: (a) $SOCl_2$ in MeOH at 0 ^{o}C to RT 14 h; (b) Bromoacetyl bromide or bromopropionyl bromide, Et_3N in THF at RT; c) MeOH/Et_3N, H_2CO_3 (pH = 8) in (1:1) ratio, 8 h; (d) LiOH in THF/H_2O, RT, 5 h.

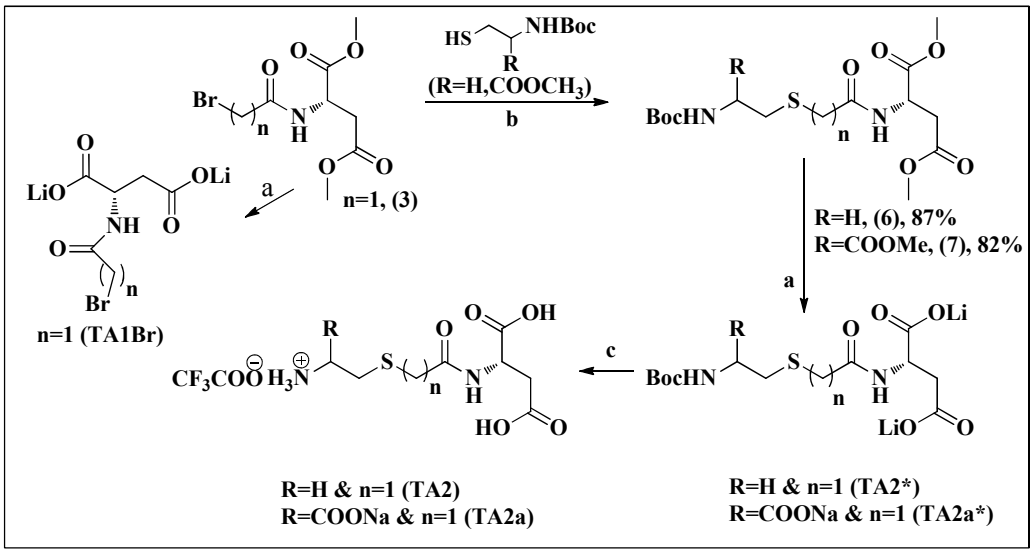
3.3 Analysis of bisubstrate analog fragments

While binding potency is an important parameter in the development of inhibitors against a target, the presence of multiple charged functional groups would likely preclude the transport of these bisubstrate analogs to the brain of CD patients. Removal of the adenosyl phosphate moiety from the intact bisubstrate analog (Figure 3) would eliminate several negatively-charged phosphate groups that contribute to the nondrug-like properties of this enzyme inhibitor. However, it is not clear how much the presence of this ADP moiety contributes to

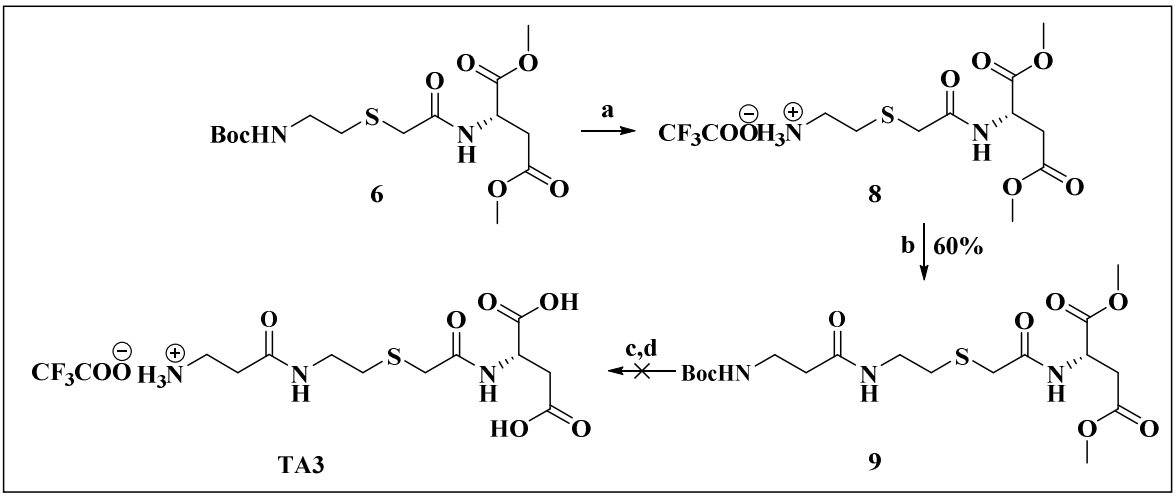
the overall binding affinity of CoA. To examine the relative contributions of the different structural elements to enzyme binding, several cofactor fragments were tested as potential inhibitors. Neither adenosine nor pantothenate shows any measureable inhibition of ANAT when tested at 2 mM concentrations. Adenosine 5′-monophosphate is a very weak inhibitor of ANAT, but increasing the number of phosphate groups on the adenosine structure does confer a slight improvement in binding affinity (Table 3). Adenosine-3′,5′-diphosphate, adenosine-5′-diphosphate and adenosine 5′-triphosphate were each observed to be weak, but slightly improved inhibitors of ANAT.

3.4 Synthesis of truncated bisubstrate analog derivatives

The overall affinity of the transition state analog mimics of the ANAT reaction are quite strong, but the affinity contributed from the adenosyl phosphate moiety by itself is fairly weak. It would be helpful to learn the importance of each of the other structural elements in contributing to the overall high affinity of these bisubstrate analogs. To accomplish this aim a series of truncated bisubstrate analogs were synthesized starting from the amino acyl end, with the structures of these truncated analogs shown in Figure 3. To compare the individual interactions/affinities of each truncated bisubstrate analog with ANAT, each fragment was systematically assembled by using linear synthetic approaches (Schemes 2 and 3).



SCHEME 2 Synthesis of truncated bisubstrate analogs TA1Br and TA2. Reagents and conditions: (a) LiOH in THF/H_2O, RT, 5 h; (b) MeOH/Et_3N, H_2CO_3 (pH = 8) in (1:1) ratio, 8 h; (c) CF_3COOH in DCM at 0 ^oC, overnight.



SCHEME 3 Attempted synthesis of the truncated bisubstrate analog TA3. Reagents and conditions: (a) CF_3COOH in DCM, overnight; (b) *N*-boc-β-alanine, EDCl, Et_3N in DCM for 5 h; (c) LiOH in THF/H_2O, RT, 5 h. (d) CF_3COOH in DCM at 0 ^oC, overnight.

3.5 Analysis of truncated bisubstrate analog inhibitors

N-acetylaspartate (TA1) is also a weak inhibitor of ANAT (K_i = 1.6 mM), with a binding affinity that is comparable to that of the ADP moiety. The *N*-chloroacetyl derivative of aspartate (TA1Cl) shows an 8-fold improvement in binding, while the *N*-bromoacetyl derivative (TA1Br) does not show any measurable affinity to ANAT (Table 2). Coupling *N*-bromoacetyl aspartate with cysteamine adds the thiol end of CoA, but this fragment (TA2) does not show any measurable inhibitor of ANAT. However, the *t*-butyloxycarbonyl (boc)-protected precursor of this fragment (TA2*) was found to be a weak inhibitor (K_i = 2.1 mM). Replacing cysteamine with cysteine introduced an additional carboxyl group (TA2a), resulting in weak binding to ANAT. As with the cysteamine adduct, the boc-protected precursor of the cysteine adduct (TA2a*) is a slightly better inhibitor (K_i = 0.87 mM) suggesting the presence of a hydrophobic pocket in the cofactor binding site. Addition of the next moiety, β-alanine, was attempted to extend the structure of the CoA analog fragment (Scheme 3, TA3) but, unfortunately, multiple attempts at deprotection of the protected intermediate (compound 9) each resulted in substantial degradation of the final product. Pantothenic acid, the middle component of the coenzyme A structure that connects ADP to β-cysteamine (Figure 3), does not bind to ANAT with any measurable affinity. So it is unlikely that extending the CoA structure by coupling pantothenate to produce an even longer fragment would lead to a substantial increase in the affinity of this truncated fragment series.

Since the expanded bisubstrate analog (BA2) is the most potent ANAT inhibitor (Table 1), a related set of truncated analogs were synthesized starting with *N*-propionyl aspartate. However, none of these compounds, including *N*-bromopropionyl aspartate, the cysteamine adduct, or the boc-protected cysteamine adduct, showed any appreciable inhibition of ANAT (data not shown).

3.6 Cooperative binding of bisubstrate analog fragments

The failure to observe improved binding affinity through the inclusion of the additional structural components of the CoA structure (TA1 and TA2) suggests that the high affinity of the bisubstrate analogs structures (BA1 and BA2) comes not from interactions with the central structural components of CoA, but from the combined interactions of the functional groups at each end of this cofactor structure. To test this idea the binding of the truncated fragment series was re-examined in the presence of high levels of several different adenosyl phosphates.

For each of the truncated analogs tested the presence of 3′,5′-ADP, 5′-ADP or ATP was examined at a minimum of five-times excess over their respective K_i values to measure any possible effects on analog binding to ANAT. The addition of 12 mM 3′,5′-ADP had no measurable effect on the binding of either TA2 or the boc-protected version (TA2*). However, for the extended analogs that included a cysteamine or cysteine moiety (TA2 and analogs), the presence of 5′-ADP or ATP led to an average of ~1.4-fold improvement in binding affinity (Table 4). The greatest cooperative effect for these analogs was seen between the binding of 5′-ADP and that of *N*-(acetylcysteinyl)aspartate (TA2a), with a 2.5-fold increase in analog affinity. For the shorter truncation analogs (TA1) the presence of ATP caused the greatest increase in binding, with a 2.8-fold and 4.7-fold improvement in the K_i values for TA1 and TA1Cl, respectively (Table 4).

The reverse experiments were also conducted to measure any possible enhancements induced in ADP or ATP binding in the presence of high levels of several TA inhibitors. Here the effects were much less pronounced, with only slight enhancements in adenine nucleotide binding in most cases. The largest increases in affinity were observed for the combinations that also gave the best improvements in analog affinity, such as ATP binding in the presence of TA1 and TA1Cl. But even here the enhancements in ATP affinity was less than 2-fold.

4. DISCUSSION

4.1 General design of bisubstrate analogs

Bisubstrate analogs, sometimes referred to as transition state analogs, were originally developed to examine the chemical mechanism of an enzyme (Jin *et al.*, 1999, Parmentier *et al.*, 1992), to measure the effects of mutations on substrate recognition (Zeringo & Bellizzi, 2014, Hengge *et al.*, 1996), and to mimic the transition state in enzyme structural studies (Lolis & Petsko, 1990, Betts *et al.*, 1994, Lowther *et al.*, 1999, Focia *et al.*, 1998). Bisubstrate analogs are designed to incorporate the binding motifs of both substrates for the target enzyme within the same molecule. As a consequence, bisubstrate analogs tend to have affinities that are significantly greater than the sum of binding energies of the individual substrates (Parang & Cole, 2002, Wolfenden, 1972). While this high affinity feature is part of the design goals, because of the difficulty in mimicking the transient structure of a reaction transition state, this enhanced affinity is not always achieved. For example, the bisubstrate inhibitors P1-(5-adenosyl)-P3-(6-glucosyl) triphosphate and P1-(5-adenosyl)-P4-(6-glucosyl) tetraphosphate, that were synthesized to mimic the transition state of the reaction catalyzed by hexokinase, were found to be only weak inhibitors with their binding affinities lower than the either of the substrates ATP and glucose (Danenberg & Danenberg, 1977). It is clearly important to understand the mechanism of the reaction that is being catalyzed, as well as the nature of the proposed transition state structure in order to design bisubstrate analogs with the optimal potency.

4.2 Bisubstrate analog inhibitors of ANAT

Identifying the active site structure of an enzyme target is an important criteria to guide the development of new inhibitors. Because the structure of ANAT is still unknown, developing selective active site-specific inhibitors against this enzyme is more challenging. There is,

however, detailed structural information about the substrates for this enzyme-catalyzed reaction that can be used to help guide inhibitor design. ANAT is an acetyltransferase, catalyzing an acylation reaction where the α-amino group of L-aspartate acts as a nucleophile attacking the carbonyl carbon of acetyl CoA (Figure 2). To achieve optimal potency a bisubstrate analog must closely mimic the transition state of the reaction. Because the exact distance between the two substrate molecules in the transition state of this reaction is not precisely known, bisubstrate analogs were designed with two different linkers between the substrates to investigate the effect of this change on the binding affinity to the enzyme. The direct coupling of the acetyl group of *N*-acetylaspartate to the thiol group of coenzyme A produces a bisubstrate analog (BA1) with mid-nanomolar level binding affinity to ANAT. The incorporation of an additional methylene carbon between the transferred acetyl group and coenzyme A (BA2) leads to a further increase in binding affinity beyond that of the already potent inhibitor with the shorter linker (Table 1). This improved affinity suggests that this enzyme-catalyzed reaction has a late transition state in which the acetyl group has been substantially transferred to L-aspartate, with the bond to the departing thiol of coenzyme A significantly elongated in the transition state.

4.3 Role of structural moieties in bisubstrate analog binding

Now that an optimized bisubstrate analog structure has been synthesized, it would be useful to identify the essential binding determinants, and also to eliminate non-essential components, to arrive at a core structure that can be used to guide the development of more drug-like ANAT inhibitors. By synthesizing truncated derivatives of this bisubstrate analog, the correlation between the affinities of these truncated structures and the functional groups that are present in each derivative can be examined. Given the importance of precisely orienting the two substrates in the active site of ANAT for efficient acetyl transfer, it was

expected that the functional groups at each end of the bisubstrate analog would make the majority of the important contributions to the overall affinity. However, neither the adenosine phosphate moiety nor the product NAA were found to have greater than millimolar affinities towards ANAT, values that are about four orders-of-magnitude weaker than the best bisubstrate analog inhibitor (Table 2). Coupling several additional structural moieties of coenzyme A, including cysteamine and β-alanine, to NAA did not confer any additional affinity to the target enzyme.

There are several possible reasons for the failure of these extended substrate analogs to show increased binding affinity to ANAT. These linear molecules are quite flexible, with multiple rotatable bonds that lead to a large number of possible conformers. This lack of rigidity decreases the population of the optimal binding conformation, leading to lower overall affinity to a given target. A similar phenomenon was observed when examining the transport and uptake properties of small molecule drugs. Drugs that are more flexible tend to accumulate to a much lower level in Gram-negative bacteria when compared to more rigid drugs with similar overall pharmacological properties (Richter *et al.*, 2017).

In addition to being quite flexible, the central pantothenate and cysteamine moieties have relatively few functional groups that can make productive binding interactions in the coenzyme A binding pocket. To identify the importance of different CoA functional groups in binding affinity, a series of structures of *N*-acetyltransferase enzymes were examined. The target sequence of ANAT was provided and a closest homolog template search was performed using the *SWISS-MODEL* server (Arnold *et al.*, 2006). The results were filtered by selecting structures with either acetyl CoA or CoA bound and any redundant structures were discarded. The top 20 structures were then downloaded from the Protein Data Bank (PDB) (Berman *et al.*, 2000), and further analyzed using *Ligplot* (Wallace *et al.*, 1995) to identify the key functional groups involved in either hydrogen bonding or hydrophobic interactions

with their receptor residues. As expected, in all of the structures studied the adenosine moiety makes multiple hydrogen bonding interactions, and the negatively charged phosphate groups make numerous electrostatic interactions with positively charged residues in the binding pocket. To eliminate these dominant interactions, subsequent analysis was limited to only the pantothenate and cysteamine moieties in order to identify the potential effect of the functional groups in each truncated analog on the binding affinity. There are five functional groups in the pantothenate and cysteamine region of the structure that can potentially participate in hydrogen bonding interactions (Figure 3). However, in half of the structures that were examined there are two or fewer hydrogen bonds made, and only two structures show more than three hydrogen bonds with these functional groups. The hydrogen bonding interactions identified from this region of the CoA structure occur primarily through the carbonyl group from the pantothenate moiety and the amide group from the cysteamine. The weak binding affinity of these truncated analogs with ANAT is likely due to a combination of the limited number of bonding interactions to this region of the cofactor structure, as well as to the higher flexibility of the truncated analogs.

4.4 Binding cooperativity to improve truncated analog binding

The presence of millimolar levels of adenosine nucleotides as metabolites should lead to some occupancy of the adenosine sub-site in the CoA binding pocket of ANAT under cellular conditions. To test the possible effects of nucleotide binding on the affinity of the set of truncated bisubstrate analogs several adenosine phosphates were included in ANAT inhibitor binding studies. While the addition of 3′,5′-ADP had no effect on truncated analog binding, both 5′-ADP and ATP did show cooperative effects on analog binding. The addition of millimolar levels of ADP had a greater effect on the binding of the longer truncated analogs (TA2), with up to a 60% improvement in the K_i value. For the shorter truncated analogs

(TA1) the presence of ATP had the greater effect, leading to as much as an 80% improvement in binding affinity. This 40-fold increase in binding affinity relative to NAA binding brings this truncated analog inhibitor (TA1Cl) to within a factor of 200 of the binding affinity of the full length bisubstrate analog (BA1). These results suggest that as improved inhibitors are developed that optimize the potential interactions in the substrate and thiol end of the CoA binding site of ANAT, the normal cellular levels of ADP and ATP could serve to enhance inhibitor binding to this target enzyme. These studies also highlight the importance of occupying both the nucleotide and the aspartate binding pockets to achieve maximum inhibition.

5 CONCLUSIONS

Bisubstrate analogs have been quite useful in a wide variety of applications, and many of these compounds have been found to be quite potent enzyme inhibitors. However, because these analogs typically incorporate charged and polar functional groups to make additional interactions at the active site, these compounds generally do not have the optimal structural properties to be considered as viable drug candidates. The general inability of acetyl-CoA and other CoA analogs to penetrate into cell plasma membranes also makes them poor starting points for drug candidate development (Robishaw & Neely, 1985). In particular, the highly charged ATP region of acetyl CoA makes any bisubstrate analogs that incorporate this moiety much less likely to cross a membrane barrier. These bisubstrate analog inhibitors of ANAT have provided information on the transition state of this reaction and established the drugability of this important enzyme. In order to develop drug-like candidates that mimic the substrate structures of ANAT it will be necessary to design inhibitors based on this core bisubstrate analog structure, but with neutral isosteric replacements of the charged functional groups.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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TABLE 1 Kinetic evaluation of ANAT bisubstrate analogs

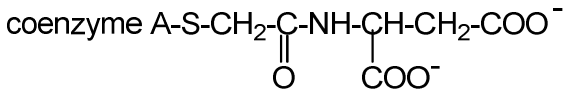
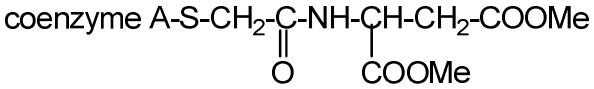
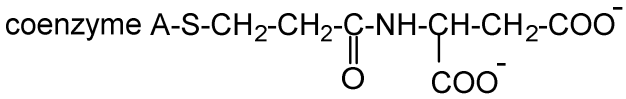
Analog number	bisubstrate structure	K_i (nM)
BA1		275 ± 10
BA1Me		18,000 ± 2,000
BA2		48 ± 8

TABLE 2 Kinetic evaluation of truncated analog inhibitors of ANAT

Analog number[a]	truncated analog name	K_i (mM)
TA1	*N*-acetyl aspartate	1.60 ± 0.12
TA1Cl	*N*-chloroacetyl aspartate	0.20 ± 0.04
TA1Br	*N*-bromoacetyl aspartate	>2
TA2	*N*-(acetylcysteaminyl) aspartate	>2
TA2*	*N*-(acetylcysteaminyl boc ester) aspartate	2.10 ± 0.37
TA2a	*N*-(acetylcysteinyl) aspartate	1.03 ± 0.20
TA2a*	*N*-(acetylcysteinyl boc ester) aspartate	0.87 ± 0.16

[a] asterisk (*) indicates the boc-protected form of the inhibitor

TABLE 3 Kinetic Evaluation of Coenzyme A Fragments as Possible Inhibitors

substrate analog	K_i (mM)
adenosine	n.i.[a]
pantothenate	n.i.[a]
adenosine 5'-monophosphate	4.0 ± 0.8
adenosine 5'-diphosphate	1.10 ± 0.15
adenosine 5'-triphosphate	0.96 ± 0.12
adenosine 3',5'-diphosphate	2.30 ± 0.24

[a] no inhibition of ANAT observed when tested at 2 mM

TABLE 4 Cooperative effect of simultaneous binding of ANAT inhibitors

Analog number	K_i (mM) (plus adenosine inhibitor)		
	none	+ 5 mM ATP	+ 5.5 mM ADP
TA1	1.61 ± 0.12	0.58 ± 0.08	0.85 ± 0.13
TA1Cl	0.20 ± 0.04	0.043 ± 0.005	0.170 ± 0.025
TA1Br	>2	----	----
TA2*	2.10 ± 0.37	1.52 ± 0.20	1.46 ± 0.19
TA2a	1.03 ± 0.20	0.66 ± 0.10	0.41 ± 0.05
TA2a*	0.87 ± 0.16	0.84 ± 0.09	0.73 ± 0.10

Figure Legends

FIGURE 1 Metabolism of *N*-acetyl-L-aspartate in the brain. Aspartate *N*-acetyltransferase (ANAT) catalyzes the transfer of the acetyl group from acetyl-coenzyme A to produce *N*-acetyl-L-aspartate (NAA). NAA is transported to oligodendrocytes by a specific dicarboxylate transporter (NaDC3) where it is hydrolyzed by aspartoacylase to release acetate and regenerate L-aspartate. Activation of acetate by coenzyme A provides the precursor for fatty acid biosynthesis.

FIGURE 2 ANAT-catalyzed reaction, showing the transfer of the acetyl group from coenzyme A to L-aspartate, and the proposed transition state for this reaction. The structure of a pair of bisubstrate analogs designed to mimic this transition state are shown, where n is either one (BA1) or two (BA2).

FIGURE 3 Truncation series of ANAT bisubstrate analog inhibitors. The sub-structures of acetyl CoA-aspartate bisubstrate analogs are indicated, from *N*-acetylaspartate (TA1), to the cysteamine adduct (TA2), the β-alanine adduct (TA3) and the bridging pantothenate.