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COMMUNICATION

Design, Synthesis and Evaluation of AdSS Bisubstrate Inhibitors.

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Abstract: Many cancers lack expression of methylthioadenosine phosphorylase (MTAP). These cancers require adenylosuccinate synthetase (AdSS) for nucleic acid synthesis. By inhibiting adenylosuccinate synthetase, we potentially have a new therapeutic agent. Bisubstrate inhibitors were synthesized and evaluated against purified AdSS. The best activity was obtained with adenosine bearing a 4-carbon linker that connects the *N*-formyl-*N*-hydroxy moiety to the 6-position of the purine nucleoside.

Cancer is a significant cause of death worldwide, with lung cancer being the most prominent in men while breast cancer is the highest in women.[1] However, in the United States death from lung cancer is the highest for both men and women.[2] In the search for a new target opportunity, we looked at the nucleotide biosynthetic pathway.[3]

During cell proliferation, adenine nucleotides for nucleic acid biosynthesis are derived through a de novo biosynthetic route that requires the key enzyme adenylosuccinate synthetase (AdSS). In the presence of guanosine triphosphate (GTP), magnesium ions, and aspartate, the AdSS enzyme catalyzes the conversion of ionosine monophosphate (IMP) to the aspartylated nucleotide adenylosuccinate monophosphate (ASMP).[4] Many cancers (approx. 25% of many common human cancers) lack the salvage pathway mediated by methylthioadenosine phosphorylase (MTAP) and rely exclusively on the de novo biosynthesis of AMP.[5] Inhibition of AdSS in such tumors would block the de novo pathway leading to selective cancer cells death. MTAP deficient cancers are hypersensitive to the cytotoxic effects of AdSS inhibitors, such as hadacidin[6], L-alanosine[7], 6-mercapto-IMP[8] and hydantocidin[9]. Inhibition of AdSS by these compounds suggests the utility of inhibitors of this enzyme in therapeutic applications. Currently, there are no commercially known direct binding inhibitors of human AdSS.[10]

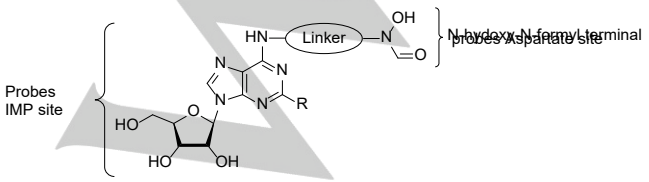
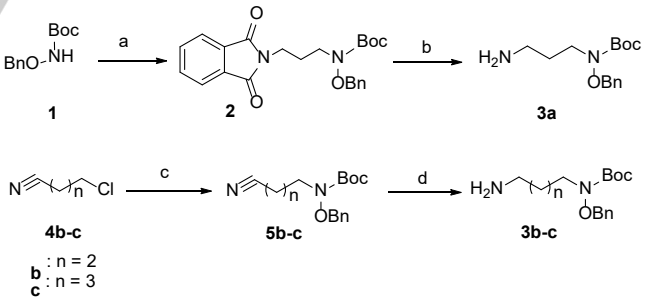


Figure 1. Design of bisubstrate inhibitors for AdSS.

To date, the majority of inhibitors investigated have been found to mimic and compete with either IMP or aspartate. In the present study, we discuss our initial results on the syntheses and evaluation of novel molecules that are designed to span the IMP site into the aspartate-binding pocket of adenylosuccinate synthetase.

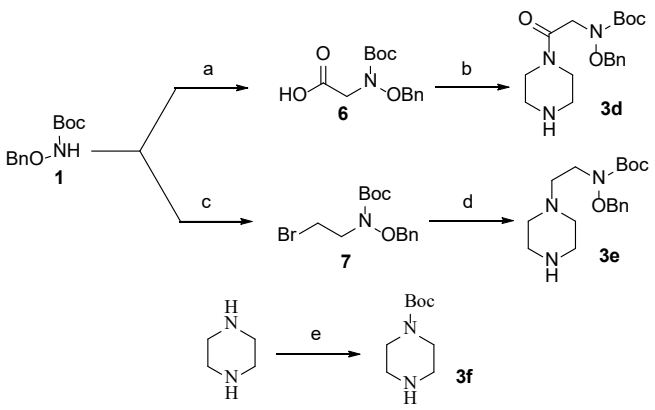
Hadacidin (*N*-formyl-*N*-hydroxylglycine), an anticancer agent is a structural analogue of aspartate that reversibly inhibits AdSS.[11] Previous studies[11,12] have shown that the *N*-formyl-*N*-hydroxy group is essential for inhibitory activity of hadacidin. We have synthesized a series of *N*-formyl-*N*-hydroxy linked purine nucleoside derivatives and evaluated these molecules as AdSS inhibitors. Results from enzymatic assays show a large dependence of binding affinity on the length of the linker between purine nucleoside and hadacidin. More conformationally constrained compounds bearing a piperazine were also prepared. The inhibitory activities of all compounds (**14a**–**f** and **15a**–**f**) were evaluated for activity against AdSS.



Scheme 1 Reagents and Conditions: (a) *N*-(3-bromopropyl)phthalimide, K_2CO_3, DMF, rt, 80%; (b) excess $NH_2NH_2.H_2O$, 92%; (c) BocNHOBn (**1**), NaH, Cat. NaI, DMF, 70 °C; 98% (d) Raney Ni, NH_3, H_2 (45psi), 60–65%

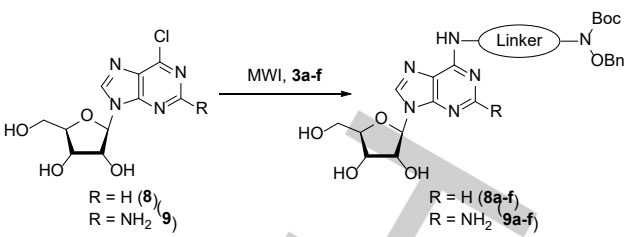
The synthesis of bisubstrate inhibitors began with construction of a spacer linked hadacidin moiety. We started with commercially available *tert*-butyl benzyloxycarbamate (**1**). Alkylation of **1** with *N*-(3-bromopropyl)phthalimide at room temperature provided **2** in 80% yield (scheme 1). The facile removal of the phthalimide protecting group by treatment with an excess of hydrazine monohydrate in ethanol afforded *N*-boc-*N*-benzyl-3-aminopropane (**3a**) in overall 92% yield.[13] Extended derivatives

3b[13] and **3c** were synthesized by initial treatment of **1** with 4-chlorobutyronitrile (**4b**) and 5-chlorovaleronitrile (**4c**) respectively to give compounds **5b** and **5c**, followed by reduction with Raney nickel under pressurized H_2 atmosphere.



Scheme 2. Reagents and Conditions: (a) Bromoacetic acid, NaH, dry THF, 95%; (b) piperazine, PyBOP, HOBt, Et_3N, DMF, 52%; (c) 1,2-dibromoethane, NaH, dry THF, 97%; (d) THF/H_2O/MeOH, 80 °C, MWI, 70%; (e) Boc_2O, DCM, 0 °C, 30%

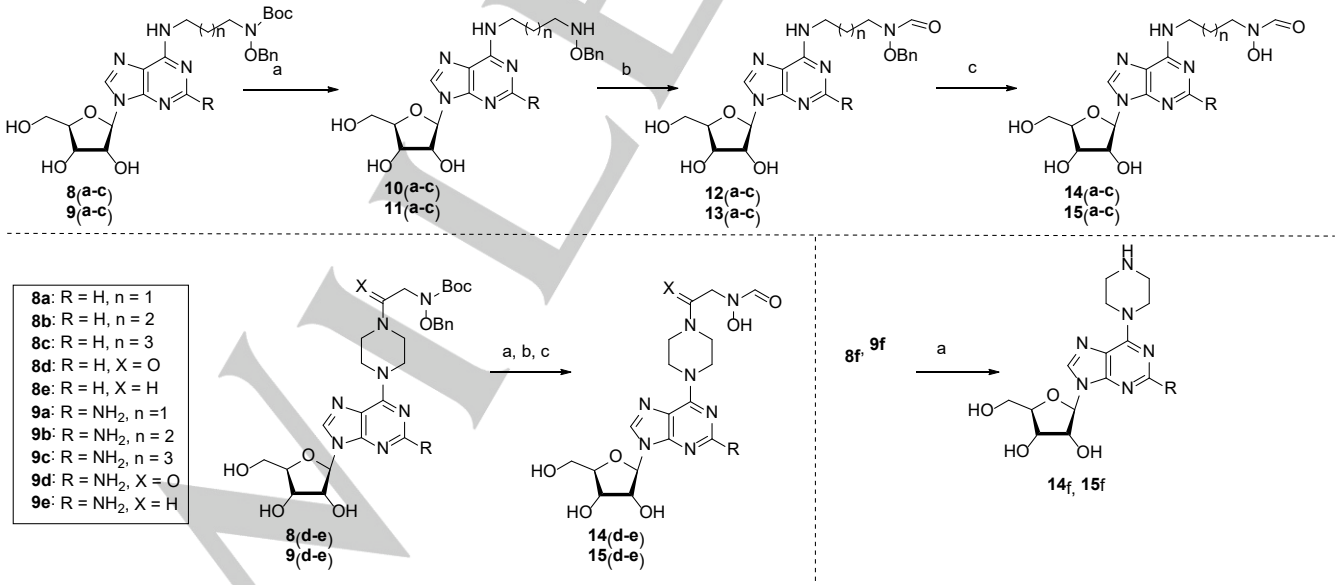
Scheme 2 represents syntheses of the more constrained, piperazine linked hadacidin moiety. Reaction of **1** with bromoacetic acid provided compound **6** in 95% yield. A successive treatment with piperazine in presence of HOBt and PyBOP provided compound **3d**. Further, alkylation of **1** with 1,2-dibromoethane was carried out effortlessly in the presence of NaH with 97% yield. Under microwave irradiation compound **7** and piperazine afforded compound **3e** in 70% yield.[14] Compound **3f** was obtained by Boc protection of piperazine at 0 °C.



Compound	Yield %	Compound	Yield %
8a	88	**9a**	73
8b	85	**9b**	70
8c	80	**9c**	57
8d	74	**9d**	50
8e	80	**9e**	80
8f	75	**9f**	55

Scheme 3. Microwave assisted syntheses of purine nucleotide linked *N*-hydroxy-*N*-formyl moieties. Reaction conditions: H_2O-methanol-THF, 100 °C, 10 min.

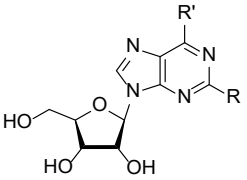
The key step in the syntheses of bisusbstrate inhibitors is the coupling of purine nucleoside with spacer linked *N*-benzyloxy-*N*-tert-butoxycarbonylamino terminal (compounds **3a**–**f**). The coupling was achieved in H_2O-methanol-THF mixture by the aid of microwave irradiation at 100 °C for 10 min (scheme 3).[14] We chose 6-chloropurine riboside (**8**) and 2-amino-6-chloropurine riboside (**9**) to provide purine end to probe IMP site. The compounds **8a**–**f** and **9a**–**f** were obtained in good to moderate yields when 2 or more equivalents of **3a**–**f** coupling partners

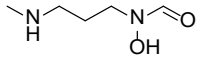
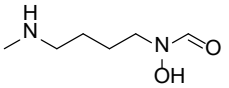
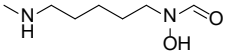
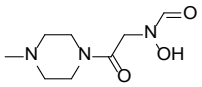
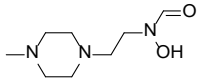
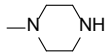


Scheme 4. Reagents and Conditions: (a) 80% TFA in CH_2Cl_2, 0 °C, 30 min, 40–50%; (b) acetic anhydride, formic acid, rt, 30–45%; (c) H_2/Pd-C, MeOH, rt, 60–70%.

were employed. The final steps towards successfully obtaining the inhibitors are demonstrated in scheme 4. Boc deprotection of coupled compounds **8a**–**c** and **9a**–**c** with 80% TFA in methylene chloride afforded **10a**–**c** and **11a**–**c** respectively. *N*-formylation was achieved by treatment with a formic acid and acetic anhydride mixture at 0 °C for an hour. It should be noted that under these conditions 5'-OH of ribose also gets formylated resulting in low yields of desired product. A final benzyl deprotection with Pd-C under H_2 at 1 atm provided desired compounds **14a**–**c** and **15a**–**c**. A similar set of reactions provided compounds **14d**–**f** and **15d**–**f** (scheme 4).

Table 1. Evaluation of bisubstrate inhibitors for activity against adenylosuccinate synthetase.



R'	Compound	R	% Inhibition[a]
DMSO	-	-	0
	**14a**	H	25
	15a	NH_2	23
	**14b**	H	40
	15b	NH_2	34
	**14c**	H	0
	15c	NH_2	0
	**14d**	H	18
	15d	NH_2	20
	**14e**	H	0
	15e	NH_2	0
	**14f**	H	0
	15f	NH_2	0
Hadacidin	-	-	100

a. Tested at 10 mM

The ability of purine nucleotide linked hadacidin derivatives to inhibit AdSS was demonstrated by HPLC detection. Adenylosuccinate synthetase catalyzes the condensation of IMP with aspartate forming adenylosuccinate monophosphate. The enzymatic reaction also requires the conversion of GTP to GDP. The mixture (100µl) contained, 20mM Hepes (pH 7.5), 50µM IMP, 25µM GTP, 500µM $MgCl_2$, 10mM test compound and 2.0µg of purified AdSS enzyme. The assay was initiated at room temperature with the addition of either 100 µM or 500 µM of aspartate. After 5 minutes, the reaction was stopped by freezing in a methanol/dry ice-bath. The solution was allowed to reach room temperature before 20µl of reaction mixture was injected onto the HPLC. The mobile phase for the assay contained 65mM potassium phosphate, 1mM PIC A, and 10% methanol. The buffer has a pH of 4.4. The HPLC assay procedure allows separation of all UV active components (GDP, GTP, and ASMP) of the mixture at 266 nm. The amount of adenylosuccinate monophosphate formed was quantified by the integration of peak areas over 3 runs.

The inhibitory capacities of the newly synthesized compounds against adenylosuccinate synthetase are shown in Table 1. As expected, compounds **14f** and **15f** were not active. With the introduction of the *N*-formyl-*N*-hydroxy group (compounds **14e** and **15e**), no inhibition of AdSS was observed. However, similar derivatives (**14d** and **15d**) with additional amide linkage showed some inhibition. The compounds with a linear linker showed better inhibition than compounds with more conformationally restricted piperazine ring. Compound **14a** with a 3-carbon linker showed 25% inhibition.

Having established the advantage for linearly linked compounds over the conformationally restricted linkers, attempts were made to increase the potency of inhibitors **14a** and **15a**. A 40% inhibition of AdSS activity was observed with addition of one methylene group (compound **14b**) to the linear chain. The result of compound **14b** prompted us to further increase the chain length. Interestingly, on increasing the distance to 5-carbon (**14c**) between two moieties resulted in drastic loss of activity. These experiments suggest that a bisubstrate inhibitor with a four-carbon linker, as in **14b** and **15b**, span both the IMP and aspartate catalytic domains more efficiently. Replacing adenosine (**14a**–**f**) with 2,6-diaminopurine (**15a**–**f**) had a subtle effect on the capabilities of these compounds to inhibit AdSS.

In summary, we have synthesized a series of bisubstrate inhibitors with a purine nucleoside and a hadacidin moiety separated by linkers of varying complexity and sizes. Investigations of the biological profile of our target compounds have shown that a linear spacer is more suitable than a cyclic spacer and that the carbon chain length has an influence on the activity. While, none of the derivatives showed significant AdSS inhibition the study nevertheless provided information about the size of linker suitable to covalently connect IMP and aspartate inhibitors that probe aspartate. The best activity was obtained with compound **14b** bearing a C-4 linker that connects the adenosine moiety to a *N*-formyl-*N*-hydroxyl group. We believe that the described bisubstrate strategy could be useful in designing novel, highly specific inhibitors for AdSS.

Acknowledgements

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Keywords: Lung cancer • small molecule • adenylosuccinate synthetase • MTAP

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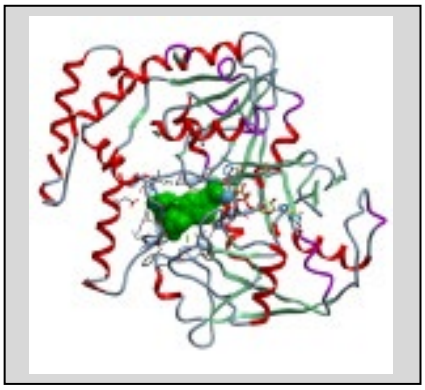

The active site of adenylosuccinate synthetase (AdSS) with a model bisubstrate inhibitor (in green) is shown. A small collection of compounds were developed to bind into the aspartic acid and inosine monophosphate pocket of AdSS. Inhibition of this enzyme affects the purine biosynthesis and is expected to cause selective cell death in lung cancer. The describe bisubstrate strategy could be useful in designing novel, highly specific inhibitors of AdSS.