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Synthesis and evaluation of potential inhibitors of human and *Escherichia coli* histidine triad nucleotide binding proteins

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ABSTRACT

Based on recent substrate specificity studies, a series of ribonucleotide based esters and carbamates were synthesized and screened as inhibitors of the phosphoramidases and acyl-AMP hydrolases, *Escherichia coli* Histidine Triad Nucleotide Binding Protein (ecHinT) and human Histidine Triad Nucleotide Binding Protein 1 (hHint1). Using our established phosphoramidase assay, K_i values were determined. All compounds exhibited non-competitive inhibition profiles. The carbamate based inhibitors were shown to successfully suppress the Hint1-associated phenotype in *E. coli*, suggesting that they are permeable intracellular inhibitors of ecHinT.

Histidine triad nucleotide binding protein (Hint) is named after the highly conserved motif related to the sequence His-X-His-X-His-XX in the active site, where X is a hydrophobic amino acid. Hint is considered to be the ancestor of the histidine triad protein superfamily and can be found in both prokaryotes and eukaryotes.[1] While eukaryotes generally express multiple forms of Hint (Hint1, Hint2 and Hint3), prokaryote genomes, including gram negative and gram-positive bacteria, typically encode one Hint gene.[2]

Recently, evidence has begun to emerge that Hint1 may function as a tumor suppressor through multiple molecular mechanisms involving triggering apoptosis,[3] regulating gene transcription and cell cycle modulation.[4,5] Hint1 deleted mice studies have revealed that an increased susceptibility to the carcinogenic DMBA induced ovarian and mammary tumors, as well as an increased occurrence of spontaneous tumors.[6] Eukaryotic Hint1 has been shown to associate with, and suppress the β-catenin Wnt signaling pathway transcriptional activity by direct interactions with Reptin and Pontin.[7] Hint1 has also been associated with transcription factors such as, TFIIH,[8] MITF,[4,9] and USF2.[5] In addition, the interactions between MITF and USF2 appear to be mediated by LysRS.[4,5,10] Recently, we have demonstrated that lysyl-adenylate (lysyl-AMP) generated by lysyl-tRNA synthetases (LysRS) are also substrates for Hints.[11]

Despite such a wide variety of possible biological functions in eukaryotes, the cellular function and rationale for the evolutionary conservation of Hint in bacteria has remained a mystery. Moreover, none of the previously proposed functions has been successfully linked to the enzymatic activity of Hints. Recently, we have shown that the expression of the catalytically active Hint is essential for the activity of the enzyme D-amino acid dehydrogenase (DadA) in *Escherichia coli* DadA.[12] Consequently, we have chosen to synthesize novel Hint1 inhibitors, which can be used as chemical probes to study the connection between the newly discovered Hint1-associated phenotype in *E. coli* and its enzymatic activity.

Hint1 has been found to be a nucleoside phosphoramidase and acyl-AMP hydrolase.[1] Probing Hint1 base recognition site, the substrate specificity for both purine and pyrimidine phosphoramidates was determined.[13] Structure–activity relationship studies revealed that Hint1 prefers purine over pyrimidine phosphoramidates. In addition, based on kinetic and crystallographic studies, the 2- and 3-hydroxyl groups of the ribose ring were shown to be required for maximal phosphoramidase efficiency.[13]

Based on the previous findings and knowledge of the general outline of substrate specificity requirements, compounds **1** and **2** were designed and synthesized following Scheme S1 (Supplementary data). Both compounds were titrated into a phosphoramidase assay using the fluorogenic substrate tryptamine 5′-adenosine phosphoramidate (TpAd) (Fig. 1a) as described previously.[13] In short, in the absence of inhibitors, turnover rates were measured by following the hydrolysis of TpAd by ecHinT and hHint1 proteins. Excitation wavelength was set at 280 nm, and fluorescence emission was measured at 360 nm. The rate of hydrolysis of the substrate was determined by measuring the increase of fluorescence intensity upon the addition of the enzyme over two minutes. The Michaelis–Menten constants, k_{cat} (s^{-1}) and K_m (μM), were determined by nonlinear regression analysis of the initial

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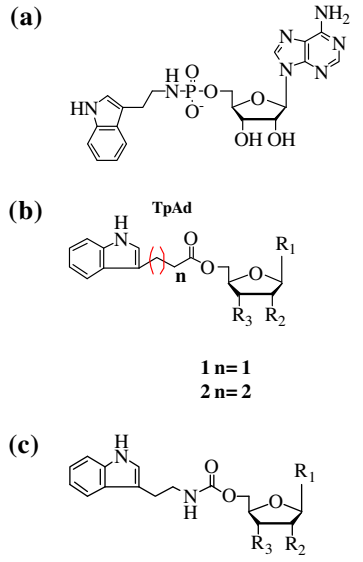

Figure 1. Structures of (a) tryptamine 5′-adenosine phosphoramidate and its (b) ester and (c) carbamate analogues.

velocity versus concentration using JMP IN 7 software. In the presence of inhibitors, the proteins were pre-incubated with the tested inhibitor for 30 s at 25 °C before the addition of the substrate. The reaction was followed under the same condition as of in the absence of inhibitor.

While Hints did not show any appreciable esterase activity against compound **1** and **2**, kinetic analysis revealed a significant and dose-dependent decrease in the apparent V_{max} of Hint1 in the presence of either compound **1** or **2** (Supplementary Fig. S1). In contrast, the K_m value for TpAd was unaltered by the presence of the inhibitor. Thus, the mechanism of inhibition was clearly of a non-competitive type, suggesting that the inhibitor and TpAd substrate do not share a common binding site on the enzyme. K_i values were estimated from the Dixon plot and are summarized in Table 1.

A second series of carbamate analogues was prepared following Schemes 2S and S3 (Supplementary data). As shown in Table 1, compound **3**, a thymidine carbamate analogue in which 3-hydroxyl group of the ribose ring was replaced with a primary amine group, inhibited Hint with a K_i value of 122 μM for ecHinT and 139 μM for hHint1. Replacement of the amine group with an azido moiety at the 3′ position, compound **4**, resulted in an inhibitor with a K_i value of 565 μM for ecHinT and 715 μM for hHint1. The five-fold enhancement in equilibrium binding conferred by the amine group at the 3′ position is most likely explained by the formation of favorable hydrogen bonding interactions between the inhibitor and its binding site in the protein structure. Adenosine carbamate, compound **5**, and guanosine carbamate,[12] compound **6**, were the most potent inhibitors prepared in this study. As shown in Table 1, K_i values for the adenosine carbamate analogue ranged between 73 μM for ecHinT and 103 μM for hHint1 while the K_i values for the guanosine carbamate analogue ranged between 42 μM for ecHinT and 34 μM for hHint1. All compounds exhibited non-competitive inhibition profiles.

Table 1
Inhibition constants determined in HEPEs buffer (pH 7.2) at 25 °C

Compds	R_1	R_2	R_3	EcHinT K_i (μM)[a]	hHint1 K_i (μM)[a]
1	Adenine	OH	OH	375 (±13)	400 (±27)
2	Adenine	OH	OH	393 (±17)	457 (±32)
3	Thymine	H	NH_2	122 (±10)	139 (±17)
4	Thymine	H	N_3	565 (±23)	715 (±35)
5	Adenine	OH	OH	73 (±4)	103 (±18)
6	Guanine	OH	OH	42 (±6)[b]	34 (±4)

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] Value was taken from Ref. 12.

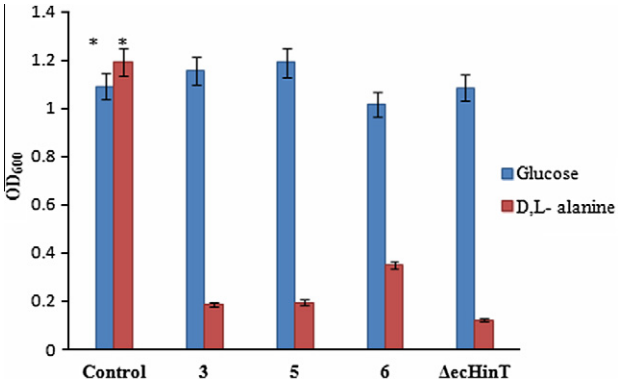

Figure 2. ecHinT inhibition resulted in impaired phenotype. *Measurements were carried out in duplicates and the standard deviation was ±2%. Results for compound **6** taken from Ref. 12.

Recently, we have been able to connect ecHinT to an established phenotype in bacteria. Using active site mutants, we demonstrated that the catalytically active ecHinT is required for *E. coli* to utilize D-alanine as a carbon source. To assess the ability of our compounds to inhibit ecHinT in vivo, we tested the ability of *E. coli* BW25113 strain to grow on D-alanine in the presence of 100 μM of either compound **3**, **5** or **6**. Cultures were grown in M9 media, supplemented with either 22 mM D,L-alanine or 10 mM glucose, at 37 °C for 40 h before OD_{600} was measured. Consistent with our previous finding, that the catalytic activity of ecHinT is required for *E. coli* to grow on D-alanine, inhibition of ecHinT in BW25113 resulted in its impaired growth when D-alanine is the sole carbon source (Fig. 2). In addition to the cell permeability properties of compounds **3**, **5** and **6**, no toxicity was observed with either inhibitor when glucose is the sole carbon source in the growth medium.[12]

In conclusion, we have designed and synthesized the first generation of cell-permeable Hint inhibitors. Previously, we have shown that Δ*hinT E. coli*, a mutant strain in which *hinT* was deleted, exhibited impaired growth when D-lanine is the sole carbon source.[12] Using the developed inhibitors as chemical probes, this work has demonstrated that inhibition of ecHinT resulted in similar impaired growth observed for Δ*hinT* in the presence of D-alanine as a sole carbon source. Guanosine carbamate, 6,[12] one of the best inhibitors reported in this study, may serve as structural template for the design and development of more potent inhibitors with enhanced inhibition constants. Ongoing crystallographic studies of the inhibitor bound form of Hint should provide a structural foundation for understanding the mechanism of inhibition and for the design of selective Hint inhibitors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.082.

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