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Bioorganic & Medicinal Chemistry Letters 13 (2003) 1985–1988

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Unexpected Inhibition of *S*-Adenosyl-L-homocysteine Hydrolase by a Guanosine Nucleoside†

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Received 18 February 2003; revised 25 March 2003; accepted 25 March 2003

Abstract—A series of shape-modified flexible nucleosides ('fleximers', **1**, **2**, and **3**) was modeled, synthesized and subsequently assayed against *S*-adenosyl-L-homocysteine hydrolase (SAHase). No inhibitory activity was observed for the adenosine fleximer, which served as a substrate, but moderate inhibitory activity was exhibited by the guanosine fleximers. This is the first known report of a guanosine nucleoside analogue possessing activity against SAHase.

One focus for our research has involved the design and synthesis of novel shape-modified nucleosides to explore fundamental aspects of nucleic acid structure, function and stability, as well as to serve as potential design tools to investigate parameters for enzymes possessing flexible binding sites. We recently reported the synthesis of a series of flexible nucleosides for this latter purpose.[1,2]

These novel fleximers feature a purine ring 'split' into the individual components; the imidazole and pyrimidine rings of adenosine, inosine and guanosine (as in **1**, **2**, and **3**; Fig. 1) are connected by a single carbon–carbon bond thereby creating a separation of 1.50 Å between the rings.

Analogous to Leonard's *dist*-benzoseparated analogues[3] these 'fleximers' are connected at the C-5 of the imidazole and the C-6 of the pyrimidine rings. This allows retention of the components necessary for molecular recognition, but introduces flexibility to the structure.

It was our thought that this approach might serve to overcome the problems typically associated with using crystallographic data in modeling when dealing with flexible enzyme binding sites; if both the enzyme and the inhibitor were allowed to achieve their lowest energy conformation, then this should result in a lower overall energy for the enzyme–inhibitor complex. This conformation should then be the most highly populated, thereby increasing the chances that this would be the conformation that would crystallize. Once the enzyme and inhibitor revealed this conformation, then the data could then be used as a modeling tool to design a second generation of compounds.

One enzyme studied extensively in our laboratories is *S*-adenosyl-L-homocysteine hydrolase (SAHase). The byproduct of all *S*-adenosylmethionine (SAM)-promoted methyltransferase reactions is *S*-adenosylhomocysteine (SAH), and SAHase cleaves SAH into its two

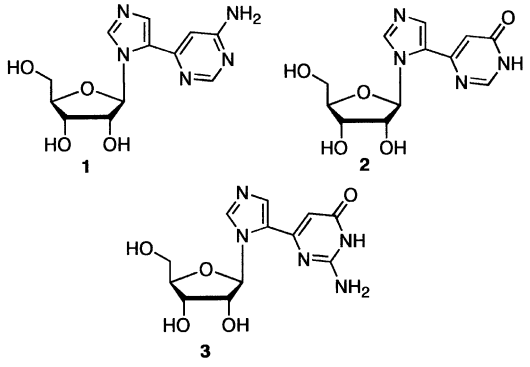

Figure 1. Parent fleximers.

†This work was initially reported at the International Roundtable on Nucleosides, Nucleotides and Nucleic Acids, Leuven, Belgium, September, 2002.

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0960-894X/03/$ - see front matter
doi:10.1016/S0960-894X(03)00331-7

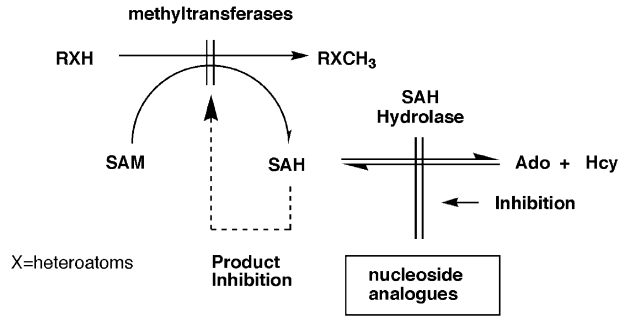

Figure 2. Inhibition of SAHase by nucleoside analogues.

components, adenosine (Ado) and homocysteine (Hcy) (as depicted in Fig. 2 on the next page). As a result, SAH is a potent competitive inhibitor of all methylations dependent upon SAM as the methyl donor, including DNA MeTase.[4] SAM is the most reactive methyl donor thus SAM-dependent methylations are considered to be most important biologically.[5]

As previously mentioned, SAHase cleaves SAH into its two cellular components, adenosine (Ado) and homocysteine (Hcy), and requires the assistance of an enzyme-bound cofactor, NADH.[6] Inhibition of SAHase by nucleoside inhibitors involves depletion of the NADH cofactor, which causes an intracellular accumulation of SAH, thereby elevating the SAH/SAM ratio. This imbalance in the SAH/SAM ratio results in cessation of SAM-dependent methylations, which leads to improperly methylated DNA.[7] Many modified nucleosides have proven to be exceptional inhibitors of SAHase, and as a consequence, methyltransferases via this biofeedback mechanism, exhibiting activity against numerous viruses including the pox viruses, arenaviruses, rhabdoviruses, reoviruses, vaccinia virus, parainfluenza virus, measles, ebola virus, sarcoma virus and the herpes viruses as well as against colon carcinoma, leukemia cells, among other cancer types.[5,7]

Given the recent report that SAHase possesses a flexible binding site,[8,9] we decided to pursue an investigation of this enzyme using our fleximers as potential design tools. The structures were optimized using RHF SCF gradient optimization at the 6-311G* level of theory from an initial model as previously reported.[1] To gauge the level of conformational flexibility in the context of an enzyme active site, all three fleximer parent compounds were modeled in SAHase. Using automated molecular docking (Insight II, Discover/Acceryls), Monte Carlo simulation, followed by simulated annealing revealed that the fleximers, with the exception of the guanosine fleximer (**3**), occupied a region in the SAHase active site in an orientation that is similar, but not identical to, the position of the known nucleoside inhibitor in the crystal structure published by Turner et al.[9] The crystallographic water molecules in the original structure for the active site were retained as they also interacted with the fleximers.

The guanosine fleximer (**3**) (as shown in Figs. 3 and 4) proved to be quite interesting; the lowest energy conformation revealed that the heterocyclic moieties had adopted a *syn*-like conformation, bending back over the sugar to form a curved structure. More significantly, the pyrimidine moiety exhibited a high degree of twist, essentially reversing the substituents to allow the fleximer to take advantage of intramolecular hydrogen bonding between the carbonyl of the pyrimidine and the 5′-hydroxyl of the sugar moiety.

Following their synthesis,[1,2] the fleximers and their corresponding parent nucleosides were assayed against SAHase.[10] As shown in Table 1, the adenosine fleximer was an alternative substrate for the enzyme, albeit worse than adenosine itself, while the inosine a worse substrate, but exhibiting weak enzymatic activity. To our surprise, the guanosine fleximer showed activity in both the synthetic and the hydrolytic directions with K_i values of 217 and 128 μM, respectively. While these values are not spectacular since aristeromycin and

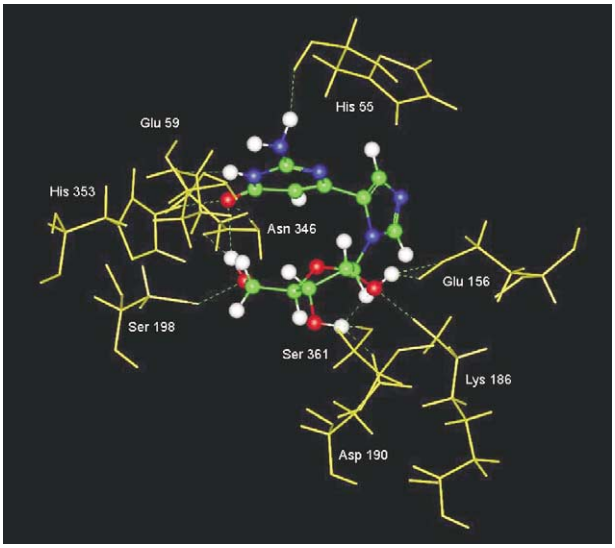

Figure 3. Guanosine-fleximer **1** in SAHase.

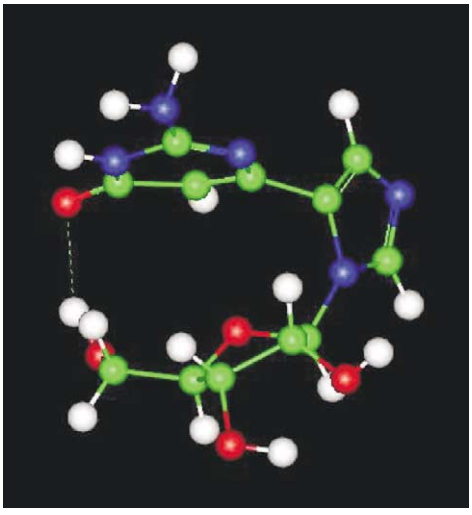

Figure 4. Guanosine-fleximer **3** exhibiting intramolecular hydrogen bonding.

Table 1. Results of the enzyme assays with SAHase, parent nucleosides and the parent fleximers[10]

Compd	K_m (μM)[a]	k_{cat} (m^{-1})[a]	K_i (μM)[a]
Synthesis direction			
Adenosine	0.82 (±0.004)	91.2 (±0.2)	nd
1	54.4 (±1.1)	0.56 (±0.04)	nd
Inosine	2.5 (±0.03)	44.2 (±0.7)	925 (±21)
2	421 (±5.7)	0.005 (±0.003)	422 (±16)
Guanosine	844 (±6.6)	0.06 (±0.02)	1472 (±32)
3	ns	ns	217 (±13)
Hydrolysis direction			
3	6.6 (±0.2)	28.3 (±1.2)	128 (±18)

na = not determined.
ns = not a substrate.
[a]Values are means of three experiments, standard deviation is given in parentheses

neplanocin have K_i values in the nanomolar range against SAHase, they are notable since, to our knowledge, no guanosine nucleosides have ever been reported to exhibit activity against SAHase.

Isothermal calorimetry was then carried out to determine whether or not the guanosine fleximer was forming a stable complex with SAHase.[12] Fleximer **3** was shown to bind to SAHase with a stoichiometry of 1:1 per monomer or four compound molecules binding per functional SAHase tetramer (note: the inosine fleximer **2** was also studied, but given that it serves as a poor substrate as well as a poor inhibitor, the data obtained was not meaningful).

The top panel depicted in Figure 5 shows the raw ITC data for the titration of the guanosine fleximer **3**, while the bottom reflects the binding isotherm produced by integrating each injection peak with respect to time. Results for the interaction between SAHase and **3** are as follows:

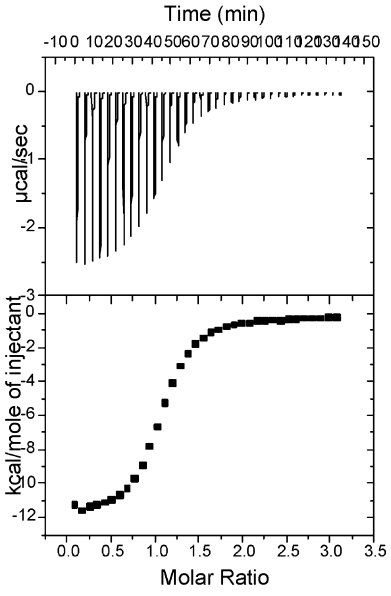

Figure 5. ITC data for **3**.

Stoichiometry:	0.995 ± 0.02
K_a (M^{-1}):	$1.65\times10^6 \pm 4.5\times10^4$
ΔH (kcal/mol):	−26.1 ± 1.45
ΔS (cal mol^{-1} K^{-1})	−11.6 ± 2.2
ΔG (kcal/mol):	−22.6 ± 2.1 (vs −24.83 theoretical)

This indicates that the interaction between the guanosine fleximer (**3**) and SAHase is enthalpically driven, that is, ΔH is negative. The reaction is not entropically favored, as evidenced by the negative value of ΔS, however, the enthalpic term is larger in magnitude than the term, $T\Delta S$, hence the overall free energy (ΔG) is negative. In that regard, we were also gratified to see that the predicted value of -24.83 kcal/mol was in good agreement with the observed value of −22.6 kcal/mol, differing only by approximately 2 kcal. As a result, the original plan to use the fleximers as design tools appears to be plausible.

While it is not surprising that the binding reactions are entropically unfavorable, since this presumably arises from the loss of configurational entropy upon binding, and this entropic loss is a hallmark of the increased degrees of freedom afforded by the separation of the purine base into its two components, we have shown that this entropic cost can be overcome by utilizing the inherent ability of the fleximers to adopt more favorable conformations which can then allow more favorable binding interactions than are possible with more rigid nucleoside analogues.

Also while we cannot be certain at this point if the unusual, albeit theoretical, conformation adopted by the guanosine fleximer in the modeling studies will prove to be real once crystallized, the activity shown by the guanosine fleximer is nonetheless an important observation. If this is indeed the case and the unusual conformation adopted by the guanosine fleximer proves to be responsible for the unexpected inhibitory activity against SAHase, clearly the ability of the fleximer to sample interactions that a normal guanosine nucleoside is incapable of will obviously be of value as well. Regardless, efforts to obtain crystallographic data are underway and we hope to report the results soon.

In conclusion, we believe this to be the first report of a guanosine nucleoside analogue to exhibit inhibitory activity of SAHase and this unusual activity has provided an important new lead in the area of enzyme inhibition.

Acknowledgements

We thank Professor Donald Doyle and Mrs. Lauren Schwimmer, Georgia Tech for their assistance with the modeling.

References and Notes

1. Seley, K. L.; Zhang, L.; Hagos, A.; Quirk, S. *J. Org. Chem.* **2002**, *67*, 3365.

2. Seley, K. L.; Zhang, L.; Hagos, A. *Org. Lett.* **2001**, *3*, 3209.
3. Leonard, N. J. *Acct. Chem. Res.* **1982**, *15*, 128.
4. Chiang, P. K.; Miura, G. A. In *Biological Methylation and Drug Design*; Borchardt, R. T., Creveling, C. R., Ueland, P. M., Eds.; Humana: Clifton, NJ, 1986; p 239.
5. Chiang, P. K. *Pharmacol. Ther.* **1998**, *77*, 115.
6. Palmer, J. L.; Abeles, R. H. *J. Biol. Chem.* **1979**, *254*, 1217.
7. De Clercq, E. *Nucleosides Nucleotides* **1994**, *13*, 1271.
8. Yin, D.; Yang, X.; Hu, Y.; Kuczera, K.; Schowen, R. L.; Borchardt, R. T.; Squier, T. C. *Biochemistry* **2000**, *39*, 9811.
9. Turner, M. A.; Yang, X.; Yin, D.; Kuczera, K.; Borchardt, R. T.; Howell, P. L. *Cell Biochem. Biophys.* **2000**, *33*, 101.
10. The SAHase activity is measured in the synthetic direction by measuring the rate of formation of *S*-adenosylhomocysteine (SAH) from adenosine and homocysteine. The enzyme was purchased from Sigma Chem. Co. (A 1705), and passed through a 4.9 $cm^2 \times 54$ cm Sephadex G100 column in 10 mM Tris–HCl (pH 7.8). Fractions containing enzyme were pooled and concentrated to 10 mg/mL via pressure filtration and stored at −70 °C. The measured specific activity of the enzyme was 27 units/mg; where a unit hydrolyzes 1 nM of SAH to adenosine and L-homocysteine per min at pH 7.8 at 37 °C. Enzyme is added to a reaction that contained (in 100 μL): 1 mM adenosine, 1 mM homocysteine, 20 mM potassium phosphate (pH 7.5), 0.25 mg/mL BSA, 1 mM EDTA, and various concentrations of fleximer. The reactions were incubated for various times (0–60 min) at 37 °C and were quenched by the addition of 100 μL of 20 mM HCl. The entire reaction was loaded onto a Cellex-P HPLC column that was equilibrated in 10 mM HCl. A linear 10 mL gradient (10 mM HCl to 100 mM HCl/200 mM NaCl) was applied to the sample after loading. Unreacted adenosine is eluted first, early in the gradient, followed by SAH towards the end of the gradient. Eluting peaks were detected at 254 nm. The peaks are integrated and compared to similarly run standards of known concentration in order to calculate K_m and k_{cat} values. The reaction is also run without adenosine in order to determine whether any of the fleximers can act as substrates for the enzyme.

The hydrolysis of SAH is determined by measuring the formation of inosine. SAH hydrolysis was assayed at 37 °C for 4 min (for the determination of initial velocities) in 0.1 mL of 25 mM potassium phosphate buffer (pH 7.2) containing 2.5–80 μM SAH, 0.4 unit of adenosine deaminase and SAHase. The reaction was initiated upon the addition of SAHase and terminated by the addition of 10 μL of 0.5 N HCl. The reaction mixture was kept on ice until HPLC analysis. The mixture is then analyzed for inosine content via HPLC at 260 nm on a C18 column (150×4.6 mm). The mobile phase is composed of 25 mM KH_2PO_4 and acetonitrile 95:5 (25 mM KH_2PO_4: acetonitrile). The flow rate is 0.5 mL/min.

Time-dependent SAHase inactivation by the target compounds is measured by incubating various concentrations of each of the inhibitors (2.5–20 μM) with 20 nM SAHase in 500 μL of 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA buffer (buffer A) at 37 °C for different times (0–20 min). The activity remaining is determined in the synthetic direction as above.[11] The pseudo-first-order rate of inactivation (k_{obs}) is obtained by plotting the logarithm of the remaining activity versus preincubation time. The K_i and k_2 values are obtained by plotting $1/k_{obs}$ versus 1/[Inhibitor], using the equation $1/k_{obs} = K_i/(k_2)[I]) + 1/k_2$.[11]
11. Ault-Riche, D. B.; Lee, Y.; Yuan, C.-S.; Hasobe, M.; Wolfe, M. S.; Borcherding, D. R.; Borchardt, R. T. *Mol. Pharmacol.* **1993**, *43*, 989.
12. Isothermal titration calorimetry (ITC) was performed with a VP-ITC instrument from MicroCal, Inc. The compound was dissolved in 20 mM cacodylate (pH 6.8), 20 mM NaCl at a final concentration of 1 mM at 10 °C. SAHase was dialyzed into the same buffer at a final concentration of 20 μM. Titrations were carried out by injecting 5 μL of an inhibitor solution (at concentration ranges from 0.5 to 2.0 mM) into the 1.4 mL stirred reaction cell. SAHase ranged in concentration from 50 to 80 μM in the cell. Both the inhibitor and the enzyme were in 20 mM sodium cacodylate (pH 5.5–7.0), 40 mM NaCl, or 20 mM Tris HCl (pH 7.0–7.5), 40 mM NaCl. Titrations were conducted between 20 and 40 °C. Typical experimental conditions for the titrations were a 10-s injection period followed by a 240-s delay between injections for a total of 40 injections. Blank titrations of inhibitor into buffer were performed in order to correct for heats of dilution and mixing. The independent set of multiple binding sites is the most common model for binding experiment evaluations. The analytical solution for the total heat is determined by:

$$Q = V\Delta H\left[[L] + \frac{1 + [M]nK - \overline{(1 + [M]nK - [L]K)^2 + 4K[L]}}{2K}\right]$$

where Q is the total heat, V is the cell volume, ΔH is the enthalpy, M is the macromolecule concentration (the binding partner in the cell), n is the binding stoichiometry, L is the ligand concentration (the binding partner in the syringe), and K is the association constant.[13] Data were fit to this model using Origin version 5 (MicroCal, Inc.). The free energy of binding is related to the binding enthalpy by: $\Delta G = \Delta H - T\Delta S$ and for all calorimetric titrations, the protein concentration is given per monomer of enzyme.
13. Freire, E. *Adv. Protein Chem.* **1998**, *51*, 255.