Communications to the Editor

Mechanism-Based Inhibition of an Essential Bacterial Adenine DNA Methyltransferase: Rationally Designed Antibiotics

Daphne C. Wahnon, Vincent K. Shier, and Stephen J. Benkovic*

The Pennsylvania State University
Department of Chemistry, 414 Wartik Laboratory
University Park, Pennsylvania, 16802

Received September 6, 2000

The emergence of bacteria resistant to available antibiotics has caused great concern in the medical community and has created a need for the discovery of novel antibiotic agents. In the past new antibiotics have been discovered by the random screening of natural products and the subsequent identification of the target protein. In addition to this drug discovery method, the recent advances in genome sequencing have made it possible to envision a complementary drug discovery method in which a bacterial enzyme target is first identified and new antibiotic compounds are discovered from the targeted inhibition of this enzyme. Successful antibiotics would be inhibitors of an essential bacterial enzyme that is unique to bacteria and has no mammalian homologue. Here we report on the progress toward the development of small-molecule selective inhibitors of an essential bacterial N-6 adenine DNA methyltransferase (MTase), using a mechanism-based multisubstrate adduct approach and demonstrate the inhibition using CcrM (cell cycle regulated DNA MTase) from the pathogenic *Brucella abortus*.

DNA methylation is an important and ubiquitous biological event that encodes an additional level of information to the "four base" genetic code. The majority of DNA MTases in bacteria belong to restriction modification systems (RM) that protect the bacterial host DNA from invading viruses. CcrM, however, lacks the cognate restriction enzyme of RM systems, serves a critical regulatory role in the correct progression of cell cycle events,[1] and is essential to cellular viability.[2] In addition, CcrM is conserved among several bacterial species including pathogens such as *B. abortus*, *Helicobacter pylori*, and *Haemophilus influenza*.[3] In mammals, DNA methylation is also critical in regulating higher functions of the genome including replication, and gene expression and is intimately linked to the biochemistry of some cancers.[4] N-6 adenine DNA MTase activity is not found in mammalian cells, however, and instead occurs at C-5 cytosine.[5] For these reasons CcrM is implicated as an ideal target for antibacterial therapy. A second regulatory bacterial N-6 DNA MTase Dam is not essential to cellular viability but has been recently demonstrated to be essential to bacterial virulence in S*almonella typhimurium*.[6] Inhibitors of N-6 adenine would result in potent compounds against bacterial strains that contain CcrM, Dam methylase, or similar regulatory adenine DNA MTases. Adenine DNA MTase inhibitors would also act on RM systems and provide further beneficial bacteriostatic effect.

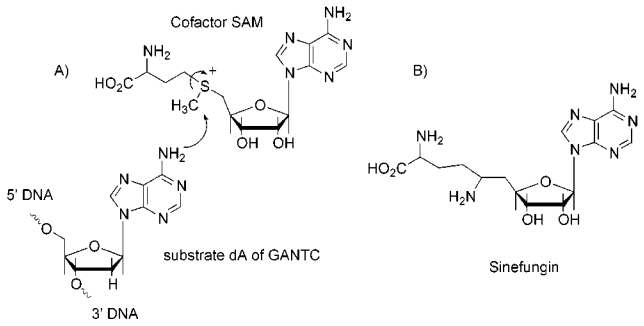


Figure 1. (A) Reaction catalyzed by CcrM: a direct methyl transfer from SAM to deoxyadenosine of GANTC. (B) Sinefungin, a natural product methyltransferase inhibitor.

Enzymatic N-6 adenine DNA methylation (Figure 1A) proceeds in a direct fashion in contrast to the mechanism observed in solution.[7] To access the exocyclic amine, which is normally involved in Watson−Crick base pairing, the target adenine is flipped away from the double helix and consequently positioned proximally to the cofactor *S*-adenosylmethionine (SAM) to allow direct methyl transfer to occur.[8] In CcrM, the target base is contained within the recognition sequence GANTC. Known inhibitors of DNA methylation are analogues of SAM, including the natural product sinefungin (Figure 1B). These compounds are reasonable inhibitors but target all methylation events in the cell. Clinical use of these compounds is limited by their toxicity.[9]

We reasoned that by extending from N-6 of the substrate adenine into the SAM binding pocket and tethering the substrate to the cofactor we might retain the inhibition observed with cofactor analogues and induce selectivity from the additional binding in the target adenine binding site. Similar multisubstrate adduct approaches (the covalent attachment of two enzyme substrates to form a single molecule) have been shown to increase binding affinity and specificity of the target enzyme.[10] Compounds **5**−**8** were designed as partial multisubstrate adducts for N-6 adenine DNA MTase.

Adapting the methodology developed for the synthesis of N-6 arylthiomethyl ribonucleosides[11] it was possible to obtain compounds **5**−**8** from adenosine, adenosine 5′-phosphate, 2′-deoxyadenosine, and 2′-deoxyadenosine 5′-phosphate. Hydrolysis of *N*-acetyl homocysteine thiolactone under deoxygenated conditions afforded the *N*-acetyl-DL-homocysteine that was used in the subsequent N-6 alkylthiomethylation step (Scheme 1). Reaction solutions were kept near pH 5 during the synthesis of the 2′-

* To whom correspondence should be addressed. Telephone: 814-865-2882. Fax: 814-865-2973.

(1) (a) Zweiger, G.; Marczynski, G.; Shapiro, L. *J. Mol. Biol.* **1994**, *235*, 472. (b) Stephen, C.; Zweiger, G.; Shapiro, L. *J. Bacteriol.* **1995**, *177*, 1662.
(2) Stephens, C.; Reisenauer, A.; Wright R.; Saphiro L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 1210.
(3) Wright, R.; Stephens, C.; Shapiro, L. *J. Bacteriol.* **1997**, *179*, 5869.
(4) Szyf, M. *Pharmacol. Ther.* **1996**, *70*, 1.
(5) (a) Malone, T.; Blumenthal, R.; Cheng, X. *J. Mol. Biol.* **1995**, *253*, 618. (b) Razin, A.; Szyf, M. *Biochim. Biophys. Acta* **1984**, *782*, 331. (c) Adams, R. *BioEssays* **1995**, *17*, 139.
(6) Heithoff, M. D.; Sinsheimer, R. L.; Low, D. A.; Mahan, M. J. *Science* **1999**, *284*, 967.
(7) (a) Pogolotti, A. L., Jr.; Subramaniam, R.; Santi, D. V. *J. Biol. Chem.* **1988**, *263*, 7461. (b) Ho, D.; Wu, J.; Santi, D.; Floss, H. *Arch. Biochem. Biophys.* **1991**, *284*, 264.
(8) (a)Allan, B.; Beechem, J.; Lindstom, W.; Reich, N. *J. Biol. Chem.* **1998**, *273*, 2368. (b) Holz, B.; Klimasauskas, S.; Seva, S.; Weinhold, E. *Nucl. Acids Res.* **1998**, *26*, 1076.
(9) Ghosh, A. K.; Liu, W. *J. Org. Chem.* **1996**, *61*, 6175. Zwerygerth, E.; Schillinger, D.; Kaufmann, W.; Roettcher, D. *Trop. Med. Parasitol.* **1986**, *37*, 255.
(10) (a) Broom, A. D. *J. Med. Chem.* **1989**, *32*, 2. (b) Inglese, J.; Blatchly, R. A.; Benkovic, S. J. *J. Med. Chem.* **1989**, *32*, 937. (c) Inglese, J.; Benkovic, S. J. *Tetrahedron* **1991**, *47*, 2351.
(11) (a) Kemal, O.; Reese, C. Synthesis, **1980**, 1025−1028. (b) Yamazaki, Y.; Suzuki, H.; Kamibayashi, A.; Watanabe, N.; Takahashi, N. *Agric. Biol. Chem.* **1979**, *43*, 1945.

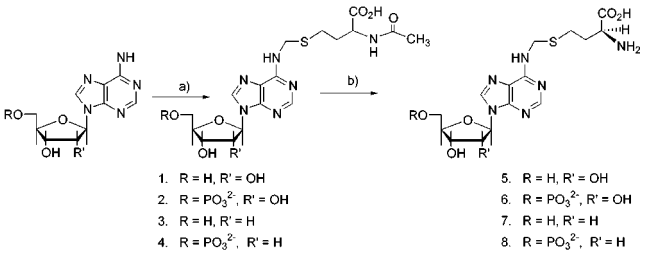
10.1021/ja003285o CCC: $20.00
Published on Web 01/13/2001

Table 1. K_i's of Compounds **5**−**8**

Compound	*K_I (μM) B.CcrM*	*K_I (μM) C.CcrM*
5	30	41.5
6	15	ND
7	7	7
8	5	1

deoxyadenosine analogues to avoid depurination side products. Compounds **1**−**4** were purified using reverse phase HPLC, cellulose chromatography or DEAE sephadex ion exchange chromatography. *N*-acetyl deprotection of **1**−**4** was achieved using acylase I and yielded enantiomerically pure **5**−**8** in a kinetic resolution step. **5**−**8** were purified by cellulose chromatography to remove salts and enzyme and were stable for a several days at 30 °C in 50 mM, pH 7.4 HEPES buffer (assay conditions) although significant decomposition was observed in both basic (>pH 8) and acidic aqueous solutions.

Scheme 1. Reaction and Conditions: (a) HOAc, 7:3 Ethanol: Water, Reflux, 2 Days; (b) Acylase I, pH 7 phosphate buffer



We have previously reported on the purification and mechanism of CcrM from *Caulobacter crescentus*.[12] We now report on the purification of CcrM from the pathogenic *B. abortus* (*B.CcrM*). *B.CcrM* was overexpressed in *Escherichia coli* as an N-terminal six histidine tagged protein (*B.CcrM*) and purified using standard Ni NTA affinity chromatography procedures. This represents the first purification of this CcrM homologue. In a typical purification, 10 mg of >95% pure protein was obtained per two liter of bacterial culture. The purified *B.CcrM* is functional and methylates the synthetic substrate hemi-methylated N-6 45/50 mer as determined by the tritium incorporation filter-binding assay previously described.

Compounds **5**−**8** were shown to inhibit *B.CcrM* activity with K_i's ranging from 5 to 30 μM (Table 1). Adenosine and homocysteine individually had no effect on MTase activity. The tethering of these two compounds results in a cooperative effect on binding to *B.CcrM* and is consistent with the active site binding of the partial multi substrate adduct inhibitor. Since **5**−**8** were assayed in the presence of their respective *N*-acetylated precursors, we assayed **1**−**4** separately to determine the effect of these compounds. We observed no inhibition for **1**−**4** at concentrations where **5**−**8** completely inhibited the enzymatic reaction. As can be seen from Table 1, inhibition was not greatly influenced by the presence of a 5′-phosphate group or the absence of a 2′-hydroxyl.

We used HhaI, a bacterial C5 cytosine DNA MTase to determine whether our inhibitors showed selectivity toward adenine DNA MTase. No inhibition of HhaI activity was observed with compounds **7** and **8** even at concentrations 10-fold greater than required to inhibit CcrM activity (Figure 2). Sinefungin, in contrast, was found to inhibit both CcrM and HhaI. To test the hypothesis that inhibition of adenine DNA MTase would lead to inhibition of bacterial cell growth we tested compounds **7** and **8** in an in vivo cell growth assay. We carried out the cell growth assays using *C. crescentus* as a model for the pathogenic *B. abortus* since CcrM from *B. abortus (B.CcrM)* has a 65% identity and 76% homology to CcrM from *C. crescentus (C.CcrM)*. Furthermore, these compounds inhibit CcrM from both bacteria with nearly identical K_I values (Table 1). Inhibition of cell growth was observed in the presence of compounds **7** and **8** with an IC_{50} of about 500 μM. It is clear from these results that CcrM is inhibited at much lower concentrations than is required to produce an antibiotic effect in vivo. It is likely that these compounds do not easily pass through the bacterial cell wall to contact the target enzyme. We are now extending this work to include the synthesis of non-nucleoside analogue inhibitors and have observed that small molecule inhibitors, which are able to pass through the cell wall, are good inhibitors of bacterial cell growth. For the most effective inhibitors cell growth inhibition data can be correlated to enzyme inhibition data supporting a mechanism-based inhibition of cell growth (*data not shown*).

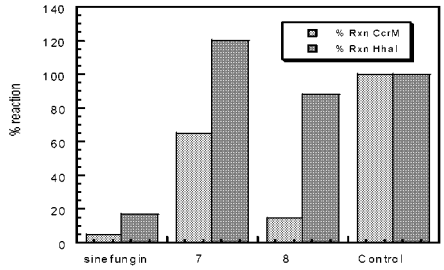


Figure 2. Selective Inhibition: Percent reaction for sinefungin, **7**, and **8** for CcrM and HhaI. CcrM Assays: [Sinefungin] = 75 μM, [I] = 100 μM. Hha I Assays: [Sinefungin] = 200 μM, [I] = 1 mM.

In conclusion, we have described the purification of a novel cell cycle regulated adenine DNA MTase from the pathogenic *B. abortus* and presented a mechanism-based strategy for the selective inhibition of this enzyme. We have observed inhibition comparable to the natural product sinefungin, but in contrast to sinefungin, the observed inhibition is selective for adenine DNA methylation. We have demonstrated that inhibitors of adenine DNA MTase have antibiotic effects in a cell strain containing a regulatory CcrM and we are now focusing on the synthesis and screening of small-molecule combinatorial libraries to develop lead antibiotic candidates. These studies demonstrate that the essential regulatory bacterial adenine DNA MTases are an attractive target for the design of novel antibiotics. As sequences of bacterial genomes are completed and as additional bacterial cell cycle regulators are discovered the available targets for rationally designed antibiotics will expand.

Acknowledgment. We thank Rachel Wright (Department of Developmental Biology, Stanford University) for the *Brucella abortus* and *Caulobacter crescentus* CcrM gene. This work was sponsored by DARPA Grant MDA972-97-1-0008.

Supporting Information Available: Experimental details and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA003285O

(12) Berdis, A. J.; Lee, I.; Coward, J. K.; Stephens, C.; Wright, R.; Shapiro, L.; Benkovic S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2874.