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A novel transition state analog inhibitor of guanase based on azepinomycin ring structure: Synthesis and biochemical assessment of enzyme inhibition

Saibal Chakraborty, Niti H. Shah, James C. Fishbein, Ramachandra S. Hosmane [†,*]

Laboratory for Drug Design & Synthesis, Department of Chemistry & Biochemistry, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250, USA

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ABSTRACT

Synthesis and biochemical inhibition studies of a novel transition state analog inhibitor of guanase bearing the ring structure of azepinomycin have been reported. The compound was synthesized in five-steps from a known compound and biochemically screened against the rabbit liver guanase. The compound exhibited competitive inhibition profile with a K_i of 16.7 ± 0.5 μM.

Guanine deaminase (EC 3.5.4.3, Guanase, or GDA), is an enzyme that hydrolyzes guanine to form xanthine that is unsuitable for DNA/RNA buildup.[1] This enzyme has been found in normal or transformed human organs and sera.[2] One of the approaches for antiviral/anticancer therapy is to design structural mimics of natural guanine as nucleic acid building blocks, with an anticipation that such analogs would be incorporated into DNA/RNA of virus or cancer cells, interrupting their normal replicative processes.[3] Indeed, there are some known guanine mimics, such as 6-thioguanine[4,5] and 8-azaguanine,[6] both of which are potent anticancer compounds. However, they both are believed to be substrates for the enzyme guanase, which converts them into their respective inactive forms 6-thioxanthine and 8-azaxanthine, respectively.[7] A potent guanase inhibitor would restore the original potency of these anticancer compounds.

A potent guanase inhibitor will also be an excellent tool for studying a number of disease-related metabolic processes in which the enzyme is involved either as an indicative or as a causative factor. There are several documented reports on detection of abnormally high levels of serum guanase activity in patients with liver diseases like hepatitis.[8–10] Therefore, the elevated enzyme activity has been suggested as a marker of hepatitis and hepatoma.[10] There are also various reports of patients developing hepatitis C when they are transfused with blood containing high levels of serum guanase activity.[8,10,11] Even though no direct cause and effect relationship has been established between increased guanase activity and post-transfusional hepatitis (PTH), it is reasonable to assume that the guanase level is responsible, in part, for the development of PTH. There are only a few and scattered reports on the specific physiological or metabolic role of guanase. Nonetheless, there are important observations made on this enzyme as reported in the literature. For example, it is known that high serum guanase activity is a clear biochemical indicator of organ rejection in liver transplant patients.[12] It has also been shown that patients with multiple sclerosis (MS) have significantly elevated levels of guanase activity in their cerebral spinal fluid (CSF), and that there is a clear correlation between the extent of disability and the level of guanase activity.[13]

Another important aspect of guanase activity is its involvement in cancerous tissues. It has long been known that carcinogenic processes and the activities of some enzymes in cancer tissues and cells are strongly interrelated. In this regard, it is important to consider reports of abnormal levels of guanase activity in various cancer tissues. An increase in guanase activity has been reported in lung,[14] gastric,[15] kidney,[16–18] and breast cancer tissues.[19] It is suggested that this difference in activity is a physiological attempt of the cancer cell to regulate the guanine and/or xanthine level, which is needed by cancer cells to accelerate their *salvage* metabolic pathway activity. Since the *de novo* pathway is mostly employed by normal cells for replication, a guanase inhibitor can preferentially check the growth of cancer cells without affecting the normal cells. Furthermore, guanase plays an important role in the detoxification

* Corresponding author. Tel.: +1 410 381 0005; fax: +1 410 455 1148.
E-mail address: hosmane@umbc.edu (R.S. Hosmane).
† Recently retired.

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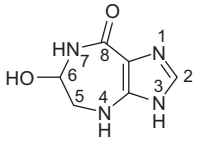
process of high amounts of toxic guanine produced from accelerated metabolism in the cancerous tissues. In any event, a guanase inhibitor can trigger cell death either due to cytotoxicity of the accumulating metabolites as proposed in the case of adenosine deaminase (ADA)[20–23] or through depletion of the nucleotide pool necessary for cell growth.

While many studies of guanase inhibition have been reported both from this lab[24–30] and others,[31–35] guanase is still one of the least studied enzymes of the nucleic acid metabolism, although it has been isolated from rabbit liver,[1] human liver,[36] and rat brain[37] years ago.

Azepinomycin[38,39] is a naturally occurring inhibitor of guanase, which has so far been studied only in tissue culture systems. So, while its IC_{50} value (the concentration of the drug required to inhibit 50% of the guanase activity) is known to be $\sim 10^{-5}$ M,[38] the K_i of azepinomycin against guanase has never been determined biochemically against the isolated pure enzyme. In our continued efforts to discover a potent guanase inhibitor, we report here the synthesis and biochemical studies of an analog azepinomycin that more closely resembles the putative transition state of the enzyme-catalyzed reaction.



Azepinomycin

The recently solved crystal structure of guanase[40] from *Bacillus subtilis* suggests that the enzyme-catalyzed reaction (Fig. 1) is assisted by an active site zinc metal ion (Zn^{+2}), which forms a tetrahedral complex with His-53, Cys-83, and Cys-86 of the protein, along with an isolated water molecule. Glutamate-55 serves as a proton shuttle, abstracting a proton from the zinc-activated water to form the necessary hydroxide nucleophile, while also enabling protonation at the N-3 site of guanine, thus resulting in the formation of the tetrahedral intermediate **2**, as shown. Glu-55 also assists in protonation of the NH_2 group of **2**, facilitating the elimination of a molecule of ammonia to form the final product xanthine.

Azepinomycin may interrupt the above hydrolytic process via coordination of its own OH group at position-6 with the active site zinc, displacing the crucial active site water molecule involved in hydrolysis. The observed weak inhibitory characteristics of azepinomycin may involve multiple factors, including, but not limited to, the fact that it does not represent the true transition state analog lacking the geminal hydroxy-amino groups attached to its 6-position so as

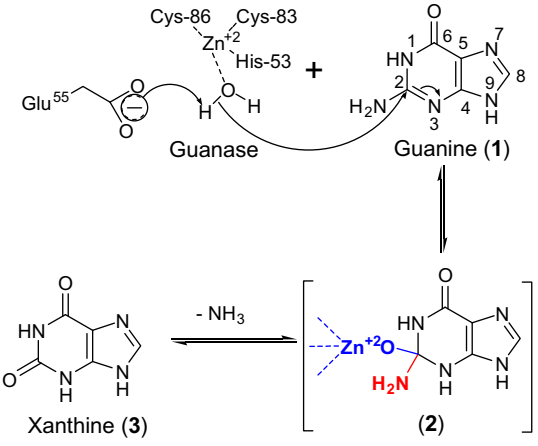
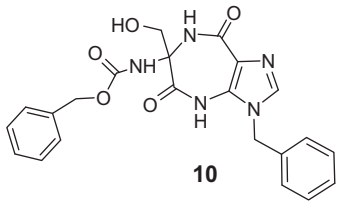


Figure 1. Zn^{+2}-assisted hydrolysis of guanine to xanthine, catalyzed by guanase.

to effectively mimic the tetrahedral intermediate (**2**) of the guanase-mediated conversion of guanine (**1**) to xanthine (**3**).

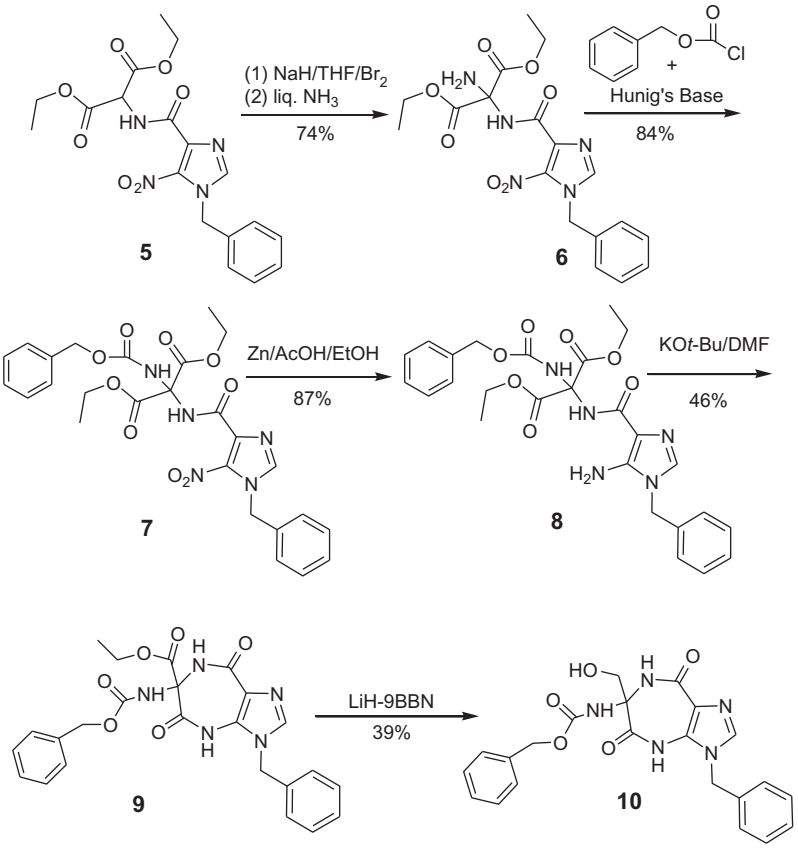
With the above rationale, we report here the design, synthesis, and biochemical evaluation of our current target molecule, namely, 3-benzyl-4,5,7,8-tetrahydro-6-hydroxymethyl-6-[(benzyloxycarbonyl)amino]imidazo[4,5-*e*][1,4]diazepine-5,8-dione (**10**). Compound **10** meets the criteria outlined above. It has the required geminal carbon at position-6 as described except that the OH group had to be removed one atom further from the geminal junction in order to avoid any potential elimination of water with anchimeric assistance from one of the two NH groups attached to the junction. For the same reason, the exonuclear NH_2 had to be protected by a benzyloxycarbonyl (CBZ) group. A benzyl group has been introduced at position-3 in order to create a hydrophobic environment in its vicinity, which has been suggested to enhance the enzyme–ligand interactions, based on our earlier studies on this enzyme.[26,28] Attachment of a benzyl group has further scope for introduction of a variety of substituents on the phenyl ring with defined dimensions, and thus can act as both hydrophobic and dimensional probes. Finally, the introduction of a carbonyl group at position-5 of the heterocycle, coupled with movement of the 6-hydroxy group away from the ring by an additional carbon atom, would allow excellent coordination of the inhibitor with Zn^{+2} to form a stable, six-membered ring structure. The latter would use two of the four metal coordination sites, while the other two would be occupied by two of the three original amino acid residues at the enzyme active site. This arrangement would further strengthen the interaction of azepinomycin with the active site Zn^{+2} ion.



10

The target **10** was synthesized (Scheme 1) in five-steps commencing from compound **5**, which has been reported from this laboratory several years ago.[41] The sequential in situ bromination of the malonate carbon of **5**, elimination of HBr, followed by conjugate addition of ammonia, gave **6** in 74% yield. The free NH_2 of **6** was protected as a benzyloxycarbonyl (CBZ) derivative by treatment with CBZ-Cl in the presence of Hünig's base to produce **7** in 84% yield. The nitro group of **7** was reduced with zinc and acetic acid to obtain **8** in 87% yield. Compound **8** was ring-closed using potassium *t*-butoxide in dimethylformamide to yield **9** in 46% yield. Finally, the ester group of **9** was selectively reduced to a primary alcohol using LiH-9BBN[42] to obtain the target **10** in 39% yield. All new compounds were completely characterized by ^1H and ^{13}CNMR and mass spectral data, coupled with either elemental microanalyses or high resolution mass spectral (HRMS) data.[43]

Guanase inhibition studies with target **10** were conducted using guanase from rabbit liver (MP Biochemicals) at 25 °C and pH 7.4 by spectroscopically measuring the rate of hydrolysis of the substrate guanine at λ_{max} 245 nm. The change in optical density at λ_{max} 245 nm per unit time is a measure of the guanase activity. A total of seven different concentrations of the substrate, ranging from 5 to 40 μM, was employed for each inhibitor concentration that was either 20 or 40 μM, while the amount of enzyme in each assay was 7.7×10^{-3} unit. The Lineweaver–Burk plots (1/V vs 1/S) (see Fig. 2) were used to calculate K_M, V_{max}, and K_i. Our biochemical results showed that K_m of enzyme with guanine as substrate was 9.5×10^{-6} M. The target compound **10** showed competitive inhibition with a K_i of $16.70 \pm 0.48 \times 10^{-6}$ M.



Scheme 1. Synthesis of the target compound **10**.

In conclusion, we have designed, synthesized, and biochemically evaluated a novel, putative transition state analog inhibitor of guanase. While the observed K_i in micromolar range is respectable, additional structure–activity relationship (SAR) studies would be necessary to further enhance the inhibitory potential. Such studies could involve introduction of various alkyl, aryl, or aralkyl substituents at N-4, N-6, and/or N-7 of **10** or attachment of atoms or groups with + or − inductive effects at various positions of the two exonuclear phenyl rings.

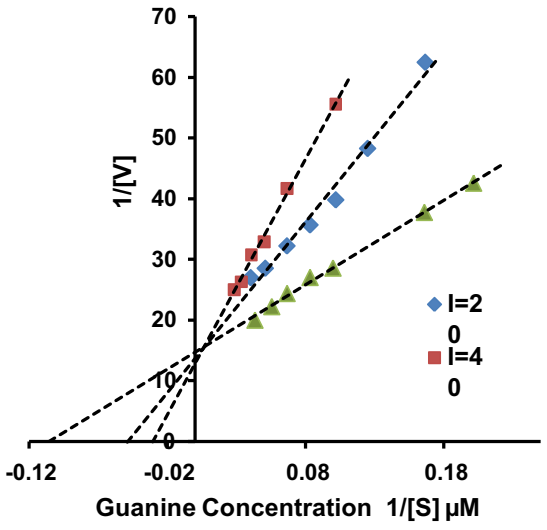


Figure 2. Lineweaver–Burk plot of target compound **10**.

Acknowledgments

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43. **Experimental**: *Organic synthesis procedure*: Preparation and physico-chemical properties of the compounds are as follows: *Diethyl 2-amino-2-[N-(1-benzyl-5-nitroimidazolyl-4-carbonyl)amino]malonate* (**6**): in a flame-dried two-neck round bottomed flask, NaH (0.68 g, 17.2 mmol) was added under nitrogen environment. Dry THF (20 mL) was then added and the flask was then cooled and maintained at −78 °C (with dry ice acetone mixture). The starting material **5** (3.5 g, 8.6 mmol) was added quickly in one portion and was allowed to stir for 10 min before bromine (liquid) (0.95 mL, 17.2 mmol) was added. After 10 min liq ammonia (2 mL) (excess) was added. The reaction mixture was allowed to stir for 1 h and then allowed to come to room temperature. After rotary evaporating the solvent it is neutralized in cold, filtered and extracted in dichloromethane. Further purification was done using column chromatography using 1–3% methanol in chloroform. Hexane wash gives yellow product. Yield 74% (2.7 g, 6.6 mmol). ^{1}H NMR (DMSO-d_6): δ 1.13 (t, 6H, CH_3), 4.17 (m, 4H, CH_2), 5.06 (s, 2H, Bn), 6.0 (s, 2H, exchangeable with D_2O, NH_2), 7.2–7.32 (m, 6H, Ar + Imd CH), 7.9 (s, 1H, exchangeable with D_2O, CONH); ^{13}C NMR (DMSO-d_6): δ 14.3, 46.2, 62.7, 83.9, 111.0, 127.8, 128.1, 129.1, 131.4, 137.1, 144.7, 163.2, 165.9; anal. calcd C, 51.55; H, 5.05; N, 16.70. Found: C, 51.44; H, 5.09; N, 16.57. *Diethyl 2-(benzyloxycarbonyl)amino-2-[N-(1-benzyl-5-nitroimidazolyl-4-carbonyl)amino]-malonate* (**7**): compound **6** (2.3 g, 5.48 mmol) was taken in a dry flask and dissolved in dry THF under nitrogen. To the stirred solution, benzylchloroformate (0.8 mL, 5.6 mmol) and Hunig's base (2.8 mL, 16.4 mmol) were added in iced condition. The reaction mixture was allowed to stir in iced condition to room temperature overnight. After the reaction was complete, the solvent was evaporated and the residue was neutralized in cold and extracted in dichloromethane. It was further purified using column chromatography with 1% methanol in chloroform. Yield 83% (2.5 g, 4.5 mmol). R_f 0.34 (chloroform/methanol, 5:0.1); ^{1}H NMR (DMSO-d_6): δ 1.12 (t, 6H, CH_3), 4.17 (m, 4H, CH_2), 5.05 (s, 2H, Bn), 5.5 (s, 2H, OBn), 7.21–7.39 (m, 10H, Ar), 8.2 (s, 1H, Imd CH), 8.4 (s, 1H, carbamate NH, D_2O exchangeable), 9.4 (s, 1H, amide NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ 14.1, 50.7, 63.5, 66.5, 70.7, 127.6, 128.1, 128.4, 128.6, 128.8, 129.3, 134.5, 135.9, 136.3, 136.9, 140.5, 155.1, 160.0, 164.6; HRMS (FAB), calcd. for $C_{26}H_{28}N_5O_9$: 554.18821 (MH^+); obsd *m*/*z* 554.18870. *Diethyl 2-(benzyloxycarbonyl)amino-2-[N-(5-amino-1-benzylimidazolyl-4-carbonyl)amino] malonate* (**8**): dried **7** (2.2 g, 3.9 mmol) was dissolved in dry ethanol. Zinc dust (5.09 g, 78 mmol) followed by acetic acid (23 mL) were added to the reaction mixture and was refluxed for an hour, when the white (zinc salts) precipitate appeared. It was then filtered through Celite followed by washing with ethanol. Evaporation of ethanol gave **8**, which was further purified by column chromatography (care should be taken to remove the acetic acid completely otherwise it can make the ring closure step problematic). Yield 87%, (1.8 g, 3.4 mmol); R_f 0.17 (chloroform/methanol, 5:0.1); ^{1}H NMR (DMSO-d_6): δ 1.11 (t, 6H, CH_3), 4.18 (m, 4H, CH_2), 5.03 (s, 2H, OBn), 5.09 (s, 2H, Bn), 5.9 (s, 2H, NH_2 exchangeable with D_2O), 7.2–7.37 (m, 11H, Ar + Imd CH), 8.07 (s, 1H, carbamate NH, exchangeable with D_2O), δ 8.1 (s, 1H, amide NH, exchangeable with D_2O); ^{13}C NMR (DMSO-d_6): δ 14.2, 46.2, 63.3, 66.5, 69.9, 111.1, 127.8, 128.1, 128.2, 128.4, 128.2, 129.2, 131.4, 136.8, 137.2, 144.4, 154.8, 163.2, 165.4; HRMS (FAB), calcd for $C_{26}H_{29}N_5O_7$: 523.20691 (M^+); obsd *m*/*z* 524.21452 (MH^+). *3-Benzyl-4,5,7,8-tetrahydro-6-(benzyloxycarbonyl)amino-6-ethoxycarbonylimidazo[4,5-e][1,4]diazepine-5,8-dione* (**9**): potassium *tert*-butoxide (24 mg, 0.21 mmol) was taken in a dry flask with nitrogen line and 5 mL of dry DMF was added and stirred vigorously. To the mixture was added **8** (70 mg, 0.13 mmol). The heterogeneous mixture immediately turns orange. The mixture was vigorously stirred for 2 h and was monitored by TLC. Care should be taken as one of the by-products is very close to the starting material in TLC using ethyl acetate as the solvent system. The resulting yellow liquid was concentrated under vacuum and neutralized and extracted with ethyl acetate. It is then purified by column chromatography using chloroform/methanol (10:1) as the solvent system. Triturating with ether gave **9** as a yellow product (30 mg, 0.06 mmol), yield 46%; R_f 0.46 (ethylacetate/methanol, 2:1); ^{1}H NMR (DMSO-d_6): δ 1.09 (t, 3H, CH_3), 4.09 (q, 2H, CH_2), 5.05 (m, 2H, OBn), 5.2 (s, 2H, Bn), 6.8 (d, 1H, NH), 7.24–7.34 (m, 11H, Ar), 7.7 (d, 1H, NH, exchangeable with D_2O), 7.8 (1H); ^{13}C NMR (DMSO-d_6): δ 14.2, 47.5, 62.7, 66.7, 99.9, 114.7, 127.7, 128.1, 128.5, 128.7, 128.9, 129.4, 135.9, 136.7, 138.6, 140.7, 150.9, 156.2, 156.9, 167.8; anal. calcd for $C_{24}H_{24}N_5O_{6.75}$: C, 60.37; H, 4.86; N, 14.67. Found: C, 58.38; H, 4.74; N, 13.96; HRMS (FAB), calcd for $C_{24}H_{23}N_5O_6$: 477.16469 (M^+); obsd *m*/*z* 478.17203 (MH^+). *3-Benzyl-4,5,7,8-tetrahydro-6-hydroxymethyl-6-[(benzyloxycarbonyl)-amino]imidazo[4,5-e][1,4]diazepine-5,8-dione* (**10**): compound **9** (280 mg, 0.58 mmol) was taken in a dry flask under nitrogen atmosphere. It was dissolved in 10 mL of freshly distilled THF. It was kept in ice-cold condition. To the cooled, well stirred solution was added drop wise through syringe 1 M solution of LiH-9BBN in THF (0.75 mL). The mixture was allowed to stir for an hour before quenching with 10 mL ethyl acetate and evaporated to dryness with small portion of silica gel. Column chromatography was done with 2% methanol in chloroform. The proper fractions were collected and evaporated to obtain **10** a was white solid (0.1 g, 0.23 mmol), yield 39%; R_f 0.26 (ethylacetate/methanol, 2:1); ^{1}H NMR (DMSO-d_6): δ 3.76 (m, 1H + 1H, CH_2), 5.02 (m, 2H + 1H, OBn + OH, OH exchangeable with D_2O), 5.2 (s, 2H, Bn), 6.5 (m, NH, 1H), 7.28–7.36 (m, 10H, Ar+), 8.28 (1H, Imidazole), 12.3 (s, NH, 1H, exchangable with D_2O); ^{13}C NMR (DMSO-d_6): δ 47.2, 60.7, 66.2, 79.7, 115.4, 127.8, 128.3, 128.4, 128.8, 129.3, 136.9, 137.2, 137.5, 140, 151.4, 155.6; HRMS (FAB), calcd for $C_{22}H_{20}O_5N_5$: 434.14413 (M^+); obsd *m*/*z* 435.15205 (MH^+). *Biochemical inhibition procedure*: guanine deaminase from rabbit liver was purchased from MP biochemicals as a suspension in 3.2 M $(NH_4)_2SO_4$, pH 6, activity = 10 mg/mL, 0.06 μmol/mL. Freshly prepared solutions of Guanine and Tris–HCl buffer (0.05 M) were used for the of biochemical studies. Enzyme solution could be kept at room temperature for a day's run. Twenty-five milligram of Guanine was dissolved in 1 mL NaOH (1 N) and volume made to 100 mL in volumetric flask with Tris–HCl buffer (0.05 M). The solution was kept at room temperature overnight. Next day, the solution was filtered and concentration was measured by UV spectrophotometer using the molar extinction coefficient 10,700 at λ 243 nm. Sixty microliter of enzyme solution was used in each 1 mL cuvette and was quickly shaken before taking the reading against air. Tris–HCl buffer was used as a reference for all measurements.