



Pergamon

Zinc(II)-Mediated Inhibition of a Ribonuclease by an *N*-Hydroxyurea Nucleotide

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Received 6 September 2002; revised 27 October 2002; accepted 2 November 2002

Abstract—The inhibition of ribonuclease Bi by 3'-*N*-hydroxyurea-3'-deoxythymidine 5'-phosphate is enhanced by 30-fold in the presence of Zn²⁺. Thus, an *N*-hydroxyurea nucleotide can recruit Zn²⁺ to inhibit the enzymatic activity of a ribonuclease. This result engenders a general strategy for the inhibition of non-metalloenzymes by metal complexes.

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Like proteases, ribonucleases are prevalent enzymes that are worthwhile targets for inhibitor development.^{1,2} In many laboratory procedures, RNA must be protected from degradation. Moreover, the neovascularization promoted by angiogenin relies on the ribonucleolytic activity of that enzyme.³ Indeed, variants of angiogenin with greater ribonucleolytic activity are more effective at promoting neovascularization,⁴ and inhibiting the ribonucleolytic activity of angiogenin could be an effective anti-angiogenesis strategy.⁵

The development of ribonuclease inhibitors lags far behind that of protease inhibitors. The most potent known small-molecule inhibitor of a ribonuclease is pUppAp, which has $K_i = 0.22 \mu\text{M}$ for the inhibition of ribonuclease A in 0.2 M Hepes buffer, pH 7.0, with no added salt.⁶ This inhibitor emerged from multiple iterations of inhibitors that closely resemble substrates.^{7,8} It is unlikely that this iterative strategy will yield substantially better ribonuclease inhibitors. UpOC₆H₄-*p*-CH₂F is a mechanism-based inactivator of ribonuclease A.⁹ Unfortunately, inactivation by UpOC₆H₄-*p*-CH₂F is not complete. Hence, new strategies for inhibiting or inactivating ribonucleases are desirable.

Zinc is the second most abundant transition metal in biology and is essential for life.¹⁰ In cells, almost all zinc is bound to proteins as zinc(II).¹¹ The ability of proteins to bind Zn²⁺ ions with high affinity portends a new strategy for ribonuclease inhibition. The efficacy of this strategy has been demonstrated with serine proteases. Using X-ray diffraction analysis, Katz and co-workers discovered that a previously known inhibitor of trypsin, bis(5 - amidino - 2 - benzimidazolyl)methane (BABIM), inhibits that enzyme by inadvertently recruiting a single Zn²⁺.^{12–14} The Zn²⁺ coordinates four heteroatoms—two from BABIM and two from enzymic side chains. The value of K_i for BABIM alone is 19 μM , and that for Zn²⁺ alone is 1 mM. Yet, the K_i for BABIM plus Zn²⁺ is 5 nM.¹²

The ability of metal complexes to inhibit non-metalloenzymes could extend beyond serine proteases.^{15–19} Herein, we report the first zinc(II)-mediated inhibitor of a ribonuclease. Our ligand is 3'-*N*-hydroxyurea-3'-deoxythymidine 5'-phosphate [pdT-3'-NHC(O)NHOH; 1]. The logic of this choice is as follows. The use of a deoxynucleoside creates additional space within the active site of the enzyme–ligand complex. This space could be necessary for Zn²⁺ binding. The use of a thymidine facilitates synthesis from a commercially available starting material (vide infra). The 5'-phosphoryl group provides another interaction with a phosphoryl group-binding subsite, as in the binding of a polymeric

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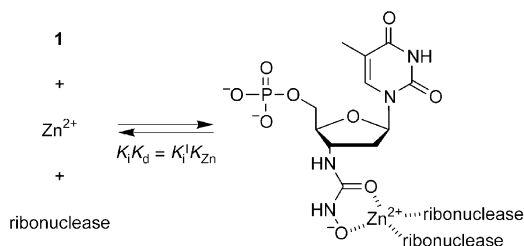
RNA substrate. Finally, hydroxamic acids are exceptional bidentate chelators of Zn^{2+} .¹⁹

As a model ribonuclease, we chose ribonuclease Bi (binase; EC 3.1.27.3). Binase is a secretory ribonuclease from *Bacillus intermedius* that catalyzes the cleavage of RNA without a need for metal ions or cofactors. The structure of crystalline binase is known at a resolution of 1.65 Å.²⁰ Analysis of this structure, along with that of a complex with a nucleoside 3'-phosphate,²⁰ suggests that Glu73 and His102 of binase act as a base and acid, respectively, during catalysis of RNA cleavage. The carboxylate and imidazole groups in the side chains of these residues could also serve as the enzymic ligands for Zn^{2+} (Scheme 1).

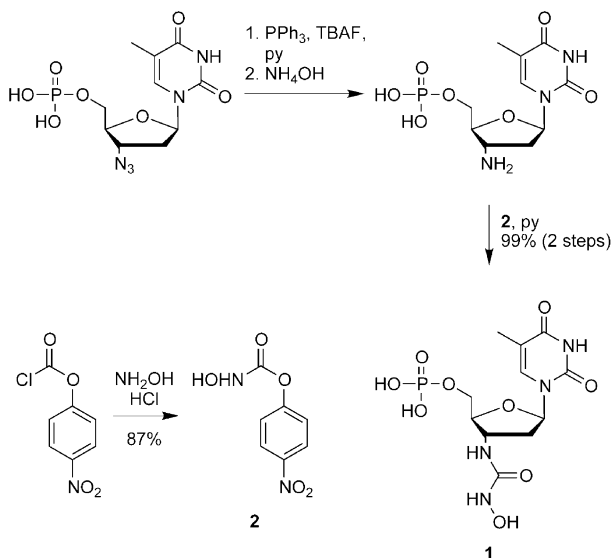
N-Hydroxyurea **1** was synthesized by the route shown in Scheme 2, which begins with the commercial reagent 3'-azido-3'-deoxythymidine 5'-monophosphate (AZT monophosphate).²¹ The ability of *N*-hydroxyurea **1** to inhibit the ribonucleolytic activity of binase was assessed in the absence and presence of Zn^{2+} .²²

The results of measurements of the binase activity inhibition by *N*-hydroxyurea **1** at different concentrations of Zn^{2+} ions are shown in Figure 1. The intercept of the lines on the ordinate is indicative of competitive inhibition.

The data in Figure 1 were used to evaluate the inhibition by *N*-hydroxyurea **1** and Zn^{2+} by using eq 1:



Scheme 1. Basis for the zinc(II)-mediated inhibition of a ribonuclease by *N*-hydroxyurea **1**.



Scheme 2. Route for the synthesis of *N*-hydroxyurea **1**.²¹

$$v = \frac{[E]_T[S]k_{cat}}{[S] + K_M^{obs}} \quad (1)$$

where

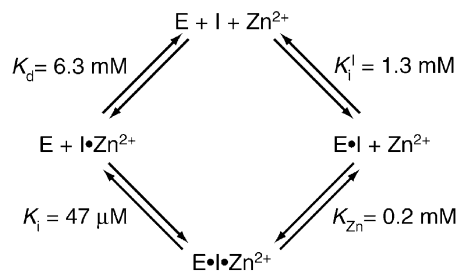
$$K_M^{obs} = K_M \left(1 + \frac{[I]}{K_i^1} + \frac{[I \cdot Zn^{2+}]}{K_i} \right) \quad (2)$$

In eqs 1 and 2, $[E]_T$, $[S]$, $[I]$, and $[I \cdot Zn^{2+}]$ are the total concentrations of the enzyme, substrate, inhibitor (*N*-hydroxyurea **1**), and $I \cdot Zn^{2+}$ complex, respectively; K_M is the Michaelis constant for the hydrolysis of poly(I); K_i^1 is the inhibition constant for the inhibitor alone; and K_i is the inhibition constant for the $I \cdot Zn^{2+}$ complex. The relationship between these two inhibition constants and K_d and K_{Zn} (which are the equilibrium dissociation constants of Zn^{2+} from the $I \cdot Zn^{2+}$ and $E \cdot I \cdot Zn^{2+}$ complexes, respectively) are depicted in Scheme 3. In the data analysis, the values of $[I]$ and $[Zn^{2+}]$ were assumed to be equal to the total concentration of inhibitor and Zn^{2+} , respectively, because the concentration of enzyme was much lower than that of inhibitor or Zn^{2+} .

The values of k_{cat} and K_M for the hydrolysis of poly(I) were 162 s⁻¹ and 79 μM, which are similar to those reported previously.^{23,24} The inhibition constant for *N*-hydroxyurea **1** alone (that is, in the absence of Zn^{2+}) was $K_i^1 = 1.3$ mM. In contrast, no inhibition of enzymatic activity was observed by Zn^{2+} alone in assays performed with $[Zn^{2+}] \leq 5$ mM (data not shown).

The application of eq 1 to the data in Figure 1 enabled the calculation of $K_i K_d$ values for different $[I]$ and $[Zn^{2+}]$. As listed in Table 1, these values were approximately constant at $K_i K_d = 3 \times 10^{-7}$ M² if $[I][Zn^{2+}] \leq 10^{-7}$ M². When $[I][Zn^{2+}]$ was increased to 1.7×10^{-6} M², the $K_i K_d$ value increased by 10-fold (Table 1). Most likely, this increase is indicative of improper usage of the total concentration of inhibitor and Zn^{2+} rather than the actual concentration. For this reason, no assays were performed with $[Zn^{2+}] \geq 5$ mM.

The affinity of the $I \cdot Zn^{2+}$ complex for the enzyme was discerned from the value of $K_i K_d$. The value of K_d reports on the affinity of I for Zn^{2+} in the assay mixture. The pK_a of a model hydroxamic acid, acetohydroxamic acid ($CH_3C(O)NHOH$), is 9.4,²⁵ indicating



Scheme 3. Scheme for zinc(II)-mediated inhibition of enzymatic activity. Values are for inhibition of ribonuclease Bi by *N*-hydroxyurea **1**.

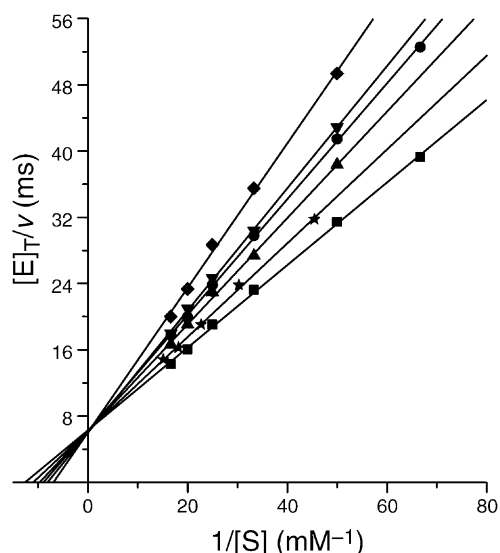


Figure 1. Lineweaver–Burk plot for the inhibition of binase by *N*-hydroxyurea **1** in the absence and presence of Zn^{2+} . Assays were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), binase (4.4×10^{-10} M), poly(I), *N*-hydroxyurea **1**, and Zn^{2+} . ■, [I]=0, [Zn²⁺]=0 (data with [Zn²⁺] ≤ 5 mM were identical); ★, [I]= 0.93×10^{-4} M, [Zn²⁺]=0.25 mM; ▲, [I]= 3.5×10^{-4} M, [Zn²⁺]=0; ●, [I]= 1.0×10^{-4} M, [Zn²⁺]=1.0 mM; ▼, [I]= 3.5×10^{-4} M, [Zn²⁺]=0.25 mM; ◆, [I]= 3.5×10^{-4} M, [Zn²⁺]=5 mM.²²

that 0.063% of acetohydroxamic acid is deprotonated at pH 6.2. Only the conjugate base of a hydroxamic acid has high affinity for Zn^{2+} ,¹⁹ and the acetohydroxamate- Zn^{2+} complex has an equilibrium dissociation constant near $10^{-5.4}$ M.^{26,27} Thus, the value of $K_d = 10^{-5.4}$ M / (0.063%) = 6.3 mM for acetohydroxamic acid at pH 6.2. Using this value of K_d as an approximation for that of the *N*-hydroxyurea **1**· Zn^{2+} complex, the value of $K_i = K_i K_d / K_d = 3 \times 10^{-7}$ M² / 6.3 mM = 47 μM. Thus, the enzyme has approximately 30-fold more affinity for the I· Zn^{2+} complex ($K_i = 47$ μM) than for I alone ($K_i^1 = 1.3$ mM).

The affinity of Zn^{2+} for the enzyme was discerned likewise. From Scheme 3, $K_{Zn} = K_i K_d / K_i^1 = 3 \times 10^{-7}$ M² / 1.3 mM = 0.2 mM. Because no inhibition of enzymatic activity was observed with [Zn²⁺] ≤ 5 mM, the enzyme· Zn^{2+} complex had an equilibrium dissociation constant of >5 mM. Thus, Zn^{2+} has >25-fold more affinity for the E·I complex than for the enzyme alone. This increase is consistent with the participation of enzymic ligands in the binding of Zn^{2+} to the E·I complex, as is depicted in Scheme 1.

In conclusion, we have demonstrated the efficacy of a new strategy for the inhibition of ribonucleases. This strategy was inspired by the inadvertent recruitment of zinc by a known protease inhibitor.^{12–14} In contrast, ribonuclease inhibition herein relies on the intentional recruitment of Zn^{2+} by an *N*-hydroxyurea moiety attached covalently to a nucleotide. The *N*-hydroxyurea moiety can present Zn^{2+} to the active-site residues of the ribonuclease, and thereby enhance binding beyond that for the inhibitor or Zn^{2+} alone. We anticipate that this strategy can be optimized further and used for the inhibition of a variety of ribonucleases, as well as other types of enzymes.

Table 1. Parameters for inhibition of ribonuclease Bi catalysis by *N*-hydroxyurea **1** and Zn^{2+} ^a

[I] (10 ⁻⁴ M)	[Zn] (10 ⁻³ M)	K_M^{obs} (10 ⁻⁴ M)	K_i [I· Zn^{2+}] (M) ²	$K_i K_d$ (10 ⁻⁷ M ²)
0.93	0.25	0.91	12.4	2.9
1.0	1.0	1.12	2.9	2.9
3.5	0.25	1.21	3.8	3.3
3.5	5.0	1.43	1.85	32

^aData are for those assays depicted in Figure 1 performed in the presence of Zn^{2+} .

Acknowledgements

We thank J. A. Hodges for contributive discussions. This work was supported by Grants GM44783 (NIH to R.T.R.), TW01058 (NIH to A.A.M), 02-04-48259 (RFBR to A.A.M), and 02-04-49110 (RFBR to G.I.Y).

References and Notes

- D'Alessio, G.; Riordan, J. F., Eds. *Ribonucleases: Structures and Functions*. Academic Press: New York, 1997.
- Raines, R. T. *Chem. Rev.* **1998**, *98*, 1045.
- Shapiro, R.; Vallee, B. L. *Biochemistry* **1989**, *28*, 7401.
- Harper, J. W.; Vallee, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *88*, 7139.
- Folkman, J. *Nature Med.* **1995**, *1*, 27.
- Russo, N.; Shapiro, R. *J. Biol. Chem.* **1999**, *274*, 14902.
- Leonidas, D. D.; Shapiro, R.; Irons, L. I.; Russo, N.; Acharya, K. R. *Biochemistry* **1997**, *36*, 5578.
- Russo, N.; Shapiro, R.; Vallee, B. L. *Biochem. Biophys. Res. Commun.* **1997**, *231*, 671.
- Stowell, J. K.; Widlanski, T. S.; Kutateladze, T. G.; Raines, R. T. *J. Org. Chem.* **1995**, *60*, 6930.
- Vallee, B. L. *Biofactors* **1988**, *1*, 31.
- Outten, C. E.; O'Halloran, T. V. *Science* **2001**, *292*, 2488.
- Katz, B. A.; Clark, J. M.; Finer-Moore, J. S.; Jenkins, T. E.; Johnson, C. R.; Ross, M. J.; Luong, C.; Moore, W. R.; Stroud, R. M. *Nature* **1998**, *391*, 608.
- Katz, B. A.; Luong, C. *J. Mol. Biol.* **1999**, *292*, 669.
- Janc, J. W.; Clark, J. M.; Warne, R. L.; Elrod, K. C.; Katz, B. A.; Moore, W. R. *Biochemistry* **2000**, *39*, 4792.
- Schirmeister, T. *Angew. Chem. Int. Ed.* **1998**, *37*, 1830.
- Thorp, H. H. *Chem. Biol.* **1998**, *5*, R125.
- Louie, A. Y.; Meade, T. *J. Chem. Rev.* **1999**, *99*, 2711.
- Nguyen, R.; Huc, I. *Angew. Chem. Int. Ed.* **2001**, *40*, 1774.
- Farkas, E.; Buglyó, P. *J. Chem. Soc., Dalton Trans.* **1990**, 1549.
- Polyakov, K. M.; Lebedev, A. A.; Okorokov, A. L.; Panov, K. I.; Schulga, A. A.; Pavlovsky, A. G.; Dodson, G. G. *Acta Crystallogr.* **2002**, *D58*, 744.
- 3'-Amino-3'-deoxythymidine 5'-monophosphate.** 3'-Azido-3'-deoxythymidine 5'-monophosphate (50 mg, 135 μmol), triphenylphosphine (50 mg, 192 μmol), and tetrabutylammonium fluoride (0.10 g, 0.36 mmol) was stirred in pyridine (20 mL) overnight at 20 °C. Aqueous NH₃ (5% v/v; 30 mL) was added, and the resulting solution was stirred for 2 h. The mixture was co-evaporated with ethanol and dried under vacuum. The mixture was used without purification directly in the next step.
- 4-Nitrophenyl *N*-hydroxycarbamate.** 4-Nitrophenyl *N*-hydroxycarbamate was synthesized by the route reported for the synthesis of phenyl *N*-hydroxycarbamate (Stewart, A. O.; Brooks, D. W. *J. Org. Chem.* **1992**, *57*, 5020).
- 3'-*N*-Hydroxyurea-3'-deoxythymidine 5'-monophosphate.** 4-

Nitrophenyl *N*-hydroxycarbamate (0.10 g, 0.50 mmol) and tetrabutylammonium fluoride (0.20 g, 0.72 mmol) was added to the crude 3'-amino-3'-deoxythymidene 5'-monophosphate and stirred overnight in pyridine (20 mL) at 20 °C. The reaction was quenched with H₂O (20 mL). The mixture was co-evaporated with ethanol and dried under vacuum. The residue was taken up in H₂O (3 mL) and purified by reversed-phase HPLC using an H₂O/acetonitrile gradient and lyophilized to give 54 mg (99% overall) of fluffy white solid. ¹H NMR (300 MHz, D₂O) δ 1.82 (s, 3H), 2.28 (m, 2H), 4.03 (m, 2H), 4.08 (m, 1H), 4.36 (m, 1H), 6.19 (t, 1.3H, NH), 7.78 (s, 1H). MS (ESI) *m/z* calcd for C₁₁H₁₆N₄O₉P (M-H) 379.07, found 379.00. 22. Enzyme kinetics. Assays of ribonucleolytic activity were performed by using UV spectroscopy to measure the cleavage of poly(inosinic acid) [poly(I)] at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), as described previously.^{23,24} Concentrations of *N*-hydroxyurea **1** were

determined by its absorbance at 267 nm using the extinction coefficient for pdT, which is $\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Dawson, R. M. C.; Elliott, W. H.; Elliott, D. C. *Data for Biochemical Research*, 3rd Edition; Clarendon Press: New York, 1989. 23. Yakovlev, G. I.; Moiseyev, G. P.; Struminskaya, N. K.; Borzykh, O. A.; Kipenskaya, L. V.; Znamenskaya, L. V.; Leschinskaya, I. B.; Chernokalskaya, E. B.; Hartley, R. W. *FEBS Lett.* **1994**, 354, 305. 24. Yakovlev, G. I.; Struminskaya, N. K.; Znamenskaya, L. V.; Kipenskaya, L. V.; Leschinskaya, I. B.; Hartley, R. W. *FEBS Lett.* **1998**, 428, 57. 25. Wise, W. W.; Brandt, W. W. *J. Am. Chem. Soc.* **1954**, 77, 1058. 26. Chang, C. A.; Sekhar, V. C.; Garg, B. S.; Guziec, F. S., Jr.; Russo, T. C., Jr. *Inorg. Chim. Acta* **1987**, 135, 11. 27. Farkas, E.; Enyedy, E. A.; Csóka, H. *J. Inorg. Biochem.* **2000**, 79, 205.