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

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Abstract—The inhibition of ribonuclease Bi by 3′-*N*-hydroxyurea-3′-deoxythymidine 5′-phosphate is enhanced by 30-fold in the presence of Zn^{2+}. Thus, an *N*-hydroxyurea nucleotide can recruit Zn^{2+} to inhibit the enzymatic activity of a ribonuclease. This result engenders a general strategy for the inhibition of non-metalloenzymes by metal complexes.

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 pdUppAp, which has $K_i = 0.22$ μM 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. $UpOC_6H_4$-*p*-CH_2F is a mechanism-based inactivator of ribonuclease A.[9] Unfortunately, inactivation by $UpOC_6H_4$-*p*-CH_2F 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 Zn^{2+} 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 Zn^{2+}.[12–14] The Zn^{2+} 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 Zn^{2+} alone is 1 mM. Yet, the K_i for BABIM plus Zn^{2+} 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 Zn^{2+} 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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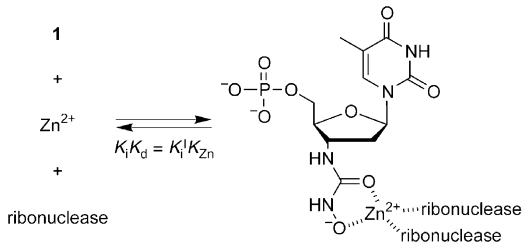
RNA substrate. Finally, hydroxamic acids are exceptional bidentate chelators of Zn^{2+}.[19]

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).

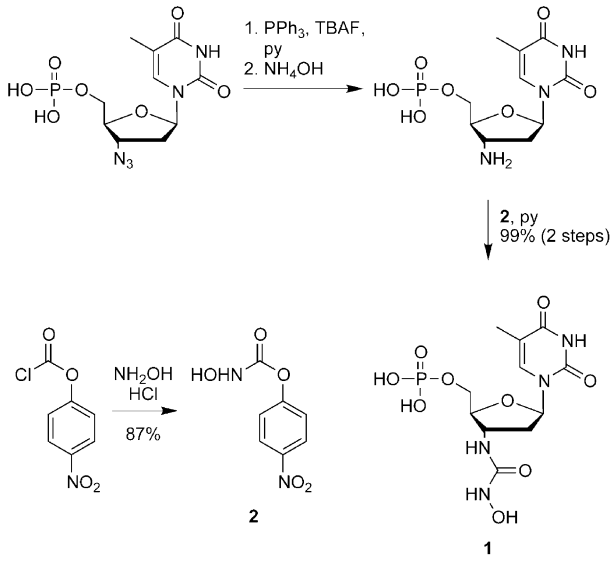
N-Hydroxyurea **1** was synthesized by the route shown in Scheme 2, which begins with the commercial reagent 3′-azido-3′-deoxythymidine 5′-monophosphate (AZT monophosphate).[21] The ability of *N*-hydroxyurea **1** to inhibit the ribonucleolytic activity of binase was assessed in the absence and presence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

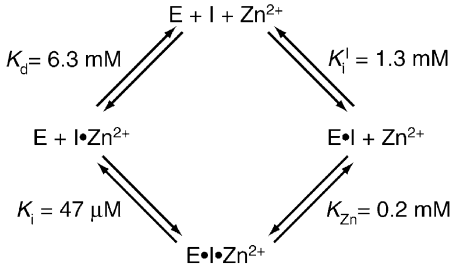
$$K_M^{obs} = K_M\left(1 + \frac{[I]}{K_i^I} + \frac{[I{\cdot}Zn^{2+}]}{K_i}\right) \quad (2)$$

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^I 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 $K_i^I = 1.3$ 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_iK_d values for different [I] and $[Zn^{2+}]$. As listed in Table 1, these values were approximately constant at $K_iK_d = 3\times10^{-7}$ M^2 if $[I][Zn^{2+}] \leq 10^{-7}$ M^2. When $[I][Zn^{2+}]$ was increased to 1.7×10^{-6} M^2, the K_iK_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_iK_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 ($CH_3C(O)NHO\underline{H}$), 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**.

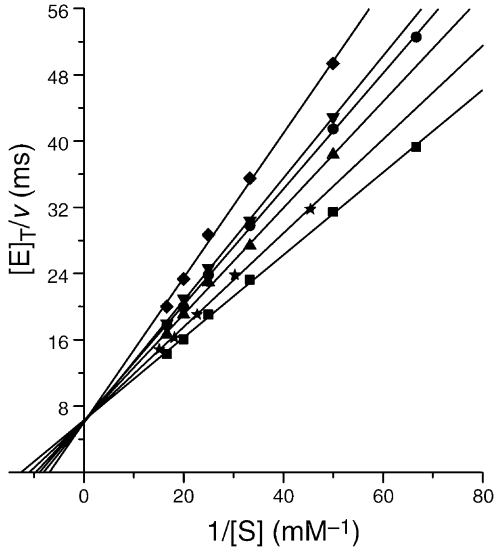


Figure 1. Lineweaver–Burk plot for the inhibition of binase by *N*-hydroxyurea **1** in the absence and presence of Zn^{2+}. Assays were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), binase (4.4×10^{-10} M), poly(I), *N*-hydroxyurea 1, and Zn^{2+}. ■, [I] = 0, [Zn^{2+}] = 0 (data with [Zn^{2+}] ≤ 5 mM were identical); ★, [I] = 0.93×10^{-4} M, [Zn^{2+}] = 0.25 mM; ▲, [I] = 3.5×10^{-4} M, [Zn^{2+}] = 0; ●, [I] = 1.0×10^{-4} M, [Zn^{2+}] = 1.0 mM; ▼, [I] = 3.5×10^{-4} M, [Zn^{2+}] = 0.25 mM; ◆, [I] = 3.5×10^{-4} M, [Zn^{2+}] = 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 $K_i = K_iK_d/K_d = 3\times10^{-7}$ M^2/6.3 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^I = 1.3 mM).

The affinity of Zn^{2+} for the enzyme was discerned likewise. From Scheme 3, $K_{Zn} = K_iK_d/K_i^I = 3\times10^{-7}$ M^2/1.3 mM = 0.2 mM. Because no inhibition of enzymatic activity was observed with [Zn^{2+}] ≤ 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_iK_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

[a]Data are for those assays depicted in Figure 1 performed in the presence of Zn^{2+}.

Acknowledgements

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21. **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 NH_3 (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 H_2O (20 mL). The mixture was co-evaporated with ethanol and dried under vacuum. The residue was taken up in H_2O (3 mL) and purified by reversed-phase HPLC using an H_2O/acetonitrile gradient and lyophilized to give 54 mg (99% overall) of fluffy white solid. ^{1}H NMR (300 MHz, D_2O) d 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 $C_{11}H_{16}N_4O_9P$ (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 $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$. Dawson, R. M. C.; Elliott, W. H.; Elliott, D. C. *Data for Biochemical Research*, 3rd Edition; Clarendon Press: New York, 1989.

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