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# Effects of Coordinating Metal Ions on the Mediated Inhibition of Trypsin by Bis(benzimidazoles) and Related Compounds

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The presence of the $Zn^{2+}$ ion dramatically enhances the inhibition of trypsin and tryptase by amidine-modified benzimidazole inhibitors via coordination to both the catalytically active Ser195 hydroxyl and His57 imidazole residues of the enzyme and the nitrogens of the amidine-modified benzimidazole inhibitor (Janc, J. W.; Clark, J. M.; Warne, R. L.; Elrod, K. C.; Katz, B. A.; Moore, W. R. *Biochemistry* **2000**, *39*, 4792–4800). Some new 5-amidino-2-substituted benzimidazoles were synthesized and compared to known related molecules to explore systematically the metal-mediated inhibition of bovine trypsin as a function of coordinating groups and metal ions. These compounds take advantage of the favorable interaction between the amidine group on one side of the inhibitor and the Asp189 carboxylate in the binding pocket of the enzyme. The 5-amidino-2-substituted benzimidazoles all demonstrated similar inhibition constants ($K_i$) of 20–50 $\mu$M in the absence of metal ions. In the presence of $Zn^{2+}$, inhibition increased to varying extents, depending upon the group substituted at the 2 position of the benzimidazole. The largest increase in inhibition in the presence of $Zn^{2+}$ was seen with (5-amidino-2-benzimidazolyl)-2-benzimidazolyl-methane with an apparent inhibition constant ($K_i'$) of 0.37 ± 0.06 nM, giving a 59 000-fold increase in inhibition when $Zn^{2+}$ is present. Other metal ions, including $Mn^{2+}$, $Sc^{3+}$, and $Hg^{2+}$, also increased the inhibition by several of the benzimidazole derivatives synthesized. The compound bis(2-benzimidazolyl)methane (BBIM) was also examined because it lacks the amidine group that provides a favorable hydrogen-bonding interaction with Asp189 in the binding pocket of trypsin. In the absence of metal ions, BBIM did not have a detectable affinity for trypsin; however, in the presence of $Zn^{2+}$, a $K_i'$ of 127 ± 3 nM was observed. This result demonstrates that an affinity for the enzyme in the absence of metal ions is not required for potent metal-mediated inhibition, greatly expanding the possibilities for metal mediation of nonmetalloenzymes.

## Introduction

Serine proteases have been linked to several disease states, including thrombosis, inflammation, and bronchoconstriction,[1−4] and there is therefore great interest in the development of serine protease inhibitors. Aromatic amidine molecules have been widely studied as competitive inhibitors of the protease enzymes[4−12] because amidine moieties bind to an aspartic acid residue in the specificity pocket adjacent to the active site of several serine proteases to produce competitive inhibitors.

Studies by Katz et al. have shown that the transition-metal ion $Zn^{2+}$ can mediate the inhibition of serine proteases by amidinobis(benzimidazoles).[1,13,14] The crystal structure in

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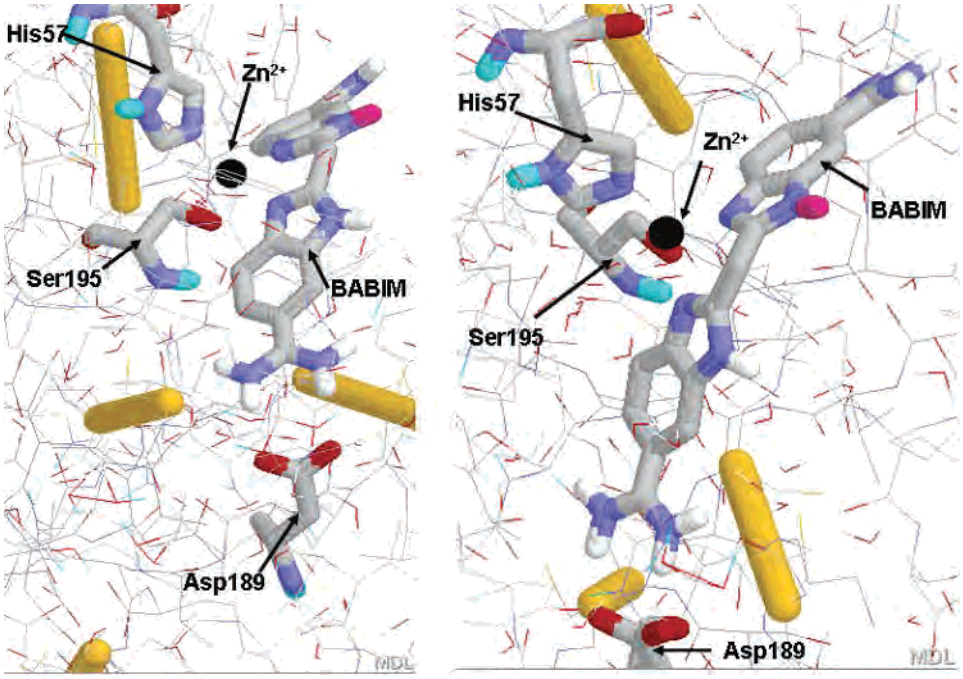


**Figure 1.** Crystal structure of tetrahedral $Zn^{2+}$ coordinated to BABIM and the serine protease enzyme, trypsin. One of the amidine groups in BABIM is hydrogen bound to Asp189 of the specificity pocket. Coordinates taken from ref 13.

Figure 1 shows one such inhibitor, bis(5-amidino-2-benzimidazolyl)methane (BABIM), bound to the active site of bovine trypsin with a $Zn^{2+}$ ion stabilizing the overall binding.[13] The $Zn^{2+}$ ion is tetrahedrally coordinated to each of the benzimidazole rings in the BABIM inhibitor, the Ser195 hydroxyl group in trypsin, and the His57 imidazole in trypsin. The amidine group of the BABIM inhibitor exhibits an additional favorable hydrogen-bonding interaction with Asp189 of trypsin, further strengthening the enzyme−inhibitor complex. The observed inhibition constant for BABIM in the presence of $Zn^{2+}$ is remarkably low (760 pM, this paper), suggesting that the metal mediation can provide for extremely potent inhibitors.

We report here a detailed study on the effects of different metal ions on the metal-mediated inhibition of trypsin with different benzamidine (AB) and benzimidazole inhibitors (Figure 2). The general 5-amidino-2-subtituted benzimidazole framework was chosen for study because of the potent inhibition of trypsin by BABIM in the presence of $Zn^{2+}$ and the available crystal structure as a guide. The amidine group anchors one benzimidazole in place, and by substitution at the 2 position, one of the ligating sites can be altered to tune the inhibition properties for particular metal ions. A much wider range of metal ions was examined than has been done previously: in addition to $Zn^{2+}$, the first-row dications $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ were evaluated along with $Sc^{3+}$, $Y^{3+}$, $Cd^{2+}$, and $Hg^{2+}$. The measured affinities reveal guiding principles that can ultimately be used to design enzyme−metal−inhibitor complexes with a range of properties.

## Experimental Section

**Synthesis.** All starting materials for synthesis were purchased from Aldrich or Fisher Scientific. The benzamidine (AB) and 4-aminobenzamidine (AAB) inhibitors were purchased from Aldrich. Ammonia and hydrochloric acid gases were purchased in 1-L lecture bottles from Aldrich. The starting materials were used without further purification. $^1$H NMR analyses were obtained on a Bruker 300-MHz spectrometer. Elemental analyses were obtained from Elemental Analysis Inc., Lexington, KY. Mass spectrometry analyses were obtained from RT Center Mass Spectrometry Labs, RTP, NC.

**(a) Synthesis of 3,4-Diaminobenzonitrile.** 3,4-Diaminobenzonitrile was synthesized by a modification of the procedure of Fairley et al.[15] A total of 2.0 g of 4-amino-3-nitrobenzonitrile and 0.4 g of 10% palladium on carbon were added to a Parr reaction bottle. Under a continuous flow of argon, 100 mL of ethyl acetate and 20 mL of methanol were added. The suspension was hydrogenated in a Parr reactor until hydrogen gas was no longer consumed. Palladium on carbon was removed by suction filtration through Celite, and the solvent was removed by rotary evaporation. The white solid was used immediately or stored under argon until further use. The reaction afforded 1.42 g of product (87.1%). The product was characterized by $^1$H NMR analysis. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 4.8 (s, 2H, $NH_2$), 5.4 (s, 2H, $NH_2$), 6.5−6.8 (m, 3H, aromatic H).

**(b) Synthesis of 4-Amino-3-nitrobenzamidine.** 4-Amino-3-nitrobenzamidine was synthesized by a modification of the procedure of Fairley et al.[15] A total of 5.2 g of 4-amino-3-nitrobenzonitrile was added to anhydrous dioxane (75 mL) and anhydrous methanol (2.5 mL) in a round-bottomed flask. The suspension was cooled

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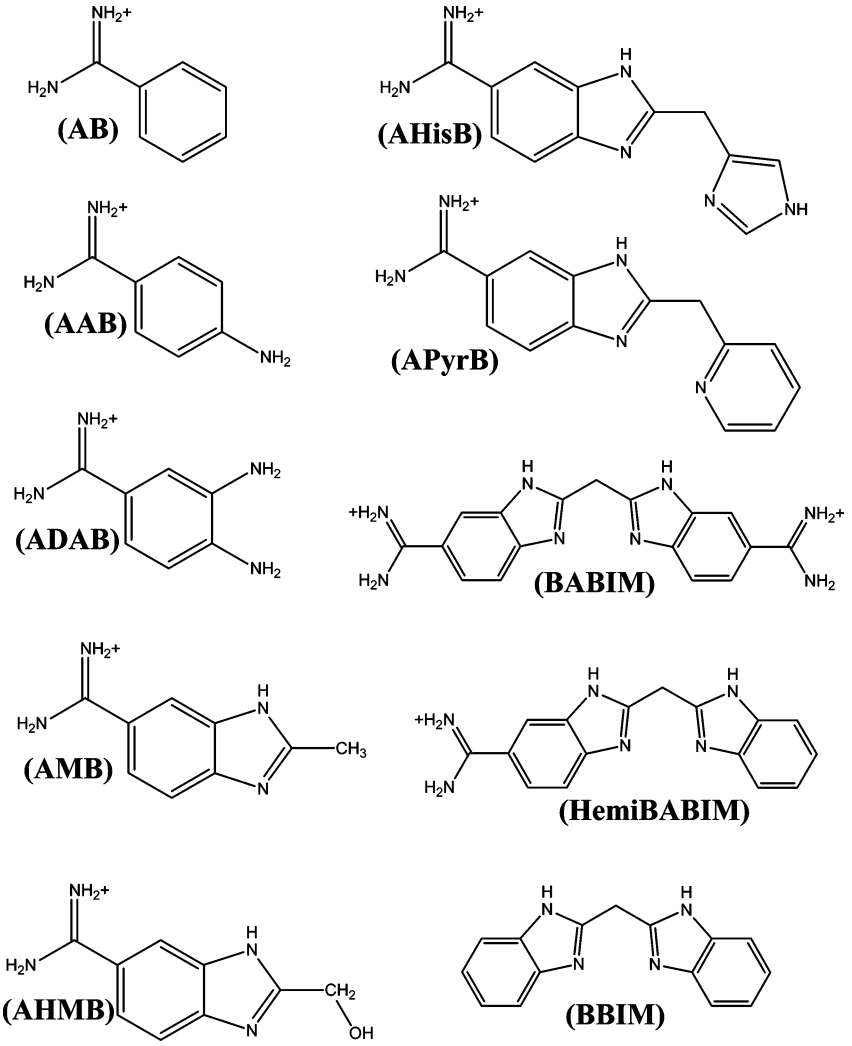


**Figure 2.** Structures of AB, AAB, ADAB, AMB, AHMB, AHisB, APyrB, BABIM, HemiBABIM, BBIM.

on ice and then saturated with anhydrous hydrochloric acid bubbled through sulfuric acid at 0 °C for 30 min. The flask was capped and stirred at room temperature for 2 days. The suspension was then cooled on ice and resaturated with anhydrous hydrochloric acid at 0 °C for 30 min. The flask was capped and stirred at room temperature for 7 days. A total of 50 mL of diethyl ether was added, and the reaction mixture was chilled on an ice bath. The light-yellow solid was collected by filtration under argon and rinsed with diethyl ether, yielding the imidate salt, which was used immediately in the next reaction step.

The imidate salt was placed in a Parr reaction bottle, and 200 proof ethanol (200 mL) was added. The solution was saturated with anhydrous ammonia for 30 min. After saturation with ammonia, the Parr bottle was clamped closed and heated at 80 °C for 12 h. The imidate salt dissolved in ethanol when heated, and the insoluble product precipitated out of solution as the reaction proceeded. The suspension was then cooled to 0 °C. The dark-yellow solid was collected by suction filtration and rinsed with diethyl ether. The product was stored under argon until use in the next reaction. After the reaction, 4.69 g of 4-amino-3-nitrobenzamidine was collected (58.3%). The resulting product was characterized by $^1$H NMR analysis. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 7.1 (d, 1H, aromatic H), 7.8 (d, 1H, aromatic H), 8.1 (s, 2H, $NH_2$), 8.6 (s, 1H, aromatic H), 9.1 (br, 4H, amidine H).

**(c) Synthesis of 3,4-Diaminobenzamidine (ADAB).** ADAB was synthesized by a modification of the procedure of Fairley et al.[15] A total of 2.25 g of 4-amino-3-nitrobenzamidine and 0.5 g of 10% palladium on carbon were added to a Parr reaction bottle. Under a continuous flow of argon, 100 mL of methanol and 25 mL of water were added. The suspension was hydrogenated in a Parr reactor until hydrogen gas was no longer consumed. Palladium on carbon was removed by suction filtration through Celite, and the solvent was removed by rotary evaporation. A total of 1.59 g (82%) of the white solid product was stored under argon until further use. The product was characterized by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 4.8 (s, 2H, $NH_2$), 5.6 (s, 2H, $NH_2$), 6.5−6.9 (m, 3H, aromatic H), 8.4 (s, 2H, amidine H), 8.7 (s, 2H, amidine H). Anal. Calcd for $C_7H_{10}N_4$·HCl: C, 45.04; H, 5.95; N, 30.02; Cl, 18.99. Found: C, 44.82; H, 5.83; N, 29.58; Cl, 19.17.

**(d) Synthesis of (5-Amidino-2-benzimidazolyl)-2-benzimidazolylmethane (HemiBABIM).** HemiBABIM was synthesized using a previously described procedure by Fairley et al.[15] The product was characterized by $^1$H NMR analysis. $^1$H NMR (300 MHz, DMSO-$d_6$, TFA): $\delta$ 5.2 (s, 2H, $CH_2$), 7.5−8.3 (m, 7H, aromatic H), 9.0 (s, 2H, amidine H), 9.4 (s, 2H, amidine H). MS: calcd for $C_{16}H_{11}N_5$, 273.010145; found, 273.10120.

**(e) Synthesis of 5-Amidino-2-methylbenzimidazole (AMB).** 5-Cyano-2-methylbenzimidazole was synthesized by a modification of the procedure of Rangarajan et al. for the syntheses of similar

5-cyano-2-substituted benzimidazoles.[16] A total of 1.33 g of 3,4-diaminobenzonitrile was refluxed with 1.70 g of sodium acetate in 4 M hydrochloric acid (12 mL) for 3 h. The dark-blue solution was basified to pH ≫ 10 with 2 M sodium carbonate, and a precipitate formed. Ethyl acetate was added to extract the desired product. The pink ethyl acetate layer was dried over sodium sulfate and filtered. The filtrate was dried by rotary evaporation. The desired product was isolated by column chromatography with a silica gel 230−400-mesh stationary phase, using first ethyl acetate as an eluant and then a 50:50 ethyl acetate/methanol mixture.

A total of 0.38 g of 5-cyano-2-methylbenzimidazole, synthesized above, was added to a round-bottomed flask with anhydrous dioxane (15 mL) and anhydrous methanol (0.5 mL). The reaction mixture was cooled on ice and then saturated with anhydrous hydrochloric acid bubbled through sulfuric acid at 0 °C for 30 min. The flask was capped and allowed to stir at room temperature for 4 days. The reaction mixture was chilled and then filtered under argon, yielding the imidate salt. The imidate salt was washed with cold diethyl ether and then transferred to a Parr reaction bottle. A total of 30 mL of 200 proof ethanol was added to the Parr bottle, and the solution was saturated with anhydrous ammonia for 30 min. After saturation with ammonia, the Parr bottle was clamped closed and heated at 80 °C for 6 h. The reaction was cooled to 0 °C, and solid was precipitated with cold diethyl ether. The solid was collected by filtration and rinsed with cold diethyl ether, yielding 0.18 g (35%). The product was characterized by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$, TFA): $\delta$ 2.8 (s, 3H, $CH_3$), 7.8−8.2 (m, 3H, aromatic H), 9.1 (s, 2H, amidine H), 9.5 (s, 2H, amidine H). Anal. Calcd for $C_9H_{10}N_4$·HCl·0.35$H_2O$: C, 49.8; H, 5.40; N, 25.8; Cl, 16.4. Found: C, 50.38; H, 5.28; N, 25.22; Cl, 15.90.

**(f) Synthesis of 5-Amidino-2-(hydroxymethyl)benzimidazole (AHMB).** AHMB was synthesized by a modification of the procedure of Phillips for the synthesis of 2-substituted benzimidazoles.[17] A total of 1.86 g of ADAB was refluxed with 0.95 g of glycolic acid in 4 M hydrochloric acid (12 mL) for 48 h. The dark-green reaction mixture was removed from heat and allowed to cool to room temperature. After cooling, the mixture was basified to pH 5 with ammonium hydroxide, resulting in a change of color to red. The solution was filtered to remove solid impurities, and the solvent was removed by rotary evaporation. The solid was suspended in 80 mL of warm methanol and collected by suction filtration. The solid was washed with copious amounts of diethyl ether before being collected. A coldfinger apparatus was set up on a vacuum line, and the solid was placed on the vacuum line and heated at or below 100 °C, allowing the ammonium chloride impurity to be collected on the coldfinger. The remaining solid was collected, yielding 0.718 g (31%). The product was characterized by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$, TFA): $\delta$ 5.0 (s, 2H, $CH_2$), 7.8−8.2 (m, 3H, aromatic H), 9.1 (s, 2H, amidine H), 9.5 (s, 2H, amidine H). Anal. Calcd for $C_9H_{10}N_4O$·HCl·0.25$H_2O$: C, 46.8; H, 4.98; N, 24.2; Cl, 15.4. Found: C, 46.90; H, 4.84; N, 23.84; Cl, 15.77.

**(g) Synthesis of Bis(5-amidino-2-benzimidazolyl)methane (BABIM).** BABIM was synthesized in a manner similar to that of Cleary et al. for bis(benzimidazoles).[18] A total of 2.5 g of malononitrile was added to a round-bottomed flask containing anhydrous dioxane (50 mL) and 200 proof ethanol (6.2 mL). The reaction mixture was cooled on ice and then saturated with anhydrous hydrochloric acid bubbled through sulfuric acid at 0 °C for 30 min. The flask was capped and allowed to stir at room temperature for 10 days. The reaction mixture was chilled and then filtered under argon, yielding the diimidate salt. The diimidate salt was washed with cold diethyl ether and dried on a vacuum line overnight. The white solid was stored in a drybox until it was used.

A total of 1.10 g of the diimidate salt was reacted with 1.77 g of ADAB in glacial acetic acid (50 mL) at 70 °C for 16 h under argon. The reaction mixture was cooled to room temperature and filtered. Diethyl ether was added to the filtrate, and a sticky oil precipitated out. The liquid was decanted, and the remaining oil was rinsed several times with diethyl ether. The oil was dissolved in hot 2-propanol (50 mL), cooled to room temperature, and filtered. Diethyl ether (150 mL) was added to precipitate the product. The solid was filtered and rinsed with copious amounts of diethyl ether. The remaining hygroscopic solid was quickly transferred to a vial and placed on a vacuum line overnight to dry, yielding 0.6 g (13%). The product was characterized by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$, TFA): $\delta$ 4.6 (s, 2H, $CH_2$), 7.6−8.3 (m, 6H, aromatic H), 9.0 (s, 4H, amidine H), 9.3 (s, 4H, amidine H). Anal. Calcd for $C_{17}H_{16}N_8$·3HCl·0.5$NH_4$Cl·$H_2O$: C, 42.0; H, 4.7; N, 24.5; Cl, 25.6. Found: C, 41.51; H, 4.19; N, 23.24; Cl, 25.60.

**(h) Synthesis of 5-Amidino-2-(pyridin-2′-ylmethyl)benzimidazole (APyrB).** APyrB was synthesized by a modification of the procedure of Verner et al. for the synthesis of 2-substituted benzimidazoles.[19] A total of 1.28 g of ADAB and 1.22 g of 2-pyridinylacetic acid hydrochloride were heated with polyphosphoric acid (20 g) at 180 °C for 3 h. The hot solution was poured into 100 mL of water and neutralized with sodium hydroxide pellets. The solid was collected and dissolved in methanol. The solution was filtered, and the filtrate was concentrated to a dark-green oil by rotary evaporation. A total of 30 mL of ethanol saturated with anhydrous hydrochloric acid was added to the oil and dried by rotary evaporation, leaving a gray solid. The product was rinsed with 100 mL of a cold 4:1 mixture of acetone/hydrochloric acid and air-dried. After purification, 0.228 g (8.5%) of solid was collected. The product was characterized by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 4.8 (s, 2H, $CH_2$), 7.5−8.2 (m, 7H, aromatic H), 9.1 (s, 2H, amidine H), 9.4 (s, 2H, amidine H). Anal. Calcd for $C_{14}H_{13}N_5$·3HCl·1.75$H_2O$: C, 42.87; H, 4.66; N, 17.86; Cl, 27.12. Found: C, 42.60; H, 4.62; N, 17.24; Cl, 27.83.

**(i) Synthesis of 5-Amidino-2-(imidazol-4′-ylmethyl)benzimidazole (AHisB).** AHisB was synthesized by a modification of the procedure of Phillips for the synthesis of 2-substituted benzimidazoles.[17] A total of 1 g of 4-imidazoleacetic acid hydrochloride and 0.933 g of ADAB were refluxed in 4 M hydrochloric acid (9 mL) for 48 h. The reaction mixture was dried by rotary evaporation. The resulting solid was dissolved in 100 mL of methanol and filtered. Diethyl ether was added to the filtrate to precipitate the desired product. The solid was collected by filtration and rinsed with copious amounts of diethyl ether. The solid was placed on a vacuum line overnight to dry. A total of 1.5 g (82%) of product was collected. The product was analyzed by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$, TFA): $\delta$ 4.8 (s, 2H, $CH_2$), 7.2−9.1 (m, 5H, aromatic H), 9.1 (s, 2H, amidine H), 9.5 (s, 2H, amidine H). Anal. Calcd for $C_{12}H_{12}N_6$·3HCl·$H_2O$:

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C, 39.2; H, 4.63; N, 22.9; Cl, 29.0. Found: C, 39.55; H, 4.51; N, 22.87; Cl, 28.72.

**(j) Synthesis of Bis(2-benzimidazolyl)methane (BBIM).** BBIM was synthesized by a modification of the procedure of Phillips for the synthesis of 2-substituted benzimidazoles.[17] A total of 1.44 g of malonic acid and 3 g of 1,2-phenylenediamine were refluxed in 4 M hydrochloric acid (12 mL) for 48 h. The reaction mixture was allowed to cool and then basified with 2 M sodium carbonate. The mixture was extracted to ethyl acetate and dried over sodium sulfate. The solid sodium sulfate was filtered, and the filtrate was removed by rotary evaporation to yield a brown solid. The solid was treated with a small amount of ethyl acetate to dissolve impurities, and the solid was collected by filtration, rinsing with diethyl ether. After rinsing, 0.16 g (9.3%) of product was collected. The product was characterized by $^1$H NMR analysis and elemental analysis. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 4.5 (s, 2H, $CH_2$), 7.1−7.6 (m, 8H, aromatic H). Anal. Calcd for $C_{15}H_{12}N_4$: C, 72.6; H, 4.87; N, 22.6; Cl, 0. Found: C, 71.86; H, 4.81; N, 22.14; Cl, 0.11.

**Enzyme Kinetics.** All kinetic data were collected on a Cary 300 Bio UV/visible spectrophotometer at 25 °C. Reactions were typically run in triplicate using the multicell changer of the spectrophotometer. Methacrylate disposable cuvettes of 1 mL were purchased from Fisher Scientific. The data collected were analyzed using Kaleidagraph version 3.52 by Synergy Software and SigmaPlot version 8.0 with the SigmaPlot Enzyme Kinetics Module 1.11 by SPSS Inc. Kinetic data were collected using the chromogenic substrate, Chromozym TH (tosylglycylprolylarginine/4-nitroanilide acetate), purchased from Roche. Chromozym TH was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1.9 mM and stored in the dark at room temperature. Unused portions were discarded after 2 weeks. Bovine trypsin was purchased from Sigma and Worthington Biochemical Corp. Bovine trypsin was diluted to stock concentrations of 14 and 20 nM in 25 mM Tris-HCl, 50 mM sodium chloride, pH 8.1 buffer, and 50% glycerol at 25 °C. These stock solutions were stored at −20 °C prior to use. All inhibitors were dissolved in 50 mM Tris-HCl, 100 mM sodium chloride, and pH 8.1 buffer at their desired concentrations prior to use. All metal chlorides were purchased from Aldrich and used without further purification. All other chemicals used were purchased from Aldrich or Fisher Scientific.

The stability of the trypsin enzyme was evaluated using an initial reaction mixture that contained 50 mM Tris-HCl, 100 mM sodium chloride, and pH 8.1 buffer at 25 °C. The enzyme was incubated in the reaction mixture over a range of time intervals from 0 min to 2.5 h. After each incubation time, the enzyme activity was initiated by the addition of the substrate, Chromozym TH, to a final concentration of 38 $\mu$M. The absorbance increase at 405 nm was collected for 4−5 min, and the linear portion of the graph was used for analysis. Using the extinction coefficient of nitroanilide at 405 nm ($\epsilon$ = 10.4 mmol$^{-1}$ L cm$^{-1}$), the slope was converted to velocity (millimoles per minute). The percentage of the maximal velocity was calculated, and data were plotted in the Kaleidagraph as a function of the incubation time of the enzyme (see the Supporting Information).

A stock solution of 1 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM Tris-HCl, 100 mM sodium chloride, and pH 8.1 buffer at 25 °C was prepared to determine inhibition constants in the absence of exogenous metal ions. The final reaction mixture contained in 1 mL of 0.8 mM EDTA, 50 mM Tris-HCl, 100 mM sodium chloride, and 10% DMSO at pH 8.1. The final enzyme concentration was 0.7 nM. The substrate concentrations were varied from 19 to 76 $\mu$M Chromozym TH. Inhibitor concentrations were varied over a broad range to determine an initial inhibition constant. Once an initial inhibition constant was determined, the inhibitor concentrations were varied within a more narrow range to determine a more exact inhibitory constant. The reaction mixture was prepared, followed by immediate addition of the substrate to initiate the reaction. The absorbance increase at 405 nm was collected for 4−5 min, and the linear portion of the graph was used for analysis. Using the extinction coefficient of nitroanilide at 405 nm ($\epsilon$ = 10.4 mmol$^{-1}$ L cm$^{-1}$), the slope was converted to velocity (millimoles per minute). The data were analyzed using a SigmaPlot Enzyme Kinetics Module 1.11 competitive inhibition model to determine an inhibition constant, $K_i$.

Stock solutions of 1 mM metal chlorides, $ZnCl_2$, $CuCl_2$, $NiCl_2$, $CoCl_2$, $MnCl_2$, $ScCl_3$, $YCl_3$, $CdCl_2$, and $HgCl_2$, were prepared in 50 mM Tris-HCl, 100 mM sodium chloride, and pH 8.1 buffer at 25 °C to determine the apparent inhibition constants in the presence of metal ions. The final reaction mixture contained 1 mL of 0.8 mM metal chloride, 50 mM Tris-HCl, 100 mM sodium chloride, and 10% DMSO at pH 8.1. The final enzyme concentration was 1 nM. Substrate concentrations varied from 19 to 76 $\mu$M. Inhibition was determined in the same way as the absence of metal ions, with the exception that the reaction mixture was allowed to preincubate for 30 min prior to the addition of the substrate to initiate enzyme activity. This is to accommodate the slow-binding nature of metal-mediated inhibition. The data were analyzed using a SigmaPlot Enzyme Kinetics Module 1.11 competitive inhibition model or tight-binding inhibition model to determine the apparent inhibition constant, $K_i'$.

**Determination of Inhibition Constants.** Inhibition was measured and defined in terms of the inhibition constant, $K_i$. $K_i$ is a dissociation constant between the inhibitor and enzyme as described by eq 1.[20] Determination of the inhibition constant was accomplished through spectroscopic assays, using a chromogenic substrate of trypsin. During examination of the inhibitors in the absence of metal ions, simple competitive inhibition was observed. However, with the addition of metal ions, two additional modes of inhibition were observed and measurements were reported as an apparent inhibition constant, $K_i'$.

$$K_i = [E][I]/[EI] \quad (1)$$

The first new mode of inhibition observed when metal ions were added to the reaction was slow-binding inhibition. Slow-binding inhibition results from the delayed establishment of equilibrium between the enzyme and inhibitor.[21,22] Because the time for equilibrium to be established between the free metal ion, inhibitor, and enzyme was on the order of several minutes, standard Michaelis−Menten kinetics do not apply.[21,22] To account for the slow-binding nature of these inhibitors in the presence of metal ions, a preincubation of the inhibitor, metal ion, and enzyme was performed before the addition of the substrate.[20] This preincubation time allowed for a steady-state equilibrium to be established before the reaction with the substrate was observed.

The second case of inhibition noted during metal-assisted studies with trypsin was tight-binding inhibition.[21−23] Tight-binding inhibition occurs when the inhibitory effects of a particular inhibitor are

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**Table 1.** Inhibition Constants of Bovine Trypsin with AB Inhibitors

| inhibitor | $K_i$ with EDTA ($\mu$M) | $K_i'$ with $Zn^{2+}$ ($\mu$M) |
|---|---|---|
| AB | $37 \pm 2$ (18.4[a]) | $102 \pm 1$ |
| AAB | $15.1 \pm 0.6$ (8.25[a]) | $50 \pm 2$ |
| ADAB | $28 \pm 2$ | $41 \pm 3$ |

[a] Experimental conditions at pH 8.15, 15 °C.[27]

seen at concentrations of inhibitor that are comparable to that of the enzyme. In the case of tight-binding inhibitors, the double-reciprocal plots used in standard Michaelis−Menten kinetics mimic noncompetitive inhibitors by intersecting at the *x* axis rather than competitive inhibitors, which intersect at the *y* axis.[20] An inhibitor is no longer considered a simple competitive inhibitor but a tight-binding inhibitor if eq 2 is satisfied.[24,25] $K_i'$ is the apparent inhibition

$$K_i' < 1000[E] \quad (2)$$

constant, and [E] is the enzyme concentration in the reaction mixture.

Not all of the metal-mediated inhibitors fall into the tight-binding class; however, all of the inhibitors were treated as slow-binding inhibitors in the presence of metal ions.

**Computer Modeling.** The crystal structure of the bovine trypsin/BABIM/$Zn^{2+}$ complex at pH 8.2 (RCSB Protein Data Bank ID number 1XUF) as reported by Katz et al. was used for all analyses.[13] Protein Explorer Version 2 was used to visualize the crystal structure. Docking studies were performed using Sybyl Version 6.9 by Tripos and the FlexX module by the German National Research Center for Information Technology (GMD). The R. L. Juliano Structural BioInformatics Core Facility at the University of North Carolina at Chapel Hill was used for modeling studies.

## Results and Discussion

The compounds ADAB, AMB, AHMB, APyrB, AHisB, BABIM, HemiBABIM, and BBIM (Figure 2) were synthesized by adaptations of procedures reported by Phillips and Fairley et al.[15,17] Detailed procedures are given in the Experimental Section.

**Inhibition of Trypsin in the Absence of Free Metal Ions.** Trypsin hydrolyzes peptides following arginine and lysine residues due to an aspartic acid (Asp189) in the specificity pocket adjacent to the catalytic active site of the enzyme. The Asp189 hydrogen bonds to arginine and lysine amino acids in large peptides, properly orienting the amide bond for hydrolysis by the active site serine, Ser195.[26] AB and AAB (Figure 2) are known competitive inhibitors of trypsin that competitively hydrogen bond to the Asp189 residue.[27−30] Binding of the amidine group is further stabilized by two other amino acid residues, Ser190 and Gly219, through additional hydrogen bonding.[29] Accordingly, AB, AAB, and ADAB all exhibit micromolar inhibition constants (Table 1) for trypsin, ranging from 15 to 40 $\mu$M. Slight modification of the AB inhibitor by the addition of amine groups does not significantly alter the $K_i$ values.

Elaborated 5-amidino-2-substituted benzimidazoles (Figure 2) also inhibit trypsin with $K_i$ values ranging from 30 to 50 $\mu$M (Table 2). The largest inhibitors examined, BABIM and HemiBABIM, exhibit $K_i$ values of $11.4 \pm 0.7$ and $22 \pm 1$ $\mu$M, respectively. As with the AB inhibitors, hydrogen bonding of the amidine group in the 5-amidino-2-substituted benzimidazoles to Asp189 provides the primary interaction with trypsin. The compound BBIM does not contain an amidine group, and accordingly, there is no detectable affinity for trypsin in the absence of metal ions (Table 2).

Figure 3 shows docking results for the modeling of the BABIM inhibitor to the active site of trypsin. As expected, one of the amidine moieties in the BABIM inhibitor is always situated into the specificity pocket and hydrogen-bonded to Asp189. The other benzimidazole group is free to shift into many different positions, provided the amidine−Asp189 interaction is preserved.

**Inhibition of Trypsin in the Presence of $Zn^{2+}$.** The addition of $Zn^{2+}$ ions changes the affinities of the inhibitors for trypsin dramatically. Free $Zn^{2+}$ inhibits trypsin with a $K_i$ of $1.19 \pm 0.09$ mM (Table 3). Interestingly, the simple AB inhibitors demonstrate an apparent *decrease* in inhibition when $Zn^{2+}$ is present compared to the inhibition observed when no $Zn^{2+}$ is present in the reaction (Table 1). This apparent decrease in inhibition of the AB compounds is likely due to $Zn^{2+}$ ions and the AB compounds inhibiting the enzyme independently of each other. Thus, the increase in the $K_i'$ value indicates that while both the AB inhibitors and $Zn^{2+}$ can occupy the active site at the same time, they do not do so synergistically.

The 5-amidino-2-substituted benzimidazole inhibitors produce a significantly different result than the simple AB inhibitors in the presence of $Zn^{2+}$. As observed by Katz et al.,[13,14] all 5-amidino-2-substituted benzimidazole inhibitors exhibit increased inhibition (decrease in $K_i'$) when $Zn^{2+}$ is added to the reaction mixture compared to the inhibition observed in the absence of metal ions (Table 4). This increase in inhibition is due to the $Zn^{2+}$ ion coordinating the active site serine and histidine, along with the benzimidazole inhibitor, forming a tighter inhibitor−enzyme complex that otherwise cannot be formed when no metal ions are present.[13] For ease of discussion, we will compare the inhibition of these compounds when no metal ions are present to the inhibition of the compounds in the presence of metal ions using the term $\Delta$,[31] which is calculated by dividing the inhibition constant of the inhibitor in the absence of metal ions by the apparent inhibition constant of the inhibitor in the presence of metal ions. Therefore, at higher values of $\Delta$, there is a higher degree of metal-assisted inhibition for the compound. When there is no detectable $\Delta$ value, the metal ion does not appear to increase the affinity of the compound for the enzyme.

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**Table 2.** Inhibition Constants of Bovine Trypsin with 2-Substituted Benzimidazole Derivatives and Selected Transition Metals[a]

| inhibitor | EDTA $K_i$ (nM) | $K_i'$ (nM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $Zn^{2+}$ | $Mn^{2+}$ | $Co^{2+}$ | $Ni^{2+}$ | $Cu^{2+}$ | $Sc^{3+}$ | $Y^{3+}$ | $Cd^{2+}$ | $Hg^{2+}$ |
| AMB | 37 000 ± 2000 | 6 100 ± 200 | 46 000 ± 5000 | 12 0000 ± 8000 | 98 000 ± 4000 | 10 6000 ± 4000 | 14 9000 ± 5000 | 87 000 ± 5000 | 65 000 ± 7000 | 9 800 ± 900 |
| AHMB | 47 000 ± 4000 | 1 610 ± 60 | 48 000 ± 4000 | 69 000 ± 9000 | 86 000 ± 6000 | 82 000 ± 7000 | 10 1000 ± 3000 | 78 000 ± 9000 | 49 000 ± 3000 | 470 ± 3 |
| APyrB | 30 000 ± 1000 (> 1 mM[b]) | 12.1 ± 0.6 (136 $\mu$M[d]) | 11 300 ± 800 | 13 000 ± 1000 | 70 000 ± 10000 | 20 000 ± 1000 | 25 000 ± 1000 | 43 000 ± 3000 | 2 100 ± 200 | 28 ± 2 |
| AHisB | 42 000 ± 5000 (273 mM[b,c]) | 4.2 ± 0.5 (666 nM[c,d]) | 2 300 ± 200 | 7 600 ± 800 | 72 000 ± 7000 | 11 300 ± 800 | 8 000 ± 600 | 6 700 ± 400 | 700 ± 100 | 54 ± 4 |
| Hemi BABIM | 22 000 ± 1000 (182 $\mu$M[b]) | 0.37 ± 0.06 (23.5 nM[d]) | 2.8 ± 0.5 | 86 ± 6 | 267 ± 2 | 356 ± 4 | 13 ± 1 | 24 ± 3 | 7 ± 2 | 44 ± 3 |
| BABIM | 11 400 ± 700 (19 $\mu$M[b]) | 0.76 ± 0.09 (90 nM[d,e]) | nd | nd | nd | nd | 133 ± 5 | 190 ± 4 | nd | nd |
| BBIM | n.o. | 127 ± 3 | nd | nd | nd | nd | nd | nd | nd | nd |

[a] nd = not determined. n.o. = none observed. [b] Human trypsin at 10 mM EDTA, pH 8.2, 22 °C.[13] [c] $K_i$ of a similar AHisB inhibitor.[13] [d] Human trypsin at 150 $\mu$M $ZnCl_2$, pH 8.2, 22 °C.[13] [e] $K_i'$ of 5.0 ± 0.3 nM with bovine trypsin at 100 nM $ZnCl_2$, pH 8.2, 22 °C.[13]



**Figure 3.** BABIM docked in the active site of bovine trypsin. The blue, yellow, and red BABIM inhibitors represent a sample of the docking results using Sybyl Version 6.9 by Tripos with the FlexX module by GMD. The multicolored BABIM inhibitor represents the location of the inhibitor when $Zn^{2+}$ is present as indicated by the crystal structure.[13]

**Table 3.** Inhibition Constants of Bovine Trypsin with Selected Metal Ions

| metal ion | $K_i$ (mM) | metal ion | $K_i$ (mM) |
|---|---|---|---|
| $Zn^{2+}$ | 1.19 ± 0.09 | $Cd^{2+}$ | 25 ± 1 |
| $Cu^{2+}$ | 2.6 ± 0.1 | $Hg^{2+}$ | 1.4 ± 0.1 |
| $Ni^{2+}$ | 10.4 ± 0.4 | $Y^{3+}$ | 5.8 ± 0.4 |
| $Co^{2+}$ | 41 ± 2 | $Sc^{3+}$ | not determined[b] |
| $Mn^{2+}$ | ≫200[a] | | |

[a] No inhibition was observed at concentrations up to 200 mM $MnCl_2$. [b] No inhibition was observed as $ScCl_3$ precipitated out of solution at concentrations above 5 mM.

This Δ effect ranges from as little as 6.1 for the methyl inhibitor, AMB, to 59 000 for the HemiBABIM inhibitor (Table 4). Because AMB can only function as a monodentate ligand to $Zn^{2+}$ through the nitrogen in the 1 position of the benzimidazole ring, it is not surprising that a modest Δ is observed. This value provides a baseline for the interaction of a single coordinating group from the inhibitor to the metal ion. The AHMB inhibitor has a higher Δ value of 20 than AMB, indicating that the pendant hydroxyl group can coordinate to $Zn^{2+}$ in addition to the benzimidazole nitrogen.

Pyridine-based compounds have been examined as zinc chelators[32] and zinc sensors.[33−36] The inhibitor APyrB has a pyridine group in the 2 position of the 5-amidino-2-substituted benzimidazole and gives a much larger Δ effect of 2500 in the presence of $Zn^{2+}$. Even larger Δ effects in the presence of $Zn^{2+}$ were observed with inhibitors that present a second imidazole-like ligand to the metal center:


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**Table 4.** Δ Values for 5-Amidino-2-substituted Benzimidazoles[a]

| inhibitor | $\Delta Zn^{2+}$ | $\Delta Mn^{2+}$ | $\Delta Co^{2+}$ | $\Delta Cu^{2+}$ | $\Delta Ni^{2+}$ | $\Delta Sc^{3+}$ | $\Delta Y^{3+}$ | $\Delta Cd^{2+}$ | $\Delta Hg^{2+}$ |
|---|---|---|---|---|---|---|---|---|---|
| AMB | 6.1 | no Δ effect | no Δ effect | no Δ effect | no Δ effect | no Δ effect | no Δ effect | no Δ effect | 3.8 |
| AHMB | 20 | no Δ effect | no Δ effect | no Δ effect | no Δ effect | no Δ effect | no Δ effect | no Δ effect | 100 |
| APyrB | 2500 | 2.6 | 2.3 | 1.5 | no Δ effect | no Δ effect | no Δ effect | 14 | 1100 |
| AHisB | 10000 | 18 | 5.5 | 3.7 | no Δ effect | 5.3 | 6.3 | 60 | 780 |
| HemiBABIM | 59000 | 7900 | 260 | 62 | 82 | 1700 | 920 | 3100 | 500 |
| BABIM | 15000 | nd | nd | nd | nd | 86 | 60 | nd | nd |

[a] nd = not determined.

the AHisB inhibitor has a Δ value of 10 000, whereas the bis(benzimidazoles) BABIM and HemiBABIM have Δ values with $Zn^{2+}$ of 15 000 and 59 000, respectively. *This 59 000-fold increase in inhibition equates to* a *370 pM* $K_i'$ *for HemiBABIM* (Table 2). Several zinc enzymes use imidazole ligands to coordinate $Zn^{2+}$ with extremely tight affinities;[37−39] thus, it is not surprising that we observed large Δ values for molecules that contain imidazole and benzimidazole ligands.

Because BBIM lacks an amidine group, there is no detectable affinity for trypsin in the absence of metal ions, as discussed above. However, when $Zn^{2+}$ ion is present, BBIM gives a $K_i'$ of 127 ± 3 nM (Table 2). This result provides a quantitative assessment of the Δ effect in the absence of assistance from amidine−carboxylate hydrogen bonding and suggests that an independent means of associating the inhibitor is not required to achieve potent $Zn^{2+}$-assisted inhibition. In all of the other inhibitors studied in our laboratory and by Katz et al.,[13,14] an amidine moiety has been present to direct the inhibitor to the active site. The nanomolar $K_i'$ value of BBIM in the presence of $Zn^{2+}$ ion that we present here indicates that the metal binding alone can produce a tight inhibitor and suggests that the metal-assisted effect might ultimately be applied to other serine proteases that lack specificity for cationic amino acids.

**Inhibition of Trypsin in the Presence of Selected First-Row Transition-Metal Ions.** Other metal ions can substitute for $Zn^{2+}$ in known $Zn^{2+}$ enzymes, such as carbonic anhydrase.[40−49] Janc et al. demonstrated that metal ions other than $Zn^{2+}$ can assist in the inhibition of the serine protease tryptase (not trypsin) by BABIM; however, BABIM only gave a Δ of 10 with $Cd^{2+}$, $Co^{2+}$, and $Mn^{2+}$, while no metal-assisted inhibition was observed in the presence of $Cu^{2+}$, $Mg^{2+}$, and $Ni^{2+}$; oxidation of $Co^{II}$ to $Co^{III}$ was not detected in our experiments or reported by Katz et al.[1] These changes in inhibition compare to a Δ of 85 for tryptase with BABIM when $Zn^{2+}$ is present.[1] This value is small compared to that of 15 000 for trypsin with BABIM in the presence of $Zn^{2+}$ (Table 4), so we determined the effect of several first-row transition-metal ions in the inhibition of trypsin by 5-amidino-2-substituted benzimidazole derivatives.

The metal ions $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ all exhibit various degrees of metal-assisted inhibition with the 5-amidino-2-substituted benzimidazoles. The AMB and AHMB inhibitors both do not show a Δ effect with the metal ions $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ (Table 4), indicating that the special affinity of $Zn^{2+}$ is required for these relatively weak inhibitors. The APyrB inhibitor shows a small degree of metal-assisted inhibition with $Mn^{2+}$, $Co^{2+}$, and $Cu^{2+}$, with Δ values of 2.6, 2.3, and 1.5, respectively. This small enhancement is less than that seen, for example, for the monodentate AMB inhibitor in the presence of $Zn^{2+}$ (Δ value of 6.1) and far less than the Δ value of APyrB with $Zn^{2+}$ of 2500. Furthermore, $Ni^{2+}$ does not enhance the inhibition properties of the APyrB inhibitor for trypsin. The AHisB inhibitor shows larger Δ effects in the presence of the metal ions $Mn^{2+}$, $Co^{2+}$, and $Cu^{2+}$ than the corresponding Δ values for APyrB. The $Mn^{2+}$ enhancement is the largest for the AHisB inhibitor with a Δ value of 18, whereas $Co^{2+}$ and $Cu^{2+}$ have Δ values of 5.5 and 3.7, respectively. However, these values are also significantly less than the $Zn^{2+}$-assisted enhancement seen with AHisB of 10 000. As with the APyrB inhibitor, AHisB does not have enhanced inhibition of trypsin in the presence of free $Ni^{2+}$.

The HemiBABIM inhibitor has a more pronounced change across the first-row transition-metal series than the other inhibitors examined (Table 4). With the HemiBABIM inhibitor, the degree of enhancement of inhibition decreases across the periodic table from $Mn^{2+}$ to $Cu^{2+}$ (Table 4). The Δ values for HemiBABIM with $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ are 7900, 260, 82, and 62, respectively. The Δ value of 7900 ($K_i' = 2.8 \pm 0.5$ nM) for HemiBABIM in the presence of $Mn^{2+}$ is larger than the Δ effect of $Zn^{2+}$ with the APyrB inhibitor (Δ value of 2500) and only slightly lower than the Δ effect of $Zn^{2+}$ with the AHisB inhibitor (Δ value of 10 000).

It is important to point out that the order of metal-assisted inhibition does not follow the Irving−Williams series, which defines the relative stabilities of ligands formed with first-row transition metals ($Mn^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$).[50,51] The large Δ effects seen with $Zn^{2+}$ over the first-row transition-metal ions ($Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$) most

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likely arise from the strong preference of $Zn^{2+}$ for tetrahedral coordination and the ability of the ternary complex to orient the two nitrogens of the inhibitor in a tetrahedral array compared to the coordinating amino acids (Figure 1).[49,52−54] This idea is most dramatically shown by the low Δ value for the $Cu^{2+}$ ion, which would be predicted to have the greatest stability from the Irving−Williams series but which prefers a predominantly square-planar geometry or in some cases a distorted four-coordinate geometry in biological molecules.[49,52,54,55] For $Cu^{2+}$ to adopt a square-planar geometry, the amidine−Asp189 interaction would have to be disrupted in the specificity pocket of the enzyme, thus decreasing the affinity of the inhibitor for the enzyme.

**Inhibition of Trypsin in the Presence of Scandium and Yttrium Ions.** Because the affinities for the first-row transition-metal ions indicated that the tetrahedral geometry was the primary determinant of $Zn^{2+}$ specificity, the effects of the $d^0$ metal ions $Sc^{3+}$ and $Y^{3+}$ on the inhibition of trypsin by the AMB, AHMB, APyrB, AHisB, HemiBABIM, and BABIM inhibitors (Figure 2) were determined. $Sc^{3+}$ and $Y^{3+}$ are harder ions than $Zn^{2+}$ because of their increased charge but exhibit a strong preference for tetrahedral coordination.[56] The weaker inhibitors AMB, AHMB, and APyrB do not demonstrate enhanced inhibition of trypsin in the presence of $Sc^{3+}$ and $Y^{3+}$; however, AHisB gave Δ values of 5.3 and 6.3 in the presence of $Sc^{3+}$ and $Y^{3+}$, respectively (Table 4), and BABIM gave Δ values of 86 and 60, respectively. As with $Zn^{2+}$, the largest increase in inhibition was with the HemiBABIM inhibitor, which has Δ values of 1700 and 920 in the presence of $Sc^{3+}$ and $Y^{3+}$, respectively. With all inhibitors, the increase in inhibition with $Sc^{3+}$ and $Y^{3+}$ is significantly less than the increase seen with $Zn^{2+}$. The only inhibitors that have an increase in inhibition with $Sc^{3+}$ and $Y^{3+}$ are the bis(imidazole)-based inhibitors AHisB, BABIM, and HemiBABIM. The higher Δ values for $Zn^{2+}$ compared $Sc^{3+}$ and $Y^{3+}$ are likely due to the borderline hard−soft status of $Zn^{2+}$ and the consequent affinity for imidazole.[56,57]

**Inhibition of Trypsin in the Presence of Cadmium and Mercury Ions.** The effects of the $d^{10}$ metal ions $Cd^{2+}$ and $Hg^{2+}$ on the inhibition of trypsin by the AMB, AHMB, APyrB, AHisB, and HemiBABIM inhibitors (Figure 2) were also determined. Like $Zn^{2+}$, $Cd^{2+}$ and $Hg^{2+}$ have $d^{10}$ configurations, which might further elucidate the preferences for metal-assisted inhibition of trypsin by 5-amidino-2-substituted benzimidazole inhibitors. The $Cd^{2+}$ and $Hg^{2+}$ ions differ from $Zn^{2+}$ in that they are larger and softer than the $Zn^{2+}$ ion.[56] The methyl-substituted inhibitor AMB does not demonstrate $Cd^{2+}$-enhanced inhibition; however, there is a 3.8-fold enhancement in the inhibition of trypsin when $Hg^{2+}$ is present (Table 4). $Hg^{2+}$ is the only metal ion other than $Zn^{2+}$ where metal-assisted inhibition of trypsin is observed with the AMB inhibitor; this is not surprising given the ability of $Hg^{2+}$ to form three-coordinate complexes.[58−60]

Although no $Cd^{2+}$-enhanced inhibition was observed with the AHMB inhibitor, a unique result occurs when the AHMB inhibitor is examined with $Hg^{2+}$. A Δ value of 100 ($K_i' = 470 \pm 3$ nM) was observed with $Hg^{2+}$ compared to the Δ value of 20 ($K_i' = 1.61 \pm 0.06$ μM) seen with $Zn^{2+}$. This is the first example of an inhibitor that demonstrates better inhibition with a metal ion other than $Zn^{2+}$. Several reasons might account for this observation. First, $Hg^{2+}$ has many valencies, which allow for more flexibility and different potential binding modes of the benzimidazole inhibitor to the metal ion.[52,54,57] Second, AHMB is the only inhibitor screened that binds in a five-membered chelate ring to the metal ion.[61] All of the other bidentate inhibitors bind in a six-membered chelate ring to the metal ion, and larger cations are known to bind with a higher stability to five-membered chelate rings, while smaller cations bind better to six-membered chelate rings.[61] The larger $Hg^{2+}$ ion has an ionic radius of 1.1 Å compared to the smaller $Zn^{2+}$ ion with an ionic radius of 0.74 Å.[62]

The APyrB inhibitor shows a 14-fold enhancement in the presence of $Cd^{2+}$ and an 1100-fold enhancement in the presence of $Hg^{2+}$. This enhancement with $Hg^{2+}$ is only slightly less than that observed in the presence of $Zn^{2+}$ with a Δ effect of 2500. It is also interesting to note that the AHisB inhibitor is a weaker inhibitor than APyrB in the presence of $Hg^{2+}$ with only a 780-fold enhancement. This observation is in contrast to all of the other metal ions, which show stronger inhibiton with AHisB than APyrB. AHisB has a 60-fold increase in inhibition when $Cd^{2+}$ is present and is consistent with the general trend of AHisB having a higher degree of metal enhancement than APyrB.

The HemiBABIM inhibitor is the only inhibitor studied that has a higher degree of metal enhancement with $Cd^{2+}$ when compared to $Hg^{2+}$ with Δ values of 3100 and 500, respectively (Table 4). In the presence of $Hg^{2+}$, the $K_i'$ of $44 \pm 3$ nM is comparable to the $K_i'$ values of APyrB ($K_i' = 28 \pm 2$ nM) and AHisB ($K_i' = 54 \pm 4$ nM).

## Conclusions

The ability of metal ions to increase dramatically the affinity of benzimidazole-based inhibitors has been pursued with applications in pharmaceutical discovery.[13,14] We report here the first systematic study with regard to the identity of the metal ion and the identity and nature of the coordinating inhibitor. The tetrahedral array afforded by the inhibitor and the amino acid residues is the primary determinant of metal-ion specificity. Within that dominating theme, the size of

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the metal ion, ability to adopt three-coordination, and hard−soft-type preferences for imidazole over pyridine or vice versa play out as expected.

The observation that the BBIM inhibitor can inhibit trypsin in the presence of $Zn^{2+}$, while having no affinity for the enzyme in the absence of the metal ion, is a remarkable result. This phenomenon suggests that metal ions can be used to produce inhibitory interactions for proteins that are otherwise difficult to inhibit through conventional enzyme−inhibitor interactions. Further, the affinity of BBIM in the presence of $Zn^{2+}$ provides a quantitative assessment of the $\Delta$ effect in the absence of a contribution from the amidine−Asp189 interaction. The ability to better understand the energetics of metal ion assistance in enzyme−inhibitor interactions could lead to new modes of inhibition for challenging problems in drug discovery.

**Supporting Information Available:** Plot of the stability of trypsin as a function of the incubation time and examples of Michaelis−Menton plots (Figures S1−S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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