(0.1%), and Me_2SO (5%). The angiotensin 1 produced by the renin was subsequently quantified with an enzyme immunoassay by comparison with an angiotensin-1 standard curve. Test compounds were dissolved and diluted in 100% Me_2SO. Inhibitory activities are expressed as IC_{50} values, which represent the concentration of compound that inhibits 50% of the maximal (uninhibited) enzyme activity. IC_{50} values for some of the inhibitors were also determined by a second method, not reported here; good agreement between the data was found.

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Protease Inhibitors Formed In Situ from Copper and Tridentate Chelates: A Generalized Approach towards Metal-Based Pharmaceuticals

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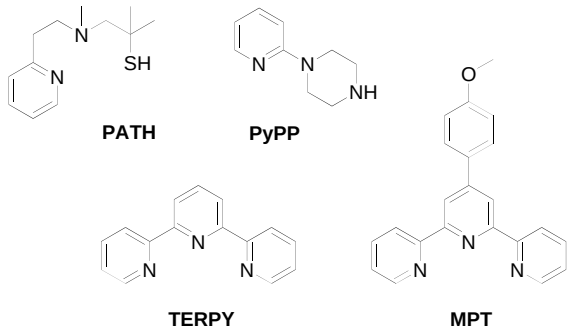
bioinorganic chemistry · chelation · drug research · furin · N ligands

Metal-based pharmaceuticals are expected to bind to their targets with high affinity, which may translate to reduced drug intake and safer treatment. Their distinct mode of action suggests they have excellent prospects in therapy against drug-resistant pathogens.[1] By their sheer number, they vastly increase the pool from which new drugs can be chosen. Metal compounds find wide use in medical diagnostics.[2] However, these compounds are coordinatively saturated and unable to bind biological molecules and bring about therapeutic effects. Of the few metallodrugs currently in use, most target DNA.[2] We therefore set out to develop a generalized methodology to obtain protein-targeted, metal-based drugs. To eliminate any fear of toxicity associated with ingesting metals, our strategy involves a prodrug intended to extract the metal from within the patient's body. Our in vitro model for this new approach involves the in situ formation of a ternary complex containing a chelate (the prodrug), a metal, and the target enzyme. We now report that the readily available tridentate chelates 2,2′:6′,2″-terpyridine (TERPY), 2-methyl-1-[methyl(2-pyridine-2-yl-ethyl)amino]-propane-2-thiol (PATH),[3] and 1-[2-pyridyl]-piperazine (PyPP) coordinate Cu^{2+} ions in situ to enhance the potency of Kex2



inhibition beyond that of the solvated metal ion. Furthermore, TERPY makes the inhibition irreversible. The variability among these chelates and the fact that none inhibits the target by itself suggest that our model applies to a range of chelates. As the single criterion we used to choose our enzyme was its inhibition by solvated metal ions (its structure is unknown), we suggest that our model has the potential to apply to additional targets. An approach *complementary* to ours, in which enzyme inactivation by a potent bidentate chelating inhibitor is enhanced by the coordination of Zn^{2+} ions, was demonstrated with trypsin-like serine proteases.[4, 5]

Kex2 is a yeast endopeptidase that converts protein precursors into biologically active proteins to be secreted from the cell.[6] Most Kex2 inhibitors also affect the mammalian homologue furin. Furin activates the envelope glycoproteins of many viruses, including Ebola, avian influenza, and rabies prior to exocytosis, thereby enabling their uptake by new host cells.[7] As furin is expressed in most body cells, its inhibitors may stop proliferation of these aggressive viruses after exposure and possibly even after the related disease symptoms appear. Furin blockers may also affect toxins of bacteria such as *Bacillus anthracis* (which causes anthrax) and Pseudomonas by hindering maturation of their protoxins during endocytosis.[7] Such antidote-like treatment is needed when toxin levels render antibiotics ineffective.

Both Zn^{2+} and Cu^{2+} ions inhibit Kex2 in vitro,[8] with Cu^{2+} ions being more potent. Likewise, chelate enhancement of inhibition was much better with Cu^{2+} ions. We report only results with copper.

In the present study we used tridentate chelates as our prodrug models. Their binding constants to metals are much higher than those of bidentates.[9] Thus, tridentates are predicted to compete more effectively for the endogenous metals against natural cellular factors. Tetradentates will bind more strongly, but are less likely to generate sufficient free sites on the metal for target coordination. We tested chelates that seemed likely to bind to Cu^{2+} ions, but target inhibition was not among the criteria in choosing them. None of our chelates inhibited Kex2 on

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their own. Micromolar concentrations of chelate in the absence of copper decreased Kex2 activity by less than 5%.

As an indication for chelate involvement in the process, we looked for improvement over the inhibition by solvated Cu^{2+} ions. A typical experiment involved incubation of chelate, copper salt, and Kex2 at 22 °C, followed by addition of the substrate analogue BOC-Arg-Val-Arg-Arg-MCA (Boc = *tert*-butoxycarbonyl, MCA = methylcoumarinamide).[10, 11] The degree of inactivation was determined from the initial formation rate of the fluorescent AMC product (AMC = 7-amino-4-methylcoumarin). Upon addition of TERPY in a 1:1 ratio to the metal, an enhancement of the Kex2 inhibition by copper was observed (IC_{50} of 20 versus 95 μM Cu^{2+} ions achieving the same effect without the chelate, Figure 1). The curves in Figure 2 were fitted by Equation (1), where P = [product], k_{obs} = apparent inactivation rate constant, v_0 = rate of uninhibited reaction, v_s = product formation rate at equilibrium with inhibitor, t = time.[12, 13]

$$P = v_s t + (v_0 - v_s)(1 - \exp^{-k_{obs}t})/k_{obs} \quad (1)$$

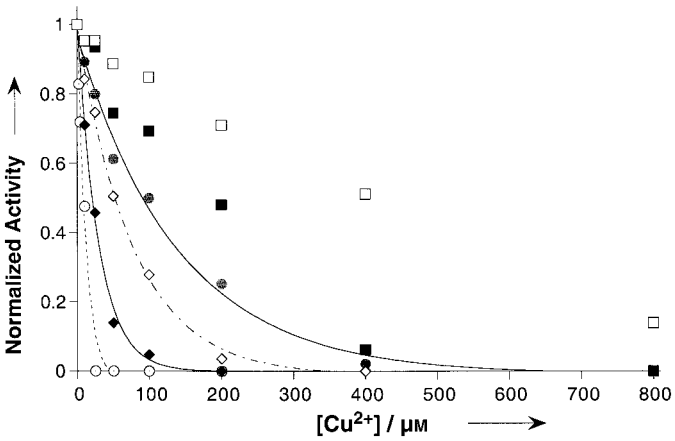

Figure 1. *Kex2 inhibition by solvated Cu^{2+} ions alone (●), and in the presence of TERPY (in the presence (◆) and absence (◇) of chloride ions), DPA (in the presence (■) and absence (□) of chloride ions), and MPT (⊙) at a 1:1 ratio to [Cu^{2+}]. Mixtures were incubated in 96-well plates at 22 °C for 2 h prior to substrate addition. Initial product formation rates were obtained from fluorescence measurements at 30 °C. [Kex2] = 1.2 nM, [substrate] = 19 μM (which is the Michaelis constant (K_m) value.[22] Buffer was 168 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bis-Tris; pH 7.4) with 0.1% Triton x-100, 3% dimethylsulfoxide (DMSO). For experiments with chloride [NaCl] = 20 mM and [$CaCl_2$] = 1 mM, but for those without chloride [Na_2SO_4] = 10 mM and [$CaSO_4$] = 1 mM. The data were fitted to the equation: $\delta P/\delta t = v_s + (v_0 - v_s)exp[-k_{obs}t]$. (The terms are explained in the text.)*

This equation simulates slow binding inhibitors. v_o was obtained from the noninhibited reaction. In the presence of TERPY, the curves are flat and were fitted best with $v_s = 0$, as is expected for irreversible inactivation.[12] With Cu^{2+} ions alone, $v_s > 0$. The fit was corrected by adding a v_{hv} term for the slow product photochemical degradation,[14] which is first order in AMC, but zero order in inhibitor. v_{hv} was obtained from the negative linear slope at steady state. The reduced inhibition rates at higher substrate concentrations (Figure 3) indicate competition between substrate and inhibitor. Enhancement of copper inhibition by TERPY was maximized at a 1:1 ratio.

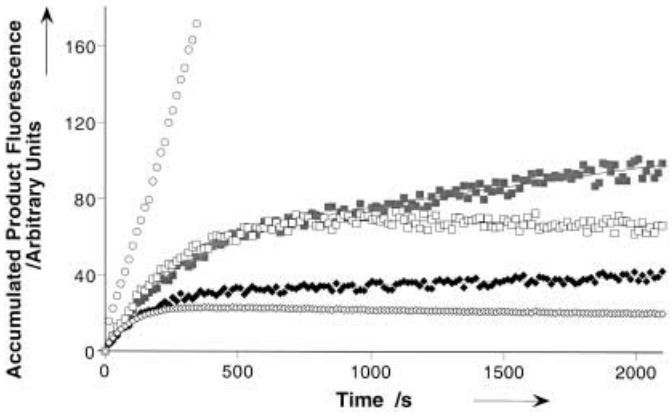

Figure 2. *Kex2 inhibition progress curves in the presence of 400 μM (■) and 610 μM (◆) 1:1 Cu^{2+} and TERPY mixture, 400 μM (□) and 610 μM (◇) solvated Cu^{2+} ions alone, and in the absence of inhibitor (⊙). Conditions were as stated in the legend of Figure 1 (in the presence of chloride), but [substrate] = 100 μM, and the reactions were initiated by enzyme addition at 30°.*

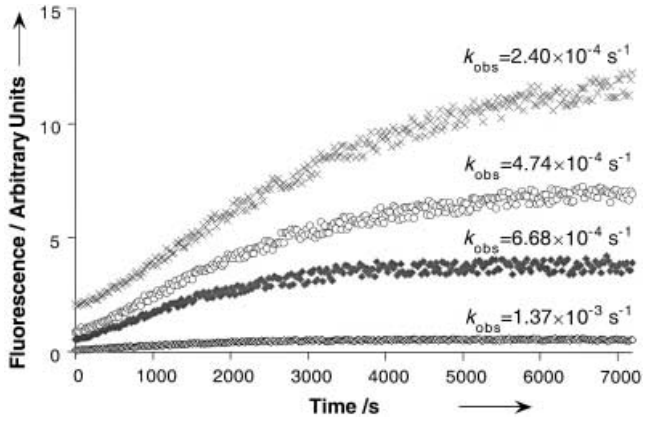

Figure 3. *Inhibition progress curves at substrate concentrations of 4.75 μM (◇), 19 μM (K_m, ◆), 38 μM (○), and 76 μM (x). Inhibitor concentration was fixed ([Cu^{2+}] = [TERPY] = 270 μM). Conditions were as stated in the legend of Figure 2, but [Kex2] = 0.06 nM (0.03 nM at [substrate] = 4.75 μM) to avoid substrate depletion.*

PyPP also enhanced the inhibition of Kex2 by copper, but less effectively than TERPY (IC_{50} of 75 μM). PATH gave similar results to TERPY, but it did not render inhibition irreversible, which suggests a different inactivation mechanism is occurring.

Other copper-binding tridentates, such as 1,4,7-triaazacyclononane ([9]aneN$_3$, IC_{50} of 125 μM), 1,4,7-trithiacyclononane (155 μM), and di[2-picolyl]amine (DPA, 165 μM, Figure 1), attenuated the inhibition. This may be due either to the formation of [Cu(κ^3-chelate)$_2$],[15] which effectively removes copper from solution (coordinatively saturated Cu^{2+} can not bind to the target) or to an interaction with Kex2 that is less favorable than that of solvated copper. The latter effect is evident in comparing [9]aneN$_3$ (IC_{50} of 125 μM) to its bulkier 1,4,7-trimethyl derivative (Me$_3$-[9]aneN$_3$, 135 μM). Upon replacement of the chloride ions in the buffer by weakly coordinating sulfate ions, chloro-bridged compounds do not hinder [Cu(κ^3-chelate)$_2$] formation, thereby rendering [9]aneN$_3$ less active than its derivative (IC_{50} of 210

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versus 115 μM for Me_3-[9]aneN_3) because it can form the bis-chelate product[15c] much more readily.[16]

Metal binding to protein is the most likely explanation for the results, because free TERPY and coordinatively saturated [Cu(TERPY)$_2$](ClO$_4$)$_2$[15a] were inactive. The maximized effect at a copper to TERPY ratio of 1:1 and the reduced inhibitory effect in the absence of chloride ions (Figure 1) imply that the active blocker is [Cu(TERPY)Cl]$^+$,[15a] which coordinates free and *N*-acetylated amino acids readily.[17, 18] Accordingly, the IC_{50} values of both [Cu(TERPY)Cl](ClO$_4$)[15a] and [Cu(TERPY)Cl$_2$][15a] were similar to that of the inhibitor formed in situ (16 versus 20 μM). The difference is explained by slow inhibitor formation. Pre-incubation (40 minutes) of Cu^{2+} and TERPY (400 μM) prior to substrate (19 μM) and enzyme addition increased k_{obs} by 15%. This also suggests formation of the active inhibitor prior to interaction with the enzyme. In the absence of chloride, [Cu(TERPY)(solvent)]$^{2+}$ (solvent = H_2O[15a] or Bis-Tris) is the likely inhibitor. As Kex2 inhibition is competitive with substrate (Figure 3), the inhibitor probably binds at the active site, of which catalytic His213 seems to be the best copper-coordinating residue. A study of Hg^{2+} inhibition of proteinase K (a homologue of Kex2) implies that nearby, buried Cys217 may also participate in copper coordination after modification of the pocket.[19] Cationic [Cu(TERPY)Cl]$^+$ and [Cu(TERPY)(solvent)]$^{2+}$ should be favored to reach the active site, which is designed for polybasic substrates.[10, 11]

Our results suggest that metal complexes could be used in vivo to block enzymes that are inhibited by metal ions in vitro. But chelates showing the desired effect must be modified for specificity and higher target affinity. The combinatorial synthesis approach is modestly demonstrated by the TERPY derivative 4′-(4-methoxyphenyl)-2,2′:6′,2″-terpyridine (MPT), with an IC_{50} value (10 μM) that is half of that of TERPY (Figure 1). Experiments to achieve specificity by tethering target-selective peptides[20] (or antibodies) are currently underway. Extracellular targets such as cell-surface furin (which processes toxin precursors) seem well suited for the outlined approach due to the presence of metal-exchanging carriers such as serum albumin.[21]

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On the Origin of Deoxypentoses: Evidence to Support a Glucose Progenitor in the Biosynthesis of Calicheamicin

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carbohydrates · deoxysugars · enzymes · natural products · metabolism

The deoxysugars have long been recognized as a unique and essential class of naturally occurring carbohydrate. These deoxygenated and often functionalized sugars present unusual

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