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# Catalytic metallodrugs*

James A. Cowan

*Evans Laboratory of Chemistry, Ohio State University, 100 West 18th Avenue, Columbus, OH 43210, USA*

*Abstract*: Drug discovery remains a top priority in medical science. The phenomenon of drug resistance has heightened the need for both new classes of pharmaceutical, as well as novel modes of action. A new paradigm for drug activity is presented, which includes both recognition and subsequent irreversible inactivation of therapeutic targets. Application to both RNA and enzyme therapeutic targets has been demonstrated, while incorporation of both binding and catalytic centers provides a double-filter mechanism for improved target selectivity and lower dosing. In contrast to RNA targets that are subject to strand scission chemistry mediated by ribose H-atom abstraction, proteins appear to be inactivated through oxidative damage to amino acid side chains around the enzyme active site. Methods to monitor both intracellular delivery and activity against RNA targets have been developed based on plasmid expression of the green fluorescent protein (GFP). Herein, the activity of representative metallodrugs is described in the context of both in vitro and cellular assays, and the mechanism of action is discussed. Studies with scavengers of reactive oxygen species (ROS) confirmed hydrogen peroxide to be an obligatory diffusible intermediate, prior to formation of a Cu-bound hydroxyl radical species generated from Fenton-type chemistry.

*Keywords*: HIV; RRE; ACE; metallodrug; catalysis.

## METALLODRUGS

Our laboratory has worked toward development of a new paradigm for therapeutic action–catalytic metallodrugs (Fig. 1). Such molecules include both a metal binding domain (to catalyze oxido-reductase chemistry on proteins and nucleic acids) and a target recognition domain (Fig. 1), and have the potential to act on therapeutic targets with regulatory or functional roles that are either essential for

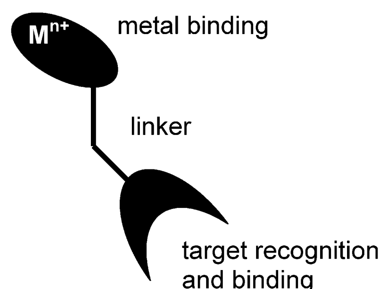


**Fig. 1** Metallodrug design. The metal binding and targeting domains may be separate (as shown), or part of the same molecular framework. The linker provides flexibility to adopt the most catalytically active conformation.

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pathogen or viral survival, or inactivation of disease-related enzymes. This paper will review both the conceptual framework underlying the approach and summarize recent progress.

A central premise underlying the design of metal complexes to mediate chemistry on proteins or nucleic acids is that the juxtaposition of a target recognition domain with a catalytic degradative moiety will result in a molecule with properties that are superior to the sum of the individual component parts; providing a novel design platform for drug development. In our studies, the N-terminal ATCUN (amino terminal Cu and Ni binding) motif was selected for metal binding. This sequence occurs naturally in certain species of albumins [1], and its physiological function is to bind and transport divalent Cu and certain other transition metals in the blood. This also suggests the metal-bound ATCUN motif to have very little cytotoxicity. ATCUN peptides have been extensively characterized and bind Cu and Ni with very high affinity ($K_D \sim 1.18 \times 10^{-16}$ M), a prerequisite for cellular use. NMR, electron paramagnetic resonance (EPR), visible spectroscopy, and X-ray crystallography are consistent with divalent Cu ion coordinated in a square planar configuration [1–4], leaving two faces accessible for binding to a target. Moreover, the ligand stabilizes the $M^{3+/2+}$ redox states and does not permit formation of the labile $Cu^+$ state. The GGH sequence shown in Fig. 2 is representative of the natural ATCUN motif, however, any peptide that contains a His residue at the third position will demonstrate binding of Cu and Ni. Other transition metals appear to have a lower affinity for the ATCUN sequence.

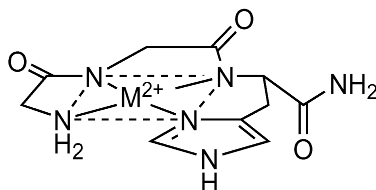


**Fig. 2** Structure of [GGH-$M^{2+}$] motif. The carboxyl terminus is shown in an amidated state, but may also be used as a free carboxylate.

## DESIGN AND FUNCTION OF CATALYTIC METALLODRUGS

Blocking the functional activity of a therapeutic target by classical competitive inhibition is well documented. Here, we are concerned with a distinct and novel strategy for mediating irreversible inactivation of target RNAs or proteins [5,6]. The potential for multiple turnover clearly differentiates this approach from the use of suicide inhibitors that has previously been documented in studies of enzyme catalysis. Moreover, target recognition is based on tertiary rather than primary structure, and so the approach stands in contrast to molecular mimics of RNA interference therapy. Since proteins and many RNAs possess tertiary structure that is required for activity and/or function, these are the two families of macromolecules that have formed the focus of our studies.

While catalytic metallodrugs may retain classical inhibitory properties, they also show the potential for catalytic degradation of the drug target (Fig. 3). Irreversible destruction of target also affords the potential for substoichiometric administration of drug, with the promise of a significant lowering of dosage and a commensurate decrease or elimination of side effects or toxicity. *This key point differentiates the activity of catalytic metallodrugs relative to the high affinity binding that is essential for the classical inhibitory mechanism of drugs currently on the market.* High affinity binding of the targeting domain may not be desirable from the viewpoint of facile release of the metallodrug following inactivation of the target. Examples that we have studied thus far have typically fallen in the low μM to nM range, though optimization of the binding affinity of the targeting domain is an issue that will require further exploration on a case-by-case basis.

A combination of reversible binding and irreversible chemistry yields a novel double-filter mechanism for target recognition that is illustrated in Fig. 3. If two proteins (or RNA motifs), A and B, are

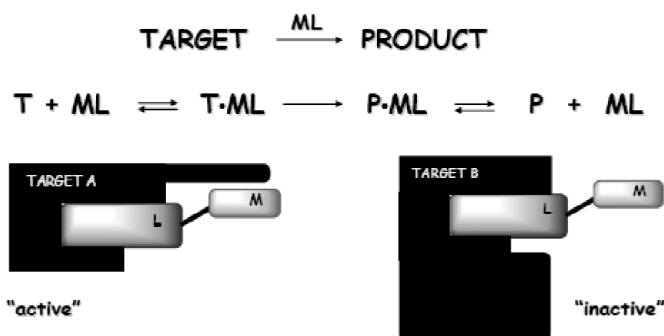


**Fig. 3** In a traditional approach to drug design, a molecule with high binding affinity for a select protein target acts by reversible inhibition of protein function. An excess of molecule is required to ensure saturation of target. The approach described herein employs a substoichiometric concentration of drug that executes catalytic irreversible inactivation of a select protein target. Selectivity is based both on target recognition *and* productive orientation of metallodrug to execute irreversible cleavage or damage of the target. Multiple turnover also distinguishes this approach from suicide inhibition.

recognized by the targeting domain of the drug, but only protein A has a suitable orientation for chemical inactivation by the catalytic metal domain, then only protein A would be irreversibly inactivated by the metallodrug (Fig. 3). The use of a substoichiometric concentration of drug ensures that the majority of protein B is not influenced by the metallodrug. Of considerable practical significance is our observation that such metal-binding peptides can be delivered in a metal-free state with subsequent recruitment of intracellular metal cofactors ([7] and later in Fig. 7), avoiding toxicity and regulatory problems that might stem from delivery of exogenous metal cofactors.

## DEVELOPMENT OF BIOMIMETIC NUCLEASES AND PROTEASES

In recent years, there has been substantial interest in the development of biomimetic nucleases, especially for DNA cleavage [8–20]. The primary motivation for such efforts reflects the desire to develop anticancer or footprinting agents, with the former most likely influenced by bleomycin, an anticancer drug that suffers from significant toxicity levels. There have been fewer efforts to mimic RNA nucleases [5,21–25], although RNA is intrinsically more sensitive to hydrolysis and is more stable to oxidative damage at the ribose ring as a consequence of the presence of the 2'-OH [26].

While progress has been made, many catalyst systems exhibit poor catalytic performance. A key design problem that we had identified several years ago stems from the absence of significant binding affinity of the catalyst for the nucleic acid substrate [14,27,28]. A significant improvement in catalyst efficiency is demonstrated when the catalytic metal center was coupled to a targeting site with at least modest affinity for the reactant [14,22,23,29,30]. These studies also demonstrated the positioning of the metal-associated reactive oxygen species (ROS), relative to the scissile bond on the ribose ring, to be the most important factor regulating oxidative cleavage activity [5,22,23].

With the exception of developments in ribozyme chemistry [31–37], catalysts for RNA cleavage are relatively rare [5,21,22,38–40]. Even more scarce are effective small-molecule mimics of protease enzymes, or catalysts that mediate chemistry on protein amino acid side chains [16,41,42]. Apparently, the barriers to achieving such reactivity are significantly greater than are found in mediating chemistry on nucleic acids. Nevertheless, the hypothesis that improving the affinity of the catalytic agent for a protein (or RNA) target should result in more effective chemistry has been borne out by recent studies in our laboratory [5,6,43]. We sought to design new families of metallotherapeutics, or metallodrugs (Fig. 4) with novel functional mechanisms, and direct these catalysts to the inactivation of therapeutically relevant RNA targets.

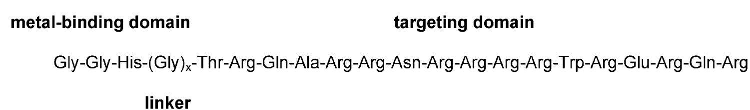


**Fig. 4** Peptide design showing metal binding, linker, and RRE RNA targeting sequences [5]. Peptides studied include Rev1 ($x = 0$), Rev2 ($x = 1$), Rev3 ($x = 2$), Rev4 ($x = 4$), and Rev5 ($x = 6$). A control Rev peptide corresponds to the targeting domain alone.

## HIV RRE RNA AS A TARGET OF METALLODRUG DEGRADATION

Traditional drug development has focused primarily on protein targets, reflecting the diverse structural and catalytic roles of cellular proteins that provide targets in metabolic and infectious disease. The complexity of protein structure also affords an opportunity for selective recognition by organic molecules, using spatial and bonding constraints to map drugs to protein targets. Likewise, many retroviruses and pathogens contain important RNA sequences that can both adopt complex tertiary structural motifs and are amenable to selective binding by drug molecules. Most important, there is no cellular repair mechanism for RNA, and so compounds that are capable of specific or selective binding to RNA should be considered in developing effective chemotherapeutic treatments

Productive HIV infection is dependent on the interaction of a regulatory protein, Rev, with a specific RNA structure known as the Rev-responsive element or RRE. The RRE is a 234-nucleotide RNA sequence embedded within the viral *env-coding* region. The high affinity Rev binding site, or core element, within the RRE consists of a stem-bulge-stem structure (Fig. 6, later). Cedergren and coworkers [44] have demonstrated in a study of the occurrence of specific viral (and other) RNA motifs in native host cells that critical viral sequences such as the core of the HIV RRE are under-represented in natural genomic sequences. Such sequences are suggested to have a detrimental influence on cell growth or viability, and so there is a minimal possibility of interference from natural sequences of similar structure. Since the RRE-Rev interaction is essential for viral replication, it is an attractive target for antiviral therapy against which there is no current commercial drug

## HIV RRE RNA TARGETS ARE CATALYTICALLY CLEAVED BY METALLODRUGS

Our concept of a catalytic metallodrug, capable of irreversible, multiturnover degradation of a therapeutic target has been tested against HIV RRE RNA, both in vitro [5,22] and in cellular assays [45] (Figs. 5–7). Figure 4 illustrates a family of N-terminal metal binding ATCUN motifs [3] that are extended to include the RRE RNA recognition peptide through a variable-length glycine linker sequence ($G_x$, $x = 0, 1, 2, 4, 6$). The binding affinity of both the Rev1 and Cu-Rev1 peptides to the fluorophor-labeled RRE RNA determined to be ~30 and 35 nM, respectively. These numbers are consistent with published data for binding of the native Rev peptide lacking the ATCUN motif [46].

RRE RNA cleavage by a Cu-Rev1 peptide complex (Fig. 5) was studied in vitro using 5' fluorescein end-labeled RNA in the presence and absence of mild reducing agents such as ascorbate, under physiological conditions, and the products of cleavage were separated on denaturing polyacrylamide gels and quantified by imaging methods. Cleavage sites were assigned by mass spectrometric studies, and the size of the product species identified by mass spectrometry matched the product sizes judged by polyacrylamide gel electrophoresis (PAGE) [5]. Combining both the PAGE and mass spectrometry data, RRE cleavage by the Cu-Rev1 peptide complex is not random, but rather, three specific cleavage sites were observed within the likely binding pocket for the metallopeptide complex (Fig. 5). The accurate masses obtained for the cleavage products are different from the calculated mass values expected for products from hydrolysis, but are consistent with those expected following strand scission by a C-1'H or C-4'H oxidative cleavage path in the presence of ascorbate [5]. No reaction was promoted by the metal-free Rev1 peptide, or by the $[GGH\text{-}Cu]^+$ or $[KGHK\text{-}Cu]^+$ ATCUN peptides. In the case of

free $Cu^{2+}$ (aq), RNA products were smeared on the gel as a result of random cleavage, and so no specific product was observed. The distinct cleavage behavior and reaction pathways for $Cu^{2+}$-ATCUN complex and $Cu^{2+}$(aq) have already been discussed in our previous work [29].

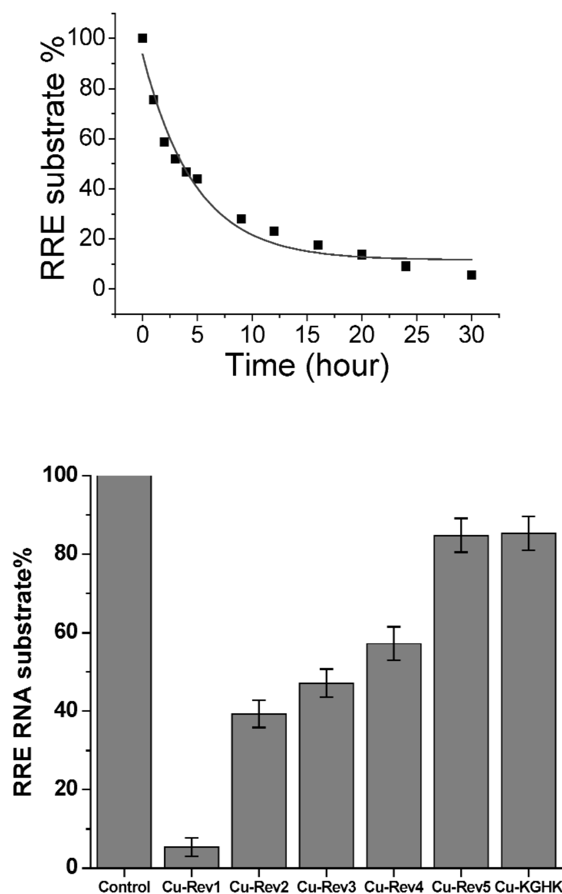


**Fig. 5** (left) Evaluation of observed rate constants for oxidative RRE RNA cleavage, monitored by following the loss of RRE RNA substrate by gel electrophoresis [5]. [RRE] = 5 μM and a [Cu-Rev1 complex]:[RRE] ratio of 1:1, $k_{obs}$ ~ 0.21 $h^{-1}$ (or $k_2$ ~ 700 $M^{-1}$ $min^{-1}$). (right) Relative cleavage efficiency for Cu derivatives of the family of peptides illustrated in Fig. 4 [7].

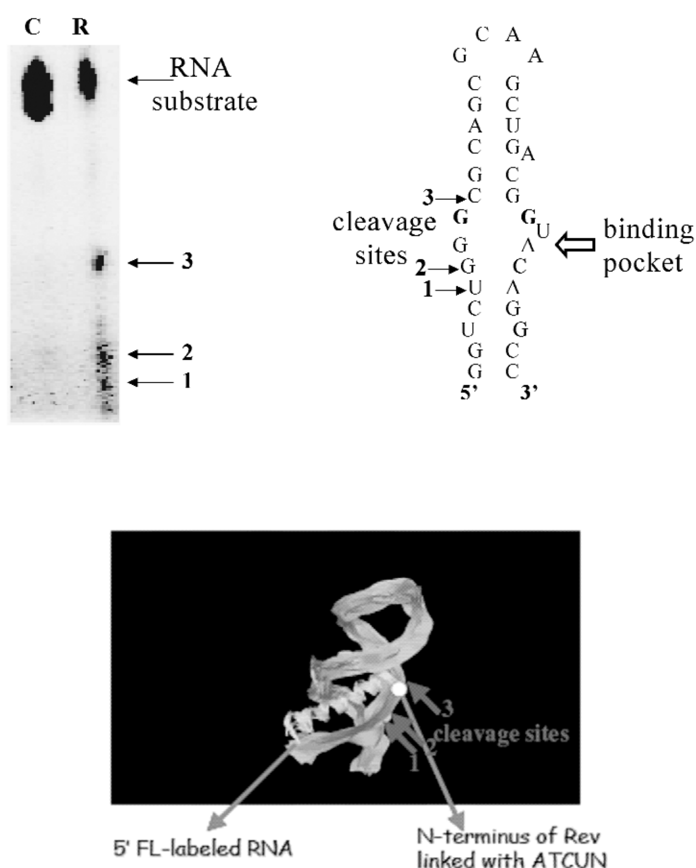


**Fig. 6** (top left) Cleavage reactions were analyzed by 8 M urea denaturing 20 % PAGE: where lane C shows results from a control reaction with 10 μM [RRE] and 100 μM ascorbate; and lane R shows results from a cleavage reaction with 10 μM [RRE], 10 μM [Cu-Rev1 complex], and 100 μM ascorbate in 20 mM HEPES with 100 mM NaCl at 37 °C for 3 h. (top right) A schematic illustration of the stem loop structure adopted by RRE RNA IIB [61], showing the cleavage sites identified by mass spectrometry studies. (bottom) Based on the solution structure [61], the position of the cleavage sites are as expected. Adapted from [5].

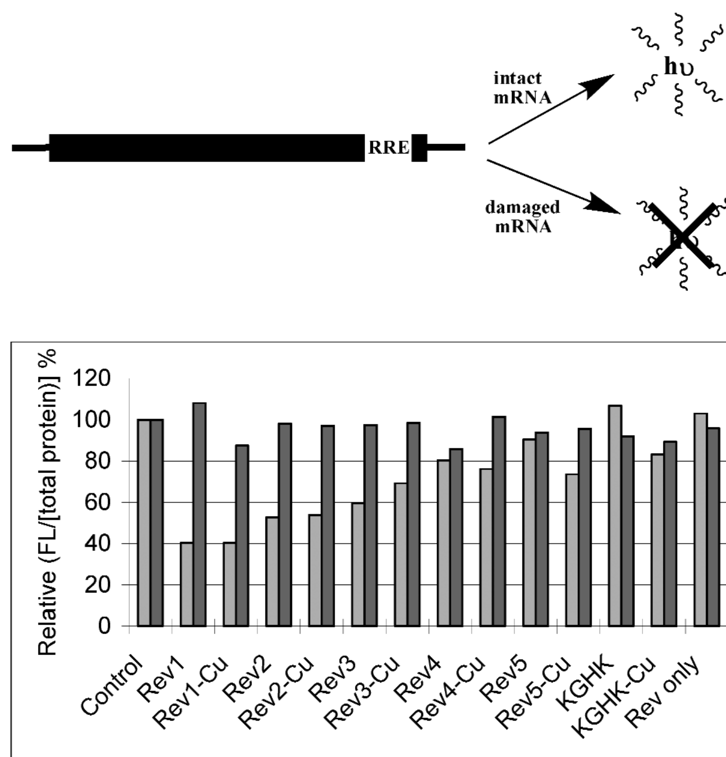


**Fig. 7** (top) A complementary DNA sequence for a portion of RRE RNA was introduced into an expression vector for the GFP [45]. The presence of the RRE insert at the C-terminus did not perturb the fluorescence response from the protein. Transcribed mRNA was assessed from the translated protein by fluorescence methods. Intact mRNA is translated to GFP with retention of fluorescence, while damaged mRNA is cleaved before the stop signal and translation of the polypeptide chain is aborted. (bottom). Influence of peptides and Cu derivatives on GFP expression in *E. coli.* Data was obtained from treatment with the relevant peptide or metallopeptide in a 1 h pre-incubation (prior to induction of GFP expression by addition of 0.5 mM IPTG) and subsequent hourly addition (up to 4 h) of aliquots (10 µM final concentration) of peptide. Light gray represents cells with plasmid encoding RRE-tagged GFP, and dark gray represents the background control experiment for nonspecific cleavage, using plasmid-encoding GFP (lacking the RRE insert) as a cytotoxicity and selectivity test. Each column is the average of at least three independent experiments. Adapted from [7].

## CELLULAR ASSAYS OF RNA CLEAVAGE

Relative to other catalysts that execute DNA and RNA cleavage chemistry [10,47–50], the design of novel inorganic drug molecules also require consideration of cellular delivery and efficacy. Prior studies have demonstrated the potential for cellular destruction of RNA targets using metalloaminoglycosides [45], using a plasmid encoding a target RNA sequence fused to the C-terminus of the green fluorescent protein (GFP). After transforming into a host cell, the relative concentration of expressed RNA is assessed by monitoring the fluorescence from GFP. These metallopeptides were designed to explicitly address the need for cellular and nuclear uptake, possessing both an arginine-rich motif (ARM) that promotes cellular uptake, and a nuclear import sequence. Figure 7 shows the results obtained by targeting the HIV RRE RNA target sequence in *E. coli* cells. Similar results were obtained in human Jurkat cells [7], and establishes the fact that the metallopeptides can effectively target cognate RNAs in cellular assays.

The activities of the metallopeptides illustrated in Fig. 4 have been screened both in vitro and in cellular assays, and consistent results were obtained with each method. Optimal activity was observed with the Rev1 metallopeptide complex. Increasing the catalyst concentration resulted in lower levels of fluorescence, reflecting more RNA cleavage. Extensive controls with ATCUN and recognition domain peptides alone were satisfactorily performed. Significantly, the cellular activity of each was similar, irrespective of whether the peptide was delivered in a metal-bound or -free state. Consequently, these peptides appear able to recruit intracellular metal cofactors, and so future use of such a class of compounds need consider delivery of the metal-free molecule avoiding potential regulatory issues that might stem from delivery of exogenous metal cofactors.

## ZINC-METALLOPROTEASES AS DRUG TARGETS IN CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) impacts over 64 400 000 Americans according to estimates made in 2001 [51], and statistics indicate a growing number of the population to be suffering from CVD. The family of zinc metallopeptidases involved in cardiovascular regulation includes angiotensin converting enzyme (ACE), endothelin converting enzyme (ECE-1), and neprilysin (NEP). ACE and ECE-1 are involved in the generation of the strong vasoconstrictors angiotensin II (Ang II) and endothelin I (ET-I) that are derived from the inactive precursors angiotensin I (Ang I) and endothelin, respectively [52], while ET-I is degraded by NEP. There is substantial interest in evolving compounds with synergistic therapeutic profiles in comparison to individual selective inhibitors [52]. Since NEP achieves the degradation of ET-I, inhibition of the former results in increased plasma levels of ET-I, thereby limiting the application of the dual NEP/ACE inhibition approach. This problem could be overcome by inhibiting ECE-1, and a common zinc metalloprotease inhibitor for ACE, NEP, and ECE-1 would be an ideal drug candidate.

Typically, cardiovascular drugs are based on inhibition of ACE, ECE, or NEP, and are blockers of receptors that depend on the product formed by these proteases (such as AT2 receptor blockers). In addition to these, a small number of drugs that act by a dual mechanism (such as the calcium channel $\beta_1$ adrenergic blockers 1, calcium channel $\alpha_1$ adrenergic blockers, 5HT 2A antagonists, endothelin and angiotensin receptor blockers, ECE and ACE inhibitors, and NEP and ECE inhibitors) increasingly present an alternative therapeutic approach [52].

## CATALYTIC INACTIVATION OF ACE BY Cu-ATCUN COMPLEXES

Inhibition of enzyme catalysis by classical competitive inhibition is well documented. Building on a strategy of developing more effective catalysts by incorporating a targeting motif in the metallodrug (Fig. 8), a similar improvement in activity has been demonstrated for molecules designed to mediate chemistry on proteins by promoting binding of the catalyst to the protein target. In particular, irreversible inactivation of human somatic angiotensin converting enzyme (hACE) by Cu-peptide complexes has been demonstrated [6]. This strategy is further supported by literature precedent for modification of peptide backbone residues by metal-promoted redox activity [53,54]. The targeting domain selectively binds to the enzyme active site of interest, while the metal center serves as the catalytic center to inactivate the target by performing redox chemistry and residue modification.

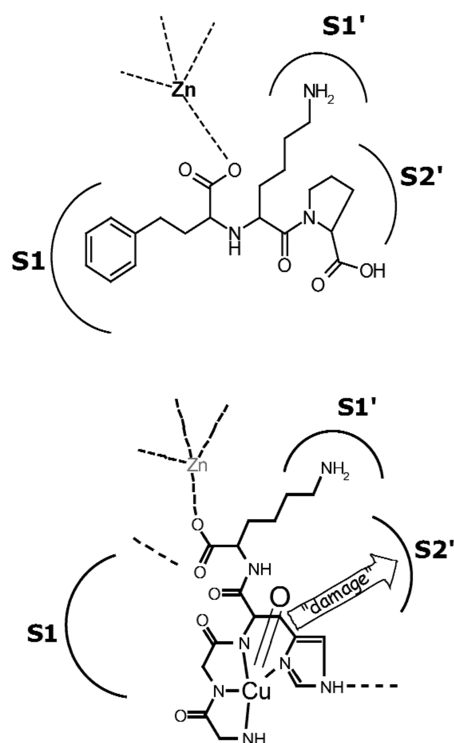


**Fig. 8** (left) ACE active site showing the association of lisinopril (inhibitor) with enzyme subsites [62]. (right) A schematic illustration of the proposed mechanism of action. A bound metallopeptide activates dioxygen, and the resulting ROS mediates irreversible damage to the target enzyme

An early lead compound, the Cu-peptide complex $[KGHK\text{-}Cu]^+$, displayed inhibitory (μM) behavior against ACE (Fig. 9). It is important to note that a high binding affinity is not a prerequisite for our drug design strategy (see section "Design and function of catalytic metallodrugs"). Figure 9 illustrates the inactivation of ACE by Cu-peptide complexes in the presence of ascorbate as an electron source. Under mildly reducing conditions, using a physiologically relevant reductant such as ascorbate with dioxygen (oxidative conditions), $[KGHK\text{-}Cu]^+$ was found to inhibit ACE activity several fold more effectively than observed under hydrolytic conditions (Fig. 9) [6,43]. $[KGHK\text{-}Cu]^+$-mediated ACE inactivation was carried out under oxidative conditions and furnished $k_{obs}$~ $2.86 \times 10^{-2}$ $min^{-1}$, for deactivation of ACE at a catalyst concentration corresponding to $K_I$. While $Cu^{2+}(aq)$ displays high levels of activity, control studies demonstrate Cu to remain bound to the ATCUN ligand [1,2], and so the activity displayed by Cu-ATCUN complexes is not a reflection of free metal ion. Moreover, physiological free Cu is strictly controlled. Accordingly, Cu-peptide motifs with tailored amino acid sequences can act not only as inhibitors of ACE, but also show excellent potential for catalytic inactivation of the enzyme by redox chemistry.

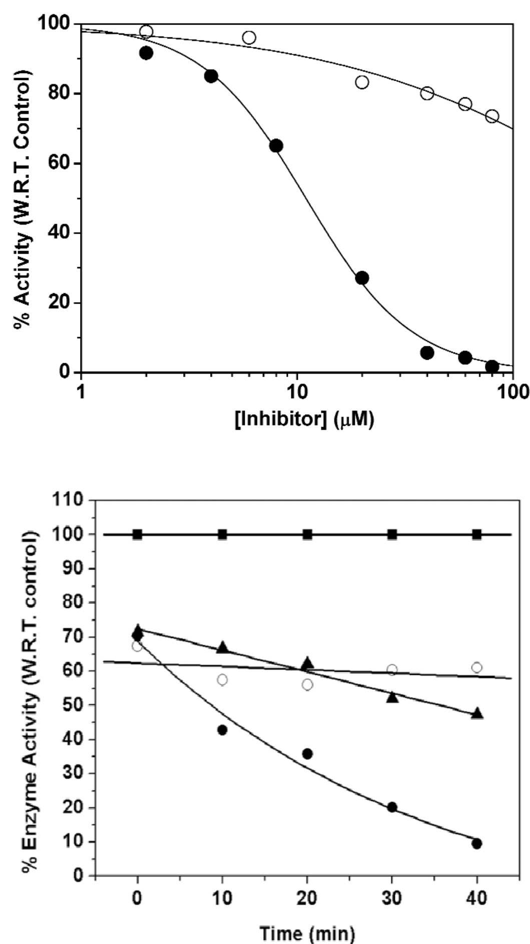


**Fig. 9** (left) Dose-dependent inhibition of ACE by $[KGHK\text{-}Cu]^+$ (●) and $[GGH\text{-}Cu]^-$ (—) under hydrolytic conditions. (right) Enzyme activity was determined at the given time intervals by taking aliquots of enzyme and determining the activity under initial velocity conditions with 10 μM substrate and 10 μM $ZnCl_2$ in 50 mM HEPES (pH 7.4) containing 300 mM NaCl, and 1 nM ACE. Conditions included (■) no inhibitor, 10 μM ascorbate; (●) with $[KGHK\text{-}Cu]^+$ (4.4 μM), 10 μM ascorbate; (○) with $[KGHK\text{-}Cu]^+$ (4.4 μM), no ascorbate; and (▲) with $Cu^{2+}$(aq) (4 μM), 10 μM ascorbate. The scatter reflects the error in the measurements. Fluorogenic substrate Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH is used in all cases to determine enzyme activity. Adapted from [6,43].

## CATALYTIC INACTIVATION OF ECE-1 BY $[(KGHK)\text{-}Cu]^+$: POTENTIAL DUAL ACTION DRUGS

Given the preliminary success of $[KGHK\text{-}Cu]^+$ against ACE, this complex has also been screened against ECE-1 to evaluate its potential as a dual inhibitor of ACE and ECE-1 [6,43]. The inhibition profile for $[KGHK\text{-}Cu]^+$ against human ECE-1 was investigated by use of an internally quenched fluorogenic substrate peptide (Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH) and a $K_I \sim 2.1$ μM was determined. Employing $[KGHK\text{-}Cu]^+ = K_I = 2.1$ μM, a time-dependent inactivation of hECE-1 was observed in the presence of ascorbate, as described previously for human ACE, with a catalytic rate, $k_{obs} \sim 1.2 \times 10^{-2}$ min$^{-1}$, observed for ECE-1 inactivation. That is, $[KGHK\text{-}Cu]^+$ shows promise as a possible dual inactivator of ACE and ECE-1.

## FORMATION OF REACTIVE OXYGEN SPECIES

Formation of ROS presumably arises through Fenton-type chemistry that gives rise to an active $Cu^{3+}$=$O^{2-}$ oxone intermediate that mediates oxidative damage to target molecules. Formation of diffusible hydroxyl radicals has been observed by use of the common rhodamine B assay, and prior studies of RNA and DNA cleavage revealed a significant reduction in diffusible hydroxyl radical concentration when substoichiometric catalyst was used to mediate damage to a target protein or nucleic acid [55]. This is consistent with formation and immediate reaction of a metal-associated ROS.

While reaction via a $Cu^{2+/+}$ couple would be expected from prototypical Cu redox chemistry, the family of ATCUN motifs is known to stabilize $Cu^{3+}$ [56–59], and is electrochemically well defined. In fact, all of the Cu-ATCUN complexes that we have studied display only the $Cu^{3+/2+}$ redox couple. The resulting $Cu^{3+}$ species is prone to C-terminal decarboxylation for tripeptides, or dehydrogenation in the case of amidated or longer peptides, although the $E_{1/2}$ for the resulting product is similar to that of the starting peptide [60], but this decomposition pathway appears to be avoided if the catalyst is actively turning over.

## CONCLUDING SUMMARY

In conclusion, it is clear that metallotherapeutics not only afford novel scaffolds for the design of new drug families, providing additional binding capabilities through coordination to the metal center, but also provide for irreversible catalytic degradation of the therapeutic target by use of the intrinsic reactivity of the metal center. The latter fact offers a new paradigm for exploration of drug design and mode of action, the ramifications of which will require further study and thought to evaluate the pros and cons of such an approach.

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