Inactivation of human angiotensin converting enzyme by copper peptide complexes containing ATCUN motifs†

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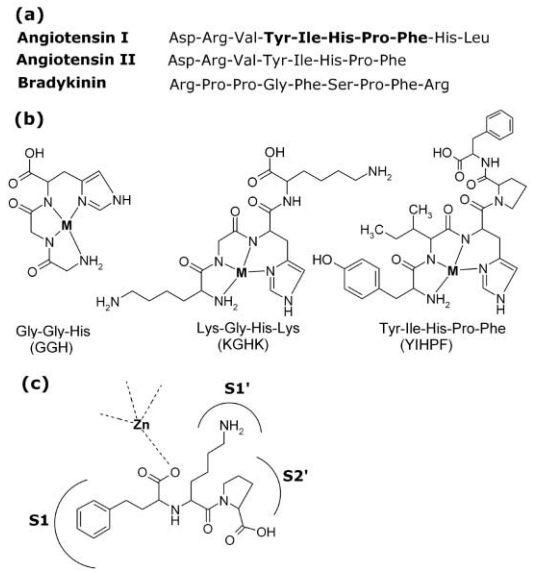
The copper complex [KGHK-Cu]$^+$ demonstrates catalytic inactivation of human angiotensin converting enzyme at sub-saturating concentrations, under oxidative conditions, with an observed rate constant $k \sim 2.9 \pm 0.5 \times 10^{-2}$ min^{-1}.

Angiotensin converting enzyme (ACE, peptidyl dipeptidase A; EC 3.4.15.1) is a zinc-containing metalloenzyme associated with the plasma membrane of epithelial and endothelial cells.[1] It is exposed at the extracellular surface and functions as a dicarboxypeptidase, converting the natural peptide substrate angiotensin I (Ang I) to angiotensin II (Ang II), (Scheme 1a) a potent vasopressor.[2] It also converts the vasodilator peptide bradykinin to an inactive form, thus playing an important role in blood pressure regulation. ACE inhibitors and angiotensin II receptor blockers are now in wide clinical use for the treatment of hypertension.



Scheme 1 (a) Peptides involved in the renin–angiotensin system (residues in bold are the ATCUN peptide fragments derived from Ang I and used in the present study). (b) Peptides used in the present study in their metal complexed forms (M = Cu^{2+}) (c) Crystallographically defined association of lisinopril (inhibitor) with one of the active sub-sites of ACE.[7] Ligand KGHK may mimic the lysine chain binding to pocket S1′.

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† Electronic supplementary information (ESI) available: enzyme optimum assay conditions and activity determinations, fixed-time inhibition profiles of metallo-peptides, dose-dependence response curves, and enzyme kinetic parameters. See DOI: 10.1039/b511081e

Lacking direct structural information on ACE active sites, the design principles for first generation ACE inhibitors were based on structure–activity studies of several snake venom peptides (BBPs, bradykinin potentiating peptides) that inhibited the metabolism of bradykinin and Ang I.[3] The enzyme possesses two homologous catalytically active sites with a conserved zinc-binding motif (HisGluXXHis);[4,5] although each of these sites demonstrates distinct substrate reactivities. The mode of action of first generation ACE inhibitors (captopril, enalapril and lisinopril) involved both tight binding to the catalytic active site,[6] and coordination to the catalytically important zinc ion as recently revealed through structural studies of the lisinopril-bound C-domain active site of ACE (Scheme 1c).[7] However, coordination of zinc by ACE inhibitors has been implicated with side effects on zinc metabolism, including mild zincuria, as well as a need for copper supplementation.[8] More recently, an ACE homologue ACE2 has been associated with the renin–angiotensin system and strikingly shown to be insensitive to all ACE inhibitors tested thus far.[9] Consequently there is a need for a new generation of ACE inhibitors, as well as inhibitors for the general class of zinc metallopeptidases involved in cardiovascular regulation.

Herein we describe an approach to drug design that is based not only on an inhibition strategy, but also seeks the sub-stoichiometric inactivation of a target enzyme. To this end we have investigated the use of metallopeptides as catalysts for irreversible inactivation of ACE activity. Specifically, we have evaluated several members of the ATCUN (amino terminal copper/nickel) family of peptides (Scheme 1b) that have previously been shown to mediate protein cleavage[10] and protein cross linking[11] in the presence of per-acids. Site-selective oxidative cleavage of DNA and RNA has also been demonstrated,[12] however, the potential for enzyme inactivation by metallopeptides is not well established.[13]

Our studies were carried out with the human somatic isoform of the enzyme (ACE) obtained from recombinant sources, corresponding to the ectodomain of somatic ACE,[4] and using fluorogenic peptide substrate Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH[14] (Mca is methyl coumarin, Dnp is dinitrophenyl). This assay system was found most suitable to elaborate the inhibitory effect of the Cu-ATCUN peptides by fluorescence spectrometry (λ_{emm} = 405 nm), relative to the conventional assay based on the detection of His-Leu upon reaction with *o*-phthalialdehyde (λ_{emm} = 500 nm).[15] The latter suffers complications from inner filter effects at higher concentrations of Cu-ATCUN peptides (λ_{max} = 525 nM).

Consistent with prior observations by Cushman and Cheung,[16] free metal ions such as Cu^{2+}(aq) and Ni^{2+}(aq) showed a dose-dependent inactivation of the enzyme following replacement

of the catalytically active native zinc cofactor [Cu^{2+}(aq), IC_{50} = 4 μM]. The potency of the peptides GlyGlyHis (GGH), LysGlyHisLys (KGHK), TyrIleHisProPhe (YIHPF), and their corresponding Cu^{2+} complexes[17] toward ACE inhibition was determined by establishing a dose-dependent inhibition curve under equilibrium conditions. The measured IC_{50} value for [KGHK-Cu]$^+$ was determined to be 11 μM[18] (Fig. 1) while all of the metal-free peptides and copper complexes [GGH-Cu]$^-$, [YIHPF-Cu]$^-$ showed $IC_{50} > 100$ μM. The weaker binding by the latter, may reflect the absence of important side-chain contacts with the enzyme pocket, including the charged lysine (Scheme 1).

A pre-incubation time of at least 45 min significantly enhanced the inhibitory effect of the metallopeptides, suggesting slow binding to the enzyme active site.[19] Under such pre-incubation conditions, and under hydrolytic conditions (no ascorbate present), the Cu^{2+}-peptide, [KGHK-Cu]$^+$ was found to be a competitive inhibitor of ACE ($K_I \sim 4.0 \pm 0.2$ μM) (Fig. 2).

A dramatic change in enzyme inactivation was observed, relative to the inhibitor metallopeptide, following a switch from hydrolytic to oxidative conditions under pre-incubation conditions. Employing the peptide complex [KGHK-Cu]$^+$ at sub-saturating concentrations ([KGHK-Cu]$^+ \leqslant K_I$) the inhibitory activity of [KGHK-Cu]$^+$ was enhanced several fold in the presence of a twofold excess of ascorbate and dioxygen, relative to inhibition under hydrolytic conditions (Fig. 3).

Two mechanisms were considered for this behavior. First, substitution of Zn^{2+} by Cu^{2+} in the enzyme active site following reductive release of copper ion from the peptide, with subsequent re-oxidation prior to binding to ACE. This mechanism was discounted after considering the distinct affinities of ACE ($\sim 10^{-6}$ M) and ATCUN peptides ($\sim 10^{-18}$ M) for Cu^{2+}. Furthermore, ~80% loss of total enzyme activity was obtained at 4 μM [KGHK-Cu]$^+$ concentration under oxidative conditions, whereas free Cu^{2+}(aq) under identical conditions (IC_{50} for Cu^{2+}(aq) ~ 4 μM) brought about only 50% loss of total enzyme activity (supplementary information†). Accordingly, an alternative mechanism of [KGHK-Cu]$^+$ inhibition under oxidative conditions was invoked through catalytic inactivation by reactive oxygen species following Fenton chemistry from either peptide-free Cu^+(aq) or [KGHK-Cu]$^+$.[20] The absence of any significant change

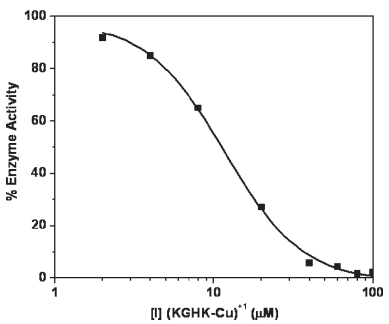

Fig. 1 Dose-dependent inhibition of ACE by [Cu(KGHK)]$^+$ under hydrolytic conditions. Reaction mixtures (100 μL) contained 50 mM HEPES buffer, 300 mM NaCl and 10 μM $ZnCl_2$ (pH 7.4), 10 μM substrate and 1 nM (13.1 ng) ACE. Concentrations of [Cu(KGHK)]$^+$ were varied from 0 to 100 μM. Prior to the start of the enzymatic reaction (by addition of substrate) the copper-peptide was pre-incubated with the enzyme for 45 min.

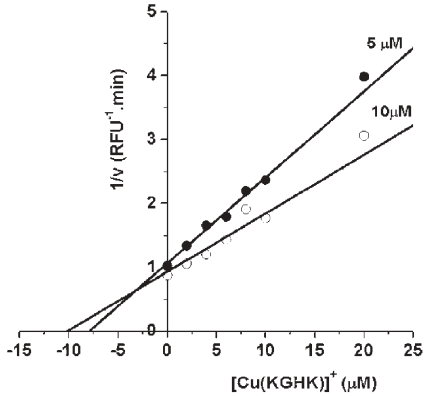

Fig. 2 Dixon plot showing competitive inhibition, where substrate (Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH) concentrations of 5 and 10 μM were used.

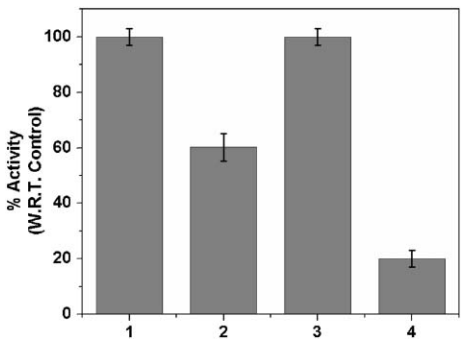

Fig. 3 A comparison of ACE inhibition activity for [KGHK-Cu]$^+$ (IC_{50} = 11 μM) evaluated under a variety of experimental conditions. The reaction solution contained 10 μM substrate (Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH), 10 μM $ZnCl_2$ in 50 mM HEPES (pH 7.4) buffer with 300 mM NaCl and 1 nM ACE. (**1**) Hydrolytic control with no inhibitor and no ascorbate. (**2**) 4 μM [KGHK-Cu]$^+$, no ascorbate. (**3**) Oxidative control with 10 μM ascorbate. (**4**) 4 μM [KGHK-Cu]$^+$ and 10 μM ascorbate. The enzyme was pre-incubated for 45 min with the metallopeptide. Relative activity was measured after 30 min using a fixed time assay under the conditions of initial velocity measurements.

in absorbance and the distinct inhibitory behavior of free copper ion *versus* the [KGHK-Cu]$^+$ complex under such solution conditions (Fig. 4) argues against any significant build-up of Cu^+(aq) species. Inasmuch as enzyme inactivation is not observed for the other copper-peptide complexes studied, a direct catalytic inhibitory effect by the action of reactive oxygen species formed by an ACE-bound [KGHK-Cu]$^+$ complex is the most plausible explanation.

The [KGHK-Cu]$^+$-mediated catalytic inactivation of ACE was followed by monitoring the progress curve for the inactivation of recombinant human ACE by [KGHK-Cu]$^+$ at a sub-saturating concentration (4.4 μM) of complex under both hydrolytic and oxidative conditions (Fig. 4 and supplementary material†). Under hydrolytic conditions, inhibition of ACE by [KGHK-Cu]$^+$ is proportional to the inhibitor concentration. However, in the presence of ascorbate (a twofold excess relative to that of inhibitor) enzyme inactivation was achieved to a significantly greater extent, relative to the inhibitor's initial concentration and that of free Cu^{2+}(aq), supporting catalytic multi-turnover inactivation.

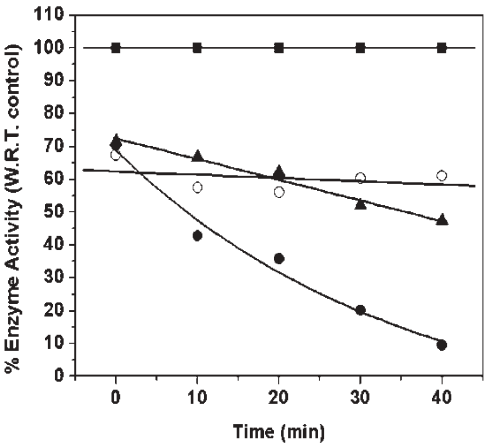

Fig. 4 Enzyme activity was determined at the given time intervals by taking aliquots of enzyme and determining the activity under initial velocity conditions with 10 μM substrate and 10 μM $ZnCl_2$ in 50 mM HEPES (pH 7.4) containing 300 mM NaCl, and 1 nM ACE. Conditions included (■) no inhibitor, 10 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.

Efficient multi-turnover inactivation of ACE at sub-saturating concentrations of $[KGHK\text{-}Cu]^+$ ($\sim K_I$) was achieved under oxidative conditions and kinetic parameters obtained by initial velocity measurements (Fig. 4) furnished a catalytic rate constant, k_{obs} $\sim 2.9 \pm 0.5 \times 10^{-2}$ min^{-1} for enzyme inactivation. Accordingly, this family of metallopeptides can act not only as classical competitive inhibitors of enzyme activity, but also show the potential for irreversible catalytic inactivation of enzyme function at sub-saturating concentrations.

The mechanism of inactivation remains unclear. Previously it has been suggested that $[KGHK\text{-}Cu]^+$ does not act as an artificial protease,[21] however other mechanisms of inactivation can be invoked, including modification of active site amino acid residues by reactive oxygen species.[22] For example the most susceptible amino acid residues to metal catalyzed oxidation include histidine, arginine, lysine, proline, methionine and cysteine.[13] Evaluation of possible pathways will be the subject of future investigations.

In conclusion, the results herein demonstrate progress toward the development of a novel class of drug molecule that exerts its action by a mechanism of catalytic inactivation that is, to the best of our knowledge, the first report of its kind. The present study summarizes our key findings in the development of an inhibitor of the angiotensin converting enzyme, although the strategy should be broadly applicable to other therapeutically-relevant proteases.‡

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Notes and references

‡ All experiments were performed in triplicate and the results show the mean values of each activity determination. **Enzyme:** A recombinant isoform of human angiotensin converting enzyme (rhACE) corresponding to the ectodomain somatic ACE was obtained from R&D systems Inc., supplied as a 0.2 μm filtered solution in 12.5 mM Tris, 75 mM NaCl, 0.5 μM $ZnCl_2$, pH 7.5 and 40% glycerol at a concentration of 0.434 mg mL^{-1}. Working stock solutions were made by diluting aliquots of the supplied stock into buffer (50 mM HEPES containing 300 mM NaCl and 10 μM $ZnCl_2$, pH 7.4). Fresh stocks were made prior to each experiment. **Peptides and metallopeptides**: Peptides containing histidine as a third residue (ATCUN motifs), such as glycylglycylhistine (GGH) (BACHEM Bioscience), lysylglycylhistidyllysine (KGHK) (BACHEM Bioscience), and tyrosylisoleusylhistidylprolylphenylalanine (YIHPF angiotensin II 4-8) (Phoenix Pharmaceutical Inc.) were chosen for the study. Peptide stock solutions were prepared in de-ionized water and complexes with Cu^{2+} were formed in 1 : 1.2 metal to peptide ratio in 50 mM HEPES buffer (pH 7.4) using $CuSO_4 \cdot 5H_2O$ as the source of the metal ion. The concentrations of the Cu^{2+} peptide complexes were determined spectrophotometrically, using $\varepsilon_{528\ nm} \sim 108$ M^{-1} cm^{-1} for $[GGH\text{-}Cu]^-$ and ~ 85 M^{-1} cm^{-1} for $[KGHK\text{-}Cu]^+$, respectively.[23,24] **Enzyme Assays**: Recombinant human ACE activity was assayed (final volume of 100 μL) at 37 °C in a buffer consisting of 50 mM HEPES containing 300 mM NaCl and 10 μM $ZnCl_2$ (pH 7.4) with 10 μM internally quenched fluorogenic peptide substrate, Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH (R&D Systems Inc.) and 1 nM (13.1 ng) rhACE. Reactions were run in the wells of polystyrene 96-well microplates (PS white, Porvair). The time-dependent increase in fluorescence following cleavage of the peptide substrate was monitored (relative fluorescence units min^{-1}, RFU min^{-1}). Fluorescence change was typically measured for 30 min with a Perkin Elmer Luminescence Spectrophotometer LS50B equipped with a fluorescence microplate reader (Perkin Elmer) at 37 °C using PE applications FL winlab software with excitation and emission wavelengths set at 320 and 405 nm, respectively. Optimum substrate concentration was identified by incubating the enzyme with 5 to 50 μM substrate.

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