## Inhibition of matrix metalloproteinase-9 by "multi-prong" surface binding groups<sup>†</sup>

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A novel strategy of blocking the active site accessibility of MMP-9 by "multi-prong" surface binding groups is described.

One of the ultimate goals of biomedical research is to design/ discover enzyme inhibitors (against pathogenic enzymes) as potential drugs for the treatment of various human diseases. Despite recent advances in the areas of genomics and proteomics, the basic approaches in drug discovery have remained more or less the same.<sup>1</sup> The prevailing idea has been to synthesize small molecular weight compounds (either *via* "rationale" or "combinatorial" methods), which could snugly "fit" into the active site pockets of putative pathogenic enzymes, precluding the accessibility of their cognate substrates.<sup>1,2</sup> However, due to marked similarity in the active site pockets of functionally similar enzymes (particularly "isozymes"), there have been major challenges in designing the isozyme selective inhibitors, which could only inhibit the pathogenic isozymes, but *not* their physiologically desirable counterparts.<sup>3</sup>

Instead of exclusively focusing on the confines of the enzyme's active site pockets, there have been efforts in designing enzyme inhibitors which interact at the enzyme's surface exposed residues as well.<sup>4</sup> We recently demonstrated that a weak inhibitor of carbonic anhydrase (*viz.*, benzenesulfonamide) can be converted into a strong inhibitor by attaching an iminodiacetate IDA-Cu<sup>2+</sup> group *via* a suitable spacer.<sup>5</sup> In such a "two-prong" inhibitor, whereas the benzenesulfonamide group binds at the active site pocket of the enzyme, the IDA-Cu<sup>2+</sup> moiety loops around and interacts with one of the surface exposed histidine residues. Since the latter are unlikely to be conserved during the course of evolution, our two-prong approach exhibits potential for designing highly potent and isozyme specific inhibitors of enzymes (particularly those harboring surface exposed histidine residues).<sup>5</sup>

Just how effective can targeting of the surface exposed histidine residues be in designing an isozyme specific inhibitor? In this pursuit, it was realized that matrix metalloproteinases (MMPs) are involved in the pathogenesis of various human diseases, such as cancer, arthritis, atherosclerosis, aneurism, periodontal disease, skin ulceration, gastric ulcer, liver fibrosis, *etc.*, and some of the isozymes (*e.g.*, MMP-9) harbor several patches of the surface exposed histidine residues.<sup>6</sup> Although both carbonic anhydrases (CAs) and matrix metalloproteinases (MMPs) are Zn<sup>2+</sup> containing enzymes, they exhibit different selectivities for "zinc binding

group" inhibitors. Whereas CAs are preferentially inhibited by the sulfonamide derivatives, MMPs are inhibited by hydroxamates.<sup>2</sup> The question arose can benzenesulfonamide (being a weak inhibitor for MMPs) be converted into a strong inhibitor for MMP-9 by attaching the IDA-Cu<sup>2+</sup> moieties as tether groups, such that the latter could interact with the surface exposed histidine residues of the enzyme. To probe this, we synthesized differently branched benzenesulfonamide derivatives (see ESI<sup>†</sup>), containing short chain spacers, which terminated into the IDA-Cu<sup>2+</sup> moieties corresponds to the conjugate number except for conjugate-**5**, which contains 4 IDA-Cu<sup>2+</sup> moieties but does not contain the benzenesulfonamide group.

Using the recombinant form of human MMP-9, we performed steady-state kinetic experiments<sup>7</sup> for the inhibition of the enzyme in the presence of the different conjugates shown in Fig. 1 (see ESI†). By measuring the initial rates of the enzyme catalyzed reaction as a function of increasing concentrations of the conjugates in Fig. 1, we determined their  $IC_{50}$  values (the



Fig. 1 Structures of differently branched IDA-Cu<sup>2+</sup> containing conjugates.

<sup>†</sup> Electronic supplementary information (ESI) available: synthetic details and inhibition studies. See http://www.rsc.org/suppdata/cc/b5/b501780g/ \*sanku.mallik@ndsu.edu (Sanku Mallik) dk.srivastava@ndsu.edu (D. K. Srivastava)

concentration of an inhibitor required to inhibit 50% of the enzyme activity), which are summarized in Table 1. An examination of the data in Table 1 revealed that the  $IC_{50}$  value of the inhibitors decreased by about 50 fold upon increase in the number of IDA-Cu<sup>2+</sup> moieties from one (conjugate-1) to four (conjugate-4). Since the difference in the  $IC_{50}$  value between conjugate-2 and conjugate-3 was about 2 fold, we regarded these inhibitors to be nearly equally potent.

Based on literature data, it is known that at neutral pH, IDA-Cu<sup>2+</sup> preferentially interacts with the imidazole group of the histidine residues.<sup>8</sup> Such an interaction is explicitly corroborated by a recent NMR spectroscopic study.<sup>8</sup> Hence, it appears likely that the inhibition of MMP-9 by the conjugates of Fig. 1 is due to the interaction of their IDA-Cu<sup>2+</sup> moieties with the enzyme's histidine residues. From the point of view of designing enzyme inhibitors, it is noteworthy that the inhibitory potencies of the above conjugates increase with an increase in the number of IDA-Cu<sup>2+</sup> moieties. Apparently, the origin of such a feature lies in the "multi-prong" attachment of the IDA-Cu<sup>2+</sup> moieties (of the conjugates) to the surface exposed histidine residues of the enzyme.

The question arose could we justify the multi-prong attachment of conjugate-4 with the surface exposed histidine residues of MMP-9 on the basis of the structural data of the protein. If so, would conjugate-4 be immune to inhibiting other MMPs, which do not possess similar (or highly abundant) patches of the surface histidine residues. Such a demonstration would attest to the selectivity of conjugate-4 for MMP-9. In this pursuit, we compared the X-ray crystallographic structures of known MMPs from the point of view of distribution of the surface exposed histidine residues, and observed that the latter was markedly different between MMP-9 and MMP-10 (Fig. 2).

Both these enzymes contain two Zn<sup>2+</sup> atoms (one catalytic and the other structural at nearly identical positions), and each of them are picket-fenced by 3 histidine residues. The major difference between these two enzymes is the number of surface histidine residues, which is more abundant in the case of MMP-9 vis à vis MMP-10. It is clearly apparent that the corresponding histidine residues (viz., H117, H118, H119, H441, and H432) of the MMP-9 structure are missing in MMP-10. Hence, the IDA-Cu<sup>2+</sup> moieties of conjugate-4 have a much higher propensity for binding to the surface (harboring the histidine residues) of MMP-9 than to that of MMP-10. Given this, we expected that conjugate-4 would serve as a more potent inhibitor for MMP-9 than for MMP-10. To probe this, we performed a comparative experiment for the MMP-9 and MMP-10 catalyzed reactions in the presence of increasing concentration of conjugate-4. As shown in Fig. 3, conjugate-4 exhibits a much pronounced inhibitory profile with MMP-9 as compared to that with MMP-10. The inhibition of conjugate-4 for the MMP-9 catalyzed reaction was judged to be competitive in

 Table 1
 IC<sub>50</sub> values for the inhibition of MMP-9

Inhibitor	IC <sub>50</sub> /μM
Conjugate-1 Conjugate-2 Conjugate-3 Conjugate-4 Conjugate-5 Conjugate-4 (without Cu <sup>2+</sup> ) Benzenesulfonamide	$\begin{array}{c} 49 \pm 7.3 \\ 3.8 \pm 0.44 \\ 9.2 \pm 1.3 \\ 1.1 \pm 0.011 \\ 3.1 \pm 0.40 \\ 79 \pm 16 \\ > 20\ 000 \end{array}$



Fig. 2 Surface topologies of MMP-9 (top) and MMP-10 (bottom) showing the surface exposed histidine residues. Created by the software GRASP. $^{10}$ 



Fig. 3 Inhibition of MMP-9 and MMP-10 by conjugate-4.

nature. The solid smooth line is the best fit to the data for a  $K_i$  value of 1.1  $\mu$ M. Although we could not reliably determine the  $K_i$  value of conjugate-4 for MMP-10, it appeared to be several orders of magnitude higher than that for MMP-9.

To determine the extent to which the benzenesulfonamide moiety of conjugate-4 contributes to the overall inhibitory profile of MMP-9, we synthesized conjugate-5 (Fig. 1). Note that the latter conjugate differs from conjugate-4 only by the absence of the



**Fig. 4** Cartoon showing the blocking of the active site pocket of MMP-9 by a multi-prong  $IDA-Cu^{2+}$  conjugate.

benzenesulfonamide moiety. The inhibition data (IC<sub>50</sub> values) reveal that conjugate-5 is only 3 fold less potent than conjugate-4 (Table 1). Hence, the benzenesulfonamide moiety of conjugate-4 makes a minuscule energetic contribution to the interaction with MMP-9. Clearly the binding affinity of the IDA-Cu<sup>2+</sup> containing a four-prong conjugate to MMP-9 is not significantly enhanced by the presence of the active site binding group, benzenesulfonamide. In other words, the multi-prong interactions of the IDA-Cu<sup>2+</sup> moieties of conjugate-4 or conjugate-5 with the surface exposed histidine residues of MMP-9 are adequate to preclude the accessibility of the substrate to the active site of the enzyme, leading to its inhibition. Since the histidine residue on the surface of MMP-10 is sparse, the inhibition of the enzyme by conjugate-4 is not so pronounced (as shown by the straight line of Fig. 3). We propose that the complementary interaction between the IDA-Cu<sup>2+</sup> moieties of multi-prong conjugates and the surface exposed histidine residues of MMP-9 form a "claw" like structure across the active site pocket of the enzyme, as schematized in Fig. 4.

To further probe the validity of the multi-prong attachment model of Fig. 4, we investigated the effectiveness of Cu<sup>2+</sup> devoid conjugate-4 on the MMP-9 catalyzed reaction. As shown in Table 1, in the absence of Cu<sup>2+</sup>, conjugate-4 functions as a fairly weak inhibitor with an IC<sub>50</sub> value of 79  $\pm$  16  $\mu$ M. However, we could not accurately determine the IC<sub>50</sub> value of "free" benzenesulfonamide as the latter did not show inhibition up to 10 mM concentration. At higher concentrations, the enzyme started precipitating due to the solubility problem. Hence, the  $IC_{50}$ value of benzenesulfonamide must be >20 mM (Table 1). Clearly, the  $Cu^{2+}$  of conjugate-4 (and possibly of the other conjugates shown in Fig. 1 as well) serves as the major determinant for inhibition of the MMP-9 catalyzed reaction. We further investigated the influence of imidazole on the inhibitory profile of MMP-9, and found that 2 mM imidazole could easily abolish the inhibitory effect of 5.5  $\mu$ M conjugate-4 (see ESI<sup>†</sup>). Clearly, imidazole competitively abolishes the interaction of IDA-Cu<sup>2+</sup> of conjugate-4 with the surface exposed histidine residues of the enzyme. We are currently in the process of building a computer graphic model of the physical interaction between conjugate-4 and the surface exposed histidine residues of MMP-9, and we will report these findings subsequently.

The experimental outcome of the present investigation provides a prototype of designed enzyme inhibitors, which blocks the accessibility of the enzyme's active sites without fitting into the

active site pockets. Such inhibitors are mechanistically similar to the binding of selective "antibodies" or charged "nanoparticles" at the active site entrance of the enzymes.9 Like our multi-prong conjugates, these "macromolecular" species interfere in the facile entrance and exit of substrates and reaction products, respectively, from the interacting enzyme sites, causing the inhibitory effects. We surmise that the inhibitory potencies of multi-prong conjugates can be significantly increased by incorporating reasonably high affinity (active site directed) ligands (e.g., hydroxamate derivative in the case of MMP-9) in the overall structure. The major advantage of such inhibitor designs would be the formulation of drugs, which would selectively inhibit the pathogenic isozymes (e.g., selected MMP isozymes) without affecting their physiologically desirable counterparts. The lack of such selectivity has been one of the major failures in developing MMP inhibitors as potential drugs for the treatment of various human diseases.3

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