COMMUNICATION

ChemComm
www.rsc.org/chemcomm

Conjugation of poor inhibitors with surface binding groups: a strategy to improve inhibition†

Bidhan C. Roy, Ryan Hegge, Theresa Rosendahl, Xiao Jia, Rachael Lareau, Sanku Mallik* and D. K. Srivastava

Department of Chemistry and Molecular Biology, North Dakota State University, Fargo, North Dakota, USA. E-mail: Sanku.Mallik@ndsu.nodak.edu; Fax: 701-231-8831; Tel: 701-231-8829

Received (in Corvallis, OR, USA) 8th May 2003, Accepted 25th July 2003
First published as an Advance Article on the web 4th August 2003

Conjugation of surface binding groups with inhibitors for carbonic anhydrase leads to the conversion of weak inhibitors to strong inhibitors.

Recognition of protein surfaces by synthetic molecules has been successfully used to disrupt protein–protein interactions,[1] alter electron transport properties,[2] inhibit receptor–ligand interactions,[3] *etc*. These ligands recognize their targets by several, simultaneous, weak interactions (*e.g.*, hydrogen bonding, ion pairing, *etc.*). Recently, we have demonstrated a protein surface recognition strategy employing strong and directional metal–ligand interactions[4] for the enzyme carbonic anhydrase (bovine erythrocyte).

Inhibition of carbonic anhydrase is important for the treatment of glaucoma and cancer.[5,6] Usually, the clinically-approved inhibitors are the sulfonamide class of compounds. Conjugation of the high-affinity sulfonamides with bile acids,[7] short peptides,[8] amino-polycarboxylate ligands and their metal complexes[9] further enhances inhibition efficiency.

Herein, we report a strategy to convert a poor inhibitor to a good inhibitor by attaching a surface-histidine recognition group to the inhibitor. Benzene sulfonamide, a rather weak inhibitor for carbonic anhydrase (K_d = 120 μM, Table 1), was converted to a very good inhibitor for the enzyme (K_d = 130 nM, Table 1) as a result of this conjugation.

To demonstrate the proof-of-concept, five Cu^{2+}-complexes (Fig. 1) were designed and synthesized. In these complexes, the benzene sulfonamide binds to the active-site Zn^{2+} ion of the enzyme. It was estimated by molecular modelling (BioMed CAChe 6.0, Fujitsu America, Beaverton, OR) that the Cu^{2+} ions of the complexes are then capable of binding to His-4 or His-17 on the surface of carbonic anhydrase (bovine erythrocyte, protein data bank file: 1g6v.pdb). The targeted histidine residues are close to the N-terminus of the enzyme. The protein backbone in this region is flexible and has a random coil structure, facilitating the binding of the cupric ions to the histidines when the benzene sulfonamide is bound to the Zn^{2+} ion in the active site.

There are literature reports of flexible peptides converted to rigid structures by coordination to transition metal ions (Cu^{2+}, Zn^{2+}).[10] These rigid peptides demonstrated enhanced biological properties (including improved inhibition of the enzyme α-amylase) compared to the flexible counterparts. However, in these reported examples, the enhancement of biological properties is due to the rigidity of the structures induced by the metal ions.

For the studies reported herein, the ligand iminodiacetic acid (IDA) was used to chelate the cupric ions (K = 10^{12} M^{-1}).[11] The length of the spacer separating the benzene sulfonamide group from IDA was varied in these complexes. Benzene sulfonamide (**6**) and the di-Cu^{2+} complex **7** (lacking the benzene sulfonamide moiety) were used as controls for these studies.

The syntheses of sulfonamide-based metal complexes are depicted in Scheme 1. The reported Na-salt of IDA (**7**)[12] was coupled with the sulfonamides (**6**) using BOP reagent (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate). The synthesis of complex **5** was carried out by reacting cyanuric chloride and 2 equiv. of amine-IDA ester (ESI†).[13] It was then combined with 4-(aminoethyl)benzene sulfonamide (experimental details are included in ESI†). Complexes **2**, **3** and **4** have two cupric ions ~8 Å apart.[14] Complex **5** is flexible and the distances between the Cu^{2+} ions was estimated to be 8–12 Å (employing BioMed CAChe, version 6.0).

The binding constants of these complexes with carbonic anhydrase (bovine erythrocyte, Sigma Chemical Company, mixture of isozymes) were determined employing isothermal titration calorimetry (25 mM HEPES buffer, pH = 7.0, Table 1). The two controls, benzene sulfonamide (control **6**) and the di-IDA-Cu^{2+} complex **7** (lacking the benzene sulfonamide group) showed weak affinity for the enzyme. Affinities of the conjugates were considerably higher compared to the controls. Complex **3** showed the highest affinity for the enzyme, three orders of magnitude higher compared to the controls. The similarity of binding constants for complexes **1** (one Cu^{2+} ion) and **3** (two Cu^{2+} ions) possibly indicates that one cupric ion is binding to one histidine on the surface of the protein. Since the enzyme preparation included a mixture of isozymes, it is

Table 1 Binding parameters of the complexes with carbonic anhydrase

Compound	Binding constant	Enthalpy/kcal mol^{-1}
Complex **1**	$(4.6 \pm 0.07) \times 10^6$	−26.4 ± 0.8
Complex **2**	$(1.9 \pm 0.03) \times 10^5$	−51.7 ± 5.8
Complex **3**	$(7.5 \pm 0.1) \times 10^6$	−36.9 ± 4.2
Complex **4**	$(5.4 \pm 0.02) \times 10^5$	−30.3 ± 2.6
Complex **5**	$(4.3 \pm 0.03) \times 10^5$	−45.5 ± 2.2
Control **6**	$(9.0 \pm 0.1) \times 10^3$	−31.2 ± 1.6
Control **7**	$(22.8 \pm 1.3) \times 10^3$	−129.0 ± 3.2

† Electronic supplementary information (ESI) available: experimental details and UV-Vis titration data. See http://www.rsc.org/suppdata/cc/b3/b305179j/

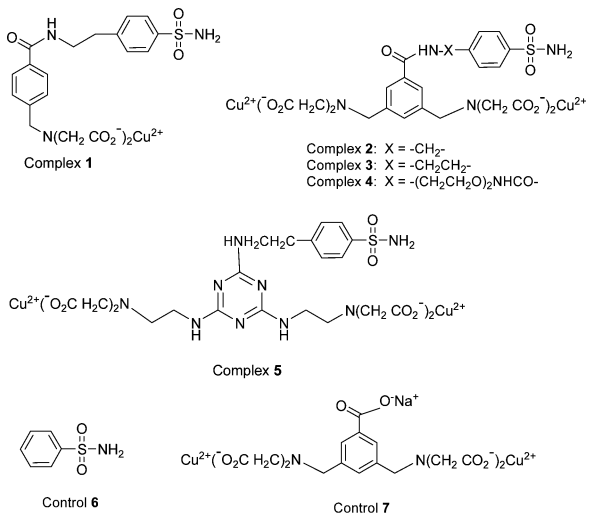


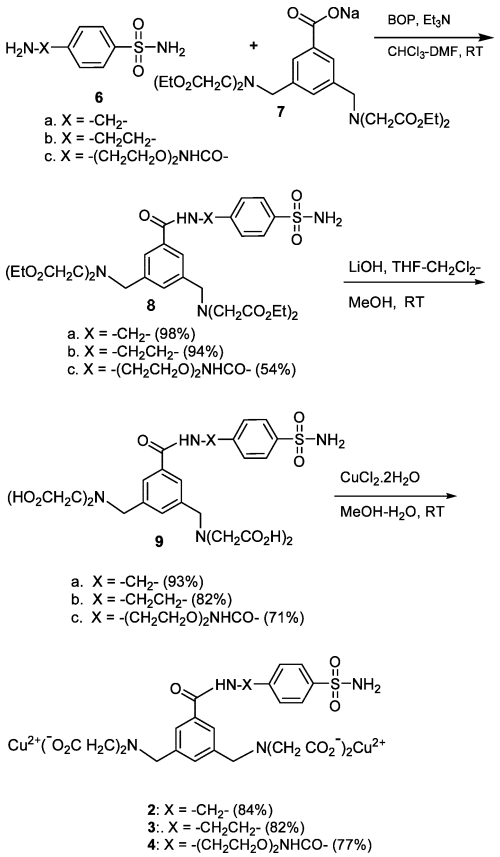
Fig. 1 The structures of the metal complexes.

DOI: 10.1039/b305179j

possible that different histidine residues from different isozymes contribute to this binding.

In order to demonstrate the binding of histidine residues to the cupric ions, the free ligands for these complexes (*i.e.*, **9a**, **9b** and **9c**) were titrated with the enzyme. The affinities were found to be much lower, similar to those of the controls **6** and **7** (data not shown). In addition, the Cu^{2+} complexes were titrated with the enzyme employing UV-Vis spectrometry.[4] The absorbance maxima for the cupric complexes were found to shift from 735 nm to 666 nm upon sequential addition of carbonic anhydrase, indicating the coordination of histidines to the cupric ions[4] (data for complex **3** are included in ESI†).

The kinetic parameters (K_m, V_{max}, and K_i) of the carbonic anhydrase catalyzed reactions were determined by measuring the hydrolysis of *p*-nitrophenyl acetate at 450 nm (Table 2). The substrate concentration dependent kinetic data in the absence and presence of inhibitors were analyzed by the non-linear regression analysis program, Grafit 4.0, and presented in the form of the double reciprocal plots (Fig. 3S, ESI†). The analyses of the kinetic data conformed to the competitive inhibition



Scheme 1 Syntheses of the copper complexes **2**, **3** and **4**. The syntheses of **1**, **4** and **6** are included in ESI.†

Table 2 Kinetic parameters of the carbonic anhydrase catalyzed reaction in the absence and presence of selected conjugates

Inhibitor	K_m/μM	V_{max} (ΔA_{450})/min^{-1}	K_i/μM
No inhibitor	15.70	0.31	
Complex **2**	28.30	0.25	0.74
Complex **3**	36.10	0.33	0.124
Complex **4**	29.20	0.31	0.814

model, and excluded other (*viz.*, non-competitive and uncompetitive) models. It should be noted that the K_i values determined by the kinetic method are similar to the dissociation constants ($K_d = 1/K_a$) of the corresponding enzyme–inhibitor complexes (Table 1, also see ESI†), determined *via* the isothermal titration microcalorimetric method.

In conclusion, we have demonstrated that the conjugation of a poor inhibitor (for the enzyme carbonic anhydrase) with a surface-binding functionality enhances the inhibitor efficiency by three orders of magnitude.

This research was supported by the National Institutes of Health grants 1R01 GM 63404-01A1 and 1P20 RR15566-01 to SM.

Notes and references

1 H. S. Park, Q. Lin and A. D. Hamilton, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 5105; Y. Wei, G. L. McLendon, A. D. Hamilton, M. A. Case, C. B. Purring, H. Park, C. S. Lee and T. Yu, *Chem. Commun.*, 2001, 1580.
2 H. Takashima, S. Shinkai and I. Hamachi, *Chem. Commun.*, 1999, 2345.
3 L. L. Kissling, J. E. Gestwicki and L. E. Strong, *Curr. Opin. Chem. Biol.*, 2000, **4**, 696; A. G. Cochran, *Chem. Biol.*, 2000, **7**, R85.
4 M. A. Fazal, B. C. Roy, S. Sun, S. Mallik and K. A. Rodgers, *J. Am. Chem. Soc.*, 2001, **123**, 6283.
5 C. T. Supuran, A. Scozzafava and A. Casini, *Med. Chem. Res. Rev.*, 2003, **23**, 146.
6 E. Kimura, *Acc. Chem. Res.*, 2001, **34**, 171.
7 A. Scozzafava and C. T. Supuran, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 1551.
8 A. Scozzafava and C. T. Supuran, *J. Med. Chem.*, 2002, **47**, 284.
9 A. Scozzafava, L. Menabouni, F. Manicone and C. T. Supuran, *J. Med. Chem.*, 2002, **45**, 1466.
10 C. A. Blasie and J. M. Berg, *Biochemistry*, 2002, **41**, 15068; W. F. DeGrado, L. DiCostanzo, S. Geremia, A. Lombardi, V. Pavone and L. Ranadaccio, *Angew. Chem., Int. Ed.*, 2003, **42**, 417; Z. Q. Tian and P. A. Bartlett, *J. Am. Chem. Soc.*, 1996, **118**, 943.
11 A. E. Martell and P. M. Smith, *Critical Stability Constants*, Plenum Press, New York, 1975, **vol. 2**, pp. 67–68. Immobilized IDA-Cu^{2+} complex is widely used for affinity chromatography purification of proteins. V. Gaber-Porekar and V. Menart, *J. Biochem. Biophys. Methods*, 2001, **49**, 335.
12 B. C. Roy and S. Mallik, *J. Org. Chem.*, 1999, **64**, 2969.
13 S. Sun, M. A. Fazal, B. C. Roy and S. Mallik, *Org. Lett.*, 2000, **2**, 911.
14 N. Shirai, Y. Watanabe and Y. Sato, *J. Org. Chem.*, 1990, **55**, 2767.