JACS COMMUNICATIONS

Published on Web 09/25/2004

Two-Prong Inhibitors for Human Carbonic Anhydrase II

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Carbonic anhydrases (EC 4.2.1.1) are ubiquitously distributed Zn(II)-containing metalloenzymes[1] that catalyze the reversible hydration of CO_2 to bicarbonate.[2] In humans, there are 14 isozymes of the α-type, some of which have been implicated in pathological conditions.[2] Thus, inhibitors of carbonic anhydrases have been employed in the treatment of glaucoma, epilepsy, and cancer.[2] Usually, these inhibitors possess a sulfonamide group, which interacts with the active site Zn^{2+}.[2] While some inhibitors have been obtained by structure-based design, capitalizing on this interaction,[3,4] there are limitations to this approach. Since the active site pocket has defined spatial dimensions, variations in inhibitor structure are not readily accommodated.[5] Also, the intrinsic flexibility in the protein allows for the binding of structurally unrelated compounds, and it is difficult to predict the nature and magnitude of such structural flexibility.[3]

Herein, we report a general strategy of inhibitor design which overcomes the limitations of the active-site based design alone. The proof-of-concept is demonstrated by converting a weak inhibitor of human carbonic anhydrase II (hCAII) into very potent inhibitors.[6] Our approach is based on attaching a metal chelating tether group (containing bound Cu^{2+}), which *extends beyond the active site pocket* of the enzyme and interacts with the surface exposed histidine residues. In this two-prong approach, the binding energy of the inhibitors is derived not only from the interactions at the active site region but also from the interactions of IDA−Cu^{2+} to the surface-exposed histidine residues.

There are some reports in the literature on linking two weak binding ligands of an enzyme to generate potent inhibitors.[7,8] In these reports, the ligands interact with the enzymes via hydrophobic, hydrogen bonding, and ion-pair interactions. There is one report on enhancing the binding affinities of carbonic anhydrase inhibitors by coordinating to His-64.[7d] This residue is situated close to the enzyme's active site pocket and is involved in catalysis.[7d] To the best of our knowledge, there are no reports of using the metal−ligand interactions involving surface amino acid residues to enhance the binding affinities of "two-prong" inhibitors.

Benzenesulfonamide (**1**, Figure 1) and *p*-aminoethylbenzenesulfonamide (**2**, Figure 1) are weak inhibitors for hCAII (K_i = 1.5 μM for **1** and 8.1 μM for **2**; Table 1).[9] These two compounds were selected as the controls in the present studies. For chelating to Cu^{2+} ions, iminodiacetate (IDA) was selected as the ligand. IDA has a very high affinity for Cu^{2+} ($K = 10^{12}$ M^{-1}).[10] In addition, CA binds to the complex IDA−Cu^{2+} via the surface-exposed histidine residues of the enzyme.[11] For the studies described herein, the weak inhibitors **1** and **2** were conjugated to the complex IDA−Cu^{2+} with a variety of spacers to generate the potent inhibitors (similar conjugates of IDA−Zn^{2+} precipitated above pH = 5.5). The structures of the resultant conjugates (**5**−**12**) are shown in Figure 1. The IDA−Cu^{2+} complexes **3** and **4** were also used as controls

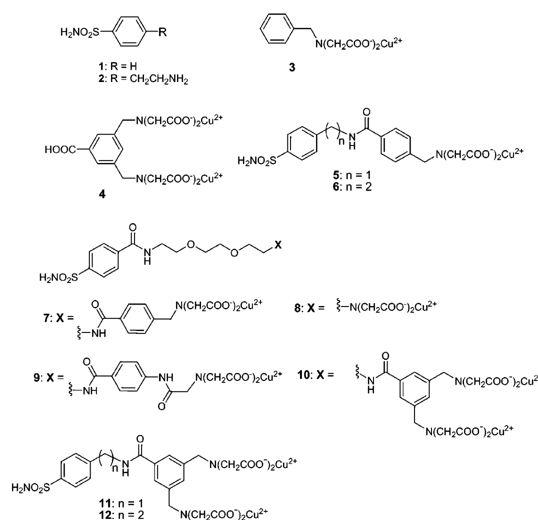

Figure 1. Structures of the conjugates synthesized (**5**−**12**) and the controls (**1**−**4**) used in this study.

Table 1. Inhibition Parameters for the Synthesized Conjugates with HCAII

conjugate	K_i (nM)	K_d (1/K_a) (ITC), nM
1	1500 ± 140	625 ± 110
2	8165 ± 312	4750 ± 1400
3	no inhibition	5500 ± 610
4	no inhibition	2130 ± 200
5	131 ± 21	37± 3
6	397 ± 30	196 ± 25
7	27 ± 6	24± 3
8	35 ± 10	55± 2
9	104 ± 17	100 ± 8
10	11 ± 3	1.0 ± 0.05
11	65 ± 9	77± 5
12	52 ± 9	13 ± 0.8

during these studies. The syntheses of these compounds are included in the Supporting Information.

The inhibitory potencies of the synthesized conjugates for the recombinant human carbonic anhydrase II (hCAII)-catalyzed reaction were determined by measuring the esterase activity of the enzyme, using *p*-nitrophenyl acetate as a substrate.[12] The inhibition constants of the enzyme−inhibitor complexes (K_i values), determined by the best fit of the experimental data, are summarized in Table 1. To ensure that the K_i values determined by the steady-state kinetic method were true measures of the binding affinities of the enzyme−inhibitor complexes, and not due to some unforeseen kinetic complexity, we performed the isothermal titration microcalorimetry (ITC) experiments (Supporting Information). The dissociation constants ($K_d = 1/K_a$) values of the individual enzyme−inhibitor complexes have been compared with their corresponding K_i values in Table 1. Note a marked similarity between the K_i and K_d values for each inhibitor. To the best of our knowledge, there is

10.1021/ja047271k CCC: $27.50 © 2004 American Chemical Society

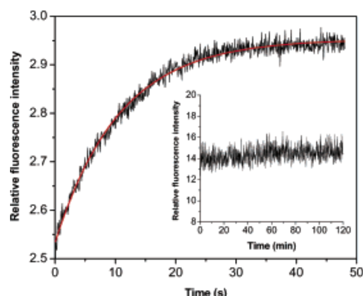

Figure 2. Dissociation "off-rates" of *p*-aminoethylbenzenesulfonamide and conjugate **11** from hCAII. The main figure shows the stopped-flow trace for the increase in the fluorescence intensity ($\lambda_{ex} = 330$ nm; cutoff filter = 335 nm) upon mixing of the enzyme-*p*-aminoethylbenzenesulfonamide complex with a high concentration of dansylamide. The after-mixing concentrations of the enzyme, inhibitor, and dansylamide were 2, 40, and 100 μM, respectively. The inset shows the spectrofluorimetric trace ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 448$ nm) upon mixing 2 μM enzyme 2.5 μM conjugate **5** and 100 μM dansylamide, respectively.

only one previous report[13a] of ITC studies on inhibitor binding to hCAII.

As apparent from the data of Table 1, all of the synthesized conjugates are potent inhibitors for the enzyme. The conjugates with triethylene glycol spacer show better inhibition as compared to the others. The conjugate **10** showed the best inhibition, 800-fold more pronounced than control **2**. It was noted that the increase in the inhibitory potencies of two Cu^{2+} ion complexes were modest as compared to the corresponding one Cu^{2+} conjugates. This feature indicates that only one of the cupric ions is binding to a histidine residue on the surface of the enzyme. Currently, we are investigating as to which histidine residue is specifically involved in the above interaction by performing the site-directed mutagenesis. However, when all the surface-exposed histidines residues are chemically modified with diethyl pyrocarbonate, the inhibition potency of conjugate **8** ($K_i = 606 \pm 59$ nM) decreased by 17-fold compared to the unmodified enzyme ($K_i = 35 \pm 10$ nM). This demonstrates the role of the surface-exposed histidine residues in the binding process.

To demonstrate the role of the cupric ions in the binding process, two different types of experiments were conducted (described in detail for the conjugate **8**). The metal free ligand of complex **8** showed a weak inhibition ($K_i = 1.5$ μM), comparable to that of the controls **1** and **2**. Similar results were obtained by the ITC titration method. In addition, conjugate **8** was titrated by the enzyme hCAII and the changes in the absorption maximum of the Cu^{2+} complex was monitored by the UV−vis spectrometry. The absorption maximum was found to shift from 730 to 665 nm, indicating the coordination of histidines to the cupric ions of **8** (data not shown).[11]

We ascertained the kinetic feasibility of dissociation of the enzyme-bound inhibitors via the competitive displacement by an active site directed ligand, dansylamide.[13] The fluorescence emission spectra of free and the enzyme-bound dansylamide ($\lambda_{ex} = 330$ nm) indicated a marked difference in the spectral profile (around 448 nm) upon binding with hCAII (Supporting Information). As an example, the dissociation off rates of the conjugate **11** and its parent compound, *p*-aminoethylbenzenesulfonamide (compound **2**), were measured via the stopped-flow assembly, configured to detect the time dependent fluorescence changes of the competitive inhibitor dansylamide ($\lambda_{ex} = 330$ nm; "cutoff" filter = 335 nm; Figure 2). The data are best fitted by the single-exponential rate equation with a rate constant of 0.094 s^{-1}. When we attempted to perform the above experiment involving conjugate **11**, practically no change in the fluorescence signal was noted up to 120 min of the reaction time, suggesting that the dissociation off rate of **11** was extremely slow. The only way to release conjugate **11** from hCAII was to add EDTA to the hCAII.**11** complex. EDTA removes the cupric ion from the conjugate **11**, and thus the resultant metal-free conjugate exhibits the off rate similar to that of control **2**.

In conclusion, we have demonstrated that weak inhibitors of hCAII can be converted to excellent inhibitors by conjugating with surface binding groups. Conjugation of IDA−Cu^{2+} (using a spacer) to benzenesulfonamides led to 800-fold improvement in the inhibition constants. It should be noted that this method can be used with other enzymes, which harbor histidine residues on their surfaces, and in close proximity to their active sites (e.g., tyrosine kinase, adenylate kinase, aldose reductase etc.). In addition, other amino acid residues (i.e., besides histidines) present on the surface of enzymes can be targeted by using other transition metal ion conjugates.[14]

Acknowledgment. This research was supported by the National Institutes of Health grants 1R01 GM 63404-01A1 to S.M. and 1R15 DK56681-01A1 to D.K.S..

Supporting Information Available: A figure showing fluorescence spectra of dansylamide and text and schemes giving synthetic details for the conjugates **5**−**12** and experimental details for the ITC experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) *The carbonic anhydrases: New Horizons*; Chegwidden, W. R., Carter, N. D., Edwards, Eds.; Birkhauser Verlag: Basel, Switzerland, 2000.
(2) (a) Vullo, D.; Franchi, M.; Gallori, E.; Antel, J.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2004**, *47*, 1272−1279. (b) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23* 146−189.
(3) (a) Moy, F. J.; Chanda, P. K.; Chen, J. M.; Cosmi, S.; Edris, W.; Levin, J. I.; Powers, R. *J. Mol. Biol.* **2000**, *302*, 671−89. (b) Yang, T. C.; Wolfe, M. D.; Neibergall, M. B.; Mekmouche, Y.; Lipscomb, J. D.; Hoffman, B. M. *J. Am. Chem. Soc.* **2003**, *125*, 7056−7066.
(4) (a) Salvatella, X.; Giralt, E. *Chem. Soc. Rev.* **2003**, *32*, 365−372. (b) Acharya, K. R.; Sturrock, E. D.; Riordan, J. F.; Ehlers, M. R. *Nat. Rev. Drug Discov.* **2003**, *2*, 891−902. (c) Benigni, R.; Zito, R. *Curr. Top. Med. Chem.* **2003**, *3*, 1289−1300.
(5) (a) Evensen, E.; Eksterowicz, J. E.; Stanton, R. V.; Oshiro, C.; Grootenhuis, P. D.; Bradley, E. K. *J. Med. Chem.* **2003**, *6*, 5125−5128. (b) Lee, A.; Breitenbucher, J. G. *Curr. Opin. Drug Discov. Dev.* **2003**, *6*, 494−508. (c) Muegge, I. *Med. Res. Rev.* **2003**, *23*, 302−321.
(6) For the preliminary results see: Roy, B. C.; Rodendahl, T.; Hegge, R.; Peterson, R.; Mallik, S.; Srivastava, D. K. *J. Chem. Soc., Chem. Commun.* **2003**, 2328−2329.
(7) For other examples with carbonic anhydrase, see: (a) Enander, K.; Dolphil, G. T.; Baltzer, L. *J. Am. Chem. Soc.* **2004**, *126*, 4464−4465. (b) Grybowski, B. A.; Ischenko, A. V.; Kim, C. Y.; Topalov, G.; Chapman, R.; Christianson, D. W.; Whitesides, G. M.; Shakhnovich, E. I. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1270−1273. (c) Boriack, P. A.; Christianson, D. W.; Kingery-Wood, J.; Whitesides, G. M. *J. Med. Chem.* **1995**, *38*, 2286−2291. (d) Scozzafava, A.; Menabouni, L.; Minicone, F.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 1466−1476.
(8) For examples with other enzymes; see: (a) Nakata, E.; Nagase, T.; Shinkai, S.; Hamachi, I. *J. Am. Chem. Soc.* **2004**, *126*, 490−495. (b) Baird, E. J.; Holowaka, D.; Coates, G. W.; Baird, B. *Biochemistry* **2003**, *42*, 12739−12748. (c) Shen, K.; Cole, P. A. *J. Am. Chem. Soc.* **2003**, *125*, 16172−16173. (d) Erlanson, D. A.; Lam, J. W.; Wiesmann, C.; Luong, T. N.; Simmons, R. L.; DeLano, W. L.; Choong, I. C.; Burdett, M. T.; Flanagan, W. M.; Lee. D.; Gordon, E. M.; O'Brien, T. *Nature: Biotechnol.* **2003**, *21*, 308−314.
(9) (a) Koike, T.; Kimura, E.; Nakamura, I.; Hashimoto, Y.; Shiro, M. *J. Am. Chem. Soc.* **1992**, *114*, 7338−7345. (b) Taylor, P. W.; King, R. W.; Burgen, A. S. V. *Biochemistry* **1970**, *9*, 2638−2645.
(10) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1975; Vol. 2.
(11) (a) Fazal, A. Md.; Roy, B. C.; Sun, S.; Mallik, S.; Rodgers, K. R. *J. Am. Chem. Soc.* **2001**, *123*, 6283−6290. (b) Pack, D. W.; Chen, G.; Maloney, K. M.; Chen, C. T.; Arnold, F. H. *J. Am. Chem. Soc.* **1997**, *119*, 2479−2487.
(12) Pocker, Y.; Stone, J. T. *Biochemistry* **1967**, *6*, 668−678.
(13) (a) Day, Y. S. N.; Baird, C. L.; Rich, R. L.; Myszka, D. G. *Protein Sci.* **2002**, *11*, 1017−1025. (b) Enander, K.; Dolphin, G. T.; Andersson, L. K.; Liedberg, B.; Lundstrom, I.; Baltzer, L. *J. Org. Chem.* **2002**, *67*, 3120−3123. (c) Thompson, R. B.; Maliwal, B. P.; Zeng, H. H. *J. Biomed. Opt.* **2000**, *5*, 17−22.
(14) Ojida, A.; Miyahara, Y.; Kohira, T.; Hamachi, I. *Biopolym. (Pept. Sci.)* **2004**, *76*, 177−184.

JA047271K