Protein crystal structures with ferrocene and ruthenocene-based enzyme inhibitors

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We have determined the protein X-ray crystal structures of four organometallic inhibitors in complex with their target enzyme carbonic anhydrase II. The barrel shaped hydrophobic ferrocene and ruthenocene moieties have provided a structure-based avenue to better occupy the hydrophobic binding patch within the enzyme active site.

Protein X-ray structures of enzymes with bound inhibitors can reveal with intricate detail how the inhibitor interacts with the enzyme active site. There are however few reports in which an organometallic inhibitor has been complexed with its target protein and a crystal structure obtained. Structures include a ruthenium-based half-sandwich complex with Pim-1 kinase (PDB ID 2BZH, 2BZI and 2BJJ),¹ a ferrocene-based ligand with the antibody 13G5 (PDB ID 1A3L),² and very recently a ruthenium d^6 -piano-stool complex with carbonic anhydrase II (PDB ID 3PYK)³ and a half-sandwich ruthenium complex with glycogen synthase kinase 3 β (PDB ID 3M1S).⁴

Metallocenes are organometallic compounds comprising a sandwich structure wherein the metal ion is located between two cyclopentadienyl (Cp) rings. The π -electrons from each Cp ring are delocalised throughout the compound and this accounts for similar chemical behaviour of ferrocenes and ruthenocenes to that of aromatic compounds such as benzenes. The ferrocenyl moiety is air and water stable, non-toxic and its metabolism is compatible with *in vivo* applications.⁵ There are numerous ferrocene-based compounds reported with good drug-like properties^{5a} and several ferrocene-based compounds have found application in drug discovery, for example ferrocenone has been used to treat iron deficiency anaemia,⁶ while hydroxyferrocifen (a Tamoxifen^{TM} analogue)⁷ and ferroquine (a chloroquine analogue)⁸ are in clinical development for the treatment of breast cancer and malaria, respectively. Ruthenocene and its derivatives are much less studied than their isostructural ferrocene counterparts, and to date only a small number of ruthenocene-based compounds appear in the literature of drug discovery.

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc metalloenzymes that are validated therapeutic targets for a number of disease states including hypoxic tumours.⁹ CAs catalyse the reversible hydration of CO₂ to generate a HCO₃⁻ and H⁺.⁹⁻¹⁰ Human CA II comprises a funnel shaped active site cavity at the base of which is a catalytic Zn²⁺ cation. This tetrahedral Zn²⁺ cation is coordinated to the imidazole sidechains of three histidine residues and to a H₂O molecule, lowering the pK_a of this water to ~6.8 (the pK_a of bulk water is ~14) to facilitate formation

of zinc bound hydroxide at physiological pH.¹⁰ Hydroxide is the nucleophile that reacts with CO₂.¹⁰ Almost all reported small molecule CA inhibitors comprise a zinc binding group (ZBG)⁹ of which the primary sulfonamide group (-SO₂NH₂) is recognised as the foremost example. The concept of the 'tail approach' for developing CA inhibitors was first described by Supuran and co-workers.¹¹ 'Tail' moieties are linked to the primary sulfonamide ZBG to give an extended CA pharmacophore of [tail]-[aromatic]-[ZBG] and compounds that have a balanced physicochemical property profile needed for *in vivo* applications.¹² We have previously reported the synthesis and CA inhibition of four metallocene-based CA inhibitors, compounds **1-4** (Fig. 1).¹³ Inhibitors **1-4** typify the [tail]-[aromatic]-[ZBG] pharmacophore, wherein the triazole-ferrocene or triazole-ruthenocene fragment comprises the 'tail' of the CA inhibitor. Inhibitors **1** and **3** are 1,4-disubstituted-1,2,3-triazoles synthesised by copper-catalysed azide-alkyne cycloaddition (CuAAC) of ethynyl ferrocene and ethynyl ruthenocene, respectively, and 4-azido benzenesulfonamide. Inhibitors **2** and **4** were synthesised from the same substrates using ruthenium-catalysed azide-alkyne cycloaddition (RuAAC) and are the corresponding 1,5-disubstituted-1,2,3-triazole regioisomers.

At hCA II (h = human) the ferrocenyl 1,5-disubstituted triazole regioisomer **2** (K_i = 36 nM) has 2-fold better CA II inhibition than the 1,4-disubstituted triazole regioisomer **1** (K_i = 80 nM).¹³⁻¹⁴ The ruthenocene derivatives, with K_i s of 9.7 nM (**3**) and 12 nM (**4**) nM, share similar hCA II inhibition and are 3- to 8-fold better hCA II inhibitors than their ferrocene counterparts. Compounds **1-4** are closely related in structure and likely to experience similar desolvation energies on binding to CA II. We therefore propose that the variable interactions of the barrel shaped hydrophobic metallocene moiety with the CA II protein may account for differences in inhibition to inform our understanding of structure-activity relationships and provide a structural base on which to direct future metallocene-based CA inhibitor design. To obtain insight into the triazole-metallocene tail group interactions

Fig. 1. Metallocene-based CA inhibitors **1-4**. Human CA II inhibition constants (K_i s) are shown.

with the CA active site amino acids we have utilised protein X-ray crystallography to determine the crystal structures of human CA II in complex with inhibitors **1-4**. The crystal structures of the CA-metalloocene-based inhibitor complexes were determined to 2.0 Å resolution (CA II:**1**), 1.5 Å resolution (CA II:**2**), 2.2 Å resolution (CA II:**3**) and 1.6 Å resolution (CA II:**4**). Data collection and refinement statistics of inhibitor-bound CA II crystal structures are provided in ESI.[†] Coordinates and structure factors have been deposited with the PDB, with accession codes 3P55 (CA II:**1**), 3P3H (CA II:**2**), 3P44 (CA II:**3**) and 3P3J (CA II:**4**). The binding mode of the sulfonamide moiety to the catalytic zinc ion is invariant as compared to other sulfonamide-bound CA II structures. The metalloocene-triazole tail groups of the inhibitors occupy space in the active site cavity of CA II without altering the configuration of residues lining the cavity. The binding cavity of CA comprises a cone-shaped active site with a hydrophobic and a hydrophilic face.¹⁰ The metallocenyl moieties are oriented towards the upper region of the hydrophobic face with both Cp rings involved in van der Waals interactions with residues constituting the hydrophobic face (Fig. 2). Specifically, the metallocenyl moieties of the 1,4-regioisomers **1** and **3** engage in interactions Val134, Phe130, Pro201 and Leu203, while the metallocenyl moieties of the 1,5-regioisomers **2** and **4** are distant from Val134 and interact only with Phe130, Pro201 and Leu203. There are no direct interactions between protein residues and the central divalent ion of the metallocenyl groups. These observations are consistent with the only prior reported protein structure with a ferrocene-based ligand (PDB ID 1A3L) wherein several van der Waals contacts with the Cp rings are also observed.² The shape of the ligands, as dictated by the regioisomeric relationship of the metallocenyl moieties, causes a tilt of the vertical axis of the benzenesulfonamide moiety of the 1,5-disubstituted-1,2,3-triazoles **2** and **4** compared to the 1,4-disubstituted-1,2,3-triazoles **1** and **3** (Fig. 3). This tilt brings the triazole of **2** and **4** into the vicinity of the active site lining residues and enables a hydrogen bond between Gln192 and this ligand. This hydrogen bond constitutes the key difference when comparing the binding interactions of **1/3** and **2/4** with the protein (Fig. 2), and the 2-fold better CA II inhibition of **2** over **1** is consistent with this additional hydrogen bond. In ligands **1** and **2**, the Cp rings of the ferrocenyl moieties adopt a distorted staggered conformation offset with an angle close to 10°. The Cp rings of the ruthenocenyl compounds **3** and **4** are in eclipsed conformation. It has been shown that rotation of the two Cp rings of ferrocene with respect to each other can occur in solution with a low energy barrier,¹⁵ and it has previously been observed that in the solid protein-bound state that the interactions between the Cp rings and their environment determines their relative orientation.² The ferrocenyl ligand of PDB ID 1A3L has substituents on both Cp rings that hydrogen bond to the antibody. Cp ring rotation appears restricted to maximize the number of specific interactions of the substituents with the antibody. The electron density provides no suggestion of multiple conformations, with the antibody acting to trap one conformer.² In contrast, the unsubstituted Cp ring in compounds **1-4** of this study form only hydrophobic interactions with the protein residues, such that a relatively unrestricted rotation of the unsubstituted Cp ring with respect to the substituted Cp ring is likely. This assumption is

supported by the electron density observed in the crystal structures of CA II:**1-4** which is less well defined for the unsubstituted Cp ring than for the substituted Cp ring.

The backbone of the 1,4-disubstituted-1,2,3-triazoles **1** and **3** adopts a near-linear topology, whereas the 1,5-disubstituted-1,2,3-triazoles **2** and **4** exhibit a corkscrew topology (Fig. 4), a phenomenon which has previously been observed with disubstituted 1,2,3-triazoles regioisomers.¹⁶ Both bound conformations are hypothesised to constitute the lowest energy conformations of the ligands in solution such that the binding of metallocenes **1-4** to human CA II does not require significant

Figure 2. Protein-ligand interactions of metallocenes **1** (A), **2** (B), **3** (C) and **4** (D) in complex with human CA II. Hydrogen bonds - dotted lines, hydrophobic interactions - dashed lines. Atomic distances are in Å.

bond rotations to accommodate the ligands. The shape complementarity of the ligands in their most favourable conformations with parts of the CA II active site, combined with the stable anchoring of the compounds to the active site zinc cation via the sulfonamide anion, are consistent with the good CA II inhibition constants observed. As well the ruthenium cation is larger than the iron cation and this increases the Cp ring separation in the ruthenocenyl ligands **3** and **4** compared to **1** and **2**. This more subtle structural variation may account for the observed difference in enzyme inhibition properties, with the ruthenocenyl ligands **3** and **4** 3- to 8-fold better CA II inhibitors than their ferrocenyl counterparts **1** and **2**.

In conclusion, there are few reports in which an organometallic inhibitor has been complexed with its target protein and a crystal structure obtained. Herein we have utilised protein X-ray crystallography to determine the crystal structures of human CA II

Fig. 3. Comparison of the space-filling in the active site of human CA II complexes with **1** (A), **2** (B), **3** (C) and **4** (D).

Fig. 4. Orthographic views depicting the linear **1** (A, B), **3** (E, F) and corkscrew **2** (C, D), **4** (G, H) geometry of metallocenes. The initial $F_0 - F_c$ difference electron density (green) is contoured at 2σ . Anomalous density (red) is contoured at 2.5σ .

II in complex with two ferrocene- (**1**, **2**) and two ruthenocene-based organometallic inhibitors (**3**, **4**), to 1.5-2.2 Å resolution. Structures (CAII:**3**) and (CAII:**4**) represent the first ruthenocene-protein structures reported. While the metallocene moiety behaves chemically like an aromatic moiety such as a phenyl group, the barrel shaped sandwich structure of metallocene permits access to 3D structural permutations that are not possible with a flat aromatic ring. This ligand attribute provides an enhanced opportunity to form a greater number of hydrophobic interactions with the hydrophobic face of the CA II binding cavity. Compounds also present two distinct ligand topologies that impact on the shape complementarity towards the conical CA II active site cavity. In the context of protein-ligand interactions this may provide a discerning avenue to develop compounds to better occupy hydrophobic protein binding pockets within enzyme active sites. The implication of binding interactions provided by these metallocene-based organometallic inhibitors has informed the on-going study of metallocene-based human CA inhibitors within our lab that have potential use as future drugs.

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Notes and references

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