

Crystal structure of human carbonic anhydrase II at 1.95 Å resolution in complex with 667-coumate, a novel anti-cancer agent

Matthew D. LLOYD*¹, Richard L. PEDERICK*, Ramanathan NATESH†, L. W. Lawrence WOO*‡, Atul PUROHIT§, Michael J. REED§, K. Ravi ACHARYA† and Barry V. L. POTTER*‡

*Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K., †Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K., ‡Sterix Ltd, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K., and §Endocrinology and Metabolic Medicine and Sterix Ltd, Imperial College, Faculty of Medicine, St. Mary's Hospital, London W2 1NY, U.K.

CA (carbonic anhydrase) catalyses the reversible hydration of carbon dioxide into bicarbonate, and at least 14 isoforms have been identified in vertebrates. The role of CA type II in maintaining the fluid and pH balance has made it an attractive drug target for the treatment of glaucoma and cancer. 667-Coumate is a potent inhibitor of the novel oncology target steroid sulphatase and is currently in Phase 1 clinical trials for hormone-dependent breast cancer. It also inhibits CA II *in vitro*. In the present study, CA II was crystallized with 667-coumate and the structure was determined by X-ray crystallography at 1.95 Å (1 Å = 0.1 nm) resolution. The structure reported here is the first for an inhibitor based on a coumarin ring and shows ligation of the sulphamate group to the active-site zinc at 2.15 Å through a nitrogen anion. The first two rings of the coumarin moiety are bound within the

hydrophobic binding site of CA II. Important residues contributing to binding include Val-121, Phe-131, Val-135, Leu-141, Leu-198 and Pro-202. The third seven-membered ring is more mobile and is located in the channel leading to the surface of the enzyme. Pharmacokinetic studies show enhanced stability of 667-coumate *in vivo* and this has been ascribed to binding of CA II in erythrocytes. This result provides a structural basis for the stabilization and long half-life of 667-coumate in blood compared with its rapid disappearance in plasma, and suggests that reversible binding of inhibitors to CA may be a general method of delivering this type of labile drug.

Key words: cancer, carbonic anhydrase inhibitor, 667-coumate, obesity, steroid sulphatase inhibitor, X-ray crystal structure.

INTRODUCTION

CA (carbonic anhydrase; EC 4.2.1.1) is a Zn(II)-dependent enzyme that catalyses the reversible hydration of carbon dioxide into hydrogen carbonate and a proton, and plays an important role in physiological anion-exchange processes and fluid balance [1]. This enzyme (molecular mass = 29 kDa) has long been a drug target for the treatment of glaucoma [2,3], but has taken on new importance as a potential target for obesity [4] and cancer [5,6] (see [7] for a comprehensive review). CA isoforms can also catalyse a series of other hydration and dehydration reactions, although it is not clear whether these reactions have any physiological significance in normal or cancerous cells [7]. At least 14 isoforms of CA have been identified in vertebrates with different physiological and pathological roles, and they can be localized in the cytosol (CA I–III and VII) or mitochondria (CA V), whereas others are secreted (CA VI) or are membrane-bound and have extracellular active-site domains (CA IV, IX, XII and XIV) [3]. There is convincing evidence for the overexpression of CA isoforms IX and XII in cancer (see e.g. [8]), and this is supposed to promote growth of the tumour by acidification of the extracellular environment. Expression is up-regulated under hypoxic conditions, which is common in solid tumours [9,10].

Breast cancer is a major cause of mortality in Western countries and is particularly prevalent in post-menopausal women. Oestrogenic steroid hormones promote growth and development of this cancer in its hormone-dependent form, and there is now a growing awareness that a key enzyme in the production of these hormones, apart from the well-recognized aromatase enzyme

complex, is steroid sulphatase. 667-Coumate (Figure 1) is a particularly potent non-steroidal inhibitor of steroid sulphatase, with an IC₅₀ value of 8 nM compared with 25 nM for the bench-mark inhibitor EMATE (oestrone-3-O-sulphamate; Figure 1) [11], the first irreversible inhibitor to be reported. 667-Coumate [11] is currently in Phase 1 clinical trials for the treatment of breast cancer. However, 667-coumate is chemically more labile in solution than EMATE. A recent pharmacokinetic study on 667-coumate in rats showed that the drug had an enhanced stability in whole blood and is protected from first pass metabolism [12]. In contrast, 667-coumate readily eliminates sulphamic acid in solution and is rapidly cleared from the plasma *ex vivo* [13]. This difference in stability between whole blood and plasma has been attributed to reversible binding of 667-coumate to CA II in erythrocytes [12] in a similar manner to that observed previously for sulphonamide drugs [14], although stability is not an issue for these drugs. The structural similarity between the sulphamate and sulphonamide moieties and the ability of their mono-anionic forms to co-ordinate with Zn(II) are the main contributing factors for this binding. The reversible binding of 667-coumate to CA II means that this steroid sulphatase inhibitor is also a reversible inhibitor of at least one CA isoform. Indeed, it is known that CA II is inhibited by 667-coumate *in vitro* with reported IC₅₀ values of 25 nM [5] and 17 nM [15]. Whether the observed anti-cancer activity of 667-coumate is due to inhibition of CA IX and XII in cancerous tissues as well as inhibition of steroid sulphatase is not clear.

X-ray crystal structures of CA II complexed with EMATE (IC₅₀ values reported as 10 nM [6], 42 nM [5] and 9 nM [15])

Abbreviations used: CA II, carbonic anhydrase II; EMATE, oestrone-3-O-sulphamate.

¹ To whom correspondence should be addressed (email M.D.Lloyd@bath.ac.uk).

Atomic co-ordinates of the reported protein structure have been deposited in the RCSB database under the accession number 1TTM.pdb.

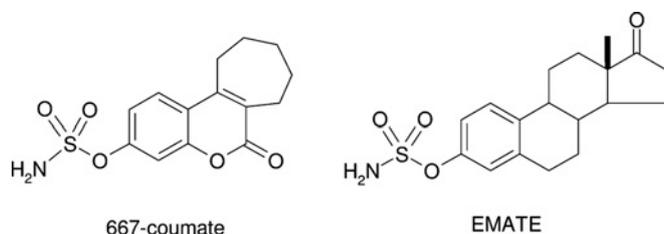


Figure 1 The structures of 667-coumate and EMATE

[6], sulphiride ($IC_{50} = 40$ nM) [16], E7070 ($K_1 = 15$ nM) [17], the 2-*N,N*-diethylaminoethylamide derivative of 5-(4-carboxy-benzenesulphonamido-1,3,4-thiadiazole-2-sulphonamide) ($K_1 = 1.4$ nM) [18] and RWJ-37947 ($IC_{50} = 36$ nM) [2] have been reported. Only EMATE from this group is an aryl sulphamate ester, one of the new classes of sulphamate ester-based drug candidates; RWJ-37947 is a sugar sulphamate related to the anticonvulsant drug, topiramate. In the present study, we report the X-ray crystal structure of CA II in complex with 667-coumate to 1.95 Å (1 Å = 0.1 nm) resolution, the first with a potent non-steroidal inhibitor based on a coumarin ring, and it is expected that a tricyclic coumarin ring mimics the steroid skeleton. This result provides a structural basis for the observed inhibition of CA II activity by this compound and its pharmacokinetic results.

EXPERIMENTAL

Materials

All chemicals and equipment were purchased from Sigma–Aldrich or Fisher and were of analytical grade or higher, and they were used without further purification. Plasmid pACA encoding for human CA II [19] was obtained from Professor C. Fierke (University of Michigan, Ann Arbor, NJ, U.S.A.). 667-Coumate was synthesized as described previously [11]. Amersham Biosciences provided protein chromatography systems and columns.

Growth and purification of enzyme

Plasmid pACA was transformed into *Escherichia coli* BL21(DE3) and cultured overnight in Luria–Bertani medium, supplemented with Kao and Michylek vitamin supplement, *E. coli* mineral supplement (5 g of EDTA, 0.5 g of $FeCl_3$, 0.05 g of $ZnCl_2$, 0.01 g of $CuCl_2$, 0.01 g of $CoCl_2 \cdot 6H_2O$, 0.01 g of H_3BO_3 and 1.6 g of $MnCl_2 \cdot 6H_2O$ in 800 ml of water, adjusted to pH 7.0 and sterile-filtered), 0.4% (w/v) glucose and 50 µg/ml ampicillin at 37°C and 170 rev./min. Starter culture (14 ml) was used to inoculate 4 × 700 ml of the same medium and was grown until $D_{600} \sim 0.8$. The temperature was decreased to 30°C and enzyme expression was induced with 0.25 mM isopropyl β-D-thiogalactoside and 1 mM $ZnSO_4$ [19]. Cells were harvested after 5 h by centrifugation at 11 300 g for 30 min at 4°C.

Cells were resuspended in 20 mM Mes/NaOH, pH 6.2 (50 ml), and lysed using the ‘one-shot’ cell disruptor (Constant Systems, Low March, Northants, U.K.). After centrifugation at 9820 g, 4°C, for 45 min, the extract was treated with 0.1% (v/v) polyethyleneimine, stirred for 15 min and centrifuged for 1 h. The crude cell extract was purified by centrifugation at 9820 g for 60 min at 4°C using an S-Sepharose XL column with a 0–500 mM NaCl gradient in 100 ml of buffer. Fractions were analysed by SDS/PAGE, and the required fractions were concentrated to approx. 10 mg/ml using a Hi-Trap S-Sepharose column, eluting with 500 mM NaCl in 10 mM Mes/NaOH (pH 6.2).

Kinetic analysis of 667-coumate inhibition

Kinetic constants were derived as described previously for tight-binding inhibitors of CA II [20,21]. Assays were conducted in a final volume of 3.0 ml in 50 mM Tris/HCl (pH 7.6) and 10 µM $ZnSO_4$ using 2.88 mM 4-nitrophenol acetate as the substrate. The substrate was dissolved in ethanol and added as a 10× stock solution. Control experiments showed that this had no effect on recombinant enzymic activity. All reagents except the enzyme were preincubated at 30°C and mixed. The formation of the 4-nitrophenol product was monitored at 348 nm for 2 min using a PerkinElmer Lambda 4.0 spectrometer with a Peltier system, and rates were derived using Kinlab software. The enzyme (0.05 ml) was added and the rate was determined as before. Inhibition constants were determined with 667-coumate (0.05 ml stock solutions in ethanol) at final concentrations of 33, 83, 166 and 250 nM, with each assay conducted at least in triplicate. The activity of the enzyme in the absence of inhibitor was 3.63 µmol · min⁻¹ · mg⁻¹, assuming a molecular mass for CA II of 29.115 kDa and $\epsilon_{348} = 5.4$ mM⁻¹ · cm⁻¹ for the product [20]. Results are expressed as means ± S.D., calculated using Excel. The concentrations of substrate [20] and enzyme [19] were determined using spectrophotometry at 348 and 280 nm respectively.

Crystallization, data collection and structural determination

The hanging drop vapour diffusion method was used for the crystallization. Protein (2.5 µl, ~10 mg/ml, ~0.3 mM) containing 0.5 mM 667-coumate and 30 mM 2-mercaptoethanol was mixed with well buffer (2.5 µl; 0.1 M Tris/HCl, pH 8.0, 1 mM $ZnSO_4$ and 2.45 M ammonium sulphate), with crystals appearing after 3–4 weeks at 4°C.

X-ray diffraction data were collected at station 9.6 at the Synchrotron Radiation Source (Daresbury, U.K.) at room temperature with a 15 s exposure and a crystal to detector distance of 160 mm, with 85 frames of 1° oscillation collected. Data were indexed and reduced with DENZO and SCALEPACK modules of the HKL suite [22] in the orthorhombic $P2_12_12_1$ space group. CA II crystallized in the $P2_12_12_1$ space group containing the active-site zinc atom without any water molecule (1UGG.pdb [23]) was used as the starting model for rigid body refinement followed by energy minimization and isotropic thermal factor (*B*-factor) refinement within CNS 1.0 [24]. Clear density for the inhibitor was observed in the difference map after this single round of refinement ($R_{free} = 0.25$; $R_{cryst} = 0.23$). Introduction of the inhibitor and alternating cycles of addition of water, manual rebuilding and energy minimization and *B*-factor refinement gave a final model with $R_{free} = 0.21$ and $R_{cryst} = 0.17$ (Table 1).

RESULTS AND DISCUSSION

Binding of 667-coumate to CA II

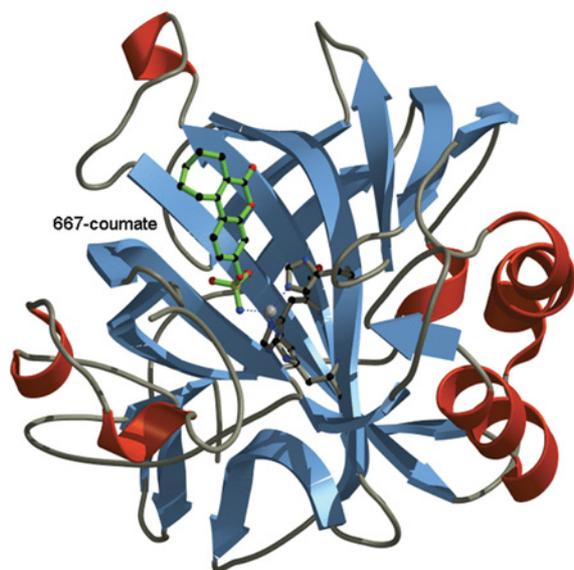
CA II was crystallized in the $P2_12_12_1$ space group in the presence of 667-coumate. The quality of crystals was better in the presence of the reducing agent 2-mercaptoethanol. The overall structure of the adduct had an R.M.S. value of 0.17 Å for the $C\alpha$ atoms when compared with the starting structure, indicating that minimal changes in the backbone position had occurred on inhibitor binding. Crystals could only be obtained in the presence of Zn(II) in the crystallization solution.

Examination of the first maps in the active-site region showed clear evidence for the anticipated ligation of the sulphamate group to the Zn(II) metal and the presence of the first two rings of the 667-coumarin moiety. 667-Coumate sits within a hydrophobic

Table 1 Crystallographic statistics of data collection and refinement for the CA II-667-coumate adduct

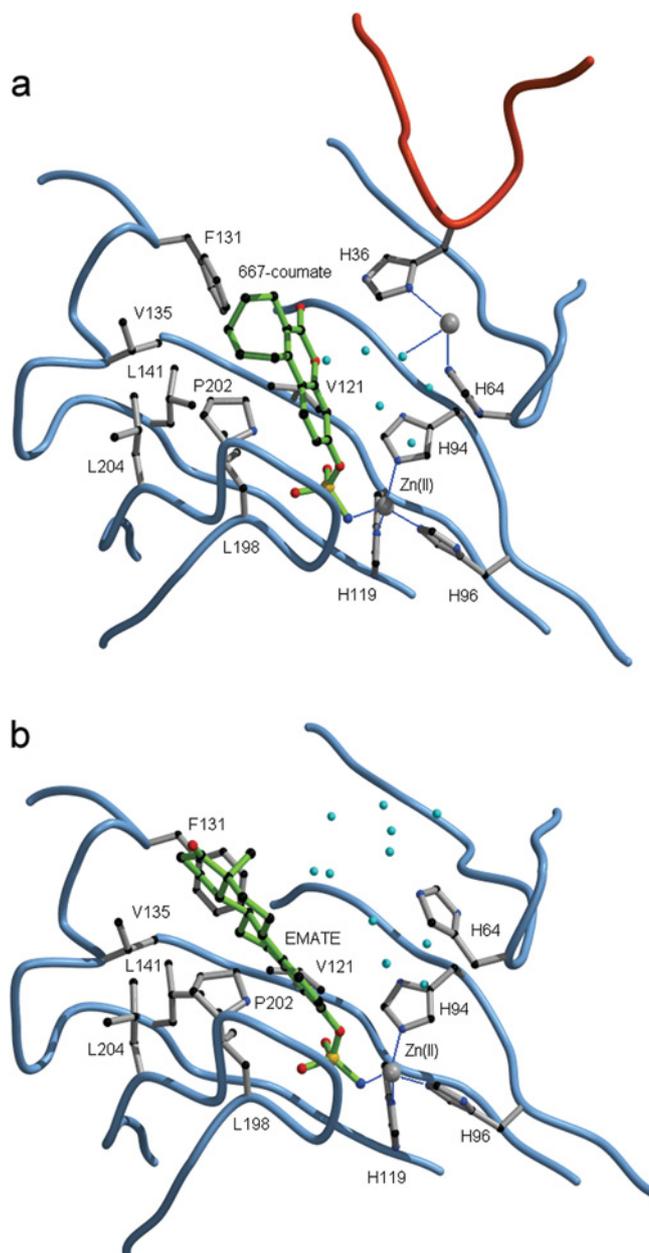
Values in parentheses are for the highest resolution shell, 2.02–1.95 Å. Note that the seven-membered ring of the ligand has conformational flexibility, as judged by its higher *B*-factors.

Parameter	Value
Resolution range (Å)	50–1.95
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell (Å)	<i>a</i> = 42.52, <i>b</i> = 72.52, <i>c</i> = 75.37
Highest resolution shell (Å)	2.02–1.95
Total reflections	73 426
Unique reflections	15 254
Completeness (%)	86.2 (83.1)
<i>R</i> _{sym} (%)	10.6 (33.9)
<i>I</i> / <i>σ</i> (<i>I</i>)	12.3 (5.2)
Refined residues	3–261
Refined water molecules	164
Refined zinc atoms	2
Resolution range in refinement (Å)	50–1.95
<i>R</i> _{free}	0.21
<i>R</i> _{cryst}	0.17
R.M.S. deviations	
Bond lengths (Å)	0.005
Bond angles (degree)	1.3
Average <i>B</i> value (Å ²)	21.0
Primary Zn ²⁺ <i>B</i> -factor (Å ²)	9.2
Secondary Zn ²⁺ <i>B</i> -factor (Å ²)	23.9
Ligand <i>B</i> -factor range (Å ²)	20.3–39.7
Ramachandran plot	
Most favoured (%)	88
Additionally allowed (%)	10.6
Generously allowed (%)	1.4
Disallowed (%)	0

**Figure 2** Overall structure of CA II with 667-coumate bound

This diagram was prepared using Bobscript [31,32].

pocket inside CA II, and is bound to the active-site zinc atom (Figure 2). There was no evidence for the decomposition of the inhibitor within the active site or covalent modification of any active-site residue. Apart from the primary zinc-binding site, examination of the structure showed a second Zn(II)-binding site on the exterior of the protein between His-64 and His-36 of a symmetry-related molecule, and this interaction appeared to be

**Figure 3** Close-up of residues in the active site of CA II binding to (a) 667-coumate and (b) EMATE [6]

Secondary structures from the protein molecule are represented by blue coils, whereas those from the symmetry-related molecule are represented by brown coils. Atom colours are black (carbon), red (oxygen), blue (nitrogen), yellow (sulphur) and cyan (water molecules). Zinc-263 is the secondary zinc metal ion from the symmetry-related protein molecule. Bonds to metal ions are represented by blue lines. This diagram was prepared using Bobscript [31,32].

involved in the stabilization of crystal-packing interactions (Figure 3a). A single water molecule is also ligated to this second zinc atom. This is in contrast with the EMATE structure (in the P2₁ space group), in which a second Zn(II)-binding site is not observed (Figure 3b). His-64 has been identified as a part of the catalytic proton shuttle that is responsible for converting the zinc-bound water molecule to hydroxide before catalysis can occur. This histidine residue is conserved in CA I, II and IV and its importance is understood by comparing the efficient catalysis by these enzymes with that of isoform III, which possesses a lysine

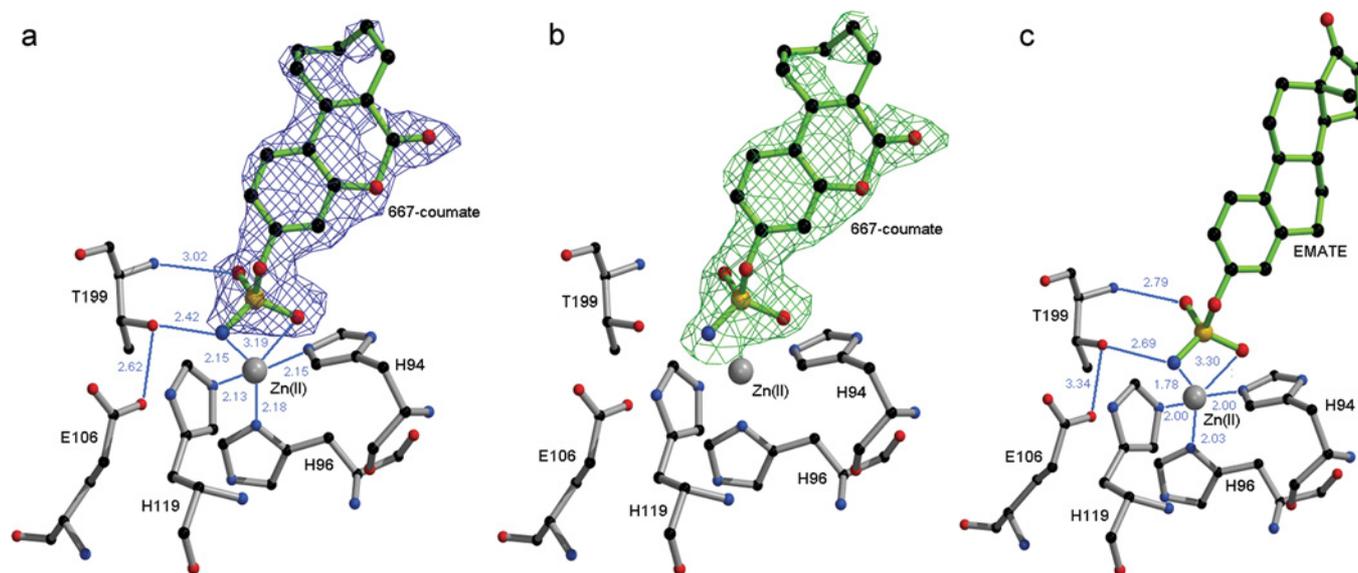


Figure 4 Binding of sulphamate drugs in the active site of human CA II

(a) Binding of 667-coumate in the active site of CA II, showing Zn(II) ligation and other interactions. Density is for the $2F_o - F_c$ map (blue) contoured at 1.0σ . Note that the seven-membered ring of 667-coumate has high conformational flexibility and, hence, lower amounts of electron density are observed in this region. (b) Identical view with density for the $F_o - F_c$ omit map (green) contoured at 2.00σ . (c) Identical view of EMATE [6] bound within the active site. Important ligand interactions are shown as blue lines, with bond lengths reported in Å. Atom colours are black (carbon), red (oxygen), blue (nitrogen) and yellow (sulphur). This figure was prepared by using Bobscript [31,32].

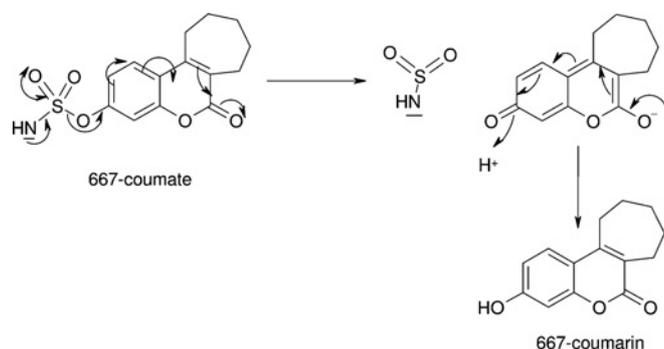
residue at this position and has only approx. 0.3% activity of CA II [7]. Interestingly, the Michigan mutant form of CA I (H67R) has been crystallized in the same $P2_12_12_1$ space group as our structure, with a Zn(II) atom ligated by His-64 [25]. The H67R CA I mutant had increased esterase activity in the presence of the second Zn(II) atom, but it is not clear whether this has any physiological or pathological consequences for either CA I or CA II activity.

As expected, the primary Zn(II) metal atom was co-ordinated by the three active-site histidine residues (His-94, His-96 and His-119), with the fourth ligand provided by the nitrogen of the inhibitor at 2.15 Å (Figure 4a). The bond angle between the inhibitor NH group, Zn(II) metal ion and histidine residues is close to 104° , and is the expected tetrahedral arrangement. The Zn(II)–nitrogen bond distance in this complex is long compared with that observed in the EMATE complex (1.78 Å; Figure 4b) [6], but is within the general range expected for metal–ligand distances. Co-ordination of the nitrogen atom to the Zn(II) metal ion appears to be required for tight binding of the inhibitor in the present structures and previously solved structures, but it is not clear if shorter metal–ligand–nitrogen distances correlate with the binding constants of the inhibitors. Examination of the active-site environment shows that inhibitor binding is stabilized by the same interactions that are observed for the EMATE and other complexes [2,6] (Figure 4). The inhibitor NH group is ligated to the active-site Zn(II) atom and is also involved in a hydrogen bond with the alcohol side chain of Thr-199 (2.42 Å), which is in turn bonded to the carboxylate of Glu-106 (2.62 Å). Inhibitor binding is also stabilized by interaction of O2 (the oxygen atom within the sulphamate group, which interacts with particular protein residues) with the active-site zinc at 3.19 Å and an interaction of O1 with the peptide backbone nitrogen at 3.02 Å.

Consideration of the metal–inhibitor bond distances reported in the present study and in [2,6] suggests that bond distance is an important factor for determining inhibition constants and that binding of the hydrophobic moiety is also important. For example,

the Zn(II)–ligand–nitrogen distance for the more hydrophobic RWJ-37497 (2.1 Å) is similar to the value reported here (2.15 Å) as well as other hydrogen-bonding interactions between Zn(II), Thr-199 and the sulphamate moiety, but the IC_{50} values differ by approx. 2-fold (17 nM [5,15] for 667-coumate compared with 36 nM for RWJ-37497 [2]). This may be accounted for by the differences in binding of the ring systems within the CA II active site. For RWJ-37497, many hydrophilic interactions are made, and binding is stabilized by hydrogen bonds. For 667-coumate, binding is predominately by hydrophobic interactions. Most notably, Asn-67 is involved in hydrogen-bonding in the RWJ-37497 structure, but in this structure, the side chain is orientated away from the coumarin ring system and does not participate in inhibitor binding.

Examination of the binding site for the 667-coumarin moiety shows that the ring structure is contained within the hydrophobic binding pocket of CA II (Figure 3a). The entire molecule is surrounded by hydrophobic residues, with close contacts being made by Val-121, Phe-131, Val-135, Leu-141, Leu-198 and Pro-202. In contrast with the EMATE structure, the active site in our structure contains few water molecules close to the ligand, the closest being at 3.64 Å (Figures 3a and 3b). The oxygen within the α,β -unsaturated lactone ring is hydrogen-bonded to the amide nitrogen of Asn-92 at 3.60 Å; the carbonyl group of this ester functionality is not hydrogen-bonded and is at least 4.5 Å away from the nearest hydrogen-bonding donors. The tricyclic ring structure of the inhibitor is in a very similar position to that occupied by the first two (A and B) rings of EMATE [6], which is bound by the same residues in a similar orientation (Figure 3b). However, minor differences do occur, the most significant of which is the position of the side chain of Phe-131. In this structure, the phenyl ring is flat, with binding stabilized by face-to-face hydrophobic interactions with the α,β -unsaturated lactone ring. In the EMATE structure [6], the phenyl ring is edge on, making an orthogonal hydrophobic interaction. The observed position of the 667-coumarin ring is close to that predicted by an earlier docking



Scheme 1 Proposed mechanism for the facile E1cB elimination of sulphamate from 667-coumate to give 667-coumarin

study [15], but in the latter the ring system is rotated by approx. 45° in the clockwise direction, when viewed from above.

The seven-membered ring of 667-coumate appears to have some conformational flexibility, as judged by its temperature factors (*B*-factors), and this is reflected by the quality of the electron density map (Figure 4a). Unexpectedly, the ring appears to be predominately in a boat conformation, in contrast with the previously predicted chair conformation [11] that was also predicted from previous modelling studies [15]. The poor density observed in the electron density map may reflect interconversion of different conformations, and this may explain the lack of density observed for one of the carbon atoms in the seven-membered ring. Examination of the binding site shows that this ring sits within the wide funnel leading to the surface of the protein, where interactions with a neighbouring molecule, including the second zinc atom, are observed (Figure 3a). In the EMATE structure, the third six-membered (C) ring and the fourth five-membered (D) ring are contained within the same area, but this structure was determined in the P2₁ space group, and interactions between the ligand and the symmetry-related protein molecules is much reduced (Figure 3b). However, this ring system is much more rigid than the seven-membered ring of 667-coumate, and this probably explains the ordering in this structure despite almost no binding of this part of the molecule by the protein.

The hydrophobic environment in which the coumarin ring system is bound probably explains the enhanced stability of 667-coumate in the presence of CA II [12]. 667-Coumate in solution will exist in its mono-anionic form under physiological and mildly basic conditions since the p*K*_a value of its N-proton was found to be 9.1 in methanol/water (1:1) [11]. It is supposed to rapidly decompose chemically in solution due to the relative ease of elimination of sulphamic acid by an E1cB mechanism, assisted by the extended conjugation present in the coumarin motif (Scheme 1). This type of reaction has been examined physicochemically using simple aryl sulphamate esters [26,27]. Binding in the anionic form to the protein disfavours this process since the lone pair of electrons on the nitrogen are less available due to co-ordination by the Zn(II) atom and, at the same time, it is expected that development of the negative charge on the carbonyl group in the transition state for the E1cB-catalysed elimination is disfavoured by the hydrophobic nature of the binding pocket.

Inhibitors of CA as therapeutic targets

The reported crystal structure demonstrates intact binding of 667-coumate in the active site of CA II. Reversible binding of this inhibitor into the hydrophobic active site of CA II probably accounts for the increased stability in blood [12] compared with

plasma [13] and protection from first pass metabolism. To characterize this binding more fully, the equilibrium dissociation constant for 667-coumate was determined to be 45 ± 6 nM. This compares favourably with the value of 200 nM measured for acetazolamide, a known inhibitor of CA II, at pH 8.45 [21].

It is probable that reversible binding of drugs to CA II in blood will be observed for other sulphamate ester drugs and other reversible inhibitors of CA with other modes of action. Indeed, the enhanced half-life of methazolamide (a methylated tautomer of acetazolamide) has been attributed to exactly this mechanism [28]. Reversible drug binding to CA II may represent a more general strategy for the delivery of many potentially labile drugs, provided that binding is reversible and that any hydrophobic moiety can be accommodated within the enzyme-active site. Moreover, other classes of less labile sulphamate-based drugs, based on 2-methoxyoestradiol, that can disrupt microtubules and have anti-angiogenic effects may also benefit by complexation through the sulphamate moiety to the central zinc atom of CA, thus engendering beneficial bio-availability and metabolic profiles [29].

Dual inhibitors of steroid sulphatase and CA such as 667-coumate represent a novel potential method for treating hormone-dependent tumours. 667-Coumate is known to inhibit CA II strongly *in vitro*, with reported IC₅₀ values of 17 [15] and 25 nM [5]. Although these values for CA are similar to that of EMATE (IC₅₀ = 10 nM) [6], 667-coumate is a more potent inhibitor of steroid sulphatase *in vitro* with an IC₅₀ of 8 nM compared with 25 nM [11]. Whether this dual inhibition activity accounts for its efficacy in treating hormone-dependent breast tumours is currently not clear, since CA II has not been implicated in cancer. However, previous studies have suggested that some good CA II inhibitors are also potent inhibitors of CA IX [17], which is overexpressed in cancerous tissues.

Previous work has shown that discrimination between at least some CA isoforms is possible (e.g. [6,30]), but the details of how this is achieved at the molecular level are not yet known. Since certain isoforms of CA appear to be overexpressed in cancerous tissues [8–10], including CA IX and XII, it seems probable that one can selectively achieve activity against cancerous tissues, although CA has a lesser effect on normal tissues. However, moderate inhibition of erythrocyte CA II needs to be retained, as this represents a protection mechanism during drug delivery.

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