Unexpected Nanomolar Inhibition of Carbonic Anhydrase by COX-2-Selective Celecoxib: New Pharmacological Opportunities Due to Related Binding Site Recognition

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By optimizing binding to a selected target protein, modern drug research strives to develop safe and efficacious agents for the treatment of disease. Selective drug action is intended to minimize undesirable side effects from scatter pharmacology. Celecoxib (Celebrex), valdecoxib (Bextra), and rofecoxib (Vioxx) are nonsteroidal antiinflammatory drugs (NSAIDs) due to selective inhibition of inducible cyclooxygenase COX-2 while sparing inhibition of constitutive COX-1. While rofecoxib contains a methyl sulfone constituent, celecoxib and valdecoxib possess an unsubstituted arylsulfonamide moiety. The latter group is common to many carbonic anhydrase (CA) inhibitors. Using enzyme kinetics and X-ray crystallography, we demonstrate an unexpected nanomolar affinity of the COX-2 specific arylsulfonamide-type celecoxib and valdecoxib for isoenzymes of the totally unrelated carbonic anhydrase (CA) family, such as CA I, II, IV, and IX, whereas the rofecoxib methyl sulfone-type has no effect. When administered orally to glaucomatous rabbits, celecoxib and valdecoxib lowered intraocular pressure, suggesting that these agents may have utility in the treatment of this disorder. The crystal structure of celecoxib in complex with CA II reveals part of this inhibition to be mediated via binding of the sulfonamide group to the catalytic zinc of CA II. To investigate the structural basis for cross-reactivity of these compounds between COX-2 and CA II, we compared the molecular recognition properties of both protein binding pockets in terms of local physicochemical similarities among binding site-exposed amino acids accommodating different portions of the drug molecules. Our approach Cavbase, implemented into Relibase, detects similarities between the sites, suggesting some potential to predict unexpected cross-reactivity of drugs among functionally unrelated target proteins. The observed cross-reactivity with CAs may also contribute to differences in the pharmacological profiles, in particular with respect to glaucoma and anticancer therapy and may suggest new opportunities of these COX-2 selective NSAIDs.

Introduction

Cyclooxygenases (COX) catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins (PGs) and thromboxane.[1] PGs are important biological mediators that signal through a family of G-protein-coupled receptors.[2] These signaling pathways result in widely divergent physiological responses including transmission of pain and inflammation as well as maintenance of kidney function and intestinal homeostasis.[3−7] Recognition of two distinct forms of cyclooxygenase has led to the hypothesis that selective inhibition of the inducible form (COX-2) while sparing the constitutive form (COX-1) may allow generation of safer drugs for controlling the pain and inflammation associated with chronic inflammatory diseases such as rheumatoid- and osteo-arthritis.[8] Indeed, discovery and development of the COX-2 selective agents celecoxib (Celebrex) and rofecoxib (Vioxx) has demonstrated that these COX-1 sparing agents yield efficacy comparable to nonselective nonsteroidal antiinflammatory agents (NSAIDs; e.g. diclofenac or naproxen), both in animal models of inflammation as well as in the clinic.[9−12] Moreover, the new COX-2 selective agents greatly reduce the incidence of serious gastrointestinal side effects associated with NSAID use attendant to COX-1 inhibition.[13,14] Overall, clinical experience with these COX-2 selective agents has been positive, demonstrating that inhibition of COX-2 is a well tolerated and efficacious therapeutic approach. Although both celecoxib and rofecoxib target COX-2 in vivo, results from several large clinical trials have provided evidence that the use of these two agents can lead to differences in clinical response patterns.[15] For example, patients treated with rofecoxib demonstrate an increased incidence of hypertension and edema that is not seen in patients treated with celecoxib.[16−18] This difference in clinical safety profiles indicates that these marketed COX-2 selective agents are not identical in their actions and suggests that celecoxib and rofecoxib possess characteristics that distinguish their pharmacodynamic and/or pharmacological responses.

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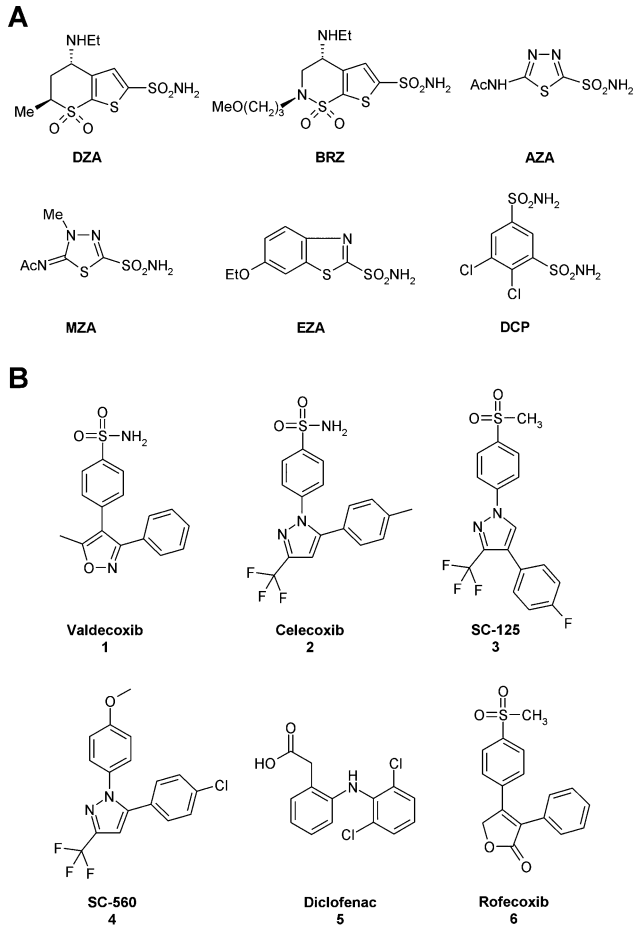

Figure 1. Chemical formulas of sulfonamide drugs dorzolamide (DZA), brinzolamide (BRZ), acetazolamide (AZA), methazolamide (MZA), ethoxzolamide (EZA), and dichlorophenamide (DCP) (A). Chemical formulas of COX-2 specific inhibitors valdecoxib **1**, celecoxib **2**, SC-125 **3**, SC-560 **4**, and diclofenac **5** used as standard during in vitro testing and rofecoxib **6** (B).

A key structural feature that distinguishes celecoxib and rofecoxib is the presence of an aryl sulfonamide moiety in celecoxib; rofecoxib possesses instead a methyl sulfone constituent but not a sulfonamide (Figure 1B). Agents containing sulfonamides are extensively employed in clinical medicine both as diagnostic tools and as therapeutics. For example, sulfonamide-containing agents are employed for inhibiting the zinc-containing enzyme carbonic anhydrase (CA), a family of 14 distinct enzymes,[19] for the treatment of glaucoma and macular edema.[19,20] Several such drugs are clinically employed including the recently developed topical agents dorzolamide (DZA) and brinzolamide (BRZ) and classical, systemically administered compounds such as acetazolamide (AZA), methazolamide (MZA), ethoxzolamide (EZA), and dichlorophenamide (DCP) which have been in clinical use for more than 45 years (Figure 1A).[19,20] The sulfonamide moiety of these CA-inhibitory agents has been shown to mediate a high affinity interaction of the aryl sulfonamide group with the Zn^{2+} ion bound to CA.[21] The presence of a sulfonamide moiety on celecoxib, therefore, may impart pharmacological properties not achieved with rofecoxib. The X-ray crystal structure of SC-558 (PDB code 6cox), a related *p*-bromo derivative of celecoxib, bound to COX-2, has been determined.[22] In this structure, SC-558's sulfonamide

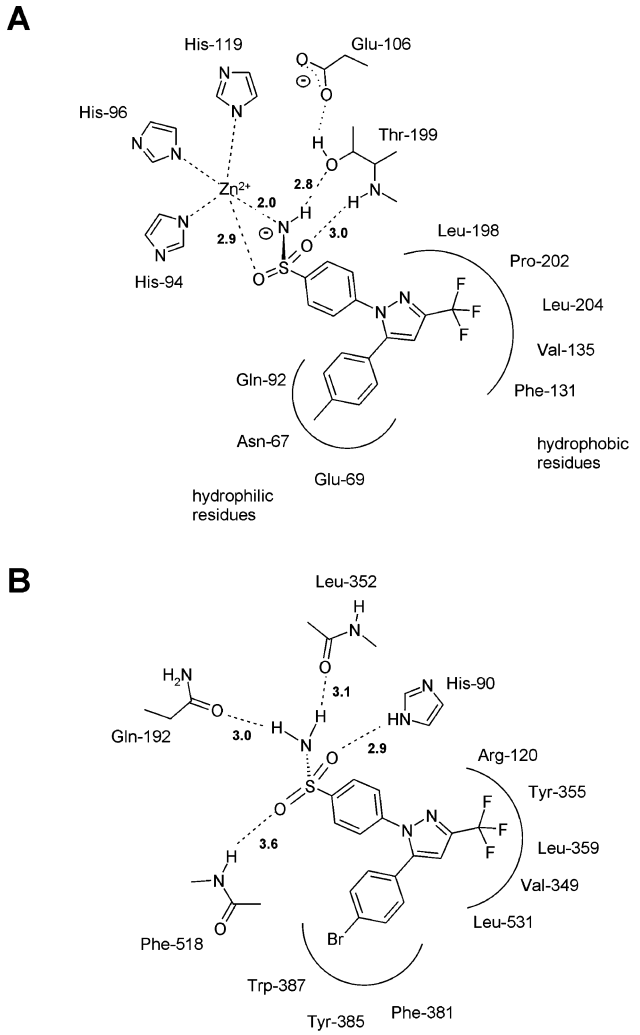

Figure 2. A. Schematic drawing of the interactions formed between CA II and celecoxib. Hydrogen bonds formed between enzyme and the inhibitor are shown as dotted lines. Bond lengths are given in Å. Residues with a distance less than 4 Å around the inhibitor are schematically drawn. They form a hydrophobic subsite (Leu-198, Pro-202, Leu-204, Val-135, and Phe-131) and a second more hydrophilic subsite (Gln-92, Asn-67, and Glu-69). B. Schematic drawing of the interactions formed between COX-2 and the bromo derivative SC-558 of celecoxib. A similar representation as in 2A shows the residues in a 4 Å sphere around the inhibitor.

group is integrally involved in mediating the binding interaction as a result of being coordinated to three principle residues corresponding to His-90, Gln-192, and Leu-352 (Figure 2B). Therefore, although COX-2 is not a Zn^{2+}-containing enzyme as is CA, the sulfonamide group of SC-558 nontheless functions as a major determinant of the protein-binding interaction. On the basis of the observation that sulfonamide moieties within pharmacological agents are important for binding to both CA and to COX-2, we investigated the interaction of celecoxib with purified CA. To obtain detailed information about the binding mode, we determined the crystal structure of CA II in complex with celecoxib at 1.5 Å resolution. Furthermore, we present a structural comparison of the binding pockets in COX-2 and CA II in an effort to determine whether physicochemical properties of the two binding pockets are conserved or resemble each other.

Table 1. CA Inhibition Data with Standard Inhibitors and the Derivatives **1**−**5**. Inhibitors Were Incubated with Enzymes for 15 Minutes Prior to Assay[25]

	IC_{50} (nM)[a]			
inhibitor	hCA I[b]	hCA II[b]	bCA IV[c]	hCA IX[d]
AZA	250	12	70	25
MZA	50	14	36	27
DZA	50000	9	43	52
DCP	1200	38	380	50
1	54000	43	340	27
2	50000	21	290	16
3	>100 μM	>100 μM	>100 μM	>100 μM
4	>100 μM	>100 μM	>100 μM	>100 μM
5	>100 μM	>100 μM	>100 μM	>100 μM

[a] Errors in the range of 5−10% of the reported value, from three determinations. [b] Human cloned isozymes, esterase assay method. [c] Isolated from bovine lung microsomes, esterase assay method. [d] Human cloned isozyme, CO_2 hydrase assay method.[25]

Results

Pharmacology. The inhibitory properties of derivatives **1**−**5** (Figure 1) as well as clinically used, classical CA inhibitors (CAI; Figure 1) versus several physiologically relevant CA isozymes are given in Table 1. As apparent from the data in Table 1, the sulfonamide-type COX-2 inhibitors **1** and **2** exhibit efficient CA inhibitory potency against CA II, with affinities comparable to those of dichlorophenamide (DCP) (a clinically used CA inhibitor for systemic antiglaucoma medication),[19,20] whereas they inhibit with moderate potency the membrane-bound isozyme CA IV. They act as very weak CA I inhibitors, similarly to dorzolamide (DZA), the clinically applied topical CA inhibitor of Merck.[23] This inhibition profile suggests both COX-2 sulfonamide-containing inhibitors may possess utility in the treatment of glaucoma. The nonsulfonamide COX inhibitors **3**, **4**, and **5** on the other hand do not possess CA inhibitory activity, likely because these inhibitors do not have a sulfonamide anchoring group that is essential for binding to CA. Strikingly, celecoxib and valdecoxib demonstrated very potent inhibition of CA IX. This tumor-associated CA isozyme is a transmembrane protein with a suggested function to either maintain acid−base balance or to interact in intercellular communication.[24] It consists of an N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region, and a short intracytoplasmic tail.[24] CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from, for example, cervix uteri, kidney, lung, esophagus, breast, and colon. In contrast, expression in normal tissues, namely in the epithelia of the gastrointestinal tract, is restricted.[24] As recently proven, some sulfonamides act as potent CA IX inhibitors, and this may be beneficial for the development of novel anticancer therapies.[19] As obvious from the data in Table 1, both celecoxib and valdecoxib act as very potent CA IX inhibitors, with inhibition constants in the range of 16−27 nM. Particularly celecoxib is among the most potent inhibitors yet reported,[25] being much more efficient than acetazolamide and methazolamide, the CAIs *par excellence*. To assess the possible use of these COX-2 inhibitors as potent CA inhibitors for glaucoma treatment, several such drugs were administered systemically to hypertensive rabbits over one week (Table 2). As apparent from Table 2, **1** and **2** demonstrate intraocular pressure (IOP) lowering properties in hypertensive rabbits, whereas the nonsulfonamides **3**, **4**, and **5** do not demonstrate this activity.

Table 2. Average Variations of Intraocular Pressure ΔIOP,[a] after One Week Administration of Different Dosages of Compounds **1**−**5** and AZA (oral administration) in Groups of Five Animals Each (dosing vehicle consisted of 0.5% methylcellulose (weight/vol) Containing 0.1% Polysorbate (Tween 80, vol/vol) in distilled water). Initial Eye Pressure Was in the Range of 31−35 mmHg

		ΔIOP (mmHg)[a]						
compound	dosage (mg/kg)	day 1	day 2	day 3	day 4	day 5	day 6	day 7
vehicle		0.13	0.16	0.12	0.15	0.24	0.29	0.30
1, valdecoxib	10	0.12	0.83	2.44	3.71	5.13	5.89	6.08
2, celecoxib	150	2.35	2.62	2.97	3.84	4.15	4.90	5.87
3, SC-125	30	0.14	0.31	0.22	0.25	0.16	0.34	0.16
4, SC-560	30	0.23	0.17	0.25	0.34	0.30	0.41	0.52
5, diclofenac	30	0.13	0.15	0.24	0.27	0.36	0.34	0.41
AZA	30	6.13	6.25	6.29	7.58	7.95	8.14	8.74

[a] ΔIOP = (average IOP before treatment) − (average IOP after adminstration of drug, measured 2 h postadministration). The mean was calculated by using five animals IOP values on each drug dosage. Errors of IOP measuring are in the range of 5−10% of the reported values.

Crystal Structure Analysis. Human CA II is located in erythrocytes where it is involved in respiration by catalyzing the reversible hydration of carbon dioxide and water to bicarbonate and a proton. The structural motif of the zinc-containing one-domain enzyme consists of a 10-stranded, twisted β-sheet. The zinc ion is located at the bottom of a cone-shaped cavity and coordinates to three histidine residues (His-94, His-96, His-119) and a solvent molecule in the native structure.[26] NMR spectroscopy shows that sulfonamides predominantly coordinate with the monoprotonated and negatively charged nitrogen of the sulfonamide to the zinc.[27] To understand the high affinity binding of celecoxib on a structural basis, we determined the crystal structure of the hCA II−celecoxib complex (PDB code 1oq5). Celecoxib adopts a similar binding mode compared to typical sulfonamide CAIs.[27] It binds with its sulfonamide toward the zinc resulting in a tetrahedral coordination. Two additional hydrogen bonds to the side chain oxygen atom of Thr-199 (2.8 Å) and the backbone nitrogen atom of Thr-199 (3.0 Å) confirm the tight binding of celecoxib (Figure 2A). The second oxygen atom of the sulfonamide group is oriented toward the zinc atom (2.9 Å), revealing a slight distortion of the tetrahedral coordination geometry. The inhibitor coordinates zinc via its likely deprotonated sulfonamide group replacing the zinc-bound water molecule (Figure 3A) thereby forming the fourth coordination site at zinc (2.0 Å). The terminal sulfonamide group has a more acidic character due to a strong induced dielectric fit in the close neighborhood of the positively charged zinc. The Thr-199 side chain hydroxyl group forms an additional hydrogen bond to the carboxylate group of Glu-106 (2.6 Å); in consequence, Thr-199 can only operate as a hydrogen-bond acceptor during inhibitor binding. The *p*-tolyl group of celecoxib is surrounded by Asn-67, Glu-69, and Gln-92, whereas the trifluoromethyl group is pointing toward the more hydrophobic residues Phe-131, Val-135, Leu-198, Pro-202, and Leu-204. The

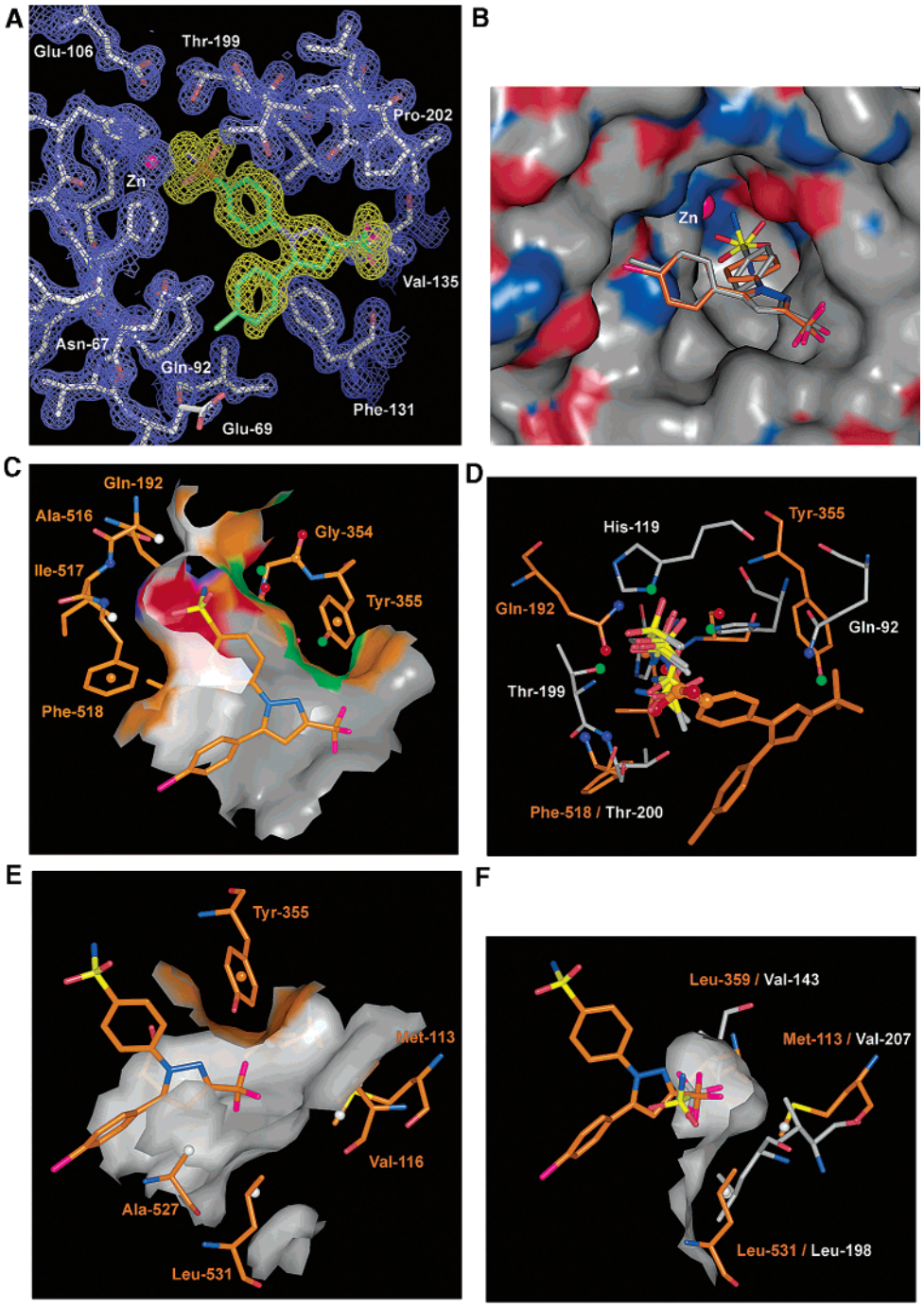

Figure 3. A. Electron density of the celecoxib inhibitor. The electron density is shown in the active site of CA II for celecoxib. The σ_A-weighted $F_o - F_c$ electron density is contoured at 3σ in yellow and shown within a radius of 2 Å around the inhibitor. Except for the phenyl moiety oriented toward the surface of the cone-shaped binding pocket, electron density unambiguously allowed placement of the inhibitor. All molecule representations are drawn in PyMOL.[41] B. Solvent accessible surface of the binding pocket of CA II hosting celecoxib colored by contacting areas of neighboring nitrogen (blue), oxygen (red), and sulfur (yellow). The ligand is shown color-coded by atom types (oxygen in red, nitrogen in blue, sulfur in yellow, halogens in magenta) in its binding mode observed in CA II. The bound conformation as observed in COX-2 is shown in orange (throughout all figures). The sulfonamide anchor adopts a different orientation in both enzymes. The sulfonamide of celecoxib coordinates to zinc (magenta). The RMSD values between both conformers is 0.37 Å neglecting the bromide atom and the terminal methyl carbon of the tolyl portion of celecoxib and the oxygen and nitrogen atoms at both sulfonamide anchors; considering all atoms reveals a RMSD = 0.81 Å. C. The subcavity around the sulfonamide group in COX-2 (PDB code 6cox)[22] is used as query cavity for similarity matching. Some amino acids are not shown for reasons of clarity. The pseudocenters, represented as colored spheres, are used to describe the physicochemical properties of the binding site. Color scheme: H-bond donor (blue), H-bond acceptor (red), ambivalent donor/acceptor (green), hydrophobic aliphatic (white), aromatic (orange). D. Local environment of the sulfonamide group of SC-558 as bound to COX-2 (orange). Superimposed are sulfonamide portions as extracted from related hCA II binding pockets. E. Subcavity around the trifluoromethyl group of SC-558 in COX-2 used as query cavity for similarity matching (for color coding see Figure 3C). F. Local environment around the trifluoromethyl group of SC-558 bound to COX-2 (orange). On rank 76 of a Cavbase search, a CA structure (PDB code 1bcd)[42] is found. Interestingly enough, this CA cavity accommodates a CF_3 group. It is shown together with the superposition of the two trifluoromethyl groups (RMSD = 0.78 Å, originating from this CA II structure and the COX-2 example). It is displayed together with its surface and the contributing residues.

Table 3. Data Collection and Refinement Statistics for the CA II Celecoxib Inhibitor Complex

	celecoxib complex
resolution range (Å)	30−1.5
space group	$P2_1$
unit cell (Å, deg for β)	$a = 42.0$, $b = 41.1$, $c = 71.9$, $\beta = 104.3$
highest resolution shell (Å)	1.53−1.5
no. of observations	123343
no. of unique reflections	35103
completeness (%)	91.5 [37.0][a]
mean I/σ_I	18.8 [2.8]
R_{sym} (%)[b]	9.3 [31.1]
refined residues	257
refined substrate atoms/Zn atom	26/1
refined water molecules	233
resolution range in refinement (Å)	10−1.5
R_{cryst} ($F_o > 4\sigma F_o$; F_o)[c]	12.7, 13.1
R_{free} ($F_o > 4\sigma F_o$; F_o)[d]	18.4, 18.9
Rms deviations	
bond lengths (Å)	0.009
bond angles (deg)	2.1
average B value protein (Å^2)	22.3
main chain (Å^2)	19.9
side chain (Å^2)	24.7
substrate/Zn (Å^2)	24.4/13.9
waters (Å^2)	34.0
Ramachandran plot[e]	
most favored (%)	87.4
additionally allowed (%)	12.1
generously allowed (%)	0.5
disallowed (%)	0.0

[a] Values in brackets are statistics for the highest resolution shell. The low completeness in the outer shell is caused by the squared format of the R-Axis 4 detector and not by any anisotropic diffraction of the crystal. However, we decided to include these reflections in the refinement due to their good statistics. [b] $R_{sym} = [\Sigma_h\Sigma_i|I_i(h) - \langle I(h)\rangle|/\Sigma_h\Sigma_i I_i(h)] \times 100$, where $\langle I(h)\rangle$ is the mean of the $I(h)$ observation of reflection h. [c] $R_{cryst} = \Sigma_{hkl}|F_o - F_c|/\Sigma_{hkl}|F_o|$. [d] R_{free} is calculated in the same manner as R_{cryst} but from 5% of the data that were not used for refinement. [e] From Procheck.[43]

electron density assigned to the *p*-tolyl group is only partially defined indicating some possible disorder or enhanced mobility of this group. Interestingly, it is oriented toward the upper rim of the conical binding pocket of hCA II, thus being in close contact to the solvent environment. Considering the observed binding mode, an additional hydrophilic group replacing the methyl group at the terminal tolyl moiety should be beneficial for binding due to polar residues of hCA II in this area of the binding pocket. In addition to the mentioned hydrogen bonds celecoxib forms 64 van der Waals contacts to CA II. The inhibitor is deeply anchored in the enzyme binding pocket burying 79.3% of the molecular surface of celecoxib (262.6 Å^2 out of 331 Å^2).

Comparison of Binding Site Geometries. For the following structural comparison of the binding pockets of COX-2 and CA II, the crystal structure of the related *p*-bromo derivative of celecoxib, SC-558, in complex with COX-2[22] and the structure determined here will be used. The *p*-methyl group and the *p*-bromo substituent can be assumed to be strictly bioisosteric in the present case.

Celecoxib possesses a fairly rigid skeleton. The bound conformation in both structures superimposes well using the coordinates of the atoms composing the three ring systems (Figure 3B). The orientation of the sulfonamide anchor deviates in both structures (Figures 2, 3B). With respect to a best plane running through sulfur and the atoms of the adjacent phenyl ring, a locally mirrored arrangement of exposed donor (NH) and acceptor (O) functionalities at sulfur toward the protein sites becomes evident. This flipped arrangement complicates an overall comparison of both binding pockets. Accordingly, we tried to match separately the three local subcavities hosting sulfonamide, trifluoromethyl, and bromophenyl group of SC-558. For this comparison we used our recently introduced algorithm Cavbase to screen and match protein binding pockets in terms of surface-exposed physicochemical properties.[28,29] In Cavbase, the amino acids of the protein binding site are represented by pseudocenters to map binding-site properties. They are subsequently used to match related binding sites. A similarity score is calculated according to the degree of overlapping surface patches to rank all mutual compared cavities.

Table 4. Pseudocenters Assigned to the Corresponding Amino Acids Shared in Common in the Subpockets Accommodating the Sulfonamide Group in CA II and COX-2

COX-2 (6cox)			CA-II (1bn4)		
type	corresponding amino acid[a]		type	corresponding amino acid[a]	
donor	F 518	p	donor	T 200	p
don_acc	Y 355	s	donor	Q 92	s
acceptor	S 353	p	don_acc	H 94	s
acceptor	L 352	p	don_acc	H 96	s
aromatic	L 352	p	aromatic	H 96	s
donor	Q 192	s	don_acc	H 119	s
acceptor	Q 192	s	don_acc	T 199	s

[a] One-letter residue name, residue number and origin (s: center originates from side chain atom(s); p: center originates from peptide backbone atom)

The cavity accommodating the sulfonamide anchor in COX-2 was used as reference cavity (Figure 3C) considering 25 pseudocenters. It has been screened against a set of 9433 ligand-containing cavities with Cavbase. From this list all COX cavities have been discarded. In total a collection of 65 cavities originating from CAs have been considered. As a result on rank 38 (means after 0.4% of the considered sample set of cavities) the first CA cavity is retrieved, followed by further examples from this enzyme class on subsequent ranks. Although Cavbase does not exploit any information about bound ligands, the orientation of the sulfonamide groups in the retrieved CAs match well with that of SC-558 bound to COX-2 (Figure 3D, Table 4). However, one complication has to be considered in this comparison: due to the locally induced pK_a shift next to Zn^{2+} in CA II, the sulfonamide group supposedly binds deprotonated,[27] whereas in COX-2 a similar dramatic pK_a shift appears unlikely regarding the local binding partners (Figure 2B). In consequence, at both sites the donor and acceptor functionalities of this sulfonamide group have to be considered as mutually equivalent. Formally in our analysis the position of the zinc coordinated to the deprotonated nitrogen of the sulfonamide group roughly coincides with the position of the second proton present at this sulfonamide group once bound to COX-2.

The subcavity accommodating the trifluoromethyl group in the COX-2 reference is defined by seven residue-assigned pseudocenters (Figure 3E). A Cavbase search was performed on the same list of 9433 cavities mentioned above. Among the 200 best ranked solutions 41 CAs are found, which bind in a similar region as the

Table 5. Pseudocenters Assigned to the Corresponding Amino Acids Shared in Common in the Subpockets Accommodating the Trifluoromethyl Group in CA II and COX-2

COX-2 (6cox)			CA-II (1bcd)		
type	corresponding amino acid[a]		type	corresponding amino acid[a]	
aliphatic	L 359	s	aliphatic	V 143	s
aliphatic	M 113	s	aliphatic	V 207	s
aliphatic	L 531	s	aliphatic	L 198	s

[a] One-letter residue name, residue number, and origin (s: center originates from side chain atom(s); p: center originates from peptide backbone atom).

trifluoromethyl group of SC-558 (Table 5). Interestingly, on rank 76 (after 0.8% of the entire probe sample set), a CA subsite is detected that hosts a CF_3-containing sulfonamide inhibitor. Its trifluoromethyl group superimposes well with that of SC-558 in COX-2 (Figure 3F). As matching residues, the exposed side chains of residues Val-143, Val-207, and Leu-198 in CA II are detected which overlap with Leu-359, Met-133, and Leu-531 in COX-2.

Finally, we tried to match the cavity accommodating the bromophenyl group of SC-558 in COX-2 with that hosting the tolyl moiety of celecoxib in CA II. In our Cavbase search we did not find any CA structure on the first ranks. This result is not surprising because the rather deeply buried COX-2 subcavity is composed of aromatic residues (Phe-518, Trp-387, Tyr-385, Phe-381; Figure 2B), whereas the open, partially solvent-exposed subcavity from CA II is formed by hydrophilic residues (Asn-67, Glu-69, Gln-92). Thus, both subcavities exhibit quite dissimilar physicochemical properties, impossible to match by our approach.

Discussion

Modern drug discovery research aims to develop safe and efficacious therapeutic agents in part by optimizing binding to a single selected protein target. This strategy is intended to minimize undesirable effects resulting from side scatter pharmacology. An appreciation of the scope of this task, however, has increased as a result of the various genome sequencing projects unveiling the existence of protein families possessing related functional binding pockets. Frequently, these proteins are involved in completely different biochemical pathways and are localized in distinct tissues and organs. Despite the challenge, selectivity can be engineered into small molecules, and the design of agents that affect COX-2 while not impacting COX-1 represents a remarkable success story.[30] In this pursuit, the focus of the task was to develop agents that affected COX-2 but spared activity of the constitutively expressed COX-1 enzyme. In this type of targeted pursuit for selectivity, interactions with proteins that are unrelated in sequence and/or function to the primary targets are difficult to predict and to assess. Similarity in the shape and exposed physiochemical properties of binding pockets on functionally diverse proteins can lead to unexpected cross reactivity. Considering the vast combinatorial multiplicity resulting from the combination of 30 to 50 atoms in a typical drug-sized molecule, the task of finding highly selective ligands would seem very achievable. Surprisingly, however, a small number of priviledged building blocks are found recurrently in successful drug molecules, making these preferred elements of the medicinal chemists' tool box. One such building block is the sulfonamide group, and as exemplified in this study the arylsulfonamide group of celecoxib is a key mediator of the interaction of this agent with both COX-2 and CA II. These two enzymes are of totally different biochemical functions; however, they obviously bind the same type of ligand with comparable binding affinities. Would such behavior be predictable? One possible approach is the systematic analysis of ligand skeletons for common building blocks that suggests multiple binding to unrelated proteins. Given the high preference of sulfonamide anchors to bind to CAs, this strategy might be evident in the present case. However, it is the protein binding site that determines the features responsible for ligand recognition and binding of a ligand.

Our recently introduced Cavbase approach to compare binding cavities in terms of related physicochemical surface properties matches in part the binding pockets of COX-2 and CA II. In both enzymes the subcavities accommodating the different functional groups of celecoxib are mutually arranged with similar topography. In the sulfonamide binding pockets a flipped orientation of exposed donor and acceptor properties is given. Using the recognition pockets of COX-2 which host the sulfonamide and trifluoromethyl group as reference allows retrieval of CA cavities that bind the same functional groups with similar orientations. Since the matching surface patches of these subcavities are rather small, they do not produce a high similarity score with the query cavity from COX-2. The commonly found patches in the matching CA pockets are mostly not scored on the most prominent ranks. Adding the separate ranks of individual subcavity matches to a total composite similarity measure accomplishes higher significance. Merging the rankings of the sulfonamide and trifluoromethyl subpockets into one combined rank places the first carbonic anhydrase entry (PDB code 1bn4,[31]) already on the third position. In summary, Cavbase seems to possess some potential to predict unexpected cross reactivity of known drugs to other functionally unrelated proteins.[32]

Conclusions

Cross reactivity of celecoxib to COX-2 and human CA II has been demonstrated by enzyme kinetics and crystal structure analysis. A comparison of the binding cavities of both enzymes indicates some relationship of the exposed recognition properties. Elimination of the essential aryl sulfonamide group as in rofecoxib **6** and SC-125 **3**, leads to a loss of the cross reactivity with CAs. In addition to providing a possible explanation for why celecoxib and rofecoxib possess distinct clinical response profiles, the present findings may suggest new opportunities. As already noted, CA inhibitors are employed clinically for the treatment of glaucoma; CA II and CA IV are both located in the glaucomatous eye, and inhibition of their activity is associated with lowering of intraocular pressure.[19,20] When administered orally to glaucomatous rabbits, celecoxib and valdecoxib lowered IOP, suggesting that these agents may have utility in the treatment of this disorder. Similarly, a number of in vitro and in vivo studies have suggested that COX-2 inhibitors possess anticancer properties,[33−35]

and celecoxib is approved clinically to reduce the number of adenomatous colorectal polyps in patients with familial adenomatous polyposis as an adjunct to usual care. Interestingly, a recent study found that celecoxib, but not rofecoxib, inhibited growth of hematopoietic and epithelial cell lines that did not express COX-2.[35] The presence of a pharmacological effect in the absence of COX-2 suggests that the response to celecoxib is derived independently of prostanoid metabolism at least in some cases. A number of cancer cell lines are known to upregulate expression of CA isoforms, and mainly CA IX,[24,25] and perhaps a celecoxib-mediated inhibition of CA is in part responsible for this type of pharmacological response, although other non-COX-2-dependent activities have been proposed.[24,25] Indeed, here we show that valdecoxib and celecoxib are very potent CA inhibitors, which has been confirmed by the CA II structure in complex with celecoxib. The cross reactivity of celecoxib with CA II and COX-2 can be explained by structural similarities across the subsites of the binding pockets in both enzymes. At these sites the inhibitor is recognized similarly by related physicochemical properties. The question is whether the lately reported case of cross-reactivity of NSAIDs inhibiting prostanoid efflux demonstrates a similarity of binding sites.[36]

Experimental Section

Biological Data. Acetazolamide (AZA) was the most effective IOP lowering agent via the systemic route, with a potent effect (of around 6 mmHg IOP lowering) already observed after the first administration, which then has been maintained for the next 2−5 days, and finally plateaued at about 8 mmHg after one week of administration. Valdecoxib **1** was also effective as a systemic IOP lowering agent at several dosages investigated here (Table 2, and data not shown). In contrast to acetazolamide, in the first 3 days of administration, **1** showed only a weak IOP lowering effect (of 2−3 mmHg), which tended to increase in the next 3 days, and then leveling off at about 6 mmHg at day 5 till the end of the study (day 7). The same type of behavior has been observed for celecoxib **2**, but this compound was slightly less effective than valdecoxib: in the first 2 days of administration the effect of celecoxib **2** was very weak; then a more potent IOP lowering was gradually achieved in the next days, arriving at a maximal effect similar to that of valdecoxib, i.e., an IOP lowering of around 6 mmHg after one week of administration.

Crystallization and Structure Determination. The CA II enzyme (5 mg/mL) crystallizes at 4 °C from 2.5 M $(NH_4)_2SO_4$, 0.1 M Tris-HCl pH 8.2 and 0.3 M NaCl in space group $P2_1$. The complex was obtained by cocrystallization of the enzyme with the inhibitor at 1 mmol/L. For cryoprotection, crystals were briefly soaked in mother liquor containing 20% glycerol. The data set was collected on a Rigaku rotating anode generator operated at 50 kV, 90 mA equipped with a Raxis 4++ imaging plate detector; 402 frames with $\delta\varphi = 0.5°$ at a crystal-to-detector distance of 100 mm were collected at −170 °C. Data were processed and scaled with Denzo and Scalepack.[37] Due to the square format of the detector, the highest resolution shell is rather incomplete; nevertheless data were included since they have good counting statistics and R_{sym} values. CA II (PDB code 1cil)[23] with the inhibitor and zinc atom coordinates omitted was used as a starting model for rigid body refinement in CNS.[38] Initial refinement was continued in CNS using positional and slow-cooling protocols followed by restraind *B*-value refinement. Refinement was then continued with SHELXL-97.[39] For each refinement step, at least 10 cycles of conjugate gradient minimization were performed, with restraints on bond distances, angles, and *B*-values. Full anisotropic refinement seemed justified based on a drop in R_{free}. In the final stages, hydrogen atoms were placed in calculated positions without use of additional parameters. Intermittent cycles of model building were done with the program O.[40] The coordinates have been deposited in the PDB (http://www.rcsb.org/pdb/) with access code 1oq5 and are available immediately from klebe@mailer.uni-marburg.de.

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