Crystal structure of rofecoxib bound to human cyclooxygenase-2

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Rofecoxib (Vioxx) was one of the first selective cyclooxygenase-2 (COX-2) inhibitors (coxibs) to be approved for use in humans. Within five years after its release to the public, Vioxx was withdrawn from the market owing to the adverse cardiovascular effects of the drug. Despite the widespread knowledge of the development and withdrawal of Vioxx, relatively little is known at the molecular level about how the inhibitor binds to COX-2. Vioxx is unique in that the inhibitor contains a methyl sulfone moiety in place of the sulfonamide moiety found in other coxibs such as celecoxib and valdecoxib. Here, new crystallization conditions were identified that allowed the structural determination of human COX-2 in complex with Vioxx and the structure was subsequently determined to 2.7 Å resolution. The crystal structure provides the first atomic level details of the binding of Vioxx to COX-2. As anticipated, Vioxx binds with its methyl sulfone moiety located in the side pocket of the cyclooxygenase channel, providing support for the isoform selectivity of this drug.

1. Introduction

The cyclooxygenase enzymes (COX-1 and COX-2) catalyze the committed step in the biosynthesis of prostaglandins, prostacyclins and thromboxanes (reviewed in Smith *et al.*, 2011). Sequential cyclooxygenase and peroxidase reactions carried out in two separate but functionally linked active sites convert arachidonic acid (AA) into prostaglandin H₂ (PGH₂). PGH₂ is subsequently metabolized by downstream tissue-specific synthases into bioactive signaling molecules. While the catalytic mechanism involved in the conversion of AA to PGH₂ is conserved between isoforms, COX-2 efficiently oxygenates a broad spectrum of fatty-acid and endocannabinoid ester substrates, whereas COX-1 preferentially oxygenates AA. In general, COX-1 is constitutively expressed in most tissues, while the expression of COX-2 is tissue-specific and is largely induced upon stimulation by cytokines and growth hormones. As such, COX-1 and COX-2 have distinct physiological roles. COX-1 is typically involved in more homeostatic functions such as the regulation of platelet aggregation and gastric acidity, whereas COX-2 is typically involved in pathological states including inflammation, pain and fever (Morita, 2002).

COX-1 and COX-2 are the pharmacological targets of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen. Inhibition of COX-1 in the bloodstream provides beneficial cardiovascular effects, including reduced platelet aggregation, as observed with low-dose aspirin regimens (Campbell *et al.*, 2007). However, prolonged inhibition of COX-1 in the gastrointestinal system can result in ulcer formation and gastric bleeding (Wallace, 2008). Injury to the gastrointestinal tract is one of the main complications

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encountered with prolonged ingestion of NSAIDs. Selective COX-2 inhibitors (coxibs), which include celecoxib (Celebrex) and rofecoxib (Vioxx), were designed to inhibit COX-2 over COX-1, thus providing the desired pain relief and anti-inflammatory properties while avoiding the gastric complications encountered with typical nonselective NSAIDs (DeWitt, 1999). Preliminary studies showed that coxibs exhibited anti-inflammatory properties that are similar to those of traditional NSAIDs, but with reduced gastrointestinal toxicity. However, subsequent clinical trials examining the efficacy of coxibs in reducing the occurrence of cancerous colon polyps revealed that these drugs increase the risk of cardiovascular complications, which led to the withdrawal of Vioxx from the market (Marnett, 2009).

COX-1 and COX-2 are virtually identical in their overall tertiary structure (Garavito *et al.*, 2002). However, one of the hallmark differences between COX-1 and COX-2 is the first-shell substitutions of the cyclooxygenase channel residues Ile434, His513 and Ile523 in COX-1 by Val434, Arg513 and Val523, respectively, in COX-2. These substitutions result in a ~25% increase in the volume of the active site and the creation of a side pocket off the main channel with Arg513 located at its base (Garavito *et al.*, 2002). The coxibs were built upon a diaryl heterocycle-based chemical scaffold, with a sulfonamide or methyl sulfone moiety designed to bind within the side pocket to provide isoform-selective inhibition (Marnett, 2009). The presence of valine instead of isoleucine at position 523 in COX-2 provides access for the coxibs to the side pocket. Indeed, substitution of Val523 in COX-2 by isoleucine abolishes isoform-selective inhibition, while substitution of Ile523 in COX-1 by valine enhances isoform-selective inhibition (Wong *et al.*, 1997; Gierse *et al.*, 1996).

NSAID and coxib binding within the cyclooxygenase channel of COX-1 and COX-2 has been extensively characterized by utilizing site-directed mutagenesis coupled to functional studies, as well as by X-ray crystallographic structure determinations (reviewed in Blobaum & Marnett, 2007). Despite the significant attention that has been paid to its development and withdrawal from the market, no structural data have been published on the binding of Vioxx within the cyclooxygenase channel of COX-2. Here, we report the identification of novel crystallization conditions for human COX-2 (huCOX-2) that allowed the X-ray crystal structure of huCOX-2 in complex with Vioxx to be determined to 2.7 Å resolution. Our structure reveals that Vioxx adopts a similar binding pose within the cyclooxygenase channel as that observed for celecoxib, with its methyl sulfone moiety inserted into the side pocket near Arg513.

2. Materials and methods

2.1. Protein production, crystallization and data collection

For crystallization, we utilized a wild-type huCOX-2 construct that had been engineered to contain a FLAG affinity tag at the N-terminus and a deletion of residues 586–612 (Δ 586) at the C-terminus (Lucido *et al.*, 2016). The resulting

FLAG Δ 586 huCOX-2 construct was used to generate purified protein in 25 mM Tris pH 8.0, 150 mM NaCl, 0.53% (w/v) *n*-octyl β -D-glucopyranoside (β OG) as described previously (Lucido *et al.*, 2016). Prior to crystallization screening, FLAG Δ 586 huCOX-2 was concentrated to 4.9 mg ml⁻¹ and reconstituted with a twofold molar excess of Co³⁺-protoporphyrin IX. A 2.5-fold molar excess of Vioxx was then added to the reconstituted enzyme and allowed to incubate on ice for 30 min before dispensing for crystallization screening.

We initially attempted to solve the crystal structure of Vioxx bound to huCOX-2 using the crystallization conditions that have previously been utilized to crystallize murine COX-2 (muCOX-2; Vecchio *et al.*, 2010, 2012; Vecchio & Malkowski, 2011a) and huCOX-2 (Lucido *et al.*, 2016). However, we found that Vioxx was extremely insoluble in these polyacrylic acid 5100-based conditions. Attempts to soak the inhibitor into the active site of huCOX-2 crystals grown under these conditions were also futile, as Vioxx itself crystallized almost instantaneously upon addition to the crystallization drop, resulting in huCOX-2 structures with empty active sites (data not shown). We then utilized high-throughput crystallization screening (Luft *et al.*, 2011) coupled to a tailored membrane-protein screen (Kozelak-Rosenblum *et al.*, 2009) in an attempt to identify leads that were amenable to maintaining the solubility of Vioxx. Multiple new crystallization leads were identified, in which the conditions predominantly consisted of ammonium phosphate or PEG 400 as the major precipitant.

One lead was subsequently optimized in the sitting-drop vapor-diffusion format at 23°C. 3 μ l protein solution was combined with 3 μ l of a drop solution consisting of 27–32% PEG 400, 300 mM ammonium phosphate, 100 mM HEPES pH 7.0 and equilibrated over a reservoir containing 0.5 ml precipitant solution. Crystals appeared as long rods (Supplementary Fig. S1) within two weeks and were subsequently harvested, followed by a soak in 32% PEG 400, 300 mM ammonium phosphate, 100 mM HEPES pH 7.0, 0.6% (w/v) β OG supplemented with 10% glycerol for 20 min before cooling directly in a gaseous nitrogen stream cooled to 100 K. Diffraction data were collected on beamline 23ID-B at the Advanced Photon Source at Argonne National Laboratory. Intensities were integrated, scaled and merged in the orthorhombic space group *I*222 using *HKL-2000* (Otwinowski & Minor, 1997). The elongated rod shape of the crystals proved to be advantageous in that diffraction data could be collected from multiple positions on the crystal using a small 20 μ m beam size. Combining data from multiple positions on the crystal mitigated the effects of radiation damage. Data-collection statistics are summarized in Table 1.

2.2. Structure solution and refinement

The structure was solved by molecular replacement (MR) using *Phaser* (McCoy *et al.*, 2007) and a truncated search model of huCOX-2 derived from PDB entry 5f19 (Lucido *et al.*, 2016), with residues 109–125, ligands, cofactors and waters removed. Two monomers were located in the crystallographic asymmetric unit. After MR, residues 109–125 were rebuilt

Table 1

Crystallographic data-collection and refinement statistics for huCOX2–Vioxx.

Values in parentheses are for the outermost resolution shell.

Space group	<i>I</i> 222
No. of molecules in asymmetric unit	2
Wavelength (Å)	1.000
Unit-cell parameters	
<i>a</i> (Å)	126.99
<i>b</i> (Å)	149.42
<i>c</i> (Å)	185.06
$\alpha = \beta = \gamma$ (°)	90.00
Resolution (Å)	30.00–2.70 (2.74–2.70)
Total No. of observations	340625
Total No. of unique observations	48415
Multiplicity	7.0 (7.1)
Completeness (%)	100.0 (100.0)
Mean <i>I</i> / σ (<i>I</i>)	17.9 (2.1)
<i>R</i> _{merge} [†] (%)	9.0 (46.6)
<i>R</i> _{meas} [‡] (%)	11.5 (97.1)
<i>R</i> _{p.i.m.} [‡] (%)	4.3 (36.4)
CC _{1/2} [‡]	0.85
Wilson <i>B</i> factor (Å ²)	43.30
No. of atoms in refinement	9344
<i>R</i> _{work} (%)	17.8 (21.0)
<i>R</i> _{free} [§] (%)	22.0 (26.4)
Average <i>B</i> factor (Å ²)	
Protein	48.5
Solvent	34.9
Vioxx monomer <i>A</i>	50.0
Vioxx monomer <i>B</i>	90.9
Mean positional error [¶] (Å)	0.36
R.m.s.d., bond lengths (Å)	0.005
R.m.s.d., bond angles (°)	1.275
Ramachandran plot	
Favored (%)	97
Allowed (%)	3
Disallowed (%)	0
Clashscore ^{††}	2.00

[†] *R*_{merge}, *R*_{meas} and *R*_{p.i.m.} are as defined in HKL-2000 (Otwinowski & Minor, 1997). [‡] CC_{1/2} in the highest resolution shell is as defined in Karplus & Diederichs (2012). [§] 5.0% of the reflections were utilized to generate the test set. [¶] Coordinate error as calculated from the Luzzati plot. ^{††} Clashscore as calculated in *MolProbity* (Chen *et al.*, 2010).

using *Coot* (Emsley & Cowtan, 2004) and the structure was initially refined utilizing *phenix.refine* (Afonine *et al.*, 2012) and applying NCS restraints. Successive rounds of manual model building and refinement were used to build in the inhibitor, protoporphyrin IX, sugars and waters. In the final rounds of refinement, NCS restraints were released and translation–libration–screw (TLS) refinement (Winn *et al.*, 2001) was applied utilizing the *TLSMD* web server (Painter & Merritt, 2006). The refinement statistics are summarized in Table 1. Structure validation was performed with *MolProbity* (Chen *et al.*, 2010) and simulated-annealing OMIT maps were calculated with *PHENIX* (Adams *et al.*, 2010).

The final model consists of residues Asn34–Pro583, Co³⁺–protoporphyrin IX, carbohydrate moieties linked to Asn144 and Asn410, and Vioxx bound in the cyclooxygenase channel of each monomer. One glycerol molecule, two ammonium ions, six phosphate ions and 154 waters were also modeled into the electron density. The canonical dimer observed within the crystallographic asymmetric unit is identical to that seen in other structures of huCOX-2 and muCOX-2 in complex with substrates and inhibitors (Lucido *et al.*, 2016; Orlando *et al.*,

2015; Vecchio & Malkowski, 2011a; Vecchio *et al.*, 2010). The domain makeup, including the N-terminal epidermal growth factor-like domain, membrane-binding domain and catalytic domain, is well resolved in each monomer. Moreover, the monomers are virtually identical when compared with each other (C α r.m.s.d. of 0.20 Å for 510 pairs), as well as when they are compared with monomers of COX-2 in complex with substrate (C α r.m.s.d. of 0.24 Å for 510 pairs; Vecchio *et al.*, 2010) and inhibitor (C α r.m.s.d. of 0.28 Å for 502 pairs; Lucido *et al.*, 2016). The conformation of Vioxx in monomer *A* will be used to describe the enzyme–inhibitor interactions and in comparison to the crystal structure of celecoxib bound to muCOX-2. As a matter of convention, cyclooxygenase residues are labeled according to the ovine COX-1 (ovCOX-1) numbering scheme (Smith *et al.*, 2011).

3. Results and discussion

3.1. Vioxx binding pose

Vioxx was observed to bind to huCOX-2 with the methyl sulfone moiety in the side pocket of the cyclooxygenase channel and the phenyl ring extended up towards the side chain of Tyr385 (Fig. 1). The inhibitor makes a total of 42 contacts with residues lining the cyclooxygenase channel (Supplementary Table S1). The only hydrophilic interactions made by Vioxx within the cyclooxygenase channel are those formed between the O atoms of the methyl sulfone moiety of Vioxx and the side-chain N atoms of His90 and Arg513 located at the base of the side pocket. All remaining enzyme–inhibitor contacts are hydrophobic in nature.

Overall, Vioxx binds in the cyclooxygenase active site in the same general conformation as is observed for celecoxib (Wang *et al.*, 2010; Fig. 2). Despite their very similar binding poses, Vioxx is approximately 60-fold more selective for COX-2 than celecoxib (Marnett, 2009). The basis for the difference in COX-2 selectivity between the inhibitors is likely to be rooted in their binding kinetics rather than in a particular enzyme–inhibitor interaction. The coxibs are slow, tight-binding inhibitors of COX-2 but are rapidly reversible inhibitors of COX-1 (Marnett, 2009). Detailed kinetic analyses have demonstrated that the binding and dissociation of coxibs to and from COX-2 is a multistep process (Lanzo *et al.*, 2000). The binding and dissociation kinetics of Vioxx and celecoxib have not yet been characterized side by side. We suspect that such analysis would provide a clear rationale for the different degree of isoform selectivity observed with Vioxx and celecoxib.

3.2. Crystal packing and flexibility of monomer *B*

X-ray crystal structures of muCOX-2 and huCOX-2 in complex with substrates and inhibitors determined from crystals derived from crystallization cocktails that utilized polyacrylic acid 5100 as the primary precipitant belong to the orthorhombic space group *I*222 (Lucido *et al.*, 2016; Orlando *et al.*, 2015; Vecchio *et al.*, 2010, 2012; Vecchio & Malkowski, 2011a,b). Despite their different morphology, the new crystal form of huCOX-2 identified in this study also crystallizes in

space group $I222$. However, the unit-cell parameters differ significantly from those of crystals obtained from polyacrylic acid 5100-based conditions, resulting in an approximately 20% larger unit-cell volume (Supplementary Table S2). The difference in unit-cell volume is the result of different crystal packing between the crystal forms obtained from the different

precipitants (Supplementary Fig. S2). Although the crystal packing differs between the two crystal forms, the derived structures are virtually superimposable (C^α r.m.s.d. of 0.22 Å for 984 pairs; Supplementary Fig. S2).

In the huCOX-2 crystal structure in complex with Vioxx, regions of monomer *B* exhibit higher *B* factors than the

Figure 1

Vioxx bound within the cyclooxygenase channel of human COX-2. Stereoview of Vioxx bound within the cyclooxygenase channel of monomer *A* of the huCOX-2-Vioxx crystal structure. $F_o - F_c$ simulated-annealing OMIT electron density (blue) contoured at 3σ is shown with the final refined model of Vioxx (yellow). Residues lining the cyclooxygenase channel are labeled accordingly. C atoms of residues lining the channel are colored green, while N, O and S atoms are colored blue, red and cyan, respectively.

Figure 2

Comparison of Vioxx and celecoxib bound within the cyclooxygenase channel of COX-2. (*a*, *b*) Chemical structures of (*a*) celecoxib and (*b*) Vioxx. Although both inhibitors share a common diaryl heterocycle scaffold, celecoxib contains a pyrazole heterocycle and a sulfonamide moiety, while Vioxx contains a furanone heterocycle and a methyl sulfone moiety. (*c*) Stereoview showing an overlay of Vioxx (yellow) and celecoxib (magenta) from PDB entry 3ln1 (Wang *et al.*, 2010) bound within the cyclooxygenase channel of COX-2 (C^α r.m.s.d. of 0.31 Å for 542 pairs). The binding mode of the two coxibs is conserved, with the sulfone/sulfonamide moieties penetrating into the COX-2 specific side pocket. C atoms of residues lining the channel are colored green for huCOX-2 and salmon for muCOX-2, while N and O atoms are colored blue and red, respectively.

equivalent regions in monomer *A* based on crystal packing (Supplementary Fig. S3). As a consequence, residues within monomer *B* located around the cyclooxygenase active-site channel exhibit higher *B* factors than those observed for the equivalent residues in monomer *A* (Supplementary Table S3), resulting in a greater degree of atomic displacement of the inhibitor and hence weaker electron density for parts of the inhibitor in monomer *B* (Supplementary Fig. S4). It should be noted that the electron density for Vioxx in monomer *B* is poor only for the phenyl ring that connects the methyl sulfone moiety and the furan ring. The methyl sulfone, furan ring and 3-phenyl moiety themselves all have well defined electron density. Thus, we primarily attribute the weaker electron density for Vioxx in monomer *B* to a higher degree of atomic displacement rather than to a lack of presence of the drug.

4. Summary

In this investigation, we have identified new crystallization conditions for huCOX-2 that are amenable to co-crystallization with Vioxx. Using these newly identified conditions, we were successful in obtaining the X-ray crystal structure of Vioxx bound to huCOX-2, revealing in atomic detail for the first time how one of the most controversial coxibs ever developed binds to its molecular target. Vioxx binds with the methyl sulfone moiety inserted into the COX-2 specific side pocket within the cyclooxygenase channel. The observed binding conformation is virtually identical to the pose adopted by Celebrex and explains why Vioxx possesses selectivity for COX-2 over COX-1.

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References

Adams, P. D. *et al.* (2010). *Acta Cryst. D* **66**, 213–221.

- Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H. & Adams, P. D. (2012). *Acta Cryst. D* **68**, 352–367.
- Blobaum, A. L. & Marnett, L. J. (2007). *J. Med. Chem.* **50**, 1425–1441.
- Campbell, C. L., Smyth, S., Montalescot, G. & Steinhubl, S. R. (2007). *JAMA*, **297**, 2018–2024.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst. D* **66**, 12–21.
- DeWitt, D. L. (1999). *Mol. Pharmacol.* **55**, 625–631.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst. D* **60**, 2126–2132.
- Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M. & Seibert, K. (1996). *J. Biol. Chem.* **271**, 15810–15814.
- Garavito, R. M., Malkowski, M. G. & DeWitt, D. L. (2002). *Prostaglandins Other Lipid Mediat.* **68–69**, 129–152.
- Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
- Koszelak-Rosenblum, M., Krol, A., Mozumdar, N., Wunsch, K., Ferin, A., Cook, E., Veatch, C. K., Nagel, R., Luft, J. R., DeTitta, G. T. & Malkowski, M. G. (2009). *Protein Sci.* **18**, 1828–1839.
- Lanzo, C. A., Sutin, J., Rowlinson, S., Talley, J. & Marnett, L. J. (2000). *Biochemistry*, **39**, 6228–6234.
- Lucido, M. J., Orlando, B. J., Vecchio, A. J. & Malkowski, M. G. (2016). *Biochemistry*, **55**, 1226–1238.
- Luft, J. R., Snell, E. H. & DeTitta, G. T. (2011). *Exp. Opin. Drug. Discov.* **6**, 465–480.
- Marnett, L. J. (2009). *Annu. Rev. Pharmacol. Toxicol.* **49**, 265–290.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Morita, I. (2002). *Prostaglandins Other Lipid Mediat.* **68–69**, 165–175.
- Orlando, B. J., Lucido, M. J. & Malkowski, M. G. (2015). *J. Struct. Biol.* **189**, 62–66.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Painter, J. & Merritt, E. A. (2006). *J. Appl. Cryst.* **39**, 109–111.
- Smith, W. L., Urade, Y. & Jakobsson, P. J. (2011). *Chem. Rev.* **111**, 5821–5865.
- Vecchio, A. J. & Malkowski, M. G. (2011a). *J. Biol. Chem.* **286**, 20736–20745.
- Vecchio, A. J. & Malkowski, M. G. (2011b). *J. Struct. Biol.* **176**, 254–258.
- Vecchio, A. J., Orlando, B. J., Nandagiri, R. & Malkowski, M. G. (2012). *J. Biol. Chem.* **287**, 24619–24630.
- Vecchio, A. J., Simmons, D. M. & Malkowski, M. G. (2010). *J. Biol. Chem.* **285**, 22152–22163.
- Wallace, J. L. (2008). *Physiol. Rev.* **88**, 1547–1565.
- Wang, J. L., Carter, J., Kiefer, J. R., Kurumbail, R. G., Pawlitz, J. L., Brown, D., Hartmann, S. J., Graneto, M. J., Seibert, K. & Talley, J. J. (2010). *Bioorg. Med. Chem. Lett.* **20**, 7155–7158.
- Winn, M. D., Isupov, M. N. & Murshudov, G. N. (2001). *Acta Cryst. D* **57**, 122–133.
- Wong, E., Bayly, C., Waterman, H. L., Riendeau, D. & Mancini, J. A. (1997). *J. Biol. Chem.* **272**, 9280–9286.