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Structure Report

The structure of NS-398 bound to cyclooxygenase-2

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ABSTRACT

The cyclooxygenases (COX-1 and COX-2) are membrane-associated, heme-containing homodimers that generate prostaglandin H_2 from arachidonic acid (AA) in the committed step of prostaglandin biogenesis and are the targets for nonsteroidal anti-inflammatory drugs (NSAIDs). N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398) was the first in a series of isoform-selective drugs designed to preferentially inhibit COX-2, with the aim of ameliorating many of the toxic gastrointestinal side effects caused by conventional NSAID inhibition. We determined the X-ray crystal structure of murine COX-2 in complex with NS-398 utilizing synchrotron radiation to 3.0 A resolution. NS-398 binds in the cyclooxygenase channel in a conformation that is different than that observed for other COX-2-selective inhibitors, such as celecoxib, with no discernible penetration into the side pocket formed in COX-2 by the isoform-specific substitutions of I434V, H513R, and I523V. Instead, the methanesulfonamide moiety of NS-398 interacts with the side chain of Arg-120 at the opening of the cyclooxygenase channel, similar to that observed for acidic, nonselective NSAIDs such as indomethacin and flurbiprofen. Our structure validates inhibitor studies that identified Arg-120 as a molecular determinant for time-dependent inhibition of COX-2 by NS-398.

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1. Introduction

Prostaglandin endoperoxide H synthases, also known as cyclooxygenase enzymes (COX-1 and COX-2), catalyze the committed step in the biosynthesis of prostaglandins, prostacyclins, and thromboxanes. Each isoform consists of two separate, but functionally linked active sites, which generate the product prostaglandin H_2 (PGH_2) from the ω-6 polyunsaturated fatty acid substrate arachidonic acid (AA) in sequential reactions (Rouzer and Marnett, 2009; Smith et al., 2000). COX-1 and COX-2 share a similar three-dimensional fold and the catalytic mechanism is conserved between isoforms (Garavito et al., 2002). The major difference between isoforms is in their observed expression patterns (Tanabe and Tohnai, 2002). In general, COX-1 is constitutively expressed in nearly every tissue, whereas COX-2 expression is tissue-specific and largely induced upon stimulation by inflammatory agents. Additionally, while both COX-1 and COX-2 preferentially oxygenate AA, COX-2 has been shown to selectively utilize eicosapentaenoic acid and a wide spectrum of AA derivatives, such as the endocannabinoids 2-arachidonoyl glycerol and arachidonoyl ethanolamide as substrates (Kozak et al., 2000; Vecchio and Malkowski, 2011; Vecchio et al., 2010; Wada et al., 2007; Yu et al., 1997).

COX-1 and COX-2 are the pharmacological targets of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), utilized for the treatment of pain and inflammation, as well as some cancers (Blobaum and Marnett, 2007). NSAID binding within the cyclooxygenase channel of COX-1 and COX-2 has been extensively studied and many crystal structures of COX-1 and COX-2 in complex with NSAIDs have been elucidated to compliment inhibition studies (Duggan et al., 2010; Harman et al., 2007; Kurumbail et al., 1996; Loll et al., 1995; Luong et al., 1996; Picot et al., 1994; Rowlinson et al., 2003; Selinsky et al., 2001; Wang et al., 2010). Classical nonselective NSAIDs (nsNSAIDs) inhibit both COX isoforms via one of three different modes of inhibition (reviewed in (Blobaum and Marnett, 2007; DeWitt, 1999)): (1) covalent modification (e.g. aspirin acetylation of Ser-530); (2) rapid, reversible binding (e.g. ibuprofen); and (3) rapid, lower affinity binding followed by time-dependent, high affinity, slowly reversible binding (e.g. flurbiprofen). The classic, acidic nsNSAIDs typically bind within the cyclooxygenase channel such that the carboxylic acid group of the inhibitor interacts with the side chain of Arg-120 at the opening of the channel (Kurumbail et al., 1996; Picot et al., 1994). However, diclofenac, which binds with its carboxylic acid group

Abbreviations: COX, cyclooxygenase; hu, human; mu, murine; ov, ovine; AA, arachidonic acid; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide; MR, molecular replacement; NSAIDs, nonsteroidal anti-inflammatory drugs; βOG, *n*-octyl β-D-glucopyranoside.

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interacting with the side chain of Tyr-385 and Ser-530 at the apex of the channel, is an exception (Rowlinson et al., 2003). COX-2 can also be selectively inhibited in a time-dependent manner by diarylheterocycle-based compounds (termed "coxibs"), which include celecoxib, rofecoxib, valdecoxib, and etoricoxib (Marnett, 2009). The coxibs exploit a side pocket, generated by the substitution Ile-434 and Ile-523 in COX-1 to Val-434 and Val-523 in COX-2, which effectively increases the overall volume of the cyclooxygenase channel of COX-2 by ~20% (Kurumbail et al., 1996; Luong et al., 1996). In addition, the substitution of His-513 in COX-1 to Arg-513 in COX-2 alters the chemical environment of this side pocket by placing a positive charge at the base of the pocket. Indeed, structure–function analyses utilizing diarylheterocyclic COX-2 inhibitors confirm both the insertion of the methylsulfonyl or sulfonamoylphenyl substituent past Val-523 into the side pocket and subsequent interaction with the side chain of Arg-513 (Gierse et al., 1996; Guo et al., 1996; Kurumbail et al., 1996; Wang et al., 2010; Wong et al., 1997).

N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS-398) was one of the earliest time-dependent COX-2-selective compounds identified, with an IC_{50} value of 0.1 μM (Futaki et al., 1993; DeWitt, 1999). Like many nsNSAIDs that preceded it, NS-398 possessed anti-inflammatory and analgesic effects *in vivo*, but elicited no significant gastrointestinal toxicity (Futaki et al., 1993, 1994). Despite the presence of a methylsulfonamide moiety and no carboxylic acid group, inhibition studies utilizing NS-398 and R120E and R120Q human (hu) COX-2 mutant constructs revealed a striking dependence on the interaction of the inhibitor with the side chain of Arg-120 as being a determinant for time-dependent inhibition instead of Arg-513 (Greig et al., 1997; Rieke et al., 1999). In addition, NS-398 was ~1000-fold less potent towards the R120E huCOX-2 mutant construct (Greig et al., 1997). Moreover, mutation of Val-523 to isoleucine completely abolished time-dependent inhibition of huCOX-2 by NS-398, but still allowed for rapid, reversible inhibition of this isoform (Gierse et al., 1996; Guo et al., 1996). We report here the X-ray crystal structure of Fe^{3+}-protoporphyrin IX reconstituted murine (mu) COX-2 in complex with NS-398 to 3.0 Å resolution. This study was designed to detail at the molecular level the binding of NS-398 within the cyclooxygenase channel of COX-2 and further investigate the dependence of NS-398 binding with the side chains of Arg-120 and Val-523.

2. Materials and methods

2.1. Crystallization and data collection

Murine COX-2 (muCOX-2) was expressed in baculovirus-infected insect cells and the apo enzyme purified as previously described (Vecchio et al., 2010). Prior to crystallization, the enzyme was concentrated to 3 mg/mL, reconstituted with a 2-fold molar excess of Fe^{3+}-protoporphyrin IX and subsequently dialyzed overnight at 4 °C against 20 mM TRIS, pH 8.0, 100 mM NaCl, and 0.6% (w/v) *n*-octyl β-D-glucopyranoside (βOG). A 5-fold molar excess of NS-398 was then added to the reconstituted enzyme. Crystallization experiments were setup at 296 K using the sitting-drop vapor diffusion method. 3 μL protein was combined with 3 μL of a drop solution consisting of 23–34% polyacrylic acid 5100, 100 mM HEPES, pH 7.5, 20 mM $MgCl_2$, and 0.6% (w/v) βOG and equilibrated over a reservoir solution of 23–34% polyacrylic acid 5100, 100 mM HEPES, pH 7.5 and 20 mM $MgCl_2$. Plate-like crystals formed in three days to a week that were brown in color. Prior to data collection, crystals were cryoprotected by soaking in 30% polyacrylic acid 5100, 100 mM HEPES, pH 7.5, 20 mM $MgCl_2$, and 0.6% (w/v) βOG supplemented with 10% glycerol. Data were collected on beamline A1 at the Cornell High Energy Synchrotron Source (Ithaca, NY) using an Area Detector Systems CCD Quantum-210. Data collection statistics are summarized in Table 1.

Table 1
Summary of the data collection and refinement statistics.

Crystallographic parameter	COX-2:NS398
Space group	I222
No. in asymmetric unit	2
Unit cell length (Å)	
a	120.43
b	131.21
c	179.57
$\alpha = \beta = \gamma$ (°)	90°
Wavelength (Å)	0.9777
Resolution (Å)	20.0–3.00
Highest resolution shell (Å)[a]	3.16–3.00
R_{merge}[b]	12.4 (47.5)
R_{pim}	7.5 (28.5)
Total observations	112276 (16511)
Total unique[c]	28681 (4158)
I/σ (I)	9.8 (3.0)
Completeness (%)	99.6 (100.0)
Multiplicity	3.9 (4.0)
Wilson B factor (Å^2)	63.7
Number of atoms in refinement	9237
R_{work}	0.176 (0.252)
R_{free}[d]	0.225 (0.288)
Average B factor, protein (Å^2)	34.0
Average B factor, solvent (Å^2)	23.8
Average B factor, inhibitor (Å^2):	
Monomer A (Å^2)	34.7
Monomer B (Å^2)	41.9
Mean positional error (Å)[e]	0.434
RMSD in bond length (Å)	0.011
RMSD in bond angle (°)	1.465
Ramachandran Plot (%)	
Favored	97.2
Allowed	2.8
Disallowed	0

[a] The values in parentheses represent the values in the outermost resolution shell.
[b] R_{merge} and R_{pim} as defined in (Evans, 2006 #27).
[c] Represents reflections with $F > 0\ \sigma F$, which were used in the refinement.
[d] 5.1% of the total reflections were used to generate the test set.
[e] Coordinate error as calculated by Luzatti plot.

2.2. Structure solution and refinement

The data were processed using MOSFLM and SCALA in the CCP4 suite of programs (Dodson et al., 1997) in the orthorhombic space group I222. The structure was solved by molecular replacement (MR) using the program PHASER (McCoy et al., 2007) and a truncated search model of muCOX-2 derived from PDB entry 1CVU (Kiefer et al., 2000), with residues 33–144, 320–325, 344–391, 500–553, and all ligands and waters removed. Two monomers constituting the muCOX-2 homodimer were found in the crystallographic asymmetric unit. Phases from MR were then input into *ARP/wARP*, utilizing the "automated model building starting from experimental phases" option (Langer et al., 2008). Given the moderate resolution of the data, ARP/wARP built only 53% (290 residues) of the model. Iterative cycles of manual model building in COOT (Emsley and Cowtan, 2004), followed by refinement in REFMAC5 (Murshudov et al., 1997) were carried out to fit all the remaining residues, ligands, and waters. Tight NCS restraints were applied to residues 33–582 in each monomer during cycles of refinement. NS-398 was built and a stereochemical dictionary was generated using SKETCHER (Potterton et al., 2003). The final model (R_{work} = 17.6% and R_{free} = 22.5%) consists of residues 33–582, Fe^{3+}-protoporphyrin IX, carbohydrate moieties linked to

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