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Research Article

Steriochemistry and validation of the structure of cyclooxygenase, preferred target of controversial non-steroidal anti-inflammatory drugs during the COVID 19 pandemic

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Abstract:
During COVID-19 infection, the scarce published data on nonsteroidal anti-inflammatory drugs is very controversial. In the literature, several models of cyclooxygenase, their preferred target, have been identified. The aim is to study their sterochemistry and validate the best structure. PDB entries were viewed using Discovery Studio 2.5 software. MolProbity was used for steriochemical analysis. The structures of COX bound to Naproxen (3NT1: 1.73 Å) and Ibuprofen (4PH9: 1.81Å) are of higher resolution. In the Ramachandran diagram, for 4PHP and 3LN1, 100.0% of the residues were in allowed regions. For the Rlibre factor the values of (19.8%) for 4PH9, (20.0%) for 3NT1 and (22.4%) for 1CVU were within the expected ranges. They were rather high for 3LN1, 1PXX and 4COX. The clashscores for 3NT1 (99th percentile) and 4PH9 (98th percentile) were at the top of the best ranges. 4PH9 (sidechain outliers: 1.5%) and 3NT1 (sidechain outliers: 1.8%) were modeled with their preferred rotamers. By comparing all metrics, 4PH9 appears to be the finest crystal structure. PDB entries do not necessarily constitute a reliable source of steriochemistry. Our study made it possible to verify the adherence of the models to the structural principles established to guarantee their quality. This is an essential step in the design of structure-based drugs.

Keywords: Steriochemistry, validation, cyclooxygenase, non-steroidal anti-inflammatory drugs, COVID 19

INTRODUCTION

Prostaglandin H synthase 2 (COX 2) is an important enzyme in the metabolism of arachidonic acid (1) . It is both constitutively expressed in several human tissues (eg kidneys and brain) and induced in various cell types (including monocytes / macrophages, vascular endothelial cells, etc.) in response to cytokines inflammatory, and growth factors (2) . The products of COX-2 activity [eg PGE2 and prostacyclin (PGI2)] are involved in various physiological and pathophysiological processes, including renal hemodynamics and blood pressure control, endothelial thromboresistance, pain and l inflammation (3) , (4) . Therefore, it is not surprising that COX-2 inhibitors exhibit multifaceted clinical effects, ranging from decreased pain and inflammation (3) to increased pressure. blood pressure (Patrono and al. 2014), an increased risk of atherothrombotic events (5) and a decreased risk of colorectal cancer (4). The crystal structures of cyclooxygenase (COX) isoforms are quite structurally homologous and consistent with high sequence identity (about 60%). The COX monomer consists of three structural domains: an epidermal growth factor-like N-terminal domain, a membrane binding domain of about 48 amino acids which anchors the protein to a leaflet of the lipid bilayer, and a large C-terminal globular catalytic domain with the COX active site which hosts the substrate or inhibitors and the peroxidase which contains the heme cofactor. These sites are distinct but interconnected functionally and structurally (6). Each COX isoform is a structural homodimer which functions as a heterodimer. One subunit, containing the required heme prosthetic group, acts as a catalytic site, while the other serves as an allosteric site (7). Previous evidence suggests that inhibitors may act at one or both sites, depending on the structure and concentration of the inhibitor (8) . Cyclooxygenase enzymes are pharmacologically important targets for nonsteroidal anti-inflammatory drugs, such as aspirin and new selective COX-2 inhibitors. Nonsteroidal anti-inflammatory drugs bind tightly to a single COX-2 dimer monomer and allosterically inhibit oxygenation of the substrate in the partner monome (8), (9). The first evidence that COX inhibition affects the risk of cardiovascular disease was found by large-scale studies examining gastrointestinal outcomes as a primary endpoint. Rofecoxib was withdrawn from the market in 2004 following the results of the Adenomatous Polyp PRevention On Vioxx (APPROVe) study, showing that patients taking rofecoxib were twice as likely to have thromboembolic events compared to a group on placebo (10). In COVID-19 infection, the rare published data on NSAIDs cited in relation to the increased risk of mortality or the need for respiratory assistance are highly controversial.(11) , (12) , (13) . These studies taken individually are biased (14) . Nevertheless, a cautious approach should be followed when initiating NSAIDs for fever or cough associated with COVID-19 due to the increased risk of well-known adverse reactions of NSAIDs in the specific setting of COVID-19 in addition to the risk of worsening the disease (14) .The objective of this study is to validate the adherence of

cyclooxygenase models to structural principles necessary for the accuracy of interpretation of crystallographic data.

Methods

To study the stereochemical properties of the different models of COX bounded to NSAID or to its natural substrate (arachidonic acid), the following parameters were used: RMS deviations, Ramashandran diagram, free R factor and clashscore . COX-2 is induced by inflammation while COX-1 is physiological. The different crystal structures of COX-1 and COX-2 in complex with a myriad of inhibitors and substrates were visualized in NGL format (15) then downloaded from the PDB (https: // www. rcsb.org/). The COX structures were transferred to Discovery software (Discovery Studio 2.5 (CDOCKER Dock, Dassault Systemes BIOVIA, United States). Discovery Studio makes it easy to examine the properties of large and small molecules. The groups of co-crystallized ligands were separated. Water molecules and heteroatoms have been removed. The targets (receptors) were saved in PDB format for use on Molprobity (16).

MolProbity (http://molprobity.biochem.duke.edu/) is largely based on the analysis of interatomic clashes. For this, the program calculates the positions of the hydrogen atoms and adds them to the coordinate files (sometimes replacing the riding H atoms that might already be present there). This program aims to verify the most probable orientation of the side chains. Once these residues have been placed in their most likely orientations and the H atoms have been added, the contacts of all the atoms are analyzed in detail. Close interatomic distances and clashes are graphically represented, providing useful information for reconstructing offending areas, or at least raising a red flag for users of the deposited structures (16) . The program provides graphs of Ramachandran angles, using a much more complete database. (17) . The Java SE Java Runtime Environment (JRE) was downloaded from www.oracle.com (the latest version (Java SE 7u40 as of September 2013). Java is a technology originally developed by Sun Microsystems and now developed and hosted by Oracle (www.oracle.com) Molprobity uses Java to display 3D information. The validation reports of the different crystal structures were downloaded from the PDB citing each ligand and its depository. These reports were used for the comparison and interpretation of the free R factor and the clashscore.

Results

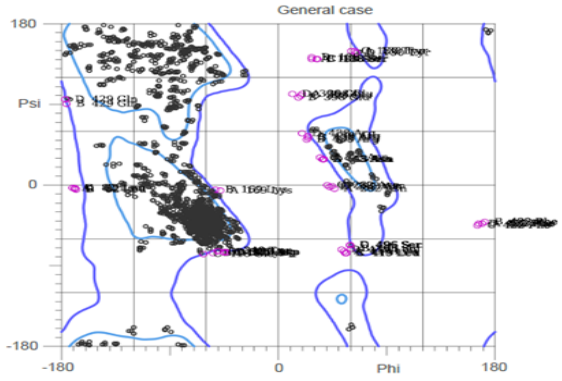
In the literature, several crystal structures of COX-1 and COX-2 in complex with a myriad of inhibitors and substrates have been identified: With arachidonic acid (their physiological substrate), classic NSAIDs such as Diclofenac, Naproxen as well as coxibs such as celecoxib. (Table 1).

Table 1: Experimental data of crystal structures of ligands-COX-2

Author/ Reference	ID PDB	Ligand	resolution	R-Value Free	R-Value Work	R-Value Observed
(Kurumbail, 1996) (18)	4COX	indométacin	2,90 Å	0.309	0.219	0.219
(Kiefer et al., 2000) (19)	1CVU	Arachidonic Acid	2,40 Å	0.235	0.204	Not determined
(Rowlinson et al., 2003) (20)	1PXX	Diclofenac	2,90 Å	0.302	0.354	Not determined
(Duggan et al., 2010) (21)	3NT1	Naproxen	1,73 Å	0.186	0.167	0.168
(Wang et al., 2010) (22)	3LN1	Célécoxib	2,40 Å	0.264	0.232	0.235
(Orlando, 2015) (23)	4PH9	Ibuprofen	1,81 Å	0.197	0.160	0.162

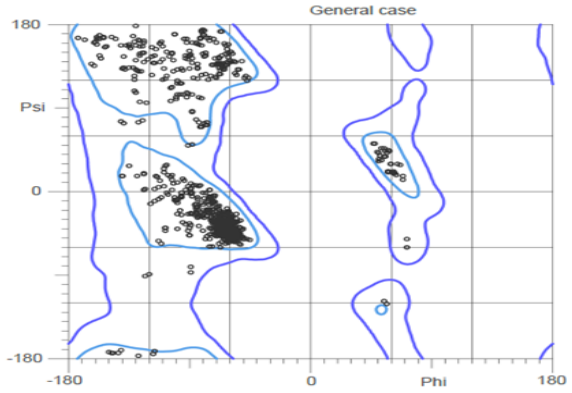
In standard steriochemistry, RMSD deviations indicate how far a refined structure deviates from targets. The allowed deviation from the targets depends on the resolution of the diffraction data used in the refinement. According to the LibreTexts Project Living Chemistry Libraries (powered by MindTouch and supported by the UC Davis Library and the California State University and Merlot Learning Program) (https://chem.libretexts.org/). 2.05 Å is the median resolution of X-ray crystallographic results in the Protein Database (135762 May 19, 2019). High digital values of resolution, such as 4 Å, mean poor resolution, while low digital values, such as 1.5 Å, mean good resolution. The structures of COX bound to Naproxen (3NT1: 1.73 Å) and Ibuprofen (4PH9: 1.81Å) are of higher resolution and likely resolve finer structural details. The backbone and most sidechains should be clear. The other structures (between 2.40 and 2.90 Å) have a less fine but rather good resolution. (Table 1).
The skeleton and many sidechains should be clear. (PROTOPEDIA resolution values interpretation guide: http://proteopedia.org/wiki/index.php/Main_Page).

4COX



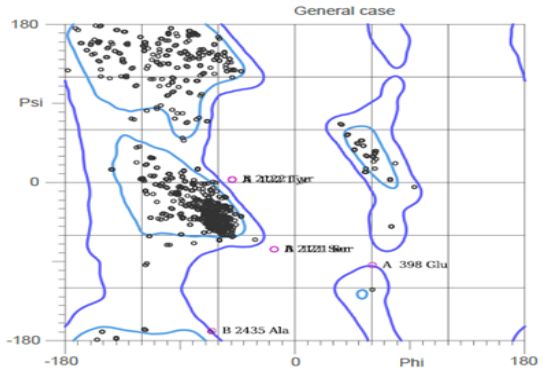
82.5% (1816/2200) of all residues were in favored (98%) regions. 96.3% (2118/2200) of all residues were in allowed (>99.8%) regions. There were 82 outliers (phi, psi):

3NT1



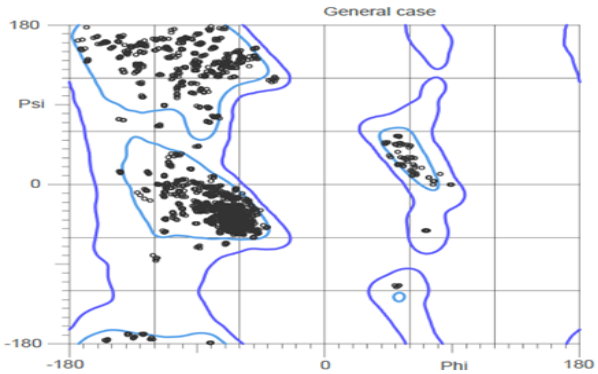
98.0% (1085/1107) of all residues were in favored (98%) regions. 100.0% (1107/1107) of all residues were in allowed (>99.8%) regions. There were no outliers.

1CVU



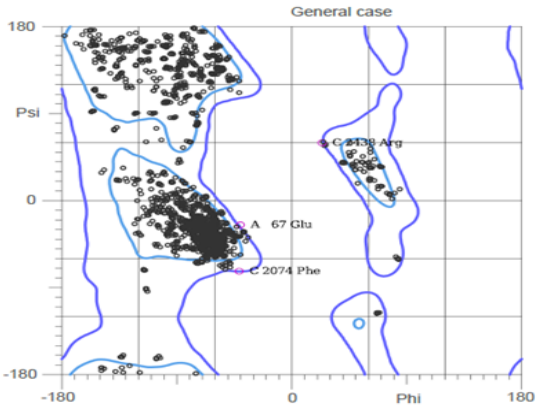
94.4% (1045/1107) of all residues were in favored (98%) regions. 99.3% (1099/1107) of all residues were in allowed (>99.8%) regions. There were 8 outliers (phi, psi):

3LN1



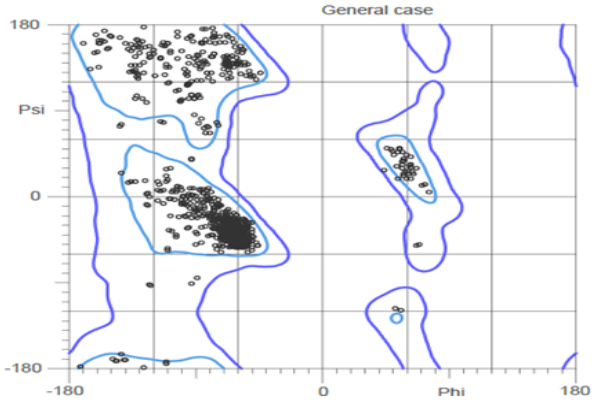
96.4% (2121/2200) of all residues were in favored (98%) regions. 100.0% (2200/2200) of all residues were in allowed (>99.8%) regions. There were no outliers.

1PXX



93.2% (2049/2199) of all residues were in favored (98%) regions. 99.7% (2193/2199) of all residues were in allowed (>99.8%) regions. There were 6 outliers (phi, psi):

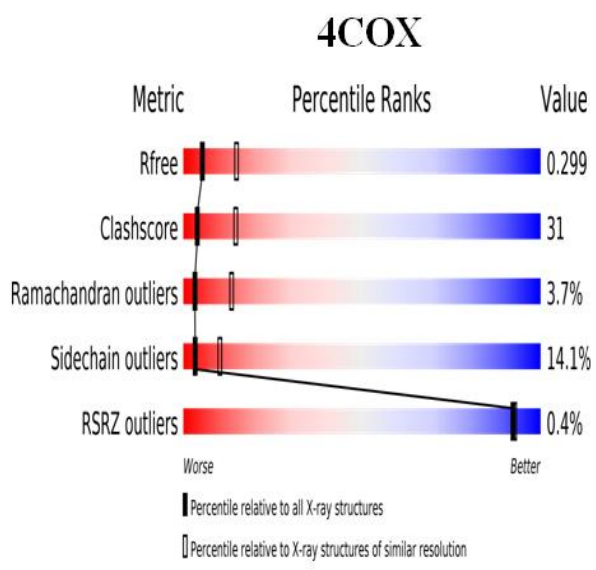
4PH9

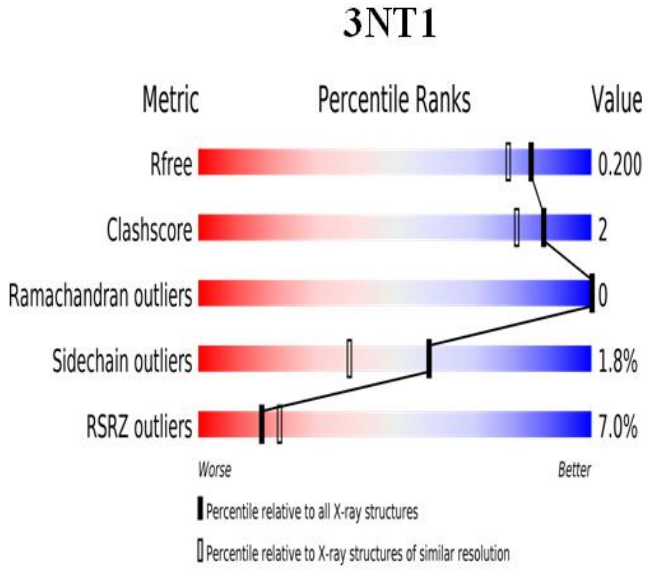


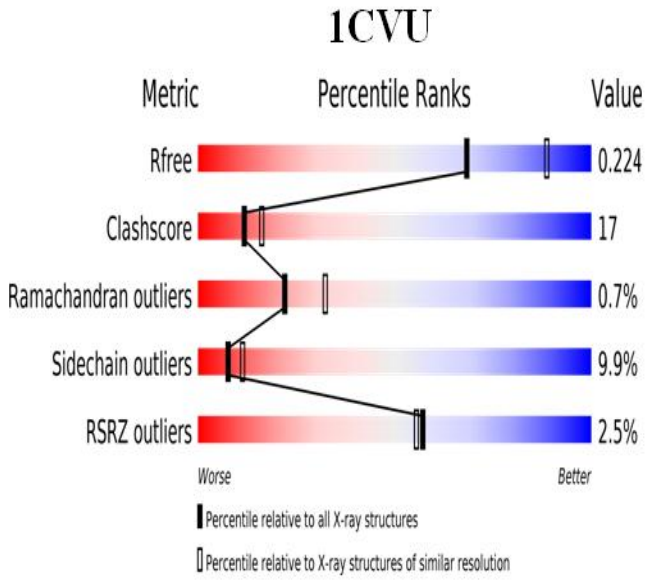
97.7% (1096/1122) of all residues were in favored (98%) regions. 100.0% (1122/1122) of all residues were in allowed (>99.8%) regions.
There were no outliers.

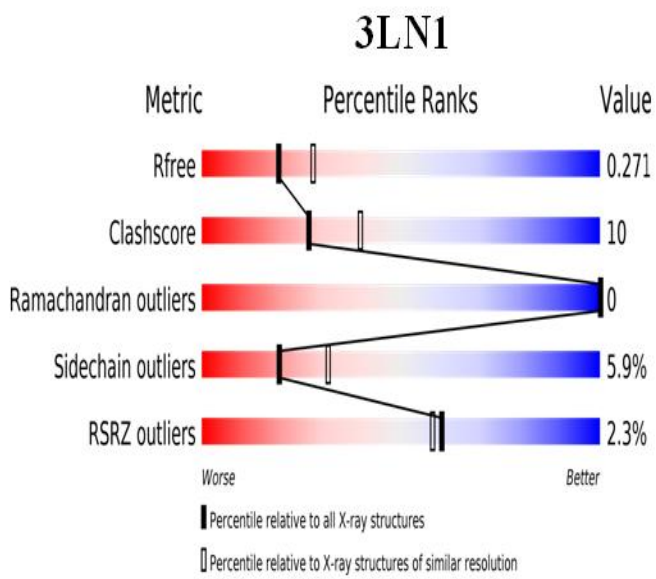
Figure 1: Ramachandran diagram of the different COX models. Number of residues in the most favored regions, allowed regions and outliers

The Ramachandran diagram is one of the main methods for validating protein structures offered on the basis of X-ray crystallographic data. The graph compares the dihedral angles selected in each amino acid found in the proposed protein. The key dihedral angles for each amino acid are located along the protein backbone and are labeled φ (phi), ψ (psi), and ω (omega). To repeat, each amino acid residue provides three rotating bonds and three distinct dihedral angles to the backbone of a peptide chain. In theory, all dihedral angles can vary in value from -180 ° to + 180 °. In practice, within a protein, the dihedral angles tend to fall within well-defined and much narrower ranges. A residue is considered an outlier of the Ramachandran plot if the combination of its torsion angles φ and ψ is unusual, as evaluated by MolProbity, (24) . The Ramachandran outlier score for an entry is calculated as the percentage of Ramachandran outliers out of the total number of residuals in the entry for which the outlier assessment is available (Figure 1). Although tracking dihedral angles in a protein can seem complex, a Ramachandran plot makes the process very simple. In the process of validating the structure assigned to a protein, amino acids that fall outside the expected shaded regions of a Ramachandran graph are of most concern. If a graph has too many of these outliers, the structure of the protein may be incorrectly assigned. In general, any protein with more than 5% of its amino acids as outliers should be treated with caution. Consequently, the structures 4COX (82 outliers / 2200: 3.72%), 1CVU (8 outliers / 1107: 0.72%), 1PXX (6 outliers / 2199: 0.27%), are quite acceptable. For structures 3NT1 (no outliers / 1107), 4PHP (no outliers / 1122), 3LN1 (no outliers / 2200), 100.0% of all residues were found in allowed regions (> 99.8%). The percentage of residues in the favored regions (98%) was 98.0%, 97.7% and 96.4%, respectively.









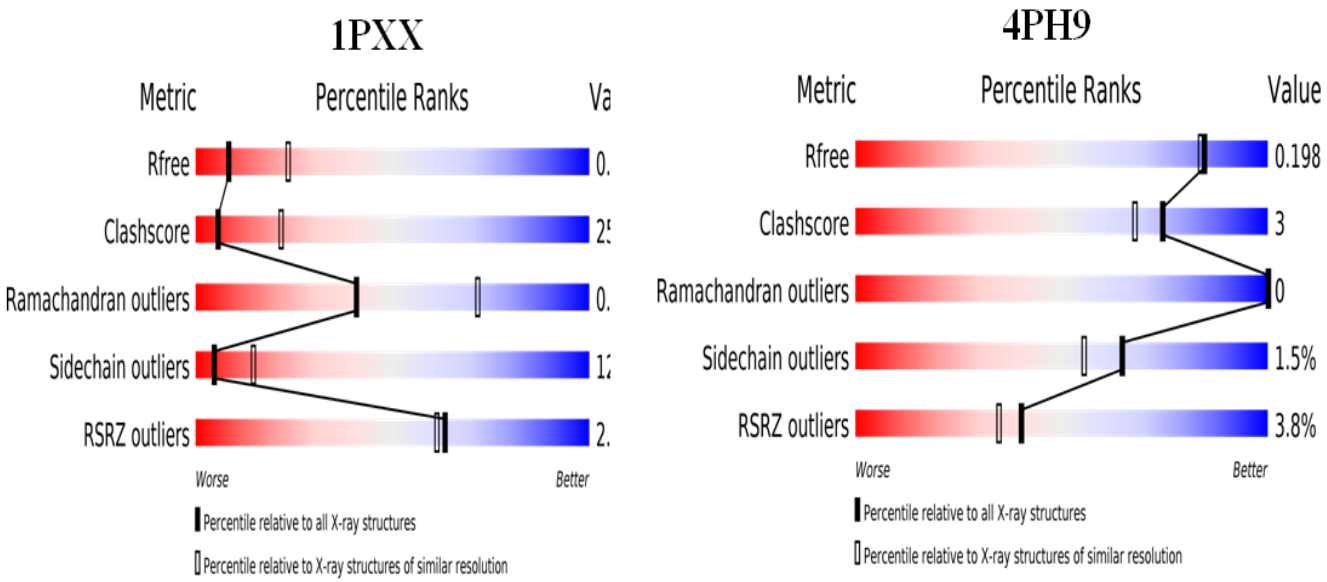


Figure 2: The metrics shown in the "slider" charts (see examples below) compare several important overall quality indicators for these structures with those of previously filed PDB entries. The comparison is carried out by calculating the percentile rank, that is to say the percentage of entries equal to or less than this structure in terms of quality indicator. The overall percentile ranks (black vertical boxes) are calculated relative to all radiographic structures available in the PDB archive until December 28, 2016. The resolution-specific percentile ranks (white vertical boxes) are calculated relative to a sub - set of X -ray entries in the same subset of the PDB archive, but considering only entries with a resolution comparable to this entry (https://www.wwpdb.org/validation/ 2016/Xray Validation Report Help)

The free R factor which appears in the metrics of all structures on PDB represents the relative deviation of the observed and calculated factors of the structures (by analogy with the conventional R factor) (17). The quoted value comes from a recalculation by the DCC program (25) . In exceptionally good cases free R can be as low as 10% for the best refined structures using ultra high resolution data; it is generally around 20-25% for medium resolution structures, but should not exceed 30% even for structures refined compared to low resolution data (17) Thus the values of (19.8%) for 4PH9 (1.81 Å), (20.0%) for 3NT1 (1.73 Å) and (22.4%) for 1CVU (2.40 Å), are within the expected ranges. It is rather high (27.1%) for 3LN1 (2.40 Å), (29.4%) for 1PXX (2.90 Å) and (29.9%) for 4COX (2.90 Å). For now, the 3NT1 and 4PHP structures are neck and neck and bring together the best qualities. Another parameter also allows you to compare the different structures: The Clashscore. This score is derived from the number of pairs of atoms in the model that are unusually close to each other. It is calculated by MolProbity, (24) and expressed in number of conflicts of this type per thousand atoms. The clashscore of 2 for 3NT1 (molprobity clashscore: 1.69, 99th percentile) and 3 for 4PH9 (clashscore: 3.44, 98th percentile) are at the top of the best ranges, which appear to be independent of resolution. For 3LN1 and 1CVU, it is 10 and 17 (around the 90th - 93th percentile) respectively. This parameter is certainly less good and the clashscores of 25 for 1PXX and 31 for 4COX are more or less acceptable (between 80 th percentile and 85 th percentile) (good mobrobity clashscore: Percentile ≥ 66). Molprobity cautions against structures between the 33 th and 66 th percentile. Watch out for structures below the 33 th percentile. Protein side chains primarily adopt certain (combinations of) preferred twist angle values (called rotamers or rotameric conformers), as do their backbone twist angles (as assessed in Ramachandran's analysis). MolProbity considers the sidechain conformation of a residue to be an outlier if its set of torsion angles are not similar to any preferred combination. The side chain outlier score is calculated as the percentage of residues with unusual side chain conformation out of the total number of residues for which the assessment is available. A percentage of side chain outliers less than or equal to 2% indicates that the residues were modeled with their preferred rotamers and were not distorted during the refinement process (17) . This is only the case for 4PH9 (sidechain outliers: 1.5%) and 3NT1 (sidechain outliers: 1.8%). The real-space R-value (RSR) is a measure of how well a part of an atomic model (in this case, a residue) fits with data in real space (26) . The RSR Z-score (RSRZ) is a standardization of the RSR specific to a type of residue and a resolution bin (27). RSRZ is calculated only for standard amino acids and nucleotides in protein, DNA and RNA chains. A residue is considered an RSRZ outlier if its RSRZ value is greater than 2. The RSRZ outlier score as shown in the slider graph is calculated as the percentage of RSRZ outliers relative to the total number of residuals for which RSRZ was calculated. This is calculated by the EDS (Electron-Density Server) component of the validation pipeline which is a reimplementation of the software used by the Uppsala EDS Server (27) . 4COX, 1CVU, 1PXX, 3LN1 all have an outliers RSRZ percentage of less than 2%. 4PH9: RSRZ = 3.8% and 3NT1: RSRZ = 7%. By comparing the five metric parameters, they are favorable to 4PH9 in 3 metrics: RSRZ outliers , sidchain ouliers and R free,

comparable to 2 metrics of 3NT1: Ramachandran outliers and clashscore. 4PH9 therefore appears to be the finest crystal structure.

Discussion

In order to minimize the chances of failure, pharmaceutical companies are constantly trying to establish the value of a target to affect a disease. This process is called target validation - ensuring that binding of the target with a molecule will provide therapeutic benefits in humans. Drugs interfere with proteins (enzymes or receptors) in biological pathways. Proteins, especially the functional parts of a protein, have a well-defined structure. Because a protein has a specific shape, drugs that work on the protein need a specific shape, a form that complements the form of protein. Therefore, if we can determine the shape of a protein (target), we can better understand the ideal shape and structure of a possible drug. This idea is very important for targeted drug discovery. There are many methods for determining the structure of a protein, and the most common is probably X-ray crystallography(28) . Enzymes are proteins that facilitate or catalyze the conversion of one molecule to another. COX 2 (ID: 1.14.99.1) catalyzes the reaction:

Hydrogen donor + arachidonic acid + 2O 2 = hydrogen acceptor + H_2O + PGH 2 (29). It is the target of several inhibitors to different degrees: Diclofenac (ID: 3033, MF: C14H11Cl2NO2, IUPAC Name: 2- [2- (2,6-dichloroanilino) phenyl] acetic acid): pIC50 = 7.7 (30) , celecoxib (ID: 2662, MF: C17H14F3N3O2S, IUPAC Name: 4- [5- (4-methylphenyl) -3- (trifluoromethyl) pyrazol-1-yl] benzenesulfonamide): pIC50 = 6.5 - 8.7 (31), (32) , naproxene (ID: 156391, MF: C14H14O3, IUPAC Name: (2S) - 2- (6-methoxynaphthalen-2-yl) propanoic acid): pIC50 = 5.6 (33) , ibuprofen (ID: 3672, MF: C13H18O2,, IUPAC Name: 2- [4- (2-methylpropyl) phenyl] propanoic acid): pIC50 = 5.9 (34) , indometacin (ID: 3715, MF: C19H16ClNO4, IUPAC Name: 2- [1- (4-chlorobenzoyl) -5-methoxy-2-methylindol-3-yl] acetic acid): pIC50 = 5.6 (32) … and many others . (https://pubchem.ncbi.nlm.nih.gov/). Cyclooxygenase enzymes are included in GtoImmuPdb because they are involved in the production of inflammatory mediators and are long-standing anti-inflammatory targets. The role of COX-2 in immuno-oncology is reviewed by Adams et al. (35). Research and validation of the target protein is an essential step in the design of structure-based drugs (36) . The Ramachandran plot, and the different metrics (Rlibre, clashscore, sidechain outliers and RSRZ outliers) make it possible to verify the sterochemical properties of the model (37). In the literature, various other model validation methods have been described (38) , (39), (40) . All entries on PDB must be rigorously verified as it does not necessarily constitute a reliable source of sterochemistry (41) . Once the protein has been validated. Molecular docking will continue by researching the sites of intercations. We used molprobity, as one of the structure validation approaches. (42) , (24). In addition to analyzing geometric parameters, MolProbity detects the positions of hydrogen atoms and adds them to coordinate parameters for the study of interatomic clashes (17).This is a new method of validating structures against PROCHECK, which has been used for a long time (43) . Examination of the different PDB entries of COX reveals the different interactions with the ligands. Most of the interactions are hydrogen bonds or hydrophobic interactions. Cyclooxygenase converts its endogenous ligand arachidonic acid (which is achiral) to prostaglandin G2 (which has five chiral centers) (19). Inhibition of prostaglandin synthesis by indomethacin is dependent on time and concentration. It is progressive and functionally irreversible. The tight binding of indometacin to cox1 requires the carboxylic group (30). Ibuprofen established a total of 21 contacts with cyclooxygenase 2 and a 22nd doubly linked to Valine-349 and Leucine-359 (23). Our results reveal that 4PH9 appears to be the finest crystal structure. In particular with R_{free} value of 19.8% for a resolution of 1.81 Å. These two parameters are closely related (44). Molpropbity places 4PH9 (Cox-bound Ibuprofen) at the 99th percentile and is assigned a percentage of side chain outliers less than or equal to 2% (4PH9: sidechain outliers: 1.5%). Ibuprofen is a competitive and severely reversible COX inhibitor (45). Rofecoxib makes a total of 42 contacts with residues lining the cyclooxygenase channel. (46) . The discovery of the crystal structure of Diclofenac in complex with COX 2 shows a bond with its carboxylate group to the hydrogen of Tyrosine -385 and Serine -530. (20). Diclofenac inhibits cox slowly and irreversibly. Modification of the carboxylic acid group of diclofenac gave potent and selective inhibitors of cox2, while incorporation of halogens at position 2 and 6 on the lower aniline ring influences the potency of cyclooxygenase inhibition (47) . Like the other compounds of the 2-arylpropionic acid family of NSAIDs, the carboxylate group of naproxene interacts with Arginine-120 and Tyrosine -355 via hydrogen bonds at the base of the active site. The rest of the interactions between naproxene and COX were Van Der Wals contacts (48). As opposed to ibuprofen which rapidly and reversibly inhibits cox, or indometacin and diclofenac which inhibit cox slowly and functionally irreversibly, naproxene inhibits cox slowly and reversibly (49). The 3NT1 structure had the best resolution (1.73 Å) with also 98.0% (1085/1107) of all residues were in favored (98%) regions 100.0% (1107/1107) of all residues were in allowed (> 99.8%) regions. There were no outliers to Ramachandran's plot. The structures 4COX (82 outliers / 2200: 3.72%), 1CVU (8 outliers / 1107: 0.72%), 1PXX (6 outliers / 2199: 0.27%), are entirely acceptable. The approach that we used for the sterochemical validation of the models is very important. It identifies the 4PH9 structure as having the best quality. The validation process used is crucial to guarantee the quality of the model. Such tests should be used before refinement of the structure.(17) . Thus the success of a molecular docking first requires the validation of the target. Very recent research has attempted to establish a link between the pathophysiology of COVID 19 infection and COX-2 inhibitors (50) . The therapeutic potential of these deriners as much as an immune modulator has not been clearly established (51). The use of nonsteroidal anti-inflammatory drugs may increase the regulation of angiotensin-converting enzyme 2 (ACE2), the cell entry receptor (52), (53), and have a direct link with the replication of SARS-Cov 2 through COX / PGE2

signaling (54). Cyclooygenase 2 was indeed induced by COVID 19 infection but did not affect viral entry or replication. (55).

Conclusion

Given the role of cyclooxygenase in the pathophysiology of COVID 19 infection. Both old and new therapies may be offered or contraindicated. In silico studies can simulate the interaction of COX with these. However, entries on PDB must be rigorously verified because they do not necessarily constitute a reliable source of sterochemistry. For a better understanding of this target we propose a practical computer method to verify the adherence of models to the structural principles established to guarantee their quality. This is an essential step in the design of structure-based drugs.

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