Ann Natl Acad Med Sci (India), **44(2)**: 97-110, 2008

Computer-Aided Design of a New Class of Potent and Selective Inhibitors of Cyclooxygenase 2(COX2), a Key Enzyme in the Inflammatory Pathway

Gita Subba Rao and Rajakrishnan Vijayakrishnan
Department of Biophysics, All India Institute of Medical Sciences,
New Delhi 110029, India.

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have been the therapeutic agents of choice for more than a century. However, their adverse gastrointestinal side-effects led to the advent of COX2 selective inhibitors, the coxibs. Recent reports on the harmful cardiovascular and renal side-effects of the conventional NSAIDs as well as the COX2 selective inhibitors valdecoxib and rofecoxib have once again led to the quest for a novel class of inhibitors. Using a structure-based approach, a small, potent and selective peptide inhibitor of COX2 has been identified. The designed peptide is predicted to have a higher activity than the most potent, known inhibitor (celecoxib), and a 800-fold selectivity for COX2 over COX1. It is thus a promising lead compound for the development of a new class of COX2 selective inhibitors.

Key words: Structure-based drug design, Cyclooxygenase, Docking, Peptide inhibitor, Anti-Inflammatory drugs.

Correspondence: Prof. Gita Subba Rao FAMS, Secretary, NAMS, New Delhi 110-029, India.
Tel: 91-11-26589289, Fax: 91-11-26588992, E-mail: nams_aca@yahoo.com.

Introduction

There are a variety of enzymes which can function as potential targets in the inflammatory pathway. Also there are a few receptors at the target site which, if scientifically analyzed, can function as potential targets for drug design. Some of the important targets for designing drugs are:

1. Phospholipase A
2. Cyclooxygenases
3. Lipoxygenases
4. Thromboxane A Synthase
5. PGF Synthase
6. PGE Synthases
7. PGD synthase
8. Receptor – PGE_2 receptor, $PGF_{2\alpha}$ receptor, etc.

Cyclooxygenase Pathway and Inflammation

One of the most important mediators of inflammation, prostaglandins (PG), belong to the group of compounds called *eicosanoids*. Prostaglandins are a group of derivatives from C_{20} polyunsaturated fatty acids in the cyclooxygenase pathway. Eicosanoids can mediate virtually every step of inflammation (1). Eicosanoids (arachidonic acid [AA] metabolites) are synthesized by two major classes of enzymes: cyclooxygenases and lipoxygenases. The cyclooxygenase (COX) pathway (Fig. 1) generates eicosanoids with a ring structure known as prostanoids (PGs, TXs, Prostacyclins) while lipoxygenase (LOX) produces open chain compounds (Leukotrienes). Further course in a particular tissue depends on the type of isoenzymes or other enzymes present in it (2). The synthesis of prostaglandin group of eicosanoids is mediated mainly by COX1 and COX2 enzymes. COX1 is a constitutive enzyme expressed all the time, whereas COX2 is inducible and is over expressed at sites of inflammation. COX1 regulates a number of house keeping functions, including gastroprotection, vascular homeostasis, renal blood flow and maintenance of glomerular function (3,4). COX2, in addition to maintenance of cardiovascular, renovascular physiological functions and platelet aggregation, is responsible for the expression of pro-inflammatory prostaglandins (5-7). Prostaglandins will in turn cause vasodilatation thus furthering the inflammatory process. The PGs are also involved in the pathogenesis of fever (temperature regulation in the hypothalamus) (1). Further, PGE_2 is hyperalgesic in that

it makes the skin hypersensitive to pain stimuli (8).

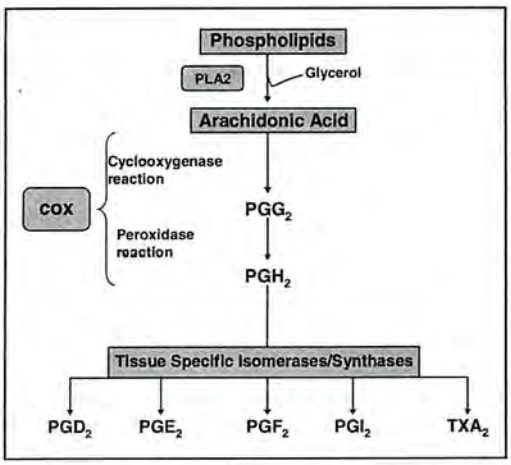

Figure 1: Cyclooxygenase (COX) pathway

Although there are clear differences in function and the DNA and mRNA structures of COX1 and COX2, there is much less difference between the protein structure and function of these enzymes. The core sequences of both enzymes, as well as their crystal structures, are 60% identical (9). Both enzymes have similar kinetics for AA. Despite these similarities, evidence indicates that COX1 and COX2 function as separate enzyme systems. COX1 is localized in the endoplasmic reticulum, whereas COX2 is localized in the endoplasmic reticulum and the nuclear membrane. In addition, COX1 and COX2 use different pools of arachidonate that are mobilized in response to different cellular stimuli for prostaglandin synthesis. PGD_2 is the major metabolite of cyclooxygenase pathway in mast cells; along with PGE_2 and $PGF_{2\alpha}$ (which is more widely distributed), it causes vasodilatation and potentiates edema. Vasodilatory function is also carried out by PGI_2 and PGE_2. PGI_2 also markedly potentiates the permeability-increasing and chemotactic effects of other mediators of inflammation.

There are many possible ways to control inflammation. The narcotic agents like morphine, are one of the most potent analgesic drugs available; but they have very serious side effects - drug abuse and dependency. The steroids are one of the most effective anti-inflammatory drugs currently available for a patient. But the large number of unwanted side effects has led to a focus on non-steroidal drugs with anti-inflammatory properties. Thus the medical field saw the advent of non-steroidal anti-inflammatory drugs (NSAIDs), which function as COX inhibitors. These drugs are free not only from the abuse potential of narcotic analgesics, but also from the steroidal side effects. The production of PGs responsible for the inflammatory process could be sufficiently controlled with conventional NSAIDs. However, these drugs, which inhibited both COX1 and COX2 were found to have adverse gastrointestinal side-effects and, therefore, drugs which selectively

inhibit COX2, such as coxibs were developed. Recent reports on the harmful cardiovascular and renal side-effects of the conventional NSAIDs as well as the COX2 selective inhibitors valdecoxib and rofecoxib have once again led to the quest for a novel class of COX2 selective inhibitors (10-12).

The coxibs (COX2 selective NSAIDs) are now finding new therapeutic applications in the field of colorectal cancer prevention as well as the prevention of progression of Alzheimer's disease (13-18). Celecoxib (19) has been approved for patients with Familial Adenomatous Polyposis (FAP) who have not had their colons removed. Coxibs have also found newer therapeutic application in lung chemoprevention (20).

The current classification of NSAIDs (Table 1) is based on the selectivity of these drugs to the two major isoforms of the COX. NSAIDs are classified as Nonselective Inhibitors [Conventional NSAIDs] (e.g., Aspirin, Indomethacin, Diclofenac etc), Preferential Inhibitors (e.g., Nimesulide, Meloxicam, Nabumetone, Etodolac), Selective Inhibitors (e.g., Celecoxib, Lumiracoxib) and Analgesic-antipyretics with poor anti-inflammatory action (e.g., Paracetamol, Nefopam) (21).

The concept of selective COX2 inhibitor drugs emerged from the need to overcome the unwanted gastrointestinal side effects of the NSAIDs currently available in the market. By definition selectivity is the degree of preference of a drug for a target with respect to another target molecule. In case of COX2 selective inhibitors, popularly known as Coxibs, biochemical assays usually reveal more than a 100-fold difference in the concentration that inhibits recombinant COX2 as compared with COX1 (23).

Table 1 : Classification of NSAIDs (2, 22)

Highly COX2 Selective	Preferential COX2 Selective	COX2 Non-Selective	NSAIDs Different Mechanism
Etoricoxib	Nimesulide	Aspirin	Acetaminophen
Lumiracoxib	Meloxicam	Diclofenac	
Parecoxib	Etodolac	Ibuprofen	
Rofecoxib		Indomethacin	
Celecoxib		Ketorolac (8)	
Valdecoxib			

Diclofenac is considered as one of the safest and has a better pharmacological profile when compared to other conventional NSAIDs (24). The lower the IC_{50} value the drug possesses, the higher is its affinity for the target enzyme. The IC_{50} values and the COX2 selectivity of various NSAIDs with human COX are given in the Table 2.

Due to the similarity of the COX isoenzymes (9), all non-selective NSAIDs also inhibit COX1 thereby blocking its constitutive function and causing various side-effects (10). So it is desirable to design a new class of selective inhibitors on the basis of the structures of COX1 and COX2. Keeping this in mind, we have used the available

Table 2 : Activity and Selectivity of NSAIDs

Drug	IC_{50} for COX1 [ì M]	IC_{50} for COX2 [ì M]	COX2 Selectivity
Aspirin	1.67	278	0.006
Sulindac	1.12	112	0.010
Indomethacin	0.03	1.68	0.017
Naproxen	9.56	5.65	1.692
Flurbiprofen (25)	0.08	0.90	0.089
Ibuprofen	0.03	1.68	0.167
Piroxicam	0.002	0.60	0.004
Diclofenac (26)	0.61	0.63	0.970
Nimesulide	70	1.27	55.118
Celecoxib (23,27)	15	0.04	375
Rofecoxib (23)	15	0.02	833.333
Nabumetone (28)	149	230	0.648

X-ray crystal structures of the complexes of COX1 and COX2 with the known inhibitors to carry out a structure-based, rational, molecular modeling approach to design small peptide inhibitors, which are both potent and selective for COX2.

Structural Details

COX is a heme containing dioxygenase bound to endoplasmic reticulum membranes. Both COX1 and COX2 catalyze two different reactions: a dioxygenase (cyclooxygenase) reaction

and a peroxidase reaction. Structural studies indicate that the active sites are spatially distinct from each other and separated by a heme group (29). COX1 and COX2 have very similar structures characterized by a membrane-binding domain comprised of amphipathic helices forming the entrance to a long hydrophobic channel. This channel leads deep inside the protein, and at its upper end comprises the cyclooxygenase active site.

The important binding residues in the active site (substrate binding) of COX (30) are as follows:

- ARG-120
- VAL-349
- SER-353
- LEU-384
- TYP-387
- VAL-523
- ALA-527
- LEU-531
- TYR-348
- LEU-352
- TYR-355
- TYR-385
- MET-522
- GLY-526
- SER-530

The active site contains mainly hydrophobic residues (9). The known inhibitor, diclofenac, makes hydrogen bonds with tyrosine 385 and serine 530 (31) in the crystal structure complex with COX2 (Fig. 2a). These hydrogen bond interactions are different from the other NSAIDs like flurbiprofen, which have hydrogen bonding to the residues R120 and Y355 as shown in Fig. 2b (found near the lower part of the defined active site of COX2) (32). Hence

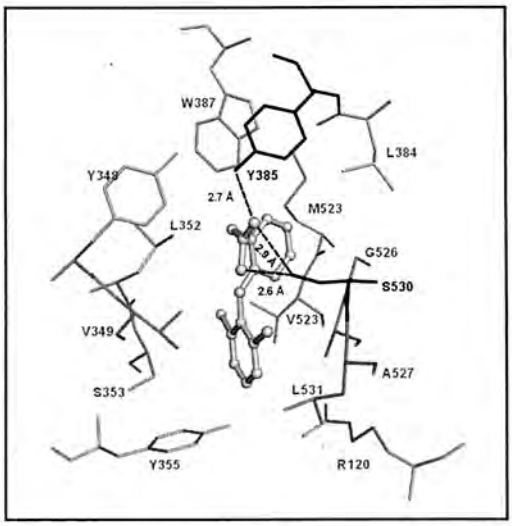

Figure 2a: Crystal structure of COX2 complex with Diclofenac (in light-grey, ball-and-stick rendering) showing the active site residues (stick rendering). Hydrogen bonds are shown as black, dashed lines together with distances. All figures were produced using PyMol v0.99 (35).

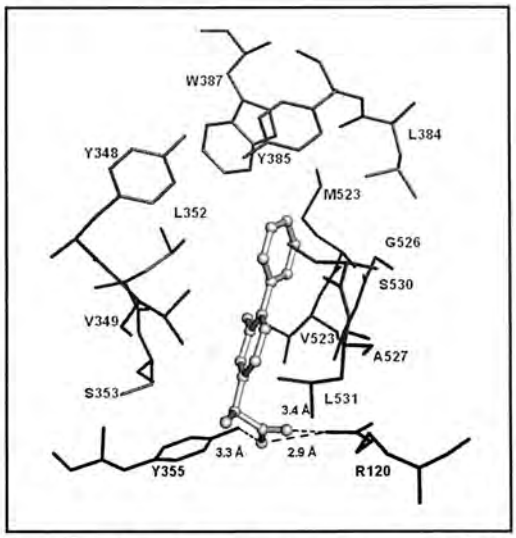

Figure 2b: Crystal structure of COX2 complex with Flurbiprofen. Coloring and rendering are the same as in Fig. 2a.

it would be challenging to design an inhibitor which has hydrogen bonding to residues both in the upper part as well as in the lower part of the active site. Aspirin binds irreversibly to serine 530 by acetylation, whereas most other NSAIDs sterically and reversibly bind to tyrosine 385 or arginine 120, thus blocking the cyclooxygenase action of the enzyme (33, 34). The ligand binding sites of COX1 and COX2 are almost identical except for the amino acid residue at position 523, which is Ile in COX1 and Val in COX2. This leads to a smaller binding site in the case of COX1 (Fig. 3), and the difference can be exploited for the design of COX2 specific inhibitors.

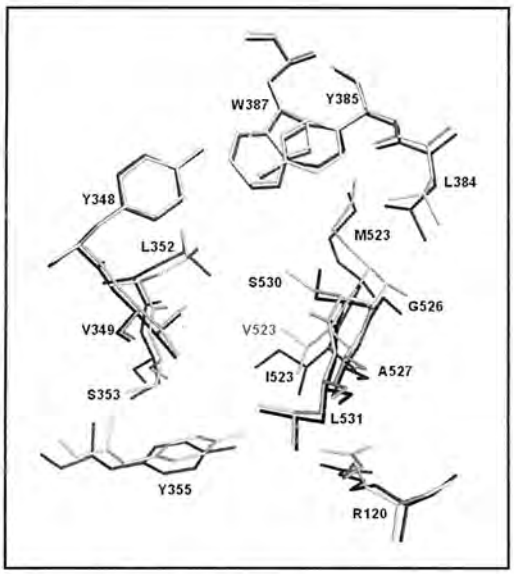

Figure 3: Superposition of COX1 (in grey) and COX2 (in black) active sites showing the difference.

The crystal structures of COX1 and COX2 complexed with several inhibitors have been determined. Our aim in this work is to design a small peptide inhibitor which has

(i) interactions with all the important active site residues,

(ii) potency higher than that of the known inhibitors, and

(iii) high selectivity for COX2

Materials and Methods

The starting point for the computational studies was the X-ray crystal structure of COX2 complexed with the ligand diclofenac [PDB code: 1PXX] (31, 36).

All computations were done using the molecular modeling software package SYBYL 6.81 (Tripos Inc.) running on a Silicon Graphics O2 Workstation. Docking of ligands to PTP1B was done with the version 3.0 of the program AutoDock (37).

Results and Discussion

Validation of the docking methodology

The AutoDock 3.0 methodology was first tested on two known inhibitors (diclofenac and flurbiprofen), which were docked in the active site of COX2, after extracting the ligand from the crystal structure. These two inhibitors

are known to bind to different regions in the active site of COX2.

Figure 4 shows the superimposition of the docked diclofenac (in grey-white) with the crystal structure position (in black). The two structures are seen to overlap very well with a positional root mean square deviation (rmsd) of 2.0 Å. The free energy of binding, ÄG was -9.44 kcal/mole, and the corresponding K_d value at 298K was 0.11μM, which agrees well with the observed value of 0.63μM (26).

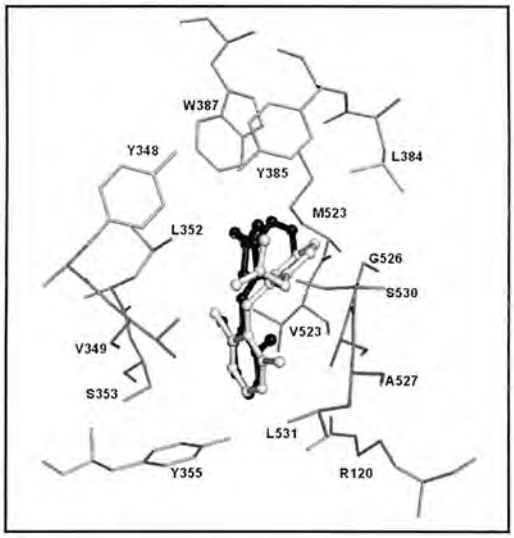

Figure 4: Docked position of diclofenac (in grey-white, ball-and-stick rendering) superimposed on the crystal structure position (in black, ball-and-stick rendering).

Figure 5 shows the superimposition of the docked flurbiprofen (in grey-white) with the crystal structure position (in black) [PDB ID: 1HT8]. It can be seen that the two structures overlap very well with a positional root mean square deviation (rmsd) of 0.6 Å. The free energy of binding, ÄG was -9.46 kcal/mole, and the corresponding K_d value was 0.16 μM, which agrees well with observed value of 0.9 +/- 0.25 μM (25).

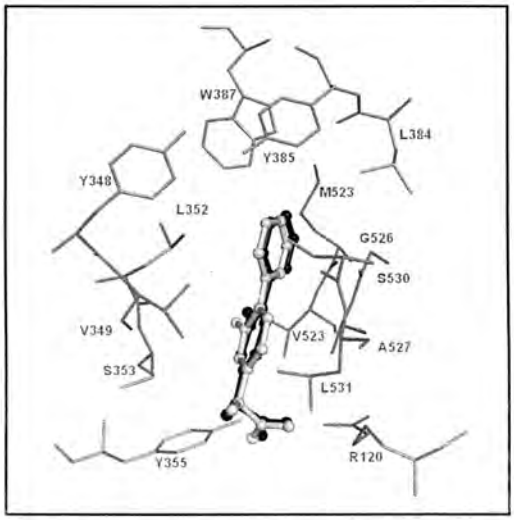

Figure 5: Docked position of flurbiprofen (in grey-white, ball-and-stick rendering) superimposed on the crystal structure position (in black, ball-and-stick rendering).

Thus the docking methodology gives results that are consistent with the observations.

Design of the Peptide Inhibitor and Modeling of the COX2 inhibitor complex

The known potent inhibitors of COX2, such as nimesulide and celecoxib (Fig. 6) have two aromatic rings and a negatively charged group.

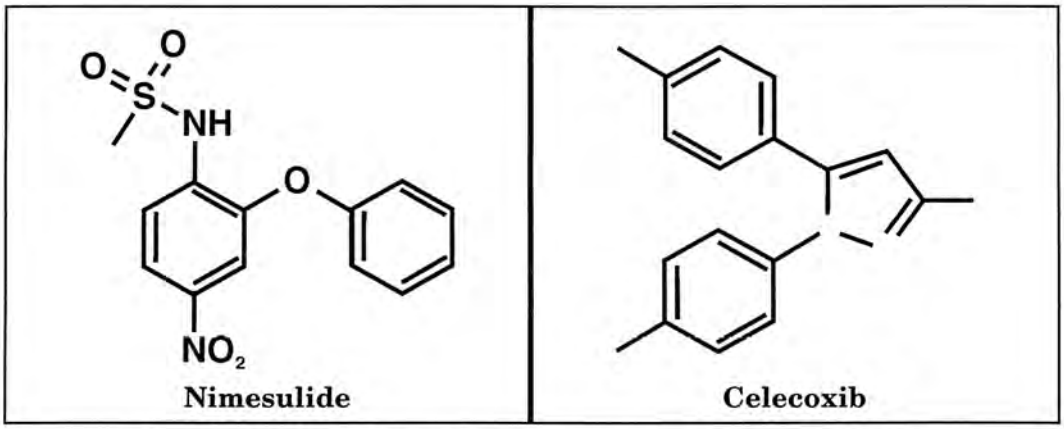

Figure 6: Chemical structure of known, potent inhibitor of COX2.

Using this information, we designed several tripeptide sequences containing two aromatic rings, and a charged residue at the C-terminal end. Each of these tripeptides was docked after a conformational energy search.

One of the most suitable ones (in terms of the docking energy and the interactions) was found to have the following sequence.

$NH3^{+}$ - YWG - COO^{-}

The final docked position of the designed inhibitor is shown in Figures 7 and 8, and the list of contacting residues (up to 4Å) is given in Table 3. It can be seen from the figures and the table that the tripeptide inhibitor occupies a position similar to that of diclofenac and flurbiprofen, but with a larger set of interacting residues. The inhibitor is seen to have hydrogen bonding with the residues Ser 530, Ala 527, Tyr 385, Arg 120 and His 90. The AutoDock3.0 results gave a free energy of binding ÄG = -13.15 kcal/mole and a corresponding K_d value of 2.28 × 10^{-4}ì M, which is about 21 times compared to the most potent known inhibitor (4.8 × 10^{-3}ì M for celecoxib (27)).

Conclusion

Using a computer modeling approach we have identified a small peptide inhibitor of COX2 which has a higher activity than that of the most potent, known inhibitor (celecoxib). In addition the designed peptide has an 800-fold selectivity for COX2 over COX1. Thus the designed inhibitor is a highly suitable lead compound for a new class of potent and selective COX2 inhibitors. Another promising potent and COX2 selective tripeptide inhibitor (WYD) has recently been identified by us (38).

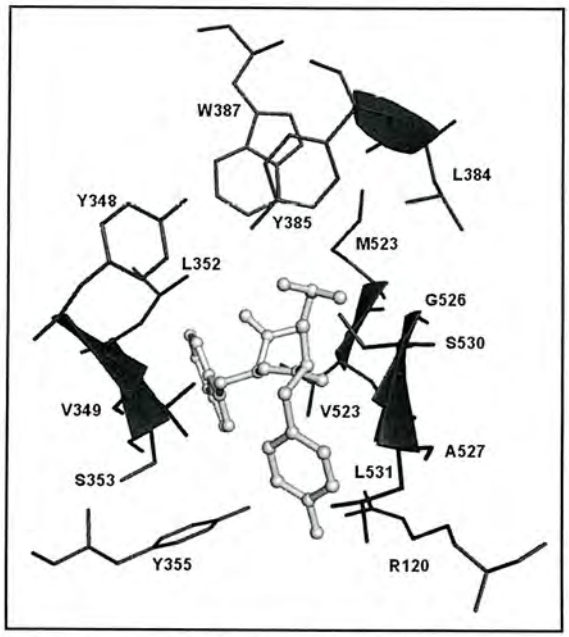

Figure 7: Final docked position of the designed peptide inhibitor, YWG (grey-white, ball-and-stick rendering) with COX2.

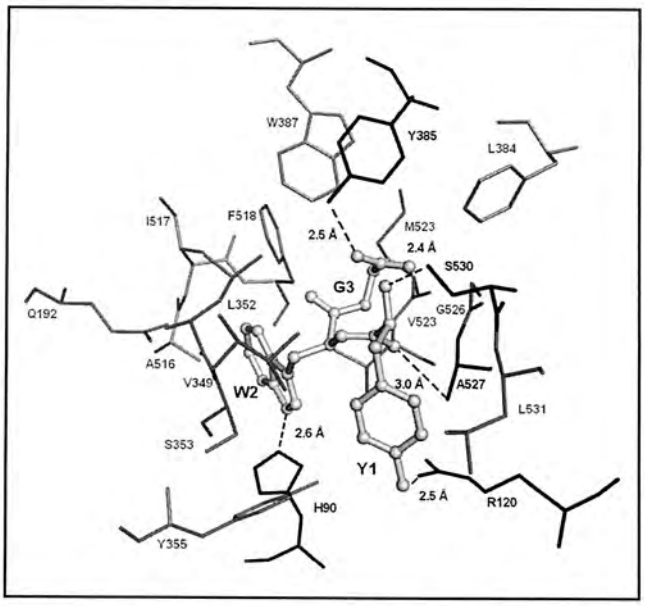

Figure 8: Final docked complex of COX2 with the designed inhibitor (grey-white, ball-and-stick rendering) showing the interacting residues (stick rendering). Hydrogen bonds are shown as black, dashed lines together with distances.

Table 3 : SYBYL6.81 Docking Results

Ligand	Docked Energy(kcal/mol)			Contacting residues (upto 4Å) in the final docked position (hydrogen bonded residues are highlighted in bold)
	Steric	Electrostatic	Total	
Diclofenac	-23.0	-0.4	-23.4	Y348, V349, L352, S353, Y355, L384, **Y385,** W387, F518, M522,V523, G526, A527, **S530**, L531.
Flurbiprofen	-20.0	-4.1	-24.1	**R120**, V349, **Y355**, L384, Y385, W387, M522, V523, G526, A527, S530, L531.
YWG	-59.9	-30.7	-90.6	**H90**, **R120**, Q192, V349, L352, S353, Y355, F381, **Y385**, W387, A516, I517, F518, M522, V523, G526, **A527**, **S530**, L531

References

1. Tucker C (2000). Acute and Chronic Inflammation. *Robbins Pathologic Basis of Diseases* **6th edition**: 50-88.
2. Tripathi KD (2003). Prostaglandins, Leukotrienes (Eicosanoids) and Platelet activating factor. *Essentials of Medical Pharmacology* **5th edition**: 156-166.
3. DeWitt DL and Smith WL (1988). Primary Structure of Prostaglandin G/H Synthase from sheep vesicular gland determined from the Complementary DNA Sequence. *Proc. Natl. Acad. Sci. USA.* **85:** 1412–1416.
4. Yang T, Schnermann JB and Josephine P (1999). Regulation of cyclooxygenase-2 expression in renal medulla by tonicity in vivo and in vitro. *Am J Physiol Renal Physiol.* **277**: F1-F9.
5. Hershman HR (1996). Prostaglandin synthase 2. *Biochim. Biophys.Acta.* **1299**: 125–140.
6. Masferrer JL, Seibert K, Zhang Y, Leahy K, Hauser S, Perkins W, Lee L, Isakson P (1994). Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA.* **91**: 3228-3232.
7. Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W,

Lee L and Isakson P (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci USA.* **91**: 12013-12017.

8. Dray A (1995). Inflammatory mediators of pain. *Br J Anaesth.* **75:** 125-131.

9. Picot D, Loll PJ and Garavito RM (1994). The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* **367**: 243-249.

10. Hinz B, Renner B and Brune K (2007). Drug Insight: cyclo-oxygenase-2 inhibitors - a critical appraisal. *Nat Clin Pract Rheumatol* **3**: 552-560.

11. Puhlmann U, Schafer D and Ziemann C (2006). Update on COX-2 inhibitor patents with a focus on optimized formulation and therapeutic scope of drug combinations making use of COX-2 inhibitors. *Expert Opinion on Therapeutic Patents* **16**: 403-430.

12. Solomon DH, Avorn J, Sturmer T, Glynn RJ, Mogun H, Schneeweiss S (2006). Cardiovascular outcomes in new users of Coxibs and nonsteroidal antiinflammatory drugs: high risk subgroups and time course of risk. *Arthritis and Rheumatism* **54**: 1378-1389.

13. Xiao Z, Luke BT, Izmirlian G, Umar A, Lynch PM, Phillips RKS, Patterson S, Conrads TP, Veenstra TD, Greenwald P, Hawk ET and Ali IU (2004). Serum proteomic profiles suggest Celecoxib-Modulated targets and response predictors. *Cancer Research* **64**: 2904–2909.

14. ADAPT Research Group (2007). Naproxen and celecoxib do not prevent AD in early results from a randomized controlled trial. *Neurology*. **68(21)**: 1800-1808.

15. Altman R (2003). Acute coronary disease Athero-Inflammation: Therapeutic approach. *Thrombosis Journal* **1**, 2.

16. Altman R, Luciardi HL, Muntaner J, Del Rio F, Berman SG, Lopez R and Gonzalez C (2002). Efficacy assessment of meloxicam, a preferential cyclooxygenase-2 inhibitor, in acute coronary syndromes without ST-segment elevation: the Nonsteroidal Anti-Inflammatory Drugs in Unstable Angina Treatment-2 (NUT-2) pilot study. *Circulation* **106**: 191-195.

17. Malmstrom K, Kotey P, Cichanowitz N, Daniels S and Desjardins PJ (2003). Analgesic efficacy of Etoricoxib in primary dysmenorrhea: Results of a randomized, controlled trial. *Gynecologic and Obstetric Investigation* **56**: 65-69.

18. Prescott WA and Johnson CE (2005). Antiinflammatory therapies for cystic fibrosis: past, present, and future. *Pharmacotherapy* **25(4)**, 555-573.

19. Henney JE (2000). New indication for Fluoxetine. *JAMA* **283**, 1131

20. Hernández-Díaz S and Rodríguez LAG (2007). Nonsteroidal anti-inflammatory drugs and risk of lung cancer. *International Journal of Cancer* **120(7)**: 1565-1572.

21. Tripathi KD (2003). Prostaglandins, Leukotrienes (Eicosanoids) and Platelet activating Factor. *Essentials of Medical Pharmacology*. **5th edition**, 167-184.

22. Davies NM and Jamali F (2004). COX-2 selective inhibitors cardiac toxicity: getting to the heart of the matter. *J Pharm Pharmaceut Sci.* **7(3)**: 332-336.

23. Rehman Q and Sack KE (1999). When to try COX-2-specific inhibitors. Safer than standard NSAIDs in some situations. *Postgraduate Medicine*. **106(4)**: 95-106.

24. Wyatt PJ, Illingworth RN, Clancy MJ, Munro PT and Robertson CE (2005). *Oxford Handbook of Accident and Emergency Medicine*. **2nd edition**, 263-310.

25. Santini G, Sciulli MG, Panara MR, Padovano R, di Giamberardino M, Rotondo MT, Del Soldato P, Patrignani P (1996). Effects of flurbiprofen and flurbinitroxy-butylester on prostaglandin endoperoxide synthases. *European Journal of Pharmacology* **316 (1)**: 65-72.

26. Blanco FJ, Guitian R, Moreno J, de Toro FJ and Galdo F (1999). Effect of antiinflammatory drugs on COX-1 and COX-2 activity in human articular chondrocytes. *J Rheumatol*. **26(6)**: 1366-1373.

27. Lu ZH, Xiong XY, Zhang BL, Lin GC, Shi YX, Liu ZG, Meng JR, Zhou YM and Mei QB (2005). Evaluation of 2 celecoxib derivatives: analgesic effect and selectivity to cyclooxygenase-2/1. *Acta Pharmacol Sin.* **26(12)**: 1505-1511.

28. Kato M, Nishida S, Kitasato H, Sakata N and Kawai S (2001). Cyclooxygenase-1 and cyclooxygenase-2 selectivity of non-steroidal anti-inflammatory drugs: investigation using human peripheral monocytes. *Journal of Pharmacy and Pharmacology* **53(12)**, 1679-1685(7).

29. Malkowski MG, Ginell SL, William LS and Garavito RM (2000). The productive conformation of arachidonic acid bound to

prostaglandin synthase. *Science* **289**: 1933-1937.

30. Bathon JM, Chilton FH, Hubbard WC, Towns MC, Solan NJ and Proud D (1996). Mechanisms of prostanoid synthesis in human synovial cells: cytokine-peptide synergism. *Inflammation*. **20:** 537-554.

31. Rowlinson SW, James RK, Prusakiewicz JJ, Pawlitz JL, Kozak KR, Kalgutkar AS, Stallings WC, Kurumbail RG and Marnett LJ (2003). A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385. *J Biol Chem*. **278:** 45763–45769.

32. Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, W. C. Stallings *et al*(1996). Structural basis for selective inhibition of cyclo-oxygenase-2 by anti-inflammatory agents. *Nature* **384:** 644-648.

33. Loll PJ, Picot D and Garavito RM (1995). The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat Struct Bio*. **2:** 637-645.

34. DeWitt DL, El-Harith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL and Smith WL (1990). The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J Biol Chem*. **265:** 5192-5198.

35. DeLano WL (2002). The PyMOL Molecular Graphics System (2002) on World Wide Web http://www.pymol.org.

36. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN and Bourne PE (2000). The Protein Data Bank. *Nucleic Acids Research*. **28**, 235-242.

37. Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Beleu RK and Olson AJ (1998). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem*. **19**: 1639-1662.

38. Vijayakrishnan R, Ramachandran MV and Subba Rao G (2008). Computer-aided, rational design of a potent and selective small peptide inhibitor of cyclooxygenase 2 (COX2). *J. Biomol. Struct. & Dynamics* **25(5)**: 535-542.