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Biochemical and Biophysical Research Communications 361 (2007) 37–42
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Surface plasmon resonance studies and biochemical evaluation of a potent peptide inhibitor against cyclooxygenase-2 as an anti-inflammatory agent

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Received 15 June 2007
Available online 5 July 2007

Abstract

Cyclooxygenase (COX) is a key enzyme in the biosynthetic pathway leading to the formation of prostaglandins, which are mediators of inflammation [D.L. Dewitt, W.L. Smith, Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence, Proc. Natl. Acad. Sci. USA 85 (1988) 1412–1416, 1]. It exists mainly in two isoforms COX-1 and COX-2 [A. Raz, A. Wyche, N. Siegel, P. Needleman, Regulation of fibroblast cyclooxygenase synthesis by interleukin-1, J. Biol. Chem. 263 (1988) 3022–3028, 2]. The conventional non-steroidal anti-inflammatory drugs (NSAIDs) have adverse gastrointestinal side-effects, because they inhibit both isoforms [T.D. Warner, F. Guiliano, I. Vojnovic, A. Bukasa, J.A. Mitchell, J.P. Vane, Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis, Proc. Natl. Acad. Sci. USA 96 (1999) 7563–7568, 3; L.J. Marnett, A.S. Kalgutkar, Cyclooxygenase 2 inhibitors: discovery, selectivity and the future, Trends Pharmacol. Sci. 20 (1999) 465–469, 4; J.R. Vane, NSAIDs, Cox-2 inhibitors, and the gut, Lancet 346 (1995) 1105–1106, 5]. Therefore drugs which selectively inhibit COX-2, known as coxibs were developed. Recent reports on the harmful cardiovascular and renovascular side-effects of the anti-inflammatory drugs have led to the quest for a novel class of COX-2 selective inhibitors. Keeping this in mind, we have used the X-ray crystal structures of the complexes of the COX-1 and COX-2 with the known inhibitors for a rational, structure based approach to design a small peptide, which is potent inhibitor for COX-2. The peptides have been checked experimentally by in-vitro kinetic studies using surface plasmon resonance (SPR) and other biochemical methods. We have identified a tripeptide inhibitor which is a potential lead for a new class of COX-2 inhibitor. The dissociation constant (K_D) determined for COX-2 with peptide WCS is 1.90×10^{-10} M, the kinetic constant (K_i) determined by spectrophotometry is 4.85×10^{-9} M and the IC_{50} value is 1.5×10^{-8} M by ELISA test.

Keywords: Peptide design; Surface plasmon resonance; Enzyme kinetics; Platelet aggregation

Treating inflammation associated with rheumatoid diseases is a challenge for the medical world. The main cause for the inflammation is the overproduction of prostaglandins, which are synthesized by cyclooxygenase enzymes. Prostaglandins are a group of derivatives from C_{20} polyunsaturated fatty acids in the cyclooxygenase pathways, known as eicosanoids. The synthesis of eicosanoids is mediated mainly by COX-1 and COX-2 enzymes. COX-1 is a constitutive enzyme expressed all the time, whereas COX-2 is inducible and is over expressed at sites of inflammation. COX-1 regulates a number of house keeping functions, including vascular homeostasis, renal blood flow and maintenance of glomerular function [6]. COX-2 in addition to maintenance of cardiovascular, renovascular physiological functions and platelet aggregation is responsible for the expression of pro-inflammatory prostaglandins [7–9]. The

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doi:10.1016/j.bbrc.2007.06.122

two enzymes are highly similar in structure and enzymatic activity. Both are homodimeric heme containing proteins with molecular weight 71 kDa. Garavito's group have determined the three-dimensional structure of cyclooxygenase offering new insights into its functional role [10]. The COX molecule consists of three-independent folding units: an EGF-like domain and the two functional domains. Within the enzymatic domain, the two functional sites (cyclooxygenase and peroxidase) are adjacent but spatially distinct. The COX active site in the protein interior is connected to the membrane by a long non-polar channel through which the substrate/inhibitor gains access. The active site of cyclooxygenases (Fig. 1) contains mainly hydrophobic residues [10].

The active site of COX-1 and COX-2 are almost identical and the only difference is in amino acid residues at positions 434 and 523 in COX-1 and COX-2, respectively. In the case of COX-1 it is isoleucine and in COX-2 it is valine. Due to this structural similarity of two isoforms of COX's, all non-selective inhibitors of COX-2 also inhibits COX-1 and ceases the production of prostaglandins thus causing various side-effects. Therefore, it is essential to design a new class of selective inhibitors on the basis of the structure of the COX-2. The known potent inhibitors of COX-2 such as SC-558 (a diaryl heterocyclic inhibitor) have 1900-fold selectivity for COX-2 over COX-1. It contains a bromophenyl ring, a pyrazole group and a phenylsulphonamide moiety. Most of the sulphur containing NSAIDs are selective COX-2 inhibitors [11] and this sulphur atom reduces the toxicity of the compound (Fig. 1).

Using this information, we have designed and synthesized several tripeptide sequences containing a hydrophobic amino acid with aromatic ring, a cysteine residue which contains sulphur atom and a charged residue at the C-terminal end. We report cloning, expression, purification of COX-2; synthesis of the designed peptide; kinetic evaluation of peptides with COX-2 by surface plasmon resonance analysis and biochemical assays.

Materials and methods

Amino acids and different reagents were obtained from Chem Impex, Qiagen, Genetix, Cayman Chemicals and Amersham Biosciences etc.

Cloning, expression, and purification of recombinant human COX-2 gene. Human COX-2 cDNA were cloned into pGEMT easy cloning vector (Promega) and sub-cloned in pET-28a bacterial expression vector (Novagen). This was expressed in BL21 (DE3) strain. COX-2 enzyme was purified by Ni–NTA affinity chromatography and finally was present in 50 mM Tris, 1% *n*-octyl-β-glucopyranoside [12].

Synthesis of peptides. The peptide was synthesized by solid phase peptide synthesizer PS3 (Protein technology, USA) using Fmoc and Wang resin chemistry [13]. The resin used was Fmoc-Ser-Wang resin and solvent used for synthesis was dimethylformamide (DMF).

In the first step, Fmoc-Ser-Wang resin was deprotected by 20% piperidine in DMF to form H_2N-Ser-Wang resin. The uronium salt 2-(1H-benzotrizole-1yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) in the presence of base *N*-methylmorpholine (NMM) activated the amino acid to form an active ester of Fmoc-Cys-OH. This was coupled with H_2N-Ser-Wang resin to get Fmoc-Cys-Ser-Wang resin. The above procedure was repeated for Fmoc-Trp-OH to get the final sequence Fmoc-Trp-Cys-Ser-Wang resin. Piperidine (20%) was then used to remove Fmoc protecting group and the resin was cleaved from the peptide with trifluoroacetic acid. The peptide was purified by reverse phase chromatography on C_{18} Pep-RPC column (1.6 × 10 cm, Amersham Bioscience). The peptide was characterized by 1H NMR spectroscopy. (400 MHz Bruker DRX-400 spectrophotometer.)

COX-2 activity and inhibition assay. Initially, the activity assay for the purified His-COX-2 was performed by the assay buffer containing 100 mM

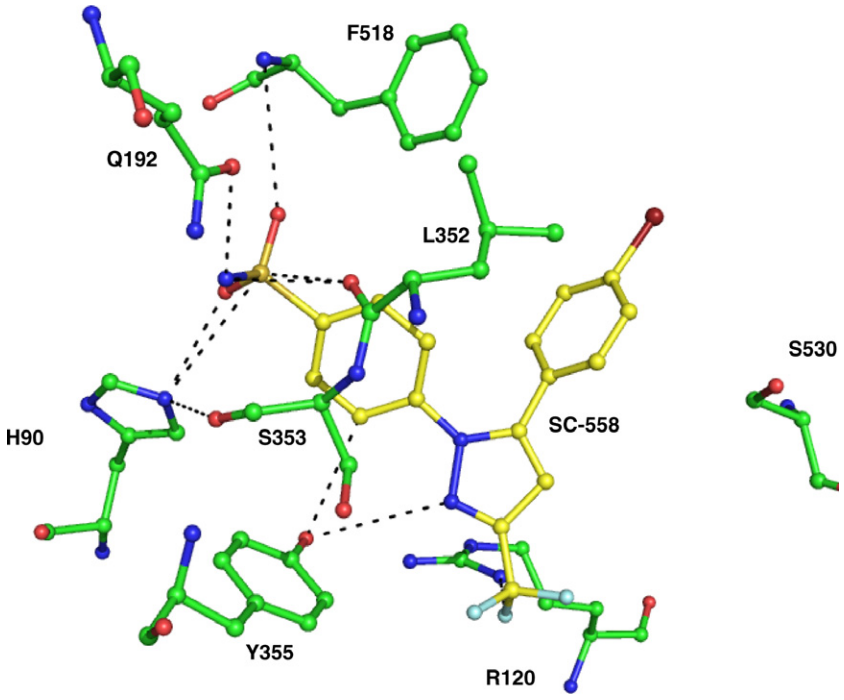

Fig. 1. Active site of cyclooxgenase-2 (COX-2) enzyme showing crystal structure of SC-558 (in yellow) Graphic figure generated using PyMOL v0.99 [30]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Tris–Cl (pH 8.0), 1 mM EDTA, and 2 μM hematin. Baseline was set at 610 nm using the assay buffer. The enzyme was incubated with the assay buffer for 45 min at room temperature. The reaction was started by the addition of the mixture of arachidonic acid (substrate) and *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine (TMPD; co-substrate) to the final concentration of 0.4 mM of arachidonic acid and 0.2 mM of TMPD [14–16]. TMPD oxidation was monitored spectrophotometrically at 610 nm and the initial velocity of the reaction was measured for first 30 s of the reaction and the activity was obtained (Fig. 2A).

Enzyme was incubated with peptide in 1:1 molar ratio in the buffer containing 100 mM Tris–Cl, pH 8.0, 1 mM EDTA, and 2 μM hematin for 45 min at room temperature. The same procedure was followed as above for the determination of initial velocity of the reaction. The percentage inhibition was calculated (Fig. 2B).

ELISA test. The initial screening of peptides for potency and selectivity with respect to COX-1 and COX-2 was done by ELISA method by evaluation of IC_{50} by using COX (ovine) Inhibitor Screening Assay kit procured from Cayman Chemicals [17,18].

The COX (ovine) Inhibitor Screening assay directly measures $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived $PGFH_2$. The prostanoid product is quantified *via* enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds. The Cayman COX assay kit includes both ovine COX-1 and COX-2 enzymes in order to screen isozyme-specific inhibitors. The IC_{50} values were determined by pre-incubation of the peptide inhibitors with enzyme for 5 min at 37 °C in EIA buffer (1 M phosphate solution, pH 7.4, containing 1% BSA, 4 M NaCl, 10 mM EDTA, and 0.1% sodium azide) and 10 μl of hematin. The reaction was stopped by adding 50 μl of 1 M HCl. The amount of prostaglandin formed was measured by ELISA (Cayman Chemical Co., Ann Arbor, MI, USA).

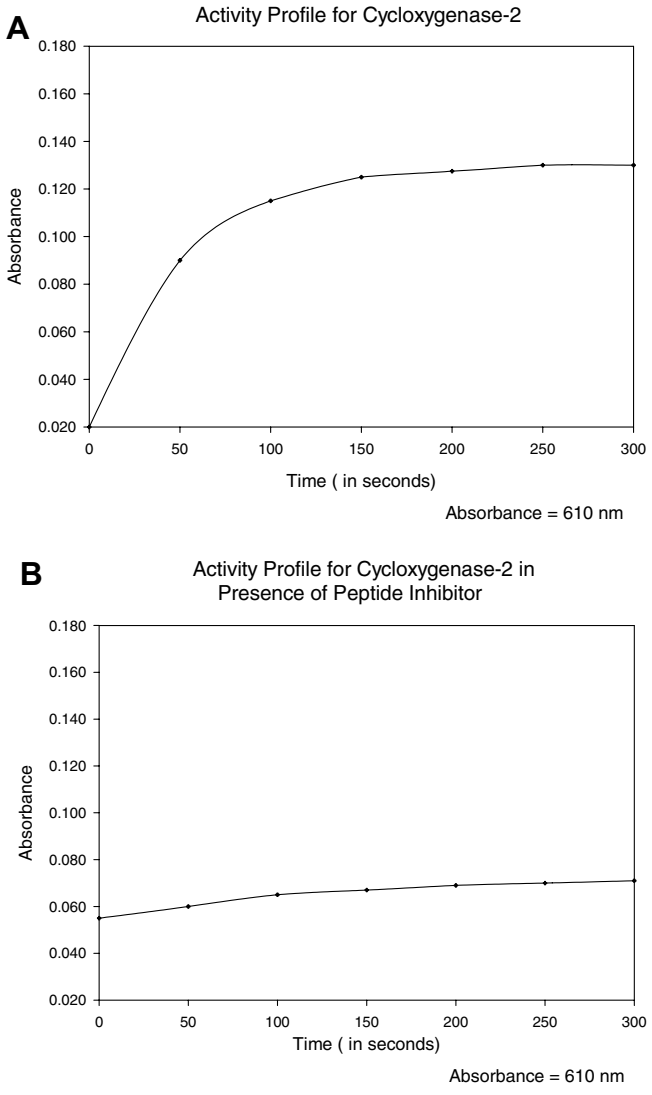

Fig. 2. (A) Activity graph for cyclooxygenase assessed by biochemical assay. TMPD oxidation was monitored spectrophotometrically at 610 nm and the initial velocity of the reaction was measured for first 30 s. (B) Activity graph for COX-2 obtained by the incubation of the enzyme with the peptide WCS in 1:1 molar ratio for 45 min at room temperature.

Kinetic studies on peptide WCS. The enzyme concentration was fixed at 0.5 μM while substrate concentration was varied from 0.4 to 1.6 mM. COX-2 was incubated separately with different concentrations of peptide ranging from 0.005 to 0.02 μM for 45 min. The reactions were initiated by the addition of substrate ranging from 0.4 to 1.6 mM, respectively, for each inhibitor concentration. The resulting products were estimated by the differences in the absorbance at 610 nm in spectrophotometer (Shimadzu Model UV-VIS 160 A). Competitive kinetic constants (K_i) were calculated graphically form double reciprocal plots of velocity versus substrate concentration from Lineweaver–Burk equation [19]. The reciprocal of velocity was plotted on *Y*-axis that of substrate concentration on *X*-axis. The negative reciprocal of the *X* intersect gives K_m.

K_i was calculated as:

$$K_{m'} = K_m\left[1 + \frac{I}{K_i}\right]$$

where $K_{m'}$ is the concentration of substrate that reduces half maximal velocity in the presence of competitive inhibitor and *I* is the concentration of peptide (inhibitor). K_m is Michaelis–Menton constant.

Surface plasmon resonance. A kinetic measurement of the interaction between COX-2 with peptides was performed using a biosensor based on surface plasmon resonance (SPR). The interaction phenomenon of two biological molecules can be monitored directly by the SPR. The phenomenon of SPR was studied by Otto [20] and Kretschmann and Raether [21] and it was used as chemical detection method by Nylander et al. [22]. An automatic instrument BIAcore 2000 (Pharmacia Bioscience) was used. Six histidine-tag which are attached to the terminal position of COX-2 is an ideal tag for immobilization due to strong rebinding effect caused by the high surface density of immobilized Ni^{2+}–nitriloacetic acid (NTA) on the chips used here, the binding of analyte i.e., the peptide in solution can be studied by monitoring the change in the resonance unit (RU) values of the sensorgram, where the progress of the interaction is plotted against time, revealing the binding characteristics.

Analysis of binding kinetics i.e., the association (K_A) and dissociation (K_D) constant for the formation of multi-molecular complex and dissociation were achieved in very short time and with a small amount of samples. 60 μl of His-COX-2 (50 μg/ml) was injected to one of the flow cell at the flow rate of 5 μl/min. Nine hundred resonance unit (RU) of COX-2 was immobilized under these conditions, where 1 RU corresponds to immobilized protein concentration of ~1 pg/mm^2. The analyte i.e., peptide inhibitors at concentration 0.005 μM was passed through the immobilized COX-2 at the flow rate of 10 μl/min and the sensorgram were run for 4 min, likewise two more concentrations 0.01 and 0.02 μM of peptides of same volume were passed over the chip and the change in sensorgram were observed. The graph shows the change in RU values with time for different concentration of peptides.

The rate constants K_A and K_D were obtained by fitting the primary sensorgram data using the BIA evaluation 3.0 software. The dissociation rate constant is derived using equation:

$$R_t = R_{t_0} e^{-k_D(t-t_0)}$$

where R_t is the response at time *t*, R_{t_0} is the amplitudes of the initial response and K_D is the dissociation rate constant. The association rate constant K_A can be derived using equation given below from the measured K_D values.

$$R_t = R_{max}[1 - e^{-(K_A C + K_D)(t-t_0)}]$$

where R_t is the response at time *t*, R_{max} is the maximum response, *C* is the concentration of the analyte in the solution. K_A and K_D are the association and dissociation rate constant, respectively.

Platelet aggregation studies. Human blood sample was obtained by venapuncture and collected in 5 ml siliconised vacutainer tubes. Platelet rich plasma (PRP) was obtained by centrifugation of the blood at 180*g* for 10 min; platelet poor plasma (PPP) was isolated by centrifugation of the blood at 1200*g* for 15 min.

Aggregation studies. Platelet aggregation studies in PRP were performed according to Born's method [23] at 37 °C with constant rate of stirring at 1000 rpm in the Chronolog Aggregometer (Dual Channel) using PPP as reference. During the experiments the optical density was continuously recorded. After a stable baseline was observed for 2 min, 20 μl inhibitor (peptide) was added and stirred for 2 min. Later 50 μl of an aggregation inducing agent (arachidonic acid) was added to PRP and change in the aggregation pattern of PRP was observed for 3 min. 5% variation was observed between the repeated measurements of platelet aggregation in PRP.

Table 1
Results for the peptides screened

S. No.	Peptide	% Inhibition	Dissociation constant (K_D)
1	YAD	⩾33	$4.67 \pm 0.1 \times 10^{-4}$
2	WYD	⩾48	$2.96 \pm 0.1 \times 10^{-6}$
3	WYS	⩾67	$1.62 \pm 0.1 \times 10^{-5}$
4	YWD	⩾42	$5.06 \pm 0.1 \times 10^{-3}$
5	GYW	⩾45	$7.86 \pm 0.1 \times 10^{-5}$
6	WYG	⩾49	$8.85 \pm 0.1 \times 10^{-6}$
7	YWY	⩾52	$7.10 \pm 0.1 \times 10^{-6}$
8	WAY	⩾68	$2.78 \pm 0.1 \times 10^{-7}$
9	WGD	⩾63	$1.67 \pm 0.1 \times 10^{-3}$
10	FCS	⩾78	$6.37 \pm 0.1 \times 10^{-9}$
11	WCS	⩾88	$1.90 \pm 0.1 \times 10^{-10}$
12	WCY	⩾70	$3.74 \pm 0.1 \times 10^{-8}$
13	WCD	⩾35	$1.74 \pm 0.1 \times 10^{-4}$
14	YCS	⩾27	$1.85 \pm 0.1 \times 10^{-3}$
15	WCA	⩾51	$1.66 \pm 0.1 \times 10^{-6}$

Fifteen peptides were checked for its inhibitory activity against COX-2. Peptide WCS was found to be the best inhibitor for COX-2. Inhibition assay was done in triplicates.

Results

Screening of peptides

The activity assay for COX-2 in presence of 15 different peptides was performed by using spectrophotometer. Table 1 shows the percentage inhibition for the 15 peptides. Peptide WCS showed inhibition ⩾85%.

ELISA test

The peptides were further screened by performing ELISA and results indicated the IC_{50} values of peptide

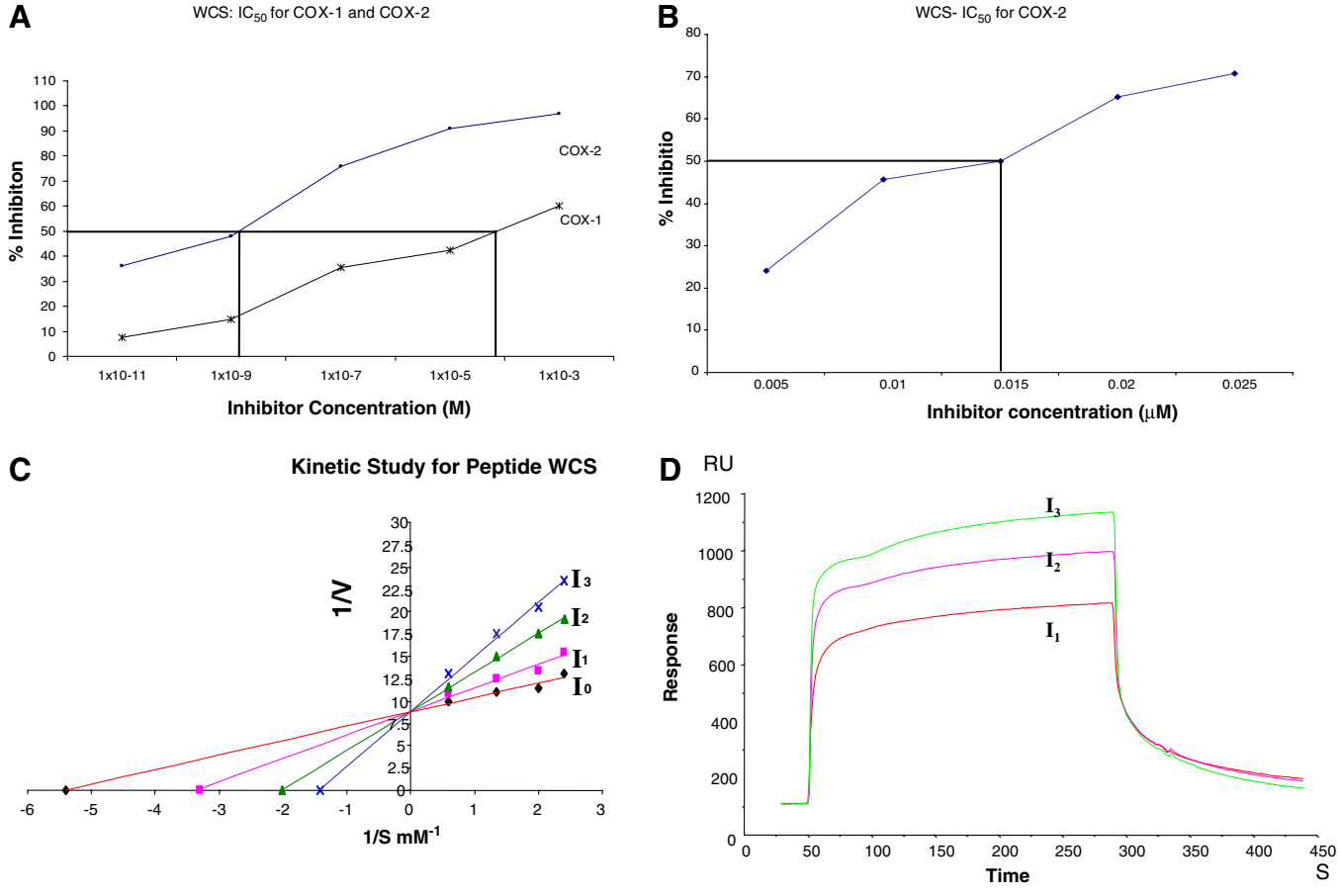

Fig. 3. (A) The IC_{50} values for native COX-1 and COX-2 with peptide inhibitor WCS were 0.85×10^{-4} M and 2.78×10^{-9} M, respectively. (B) The IC_{50} value for His-COX-2 with peptide inhibitor WCS was calculated to be 1.5×10^{-8} M. (C) Sensorgram showing binding of different concentrations of peptide WCS ($I_1 = 0.005$ μM, $I_2 = 0.01$ μM, and $I_3 = 0.02$ μM) on the Ni^{2+}–NTA chip immobilized with His-COX-2. (D) Lineweaver–Burk plot for the kinetics of peptide WCS synthesized against COX-2. COX-2 was incubated separately with different concentration of peptides $I_1 = 0.005$ μM, $I_2 = 0.01$ μM, and $I_3 = 0.02$ μM for 45 min.

WCS for COX-1 and COX-2 (without His-tag) obtained from the plot (a plot of % inhibition versus peptide concentrations) were 0.85×10^{-4} and 2.78×10^{-9} M, respectively (Fig. 3A). The IC_{50} ratio (COX-2/COX-1) for peptide WCS was $\sim 10^4$ times which shows that the peptide is a specific inhibitor for COX-2. The IC_{50} value for peptide WCS with His-COX-2 was calculated to be 1.5×10^{-8} M (Fig. 3B).

Surface plasmon resonance

The plot shows the sensorgram (Fig. 3C) for the binding of the varying concentrations of the peptide. The changes in RU with varying concentration shows the change of mass on the COX-2 immobilized on chips with time. The binding of peptide WCS with COX-2 is the strongest due to the faster on (association) as well as slower off rate (dissociation).

$K_A = 5.26 \times 10^9\ M^{-1}$

$K_D = 1.90 \times 10^{-10}$ M

Determination of kinetic constant (K_i)

Competitive kinetic constants of the best peptide WCS were calculated by using different substrate concentrations. The value of $K_i = 4.85 \times 10^{-9}$ M were obtained from Lineweaver–Burk equation (Fig. 3D).

Aggregation test

Platelet aggregation was highly induced by the addition of 50 μl aggregation inducing agent i.e., arachidonic acid to the PRP (Fig. 4A). In another set of reaction, PRP and 20 μl peptide inhibitor (50 μg/ml) were incubated for 10 min and after that arachidonic acid was added. No aggregation of platelets was observed (Fig. 4B). Celecoxib, a COX-2 selective inhibitor was used as positive blank, which showed no platelet aggregation in presence of arachidonic acid.

Discussion

Human COX-2 has been expressed in bacterial system for the first time and purified with nickel ion affinity chromatography. This prokaryotic expression system was chosen for rapid bacterial growth, stability, inexpensiveness, easiness to operate and high level of protein expression [24]. Though the expression of this eukaryotic protein in a bacterial system does not support post-translational modifications as glycosylation, but still protein was showing activity in the presence of heme which is comparable to that of native protein. This observation indicates that the glycosylation is necessary to attain the native conformation of the protein, but it is not essential for maintaining the catalytically active conformation of the enzyme [25].

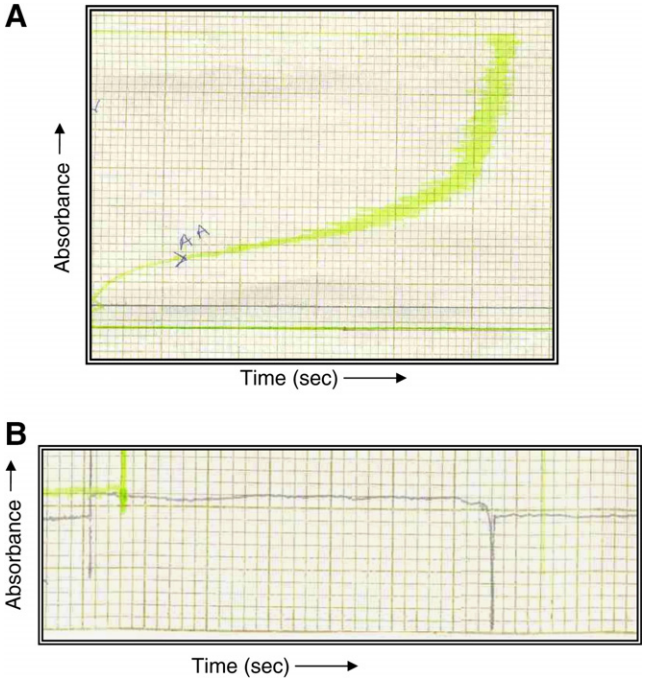

Fig. 4. (A) Effect of arachidonic acid on the platelet aggregation. (B) Platelet aggregation was not observed in the presence of 20 μl of peptide inhibitor at concentration of 50 μg/ml.

The addition of six histidine residues (His-tag) to the amino terminus simplifies the purification procedure, by binding tightly to nickel and thus can act as an affinity tag. The enzyme activity obtained was also not lost due to the addition of His-tag. The sensitivities (IC_{50}) of the His-tagged and native enzymes for non-steroidal drugs and peptide WCS fall within the range (Table 2). Thus, His-tag on N-terminus of COX-2 has no effect on the catalytic properties or inhibitor sensitiveness of the enzyme [14].

The screening of peptides by ELISA test showed that WCS inhibits COX-2 more than 85%. Platelet aggregation was negatively influenced by the use of peptide inhibitor even in presence of aggregation inducing agent arachidonic acid. The binding constant (K_D) and kinetic constant (K_i) of WCS are in nanomolar concentration which is similar to values of known, preferential selective NSAID of COX-2 like SC-558 [11,26]. Though the chemical nature of sulphur atom of sulphonamide group in SC-558 and in cysteine residue is different still similarities in binding con-

Table 2
Comparison of IC_{50} values for His-tagged COX-2 and native COX-2 with known NSAID's and with current peptide WCS

NSAID's/ inhibitor	IC_{50} (M) His-COX-2	IC_{50} (M) native COX-2
Flurbiprofen	380×10^{-9}	$7 \times 10^{-6} - 3 \times 10^{-9}$
Ibuprofen	47×10^{-6}	$56 \times 10^{-6} - 67 \times 10^{-8}$
Diclofenac	10^{-7}	40×10^{-6}
Nimesulide	7.1×10^{-8}	2.6×10^{-8}
WCS	1.5×10^{-8}	2.78×10^{-9}

This table has been compiled from the data previously reported (Refs. [27–29]).

stant of peptide with known NSAIDs SC-558 were observed. Thus, preventing the reaction of substrate arachidonic acid with the enzyme supports the possibility of peptide WCS as potent and competitive inhibitor of COX-2. As the phenyl ring of SC-558 interacts with residues in hydrophobic cavity of COX-2 formed by Phe 381, Leu 384, Tyr 385, Trp 387, Phe 518, and Ser 530, (Fig. 1) it can be assumed that the aromatic ring of tryptophan residue of peptide will also interact with residues in hydrophobic cavity. The free carboxylate group of the peptide can form the charge–charge interactions with Arg 120. However, this needs to be confirmed by the crystal structure of the complex and *in vivo* selectivity studies under physiologically relevant conditions. The designed peptide inhibitor can be considered as a potential lead compound for developing a new class of COX-2 inhibitors.

Acknowledgments

Authors acknowledge financial support from the Department of Science and Technology and Council of Scientific and Industrial Research, Government of India and IRCH, AIIMS for platelet aggregation studies and are thankful to Dr. Prescott, Huntsman Cancer Institute, USA for providing COX-2 cDNA.

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