

A Single Amino Acid Difference between Cyclooxygenase-1 (COX-1) and -2 (COX-2) Reverses the Selectivity of COX-2 Specific Inhibitors*

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Nonsteroidal anti-inflammatory drugs (NSAIDs) currently available for clinical use inhibit both COX-1 and COX-2. This suggests that clinically useful NSAIDs inhibit pro-inflammatory prostaglandins (PGs) derived from the activity of COX-2, as well as PGs in tissues like the stomach and kidney (via COX-1). A new class of compounds has recently been developed (SC-58125) that have a high degree of selectivity for the inducible form of cyclooxygenase (COX-2) over the constitutive form (COX-1). This unique class of compounds exhibit a time-dependent irreversible inhibition of COX-2, while reversibly inhibiting COX-1. The molecular basis of this selectivity was probed by site-directed mutagenesis of the active site of COX-2. The sequence differences in the active site were determined by amino acid replacement of the COX-2 sequences based on the known crystal structure of COX-1, which revealed a single amino acid difference in the active site (valine 509 to isoleucine) and a series of differences at the mouth of the active site. Mutants with the single amino acid substitution in the active site and a combination of three changes in the mouth of the active site were made in human COX-2, expressed in insect cells and purified. The single amino acid change of valine 509 to isoleucine confers selectivity of COX-2 inhibitors in the class of SC-58125 and others of the same class (SC-236, NS-398), while commonly used NSAIDs such as indomethacin showed no change in selectivity. Substitutions of COX-1 sequences in COX-2 at the mouth of the active site of COX-2 did not change the selectivity of SC-58125. This indicates that the single amino acid substitution of isoleucine at position 509 for a valine is sufficient to confer COX-2 selectivity in this example of a diaryl-heterocycle COX inhibitor.

Prostaglandin synthase catalyzes two separate reactions; the first being the cyclooxygenase function, which is the addition of molecular oxygen to arachidonic acid to form the unstable PGG_2 ,¹ and the second the further conversion of PGG_2 to the more stable PGH_2 by a peroxidase function. Hence, this “cy-

clooxygenase” (COX) enzyme performs the critical initial reaction in the arachidonic metabolic cascade leading to the formation of the prostaglandins, thromboxane, and prostacyclin (1).

Recently, a second form of the COX enzyme was isolated whose expression is inducible by cytokines and growth factors (COX-2) (2–6). This inducible COX-2 is linked to inflammatory cell types and tissues and is believed to be the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs (NSAIDs) (7–12). NSAIDs currently available for clinical use inhibit both COX-1 and COX-2 (13, 14). This suggests that clinically useful NSAIDs inhibit pro-inflammatory PGs derived from the activity of COX-2, as well as PGs in tissues like the stomach and kidney (via COX-1). These homeostatic PGs are linked to normal gastric and renal function (15). It is possible that a selective COX-2 inhibitor may eliminate the side effects associated with COX-1 inhibition while providing anti-inflammatory COX-2 inhibition.

A new class of compounds has been described that are all selective inhibitors of COX-2. Compounds such as NS-398, DUP-697, and SC-58125 have been key in elucidating the physiological role of the two types of enzymes. These compounds have both *in vivo* and *in vitro* selectivity (9, 16). Recently, the mechanism of inhibition has been elucidated. It has been demonstrated that the selective COX-2 inhibitor NS-398 exhibits a time-dependent inactivation of COX-2 and no time dependence versus COX-1. The mechanism of this time dependence is unknown. Inhibitor enzyme complexes can be separated after protein denaturation with no change in either the inhibitor or protein (17). Thus, it is apparently a very tight binding interaction and not a covalent one.

Sequence alignment of the two enzymes offers little insight into the differences between the two enzymes at or around the active site. Recently, the x-ray crystal structure of sheep seminal vesicle COX-1 has been solved (18), which gives insight into the structure of the active site. The sequence of COX-2 can be overlaid on the structure, and the differences can be mapped. Major sequence differences were found in the mouth of the substrate access channel and one difference in the active site channel.

Therefore, mutants of COX-2 were constructed and expressed to evaluate specific residues for their contribution to the selective inhibition of compounds.

EXPERIMENTAL PROCEDURES

Materials

Arachidonic acid was purchased from Nu-Chek-Prep Inc. (Elysian, MN); CHAPS, hemin chloride, TMPD, Tris, and heme (bovine hemin chloride) were purchased from Sigma; all other reagents were purchased from Fisher Scientific. NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide) was provided by Dr. John Talley and Dup-697 (5-bromo-2-[4-fluorophenyl]-3-[4-methylsulfonylphenyl]-thiophene) was provided by Dr. Len Lee, both of Monsanto Corporate Research, St. Louis.

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¹ The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drugs; ssv, sheep seminal vesicle; h, human; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TMPD, *N,N,N,N*-tetramethyl-*p*-phenylenediamine; KPO₄, potassium phosphate buffer; ELISA, enzyme-linked immunosorbent assay.

TABLE I
Significant cyclooxygenase 1 and 2 active site differences

Substrate channel entrance				Upper channel			
COX-1	89	92	112	115	119	357	523
Sheep-1	I _{np} ^a	L _{np}	L _{np}	L _{np}	V _{np}	L _{np}	I _{np}
Human-1	T _{ucp}	L _{np}	L _{np}	L _{np}	V _{np}	L _{np}	I _{np}
COX-2	74	77	98	101	105	343	509
Human-2	V _{np}	I _{np}	I _{np}	Y _{ucp}	S _{ucp}	F _{np}	V _{np}

^a Subscripts np = nonpolar and ucp = uncharged polar.

Molecular Modeling

Differences between hCOX-1 and hCOX-2 were identified using a combination of multiple sequence alignment and molecular graphics. ssvCOX-1 (19), hCOX-1 (20), and hCOX-2 (2) sequences were obtained from the Swiss-Prot data base (21) and were aligned using the CLUSTAL W (European Molecular Biology Laboratory, Heidelberg, Germany) package (22). The alignment used the percentage scoring method, a gap penalty of 3, K-tuple of 1, and a window size of 5. Differences between hCOX-1 and hCOX-2 were mapped onto the ssvCOX-1 x-ray coordinates using the Insight II (Molecular Simulations Inc., San Diego, CA) molecular graphics package. Residues for mutagenesis were selected based on a visual examination of side chains both in close proximity to the ssvCOX-1 x-ray structure of the inhibitor, flurbiprofen and along the channel reported to be the putative path of inhibitor binding.

hCOX-2 Mutagenesis

The coding region of hCOX-2 (16) was subcloned into the plasmid pALTER-1, and *in vitro* mutagenesis was performed using an Altered Sites[®] II kit (Promega). One pmol of mutant primer (5'-TGAAACCAT-GAT AGA- AGTTGGAG-3', Midland Certified Reagent Company, Midland, TX) was annealed to 100 ng of single-stranded template, and mutagenesis was performed following the manufacturer's instructions. One-half of the final mutagenesis reaction was used to transform competent BMH 71-18 mutS cells (Clontech, Palo Alto, CA) which were grown overnight in LB broth without ampicillin. Plasmid DNA was isolated from 50-ml cultures using a Qiagen kit (Qiagen, Chatsworth, CA). 10 ng of mutant plasmid was used to transform competent DH10B cells (Life Technologies, Inc., Gaithersburg, MD). Plasmid DNA was isolated from ampicillin-resistant colonies and screened for the presence of the desired mutation by sequencing with a Prism[®] Cycle Sequencing Kit and 373 DNA Sequencer (Applied Biosystems, Foster City, CA). Human COX-2 inserts containing the desired mutation with no secondary misincorporations were isolated with Qiaex resin (Qiagen, Studio City, CA) and subcloned into baculovirus transfer vector pVL 1393 for expression in SF-9 insect cells.

Generation of Recombinant Baculoviruses

Plasmid DNA containing the gene of interest was prepared by alkaline lysis of bacterial cultures and purified over a Qiagen column as described by the manufacturer. Recombinant baculoviruses were generated by transfecting 5 µg of plasmid DNA with 400 ng of linearized baculovirus DNA (Pharmingen, San Diego, CA) using the calcium phosphate procedure. Using the linearized DNA, the transfection mixture yields 99.0% or more recombinants. A stock of the recombinant baculoviruses was made from the transfection supernatant. Cells in a 1-liter spinner flask were grown to 0.5×10^6 cells/ml in serum-containing medium (IPL41, tryptose phosphate, 10% fetal bovine serum) and infected with COX recombinant baculoviruses inoculum at a multiplicity of infection of 0.1 at 27 °C. The cells were harvested at 4 days postinfection by centrifugation at $3,000 \times g$.

COX Purification from Infected Insect Cells

The purification has been described previously (16) and is summarized here. 4–6 g of wet weight cells were homogenized in 10 volumes of 25 mM Tris-HCl, pH 8.1, 0.25 M sucrose. After centrifugation at $10,000 \times g$ and resuspension in the same buffer, CHAPS detergent was added to 1% (w/v). The supernatant was removed following a $50,000 \times g$ centrifugation and loaded directly onto a 5-ml anion exchange column (Macro-Prep High Q; Bio-Rad), equilibrated with 20 mM Tris-HCl, 0.4% CHAPS, pH 8.1. COX enzyme was eluted with a linear gradient of increasing salt concentration to 0.3 M. The pool was concentrated 10× with a stirred cell (Amicon, Beverly, MA) YM-30 membrane to 1 ml and loaded onto a 25-ml Superose-12 column (Pharmacia Biotech Inc.) equilibrated with 25 mM Tris-HCl, 150 mM NaCl, 0.4% CHAPS, pH 8.1, and

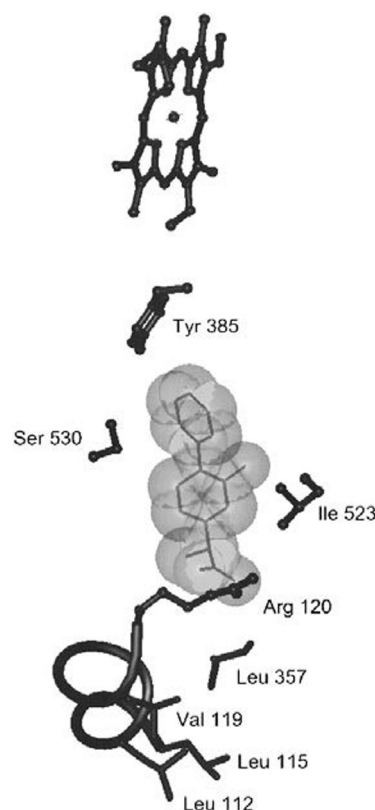


FIG. 1. **View of the ovine COX active site (1).** The heme group is shown at the top of the figure. The inhibitor, flurbiprofen, is rendered as a shaded Corey-Pauling-Koltun. Several residues have been annotated. Annotated residues are rendered as ball and stick. Arg-120, Ser-530, and Tyr-385 (ovine COX numbering) are common among ssvCOX-1, hCOX-1, and hCOX-2 and are shown to orient the reader. The remaining annotated residues represent active site differences between hCOX-1 and hCOX-2, mapped onto the ssvCOX-1 structure. Using a residue numbering scheme of ssvCOX-1 (hCOX-1 → hCOX-2), these changes include: Leu-112 (Leu-111 → Ile-98), Leu-115 (Leu-114 → Tyr-101), Val-119 (Val-118 → Ser-105), Leu-357 (Leu-356 → Phe-343), and Ile-523 (Ile-522 → Val-509).

eluted with the identical buffer at a flow rate of 0.5 ml/min. Pools were aliquoted and stored at -80°C for further use.

Enzyme Assays

Spectrophotometric Assay—Half-maximal inhibition (IC_{50}) was determined by measuring the turnover of TMPD used in a spectrophotometric assay. Arachidonic acid was used, as a hydroperoxide source, along with the peroxidase substrate *N,N,N'*-tetramethyl-*p*-phenylenediamine (TMPD) as a cosubstrate (10). Inhibitors were incubated for 1 min with purified enzyme in 1 µM heme, 0.1 M Tris-HCl, pH 8.1. The reaction was started by addition of 100 µM arachidonic acid, 170 µM TMPD and measured by a change in absorbance at 611 nm. Either the initial rate (linear for approximately 10 s) was measured or time points at 1 and 5 min were taken. Time-dependent inactivation curves were made by incubating 10 µM inhibitor with enzyme for 5 s to 2 min.

PG ELISA—Inhibitory profiles were also assessed by PGE_2 determination. Inhibitors (0.001–100 µM) were preincubated with enzyme for 20 min in 50 mM KPO_4 , pH 7.5, 1 µM heme, 0.01% phenol, 0.3 mM epinephrine. Following a 10-min incubation of arachidonic acid, PGE_2

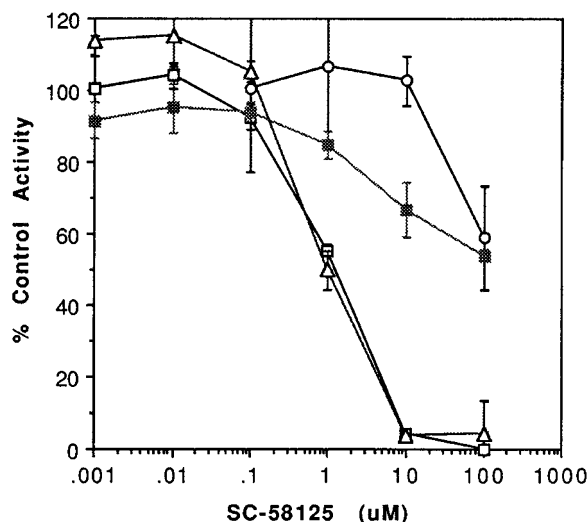


FIG. 2. Inhibition curves of the selective COX-2 inhibitor SC-58125 versus hCOX-1, hCOX-2, and the mutants of hCOX-2: V509I and the triple mutant Y101L,S105V,F343L. The selective COX-2 inhibitor SC-58125 was incubated with 4 μ g of either hCOX-2 (open squares), hCOX-1 (circles), triple mutant of hCOX-2 (triangles), or V509I hCOX-2 mutant (closed squares) for 1 min. Initial rate of the peroxidase reaction was measured.

formed as a function of the COX activity was detected by ELISA (Caymen, Ann Arbor, MI).

RESULTS

COX-1 and -2 are 63% identical and 77% similar at the amino acid level. A cursory look at the sequence alignment of human COX-1 and COX-2 reveals that most of the major differences are in the N-terminal and C-terminal regions. The catalytic domain (117–587) is highly conserved, with the major residues known to be involved in catalysis or heme binding; Arg-120, His-206, Tyr-385, His-386, and His-388 all conserved, along with the residue which is acetylated by aspirin (Ser-530). Differences that could be responsible for selectivity are most likely found in the cyclooxygenase active site rather than the peroxidase site, due to the fact that the known selective inhibitors, as do most common NSAIDs, inhibit the cyclooxygenase activity and not the peroxidase activity.

With the availability of the ram COX-1 structure, a better view of the cyclooxygenase active site is now available. The residues of COX-2 were overlaid onto the COX-1 structure. The subsequent alignment revealed a number of amino acid differences at the mouth of the cyclooxygenase substrate channel, where a series of amino acids on three α -helices surrounding the mouth of the channel that have residue changes at approximately every turn in the helix (3 amino acids) and are all on the solvent side of the substrate channel (Table I). The first helix contains two changes; threonine 89 to valine 74 and leucine 92 to isoleucine 77. The helix directly on the opposite side of the channel contains three consecutive changes: leucine 112 to isoleucine 98, leucine 115 to tyrosine 101, and valine 119 to serine 105. A third helix located between the previous two contains one change: phenylalanine 357 to leucine 343. The first three changes (89, 92, 112) are rather benign, with nonpolar residues being exchanged for nonpolar residues of about the same size. More significant changes occur at position 115, where a nonpolar leucine is replaced by an uncharged polar tyrosine; 119 where a nonpolar valine is replaced by an uncharged polar serine; and 357 where a nonpolar leucine is replaced by the much larger nonpolar phenylalanine. One difference is observed at the cyclooxygenase active site, where the isoleucine at position 523 in COX-1 is a valine in COX-2.

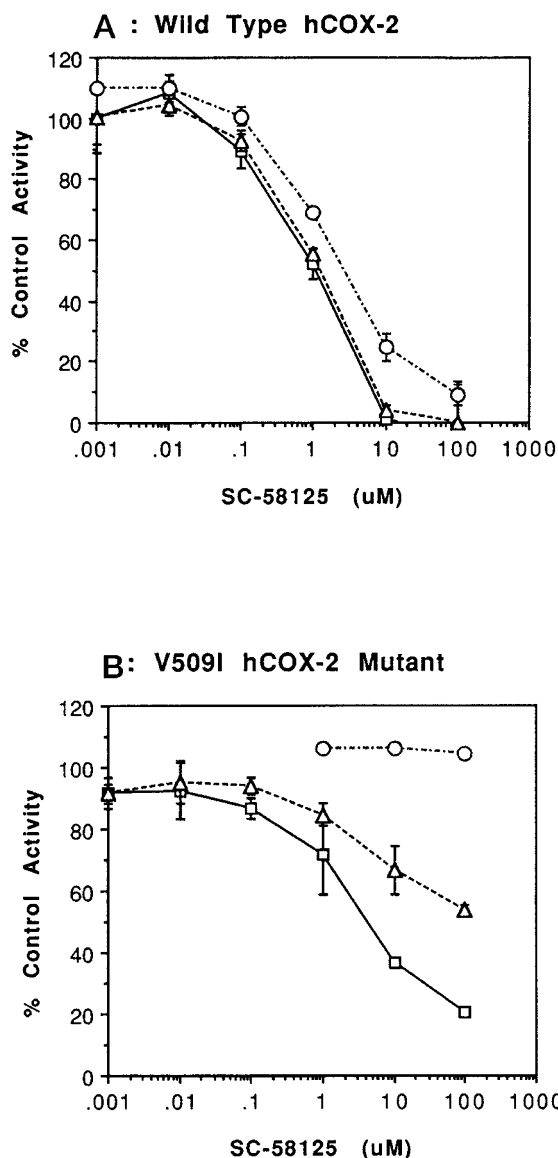


FIG. 3. Inhibition curves, with variable assay time of the selective COX-2 inhibitor SC-58125. A, hCOX-2; B, the hCOX-2 mutant V509I. 1-min preincubation of inhibitor and enzyme. Assays were performed using peroxidase substrate TMPD and arachidonate. Initial velocity (squares) at A_{611} is measured among A_{611} readings at 1 min (triangles) and 5 min (circles).

A model of sheep COX-1 indicating the relative position of the differing residues and other residues of importance is shown graphically in Fig. 1. The substrate channel is oriented from top to bottom with heme at the top and residues 112, 115, and 119 at the bottom. A little further up the channel, but slightly below the catalytic site and NSAID binding pocket sits leucine 357, which is a phenylalanine in COX-2. Residues crucial for catalysis (Tyr-385, Arg-120) are found in close contact with flurbiprofen, which is presumed to be bound in the same space as substrate. Serine 530, which is acetylated by aspirin, is shown next to flurbiprofen. The only amino acid difference in the catalytic site, isoleucine 523, is found on the opposite side.

Two hCOX-2 mutants were constructed by introducing hCOX-1 amino acids into a selected site(s); the first combined the three significant changes near the mouth of the substrate channel, tyrosine 101 to leucine, serine 105 to leucine, and phenylalanine 343 to leucine; the second is the single amino acid change, valine 509 to Isoleucine found in the active site. Insect cell expression levels of the mutants were similar to wild

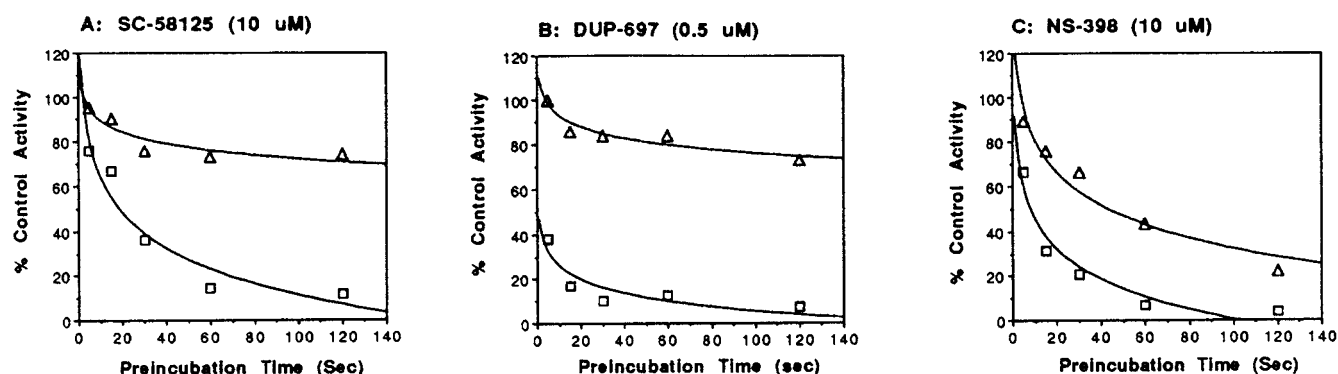


FIG. 4. Time-dependent inactivation of hCOX-2 and the mutant V509I hCOX-2 by SC-58125, DUP-697, and NS-398. 4 μ g of either hCOX-2 (squares) or the mutant V509I hCOX-2 (triangles) preincubated from 5 s to 2 min with inhibitor before addition of the peroxidase substrate TMPD and arachidonate. A_{611} was measured at 1 min. Data were fit to a logarithmic least squares curve at a correlation of >0.95 .

type without optimization at the 1-liter level. The purification scheme described under "Experimental Procedures" produced approximately 4 mg of protein from 1 liter at 50% purity. All assays were done with enzyme of this purity. V_{\max} of both mutants appears to be close to wild type hCOX-2 expressed in insect cells (data not shown).

Using the selective COX-2 inhibitor SC-58125, both wild type hCOX-2 and the triple mutant demonstrated similar inhibition profiles ($IC_{50} = 1 \mu M$). The single V509I mutation increased the IC_{50} to $>100 \mu M$, which looks like COX-1 at $>100 \mu M$ (Fig. 2). The assay protocol influences the selectivity of the compound for hCOX-2 and the mutants. Wild type hCOX-2 has an IC_{50} of 1–2 μM with the assay run as an initial rate or as an end point assay at 1 min read time or 5 min read time. The V509I mutant, however, exhibited a much different behavior. At the initial velocities, there is only a slight right shift in the IC_{50} curve (to 4 μM). At 1 min, the curve is further shifted to $>100 \mu M$ and, finally, at 5 min, there is no hint of inhibition at 100 μM , looking completely like COX-1 (Fig. 3).

Time dependent inhibition of COX-2 selective inhibitors was measured for hCOX-2 and the V509I hCOX-2 mutant. Inhibition of wild type enzyme by 10 μM SC-58125 is time-dependent and is complete by 1 min, with a half-maximal inhibition at 20 s (Fig. 4A). The mutant exhibits time-dependent behavior, as evidenced by a logarithmic decay in activity over time, only there is a distinct decrease in the rate at which the enzyme is inhibited over time, with only 20% inhibition at 2 min. DUP-697 at 0.5 μM shows a very similar profile with half-maximal inhibition at 5 s and maximal inhibition of 20% at 2 min (Fig. 4B). NS-398 at 10 μM exhibited half-maximal inhibition at 10 s with wild type that increased to 40 s with the mutant (Fig. 4C).

Half-maximal inhibition profiles were determined by the prostaglandin ELISA assay, which favors compounds with a time-dependent component, for a wide range of selective COX-2 inhibitors and known NSAIDs (Table II). All selective COX-2 inhibitors tested (SC-58125, SC-236, DUP-697, and NS-398) demonstrated hCOX-1-like activities with the mutant enzyme. The other commonly used NSAIDs, indomethacin, diclofenac, mefenamate, and flurbiprofen, showed no change in selectivity with the mutant. The only compound that did not follow the trend, Naproxen, had no inhibitory effect with the mutant compared to COX-1 and -2.

DISCUSSION

With the identification of a second COX isoform, numerous groups have embarked on efforts to identify selective inhibitors of COX-2. Prior to their discovery, the ability to identify selective inhibitors of COX-2 was not obvious as the enzymes utilize the same substrate and have a high degree of sequence homol-

TABLE II
Selective pharmacology of NSAIDs

Each inhibitor (0.001–100 μM) was preincubated for 10 min with enzyme before addition of arachidonic acid (10 μM) for 10 min. COX activity was measured as PGE_2 formed $\text{min}^{-1} \text{mg}^{-1}$.

	IC_{50}		
	hCOX-1	hCOX-2	V509I hCOX-2
		μM	
Indomethacin	0.08	0.5	0.56
Mefenamate	3.6	122	41
Diclofenac	0.02	0.01	0.04
Flurbiprofen	0.4	2.7	2.6
Naproxen	21	88	>1000
DUP-697	0.9	0.001	0.01
NS-398	>100	0.05	>100
SC-58125	>100	0.04	>100
SC-236	17	0.005	15

ogy. Nonetheless, we and others have reported the identification of selective inhibitors of COX-2 (16, 23). The mechanism of inhibition of COX-2 has been described as "time-dependent," involving a tight binding component, while these compounds are reversible inhibitors of COX-1. (17). This time-dependent component is the apparent basis for selectivity of this new class of COX-2 inhibitors, since these compounds are reversible inhibitors of COX-1. To further understand the molecular basis of this differential inhibition, a series of mutants was evaluated. We observed that a single amino acid change in COX-2 confers a COX-1 inhibitory profile for these COX-2 selective inhibitors. Specifically, the data suggest that the mechanism for selectivity is intimately associated with the removal of a single methyl group at position 523. Therefore, the time-dependent step could possibly be inhibited by the presence of an additional methyl group in the substrate channel.

How do these subtle differences in the active site translate to two functionally distinct enzymes? Recombinant COX-1 and -2 demonstrate a similar K_m and V_{\max} for their common substrate, arachidonic acid (23). Both enzymes appear to reside on the luminal side of the endoplasmic reticulum (24). Likewise, their catalytic roles appear to be the same. Therefore, the major differences between these isoenzymes may be a function of the regulatory mechanisms by which the two enzymes are expressed. For instance, COX-2 expression is induced in discrete cell population response to an inflammatory stimulus, while COX-1 is expressed constitutively in most tissue (9). Recently, it has been suggested that COX-1 and -2 have different requirements for hydroperoxide activation and that the hydroperoxide environment in the cell may influence the regulation of the enzyme activity (25), a mechanism that is distinct from that of all known NSAIDs and COX-2 selective inhibitors.

Currently, our understanding of COX-2 structure has been

deduced from the known COX-1 crystal structure. Given the high degree of homology between the two enzymes, replacement of the COX-2 residues with those predicted from COX-1 may provide a model of a COX-2 active site. Confirmation of the crystal structure of COX-2 will provide the final evidence as to the exact placement of valine 509 in the active site and the relationship of specific inhibitors in the active site of the enzyme.

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