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Characterization of Celecoxib and Valdecoxib Binding to Cyclooxygenase

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ABSTRACT

Two compounds (celecoxib and valdecoxib) from the diarylheterocycle class of cyclooxygenase inhibitors were radiolabeled and used to characterize their binding to cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), several single-point variants of COX-2 (Val523Ile, Tyr355Ala, Arg120Ala, Arg120Gln, Arg120Asn) and one triple-point variant of COX-2 [Val523Ile, Arg513His, Val434Ile (IHI)]. We demonstrate highly specific and saturable binding of these inhibitors to COX-2. Under the same assay conditions, little or no specific binding to COX-1 could be detected. The affinity of [^{3}H]celecoxib for COX-2 (K_D = 2.3 nM) was similar to the affinity of [^{3}H]valdecoxib (K_D = 3.2 nM). The binding to COX-2 seems to be both rapid and slowly reversible with association rates of 5.8×10^6/M/min and 4.5×10^6/M/min and dissociation rates of 14×10^{-3}/min ($t_{1/2}$ = 50 min) and 7.0×10^{-3}/min ($t_{1/2}$ = 98 min) for [^{3}H]celecoxib and [^{3}H]valdecoxib, respectively. These association rates increased (4- to 11-fold) when the charged arginine residue located at the entrance to the main hydrophobic channel was mutated to smaller uncharged amino acids (Arg120Ala, Arg120Gln, and Arg120Asn). Mutation of residues located within the active site of COX-2 that define a 'side pocket' (Tyr355Ala, Val523Ile, IHI) of the main channel had a greater effect on the dissociation rate than the association rate. These mutations, which modified the shape of and access to the 'side pocket', affected the binding affinity of [^{3}H]valdecoxib more than that of [^{3}H]celecoxib. These binding studies provide direct insight into the properties and binding constants of celecoxib and valdecoxib to COX-2.

Prostaglandins are synthesized from arachidonic acid by the enzyme prostaglandin H synthase (also referred to as cyclooxygenase, COX). There are, at present, two identified forms of this enzyme, COX-1 and COX-2, each distinctly regulated. COX-1 is expressed in many tissues, including the gastrointestinal tract, kidney, and platelets, whereas COX-2 is expressed at sites of inflammation, the hippocampus, female reproductive tissue, and many cancers (Sirois and Richards, 1993; Yamagata et al., 1993; Masferrer et al., 1994; Seibert et al., 1994; Turini and DuBois, 2002). Evidence indicates that COX-2–derived prostaglandins are involved in the signs and symptoms of arthritis and some forms of pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2 (Mitchell et al., 1994; Seibert et al., 1994; Gierse et al., 1995), and cause gastrointestinal injury, presumably caused by inhibition of COX-1. Selective inhibitors of COX-2 (the coxibs: celecoxib, rofecoxib, valdecoxib) were developed to avoid the side effects of NSAIDs caused by COX-1 inhibition (Hawkey, 1999).

Nonspecific NSAIDs inhibit COX-1 and COX-2 with varying potencies and apparent selectivity (Patrignani et al., 1997; Warner et al., 1999). Some NSAIDs have been reported to preferentially inhibit COX-1 (e.g., indomethacin) and others, COX-2 (e.g., meloxicam, nimesulide); generally, however, the selectivity for COX-1 versus COX-2 as measured by inhibition of enzyme activity varies widely depending on the assay system used (Meade et al., 1993; Barnett et al., 1994; Mitchell et al., 1994; Gierse et al., 1999); thus, a means for directly assessing binding of NSAIDs and coxibs to COX isoforms would be useful, particularly to determine true kinetic rate constants. The binding site of NSAIDs on COX-1, COX-2, and COX-2 variants has been visualized crystallographically (Picot et al., 1994; Kurumbail et al., 1996); the primary active site residue differences between COX-1 and COX-2 are located at Ile523, Ser516, His513, and Ile434, using the COX-1 numbering system (Kurumbail et al., 1996). Arg120, located near the entrance to the main channel, appears in COX-1 to be located within ionic bond distance of the substrate arachidonic acid and to be important for catalytic efficiency (Bhattacharyya et al., 1996). In COX-2, Arg120 has considerably less kinetic influence (Rieke et al., 1999). In general, Arg120 forms an ion pair with the carboxylate moiety of NSAIDs, and mutation of Arg120 significantly decreases the inhibitory potency of these NSAIDs but increases the potency of nonacid inhibitors of COX-1 (Mancini et al., 1995). Tyr355 and Ile523 form part of the entrance to a side

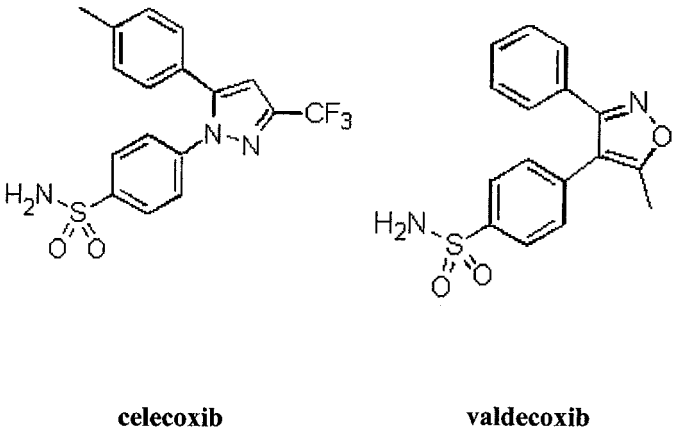
ABBREVIATIONS: COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; DEDTC, diethyldithiocarbamate; C10M, decyl maltoside; D-PBS, Dulbecco's phosphate-buffered saline; PG, prostaglandin; IHI, Val523Ile, Arg 513His, Val434Ile.

pocket within the active site. Moreover, the isoleucine/valine difference at position 523 of COX-1/COX-2 seems to contribute significantly to the COX-2 selectivity of members of a new class of inhibitors, diarylheterocycles (e.g., celecoxib, SC-236,). Replacement of Val523 in COX-2 with isoleucine (Val523Ile) slows the time-dependent inhibition produced by some members of the diarylheterocycle class (Gierse et al., 1996; Guo et al., 1996; Wong et al., 1997). The Ile523 of COX-1 seems to make the side pocket more restrictive and possibly less accessible than in COX-2.

Although specific residues important for binding have been identified, the relative contribution of these residues to the kinetics of binding of the diarylheterocycle class has not been determined. Using a fluorescent diarylheterocyclic oxazole, Lanzo et al. (2000) measured the kinetic rate constants for binding of this oxazole to native COX-1 and COX-2 by fluorescence quenching. Furthermore, some elegant attempts have been made using a mathematical model to discern the kinetic rate constants for inhibitor binding to COX-1 and COX-2 (Callan et al., 1996; So et al., 1998). In general, studies have suggested that the slow dissociation of the diarylheterocyclic inhibitors from COX-2 accounts for their selectivity. In this study, using radiolabeled celecoxib and valdecoxib, we provide a kinetic analysis of the binding of these two diarylheterocyclic compounds to COX-1, COX-2, and several COX-2 variants.

Materials and Methods

Materials. Hemin, indomethacin, naproxen, mefenamic acid, ibuprofen, and piroxicam were purchased from Sigma Chemical Co. (St. Louis, MO). Nimesulide and meloxicam were prepared in-house by the Searle Medicinal Chemistry Department. Arachidonic acid was obtained from Nu-Chek Prep, Inc (Elysian, MN), and the other fatty acid substrates, γ-linolenic acid, docosatetranoic acid, eicosapentanoic acid, and docosapentanoic acid were obtained from Cayman Chemical (Ann Arbor, MI). For the preparation of radiolabeled inhibitors,4-[5-(4-methylphenyl)-3-(trifluoromethyl-4-bromo)-1H-pyrazol-1-yl]-benzenesulfonamide and 4-[3-(4-bromophenyl)-5-methyl-isoxazol-4-yl]-benzenesulfonamide (Fig. 1) were treated with tritium gas in the presence of a palladium catalyst to obtain celecoxib tritiated at the 4-position of the pyrazole ring ([^{3}H]celecoxib; specific radioactivity, 3 Ci/mmol) and valdecoxib labeled at the 4-position of the 3-position benzene ring ([^{3}H]valdecoxib; specific radioactivity, 10 Ci/mmol), respectively.



celecoxib valdecoxib

Fig. 1. Chemical structure for the diarylheterocyclics celecoxib and valdecoxib.

Enzyme Preparation. COX-1 purified from sheep seminal vesicles and recombinant murine COX-2 expressed in insect cells was obtained as described previously (Gierse et al., 1999). Briefly for COX-1, sheep seminal vesicles were homogenized into 50 mM Tris-HCl, pH 8.0, 1 mM diethyldithiocarbamate (DEDTC), 1 mM EDTA, and 0.01% sodium azide and then centrifuged at 10,000*g* to remove cell debris. The supernatant was centrifuged at 200,000*g* to collect the microsomal fraction, which was washed once with homogenization buffer containing 115 mM sodium perchlorate and then extracted with 1.5% decyl-maltoside (C10M). The extract was centrifuged, and the resulting supernatant was concentrated by ultrafiltration using a 30-kDa molecular mass cutoff membrane and applied to a Sephacryl S-300 (Amersham Biosciences, Piscataway, NJ) gel filtration column equilibrated with 50 mM Tris-HCl, pH 8.0, 0.1 mM DEDTC, 0.1 mM EDTA, and 0.15% C10M. Protein fractions were eluted and then applied to a DEAE Trisacryl (Biosepra, Marlborough, MA) anion exchange column equilibrated with 20 mM Tris-HCl, pH 8.0, 0.1 mM DEDTC, 0.1 mM EDTA, and 0.15% C10M. The column was developed with the same buffer, adjusted to pH 5.3. Active fractions were applied to an Ultragel ACA 54 (Biosepra) gel filtration column equilibrated with 20 mM potassium phosphate, pH 7.4, 0.1 mM DEDTC, 0.1 mM EDTA, and 0.3% *n*-octyl β-D-glucopyranoside. The final material was pooled and stored at −80°C until use.

Baculovirus-infected insect cells expressing recombinant murine COX-2 were suspended in 25 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 1 mM DEDTC, and 1 mM EDTA, washed once, and extracted into the same buffer containing 1.5% C10M. The extract was centrifuged at 28,000*g* for 30 min, then applied to a Macro-Prep High-Q (Bio-Rad, Hercules, CA) anion exchange column equilibrated with 25 mM Tris-HCl, pH 8.0, 0.1 mM DEDTC, 0.1 mM EDTA, and 0.15% C10M and eluted with a linear gradient to 0.3 M NaCl. Fractions were concentrated by ultrafiltration using a 30-kDa molecular mass cutoff membrane and applied to a S-200 Sephacryl gel filtration column equilibrated with 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM DEDTC, 0.1 mM EDTA, and 0.3% *n*-octyl β-D-glucopyranoside. The active fractions were pooled and stored at −80°C until further use.

Antibody Preparation. COX-1 murine monoclonal antibody M584-7f4 and COX-2 murine monoclonal antibody R6 were affinity purified using protein-A agarose. To cell culture supernatants, sodium borate was added to 100 mM and pH adjusted to 8.9 with NaOH. These were then passed over a protein-A agarose column (Repligen, Needham, MA) equilibrated with PBS. Initially, the column was washed with 3 M NaCl, 100 mM sodium borate, pH 8.9, then by 3 M NaCl, 10 mM sodium borate, pH 8.9. The antibodies were eluted with 100 mM glycine, pH 3.0. Fractions were immediately pH neutralized with the addition of 1/10 (v/v) 1 M Tris-HCl, pH 8.1. The antibodies were then dialyzed against PBS and stored at −80°C until use.

COX-2 Mutagenesis and Expression. Site-directed mutagenesis (Val523Ile, Tyr355Ala, Arg120Ala, Arg120Asp, Arg120Gln, and His207Ala) and Val523Ile, Arg513His, Val434Ile (IHI) mutagenesis on a murine COX-2 pBlueScript(+) vector (Stratagene, La Jolla, CA); subcloning into the mCOX-2 pVL1393 baculovirus expression vector (BD Biosciences Pharmingen, San Diego, CA); and expression by homologous recombination with Baculogold vector (BD Biosciences Pharmingen) in SF-9 cells (Novagen, Madison, WI) was performed as described previously (Rowlinson et al., 1999). Purification of the COX-2 mutants was performed similarly to that described above for the native murine COX-2 enzyme.

Binding Assay. COX-1 or COX-2 specific antibodies (M584-7f4 or R6, respectively) at 10 μg/ml in 100 mM $NaHCO_3$, pH 8.2, were coated (100 μl/well) onto 96-well Immulon 2 microtiter plates (Dynex Technologies Inc., Chantilly, VA) by incubating overnight at room temperature in a humidified chamber. The coated plates were washed with Dulbecco's phosphate-buffered saline, without $CaCl_2$ and $MgCl_2$, pH 7.4 (D-PBS; Invitrogen, Carlsbad, CA) and then treated with a blocking reagent consisting of 10% skim milk in

D-PBS (0.2 ml) for 90 to 120 min at 37°C to decrease nonspecific binding to the plate. The coated and blocked plates were washed, COX enzyme was added at 20 to 35 μg/ml in 50 μl of binding buffer (100 mM Tris, 1 μM hemin, pH 8.0), and then incubated at room temperature for 60 to 120 min. Finally, these antibody-captured, enzyme-coated plates were washed with D-PBS and aspirated to dryness immediately before the binding assay.

For the binding assay, the enzyme-coated plate wells contained 85 μl of binding buffer, 5 μl of dimethyl sulfoxide, and 10 μl of radiolabeled ligand ([^{3}H]valdecoxib or [^{3}H]celecoxib), which was added last at 25°C to initiate the binding reaction. For saturation binding experiments, various concentrations of either [^{3}H]valdecoxib (0.3–3400 nM) or [^{3}H]celecoxib (0.9–350 nM) were added. Then, after a 2- to 3-h period of incubation, the wells were aspirated, rapidly washed (<2 s) with 250 μl of ice-cold D-PBS, and bound radioligand was quantitated. Data were best fit to the equation $(RL) = (R)_t\,(L)/(K_D + L)$ where (RL) is the amount of bound radiolabeled ligand, $(R)_t$ is the total enzyme concentration, L is the ligand concentration, and K_D is the equilibrium dissociation constant. For dissociation time-course experiments, the enzyme was initially incubated with radiolabeled ligand for 120 min. To initiate dissociation, the wells were aspirated, and 150 μl of excess unlabeled ligand (10 μM) was added. After various periods of time, the dissociated radiolabeled compound and the excess unlabeled compound were removed, and the remaining bound radioligand was quantitated. Data were best fit to the equation $(RL) = (RL)_0\,e^{(-k \times t)}$ where t is time and k is the dissociation rate constant. For association time-course experiments, after the addition of ^{3}H-labeled ligand, the incubations were halted at various time points by aspiration, rapidly washed, and bound radioactivity was quantitated. Data were best fit using an equation described by Rodbard and Weiss (1973). Lastly, for the competitive binding assays, a 96-well dilution plate was used to initially make dilutions of the compound. To each of these different concentrations was added a trace amount of ^{3}H-labeled ligand; then, the complete mixture of unlabeled test compound and trace radiolabel was added to the enzyme-coated plate. This method would ensure that there was no preincubation of either the test compound or the radiolabeled ligand with the enzyme. K_i values were derived from the concentration of competing compound that inhibited 50% of the binding observed in the absence of any inhibitor (IC_{50}) using the equation of Cheng and Prusoff (1973). In assays in which different COX substrates were examined, the His207Ala variant of COX-2 was employed. In addition, heme was omitted from the assay buffer. Other investigators have shown that the His207Ala variant is devoid of peroxidase activity (Landino et al., 1997). Data were transformed into logit-log analysis, and K_i values are reported. In all experiments, bound ^{3}H-labeled ligand was eluted from the plate using 10% SDS in D-PBS and quantitated using liquid scintillation counting. All assays were done at room temperature unless otherwise specified. Nonspecific binding was defined as the residual binding in the absence of enzyme.

Results

[^{3}H]Celecoxib and [^{3}H]Valdecoxib Binding to COX-1 and COX-2. The diarylheterocycle compounds [^{3}H]celecoxib and [^{3}H]valdecoxib were tested for their ability to specifically bind to COX-1 and COX-2 enzymes that had been captured by non-neutralizing antibodies onto a microtiter plate. Under these conditions, enzymatic analysis of both the COX-1- and COX-2–coated plates, using arachidonic acid as substrate, revealed that the plates did contain active enzyme sufficient to produce measurable quantities of prostaglandin E_2 (PGE_2); COX-2 produced 1.4 ng/ml, whereas COX-1 produced 0.9 ng/ml PGE_2 in 10 min at room temperature with 10 μM arachidonic acid. The production of PGE_2 by COX-1 on the coated plate could be inhibited by indomethacin, and a pharmacological analysis of COX-1 and COX-2 enzymes from both sheep and murine sources revealed similar inhibitory profiles (data not shown).

Figure 2, A and B, shows a representative saturation experiment in which increasing concentrations of [^{3}H]celecoxib (0.9–160 nM) and [^{3}H]valdecoxib (0.35–150 nM) were ex-

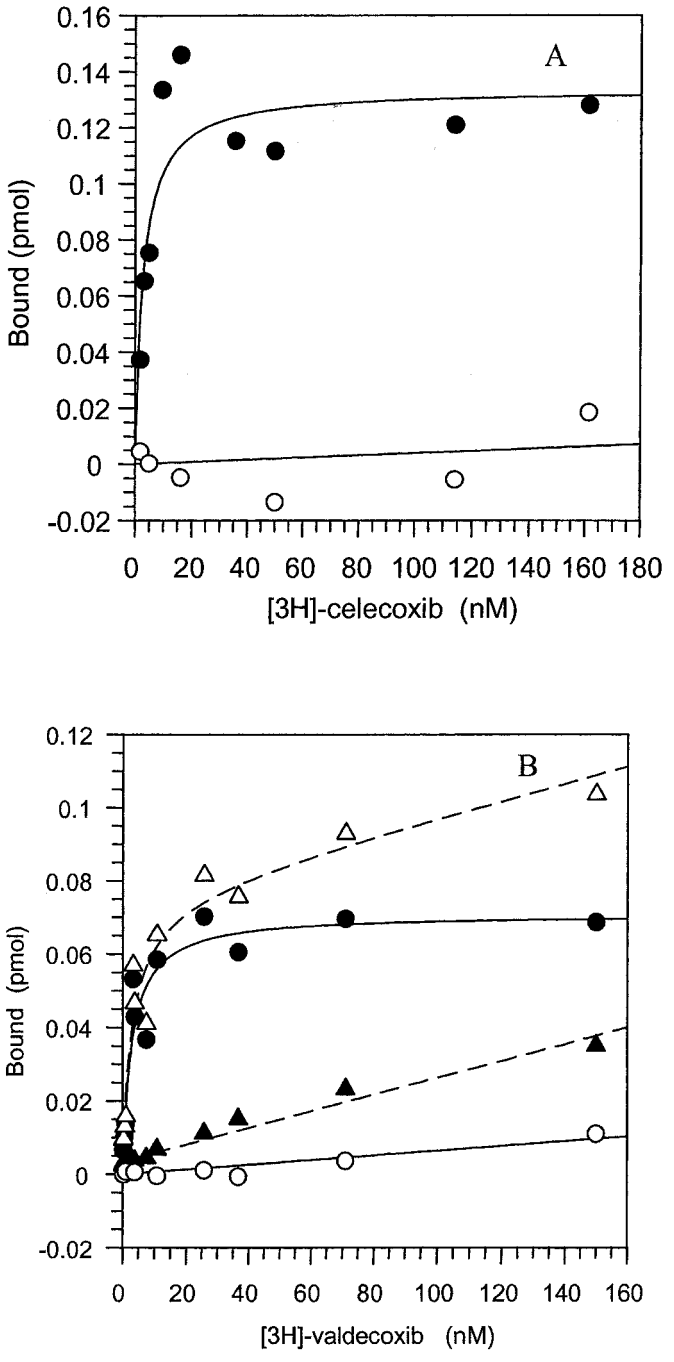


Fig. 2. Saturation binding curve of [^{3}H]celecoxib (A) and [^{3}H]valdecoxib (B) to either COX-1 (○) or COX-2 (●). Plates were coated with either COX-1 or COX-2 and then incubated with various concentrations of [^{3}H]celecoxib or [^{3}H]valdecoxib for 120 min at room temperature. The free unbound radioligand was aspirated off, the plate was rapidly washed, and the remaining amount bound was determined using liquid scintillation counting. Data shown are from one representative experiment using either [^{3}H]celecoxib or [^{3}H]valdecoxib. For clarity purposes, the nonspecific (▲) and total (△) binding to COX-2 are only shown for [^{3}H]valdecoxib. Nonspecific binding for [^{3}H]celecoxib at ≥50 nM ranged from 75 to 98% of total.

posed to both COX-1 and COX-2 for 180 min at room temperature. The data show that both [^{3}H]celecoxib and [^{3}H]valdecoxib bound to COX-2 in a saturable manner, with high specificity and low nanomolar affinity, and exhibited little or no reproducible specific binding to COX-1. In one experiment, we tested the binding of [^{3}H]valdecoxib to COX-1 at concentrations up to 3.4 μM, and no specific binding to COX-1 was observed. In the particular experiment shown, the K_D for [^{3}H]celecoxib and [^{3}H]valdecoxib binding to wild-type COX-2 were 1.9 and 2.1 nM, respectively. Scatchard transformation of the [^{3}H]valdecoxib binding data (Fig. 3) suggested that the binding was to a single, noninteractive site and did not suggest the presence of a second binding site under the conditions of this assay.

To more closely examine the binding of [^{3}H]celecoxib and [^{3}H]valdecoxib to COX-2, we studied the time courses for both association and dissociation. Kinetic rate constants derived from time-course experiments solidified the results obtained with saturation experiments. Furthermore, the results from these experiments may reveal different kinetic rate constants for compounds that seem to have similar binding affinities. A representative association time course for [^{3}H]valdecoxib (5 nM) demonstrates that the ligand binding to COX-2 reaches steady state within 60 min (Fig. 4). In other similar experiments using [^{3}H]celecoxib, as well as [^{3}H]valdecoxib, we extended the association time course to 19 h and did not observe any further increase in binding over that seen at 60 min, either in the presence or absence of an unlabeled inhibitor (data not shown). Both [^{3}H]celecoxib and [^{3}H]valdecoxib seemed to have similar association rates (5.8 and 4.5 $\times$ 10^6/M/min, respectively) for COX-2. Dissociation time-course studies demonstrated that this binding was slowly but readily reversible. Figure 5, A and B, shows the dissociation time course for both [^{3}H]celecoxib and [^{3}H]valdecoxib and demonstrates that the binding to COX-2 is readily reversible with values for $t_{1/2}$ of 50 and 98 min, respectively. Also shown is the dissociation from the COX-2 mutant Val523Ile with $t_{1/2}$ of 14 and 5 min, respectively, suggesting that [^{3}H]valdecoxib binding is more affected by this particular amino acid substitution. The dissociation rate constants (k_{-1}) from wild-type COX-2, taken in conjunction with the association rate constants (k_{+1}), translate into a kinetically derived affinity constant ($K_D = k_{-1}/k_{+1}$) of 2.4 and 1.6 nM for [^{3}H]celecoxib and [^{3}H]valdecoxib, respectively. These kinetically derived affinity constants are in excellent agreement with the K_D derived from the saturation binding experiments, and further substantiate the high affinity of these inhibitors for COX-2.

Pharmacological Characterization of [^{3}H]Celecoxib and [^{3}H]Valdecoxib Binding to COX-2. Although we have demonstrated that the binding of [^{3}H]celecoxib and [^{3}H]valdecoxib to COX-2 is saturable, reversible, specific, and of high affinity, the pharmacological relevance of this binding site remained uncertain. Consequently, we tested the ability of known COX-2 inhibitors and substrates to compete for binding of these radioligands. Some of these inhibitors have been described as exhibiting time-dependent characteristics in enzymatic assays. In the binding assay, both the cold competitor test compound and radiolabeled ligands were added simultaneously to the enzyme. When substrates were tested as competitors, we used the His207Ala mutant of the wild-type COX-2, which is devoid of peroxidase activity; thus, the cyclooxygenase reaction is indirectly prevented as well (Landino et al., 1997). The fatty acids tested, which are purported to be substrates of COX-2, inhibited the binding of both [^{3}H]celecoxib and [^{3}H]valdecoxib; arachidonic acid was the most potent (Table 1). In addition, all of the NSAIDs tested inhibited the binding of either [^{3}H]celecoxib or

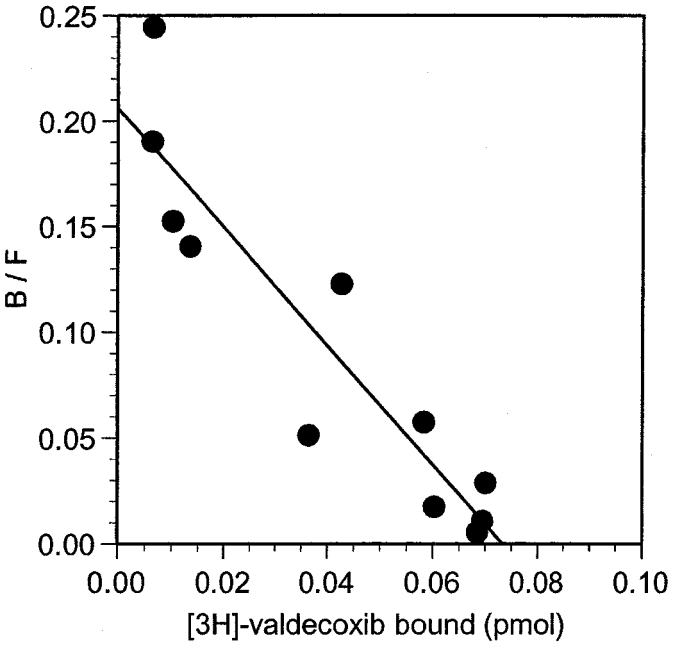


Fig. 3. Scatchard transformation of the data for [^{3}H]valdecoxib shown in Fig. 2.

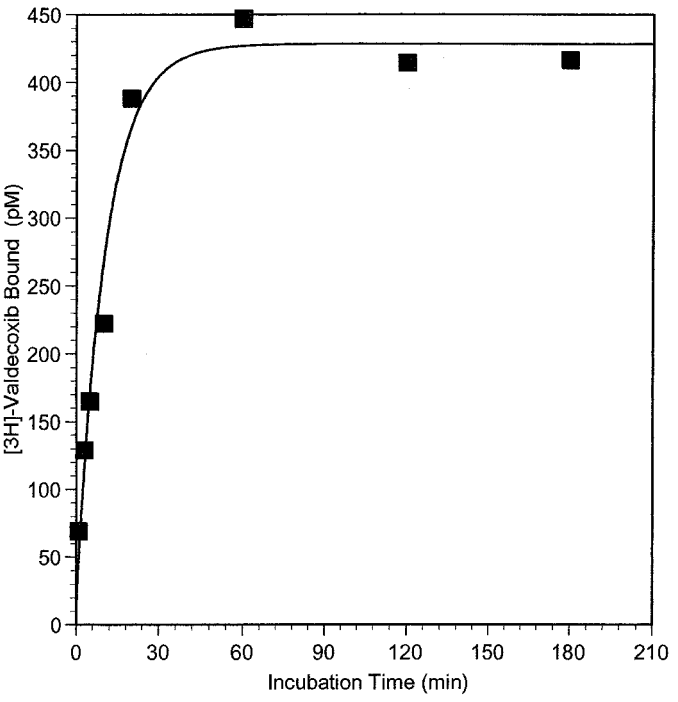


Fig. 4. Association time course for [^{3}H]valdecoxib binding to wild-type COX-2. Plates coated with wild-type COX-2 were incubated with [^{3}H]valdecoxib (5 nM) for various lengths of time. After the incubation period, the free unbound radioligand was aspirated, the plate was rapidly washed, and the remaining amount bound was determined using liquid scintillation counting. Data shown are from one representative experiment; k_{+1} = 4.0 $\times$ 10^6/M/min.

[^{3}H]valdecoxib to COX-2. In general, each NSAID seemed to maximally inhibit more than 90% of the radioligand binding, with logit-log slopes approximating one. Furthermore, the rank-order potency for inhibition of radioligand binding to COX-2 roughly corresponds to that rank-order potency observed for inhibition of COX-2 mediated PGE_2 production (Gierse et al., 1999). Thus, the binding of [^{3}H]celecoxib and [^{3}H]valdecoxib seems to be to a single pharmacologically relevant site.

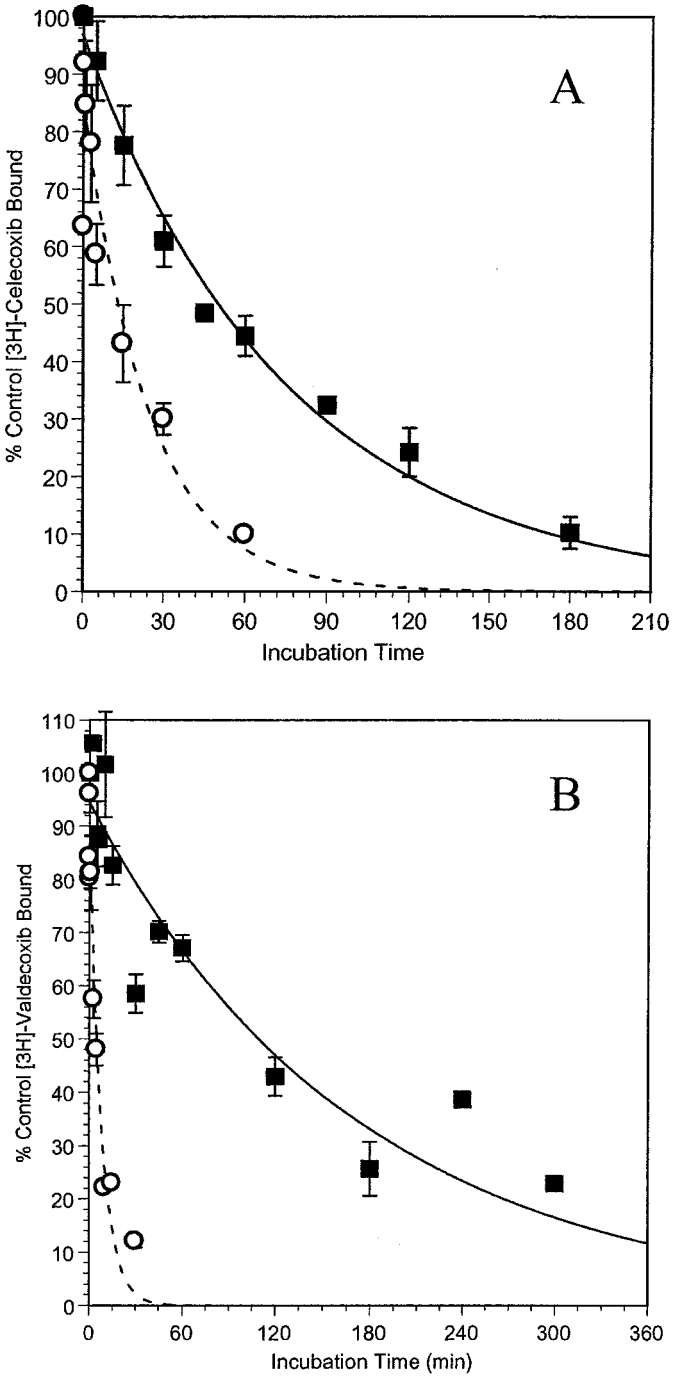


Fig. 5. Dissociation time course for [^{3}H]celecoxib (A) and [^{3}H]valdecoxib (B) binding to wild-type COX-2 (■) and the mutant Val523Ile (○). Plates were coated with either wild-type COX-2 or the mutant Val523Ile and incubated with [^{3}H]celecoxib or [^{3}H]valdecoxib for 120 min. After this initial incubation period, the free unbound radioligand was aspirated off and 150 μl of excess cold ligand (10 μM) was added to initiate the dissociation time course. Then, at various times, the wells were aspirated and the remaining amount bound was determined using liquid scintillation counting. Data shown are the mean ± S.E.M. from at least three experiments each performed in triplicate.

Binding of [^{3}H]Celecoxib and [^{3}H]Valdecoxib to Variants of COX-2. Various studies have shown that specific residues of COX-2 are critical for the inhibitory potency of diarylheterocyclics and may be, in part, responsible for the selectivity of these inhibitors relative to COX-1. Consequently, specific residues within the main channel of COX-2 were mutated and the resulting mutated COX-2 enzymes tested for their ability to bind [^{3}H]celecoxib and [^{3}H]valdecoxib. Diarylheterocycle inhibitors of COX-2 interact with residues in the COX side pocket, three of which (Val523, Arg513, and Val434) differ between COX-1 and COX-2. To closely mimic this region of COX-1, these residues in COX-2 were mutated to the corresponding amino acids of COX-1; isoleucine, histidine, and isoleucine, respectively (variants Val523Ile and IHI). Tyr355 is a unique residue in the cyclooxygenase active site because it lies at the entrance to both the side pocket and the main channel. To evaluate its impact on inhibitor binding, Tyr355 was mutated to alanine (Tyr355Ala). Because Arg120 has been shown to form an ion pair with all NSAIDs containing a carboxylate group that have been visualized crystallographically (Picot et al., 1994; Kurumbail et al., 1996) and is thought to form an ion pair with arachidonic acid (Bhattacharyya et al., 1996; Smith et al., 1996; Kiefer et al., 2000; Malkowski et al., 2000), we also examined mutants of Arg120 (Arg120Asn, Arg120Ala, and Arg120Gln) to explore their effect on the binding of [^{3}H]celecoxib and [^{3}H]valdecoxib. The saturation experiments revealed that the binding to all mutants was saturable and highly specific.

In addition to determining the K_D of [^{3}H]celecoxib and [^{3}H]valdecoxib by saturation analysis, we determined the kinetic association and dissociation rate constants for the binding of these radioligands to the mutant enzymes. [^{3}H] Celecoxib (Table 2) and [^{3}H]valdecoxib (Table 3) demon-

TABLE 1

Competitive inhibition of [^{3}H]celecoxib and [^{3}H]valdecoxib binding to COX-2

Various concentrations of the indicated compounds were incubated with either [^{3}H]celecoxib or [^{3}H]valdecoxib and allowed to compete for binding to COX-2 for 120 min. Testing of the different substrates was performed on the COX-2 mutant His207Ala. K_i values were determined from logit-log transformations of the binding data. Each value is the mean ± S.E.M. from the number of experiments indicated in parentheses. Celecoxib and valdecoxib K_i in H207A against [^{3}H]valdecoxib are 3.9 and 2.4 nM, respectively.

Compound	K_i	
	[^{3}H]Celecoxib	[^{3}H]Valdecoxib
	nM	
NSAIDs		
Indomethacin	66 ± 22 (3)	134 ± 34 (4)
Nimesulide		174 ± 47 (3)
Mefenamic acid	128 ± 67 (7)	
Naproxen	219 ± 7 (3)	230 ± 48 (3)
Ibuprofen	475 ± 97 (3)	508 ± 115(3)
Meloxicam	491 ± 76 (3)	1047 ± 235(6)
Piroxicam	3660 ± 928(7)	2403 ± 190(3)
Substrates		
Arachidonic acid	28 ± 4 (5)	55 ± 9 (7)
γ-Linolenic acid	123 ± 43 (3)	164 ± 36 (3)
Docosatetranoic acid	226 ± 68 (3)	274 ± 104(3)
Eicosapentanoic acid	69 ± 6 (3)	80 ± 12 (3)
Docosapentanoic acid	259 ± 50 (3)	338 ± 68 (3)

strated specific binding to all of the COX-2 mutant enzymes. In general, mutation of residues near the entrance to the main channel (Tyr355 and Arg120) significantly increased the association rate of both [^{3}H]celecoxib and [^{3}H]valdecoxib binding similarly (6- to 11-fold). Also, mutating Arg120 to alanine, glutamine, or asparagine increased the dissociation rate approximately 2- to 5-fold; the overall net effect of these increases was a slight, ~3-fold increase in affinity. On the other hand, the side pocket mutations tended to more greatly affect the dissociation rate than the association rate, with a greater effect on [^{3}H]valdecoxib than [^{3}H]celecoxib binding. For example, the dissociation rate of [^{3}H]valdecoxib increased nearly 19-fold in Val523Ile, 25-fold in IHI, and 253-fold in Tyr355Ala but increased only 4-, 2-, and 19-fold, respectively for [^{3}H]celecoxib binding. Dissociation time courses demonstrated that the binding was also readily reversible as evidenced by the fact that none of the radiolabeled inhibitor binding had a $t_{1/2}$ for dissociation of greater than 100 min. When viewed in combination with the association rate, a kinetically derived affinity constant is determined that closely matches the K_D derived from the saturation experiments. Thus, the binding of [^{3}H]celecoxib and [^{3}H]valdecoxib to the COX-2 mutants seems to be saturable, specific, and readily reversible.

Discussion

The ability to develop selective inhibition of COX-2 with diarylheterocycle compounds has proven beneficial therapeutically. These compounds significantly reduce the risk of gastric ulcerations typically observed with NSAIDs (Simon et al., 1999; Bombardier et al., 2000). In this manuscript, we directly characterized the binding of two diarylheterocycle compounds, [^{3}H]celecoxib and [^{3}H]valdecoxib, to COX-2 and further established their selective nature. We demonstrated that they bind in a saturable, highly specific, and readily reversible manner to a single site that correlates pharmacologically with the cyclooxygenase active site of COX-2. Furthermore, we have confirmed the highly selective COX-2 inhibitor profile of [^{3}H]celecoxib and [^{3}H]valdecoxib by demonstrating their inability to bind specifically to COX-1 within limits of the assay used. Thus, the radioligand binding assay described here could be used to screen the binding potency of potential COX-2 inhibitors.

In general, the binding of [^{3}H]celecoxib and [^{3}H]valdecoxib was very similar to both wild-type COX-2 and COX-2 variants. Competition experiments with either NSAIDs or fatty acid substrates gave comparable results. Also, both [^{3}H]celecoxib and [^{3}H]valdecoxib demonstrate a similar association

TABLE 2

Kinetic and equilibrium constants for binding of [^{3}H]-celecoxib to COX-2 mutants

Wild-type COX-2 or the specified mutant enzyme was captured by an antibody onto a plate and then saturation, dissociation, and association type binding experiments performed as described under *Materials and Methods*. Data are expressed as the mean ± S.E.M. from the number of experiments shown in parentheses.

	Dissociation Rate (k_{-1})	Association Rate (k_{+1})	Saturation (K_D)	K_D (k_{-1}/k_{+1})
	$min^{-1} \times 10^{-3}$	$min^{-1} \times 10^{-6}$	*nM*	*nM*
COX-2	14 ± 1 (4) $t_{1/2}$ = 50 min	5.8 ± 1.8 (4)	2.3 ± 0.2 (3)	2.4
Val523Ile	50 ± 6 (5) $t_{1/2}$ = 14 min	7.2 ± 1.8 (4)	3.3 ± 0.9 (4)	6.9
IHI	30 ± 4 (4) $t_{1/2}$ = 23 min	5.7 ± 1.4 (4)	6.3 ± 1.7 (3)	5.3
Arg120Asn	27 ± 1 (3) $t_{1/2}$ = 26 min	38 ± 11 (4)	0.85 ± 0.14 (3)	0.71
Arg120Gln	53 ± 3 (3) $t_{1/2}$ = 13 min	63 ± 5 (3)	0.85 ± 0.14 (2)	0.84
Arg120Ala	69 ± 14 (6) $t_{1/2}$ = 10 min	39 ± 16 (4)	3.0 ± 0.8 (3)	1.8
Tyr355Ala	261 ± 23 (4) $t_{1/2}$ = 2.7min	53 ± 33 (5)	4.0 ± 1.1 (4)	4.9

TABLE 3

Kinetic and equilibrium constants for binding of [^{3}H]valdecoxib to COX-2 mutants

Wild-type COX-2 or the specified mutant enzyme was captured by an antibody onto a plate, and then saturation, dissociation, and association binding experiments were performed as described under *Materials and Methods*. Data are expressed as the mean ± S.E.M. from the number of experiments shown in parentheses.

	Dissociation Rate (k_{-1})	Association Rate (k_{+1})	Saturation (K_D)	K_D (k_{-1}/k_{+1})
	$min^{-1} \times 10^{-3}$	$min^{-1} \times 10^{-6}$	*nM*	*nM*
COX-2	7.0 ± 0.6 (5) $t_{1/2}$ = 98 min	4.5 ± 0.6 (3)	3.2 ± 0.8 (5)	1.6
Val523Ile	131 ± 10 (3) $t_{1/2}$ = 5 min	1.5 ± 0.2 (4)	27 ± 6 (3)	87
IHI	172 ± 10 (3) $t_{1/2}$ = 4 min	1.4 ± 0.3 (3)	55 ± 16 (4)	123
Arg120Asn	16 ± 3 (3) $t_{1/2}$ = 44 min	49 ± 4 (3)	0.99 ± 0.12 (3)	0.33
Arg120Gln	33 ± 4 (3) $t_{1/2}$ = 21 min	40 ± 19 (3)	1.1 ± 0.3 (3)	0.8
Arg120Ala	19 ± 2 (3) $t_{1/2}$ = 36 min	27 ± 7 (4)	1.8 ± 0.4 (3)	0.7
Tyr355Ala	1774 ± 417 (4) $t_{1/2}$ = 0.4min	50 ± 14 (5)	55 ± 16 (4)	35

rate and a very slow dissociation rate from wild-type COX-2. The dissociation rates, as well as the association rates, we obtained are in close agreement to those reported by Lanzo et al. (2000) for the diarylheterocycle SC-299 ($k_{-1} = 15 \times 10^{-3}$/min and $k_{+1} = 7.2 \times 10^{6}$/M/min). Both of these kinetic rates for [^{3}H]celecoxib and [^{3}H]valdecoxib were increased by the mutation at Arg120 to a smaller amino acid; the association rate was significantly more affected at nearly 10-fold. Mutation of Arg120 to smaller neutral amino acids may provide more flexibility at the entrance to the main channel by disrupting the salt bridge between Arg120 and Glu524, potentially expanding the entrance to the main hydrophobic channel. This could allow more rapid access to the diarylheterocycle-binding site and give rise to the observed increase in association rate. If the entrance has been perturbed by these mutants, the increase in association rate may be somewhat offset by a more rapid dissociation of the ligand from the binding site because of the loss of van der Waals contacts and loss of the potential ion pair with Arg120. The overall net effect being a slight increase in affinity of both [^{3}H]celecoxib and [^{3}H]valdecoxib for the Arg120 mutants.

A decrease in affinity for both [^{3}H]celecoxib and [^{3}H]valdecoxib was found with the side pocket mutants Val523Ile and IHI, compared with wild-type COX-2. These mutations produced little change in the association rate of [^{3}H]celecoxib and, at most, a 3-fold decrease in the association rate of [^{3}H]valdecoxib. On the other hand, these side pocket mutations created nearly a 19-fold increase in the dissociation rate of [^{3}H]valdecoxib, whereas increasing the dissociation rate of [^{3}H]celecoxib only 2- to 4-fold over wild-type COX-2. The difference in the magnitude of the effect may be related to the decrease in volume of the side pockets of the mutants, which may prevent the optimal binding conformation of the inhibitors from being obtained. So et al. (1998) reported that the binding of a diarylheterocyclic inhibitor, SC-57666, to a Tyr355Phe mutant of COX-2 was rapidly reversible, in contrast to the slow dissociation from wild-type COX-2. Similarly, we found the Tyr355Ala mutant increases both [^{3}H]celecoxib and [^{3}H]valdecoxib dissociation rates. The Tyr355Ala mutation produced a significantly larger change in the dissociation rate of [^{3}H]valdecoxib than [^{3}H]celecoxib, 253- and 19-fold, respectively, consistent with the results of the Val523Ile and IHI side pocket mutants. We also observed a 10-fold increase in the association rate with the Tyr355Ala mutant, suggestive of less steric hindrance at the entrance and easier access to the binding site. Thus, the tyrosine-to-alanine mutation at position 355 may have the effect of enlarging both the entrance to the main channel and the entrance into the side pocket. These kinetic results are consistent with previous reports of the importance of the side pocket in diarylheterocycle potency and selectivity (Gierse et al., 1996; Guo et al., 1996; Wong et al., 1997). Moreover, based upon the side pocket and Tyr355 mutation results, one could envision that [^{3}H]valdecoxib may provide a slightly greater selectivity for COX-2 over COX-1 than [^{3}H]celecoxib. Because [^{3}H]valdecoxib has a 5-methyl group on its central heterocycle, this methyl group would probably contact Tyr355 in the binding site. Consequently, mutation at this position would have a greater impact on the binding of [^{3}H]valdecoxib than [^{3}H]celecoxib. These additional contacts between valdecoxib and the side pocket may make that inhibitor more sensitive to amino acid differences in the side pocket between COX isoforms, perhaps contributing to its increased selectivity relative to celecoxib.

We were unable to detect any reproducible specific binding of [^{3}H]celecoxib and [^{3}H]valdecoxib to COX-1 at the concentrations tested. However, because of the high degree of nonspecific binding we observed at the higher concentrations, we cannot exclude the possibility that these inhibitors may bind to COX-1 with extremely low affinity ($K_D > 1000$ nM). This result is not surprising given the low potency for inhibition of COX-1 mediated PGE_2 production that these compounds display (Talley et al., 2000). Somewhat surprisingly, the triple mutant of COX-2, IHI, which should mimic COX-1 at the critical side pocket residues, produced only 3- and 17-fold decreases in affinity of [^{3}H]celecoxib and [^{3}H]valdecoxib, respectively. These data suggest that other important differences between COX-1 and COX-2, besides the side pocket, enhance the selectivity of these diarylheterocycle inhibitors. One potential area influencing selectivity is near the entrance to the main hydrophobic channel, where Arg120, Tyr355, Glu524, and helix D reside. When these residues have been mutated, several studies described dramatic effects on substrates and the potency of inhibitors (Greig et al., 1997; Rieke et al., 1999), suicide inactivation (Bhattacharyya et al., 1996), and allosteric activation (So et al., 1998). Lanzo et al. (2000) have described this area as a lobby region in which two or three stages of interaction with inhibitors could occur.

In this report, we have characterized the binding of celecoxib and valdecoxib to wild-type COX-2 and various mutants of COX-2. However, we could not obtain any specific binding to wild-type COX-1 using assay conditions that demonstrate high-affinity saturable binding to COX-2. Using a COX-2 mutant (IHI) in which selective substitutions were designed to hinder side pocket accessibility and mimic COX-1, we could demonstrate specific binding. These data suggest that the reduced side pocket volume of COX-1 is not the only source of diarylheterocycle selectivity. Additional features of COX-2, besides the side pocket, must exist that impact the specificity and selectivity of COX-2 binding by this class of inhibitors. We believe that this radioligand binding assay offers both a unique perspective to characterize the binding of celecoxib and valdecoxib and a rapid means in which potential COX-2 inhibitors may be tested for their potency.

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References

Barnett J, Chow J, Ives D, Chiou M, Mackenzie R, Osen E, Nguyen B, Tsing S, Bach C, Freire J, et al. (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim Biophys Acta* **1209:**130–139.

Bhattacharyya DK, Lecomte M, Rieke CJ, Garavito RM, and Smith WL (1996) Involvement of arginine 120, glutamate 524 and tyrosine 355 in the binding of arachidonate and 2-phenylpropionic acid inhibitors to the cyclooxygenase active site of ovine prostaglandin endoperoxide H synthase-1. *J Biol Chem* **271:**2179–2184.

Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, et al. (2000) Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* **343:**1520–1528.

Callan OH, So O, and Swinney DC (1996) The kinetic factors that determine the affinity and selectivity for slow binding inhibition of human prostaglandin H synthase 1 and 2 by indomethacin and flurbiprofen. *J Biol Chem* **271:**3548–3554.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Gierse JK, Hauser SD, Creely DP, Koboldt C, Rangwala SH, Isakson PC, and Seibert K (1995) Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase. *Biochem J* **305:**479–484.

Gierse JK, McDonald JJ, Hauser SD, Rangwala SH, Koboldt CM, and Seibert K (1996) A single amino acid difference between cyclooxygenase-1 (COX-1) and -2 (COX-2) reverses the selectivity of COX-2 specific inhibitors. *J Biol Chem* **271:** 15810–15814.

Gierse JK, Koboldt CM, Walker MC, Seibert K, and Isakson PC (1999) Kinetic basis for selective inhibition of cyclo-oxygenases. *Biochem J* **339:**607–614.

Greig GM, Francis DA, Falgueyret JP, Ouellet M, Percival MD, Roy P, Bayly C, Mancini JA, and O'Neill GP (1997) The interaction of arginine 106 of human prostaglandin G/H synthase-2 with inhibitors is not a universal component of inhibition mediated by nonsteroidal anti-inflammatory drugs. *Mol Pharmacol* **52:**829–838.

Guo Q, Wang L, Ruan K, and Kulmacz RJ (1996) Role of Val^{509} in time-dependent inhibition of human prostaglandin H synthase-2 cyclooxygenase activity by isoform-selective agents. *J Biol Chem* **271:**19134–19139.

Hawkey CJ (1999) COX-2 inhibitors. *Lancet* **353:**307–314.

Kiefer JR, Pawlitz JL, Moreland KT, Stegeman RA, Hood WF, Gierse JK, Stevens AM, Goodwin DC, Rowlinson SW, Marnett LJ, et al. (2000) Structural insights into the stereochemistry of the cyclooxygenase reaction. *Nature (Lond)* **405:**97–101.

Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, et al. (1996) Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature (Lond)* **384:**644–648.

Landino LM, Crews BC, Gierse JK, Hauser SD and Marnett LJ (1997) Mutational analysis of the role of the distal histidine and glutamine residues of prostaglandin-endoperoxide synthase-2 in peroxidase catalysis, hydroperoxide reduction and cyclooxygenase activation. *J Biol Chem* **272:**21565–21574.

Lanzo CA, Sutin J, Rowlinson S, Talley J, and Marnett LJ (2000) Fluorescence quenching analysis of the association and dissociation of a diarylheterocycle to cyclooxygenase-1 and cyclooxygenase-2: Dynamic basis of cyclooxygenase-2 selectivity. *Biochemistry* **39:**6228–6234.

Malkowski MG, Ginell SL, Smith WL, and Garavito RM (2000) The productive conformation of arachidonic acid bound to prostaglandin synthase. *Science (Wash DC)* **289:**1933–1937.

Mancini JA, Riendeau D, Falgueyret JP, Vickers PJ, and O'Neill GP (1995) Arginine 120 of prostaglandin G/H synthase-1 is required for the inhibition by nonsteroidal anti-inflammatory drugs containing a carboxylic acid moiety. *J Biol Chem* **270:** 29372–29377.

Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, and Seibert K (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA* **91:**3228–3232.

Meade EA, Smith WL, and DeWitt DL (1993) Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other nonsteroidal anti-inflammatory drugs. *J Biol Chem* **268:**6610–6614.

Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, and Vane JR (1994) Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci USA* **90:**11693–11697.

Patrignani P, Panara MR, Sciulli MG, Santini G, Renda G, and Patrono C (1997) Differential inhibition of human prostaglandin endoperoxide synthase-1 and -2 by nonsteroidal anti-inflammatory drugs. *J Physiol Pharmacol* **48:**623–631.

Picot D, Loll PJ, and Garavito RM (1994) The x-ray crystal structure of the membrane protein prostaglandin H_2 synthase-1. *Nature (Lond)* **367:**243–249.

Rieke CJ, Mulichak AM, Garavito RM, and Smith WL (1999) The role of arginine 120 of human prostaglandin endoperoxide H synthase-2 in the interaction with fatty acid substrates and inhibitors. *J Biol Chem* **274:**17109–17114.

Rodbard D and Weiss GH (1973) Mathematical theory of immunoradiometric (labeled antibody) assays. *Anal Biochem* **52:**10–44.

Rowlinson SW, Crews BC, Lanzo CA, and Marnett LJ (1999) The binding of arachidonic acid in the cyclooxygenase active site of mouse prostaglandin endoperoxide synthase-2 (COX-2). *J Biol Chem* **274:**23305–23310.

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.

Simon LS, Weaver AL, Graham DY, Kivitz AJ, Lipsky PE, Hubbard RC, Isakson PC, Verburg KM, Yu SS, Zhao WW, et al. (1999) Anti-Inflammatory and upper gastrointestinal effects of celecoxib in rheumatoid arthritis—a randomized controlled trial. *J Am Med Assoc* **282:**1921–1928.

Sirois J and Richards JS (1993) Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. *J Biol Chem* **268:**21931–21938.

Smith WL, Garavito RM, and DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* **271:**33157–33160.

So O, Scarafia LE, Mak AY, Callan OH, and Swinney DC (1998) The dynamics of prostaglandin H synthases: studies with prostaglandin H synthase 2 Y355F unmask mechanisms of time-dependent inhibition and allosteric activation. *J Biol Chem* **273:**5801–5807.

Talley JJ, Brown DL, Carter JS, Graneto MJ, Koboldt CM, Masferrer JL, Perkins WE, Rogers RS, Shaffer AF, Zhang YY, et al. (2000) 4-[5-Methyl-3-phenylisoxazol-4-yl]-benzenesulfonamide, valdecoxib: a potent and selective inhibitor of COX-2. *J Med Chem* **43:**775–777.

Turini ME and DuBois RN (2002) Cyclooxygenase-2: a therapeutic target. *Annu Rev Med* **53:**35–57.

Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA, and Vane JR (1999) 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:**7563–7568.

Wong E, Bayly C, Waterman HL, Riendeau D, and Mancini JA (1997) Conversion of prostaglandin G/H synthase-1 into an enzyme sensitive to PGHS-2-selective inhibitors by a double $His^{513} \rightarrow Arg$ and $Ile^{523} \rightarrow Val$ mutation. *J Biol Chem* **272:**9280–9286.

Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, and Worley PF (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* **11:**371–386.

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