Conversion of Prostaglandin G/H Synthase-1 into an Enzyme Sensitive to PGHS-2-selective Inhibitors by a Double His$^{513}$ → Arg and Ile$^{523}$ → Val Mutation*

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Modeling of the active site of prostaglandin G/H synthase-2 (PGHS-2) onto PGHS-1 utilizing the known crystal structure of PGHS-1 shows that the only residues impinging directly on the active site that were not conserved in the two enzymes are His$^{513}$ and Ile$^{523}$ of PGHS-1 (Arg and Val of PGHS-2). These residues of human PGHS-1 were each mutated to the corresponding PGHS-2 residues (His$^{513}$ → Arg and Ile$^{523}$ → Val) and a double mutant (His$^{513}$ → Arg, Ile$^{523}$ → Val) containing both residues was also constructed. The mutant enzyme forms were expressed in COS-7 cells, and their properties were compared with those of the normal isozymes using microsomal membranes. The mutated enzyme forms all had apparent $K_m$ values within 1.4-fold that of the wild type enzyme, and the specific activity of the mutants were within 2-fold of that of PGHS-1. DuP697, NS-398, DFU, and SC-58125 are selective PGHS-2 inhibitors that act as time-dependent inhibitors of PGHS-2 and rapidly reversible competitive inhibitors of PGHS-1. The single Ile$^{523}$ → Val mutation increased the sensitivity to each of these selective inhibitors with most of the effect detected using instantaneous inhibition assays, except for DuP697, whose potency was further increased by preincubation with the enzyme. The double PGHS-1 His$^{513}$ → Arg, Ile$^{523}$ → Val mutant became more sensitive to inhibition by NS-398 and DFU than the single IV mutant, and time-dependent inhibition was observed. In contrast, the single His mutation did not increase the sensitivity to inhibition by the selective PGHS-2 inhibitors. The potency of a selective PGHS-1 inhibitor, L-745,296, was decreased 5- and 13-fold in the HR and HR-IV mutants, respectively. All the results indicate that mutations of His$^{513}$ and Ile$^{523}$ residues of PGHS-1 can strongly increase sensitivity to selective PGHS-2 inhibition and restore time-dependent inhibition. They also suggest that the corresponding Arg$^{519}$ and Val$^{529}$ residues of PGHS-2 are essential determinants in differentiating between the interaction of non-selective NSAIDs and selective PGHS-2 inhibitors and their mechanism of action.

Prostaglandins are derived from arachidonic acid and act as mediators of pain, fever, and other inflammatory responses (1). Their regulation by the cyclooxygenase enzymes (COXs) is the basis for their pharmacological activity (2, 3). Prostaglandin G/H synthase (PGHS)1 converts arachidonic acid into prostaglandin G$_2$ by the addition of molecular oxygen (a cyclooxygenase step) and then catalyzes the conversion of prostaglandin G$_2$ to prostaglandin H$_2$ by a peroxidease reaction (2, 3). Prostaglandin H$_2$ is the precursor to the formation of all prostaglandins, thromboxane, and prostacyclin. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, abrogate prostaglandin synthesis through inhibition of the cyclooxygenase reaction of PGHS (4).

A second isoform of PGHS has been discovered (PGHS-2) that is induced in inflammatory situations in response to cytokines or growth factors (5–10). This has lead to the development of selective PGHS-2 inhibitors, which have demonstrated that inhibition of PGHS-2 alone is sufficient to obtain an anti-inflammatory effect while eliminating the gastric ulceration seen in animal models with NSAIDs, which inhibit both PGHS-1 and -2 without a large degree of selectivity (9, 11, 12). The observation with selective PGHS-2 inhibitors and the difference in the regulation of the expression of the two isoforms has led to the suggestion that PGHS-1 is responsible for normal physiological PG synthesis, whereas PGHS-2 is the main isoform responsible for elevated PG production during inflammatory responses (9). Recent examples of selective PGHS-2 inhibitors are DuP697 (13), NS-398 (14), SC-58125 (9), and DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone).2 These compounds have similar mechanisms of action, and they inhibit PGHS-2 by a time-dependent mechanism, whereas inhibition of PGHS-1 is through a time-independent and competitive mechanism (13, 16). The molecular basis for this time-dependent inhibition has not been elucidated.

The cDNA and corresponding amino acid sequences of both human PGHS isoforms have been published, and the two enzyme forms have 63% sequence identity (17). The x-ray crystal structure of sheep seminal vesicle PGHS-1 has demonstrated that the cyclooxygenase active site is comprised of a long hy

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1 The abbreviations used are: PGHS, prostaglandin G/H synthase; PGE$_{1}$, prostaglandin E$_{1}$; NSAID, nonsteroidal anti-inflammatory drug; DuP697, 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl)thiophene; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; SC-58125, 1-[(4-methylsulfonyl)phenyl]-3-tri-fluormethyl-5-(4-fluorophenyl)pyrazole; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone; L-745,296, 3-(4-cyano phenyl)-2-(4-fluorophenyl)thiophene; HR, His$^{513}$ → Arg; IV, Ile$^{523}$ → Val; HETE, hydroxyeicosatetraenoic acid.

2 Rieudeau, D., Percival, M. D., Boyce, S., Brideau, C., Charleson, S., Cronlish, W., Evans, J., Falgueyret, J.-P., Ford-Hutchinson, A. W., Gordon, R., Greig, G., Gresser, M. J., Guay, J., Kargman, S., Mancini, J. A., O’Neill, G. P., Ouellet, M., Rodger, I. W., Wang, Z., Wong, E., Xu, L., Young, R. N., Zamboni, R., Prasit, P., and Chan, C.-C. (1997) Brit. J. Pharmacol., in press.
drophobic channel that is also the site for binding of NSAIDs (18). Several distinctive features of the active site are: 1) an Arg<sup>120</sup> residue at the mouth of the channel that has been demonstrated to be important for binding of arachidonic acid and NSAIDs containing a carboxylic acid residue (19, 20); 2) a Tyr<sup>385</sup> residue at the upper portion of the active site that is involved in the formation of a radical at the C-13 position of arachidonic acid (21); and 3) a Ser<sup>530</sup> residue (just below Tyr<sup>385</sup>) is the residue that is the site of acetylation by aspirin (22). The aspirin acetylated PGHS-2 results in an enzyme form that oxidizes arachidonic acid to 15-HETE (23) with altered sensitivity to inhibition by certain NSAIDs (24). Molecular modeling of the active site of PGHS-2 using the coordinates of PGHS-1 and site-directed mutagenesis have identified Val<sup>509</sup> of PGHS-2 as essential for inhibition by PGHS-2 selective inhibitors (25, 26). We have utilized molecular modeling of the active site of PGHS-2 to determine residues of PGHS-1 that could be mutated to recover inhibition of PGHS-2 selective inhibitors on a modified form of PGHS-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Diclofenac was obtained from Sigma and indomethacin and arachidonic acid were purchased from Cayman Chemical Co. Sulindac sulfide, DFU, DuP697, NS-398, SC-58125, and L-745,296 (compound 23) (27) were synthesized by the Medicinal Chemistry Department of the Merck Frosst Center for Therapeutic Research. Hematin and glutathione were purchased from Sigma; phenol was obtained from Life Technologies, Inc.

**Molecular Modeling**—Human PGHS-2 was homology built based
the manufacturer’s instructions. Oligonucleotides were obtained from Research Genetics (Huntsville, AL) with a one- or two-nucleotide mismatch. The oligonucleotides were hybridized to the single stranded DNA, and mutants were generated according to the manufacturer’s instructions. The mutants were confirmed by sequencing using a Prism Dye Terminator Kit (Applied Biosystems) and an ABI 373 DNA sequencer. The correct mutants were completely sequenced on both cDNA strands to confirm no misincorporation of nucleotides during the mutagenesis procedure. The cDNA of interest was subcloned into the pcDNA3 vector (Invitrogen) for transient expression in COS-7 (ATCC) cells.

Expression of PGHS-1 Recombinant Proteins—PGHS-1 cDNA constructs were transfected into COS-7 cells using a calcium phosphate transfection kit (Life Technologies, Inc.). The cells were washed with fresh medium 24 h after transfection and harvested 72 h post-transfection. The harvested cells were washed with phosphate-buffered saline (Life Technologies, Inc.) and resuspended in 100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml soybean trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride. The cells were disrupted with a Kontes Micro Ultrasonic Cell Disruptor for 3 × 10 s. The crude homogenate was centrifuged at 1000 × g for 10 min at 4 °C. The supernatant fraction was then subjected to a 100,000 × g spin for 1 h at 4 °C. The resulting microsomal pellet fraction for each mutant and the wild type PGHS-1 was resuspended in 100 mM Tris-HCl, pH 7.4, 10 mM EDTA. The protein concentration was quantitated by using the Pierce Coomassie protein assay as described by the manufacturer. Protein expression was quantitated by immunoblot analysis. Protein samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. The PGHS-1 protein was detected with a PGHS-1 polyclonal antiserum (30), and a secondary antibody developed with enhanced chemiluminescence (DuPont NEN). The immunoblot was exposed to Kodak Biomax MR film. The developed film was analyzed for expression levels by laser densitometric scanning using a Molecular Dynamics Laser Densitometer.

Enzyme Assays—PGHS activity was determined based on the conversion of arachidonic acid to PGE2 by radiimmunoassay. Microsomal membrane protein preparations (final concentration, 15 μg/ml) of PGHS-1 were incubated in the absence or the presence of inhibitor for various times in 100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM glutathione, 0.5 mM phenol, 100 μM hematin. The reaction was initiated with either 2 or 10 μM arachidonic acid and was terminated at 1 or 3 min by acidification with 0.1 N HCl (final concentration). This mixture was neutralized with an equivalent amount of NaOH and analyzed for PGE2 formation using the methyl oximated PGE2 RIA kit (Amersham Corp.). A time course of PGE2 formation for all of the microsomal preparations was obtained and found to result in a rapid product formation that was linear for the first minute and reached a maximum after 3–5 min. The Km was determined using a reaction time of 1 min over a broad range of arachidonic acid concentrations (0.1–10 μM) and was calculated using a hyperbolic regression program created by Dr. J. S. Easterby (University of Liverpool, UK). The IC50 calculations of the inhibitors tested were determined ± standard error from at least four values. Microsomal PGHS-2 was prepared from COS-7 cells infected with a vaccinia virus PGHS-2 construct as described previously (12).

RESULTS

Molecular Modeling of the Active Site of PGHS-2—The development of selective PGHS-2 inhibitors has led to attempts to identify residues of PGHS-2 involved in the binding of these inhibitors. Molecular modeling of the three-dimensional structures of the human isoforms was performed utilizing the published crystal structure of sheep PGHS-1 (18) to identify residues of PGHS-1 that can be converted to PGHS-2 to mimic the inhibitor binding site of PGHS-2. The predicted structure of PGHS-2 demonstrates that the majority of the changes between the two isoforms occur at the N-terminal region defining the amphipathic helical end at the C-terminal tail. The cytoplasmic active site of PGHS-1 and 2 are very similar. The important features of the active site are depicted in Fig. 1 with Tyr265 (PGHS-1 residues, Fig. 1A) representing the upper part of the active site located just below the heme moiety and presumably responsible for the abstraction of the hydrogen atom at the C-13 position of arachidonic acid. The Ser530 residue is the site of aspirin acetylation and is equivalent to Ser416.
in PGHS-2 (Fig. 1B). Arg^{120} (Arg^{106} in PGHS-2) is the proposed interaction site of the acid moiety of several NSAIDs and of the carboxylic acid group of arachidonic acid. His^{513} of PGHS-1 (Arg^{499} of PGHS-2) and Ile^{523} of PGHS-1 (Val^{509} of PGHS-2) are two residues that impinge on the active site and that are not conserved between the two isoforms. The solvent accessible space in the active site of these isoforms (depicted as blue dots in Fig. 1) shows that the His^{513} and Ile^{523} of PGHS-1 allow for

**TABLE II**

Sensitivity of PGHS-1, PGHS-2, and mutant forms of PGHS-1 to inhibition by NSAIDs and selective PGHS-2 inhibitors

Inhibition of PGHS activity for each recombinant enzyme was determined by using a 15-min preincubation with inhibitor followed by initiation of the reaction with 2 μM arachidonic acid. IC\textsubscript{50} values represent the average of at least four experiments ± S.E.

| Inhibitor    | PGHS-1 | PGHS-1 H513R | PGHS-1 I523V | PGHS-1 H513R, I523V | PGHS-2 |
|--------------|--------|--------------|--------------|---------------------|--------|
| Sulindac sulfide | 80 ± 40 | 140 ± 5 | 6 ± 2 | 60 ± 20 | 310 ± 40 |
| Indomethacin  | 95 ± 15 | 50 ± 2 | 19 ± 2 | 40 ± 20 | 1,000 ± 300 |
| Diclofenac    | 21 ± 1 | 18 ± 5 | 11 ± 3 | 11 ± 1 | 62 ± 10 |
| DuP697       | 110 ± 30 | 96 ± 15 | 0.3 ± 0.1 | 0.4 ± 0.1 | 5 ± 3 |
| NS-398       | 32,000 ± 13,000 | 16,000 ± 5,000 | 7,500 ± 1,600 | 940 ± 300 | 80 ± 8 |
| SC-58125     | >50,000 | >50,000 | 3,400 ± 400 | 1,600 ± 500 | 190 ± 40 |
| DFU          | >50,000 | >50,000 | 21,000 ± 5,000 | 1,800 ± 200 | 700 ± 200 |
| L-745,296    | 110 ± 20 | 510 ± 110 | 24 ± 6 | 1,400 ± 140 | >50,000 |

**FIG. 3.** Comparison of inhibition of PGE\textsubscript{2} production by PGHS-1 and -2 and several PGHS-1 mutants. Microsomal membrane preparations of COS-7 cells expressing PGHS-1 or -2 or PGHS-1 mutants were prepared and assayed for PGE\textsubscript{2} production in the presence or the absence of the selective PGHS-2 inhibitors, DuP697 (A), NS-398 (B), SC-58125 (C), and DFU (D). These compounds were preincubated with the enzyme preparation for 15 min prior to initiation of the reaction with 2 μM arachidonic acid. Each data point is an average of duplicates and is reported as a percentage of inhibition of the control reaction.
only a small available space in the vicinity of these residues for inhibitor interaction. Specifically, Ile<sup>523</sup> appears to severely restrict access to this space. In contrast, the Val<sup>509</sup> and Arg<sup>499</sup> of PGHS-2 result in the creation of a larger active site of PGHS-2 accessible to substrates and inhibitors. We have replaced the residues His<sup>513</sup> and Ile<sup>523</sup> of PGHS-1 with the corresponding residues of PGHS-2 to mimic the inhibitor binding site of PGHS-2.

Expression and Activity of Mutants of PGHS-1—The double mutant of PGHS-1 (PGHS-1 His<sup>513</sup> → Arg, Ile<sup>523</sup> → Val (HR-IV)) was constructed to completely mimic the lower end of the active site of PGHS-2 and the single mutants (PGHS-1 His<sup>513</sup> → Arg (HR) and PGHS-1 Ile<sup>523</sup> → Val (IV)) were constructed to establish the contribution of the single residue on the inhibitor selectivity. Levels of PGHS protein expression of the various mutants in microsomal membranes from transfected COS-7 cells were quantitated by immunoblot analysis using a specific anti-PGHS-1 antiserum (Fig. 2). Laser densitometric scanning indicates that the level of expression of each of the various proteins was very similar with relative levels of 0.9-, 0.6-, and 0.9-fold that of PGHS-1 for HR, IV, and HR-IV mutants, respectively. The PGHS activity of the various mutants, as assessed by conversion of arachidonic acid to PGE<sub>2</sub>, ranged from 0.6 to 2-fold that of the wild type PGHS-1 (Table I). Membranes from mock transfected COS-7 cells contained less than 3% of the PGHS activity measured for any of the recombinant PGHS proteins. The time course of PGE<sub>2</sub> production was similar for all recombinant PGHS preparations with a rapid substrate conversion over the first minute of the reaction and a plateau of product formation after a 3–5-min reaction as previously reported (19). The apparent K<sub>m</sub> values for arachidonic acid were determined and found to be very similar for PGHS-2, PGHS-1, and the mutants of PGHS-1 (0.5–0.9 μM) (Table I).

Altered Sensitivity of Mutated Enzyme Forms to PGHS-1 and PGHS-2 Selective Inhibitors—The effect of the various mutations introduced into PGHS-1 on inhibitor sensitivity was evaluated using selective PGHS-2 inhibitors (DuP697, NS-398, SC-58125, and DFU), nonselective NSAIDs (sulindac sulfide, indomethacin, and diclofenac), and a selective PGHS-1 inhibitor (L-745,296). The results are summarized in Table II, and titration curves for the selective PGHS-2 inhibitors are shown in Fig. 3. The HR mutant resulted in an enzyme form that was inhibited by NSAIDs with similar potencies as observed for wild type PGHS-1, and there was no significant change in sensitivity to PGHS-2 selective inhibitors. The most significant change for the HR mutant was a 5-fold increase in the IC<sub>50</sub> for the selective PGHS-1 inhibitor, L-745,296. In contrast to the modest effect of the HR mutation on inhibitor sensitivity, the IV mutant was found to have an increased sensitivity to all of the PGHS-2 inhibitors. For example, DuP697 was about 300-fold and SC-58125 at least 15-fold more potent at inhibiting the IV mutant than the wild type PGHS-1. The IV mutant was also more sensitive to inhibition by NS-398 or DFU, and the IC<sub>50</sub> for these selective inhibitors was at least 4-fold lower than that observed for PGHS-1. The double mutant HR-IV was more sensitive to inhibition by the selective PGHS-2 compounds, NS-398, SC-58125, and DFU, than either of the single point mutants, DFU and SC-58125, which both have IC<sub>50</sub> of >50 μM for the inhibition of PGHS-1, inhibited HR-IV with IC<sub>50</sub> values of 1.6 and 1.8 μM, respectively. The HR-IV mutant remained sensitive to inhibition by the PGHS-1 selective inhibitor L-745,296, although the potency of this inhibitor was decreased by more than 10-fold as compared with native PGHS-1.

Mechanism of Inhibition of Mutant Forms of PGHS-1—The inhibition of PGHS-2 by selective compounds such as DuP697 is time-dependent for PGHS-2 and rapidly reversible for PGHS-1 (13, 16). This difference is readily observed with microsomal preparations where only the inhibition of PGHS-2 was found to depend on the time of preincubation with enzyme as shown in Fig. 4 (A and B). We examined this time dependence for inhibition of the IV mutant, and as shown in Fig. 4C, inhibition of this mutant by DuP697 follows a similar pattern as that seen for inhibition of PGHS-2. DuP697 was also found...
to be a time-dependent inhibitor of HR-IV (data not shown). SC-58125 was a time-independent inhibitor of both the IV and HR-IV mutant (data not shown).

Both instantaneous inhibition assays in which the inhibitor was added at the time of initiation of the reaction and time-dependent inhibition have been used to evaluate the effect of NSAIDs and PGHS-2 inhibitors on enzyme activity (31). IC_{50} values were determined for both the IV and HR-IV mutants using assays with either no preincubation or a 15-min preincubation of the inhibitor prior to initiation of the enzyme reaction. These results are summarized in Table III for DuP697, DFU, and NS-398. The single IV mutation caused only a slight increase in inhibitor potency as determined for instantaneous inhibition, which was not further modified by increasing the preincubation time in the case of DFU and NS-398. In contrast, DuP697, for which an IC_{50} value of 0.1 \mu M for PGHS-1 is observed independently of the time of preincubation, was a more potent inhibitor of the IV mutant after a 15-min preincubation (IC_{50} = 0.3 nM), consistent with the time-dependent inhibition observed in Fig. 4C. The introduction of the second HR mutation did not further change the parameters for the inhibition by DuP697. For DFU and NS-398, the second HR mutation caused a further decrease in IC_{50} values for instantaneous inhibition to 5.2 and 2.6 \mu M, respectively. In addition, the IC_{50} values for these two inhibitors were about 3-fold lower using the 15-min preincubation assay. All of these selective PGHS-2 inhibitors are essentially inactive when tested for using the 15-min preincubation assay. All of these selective inhibitors with DFU and NS-398 now showing time-dependent inhibition of PGHS-1 by DuP697, time-dependent inhibition would be consistent with a two-step mechanism as shown above for PGHS-2, although alternate mechanisms cannot be ruled out. The introduction of the second HR mutation to generate the HR-IV mutant resulted in an enzyme mechanism of inhibition (rapidly reversible) for DFU, SC-58125, and NS-398 with a further increase in the potency of DuP697 by preincubation of the inhibitor with enzyme before the measurement of activity. These results suggest that the IV mutation has increased the affinity of PGHS-1 for all selective PGHS-2 inhibitors but without a change in the mechanism of inhibition (rapidly reversible) for DFU, SC-58125, and NS-398. However, this single mutation was sufficient to change the rapidly reversible mechanism of inhibition of PGHS-1 by DuP697 to the time-dependent mechanism characteristic of PGHS-2 inhibition. Reconstitution of time-dependent inhibition would be consistent with a two-step mechanism as shown above for PGHS-2, although alternate mechanisms cannot be ruled out. The introduction of the second HR mutation to generate the HR-IV mutant resulted in an enzyme form very sensitive to inhibition by all four of the PGHS-2 selective inhibitors with DFU and NS-398 now showing time-dependent inhibition in addition to DuP697. It is of interest to note that the changes of the HR mutation on inhibitor potency were observed on the IV mutant of PGHS-1 rather than on PGHS-1, demonstrating the advantage of combining mutations to show the importance of the various residues. Another difference in the active site of PGHS-2 as compared with PGHS-1 is the increased capacity of PGHS-2 to synthesize 15-R-HETE from arachidonic acid when acetylated by aspirin (23, 24). Both the single IV and the double mutant HR-IV did not synthesize any appreciable 15-R-HETE as compared with PGHS-2 when treated with 100 \mu M aspirin (data not shown). This suggests that the major determinants for the binding of selective PGHS-2 inhibitors by the PGHS-1 mutants are not sufficient to restore all of the properties of the active site of PGHS-2.

Two recent reports have shown that mutation of Val^{509} of PGHS-2 to the corresponding Ile^{523} in PGHS-1 results in the

**DISCUSSION**

The discovery of a second isoform of PGHS several years ago has led to the identification of several selective inhibitors of PGHS-2 that have anti-inflammatory properties and a significantly decreased capacity for gastric ulceration. The mechanism of action of these selective PGHS-2 inhibitors is through a two-step mechanism. The first step involves the formation of an enzyme inhibitor complex (EI) followed by the slower formation of a tightly bound EI* complex as shown in the equation below.

\[
E + I \rightleftharpoons EI \xrightarrow{k_2} EI^* 
\]

(Eq. 1)

The method of inhibition for PGHS-1 by PGHS-2 selective inhibitors is via a competitive rapidly reversible mechanism (13, 16). Molecular modeling of the active site of PGHS-2 based on the known structure of PGHS-1 was performed to predict the major differences in the active sites of the two enzymes. The model suggests that in PGHS-2, the solvent accessible surface area is increased by the residues Val^{509} and Arg^{499} of PGHS-2 (Fig. 1), resulting in the ability of PGHS-2 to utilize a variety of lipid substrates (16) and a larger active site for inhibitor binding. Because the only primary structure of the differences in the active site were localized to these latter residues and the equivalent PGHS-1 residues are His^{512} and Ile^{523}, we constructed mutants of PGHS-1 in attempts to convert the cyclooxygenase active site of PGHS-1 into that of PGHS-2. Because the apparent K_{m} values of the mutants are within 1.4-fold and the specific activity is within 2-fold of that of PGHS-1 and the mutants retained sensitivity to inhibition by all NSAIDs tested, the mutations have not significantly altered the cyclooxygenase active site of prostaglandin synthase.

The single IV mutation in PGHS-1 results in an enzyme form that is more sensitive to inhibition by all four of the PGHS-2 selective inhibitors, although NS-398 and DFU are much weaker inhibitors of this mutant compared with either the double HR-IV mutant or PGHS-2. The single IV mutant was also more sensitive to inhibition by nonselective NSAIDs and the selective PGHS-1 inhibitor L-745,296, although the effect was most pronounced with the PGHS-2 selective inhibitors. The increase in potency for the IV mutant as compared with the wild type PGHS-1 is observable at instantaneous inhibition for DFU, SC-58125, and NS-398 with a further increase in the potency of DuP697 by preincubation of the inhibitor with enzyme before the measurement of activity. These results suggest that the IV mutation has increased the affinity of PGHS-1 for all selective PGHS-2 inhibitors but without a change in the mechanism of inhibition (rapidly reversible) for DFU, SC-58125, and NS-398. However, this single mutation was sufficient to change the rapidly reversible mechanism of inhibition of PGHS-1 by DuP697 to the time-dependent mechanism characteristic of PGHS-2 inhibition. Reconstitution of time-dependent inhibition would be consistent with a two-step mechanism as shown above for PGHS-2, although alternate mechanisms cannot be ruled out. The introduction of the second HR mutation to generate the HR-IV mutant resulted in an enzyme form very sensitive to inhibition by all four of the PGHS-2 selective inhibitors with DFU and NS-398 now showing time-dependent inhibition in addition to DuP697. It is of interest to note that the changes of the HR mutation on inhibitor potency were observed on the IV mutant of PGHS-1 rather than on PGHS-1, demonstrating the advantage of combining mutations to show the importance of the various residues. Another difference in the active site of PGHS-2 as compared with PGHS-1 is the increased capacity of PGHS-2 to synthesize 15-R-HETE from arachidonic acid when acetylated by aspirin (23, 24). Both the single IV and the double mutant HR-IV did not synthesize any appreciable 15-R-HETE as compared with PGHS-2 when treated with 100 \mu M aspirin (data not shown). This suggests that the major determinants for the binding of selective PGHS-2 inhibitors by the PGHS-1 mutants are not sufficient to restore all of the properties of the active site of PGHS-2.

Two recent reports have shown that mutation of Val^{509} of PGHS-2 to the corresponding Ile^{523} in PGHS-1 results in the

**Table III**

**Instantaneous and time-dependent inhibition of PGHS mutants by selective PGHS-2 inhibitors**

Selectiv PGHS-2 inhibitors were incubated with the indicated form of PGHS-1 for 0 or 15 min prior to initiation of the reaction with 10 \mu M arachidonic acid. The IC_{50} values presented are the average of at least two determinations.

| Compound | PGHS-1 | PGHS-1 I523V | PGHS-1 H513R, I523V |
|----------|--------|-------------|---------------------|
|          | IC_{50} | 15 min | 0 min | IC_{50} | 15 min | 0 min | IC_{50} | 15 min | 0 min |
| DuP697   | 32,000 | n.d.   | n.d.   | 0.3 | 0.3 | 0.01 | 0.4 | 0.4 |
| DFU      | >50,000| 22,000 | 3,000  | 3,500 | 5,000 | 5,200 | 1,800 | 1,800 |
| NS-398   | 6,300  | 5,000  | 1,600  | 7,500 | 2,600 | 940  | 1,000 | 2,500 |
loss of sensitivity to PGHS-2 inhibition (25, 26). Our results suggest that the converse in PGHS-1 is sufficient to gain partial sensitivity to PGHS-2 inhibitors, but the double HR-IV mutation results in an enzyme form that is more sensitive to a greater number of PGHS-2 selective inhibitors from three diverse structural classes, with most having a similar mechanism of inhibition. These data suggest that the molecular modeling of PGHS-2 has accurately predicted residues that are important for PGHS-2 inhibitor selectivity. During preparation of this manuscript, the crystal structure of PGHS-2 was published and confirms the major points depicted by the molecular modeling (15). The crystal structure data demonstrate the importance of Val509 as the residue that provides access to a larger active site for PGHS-2 as compared with PGHS-1. In conclusion, we have demonstrated that the combination of molecular modeling, mutagenesis, and inhibitor characterization have delineated both residues Arg499 and Val509 to be essential determinants in the differentiation between interaction of non-selective NSAIDs and selective PGHS-2 inhibitors and their mechanism of inhibition.

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