

# Polymorphic human prostaglandin H synthase-2 proteins and their interactions with cyclooxygenase substrates and inhibitors

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The cyclooxygenase (COX) activity of prostaglandin H synthase-2 (PGHS-2) is implicated in colorectal cancer and is targeted by nonsteroidal anti-inflammatory drugs (NSAIDs) and dietary *n*-3 fatty acids. We used purified, recombinant proteins to evaluate the functional impacts of the R228H, E488G, V511A and G587R PGHS-2 polymorphisms on COX activity, fatty acid selectivity and NSAID actions. Compared to wild-type PGHS-2, COX activity with arachidonate was ~20% lower in 488G and ~20% higher in 511A. All variants showed time-dependent inhibition by the COX-2-specific inhibitor (coxib) nimesulide, but 488G and 511A had 30–60% higher residual COX activity; 511A also showed up to 70% higher residual activity with other time-dependent inhibitors. In addition, 488G and 511A differed significantly from wild type in  $V_{\max}$  values with the two fatty acids: 488G showed ~20% less and 511A showed ~20% more discrimination against eicosapentaenoic acid. The  $V_{\max}$  value for eicosapentaenoate was not affected in 228H or 587R, nor were the  $K_m$  values or the COX activation efficiency (with arachidonate) significantly altered in any variant. Thus, the E488G and V511A PGHS-2 polymorphisms may predict who will most likely benefit from interventions with some NSAIDs or *n*-3 fatty acids. *The Pharmacogenomics Journal* (2011) 11, 337–347; doi:10.1038/tpj.2010.49; published online 15 June 2010

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## Introduction

The cyclooxygenase (COX) activity of prostaglandin H synthase isoforms 1 and 2 (PGHS-1 and PGHS-2) catalyzes a key step in the biosynthesis of prostaglandins from *n*-6 and *n*-3 polyunsaturated fatty acids such as arachidonic acid (AA) and eicosapentaenoic acid (EPA).<sup>1</sup> The prostaglandins, which include prostaglandin E, prostacyclin and thromboxane, serve as potent signaling molecules in many pathophysiological processes.<sup>2</sup> The two PGHS isoforms are functionally specialized, with PGHS-1 generally considered a constitutive, housekeeping enzyme, whereas PGHS-2 is strongly induced by cytokines and mitogens in a variety of cells and has been linked to the initiation and resolution phases of inflammation and to cell proliferation.<sup>3,4</sup> PGHS-2 levels are elevated in many tumors and COX-2 activity has been implicated in the development of several types of cancer, including colorectal cancer.<sup>5,6</sup>

COX catalysis is strongly regulated by the availability of unesterified fatty acid substrate, the structure of the substrate (for example, *n*-6 vs. *n*-3) and the level

of peroxide, which is required to generate the protein-based radical at Tyr385 that begins the catalytic cycle.<sup>1</sup> In addition, COX catalysis is the primary target of NSAIDs (nonsteroidal anti-inflammatory drugs); these agents include aspirin, ibuprofen, naproxen and the COX-2 inhibitors (coxibs), which are selective for inhibition of COX-2.<sup>3</sup> NSAID use is linked to significantly decreased risk of colorectal cancer in humans.<sup>5</sup>

A limited number of nonsynonymous polymorphisms have been identified in *PTGS2*, the gene that encodes human PGHS-2 (hPGHS-2); these polymorphisms include R228H, E488G, V511A and G587R.<sup>7,8</sup> The prevalence of these polymorphisms is low, with a minor allele frequency ranging from 0.8 to 5% in the populations studied, suggesting that there is strong selective pressure on *PTGS2*. Epidemiological studies have examined the relation of the V511A polymorphism to the risks of colorectal adenoma or cancer,<sup>8–10</sup> breast cancer<sup>11</sup> and cardiovascular disease.<sup>12</sup> Because these studies were generally small ( $N \sim 400$ – $1000$ ) and the variant allele is rare ( $\sim 4\%$ ), none of the studies found a statistically significant association with risk. However, two of the colorectal cancer studies reported inverse associations with the A allele and some suggestion of an NSAID interaction.<sup>8,10</sup> Similarly, gene–NSAID interactions and pharmacogenetic relationships have been reported in noncoding *PTGS2* single-nucleotide polymorphisms (SNPs).<sup>13</sup> as well as in other genes related to prostaglandin synthesis.<sup>14,15</sup>

There have been few published studies on the nonsynonymous polymorphisms listed above,<sup>7–11,16,17</sup> and the V511A polymorphism is the only nonsynonymous *PTGS2* variant that has been studied for functional effects so far. Fritsche *et al.*<sup>7</sup> observed no major differences between wild-type hPGHS-2 and the V511A variant for metabolism of AA or linoleic acid, nor any differences between the alleles for inhibition by indomethacin or celecoxib. Lin *et al.*<sup>8</sup> reported that the major and minor alleles had similar  $V_{max}$  and  $K_m$  values, but noted it was premature to conclude that the V511A variant had no functional effect without a more thorough enzymological analysis. Several important aspects of COX-2 catalysis were not examined in the earlier studies. These aspects include: discrimination between  $n-6$  and  $n-3$  substrates, which has considerable potential for diet-driven modulation of prostanoid signaling;<sup>18</sup> peroxide-dependent feedback activation, which contributes to regulation of COX activity;<sup>19</sup> and time-dependent COX inhibition effects, which characterize the more potent pharmacological agents.<sup>20</sup> The present studies were undertaken to address these important aspects of COX functionality in V511A and three other variants of hPGHS-2 using the purified recombinant proteins.

## Materials and methods

High-purity AA and EPA were obtained in sealed ampules from Nu Chek Prep (Elysian, MN, USA) and handled under conditions found to minimize peroxide formation.<sup>21</sup> Briefly,

stocks of the fatty acids were dissolved in toluene, butylated hydroxytoluene (0.001 mol/mol) was added and the solutions stored at  $-20^\circ\text{C}$ . Working suspensions (3–30 mM) in 0.1 M Tris (pH 8.5) were prepared fresh each day and kept on ice. Tween 20 was from Anatrace (Maumee, OH, USA). Coxibs were from Cayman Chemical (Ann Arbor, MI, USA) or Tocris Bioscience (Ellisville, MO, USA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA, USA). Reagents for DNA manipulation were from Promega (Madison, WI, USA). Plasmid transfer vector pAcSG2 and BaculoGold linearized baculovirus DNA were from Pharmingen (San Diego, CA, USA). QuikChange single and multisite-directed mutagenesis kits and *Escherichia coli* strain XL-10 were from Stratagene (La Jolla, CA, USA). *Sf9* insect cells (from *Spodoptera frugiperda*), *E. coli* strain DH5 $\alpha$ , Grace's supplemented medium and fetal bovine serum were from Invitrogen (Carlsbad, CA, USA). Ni-NTA agarose was purchased from Qiagen (Valencia, CA, USA). The integrity of each plasmid described below was verified by restriction enzyme digestion and by DNA sequencing at the Microbiology and Molecular Genetics Core Facility, UT Health Science Center at Houston. All other reagents were obtained from Sigma (St Louis, MO, USA).

### Construction of plasmid for recombinant, 6 $\times$ His-tagged hPGHS-2

The 6  $\times$  His tag sequence was introduced after the signal peptide cleavage site near the N terminus of the hPGHS-2 wild type (major allele at all targeted positions), as previously reported.<sup>22</sup> Nucleotides coding for the six histidine residues were inserted into the hPGHS-2/pAcSG2 vector using PCR with PfuUltra HF DNA polymerase (Stratagene) and the following 5'-primers: sense, 5'-pCAC CATCACCTTGCTGTTCCACCCATGTCAAACC-3'; anti-sense, 5'-pATGGTGTATGATTTGCTGTATGGCTGAGCGCCAGGACC-3'.

The PCR mixture was digested with *DpnI*; the resulting 7.4 kb product was separated by agarose gel electrophoresis and ligated with T4 DNA ligase. The resulting plasmid was transformed into *E. coli* strain DH5 $\alpha$ , and ampicillin-resistant colonies were analyzed by restriction enzyme digest and DNA sequencing.

### Construction of plasmid for hPGHS-2 with R228H, E488G, V511A or G587R minor alleles

Plasmids coding for hPGHS-2 with the desired point mutations were constructed with the QuikChange mutagenesis kit using the pAcSG2 plasmid containing the 6  $\times$  His-tagged, wild-type hPGHS-2 cDNA as template. Primer pairs were (base changes underlined) as follows: R228H-f, 5'-C TGGCTAGACAGCATAAACTGCGCCTTTTC-3'; R228H-r, 5'-G AAAAGGCGCAGTTTATGCTGTCTAGCCAG-3'; E488G-f, 5'-C ATCGATGCTGTGGGGGCTGTATCCTGCCC-3'; E488G-r, 5'-GG GCAGGATACAGCCCCACAGCATCGATG-3'; V511A-f, 5'-GA AACCATGGTAGAAGCTGGAGCACCATTCTC-3'; V511A-r, 5'-G AGAATGGTGCTCCAGCTTCTACCATGGTTTC-3'; G587R-f,

5'-AGTTCTTCCCGCTCCCGACTAGATGATATC-3'; G587R-r, 5'-GATATCATCTAGTCGGAGCGGGAAGAACT-3'.

#### *Baculovirus generation and expression of recombinant proteins*

Generation, amplification and titer determination of recombinant baculovirus containing the desired hPGHS-2 cDNA, and expression of the recombinant proteins used previously published techniques;<sup>23</sup> procedures for expression of hPGHS-2 have been described.<sup>24</sup>

#### *Purification of recombinant proteins*

Purification of hPGHS-2 lacking a His tag has been described.<sup>24</sup> The purification procedure for His-tagged hPGHS-2 wild-type and mutant proteins was modified from a published protocol.<sup>25</sup> The *Sf9* cells expressing the recombinant protein were suspended in 25 mM NaP<sub>i</sub>, 20 mM imidazole, 1.0 mM phenol (pH 7.4) and sonicated; the homogenate was centrifuged at 150 000 *g* at 4 °C for 1 h. The resulting pellet was resuspended with a Dounce homogenizer in 100 ml of 25 mM NaP<sub>i</sub>, 100 mM NaCl, 20 mM imidazole, 0.1 mM phenol (pH 7.4), 1% v/v Tween 20 and stirred for 1 h at 4 °C. Unextracted membrane residue was removed by centrifugation. The rest of the purification was as described,<sup>25</sup> except that buffers were supplemented with 0.1 mM phenol. Holoenzyme was reconstituted by addition of heme.<sup>26</sup>

#### *Protein characterization*

Total protein concentrations were determined by a modified Lowry method.<sup>27</sup> hPGHS-2 mutants were analyzed by polyacrylamide gel electrophoresis under denaturing conditions,<sup>28</sup> visualization with Coomassie staining of the gel and densitometry using ImageJ<sup>29</sup> to determine the fraction of total protein present as recombinant hPGHS-2.

#### *COX activity*

Oxygen uptake was measured polarographically in standard assays at 30 °C in 100 mM KP<sub>i</sub> (pH 7.2) with 1.0 mM phenol, 1.0 μM heme and 0.025% Tween 20, using a 'Standard' electrode membrane (YSI, Yellow Springs, OH, USA).<sup>30</sup> One unit of COX activity has an optimal velocity of 1 nmol O<sub>2</sub> per min. The specific activity of each purified protein preparation was calculated by dividing the observed COX activity by the concentration of recombinant hPGHS-2 present. COX *K<sub>m</sub>* and *V<sub>max</sub>* values were determined by measuring the activity with 2–100 μM fatty acid at 25 °C with a YSI 'High Sensitivity' electrode membrane, in the presence of 100 μM HOOH; these assay conditions ensure full activation and minimize dampening effects from the electrode membrane.<sup>31</sup> The velocities were normalized to those for the same batch of enzyme with 100 μM AA without added peroxide and fitted to the Michaelis–Menten equation using KaleidaGraph (Synergy Software, Reading, PA, USA) to estimate the *V<sub>max</sub>* and *K<sub>m</sub>* values.

#### *COX self-inactivation*

COX self-inactivation rates for reactions in 100 μM AA were calculated by dividing the optimal velocity (nmol O<sub>2</sub> per

min) by the reaction extent at complete self-inactivation (nmol O<sub>2</sub>).<sup>32</sup>

#### *COX activation efficiency*

Feedback activation by peroxide was assessed by measuring the COX activity of a fixed amount of enzyme (45–55 U) in the presence of varying amounts of GPx-1.<sup>23</sup> Data for the decline in the fraction of expressed COX activity with increasing peroxide scavenger were fitted to a straight line to obtain the slope; the slope obtained for hPGHS-2 wild type in a given experiment was divided by the slope obtained for hPGHS-2 with each minor allele to calculate the relative activation efficiency for that variant.

#### *COX inhibition*

COX inhibition kinetics were assessed at 30 °C with a 'Standard' electrode membrane in the standard reaction mixture. Two experimental designs were used. The first focused on the forward reactions and involved timed preincubation of enzyme with inhibitor before the COX reaction was started by addition of AA. In this design, the enzyme (at ~10<sup>-8</sup> M) was preincubated with the desired level of inhibitor in the reaction cuvette for various times before injection of 100 μM AA to determine the amount of enzyme that had not progressed to tight inhibitor–protein complexes. Inhibitors were dissolved in ethanol; the final ethanol concentration was below 1% and did not affect activity. AA was suspended at 30 mM in 0.1 M Tris-HCl (pH 8.5). The second experimental design focused on the kinetics of conversion of tight inhibitor–protein complexes to looser complexes and dissociation of inhibitor. For this, the enzyme (~10<sup>-5</sup> M) was first incubated with a small excess of inhibitor (50 μM) to form an equilibrium mixture of tight and looser inhibitor complexes. Aliquots of this mixture were then diluted 1500-fold into the reaction cuvette and re-equilibrated for various lengths of time before the amount of enzyme in looser inhibitor complexes or without inhibitor bound was determined by addition of 100 μM AA.

#### *Peroxidase activity*

The stirred assay mixture contained 2.5 ml of 0.10 M Tris-HCl (pH 8.0) with 0.50 mM guaiacol, 1.0 μM heme and 0.40 mM HOOH at room temperature.<sup>33</sup> Reaction was started by injection of enzyme and the initial velocity calculated from the rate of increase in A<sub>436</sub> due to oxidized guaiacol ( $\epsilon_{436} = 6.39 \text{ (mM oxidized guaiacol)}^{-1} \text{ cm}^{-1}$ ). One unit of peroxidase (POX) activity has a velocity of 1 nmol guaiacol oxidized per min.

#### *Statistical analysis*

The results of replicate experiments are presented as mean ± s.d. of the mean. In most cases an unpaired, two-tailed *t*-test was used to assess statistical differences between individual variants and wild-type PGHS-2 (GraphPad Software, La Jolla, CA, USA). A *P*-value below 0.05 was considered statistically significant.

## Results

### Expression and purification

Several expression runs, totaling 3–41 of baculovirus-infected *Sf9* cell culture, were pooled to prepare homogenates for purification of each recombinant protein. Wild-type hPGHS-2 and the four polymorphic proteins expressed well in the baculovirus system, as assessed by the yield of COX activity in the insect cell homogenate (Table 1). All of the proteins were readily solubilized by Tween 20 and purified to 75–90% electrophoretic homogeneity, with 38–64% recovery of COX activity (Table 1). The COX-specific activities of the purified enzymes, calculated based on the amount of recombinant PGHS protein determined by assay of total protein and electrophoretic analysis, are shown in Table 1. The specific activities of the 228H, 488G and 587R variants were 19–30% lower than the wild type, whereas the value for the 511A variant was 55% higher than the wild type. With the exception of the 511A variant, these specific activities fall in the range of 15–30 kU mg<sup>-1</sup> observed for purified PGHS-2 in our laboratory.<sup>31,34,35</sup> To focus on possible effects of the polymorphic alleles that are specific for the COX activity, we normalized the COX activity for each of the purified proteins to the corresponding POX activity determined on the same day (Table 1). The COX/POX ratio was 2.7 for wild-type PGHS-2, and the values for the 228H and 587R variants were not significantly different. In contrast, the COX/POX ratio for the 511A variant was 20% higher, and the ratio for 488G ~20% lower, than the wild-type value; the difference was statistically significant for both of these variants. Thus, the COX-specific activity and the COX/POX ratio were both elevated for 511A and both decreased for 488G (Table 1). It is therefore likely that these two variant alleles have direct or indirect effects on COX catalytic efficiency.

### COX $V_{max}$ and $K_m$ parameters

The COX kinetics of wild-type hPGHS-2 and the four variants were evaluated with prototypical substrates from the *n*-6 (AA) and *n*-3 (EPA) families, with precautions taken to ensure reliable COX activity measurements reflecting the dependence on substrate concentration.<sup>31</sup> COX activity was

measured at 25 °C with a ‘High-sensitivity’ oxygen electrode membrane to minimize dampening effects that can significantly attenuate velocity values. HOOH (100 μM) was included in each reaction to ensure full COX activation, avoiding complications due to partial COX activation; this is particularly important for reactions with EPA where artifactually low COX velocities occur in the absence of exogenous peroxide.<sup>31</sup> Batch-to-batch variations in expression and purification of recombinant PGHS-2 protein can lead to differences in COX-specific activity and confound comparison of COX activity in wild-type and mutant proteins. To control for such variations in specific activity, we normalized the COX velocities for a particular protein reacted with a range of AA or EPA levels in the presence of HOOH to the average velocity of the same protein on the same day in replicate reactions with 100 μM AA without exogenous peroxide. This level of AA produces full COX activation without added peroxide.

Saturable responses of COX-2 reaction rate to AA and EPA concentration were observed for wild-type PGHS-2 and each of the variants, so values of  $V_{max}$  and  $K_m$  were estimated by fitting individual data sets to the Michaelis–Menten equation (examples are shown in Supplementary Figures S1–S5). The average  $V_{max}$  values with AA for wild-type and polymorphic hPGHS-2 proteins were all near unity (Figure 1a), as expected because the rate with saturating AA was used for normalization. The average  $K_m$  value for wild-type hPGHS-2 was 2.1 μM AA (Figure 1b), quite comparable to the value of 1.7 ± 0.4 μM reported under the same reaction conditions for hPGHS-2 without a His tag.<sup>31</sup> None of the mutations produced a significant difference in the average  $K_m$  for AA or for EPA (Figure 1b). The average  $V_{max}$  value for wild-type hPGHS-2 with EPA was 0.78, or almost 80% of the  $V_{max}$  with AA (Figure 1a). This shows that the fully activated enzyme oxygenates EPA more slowly than AA. The average  $V_{max}$  values with EPA for the 228H and 587R variants were indistinguishable from the wild-type value (Figure 1a). However, the average  $V_{max}$  value with EPA for 488G was 0.94, significantly higher than wild type and approaching the  $V_{max}$  value obtained with AA, and 511A had an average  $V_{max}$  value of 0.65 with EPA, significantly less than the wild-type protein (Figure 1a). Thus, the 488G variant led to less,

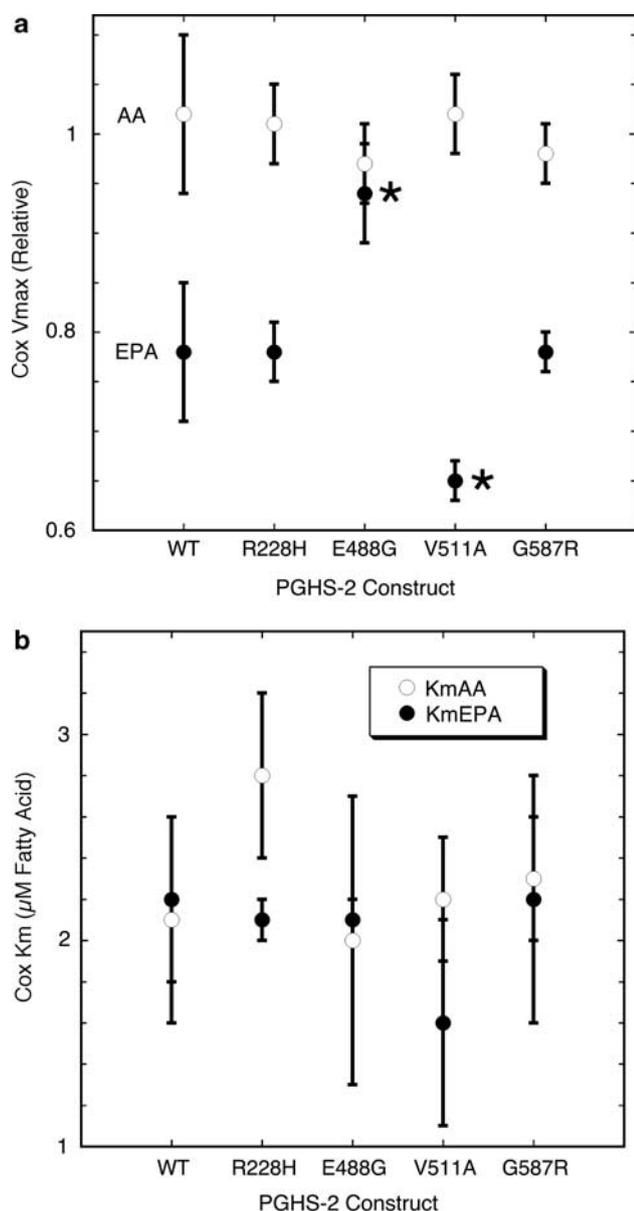
**Table 1** Expression, recovery and enzymatic activities of recombinant hPGHS-2 proteins

hPGHS-2 construct <sup>a</sup>	COX in homogenate (kU/3l culture)	Yield of COX after purification (%)	Electrophoretic homogeneity (%)	COX-specific activity <sup>b</sup> (kU mg <sup>-1</sup> PGHS-2)	COX/POX <sup>b</sup> (U/U)
Wild type <sup>c</sup>	440	64	87	30 ± 1	2.7 ± 0.1
R228H	480	38	87	22 ± 0	2.5 ± 0.1 ( <i>P</i> = 0.07)
E488G	300	46	75	21 ± 1	2.1 ± 0.1 ( <i>P</i> = 0.002)
V511A	600	42	86	46 ± 1	3.2 ± 0.2 ( <i>P</i> = 0.018)
G587R	550	52	90	24 ± 2	2.5 ± 0.1 ( <i>P</i> = 0.07)

<sup>a</sup>Variant alleles are underlined.

<sup>b</sup>Mean ± s.d. for triplicate determinations in standard assays.

<sup>c</sup>Major allele at all targeted residues.



**Figure 1** COX kinetic parameters for PGHS-2 wild type (WT) and variants with AA (open circles) and EPA (filled circles). Values for  $V_{max}$  relative to the rate with  $100\ \mu\text{M}$  AA and no HOOH (a) and  $K_m$  (b) represent the average  $\pm$  s.d. for triplicate determinations. Asterisks indicate values significantly different from wild type ( $P < 0.05$ ).

and the 511A variant led to more, COX catalytic discrimination against EPA relative to AA.

#### COX activation efficiency

Titration with a peroxide scavenger, glutathione peroxidase, were used to quantitatively compare the COX activation efficiencies of the four variant PGHS-2 proteins to the wild-type enzyme. The results, shown in Table 2, indicate that the COX activation efficiency of the 511A and

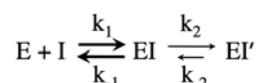
**Table 2** COX activation efficiencies of purified hPGHS-2 proteins with AA as substrate

hPGHS-2 construct <sup>a</sup>	Activation efficiency <sup>b</sup> (relative to wild type)
Wild type <sup>c</sup>	1.0
<u>R228H</u>	$1.2 \pm 0.2$ ( $n = 3$ ) $P = 0.31$
<u>E488G</u>	$1.2 \pm 0.2$ ( $n = 4$ ) $P = 0.19$
<u>V511A</u>	$1.0 \pm 0.1$ ( $n = 4$ ) $P = 0.65$
<u>G587R</u>	$1.0 \pm 0.1$ ( $n = 2$ ) $P = 0.50$

<sup>a</sup>Variant alleles are underlined.

<sup>b</sup>Values represent the average  $\pm$  s.d. for the indicated number of determinations;  $P$ -values were determined by one-sample  $t$ -test.

<sup>c</sup>Major allele at all targeted residues.



**Scheme 1** Mechanistic scheme for interaction of PGHS (E) with COX inhibitor (I).

the 587R variants was the same as the wild type. Conversely, both 228H and 488G had slightly higher activation efficiencies, though the differences were not statistically significant.

#### COX self-inactivation

The rate constant for self-inactivation was  $2.5\ \text{min}^{-1}$  for wild-type hPGHS-2 and the values for the variants ranged from 2.5 to  $2.8\ \text{min}^{-1}$ , indicating that none of the variants caused a major change in the COX self-inactivation process.

#### COX inhibition kinetics

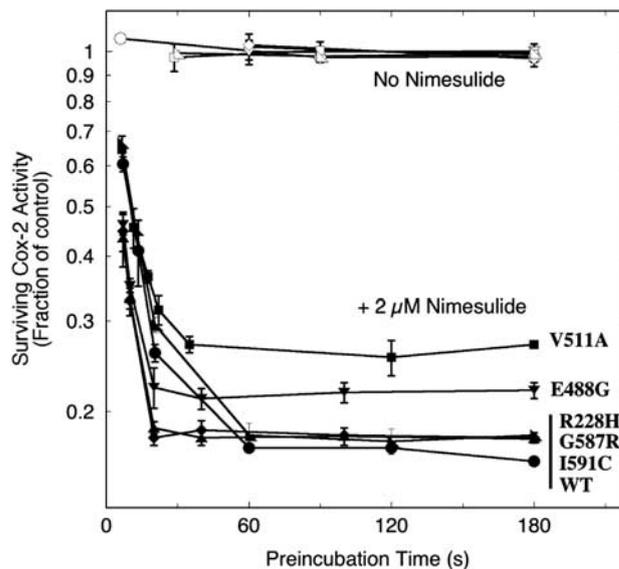
We analyzed the interactions of wild-type hPGHS-2 and the variant proteins with nimesulide, a COX-2-specific inhibitor that follows competitive, time-dependent kinetics and has proven useful as a probe of active site structure.<sup>36</sup> The action of such inhibitors can be described by a two-step mechanism such as that shown in Scheme 1. The initial binding of inhibitor (I) to the COX site of enzyme (E) to form a lower affinity complex (EI) is governed by a forward rate constant ( $k_1$ ) and a reverse rate constant ( $k_{-1}$ ). This first interaction with inhibitor typically equilibrates very rapidly, and a typical  $K_i$  value ( $k_{-1}/k_1$ ) is  $\sim 10^{-5}\ \text{M}$ . In the case of time-dependent inhibitors, the EI complex undergoes a relatively slow transition to a higher affinity complex (EI'), governed by rate constant  $k_2$ . Unless a covalent bond is formed in EI', the complex can revert back to EI by the  $k_{-2}$  process.

Two types of experimental approach were used to analyze the interactions of wild-type hPGHS-2 and the variant proteins with coxibs. The first involves preincubation of enzyme with inhibitor in the absence of substrate for various lengths of time before injection of saturating level of AA to quantitate the surviving COX activity. With shorter preincubation periods, this approach focuses on the rate of

progression to the EI' complex, which is only slowly reversible and thus does not contribute to COX catalysis when AA is added. When the preincubation is long enough, this approach provides an indication of the distribution of enzyme between E/EI and EI' at equilibrium. It is important to note that the full inhibitory potency of time-dependent agents, such as nimesulide against COX-2, cannot be reliably assessed from conventional IC<sub>50</sub> measurements because the IC<sub>50</sub> value is determined both by the affinity of the rapid, reversible initial binding ( $E + I \rightleftharpoons EI$ ; Scheme 1) and by the rates of the slower  $EI \rightleftharpoons EI'$  and  $EI \leftarrow EI'$  steps. On the other hand, the residual activity at long preincubation times is a useful measure of the maximal inhibition possible with a given agent, or conversely, what fraction of COX activity escapes inhibition even after equilibration with the agent (roughly mimicking the extended exposures *in vivo*). Thus, the ratio of the residual activities for variant and wild type gives a practical, quantitative index of any changes in COX-2 leakiness toward a given inhibitor.

Nimesulide was chosen for the screening because it has been established as a competitive, time-dependent inhibitor that is selective for PGHS-2,<sup>36</sup> and it is readily available. Range-finding assays with wild-type hPGHS-2 at a variety of nimesulide concentrations indicated that equilibration was reached in a reasonable length of time with 2 μM inhibitor, so this level was used for the comparison with the variants. The results of this comparison are shown in Figure 2. The COX activity of each of the proteins was stable for at least 3 min of preincubation in control reactions without inhibitor. With nimesulide present, wild-type hPGHS-2 and the four variants all progressively lost COX activity with increasing preincubation time until the surviving activity plateaued as equilibrium was reached. The plateau fraction of surviving COX activity in the wild type was 0.17, and essentially the same values were found with the 228H and 587R variants (Figure 2 and Table 3). The surviving COX activity plateaued at a markedly higher level (0.27) for the 511A variant. This is some 60% higher than with wild-type hPGHS-2 and indicates that the structural change at residue 511 in the variant shifts the equilibrium significantly away from the tight EI' complex toward the looser EI complex, weakening the COX inhibition. The plateau level of COX activity in the E488G protein equilibrated with 2 μM nimesulide was 0.22, also significantly above the wild-type value (Figure 2 and Table 3). Thus, formation of the EI' complex was decreased in the 488G variant, though to about half the degree seen in the 511A variant. In all proteins examined, the initial decay in COX activity followed first-order kinetics (as indicated by the early linear drops in the diagnostic semi-log plot in Figure 2), as is expected from the mechanism shown in Scheme 1, but the decline was too rapid at this level of inhibitor for a detailed comparison of *k*<sub>2</sub> values.

The same approach was used to assess the surviving COX activities of the wild-type and 511A hPGHS-2 proteins after equilibration with 2 μM levels of seven other coxibs. CAY-10404, tenidap, NS-398, valdecoxib and diclofenac exhibited time-dependent inhibition of COX-2 in semi-log plots



**Figure 2** Kinetics of COX inhibition in hPGHS-2 wild type (WT) and variants during preincubation with 2 μM nimesulide. Aliquots of each protein (10<sup>-8</sup> M) were preincubated with (filled symbols) or without (open symbols) inhibitor for the indicated lengths of time before the surviving COX activity (i.e., enzyme not in the EI' form) was assayed by injection of 100 μM AA. The values represent averages ± s.d. for triplicate measurements.

**Table 3** Residual COX activities of nimesulide complexes of hPGHS-2 and the four polymorphic variants

hPGHS-2 construct <sup>a</sup>	Residual COX activity (fraction of control) <sup>b</sup>
Wild type <sup>c</sup> (n = 9)	0.17 ± 0.01
R228H (n = 9)	0.18 ± 0.00
E488G (n = 9)	0.22 ± 0.01 <sup>d</sup>
V511A (n = 6)	0.27 ± 0.01 <sup>d</sup>
G587R (n = 9)	0.18 ± 0.01

<sup>a</sup>Variant alleles are underlined.

<sup>b</sup>After equilibration with 2 μM nimesulide, the average fraction (± s.d.) of COX activity surviving in the plateau region was calculated for each protein from the data in Figure 3.

<sup>c</sup>Major allele at all targeted residues.

<sup>d</sup>Significantly different from wild type (P < 0.05).

similar to that in Figure 2, whereas COX-2 inhibition by FR122047 and SC-560 was not time dependent (data not shown). The residual COX-2 activities after equilibration with the inhibitors are presented in Table 4. With FR122047 and SC-560 the 511A variant was inhibited the same or slightly more than wild-type enzyme. Conversely, the residual activity for the V511A variant was 10–70% greater than wild type for all but one of the time-dependent agents tested. The exception to this pattern was diclofenac, which completely inhibited both enzymes.

The second experimental approach to comparing interactions of the hPGHS-2 wild type and the 488G and 511A

**Table 4** Residual COX activities after equilibration of hPGHS-2 and the V511A variant with selected COX inhibitors

Inhibitor	Wild-type <sup>a</sup> residual COX activity (fraction of control) <sup>b</sup>	V511A residual COX activity (fraction of control) <sup>b</sup>	V511A/wild type
FR122047	0.96 ± 0.03 (n = 5)	0.97 ± 0.04 (n = 5)	1.0
CAY-10404 <sup>c</sup>	0.78 ± 0.03 (n = 6)	0.88 ± 0.01 <sup>d</sup> (n = 8)	1.1
Tenidap <sup>c</sup>	0.45 ± 0.01 (n = 5)	0.60 ± 0.01 <sup>d</sup> (n = 5)	1.3
SC-560	0.40 ± 0.01 (n = 12)	0.37 ± 0.01 <sup>d</sup> (n = 10)	0.9
Nimesulide <sup>c</sup>	0.17 ± 0.01 (n = 9)	0.27 ± 0.01 <sup>d</sup> (n = 6)	1.6
NS-398 <sup>c</sup>	0.035 ± 0.003 (n = 6)	0.058 ± 0.002 <sup>d</sup> (n = 6)	1.7
Valdecoxib <sup>c</sup>	0.035 ± 0.003 (n = 5)	0.042 ± 0.001 <sup>e</sup> (n = 5)	1.2
Diclofenac <sup>c</sup>	0 (n = 2)	0 (n = 2)	—

<sup>a</sup>Major allele at all targeted residues.

<sup>b</sup>The values shown represent the averages from reactions with 2 μM inhibitor.

<sup>c</sup>Time-dependent COX inhibition with PGHS-2.

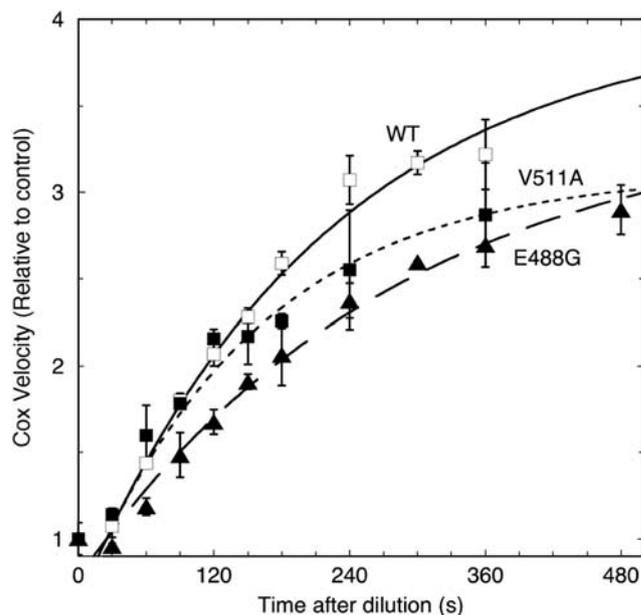
<sup>d</sup>*P* < 0.0001.

<sup>e</sup>*P* < 0.002.

variants with inhibitor involved pre-equilibration of a concentrated solution of enzyme with a small excess of nimesulide to convert the enzyme to a mixture of EI and EI' forms. This mixture was then diluted 1500-fold into buffer without substrate and preincubated for varying lengths of time to allow redistribution of the EI' complex into EI and E forms. Finally, a saturating level of AA was added to assay the increase in EI and E. The results are presented in Figure 3, normalized to the corresponding reference enzyme/inhibitor mixture that was assayed immediately on dilution. After a lag of ~25 s, the wild-type COX activity increased in a first-order manner, with a rate constant of  $4.6 \times 10^{-3} \text{ s}^{-1}$  and a maximal increase of 4.0-fold. The COX activity of the 488G variant increased at  $3.5 \times 10^{-3} \text{ s}^{-1}$ , ~25% slower than wild type, to eventually reach a 3.5-fold increase in activity. The increase in COX activity on dilution was ~40% faster in the V511A variant ( $6.4 \times 10^{-3} \text{ s}^{-1}$ ) than in wild type, reaching a 3.1-fold increase. The clear differences in the rate of recovery of COX activity on dilution of the enzyme-inhibitor mixtures indicate that redistribution from the EI' complex is faster in the 511A variant, and slower in the 488G variant, compared to wild-type PGHS-2. It is worth noting that the magnitude of the COX increase observed on dilution of the enzyme-nimesulide mixtures decreased in the order, wild type > 488G > 511A, as might be expected from the opposite rank order of residual activity observed in the experiments in Figure 2. Overall, the results of the second approach (Figure 3) corroborate the results from the first approach (Figure 2 and Tables 3 and 4), and establish that the 488G and 511A variants alter the interaction of hPGHS-2 with one or more COX-2 selective inhibitors.

## Discussion

The results of this study show that two (488G and 511A) of the four hPGHS-2 variants tested have significant functional



**Figure 3** Kinetics of re-equilibration of tightly bound nimesulide complexes of hPGHS-2 wild type (WT) and the E488G and V511A variants upon 1500-fold dilution. The diluted samples were subsequently incubated for the indicated lengths of time before the COX activity was assayed by injection of 100 μM AA. Activities were normalized to control values obtained by dilution into buffer already containing 100 μM AA. Averages ± s.d. for two separate experiments are presented. Each set of data points was fitted to a single exponential equation with a 25 s lag.

effects on the COX activity with arachidonate, on the enzyme's substrate selectivity and on the interactions with COX-2 selective inhibitors. These functional effects are in the 20–70% range, consistent with the selective pressures expected for an enzyme that figures in several major pathophysiological processes. The *in vivo* impacts of polymorphisms are more

readily evaluated when they result in large changes in enzyme kinetics. Nevertheless, there are a number of cases where physiological or clinical effects are linked to variants whose kinetic parameters after purification are at least 30% of the wild-type  $V_{\max}$  and less than three times the wild-type  $K_m$ . Examples include glucose-6-phosphate dehydrogenase L382P in *Drosophila melanogaster*,<sup>37,38</sup> chicken amylase S,<sup>39,40</sup> human ubiquitin C-terminal hydrolase L1 I93M,<sup>41</sup> several variants of human propionyl-CoA carboxylase,<sup>42</sup> human serine hydroxymethyl transferase S394N,<sup>43</sup> human P450 oxidoreductase A284P and V605F,<sup>44</sup> and human coproporphyrinogen oxidase A814C.<sup>45</sup> Given these precedents and the importance of COX-2 to prostanoid signaling pathways, it would seem worthwhile to evaluate more closely the impact of the V511A and E488G variants on the effectiveness of therapeutic intervention with coxibs or elevated  $n-3$  fatty acid intake.

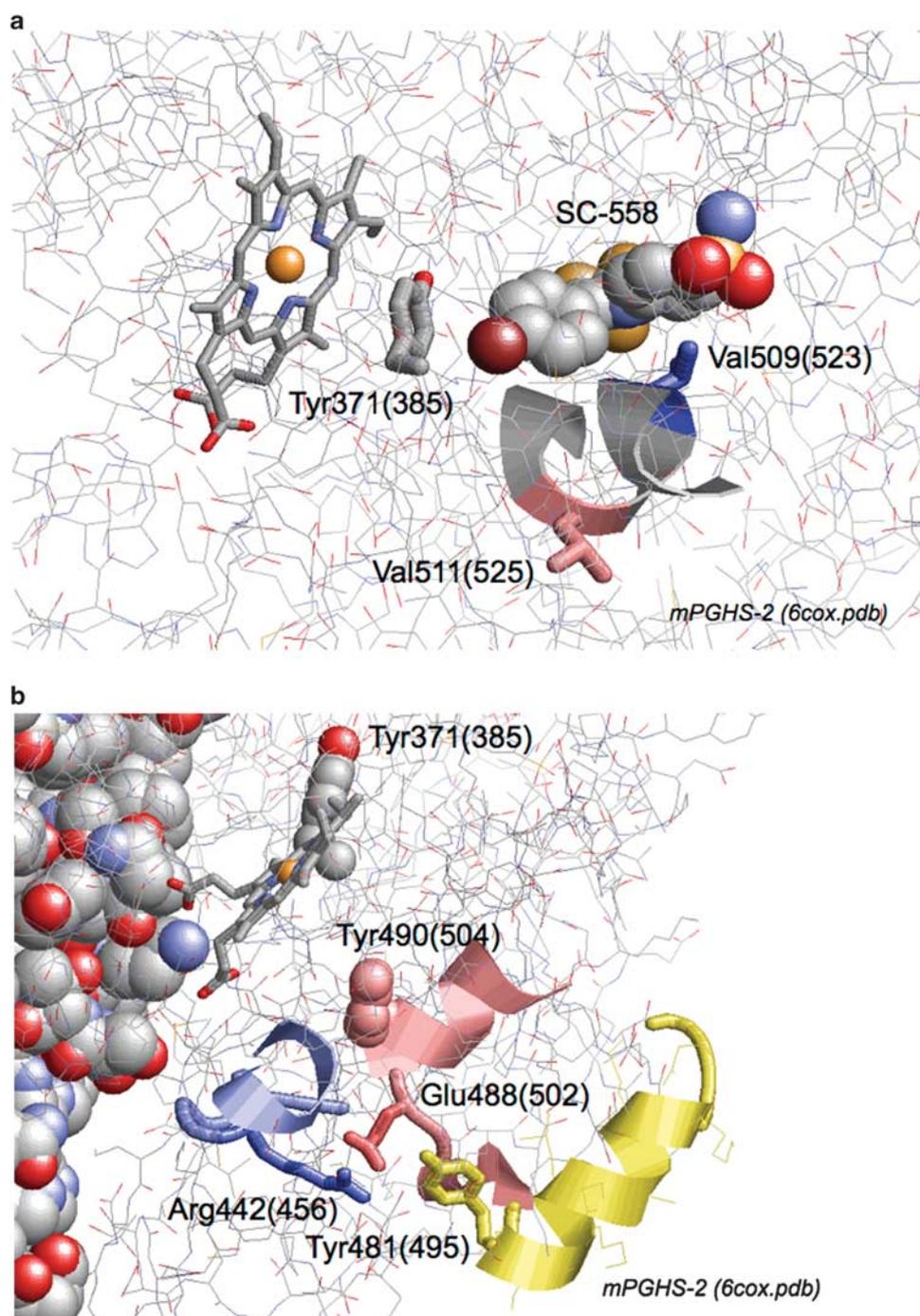
The V511A polymorphism has been previously examined for functional changes and no major differences with wild type in COX kinetic parameters, product profile or inhibition by NSAID were reported.<sup>7,8</sup> Despite this outcome, it was recognized that more enzymological analysis was needed to fully characterize possible functional impacts of the 511A allele.<sup>8</sup> The present studies incorporate several significant methodological improvements for examination of V511A and three other PGHS-2 polymorphisms. These include the use of purified recombinant proteins to avoid host cell lipids that might modify the interactions with hydrophobic substrates and inhibitors, quantitation of the COX/POX ratio as a sensitive index of COX-selective effects, examination of substrate discrimination between the two major prostanoid precursors (AA and EPA) and use of inhibitor assays focused on the time-dependent inhibition that is the major mode of action of selective coxibs.<sup>46</sup> These new approaches revealed some functional effects of the 511A variant that could not be detected in the earlier studies.

As depicted in Figure 4a, the V511A polymorphism is in a segment that forms part of the COX active site.<sup>47</sup> Although the side chain of residue 511 is not positioned to interact with bound fatty acid or inhibitor in crystallographic structures of PGHS-2,<sup>47</sup> a Val $\Rightarrow$ Ala substitution at this position could plausibly change the protein packing and the position of bound substrate with respect to the catalytic radical at Tyr371 (Tyr385 in conventional ovine PGHS-1 numbering), indirectly affecting the COX catalytic rate. AA and EPA have differences in binding interactions in PGHS-1<sup>48</sup> and the situation is likely to be similar in PGHS-2; such differences might well underlie the differential impact of V511A on the COX  $V_{\max}$  values with EPA and AA as substrate (Figure 1a). Most coxibs compete with fatty acid binding at the COX site,<sup>49</sup> so the structural changes just described can be invoked to explain the observed changes in inhibitor interactions in the 511A variant. An important structural determinant for COX-2 time-dependent inhibition has been identified just two residues away at Val509 (Figure 4a).<sup>36,50</sup> This may have some connection to the observation that the V511A/wild-type differences tended to be larger with time-dependent coxibs than with SC-560 and

FR122047, which showed only simple reversible inhibition of COX-2 (Table 4).

Crystallographic data for PGHS-2<sup>47</sup> show that Glu488 (Glu502 in ovine PGHS-1 numbering) is positioned at some distance from the COX active site (Figure 4b). Thus, the perturbation of substrate selectivity and inhibitor interactions observed in the 488G variant is not readily explained by local structural changes. However, the side chain of Glu488 appears to form a salt bridge with Arg442 and to be hydrogen bonded to the side chain of Tyr481. These interactions would be lost in the 488G variant, with the potential for long-range structural changes affecting active site events, such as the EI $\Leftrightarrow$ EI' transition. In addition, Glu488 is just two residues away from Tyr490 (Tyr504 in ovine PGHS-1 numbering; Figure 4b), the site of the other tyrosyl radical in PGHS-2.<sup>34</sup> It will thus be of interest to examine tyrosyl radical dynamics in the 488G variant.

Aspirin and coxibs are potent chemopreventive agents for colorectal adenoma recurrence;<sup>51-55</sup> aspirin has also been shown to prevent colorectal cancer with about a 10-year delay.<sup>56</sup> As PGHS-2 is a major target of NSAIDs and coxibs as well as an important mediator of inflammation, polymorphisms in *PTGS2* have been extensively examined for their association with colorectal adenoma or cancer risk.<sup>8,9,13,57-71</sup> Although several studies have shown associations with noncoding *PTGS2* polymorphisms, few studies have evaluated potential SNP-NSAIDs interactions.<sup>57,59,60,63,67,70,71</sup> The V511A polymorphism of *PTGS2* has significant prevalence only in the African-American population (thus far), and the E488G polymorphism appears to be very rare.<sup>7,8</sup> The polymorphisms studied here exist with allele frequencies between 0.8 and 5% in the African-American and Asian populations. In prior studies, the populations carrying a variant allele were up to 5%, suggesting that a significant proportion of African Americans can experience different NSAID pharmacokinetics. Differing effects among men and women were not observed in one study,<sup>61</sup> but should be evaluated further. Nevertheless, these polymorphisms could have important pharmacogenetic implications, given the role of NSAIDs in colorectal adenoma prevention. Coxibs (PGHS-2-specific inhibitors) are potent chemopreventive agents for colorectal adenoma recurrence, but their use is associated with increased risk of myocardial infarction and other cardiovascular events.<sup>72</sup> Evaluations of these polymorphisms in relation to coxibs may provide information on who is most likely to benefit from coxib use and who may be at risk of adverse events.<sup>6</sup> Given that the incidence of colorectal cancer is elevated in African Americans compared to Caucasians<sup>73</sup> and that Japan has one of the highest colorectal cancer incidences in the world,<sup>74</sup> further study is required to determine whether nonsynonymous polymorphisms in *PTGS2* can influence the chemopreventive efficacy of NSAIDs in populations that are likely to carry these polymorphisms. This information may be very useful for the tailoring of therapy in these populations so as to minimize the serious adverse events associated with NSAID use.<sup>6</sup>



**Figure 4** Crystallographic structure of murine PGHS-2 (6cox.pdb), with views focused on Val511 (a) and Glu488 (b). Residue numbers are for human PGHS-2, with ovine PGHS-1 equivalents in parentheses. Other highlights include heme, bound inhibitor (SC-558), the tyrosyl radical sites at Tyr371(385) and Tyr490(504), the coxib specificity determinant at Val509(523), and the Arg442(456) and Tyr481(495) residues that interact with Glu488.

The 511A variant has a lower relative  $V_{max}$  for EPA than does the wild type (Figure 1a), indicating that this variant discriminates more strongly against the  $n-3$  substrate relative to the  $n-6$  substrate. Increased dietary intake of  $n-3$  fatty acids such as EPA has been found to bring health benefits.<sup>75</sup> One proposed mechanism is the lower COX

activity for EPA compared to AA seen with PGHS-1 and to a lesser extent with PGHS-2.<sup>1</sup> Accordingly, individuals carrying the 511A variant of *PTGS2* might be expected to benefit more from dietary intervention designed to increase the  $n-3/n-6$  ratio in tissue lipids than would individuals with the wild-type allele. A recent study<sup>12</sup> reported no

association between the V511A polymorphism and risk of chronic heart disease or ischemic stroke, but dietary fatty acid intake was not examined as an independent variable.

In summary, our biochemical studies show that two genetic variants in PGHS-2 can affect enzyme activity, substrate selectivity and inhibition with coxibs. Further studies are needed to evaluate whether these functional differences affect the efficacy of dietary and pharmacological interventions, particularly in African Americans, the population in which these two variants have been observed.

### Conflict of interest

The authors declare no conflict of interest.

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