Cyclooxygenase (COX) is an enzyme that is responsible for formation of important biological mediators called prostanoids, including prostaglandins, prostacyclin and thromboxane. Three COX isoenzymes are known: COX-1, COX-2, and COX-3 which is a splice variant of COX-1.1 COX-1 is considered a constitutive enzyme, being found in most mammalian cells. On the other hand, COX-2, undetectable in normal tissues, and induced during inflammation, hypoxia and Wnt-signalling, is present in many cancers.2 Classical non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin block both COX-1 and COX-2. When the COX-1 is inhibited, inflammation is reduced, but the protection of the lining of the stomach is also lost. This can cause stomach upset as well as ulceration and bleeding from the stomach and even the intestines.3 Therefore, selective COX-2 inhibitors such as celecoxib and rofecoxib had been developed and prescribed.4 As COX-2 is usually specific to inflamed tissue, there is much less gastric irritation associated with COX-2 inhibition together with the decreased risk of peptic ulceration.5 However, coxib drugs such as rofecoxib (Vioxx®) and valdecoxib (Bextra®) were withdrawn from the market in 2004 and 2005, respectively, because they excessively increased the risk of heart attacks and strokes with long term use.6 Celecoxib (Celebrex®) is the only COX-2 inhibitor available in the United States. On the other hand, some studies have suggested that rofecoxib’s adverse cardiac events may not be a class effect but rather an intrinsic chemical property related to its metabolism.7 For this reason, novel scaffolds with selective COX-2 inhibitory activity needs to be found and evaluated for their anti-inflammatory effects. Recently, we reported that 1H-pyrrole-2,5-dione derivative 2l from our compound library showed an inhibitory activity, with IC_{50} value of 0.61 μM against LPS-induced PGE_2 production in RAW 264.7 macrophages (Fig. 1).7 In this study, compound 2l was further biologically evaluated in the hope that this may be further explored as selective COX-2 inhibitor and non-adverse anti-inflammatory lead compound.

The synthetic procedures and reaction conditions for 3-(4-sulfamoylphenyl)-4-phenyl-1H-pyrrole-2,5-dione 2l are shown in Scheme 1: 4-Chlorosulfonylphenylacetic acid (4) was prepared from the reaction of phenylacetic acid (3) with ClSO_2H, and then treated with SOCl_2 and subsequent NH_3 gas in CH_3CN to provide 4-chlorosulfonylphenylacetamide (5), which was condensed with ethyl benzoylformate (2) using NaH condition to yield 3-(4-sulfamoylphenyl)-4-phenyl-1H-pyrrole-2,5-dione (2l).8 Compound 2l was further evaluated for an inhibitory activity against both peptidoglycan- and poly(I:C)-induced PGE_2 productions in RAW 264.7 macrophages together with NS-398 as positive control as described previously.9 This compound generally showed the inhibitory trend similar to those of LPS-related assay on both dose- and time-dependent responses, respectively. It exhibited particularly equal or superior activity to ibuprofen and meloxicam at high concentrations (> 5 μM) against three TLR ligand-induced assay systems (Supplementary materials: Fig. 1). The IC_{50} values of ibuprofen and meloxicam were determined to be 0.86 and 0.09 μM, respectively, compared to 0.61 μM of 2l under our assay system (Table 1). The study of the ability of compound 2l to inhibit ovine COX-1 and human recombinant COX-2 activity were carried out using an enzyme immunoassay (EIA) kit (kit catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA).10 The efficacy of compound 2l was determined as the concentration causing 50% enzyme
inhibition (IC\textsubscript{50}) (Table 1). The selectivity index (SI values) was calculated as IC\textsubscript{50} (COX-1)/IC\textsubscript{50} (COX-2), and compared with those of ibuprofen and meloxicam. As a result, compound 21 exhibited time-dependent inhibition at 1 μM concentration but time-independent inhibition of human recombinant COX-2 at higher concentrations (10 and 50 μM) (Supplementary materials: Fig. 2). In our assay system, the IC\textsubscript{50} values of 21 on COX-1 and COX-2 were determined to be 126.78 and 9.10 μM, respectively. Thus, the selectivity index (SI) for 21 was found to be 13.93, indicating that 21 is slightly potent and selective COX-2 inhibitor compared to 1.15 of ibuprofen, 236.06 of meloxicam, respectively and it formed six hydrogen bonds with His75 (3.48 Å, C=O), Ser516 (3.13 Å, -NH\textsubscript{2}), Ser339 (3.13 Å, -NH\textsubscript{2}), Arg499 (3.29 Å, SO\textsubscript{2}), Phe504 (3.57 Å, SO\textsubscript{2}) and Phe504 (3.19 Å, -NH\textsubscript{2}) respectively.

The COX-1 and COX-2 inhibitory activity of 21 prompted us to perform molecular docking studies to understand the ligand-protein interactions, and COX-1/COX-2 selectivity in detail. The docking studies were carried out using the crystal structures of COX-1 (1EQH)\textsuperscript{14} and COX-2 (3LN1)\textsuperscript{15} complexed with flurbiprofen and celecoxib, respectively. The active site of the enzyme was defined to include residues within a 10.0 Å radius to each inhibitor atoms. The docking wizard of MVD2010.4.2 was used to dock all compounds in Table 1 on the active sites of COX-1 and COX-2 enzymes. The most stable docking model was selected according to the best Rerank score conformation predicted by the MVD scoring function (Table 2) for each crystal structure. The compound 21 was found to dock into the active site of COX-2 with higher Rerank score of −133.03 compared to −67.93 score for COX-1 (Supplementary data: Fig. 3). It formed five hydrogen bonds with Ser516 (3.13 Å, C=O), Ser339 (3.13 Å, -NH\textsubscript{2}), Gln178 (3.13 Å, -NH\textsubscript{2}), Arg499 (3.29 Å, SO\textsubscript{2}) and Phe504 (3.57 Å, SO\textsubscript{2}) into the active site of COX-2 (Fig. 2(a)); Hydrogen bonds are shown in blue). On the other hand, docked-celecoxib showed the Rerank score of −22.72 and −135.08 into the active site of COX-1 and COX-2, respectively and it formed six hydrogen bonds with His75 (3.48 Å, -NH\textsubscript{2}), Ser339 (3.15 Å, -NH\textsubscript{2}), Gln178 (3.13 Å, -NH\textsubscript{2}), Arg499 (3.50 Å, SO\textsubscript{2}), Ile503 (3.55 Å, SO\textsubscript{2}) and Phe504 (3.19 Å, SO\textsubscript{2}) into the active site of COX-2 [Fig. 2(b)]. In addition, its docked-configuration was nearly overlapped with that of celecoxib (red color) in COX-2 complex in PDB 3LN1. These Rerank score differences between COX-1 and COX-2 structures appropriately support the experimental selectivity indexes in Table 1 with exceptions of meloxicam. On the other hand, the comparison of docking conformation of 21 with celecoxib, co-crystallized with COX-2, illustrated in Figure 2(b), shows that compound 21 can bind into the active site of COX-2 enzyme in almost the same fashion as celecoxib with exceptions of two van der Waals interactions (blue circles) of celecoxib, which can provide rationale for higher potency and selectivity of celecoxib against COX-2.

Finally, compound 21 was investigated for its potential effect on both LPS-induced nitric oxide (NO) production and iNOS expression in RAW 264.7 cells. In addition, effects of compound 21 on LPS-induced production of TNF-α and mRNA expression of TNF-α, IL-1β, and IL-6 in RAW 264.7 cells was also investigated. Compound 21 showed little effect on these biological events (Supplementary materials: Fig. 4 and 5). These overall results mean that

\begin{table}[h!]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Compounds & \textbf{IC}\textsubscript{50} (µM) & \textbf{Selectivity index (SI)} & \textbf{Rerank Score} \\
\hline
\textbf{COX-1} & \textbf{COX-2} & \textbf{COX-1/COX-2: IC}\textsubscript{50} & \\
\hline
Ibuprofen & 0.86 & 26.21 & 30.22 & 1.15 \\
Meloxicam & 0.09 & 73.18 & 0.31 & 236.06 \\
\hline
\end{tabular}
\caption{Docking results of compound 21, ibuprofen, meloxicam and celecoxib into the active sites of COX-1 and COX-2}
\end{table}

\begin{table}[h!]
\centering
\begin{tabular}{|c|c|c|}
\hline
Compounds & \textbf{No. H-bond} & \textbf{Rerank Score} \\
\hline
\textbf{COX-1} & \textbf{COX-2} & \\
\hline
Ibuprofen & 2 & -67.93 \\
Meloxicam & 4 & -69.02 \\
Celecoxib\textsuperscript{a} & 3 & -22.72 \\
\hline
\end{tabular}
\caption{Docking results of compound 21, ibuprofen, meloxicam and celecoxib into the active sites of COX-1 and COX-2}
\end{table}
compound 2l prohibited the PGE₂ production in RAW 264.7 cells by inhibiting only the COX-2 enzyme.

In conclusion, 1H-pyrrole-2,5-dione 2l were synthesized and screened for COX-1/COX-2 inhibition. The biological results showed that 2l showed moderate inhibitory and selective profiles against COX-2, which is consistent with the molecular docking result of 2l inside the COX-2 active site. Thus, the molecular docking results suggest us that 1H-pyrrole-2,5-dione derivatives with appropriate substitutions which can fill the adjunct pocket and interact with the other residues may be useful to propose new molecules with enhanced selectivity towards COX-2. The overall findings of this study inferred that moderate inhibition of compound 2l rendered it as a hit molecule for further development of more potent and selective COX-2 inhibitor. We are in progress of synthesizing a number of new derivatives based on this molecular docking result.

Experimental Section

In vitro Cyclooxygenase (COX) Inhibition Assay. The ovine COX-1 and human recombinant COX-2 activity directly measures PGF₂α produced by SnCl₂ reduction of COX-derived PGH₂. The prostanoïd product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds using COX Inhibitor Screening Assay (Cayman Chemical, Ann Arbor, MI, USA). Briefly, control value was obtained in the absence of compound. COX enzyme was mixed with different concentration of each tested compound and heme, and incubated for 10 min at 37 °C. The reaction was initiated by adding arachidonic acid and all tubes were incubated for another 2 min at 37 °C. NS-398 (100 nM) were used as a positive control COX-2 inhibitor. The efficacy of compound was determined as the concentration causing 50% enzyme inhibition (IC₅₀). The selectivity index (SI values) was calculated as IC₅₀(COX-1)/IC₅₀(COX-2).

Docking Methodology. Docking studies have been performed using MVD 2010.4.2. With this purpose, crystal structures of COX-1/flurbiprofen and COX-2/celecoxib complex (PDB codes: 1EQH and 3LN1) were obtained from the Protein Data Bank in order to prepare the protein for docking studies. Docking procedure was followed using the standard protocol implemented in MVD 2010.4.2 and the geometry of resulting complexes was studied using the MVD's Pose Viewer utility.

Acknowledgments. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0027401).

References and Notes


16. MVD 2010.4.2 for Windows in Molegro ApS.
