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Binding of Ibuprofen, Ketorolac, and Diclofenac to COX-1 and COX-2 Studied by Saturation Transfer Difference NMR

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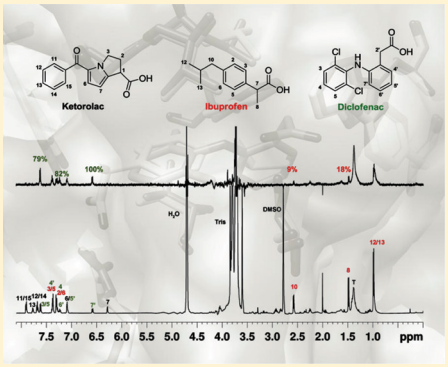
Supporting Information

ABSTRACT: Saturation transfer difference NMR (STD-NMR) spectroscopy has emerged as a powerful screening tool and a straightforward way to study the binding epitopes of active compounds in early stage lead discovery in pharmaceutical research. Here we report the application of STD-NMR to characterize the binding of the anti-inflammatory drugs ibuprofen, diclofenac, and ketorolac to COX-1 and COX-2. Using well-studied COX inhibitors and by comparing STD signals with crystallographic structures, we show that there is a relation between the orientations of ibuprofen and diclofenac in the COX-2 active site and the relative STD responses detected in the NMR experiments. On the basis of this analysis, we propose that ketorolac should bind to the COX-2 active site in an orientation similar to that of diclofenac. We also show that the combination of STD-NMR with competition experiments constitutes a valuable tool to address the recently proposed behavior of COX-2 as functional heterodimers and complements enzyme activity studies in the effort to rationalize COX inhibition mechanisms.



1. INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed therapeutics for the treatment of arthritis, pain, and inflammation. The evidence that the mechanism of action of NSAIDs is the inhibition of prostaglandin synthesis was the key to understanding both the therapeutic effect and the gastrointestinal toxicity of these drugs. This inhibition occurs via blockage of prostaglandin endoperoxide-H synthase-1 and -2 (PGHSs) also known as cyclooxygenases (COX),[1−3] by sterically hindering the entrance of the physiological substrate − arachidonic acid (AA). The discovery of two COX isoforms[4−7] associated with different functions (COX-2, inflammation; COX-1, homeostatic functions such as maintaining normal gastric mucosa) demonstrated that the anti-inflammatory and analgesic properties of traditional NSAIDs, such as aspirin, ibuprofen, or flurbiprofen (Figure 1), are due to COX-2 inhibition, whereas the ulcerogenic side effects are associated with inhibition of COX-1. To overcome the side effects associated with COX-1 inhibition, selective COX-2 inhibitors have been developed such as celecoxib and rofecoxib (diarylheterocycle compounds known as "coxibs"). However, the long-term use of both traditional NSAIDs and coxibs has been reported to cause significant cardiovascular effects, myocardial infarctions, and strokes. Indeed the removal of rofecoxib from the market because of its cardiac toxicity limited the usefulness of these otherwise efficacious drugs.[8]

The search for COX selective inhibitors is complicated by the close structural similarities between COX-1 and COX-2. COXs are homodimers of 70 kDa subunits composed of a membrane-binding domain and two catalytic domains. These domains catalyze two different reactions: a cyclooxygenase (COX catalytic domain) reaction and a peroxidase (POX catalytic domain) reaction. COX catalytic domains have very similar active sites that differ in the presence of a side pocket that is augmented in COX-2 when compared to COX-1. This overall difference in the active site shape and size is due to the presence of a valine (Val-523) in COX-2 instead of an isoleucine (Ile-523) in COX-1.[9−11] This variation enables a tighter binding of the sulfonamide or sulfone group of the coxibs (celecoxib or rofecoxib, respectively) in the side pocket of COX-2.[12]

X-ray crystallography, kinetic studies, and molecular modeling are among the most explored tools to understand both the selectivity and the binding mode of nonselective and selective inhibitors. Therefore, these approaches constitute the primary source of information toward the rational drug design of novel selective COX-2 inhibitors.[9,13]

A number of kinetic studies support a general model for COX inhibition where multiple equilibriums between free enzyme, inhibitor, and two or three enzyme−inhibitor complexes are considered.[9] There are examples of reversible competitive inhibition characterized by a single-step mechanism, usually associated with the kinetic profile of noncovalent inhibitors, as well as more complex time dependent irreversible inhibition mechanisms involving multiple steps and a time dependent change in the enzyme/inhibitor complex.[9] Most COX-2 selective inhibitors initially bind in a competitive,

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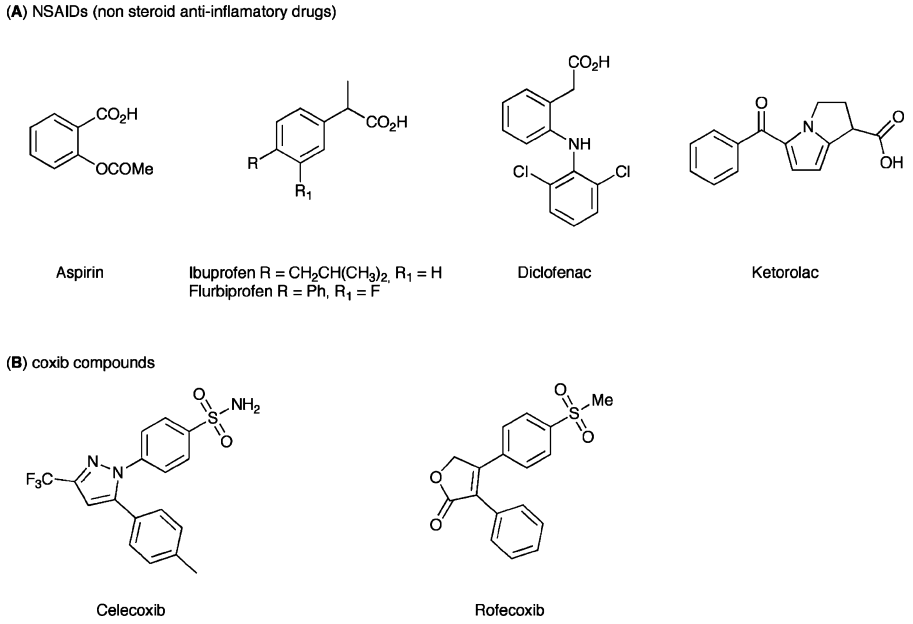

Figure 1. Nonselective (A) and selective (B) COX-2 inhibitors.

reversible binding followed by a time-dependent intermediate to a tightly bound complex (enzyme−inhibitor).

Recent studies point to the fact that only one subunit of the COX homodimer is active at a time and exhibits activity with AA and that many NSAIDs bind to a single subunit of a COX dimer to inhibit the activity of the entire dimer.[14] There is evidence that the COX active site that has a catalytic function is modulated by the nature of the ligand occupying the COX site of the partner monomer.[15] These studies show that COX functions as allosteric/catalytic couples, having a catalytic monomer (E_{cat}) and an allosteric monomer (E_{allo}). Therefore, they behave as conformational heterodimers during catalysis and inhibition, with E_{cat} being regulated by E_{allo}. Then COX inhibition depends on whether the inhibitor binds E_{allo} or E_{cat} or both. This mechanism of inhibition has been used to explain the action of widely used NSAIDs.[10] It was proposed that time-independent competitive COX inhibitors, like ibuprofen, bind to both COX sites, with inhibition of AA oxygenation only being affected after binding to the second site. Dong et al.[15] speculate also that the fact that the binding of one molecule of diclofenac[16] or indomethacin[17] per COX-2 is responsible for the complete inhibition of human COX may be due to the fact that diclofenac and indomethacin bind to E_{cat}.

In spite of the kinetic data that support the various inhibition models, the extensive data on the inhibitory effects of different classes of compounds in COX activity, and the crystallographic analysis of selective COX inhibitors, there is still a lack of structural information to correlate with the various models and studies.

Nuclear magnetic resonance spectroscopy is a unique tool to study molecular interactions in solution and to obtain information about the interactions of small ligands with biologically relevant macromolecules. However, to the best of our knowledge, NMR spectroscopy has not yet been reported as a methodology to examine COXs inhibition. Thus, this work represents the first report of the use of NMR to investigate inhibition of COX enzymes by NSAIDs.

In this manuscript we report the application of the STD-NMR experiment to screen for ligand−COX complexes and to characterize the binding epitope of widely used NSAIDs. STD-NMR is based on the nuclear Overhauser effect and on the observation of the ligand resonance signals,[18] and its ability to detect binding of low molecular weight compounds to large biomolecules is well documented.[18−20] Our aim was to test the applicability of the STD method as a tool to study NSAID binding to COXs using well-studied COX inhibitors.

To study the applicability of the STD-NMR technique to detect binding with COX-1 and COX-2 and to optimize the methodology, we have used ibuprofen. This is a nonselective COX inhibitor that displays a competitive and rapidly reversible inhibition of the COX activity, characterized by a single-step mechanism,[9,21] with inhibition constant K_I = 9 μM for COX-1 and COX-2.[22] The binding of COX-2 with inhibitors possessing different affinities and different proposed inhibition mechanisms, diclofenac and ketorolac, was also investigated in simple binding experiments and in competition binding experiments with ibuprofen.[23]

2. EXPERIMENTAL SECTION

2.1. Materials. COX-1 from ram seminal vesicles and COX-2 from sheep placenta were purchased from Cayman Chemical (Ann Arbor, MI, U.S.). The proteins are supplied in 80 mM Tris-HCl, pH 8.0, 0.1% Tween 20, and 300 μM diethyldithiocarbamate (DDC) and were used as such. Ibuprofen and (±)-ketorolac thrometamin were purchased from Sigma, and diclofenac was purchased from Merck and used as such.

For the STD-NMR experiments, ibuprofen, ketorolac, and diclofenac stock solutions (20 mM) were prepared in DMSO-d_6. From these, an amount of 15 μL was added to the COX solution directly in the NMR tube. Then 80 mM Tris-HCl buffer at pH 8.0 was used to adjust the volume to 200 μL. Final concentrations of COX and ibuprofen were 3 and 300 μM, respectively. For the competition binding experiments, the ratio of inhibitors/protein was kept to 100:1 and the solutions were prepared as above.

2.2. NMR Spectroscopy. All STD-NMR experiments were acquired at 37 °C in a Bruker Avance III spectrometer operating at 600 MHz, with a 5 mm triple resonance cryogenic probe head. The STD-NMR spectra were acquired with 1024 transients in a matrix with 32K data points in t_2 in a spectral window of 12019.23 Hz centered at 2814.60 Hz. Excitation sculpting with gradients was employed to

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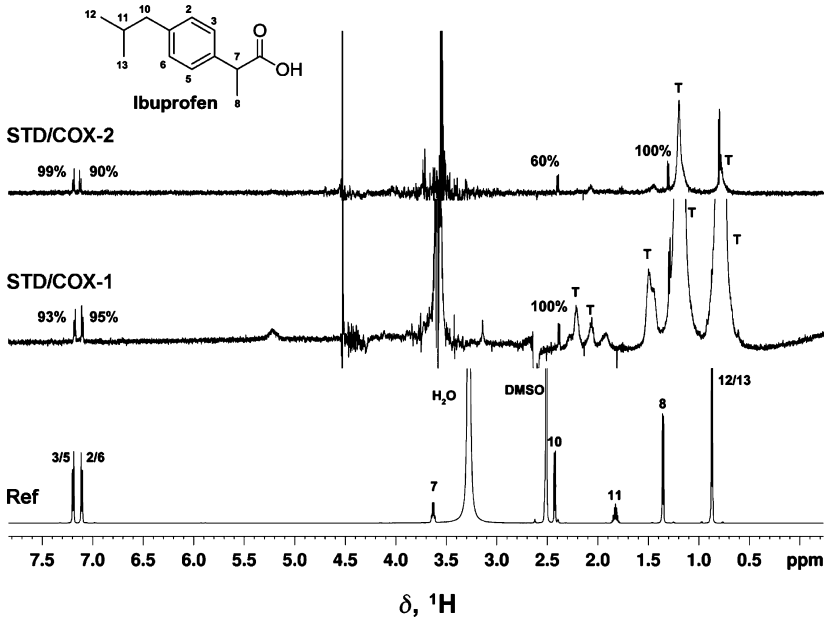

Figure 2. ^{1}H STD-NMR spectra of ibuprofen in the presence of COX-1 and COX-2, at 600 MHz and 37 °C.

suppress the water proton signals. A spin lock filter ($T_{1\rho}$) with a 2 kHz field and a length of 20 ms was applied to suppress protein background. Selective saturation of protein resonances (on resonance spectrum) was performed by irradiating at −300 Hz using a series of 40 Eburp2.1000 shaped 90° pulses (50 ms, 1 ms delay between pulses) for a total saturation time of 2.0 s. For the reference spectrum (off resonance) the samples were irradiated at 20 000 Hz. Proper control experiments were performed with the reference samples in order to optimize the frequency for protein saturation (−0.5 ppm) and off-resonance irradiation, to ensure that the ligand signals were not affected.

The STD effect was calculated using $(I_0 - I_{STD})/I_0$, in which $(I_0 - I_{STD})$ is the peak intensity in the STD spectrum and I_0 is the peak intensity in the off-resonance spectrum. The STD intensity of the largest STD effect was set to 100% as a reference, and the relative intensities were determined.[20,24]

3. RESULTS

3.1. STD-NMR of COX-1 and COX-2 with Ibuprofen.

Ibuprofen was chosen to test the applicability of STD-NMR to detect ligand-COX binding. Previous to the STD-NMR study, ^{1}H NMR spectra of COX-1 and COX-2 were acquired using the commercial enzyme solutions at 37 °C. The spectra are dominated by a strong signal at 3.8 ppm from the methylene protons of the Tris buffer. Other signals originating from DDC and Tween 20 can also be observed in the region between 0.5 and 3.5 ppm (Supporting Information). As the strong signals from the buffer and the additives (Tween and DDC) are likely to overlap with the ligand resonance peaks, these conditions are not ideal for NMR. However, attempts to resuspend the protein in different buffers resulted in precipitation and the studies were conducted with the proteins as supplied.

In Figure 2 the STD-NMR spectra obtained for the mixtures of COX-1/ibuprofen and COX-2/ibuprofen are presented (see Supporting Information for additional spectra, Figures S1 and S2).

Since the STD-NMR spectrum is obtained by subtracting a spectrum in which the protein is saturated from one without protein saturation, the difference spectra presented in Figure 2 contain only signals of the protons that received saturation from the protein. Proper control experiments were performed with the reference samples in order to optimize the frequency for protein saturation (−0.5 ppm) and off-resonance irradiation, to ensure that the ligand signals were not affected. The aromatic resonances of ibuprofen, clearly visible in the two STD spectra, are a clear indication that the drug binds reversibly to both COX isoforms. In the spectral region between 1 and 3 ppm some protons of the Tween 20 also appear in the difference spectra, showing that the surfactant also interacts with the proteins. This is not unexpected, since the surfactant is added to help stabilize and solubilize the protein and some degree of association and interaction between the enzymes and the surfactant is expected to occur.

The signals originating from the Tween 20 partially mask the ibuprofen proton resonances in the aliphatic region, since they overlap with the alkyl region of ibuprofen. However, in spite of this overlap, STD signals from the methylene protons (H10) and from the methyl group (H8) close to the carboxylic acid moiety are still detectable, reflecting a close contact of the entire molecule with both enzyme isoforms. In the STD spectrum with COX-2, we are even able to detect the chemical shifts of protons from the methyl groups of the isobutyl group (H12/13) that appear around 0.9 ppm.

Overall, the STD-NMR spectra obtained for COX-1 and COX-2 are very similar. Because of the spectral overlap with the Tween 20 resonances, complete epitope mapping of the ibuprofen resonances is not possible. Nevertheless, it is still possible to integrate the resonances of the resolved proton signals and to determine relative STD effects for H2/6, H3/5, and H10 for COX-1 and H2/6, H3/5, H8, and H10 for COX-2 (Figure 2). For both isoforms high STD percentages are found for the aromatic protons. Significant differences are observed for H10, 100% in COX-1 and 60% in COX-2, especially when compared with the aromatic protons that are not that significantly different for both isoforms. Also the difference in STD intensity between protons H2/6 and H3/5 is more significant in COX-2 (99% vs 90%) than in COX-1 (93% vs 95%). This might reflect a distinctive contact due to the

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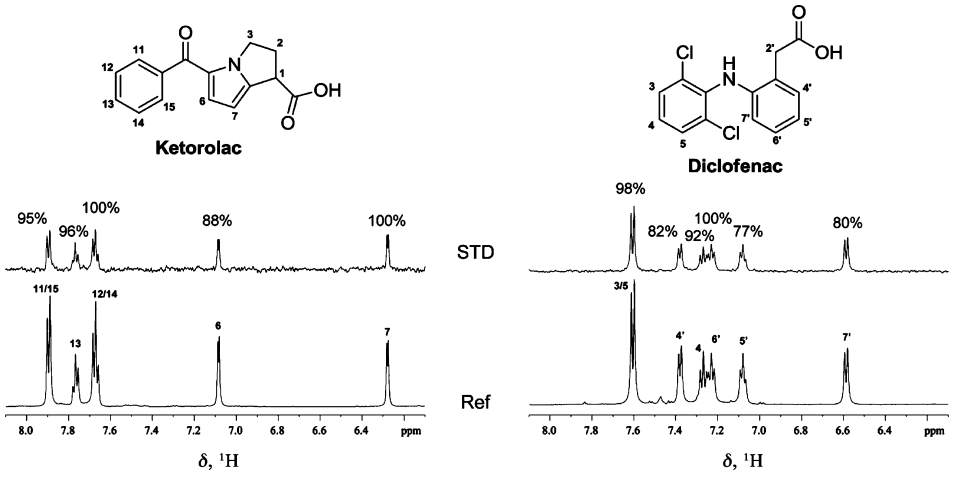

Figure 3. Expansion of the aromatic region of the ^{1}H STD-NMR spectra of ketorolac (left) and diclofenac (right) in the presence of COX-2, at 600 MHz and 37 °C with the corresponding reference spectra.

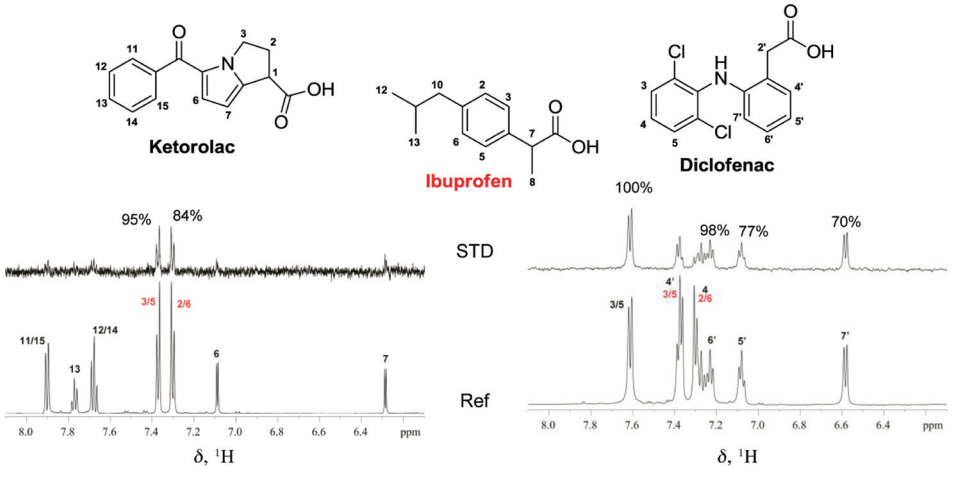

Figure 4. Expansion of the aromatic region of the ^{1}H STD-NMR spectra of ibuprofen/ketorolac (left) and ibuprofen/diclofenac (right) in the presence of COX-2, at 600 MHz and 37 °C with the corresponding reference spectra.

differences in the relative shape and size of the binding pocket of both isoforms.

3.2. STD-NMR Studies with COX-2. To investigate the relationship of inhibitory activity to binding to COX-2, the binding epitopes of ketorolac and diclofenac were examined both alone and in the presence of ibuprofen, in competition STD-NMR experiments.

Prior to the competition experiments, the drugs were examined individually for binding to COX-2, and STD-NMR spectra were obtained for ketorolac and diclofenac (Figure 3). Strong STD responses were obtained for both compounds, indicating reversible binding to the enzyme, just like for ibuprofen. All the aromatic protons of ketorolac and diclofenac received high degrees of saturation, revealing that in both cases there is a close contact with the protein and that almost the entire surface of the molecules interacts with the enzyme. Most interesting is the fact that in both compounds no STD could be detected with the aliphatic protons close to the carboxylic acid moiety, H2′ in diclofenac and H1, H2, and H3 in ketorolac (see full spectra in Supporting Information, Figure S3 and Figure S4). Relative STD intensities were calculated for all the protons that received saturation transfer and are above 90% with the exception of protons H4′, H5′, and H7′ in diclofenac that show STD intensities close to 80%.

According to reported IC_{50} values, diclofenac (IC_{50} of 60–220 nM)[22] is a more potent inhibitor of COX-2 than ibuprofen (IC_{50} = 1.53 μM).[25] For ketorolac substantially different IC_{50} values are reported depending on the experimental conditions of the activity measurements; values as low as 0.12 μM [26] have been reported if the COX-2 activity is measured after a period of preincubation of the enzyme with the NSAID while higher values such as 60.5 μM [27] were determined for the instantaneous inhibition. This time-dependent inhibition is well-known for some NSAIDs.[28]

STD-NMR experiments were performed using ibuprofen as the STD spy molecule in the competition experiments. Ibuprofen and diclofenac or ketorolac were used in equimolar amounts, and the ligand/protein ratio was 100:1 for both ligands. Since the STD-NMR experiment by itself does not allow discrimination of the binding site, in order to determine if the drugs compete for the same binding pocket, the experiments were performed twice with reverse order of ligands addition. In the first round of experiments ibuprofen was added previously, followed by the addition of ketorolac or diclofenac and acquisition of the STD spectra of the mixture. In the second set of experiments ketorolac or diclofenac was added previous to the ibuprofen, and STD-NMR spectra were recorded after the addition of each compound. The results of

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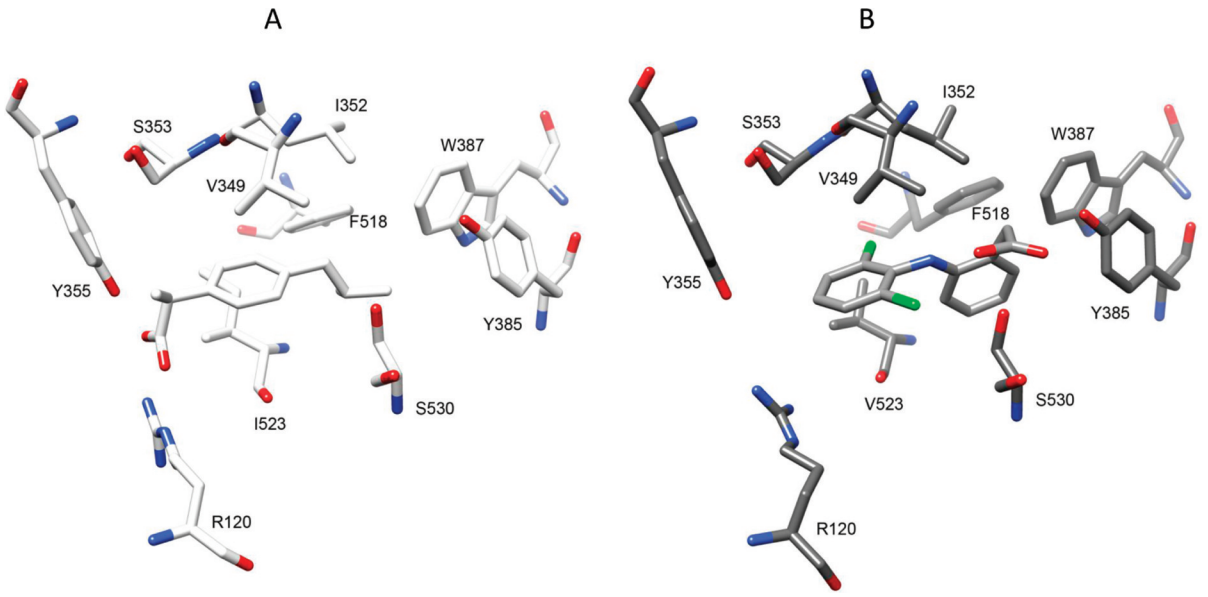

Figure 5. (A) Ibuprofen bound in the active site of ovine COX-1 with the carboxylate coordinated to Arg-120 and Tyr-355.[29] (B) Diclofenac in the active site of murine COX-2 with the carboxylate coordinated to Tyr-385 and Ser-530.[30]

both experiments were identical. Figure 4 illustrates the STD response obtained in the experiments with ibuprofen/ketorolac-COX-2 and ibuprofen/diclofenac-COX-2.

In the STD competition experiment with ibuprofen and ketorolac only residual STD signals, slightly above the noise level, are detected for ketorolac in the presence of ibuprofen (Figure 4, left). The result is independent of the order of addition of the compounds, indicating that ibuprofen and ketorolac compete for the same binding site and reflect the fact that ibuprofen has a higher association constant than ketorolac and binds preferably to COX-2. This result is in accordance with the reported differences in IC_{50} if we consider the value reported for ketorolac for the instantaneous inhibition assay.[27]

With the exception of proton H10 of ibuprofen, which seems to receive relatively less saturation, the binding epitope of ibuprofen in the presence of ketorolac is very similar to the one obtained for ibuprofen alone (compare Figure 4 with Figure 2). A lower saturation is transferred for the aromatic protons H2/6 when compared to H3/5, and the maximum STD is found for proton H8 (see Supporting Information, Figure S5 and Figure S6).

In the competition experiment with diclofenac and ibuprofen the result is quite different. It was expected that diclofenac would suppress almost completely the STD response from ibuprofen because of its lower IC_{50}. What can be observed in Figure 4 (right) is a relatively lower response from ibuprofen when compared to diclofenac, but still a considerable STD effect is detected. Once more the results are independent of the order of addition. Unfortunately, because of spectral overlap in the aromatic region, it is difficult to separate the STD responses from diclofenac and ibuprofen in order to compare with the individual experiments. However, the well resolved H10 (29% relative STD) signal from ibuprofen (see full spectra in Supporting Information) allows one to approximately quantify the ibuprofen STD response as $^1/_2$ of that of diclofenac. The binding epitope of diclofenac does not seem to be affected by the competition with ibuprofen, and similar relative values are obtained for the resolved resonances when compared with diclofenac alone (100% vs 98% for H3/5, 78% vs 77% for H5′, and 70% vs 80% for H7′).

4. DISCUSSION

The STD technique offers several advantages over other methods to detect binding activity. First, inhibitor association/dissociation is detected independently of monitoring enzyme activity and the binding component can usually be directly identified, even from a mixture (see Figure 4). Second, the region of the ligand having the strongest contact to the protein shows the most intense NMR signals, enabling epitope mapping of the ligand interactions. And third, very important for a NMR-based detection system, its high sensitivity allows using as little as 1 nmol of protein with a molecular weight of >10 kDa.[18]

One of the requirements for a successful STD-NMR experiment is the occurrence of exchange between free and bound inhibitor, allowing the use of this technique for screening ligands with dissociation constants K_D ranging from about 10^{-8} to 10^{-3} M, i.e., reversible binding and a fast exchange on the NMR time scale.

The STD results obtained for ibuprofen and COX-1 and COX-2 are consistent with the competitive and rapidly reversible mechanism accepted for COX inhibition.[9] Since the experiments with COX-1 and COX-2 were performed under the same conditions, the fact that strong STD signals could be observed for both isoforms reflects a similar rate of access/exit to both enzyme active sites. This observation is in accordance with the suggestion of an inhibition mechanism, common to COX-1 and COX-2 and for the vast majority of NSAIDs, involving first the binding of inhibitor to the enzyme near the solvent-accessible opening of the hydrophobic channel followed by a fast translocation of the inhibitor along the length of the channel and association within the COX active site.[21]

Slight differences were observed for the STD intensities of ibuprofen with COX-1 and COX-2, particularly in the relative intensity of the saturation transferred for the aromatic protons and the methylene protons. In the crystal structure of COX-1 with ibuprofen (PDB code 1EQG) the inhibitor binds in the

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COX-1 active site with the carboxylate making an ion pair with Arg-120 and a hydrogen bond with Tyr-355, with two of the aromatic protons from one side of the ring very close to Ile-523 (Figure 5A).[29]

There is no available experimental structure for the ibuprofen/COX-2 complex, but if one considers that the orientation of the ligand is similar to that of COX-1 and that the carboxylate makes the same type of contacts, then the reduction of STD intensity for H10 might be explained by the fact that in COX-2, Ile-523 is replaced by Val-523, augmenting the space available in COX-2 and reducing slightly the interaction with the methylene protons.

In the STD-NMR experiment the transfer of saturation occurs from the protein to the bound inhibitor and the detection is done after complex dissociation, being independent of the location of binding site. Therefore, the possibility that ibuprofen binds to both the COX and POX sites cannot be ruled out only by these experiments.

The relative STD intensities obtained for diclofenac with COX-2 (Figure 3, right) are very interesting to analyze in detail because of the fact that diclofenac binds the active site of COX-2 in an inverted binding mode when compared with most of the crystal structures of COX enzymes with carboxylic acid containing NSAIDs.[30] Diclofenac binds to COX-2 with its carboxylic acid moiety hydrogen-bonded to Ser-530 and Tyr-385 (Figure 5B) instead of the usual coordination to Arg-120 found for most of the carboxylated NSAIDs.

The fact that no STD is detected for H2′ of diclofenac (see full spectra in Supporting Information, Figure S3) contrasts with the high STD found for H8 in ibuprofen (Figure 2) and by itself could already constitute an indication of a different orientation in the binding pocket. This is further supported by the STD intensities of the phenylacetic acid ring, which with the exception of proton H6′ receive considerably less saturation transfer than the dichlorophenyl group (Figure 3). According to the superposition of the crystallographic structures (Figure 6), this ring is exactly located in the same region of the isobutyl group of ibuprofen that also receives less saturation transfer. The dichlorophenyl group presents the highest STD intensity and presents van der Waals contacts with Val-349, Ala-527, and Leu-531,[9] being located almost exactly in the same region as the aromatic ring of ibuprofen.

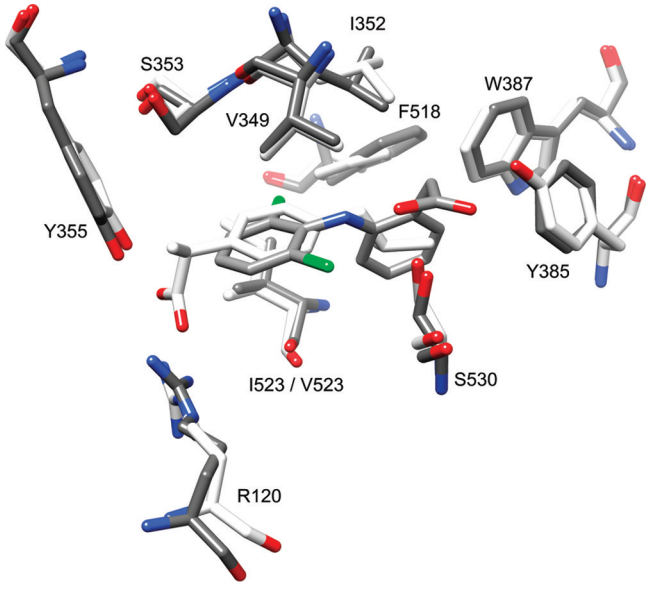

Figure 6. Superposition of the crystallographic structures of ibuprofen (light gray) in the active site of ovine COX-1 with diclofenac (dark gray) in the active site of murine COX-2. The aromatic ring of ibuprofen and the dichlorophenyl ring of diclofenac occupy the same region and give similar STD relative responses.

As far as we know, there is no information available in the literature concerning the structure of ketorolac bound to COX-2. Nevertheless, the previous analysis seems to indicate that the ligand moieties directed toward Arg-120 and Tyr-355 are associated with higher STD intensities than the moieties directed toward Ser-530 and Tyr-385. Following this reasoning and considering the STD intensities obtained for ketorolac, we can propose a binding orientation similar to that of diclofenac. The absence of STD intensities for H1, H2, and H3 seems to indicate that most probably the carboxylate adopts a conformation that favors an interaction with Ser-530 and Tyr-385 instead of Arg-120 and Tyr-355, just like the one observed in diclofenac. The high STD intensities obtained for the phenyl ring are also consistent with this arrangement and with the positioning of the aromatic ring similar to that of the phenyl ring of ibuprofen and the dichlorophenyl group of diclofenac.

Competition STD-NMR has been reported as a means to overcome one of the major limitations of the STD technique. For high-affinity ligands (k_{off} rates in the range of 0.1−0.01 s^{-1}) the saturation cannot be transferred effectively to solution, resulting in no observable STD effect.[19] Therefore, if two ligands compete for the same site, then the combination of STD-NMR with competition binding experiments allows detection of high affinity ligands by the disappearance or reduction of the STD signal of a low affinity indicator ligand.[31]

In the STD-NMR competition experiments with ketorolac and ibuprofen only residual STD signals could be detected for ketorolac while the ibuprofen signals are clearly visible (Figure 4, left). This indicates a lower affinity of ketorolac when compared to ibuprofen. The competition STD-NMR result correlates well with the differences of IC_{50} for ibuprofen (1.35 μM)[25] and that determined for the instantaneous inhibition assay of ketorolac (60.5 μM),[27] implying that the much lower IC_{50} for ketorolac in the assays with preincubation cannot be explained because of a higher binding affinity. If this were the case we would have obtained exactly the opposite result in the STD competition experiment, residual STD signals for ibuprofen and strong STD signals for ketorolac. Moreover, since the competition STD-NMR results were independent of the order of the addition of the drugs, this suggests that the time dependent inhibitory effect of ketorolac is more complex than a simple blockage of the binding sites due to competition. These results show that a relative rank of order of COX inhibition by NSAIDs may be determined from the comparison of IC_{50} values but that these values alone provide no indication of the mechanism of enzyme inhibition and require careful consideration especially when the inhibition is time dependent. On the other hand the competition STD-NMR experiment gives a direct report about the relative binding affinity of the competing ligands (if the ligands have overlapping binding sites) and can be a valuable tool to complement enzyme activity studies.

The fact that a relatively high STD response was observed for ibuprofen in the presence of diclofenac may be indicative that diclofenac and ibuprofen are not competing for the same binding site. While only qualitative, these results are in

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accordance with the recent proposal of Dong et al.[15] of a conformational heterodimer behavior for human COX-2. Accordingly, if we consider that ibuprofen binds to both sites, designated as E_{allo} and E_{cat}, and that diclofenac binds to E_{cat}, then a complete suppression of the ibuprofen signals by the presence of diclofenac would not be possible. Even with diclofenac having a much higher affinity for E_{cat} and competing effectively with ibuprofen, there will always be ibuprofen binding to E_{allo}.

One of the key issues addressed when concerning selective COX-2 inhibition is the rates of association and dissociation of an inhibitor from the enzyme. There seems to be a correlation between the relative rates of dissociation and the selectivity of the isoenzyme inhibition. For some selective inhibitors it was observed that while the association with COX-1 and COX-2 occurs at similar rates, a slower dissociation from COX-2, due to a more stable binding mode, explains the selectivity observed. Our STD results obtained for the NSAIDs studied are in accordance with these observations. STD is sensitive to the k_{off} rate, and for high-affinity ligands (k_{off} rates in the range of 0.1−0.01 s^{-1}) the saturation cannot be transferred effectively to solution, resulting in no observable STD effect. This has to do with the fact that the ligand has to dissociate more quickly than the magnetization relaxation rate; otherwise relaxation occurs and the magnetization is lost. The STD-NMR results obtained in this study for ibuprofen, ketorolac, and diclofenac show that the binding of these compounds to the enzyme is reversible and that a relatively fast dissociation rate is observed, the dissociation must occur with k_{off} rates above 0.1 s^{-1}.

5. CONCLUSIONS

In this work we have shown for the first time the use of NMR spectroscopy as a technique to characterize the binding epitope of COX-1 and COX-2 inhibitors. STD-NMR was successfully applied to study the binding of ibuprofen to COX-1/COX-2 and the binding of ketorolac and diclofenac to COX-2. The results obtained are consistent with the accepted mechanism of rapid and reversible inhibition.

The study of the binding epitopes of ibuprofen and diclofenac with COX-2 and the comparison with the crystallographic structures seem to indicate that the ligand moieties directed toward Arg-120 and Tyr-355 are associated with higher STD intensities than the moieties directed toward Ser-530 and Tyr-385. This fact allowed us to propose a binding mode for ketorolac similar to that of diclofenac with COX-2.

Our STD-NMR results open a new window of research to study the relation between multiple COX-2 binding sites, concerning not only inhibitors but also other nonsubstrate molecules, like fatty acids. It is envisaged that even conformational modifications of the E_{cat} site due to the binding to the E_{allo} may be probed by the careful study of the binding epitopes of known inhibitors in the presence of different nonsubstrate molecules.

These exploratory studies show that NMR is a valuable tool to complement enzyme activity studies in the effort to rationalize COX inhibition mechanisms.

■ ASSOCIATED CONTENT

Supporting Information

STD-NMR spectra of compounds in the presence of cyclooxygenase. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AA, arachidonic acid; COX, cyclooxygenase; DDC, diethyldithiocarbamate; NSAID, nonsteroidal anti-inflammatory drug; PGHS, prostaglandin endoperoxide-H synthase; POX, peroxidase; STD-NMR, saturation transfer difference nuclear magnetic resonance

■ REFERENCES

(1) Vane, J. R.; Botting, R. M. Anti-inflammatory drugs and their mechanism of action. *Inflammation Res.* **1998**, *47* (Suppl. 2), S78−S87.
(2) Vane, J. R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* **1971**, *231*, 232−235.
(3) Vane, J. R. Introduction: mechanism of action of NSAIDs. *Br. J. Rheumatol.* **1996**, *35* (Suppl. 1), 1−3.
(4) Xie, W. L.; Chipman, J. G.; Robertson, D. L.; Erikson, R. L.; Simmons, D. L. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 2692−2696.
(5) Marnett, L. J.; Rowlinson, S. W.; Goodwin, D. C.; Kalgutkar, A. S.; Lanzo, C. A. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J. Biol. Chem.* **1999**, *274*, 22903−22906.
(6) Kurumbail, R. G.; Stevens, A. M.; Gierse, J. K.; McDonald, J. J.; Stegeman, R. A.; Pak, J. Y.; Gildehaus, D.; Miyashiro, J. M.; Penning, T. D.; Seibert, K.; Isakson, P. C.; Stallings, W. C. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* **1996**, *384*, 644−648.
(7) Smith, W. L.; Garavito, R. M.; DeWitt, D. L. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.* **1996**, *271*, 33157−33160.
(8) Grosser, T.; Fries, S.; FitzGerald, G. A. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J. Clin. Invest.* **2006**, *116*, 4−15.
(9) Blobaum, A. L.; Marnett, L. J. Structural and functional basis of cyclooxygenase inhibition. *J. Med. Chem.* **2007**, *50*, 1425−1441.
(10) Dannhardt, G.; Kiefer, W. Cyclooxygenase inhibitors—current status and future prospects. *Eur. J. Med. Chem.* **2001**, *36*, 109−126.
(11) Chakraborti, A. K.; Garg, S. K.; Kumar, R.; Motiwala, H. F.; Jadhavar, P. S. Progress in COX-2 inhibitors: a journey so far. *Curr. Med. Chem.* **2010**, *17*, 1563−1593.
(12) Marnett, L. J. The COXIB experience: a look in the rearview mirror. *Annu. Rev. Pharmacol. Toxicol.* **2009**, *49*, 265−290.
(13) Limongelli, V.; Bonomi, M.; Marinelli, L.; Gervasio, F. L.; Cavalli, A.; Novellino, E.; Parrinello, M. Molecular basis of cyclooxygenase enzymes (COXs) selective inhibition. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 5411−5416.
(14) Rimon, G.; Sidhu, R. S.; Lauver, D. A.; Lee, J. Y.; Sharma, N. P.; Yuan, C.; Frieler, R. A.; Trievel, R. C.; Lucchesi, B. R.; Smith, W. L. Coxibs interfere with the action of aspirin by binding tightly to one monomer of cyclooxygenase-1. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 28−33.

(15) Dong, L.; Vecchio, A. J.; Sharma, N. P.; Jurban, B. J.; Malkowski, M. G.; Smith, W. L. Human cyclooxygenase-2 is a sequence homodimer that functions as a conformational heterodimer. *J. Biol. Chem.* **2011**, *286*, 19035−19046.
(16) Sharma, N. P.; Dong, L.; Yuan, C.; Noon, K. R.; Smith, W. L. Asymmetric acetylation of the cyclooxygenase-2 homodimer by aspirin and its effects on the oxygenation of arachidonic, eicosapentaenoic, and docosahexaenoic acids. *Mol. Pharmacol.* **2010**, *77*, 979−986.
(17) Kulmacz, R.; Lands, W. Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti-inflammatory agents. *J. Biol. Chem.* **1985**, *260*, 12572−12578.
(18) Meyer, B.; Peters, T. NMR spectroscopy techniques for screening and identifying ligand binding to protein receptors. *Angew. Chem., Int. Ed.* **2003**, *42*, 864−890.
(19) Mayer, M.; Meyer, B. Characterization of ligand binding by saturation transfer difference NMR spectroscopy. *Angew. Chem., Int. Ed.* **1999**, *38*, 1784−1788.
(20) Viegas, A.; Manso, J.; Nobrega, F. L.; Cabrita, E. J. Saturation-transfer difference (STD) NMR: a simple and fast method for ligand screening and characterization of protein binding. *J. Chem. Educ.* **2011**, *88*, 990−994.
(21) Prusakiewicz, J. J.; Duggan, K. C.; Rouzer, C. A.; Marnett, L. J. Differential sensitivity and mechanism of inhibition of COX-2 oxygenation of arachidonic acid and 2-arachidonoylglycerol by ibuprofen and mefenamic acid. *Biochemistry* **2009**, *48*, 7353−7355.
(22) Johnson, J. L.; Wimsatt, J.; Buckel, S. D.; Dyer, R. D.; Maddipati, K. R. Purification and characterization of prostaglandin H synthase-2 from sheep placental cotyledons. *Arch. Biochem. Biophys.* **1995**, *324*, 26−34.
(23) Walker, M. C.; Kurumbail, R. G.; Kiefer, J. R.; Moreland, K. T.; Koboldt, C. M.; Isakson, P. C.; Seibert, K.; Gierse, J. K. A three-step kinetic mechanism for selective inhibition of cyclo-oxygenase-2 by diarylheterocyclic inhibitors. *Biochem. J.* **2001**, *357*, 709−718.
(24) Yuan, Y.; Wen, X.; Sanders, D. A.; Pinto, B. M. Exploring the mechanism of binding of UDP-galactopyranose to UDP-galactopyranose mutase by STD-NMR spectroscopy and molecular modeling. *Biochemistry* **2005**, *44*, 14080−14089.
(25) Barnett, J.; Chow, J.; Ives, D.; Chiou, M.; Mackenzie, R.; Osen, E.; Nguyen, B.; Tsing, S.; Bach, C.; Freire, J.; Chan, H.; Sigal, E.; Ramesha, C. Purification, characterization and selective-inhibition of human prostaglandin-G/H synthase-1 and synthase-2 expressed in the baculovirus system. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1994**, *1209*, 130−139.
(26) Waterbury, L. D.; Silliman, D.; Jolas, T. Comparison of cyclooxygenase inhibitory activity and ocular anti-inflammatory effects of ketorolac tromethamine and bromfenac sodium. *Curr. Med. Res. Opin.* **2006**, *22*, 1133−1140.
(27) Laneuville, O.; Breuer, D. K.; Dewitt, D. L.; Hla, T.; Funk, C. D.; Smith, W. L. Differential inhibition of human prostaglandin endoperoxide-H synthase-1 and synthase-2 by nonsteroidal antiinflammatory drugs. *J. Pharmacol. Exp. Ther.* **1994**, *271*, 927−934.
(28) Gierse, J. K.; Koboldt, C. M.; Walker, M. C.; Seibert, K.; Isakson, P. C. Kinetic basis for selective inhibition of cyclo-oxygenases. *Biochem. J.* **1999**, *339*, 607−614.
(29) Selinsky, B. S.; Gupta, K.; Sharkey, C. T.; Loll, P. J. Structural analysis of NSAID binding by prostaglandin H2 synthase: time-dependent and time-independent inhibitors elicit identical enzyme conformations. *Biochemistry* **2001**, *40*, 5172−5180.
(30) Rowlinson, S. W.; Kiefer, J. R.; Prusakiewicz, J. J.; Pawlitz, J. L.; Kozak, K. R.; Kalgutkar, A. S.; Stallings, W. C.; Kurumbail, R. G.; Marnett, L. J. A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser-530 and Tyr-385. *J. Biol. Chem.* **2003**, *278*, 45763−45769.
(31) Wang, Y. S.; Liu, D. J.; Wyss, D. F. Competition STD NMR for the detection of high-affinity ligands and NMR-based screening. *Magn. Reson. Chem.* **2004**, *42*, 485−489.