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# Enantiospecific, Selective Cyclooxygenase-2 Inhibitors

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**Abstract**—Cyclooxygenase inhibition studies with novel indomethacin alkanolamides demonstrate the potential for dramatic differences in inhibitor properties conferred by subtle structural modifications. The transformation of non-selective α-(*S*)-substituted indomethacin ethanolamides to potent, COX-2 selective inhibitors by simple stereocenter inversion highlights this property. 

Cyclooxygenases (COXs) catalyze the bis-dioxygenation of arachidonic acid to provide prostaglandin (PG) $H_2$, the precursor to the primary PGs and thromboxanes (TXs).[1] The therapeutic efficacy of nonsteroidal antiinflammatory drugs (NSAIDs) derives from their inhibition of COXs.[2] Two COX isoforms exist. COX-1 is a constitutive enzyme, whereas COX-2 is inducible and highly regulated by a range of agonists. COX-1 activity accounts for PG and TX production in gastric mucosa, kidney, and platelets. COX-2 activity is thought to be responsible for elevated PG levels associated with inflammation.[3] The link between COX-2 activity and inflammation has been dramatically confirmed by the clinical efficacy of two antiinflammatory, selective COX-2 inhibitors, rofecoxib and celecoxib.

We recently demonstrated that modification of the nonselective NSAID indomethacin by amidation provides COX-2-selective inhibitors. Initial results indicated that sterically demanding amines or alcohols could be coupled to indomethacin without significant losses in potency or selectivity.[4] This observation led to an effort to develop similar compounds that possess readily functionalizable moieties to allow for the attachment of additional pharmacophores. In addition, the potential for amidase/esterase metabolism of indomethacin derivatives providing the ulcerogenic parent NSAID prompted related efforts to increase steric hindrance near the amide/ester bond, potentially enhancing metabolic stability. This strategy is similar to that used in the development of anandamide analogues with enhanced metabolic stability (e.g., (*R*)-methanandamide).[5] In the course of these investigations, we have synthesized and evaluated several novel indomethacin amides with substitutions near the amide linkage. The results demonstrate that subtle structural modifications of indomethacin amides can result in profound changes in inhibitory properties and have identified an unique enantioselective capacity of COX-1.

Indomethacin amides were prepared using standard methods. Briefly, amides were generated by EDCI- or BOP-Cl-mediated coupling of indomethacin with the appropriate amine and purified by silica gel chromatography. Enantiomeric purity was determined by chiral, high-performance liquid chromatography and established that no racemization occurred during coupling reactions (Chiralpak AD, 250×4.6 mm, Chiral Technologies Inc., Exton, PA). (*R*)-2-Methoxy-1-methylethylamine was prepared by methylating (*R*)-2-*tert*-butoxycarbonylamino-1-propanol with MeI in the presence of NaH followed by BOC deprotection with gaseous HCl. COX-1 was purified from ram seminal vesicles and apoenzyme was prepared as described.[6,7] Site-directed mutagenesis, expression, and purification of COX-2 enzymes was performed as previously described.[8] COX activity was quantified as reported previously.[9]

To examine the effects of amide substituents on COX-2 selectivity and potency of indomethacin amides, initial modifications involved coupling the free acid with aminoalkanols of varied chain length. Neither COX-2

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selectivity nor potency was significantly affected by increases in alkyl chain length (Table 1). As reference, indomethacin displays $IC_{50}$ values of 50 nM and 210 nM under these conditions for oCOX-1 and mCOX-2, respectively.[4]

Indomethacin was then amidated with an array of readily available and inexpensive 1,2-amino alcohols to expand this class of inhibitors. Initially, indomethacin was coupled to alaninol to provide enantiomers **6** and **7** (Table 2). Interestingly, both enantiomers demonstrated COX-2 inhibitory potency, but, the (*S*)-enantiomer, **7**, also proved to be a potent inhibitor of COX-1.

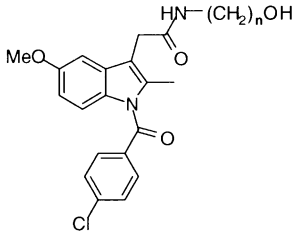
To test if this chiral discrimination was a general property of substituted ethanolamides of indomethacin, a series of chiral derivatives was synthesized and evaluated against wild-type cyclooxygenases. As seen in Table 2, the enantioselective inhibition of COX-1 by (*S*) enantiomers of α-substituted indomethacin ethanolamides holds for a wide range of substituents.

An exception to this general pattern is seen with prolinol amides of indomethacin. Both enantiomers displayed attenuated hCOX-2 inhibitory potency ($IC_{50} = 1.7$ and 1.8 μM for (*R*) and (*S*), respectively) and neither enantiomer inhibited oCOX-1 ($IC_{50} > 80$ μM).

To determine if the regiochemistry of substitution was critical in enantiospecific COX-2-selective inhibition, the effects of β-substitution were examined (Table 3). Similar to the α-substituted indomethacin ethanolamides, all β-substituted compounds retained COX-2 inhibitory potency. However, in contrast to α-substituted indomethacin ethanolamides, no tested β-substituted indomethacin ethanolamide possessed potency against COX-1. Thus, substitution at the α position is required for the observed enantiospecifity.

An effort to establish the structural requirements for enantiospecific COX-1 inhibition was undertaken by systematically evaluating derivatives of **6** (Table 4). As discussed above, α-substitution is required for enantioselective COX-1 inhibition (compare **6**/**7** with **21**/**22**). In addition, the presence of the hydroxyl moiety in **6**/**7** is required for the enantioselective pattern of inhibition observed in Table 2. Inhibitors that include methoxy (**25**/**26**) or methyl (**27**/**28**) substitutions for the hydroxyl moiety in **6**/**7** displayed altered chirality-dependent inhibition of COX isoforms. In contrast to the related alcohols, the (*R*)-enantiomers, **25** and **27**, possessed greater inhibitory potency toward COX-1 than the corresponding (*S*)-enantiomers, **26** and **28**, and are thus less

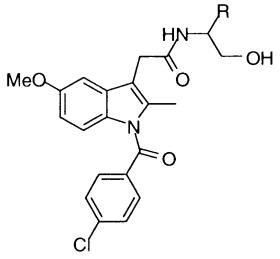
**Table 1.** Chain length SAR: *n*-amino-1-alkanol indomethacin amide inhibition of wild-type cyclooxygenases




| Compd | *n* | oCOX-1 inhibition $IC_{50}$ (μM)[a] | hCOX-2 inhibition $IC_{50}$ (μM)[a] | $IC_{50}$ (COX-1)/ $IC_{50}$ (COX-2) |
|---|---|---|---|---|
| **1** | 2 | >66 | 0.16 | >410 |
| **2** | 3 | 64 | 0.22 | 290 |
| **3** | 4 | 86 | 0.28 | 310 |
| **4** | 5 | >83 | 0.29 | >290 |
| **5** | 6 | >63 | 0.21 | >300 |

[a]Values represent the average of at least two determinations.

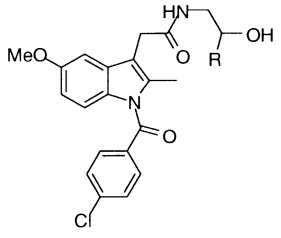
**Table 2.** Inhibition of wild-type cyclooxygenases by α-substituted indomethacin ethanolamides




| Compd | R | oCOX-1 inhibition $IC_{50}$ (μM)[a] | hCOX-2 inhibition $IC_{50}$ (μM)[a] | $IC_{50}$ (COX-1)/ $IC_{50}$ (COX-2) |
|---|---|---|---|---|
| **6** | (*R*)-$CH_3$ | 33 | 0.17 | 190 |
| **7** | (*S*)-$CH_3$ | 0.59 | 0.27 | 2.2 |
| **8** | (*R*)-$CH_2CH_3$ | 59 | 0.10 | 590 |
| **9** | (*S*)-$CH_2CH_3$ | 0.35 | 0.28 | 1.3 |
| **10** | (*R*)-$CH(CH_3)_2$ | >63 | 0.11 | >570 |
| **11** | (*S*)-$CH(CH_3)_2$ | 0.40 | 0.37 | 1.1 |
| **12** | (*R*)-$CH_2CH(CH_3)_2$ | >63 | 0.50 | >130 |
| **13** | (*S*)-$CH_2CH(CH_3)_2$ | <3.9 | 0.17 | <23 |
| **14** | (*R*)-$CH_2CH_2SCH_3$ | 51 | 0.25 | 200 |
| **15** | (*S*)-$CH_2CH_2SCH_3$ | 2.3 | 1.0 | 2.3 |
| **16** | (*R*)-$C_6H_5$ | 47 | 0.44 | 110 |
| **17** | (*S*)-$C_6H_5$ | <1.3 | 0.085 | <15 |
| **18** | (*R*)-$CH_2C_6H_5$ | >50 | 0.22 | >230 |
| **19** | (*S*)-$CH_2C_6H_5$ | <3.9 | 0.085 | <46 |
| **20** | (*S*)-$CH(CH_3)CH_2CH_3$ | 0.48 | 0.11 | 4.4 |

[a]Values represent the average of at least two determinations.

**Table 3.** Inhibition of wild-type cyclooxygenases by β-substituted indomethacin ethanolamides




| Compd | R | oCOX-1 inhibition $IC_{50}$ (μM)[a] | hCOX-2 inhibition $IC_{50}$ (μM)[a] | $IC_{50}$ (COX-1)/ $IC_{50}$ (COX-2) |
|---|---|---|---|---|
| **21** | (*R*)-$CH_3$ | 52 | 0.33 | 160 |
| **22** | (*S*)-$CH_3$ | 32 | 0.32 | 100 |
| **23** | (*R*)-$C_6H_5$ | >63 | 0.21 | >300 |
| **24** | (*S*)-$C_6H_5$ | 56 | 0.047 | 1200 |

[a]Values represent the average of at least two determinations.

COX-2 specific. This contrasts with the greater COX-2 specificity observed for (*R*)-enantiomers in the α-substituted indomethacin ethanolamide series. Surprisingly, the dimethyl substituted derivative, **29**, maintained COX-1 inhibitory potency suggesting that the inability of (*R*)-enantiomers in this series to significantly inhibit COX-1 is not simply attributable to an unfavorable steric interaction between the alkyl substituent and the enzyme.

In order to identify enzyme residues responsible for the observed enantiospecific inhibitory characteristics of these compounds, several site-directed mutant murine COX-2 enzymes were evaluated with **6** and **7**. We anticipated identifying a mutant which retained sensitivity to inhibition by **7** but was resistant to inhibition by **6**. Initially, protein regions previously identified as critical in conferring isoform selectivity upon known COX-2 inhibitors were evaluated.

The COX-2 side-pocket, which accounts for the COX-2 selectivity of diarylheterocycles, was investigated with a V523I single mutant as well as the V523I/R513H/V434I triple mutant. In contrast to diarylheterocycles which do not inhibit these mutants, both enzymes remained sensitive to inhibition by **6** and **7** (Table 5).[10,11] The substitution of a 4-bromobenzyl moiety for the 4-chlorobenzoyl group of indomethacin provides a COX-2 selective inhibitor. The selectivity of this indomethacin derivative depends on the additional COX-2 active site volume provided by the presence of a leucine at position 503 in COX-2 instead of a phenylalanine as in COX-1. Consequently, the L503F mutant is not inhibited by the 4-bromobenzyl derivative.[4,12] In contrast, the L503F mutant enzyme was potently inhibited by both **6** and **7** (Table 5). Finally, the serine residue acetylated by aspirin, Ser 530, is required for inhibition by the COX-2 selective covalent modifier, *O*-(acetoxyphenyl)hept-2-ynyl sulfide (APHS). APHS fails to inhibit mCOX-2 containing the S530A mutation.[13] This mutation had essentially no effect on the potency of **6** or **7** (Table 5).

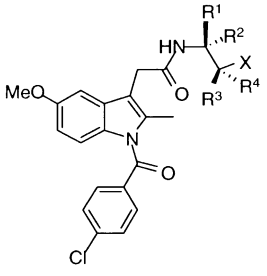
As COX-2 regions known to be involved in interactions with other COX-2 selective inhibitors appeared to play no role in the COX-2 inhibitory potency or enantiospecificity of indomethacin ethanolamides, additional active site mutations were examined. R120, Y355, and E524 form a hydrogen bonding network at the opening of the substrate access channel within COX enzymes. Mutations in R120 and E524 (R120Q, R120A, and E524L) failed to render the enzyme resistant to inhibition by **6** or **7** (Table 5). In contrast, substitution of Y355 with an alanine or phenylalanine provided mutant enzymes which were not inhibited by either enantiomer (<10% inhibition at 2.4 μM). The importance of Y355

**Table 5.** Inhibition of wild-type and mutant murine COX-2 by **6** and **7**[a]

| Enzyme | **6** Inhibition $IC_{50}$ (nM)[a] | **7** Inhibition $IC_{50}$ (nM)[a] |
|---|---|---|
| Wild-type | 540 | 170 |
| V523I | 690 | 220 |
| VRV | 340 | 110 |
| L503F | 300 | 160 |
| S530A | 570 | 150 |
| R120A | 50 | 60 |
| R120Q | 90 | 75 |
| E524L | 560 | 220 |
| V89I | 250 | 85 |
| I92L | 200 | 200 |
| Y115L | 325 | 390 |
| S119V | 240 | 200 |
| Y115L/S119V | 290 | 210 |
| H122N | 690 | 300 |
| F357L | 250 | 250 |

[a]Values represent the average of at least two determinations.

**Table 4.** Structure–activity relationship: methyl substituted indomethacin ethanolamides and derivatives




| Compd | X | $R^1$ | $R^2$ | $R^3$ | $R^4$ | oCOX-1 inhibition $IC_{50}$ (μM)[a] | hCOX-2 inhibition $IC_{50}$ (μM)[a] | $IC_{50}$ (COX-1)/ $IC_{50}$ (COX-2) |
|---|---|---|---|---|---|---|---|---|
| **6** | OH | H | $CH_3$ | H | H | 33 | 0.17 | 190 |
| **7** | OH | $CH_3$ | H | H | H | 0.59 | 0.27 | 2.2 |
| **21** | OH | H | H | H | $CH_3$ | 52 | 0.33 | 160 |
| **22** | OH | H | H | $CH_3$ | H | 32 | 0.32 | 100 |
| **25** | $OCH_3$ | H | $CH_3$ | H | H | 6.8 | 0.14 | 49 |
| **26** | $OCH_3$ | $CH_3$ | H | H | H | >60 | 0.24 | >250 |
| **27** | $CH_3$ | H | $CH_3$ | H | H | 0.18 | 0.13 | 1.4 |
| **28** | $CH_3$ | $CH_3$ | H | H | H | 4.7 | 0.11 | 42 |
| **29** | OH | $CH_3$ | $CH_3$ | H | H | 0.80 | 0.19 | 4.2 |

[a]Values represent the average of at least two determinations.

in indomethacin ethanolamide inhibition agrees with previous results with other indomethacin amides.

Several additional protein residues were examined by site-directed mutagenesis. Unfortunately, no residue substitution resulted in a mutant enzyme sensitive to inhibition by **7** but resistant to inhibition by **6** (Table 5). The structural basis for the enantioselective inhibition of COX-1 remains unclear.

It is well established that interactions between chiral small molecules and enzymes can display marked stereospecificity. The demonstration that α-substituted ethanolamides of indomethacin can be transformed from nonselective to highly COX-2-selective inhibitors by the simple inversion of stereochemistry at the single chiral center in these compounds dramatically affirms this principle. Although COX enzyme structure has been extensively studied, the combination of site-directed mutagenesis and SAR studies suggests that subtle and pharmacologically exploitable COX isoform differences remain uncharacterized.

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