Structural Determinants of Arylacetic Acid Nonsteroidal Anti-Inflammatory Drugs Necessary for Binding and Activation of the Prostaglandin D2 Receptor CRTH2

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ABSTRACT

The chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptor, a G protein-coupled receptor that mediates chemotaxis of inflammatory cells in response to prostaglandin D2 (PGD2), is hypothesized to play a role in Th2-mediated allergic disease. In addition to PGD2, CRTH2 can be activated by indomethacin, a nonselective cyclooxygenase inhibitor and widely used nonsteroidal anti-inflammatory drug (NSAID). To evaluate the structural features that confer CRTH2 binding selectivity, structure-activity relationship analysis of arylacetic acid class NSAIDs as CRTH2 receptor ligands was performed. Indomethacin, sulindac sulfaide, and zomepirac displaced [3H]PGD2 binding at the mouse CRTH2 receptor with comparable affinity (Ki = 1.5 ± 0.1, 2.5 ± 0.4, and 3.3 ± 0.3 μM, respectively). The indomethacin metabolite 5′-O-desmethyl indomethacin (5′-DMI) possessed binding affinity similar to indomethacin; however, elimination of the 2-methyl substituent on the indole ring resulted in a 10-fold decrease in binding affinity. No binding was detected for indole acetic acid and indole derivatives such as tryptophan, serotonin, and 5-hydroxy indole acetic acid, demonstrating the importance of the N-acyl moiety of indomethacin. Neutral derivatives of indomethacin also failed to bind to mCRTH2, suggesting that the negatively charged carboxylate moiety participates in a key ligand-receptor interaction. Despite similar binding affinities, NSAID-type mCRTH2 ligands exhibited variable potencies as mCRTH2 agonists. Sulindac sulfide and 5′-DMI inhibited intracellular cyclic AMP ([cAMP]) generation and stimulated cell migration comparable with indomethacin. In contrast, zomepirac did not inhibit [cAMP] generation or stimulate cell migration but weakly antagonized the effects of indomethacin on [cAMP]. Together, these results reveal structural features of arylacetic acid NSAIDs that may be exploited for the development of selective CRTH2 ligands.

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ABBREVIATIONS: PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; DP, D protein receptor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; Th2, T helper 2; COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; mCRTH2, mouse chemoattractant receptor-homologous molecule expressed on Th2 cells; 5′-DMI, 5′-O-desmethyl indomethacin; DME, Dulbecco’s modified Eagle’s medium; hCRTH2, human chemoattractant receptor-homologous molecule expressed on Th2 cells; PBS, phosphate-buffered saline; IA, indole-3-acetic acid; 2-DMI, 2-desmethyl indomethacin; PBl, p-bromobenzyl indomethacin; FSK, forskolin; FMLP, f-Met-Leu-Phe; 5-HIAA, 5-hydroxy indole acetic acid.
response to PGD$_2$ generated in the setting of allergic inflammation.

Although the precise role of CRTH2 in allergic disease has yet to be fully elucidated, emerging data suggest that it may mediate proinflammatory effects of PGD$_2$. Therefore, the generation of selective and specific CRTH2 antagonists is of interest as potential therapeutic agents for the treatment of allergic disease. It was recently reported that ramatroban, initially described as a thromboxane receptor antagonist and marketed for allergic rhinitis in Japan, has significant CRTH2 antagonist activity and can inhibit PGD$_2$-stimulated eosinophil migration in vitro (Sugimoto et al., 2003). Consistent with this finding, ramatroban was observed to inhibit antigen-induced mucosal eosinophilia in sensitized guinea pigs (Narita et al., 1996), an effect unlikely to be caused by direct inhibition of thromboxane receptor receptors on eosinophils. At present, however, no truly CRTH2-selective antagonist has been reported.

Indomethacin is an arylacetic acid class nonselective cyclooxygenase (COX) inhibitor and widely prescribed nonsteroidal anti-inflammatory drug (NSAID). In addition to suppressing prostaglandin formation by inhibiting COX, indomethacin is known to possess COX-independent activity such as suppression of malignant transformation of embryonic fibroblasts (Zhang et al., 1999) and activation of the peroxisome proliferator-activated receptors $\alpha$ and $\gamma$ (Lehmann et al., 1997). Indomethacin has also been shown to be an agonist at the CRTH2 receptor and to stimulate shape change, CD11b up-regulation, and chemotaxis of eosinophils (Hiirai et al., 2002; Stubbs et al., 2002). This novel activity of indomethacin is not shared by other NSAIDs such as aspirin or ibuprofen. We have reported that indomethacin and sulindac, also an arylacetic acid NSAID, can bind to the mouse CRTH2 receptor (mCRTH2), suggesting that arylacetic acid class NSAIDs possess a unique structural motif that allows binding to CRTH2 (Hata et al., 2003). Whereas the structure-activity relationships of indomethacin derivatives as selective inhibitors of the cyclooxygenase isoforms have been extensively studied (Kalgutkar et al., 2000a,b), little is known about these molecules as CRTH2 ligands. Based on our initial observations, we hypothesized that the arylacetic acid core provides a fundamental structural motif that allows this class of NSAIDs to bind and activate the CRTH2 receptor. To define the structural features required for the novel CRTH2 activity of arylacetic acid NSAIDs, we performed structure-activity relationship analysis on a series of arylacetic acid NSAIDs and indomethacin derivatives as mCRTH2 agonists.

Materials and Methods

Reagents. Indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid], NSAIDs, indole derivatives, forskolin, and 3-isobutyl-1-methylxanthine were from Sigma-Aldrich (St. Louis, MO). Indomethacin derivatives were synthesized as described previously (Kalgutkar et al., 2000b; Prusakiewicz et al., 2004). 5'-O-desmethyl-Indomethacin (5'-DMI) was obtained from Merck Research Labs (West Point, PA). Ramatroban [((+)-(3R)3-(4-fluorobenzensulfonylamo)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid] was obtained from Bayer AG (Kyoto, Japan). $[^{3}H]$PGD$_2$ was purchased from Amersham Biosciences Inc. (Piscataway, NJ). DMEM and Opti-MEM were from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). G418 (Geneticin) was purchased from Mediatech (Herndon, VA). l-Glutamine and penicillin/streptomycin were from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Ponasterone A was purchased from Stratagene (La Jolla, CA).

Expression of CRTH2 in HEK293 and ER293 Cells. Transient expression of mCRTH2 in HEK293 cells and generation of the ER293/mCRTH2 cell line has been described previously (Hata et al., 2003). The human CRTH2 receptor (hCRTH2) expression plasmid was obtained from the Guthrie DNA Resource Center (Sayre, PA) and transiently transfected into HEK293 cells in a similar manner. Cells were maintained at 37°C in humidified air containing 5.5% CO$_2$ in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin. Media for ER293 cells was additionally supplemented with 300 $\mu$g/ml G418 and 100 $\mu$g/ml hygromycin B. Expression of mCRTH2 in ER293/mCRTH2 cells was induced by addition of 10 $\mu$M ponasterone A 24 h before all experiments.

Radioisotype Binding Assay. HEK293/mCRTH2 and HEK293/ hCRTH2 membranes for radioligand binding experiments were harvested as described previously (Hata et al., 2003). Membranes (30 $\mu$g of membrane protein) were incubated with $[^{3}H]$PGD$_2$ at 4°C for 1.5 h in binding buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl$_2$, 140 mM NaCl, and 5 mM KCl). The binding reaction was terminated by the addition of 3 ml of ice-cold binding buffer and rapidly filtered under vacuum over Whatman GF/F filters. Filters were washed three times with 3 ml of ice-cold binding buffer, dried, and counted in 4 ml of Ultima Gold scintillation fluid (PerkinElmer Life and Analytical Sciences, Groningen, The Netherlands).

$[^{3}H]$PGD$_2$ binding assay. HEK293/mCRTH2 cells were grown to 80% confluence in six-well plates and incubated for 24 h in the presence of 10 $\mu$M ponasterone A. Thirty minutes before addition of ligands, media were replaced with Opti-MEM I containing 0.5 mM 3-isobutyl-1-methylxanthine. Cells were incubated with ligands for 10 min, washed once with PBS, and the reaction was terminated by the addition of 0.1 M HCl. Cells were scraped free, and the resulting cell suspension was centrifuged for 10 min at 1000g. Supernatants were assayed for protein content by bicinchoninic acid assay (Pierce Chemical, Rockford, IL). After normalization to protein content, $[^{3}H]$PGD$_2$ levels were determined by an enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cayan Chemical, Ann Arbor, MI).

Cell Migration Assay. ER293/mCRTH2 cells were incubated with 10 $\mu$M ponasterone A for 24 h before harvesting. Cells were trypsinized, washed three times in PBS, and resuspended in DMEM.
Cells (100,000) were added to the upper chamber of 24-well 8.0-μm polycarbonate transwell inserts (Costar, Cambridge, MA) that had been previously treated overnight with 5 μg/ml Matrigel (BD Biosciences, Bedford, MA) in PBS at 4°C and blocked in the presence of 2% bovine serum albumin in PBS for 1 h at 37°C. Ligands were diluted in DMEM and added to the lower chamber. After incubating for 4 h at 37°C, inserts were removed and cells adhering to the top of the membrane were removed with a cotton swab. Cells on the bottom of the membrane were fixed with 3.7% formaldehyde for 1 h, washed twice with PBS, and stained overnight with crystal violet. For each insert, five independent fields were counted in blinded manner at 200× magnification.

Cyclooxygenase Activity Assay. Time-dependent inhibition of ovine cyclooxygenase-1 and murine cyclooxygenase-2 was measured as described previously (Kalgutkar et al., 2000b).

Molecular Modeling of mCRTH2. The transmembrane spanning α-helical bundle of mCRTH2 was constructed with homology modeling methods, using a current β2-adrenergic receptor model as a template (Furse and Lybrand, 2003). Amino acid side chains in the β2-adrenergic receptor model were replaced to generate the mCRTH2 sequence, using a backbone-dependent side chain rotamer library. Limited energy minimization was used to relieve unfavorable steric interactions after side chain replacement. The extracellular and cytosolic loops were generated de novo. The amino and carboxy terminal segments of each loop were constructed as extended peptides and attached to the end of the appropriate transmembrane helix. Weak harmonic constraints were then applied gradually during short (10–20 ps), low-temperature (30 K) molecular dynamics simulations to connect appropriate loop segments with a trans peptide bond. A putative disulfide cross-link between the extracellular region of transmembrane helix III and extracellular loop 2 was generated by applying additional constraints during the generation of extracellular loop 2. To simplify model construction, the C-terminal tail was truncated at Val321. The intact mCRTH2 receptor model was then refined further with limited energy minimization and low-temperature molecular dynamics simulation to relieve any peptide backbone conformational strain or residual bad steric interactions. Indomethacin was then docked into the putative ligand-binding pocket using an automated ligand docking algorithm. All structural refinement calculations were performed in vacuo with a distance-dependent dielectric model, using standard AMBER all-atom potential functions. Energy minimization and molecular dynamics calculations were performed with the AMBER package. Ligand docking was performed using the automated docking module in MOE (Chemical Computing, Inc., Montreal, QC, Canada).

Data Analysis. All data are presented as the mean ± S.E.M. Competition binding curves and IC50 values were calculated using a one-site competition model (Prism 4; GraphPad Software Inc., San Diego, CA). K_i values were calculated according to the method of Cheng and Prusoff (1973). EC50 values for [cAMP]i dose-response experiments were calculated using a fixed slope sigmoidal dose-response model (Prism). Differences between means were tested for statistical significance using two-tailed unpaired t test for [cAMP]i and chemotaxis experiments, with p < 0.05 considered significant (InStat 3; GraphPad).

Fig. 2. Structures of arylacetic acid NSAIDs, indole, and indomethacin derivatives tested in these studies.
Results

A panel of NSAIDs was screened for their ability to displace [3H]PGD$_2$ from the mouse CRTH2 receptor expressed in HEK293 cells. In addition to indomethacin and sulindac (Hata et al., 2003), the arylacetic acid sulindac sulfate and zomepirac also displaced [3H]PGD$_2$ binding with high affinity ($K_i$ = $1.5 \pm 0.1, 2.5 \pm 0.4$, and $3.5 \pm 0.3$ μM for indomethacin, sulindac sulfate, and zomepirac, respectively); in contrast, diclofenac did not display detectable binding (Fig. 1). The binding of indomethacin to the human CRTH2 receptor (hCRTH2) has been previously characterized (Hirai et al., 2002; Savery et al., 2002); our binding assays confirmed that the affinity of indomethacin for hCRTH2 ($K_i$ = 3 ± 1 μM; data not shown) is similar to the affinity reported by Hirai and coworkers and similar to that of the mouse ortholog.

The arylacetic acid core of indomethacin is analogous to indole-3-acetic acid (IAA), a plant auxin that modulates a number of growth processes (Goldsmith, 1993). In addition to IAA, several other biologically active molecules share an indole or IAA core, most notably serotonin and its immediate precursors and metabolites (Fig. 2). To investigate the importance of the IAA core in conferring affinity for mCRTH2, we tested whether these molecules were capable of binding to mCRTH2. These compounds were unable to displace [3H]PGD$_2$ at concentrations up to 30 μM (Fig. 3A). Thus, the IAA core of indomethacin and other arylacetic acid NSAIDs is not in itself sufficient to confer binding to mCRTH2.

In vivo, indomethacin undergoes demethylation of the 5’-O-desmethyl substituent to yield 5’-DMI, which has been reported to lack anti-inflammatory properties (Shen, 1964; Van Dyke et al., 1982). In contrast, 5’-DMI exhibited comparable mCRTH2 binding affinity ($K_i$ = 3.9 ± 1.2 μM) to indomethacin (Fig. 3B). Removal of the 2-methyl substituent (2-desmethyl indomethacin; 2-DMI) resulted in a 10-fold decrease in binding affinity ($K_i$ = 15 ± 4 μM). Thus, the 2-methyl but not the 5’-O-methyl substituent contributes to the CRTH2 binding affinity of indomethacin. In addition, indomethacin possesses a p-substituted N-acyl moiety, a structural feature that is present in sulindac sulfate and zomepirac. Comparison of the binding affinities for 2-DMI and 5-hydroxy indole acetic acid (5-HIAA), which differs principally in the absence or presence of the N-acyl moiety, strongly suggests that this substituent is essential for high-affinity binding to mCRTH2. The p-bromobenzyl indomethacin derivative (PBI) also bound to mCRTH2 with similar affinity as indomethacin ($K_i$ = 1.2 ± 0.3 μM).

To investigate the role that the carboxylate group plays in indomethacin-CRTH2 interaction, we assessed the ability of the primary amide and methyl ester derivatives of indomethacin to bind to mCRTH2. Neither the methyl ester nor primary amide derivative showed significant binding to mCRTH2 at concentrations up to 30 μM (Fig. 3C), indicating that the carboxylic moiety is critical for binding.

Ramatroban was recently reported to be a CRTH2 antagonist (Sugimoto et al., 2003) and shares structural similarity with arylacetic acid NSAIDs (Fig. 2). Ramatroban displaced [3H]PGD$_2$ binding with high affinity in our binding assays ($K_i$ = 28 ± 6 nM; data not shown), which is in good agreement with the reported $K_i$ values of 47 ± 13 nM and 60 ± 13 nM for human and mouse CRTH2 receptors expressed in L1.2 mouse B cell leukemia cells (Shichijo et al., 2003). We next investigated the agonist activity of compounds having significant binding activity at mCRTH2 in [cAMP] inhibition and chemotaxis assays. Indomethacin, 5’-DMI, and PBI (1 μM) fully activated mCRTH2, leading to inhibition of [cAMP]$_{i}$ generation in ER293/mCRTH2 cells as demonstrated by a maximal inhibitory effect equivalent to that of the endogenous agonist PGD$_2$ (Fig. 4A). Sulindac sulfate was also capable of achieving a maximal inhibitory effect at higher concentrations (10 μM; data not shown). Similar to the rank order of relative binding affinities, 5’-DMI exhibited similar potency as indomethacin (IC$_{50}$ = 7 ± 3 and 2.5 ± 0.9 nM, respectively), whereas 2-DMI was 10-fold less potent (IC$_{50}$ = 50 ± 20 nM; Fig. 4B). In contrast, concentrations of zomepirac up to 50 μM did not significantly decrease [cAMP]$_{i}$ levels (Fig. 4C) despite its similar binding affinity to indomethacin. To determine whether zomepirac is a mCRTH2 antagonist, we evaluated whether it was capable of antagonizing indomethacin-induced inhibition of [cAMP]$_{i}$. At high concentrations, zomepirac partially antagonized the effects of indomethacin (Fig. 4D), whereas the CRTH2 antagonist ramatroban fully reversed the indomethacin-induced inhibition of [cAMP]$_{i}$ (ramatroban IC$_{50}$ = 120 ± 50 nM; data not shown). Consistent with their ability to act as mCRTH2 agonists and inhibit [cAMP]$_{i}$, indomethacin, PBI, 5’-DMI, 2-DMI, and sulindac sulfate but not zomepirac (100 nM)
stimulated migration of mCRTH2-transfected ER293 cells (Fig. 5).

To confirm previous reports that 5'-DMI lacks COX inhibitory activity (Shen, 1964; Van Dyke et al., 1982), we tested 5'-DMI for its ability to directly inhibit COX-1 and -2 in vitro. It is surprising that 5'-DMI showed identical inhibition of mouse COX-2 compared with indomethacin (IC50, 250 nM for both) and slightly lower activity in inhibiting sheep COX-1 (IC50 = 210 and 35 nM, respectively).

To visualize potential ligand-receptor interactions, we modeled the mCRTH2 receptor using a combination of de novo and homology modeling techniques starting from the transmembrane helical bundle of a previously described model of the β2-adrenergic receptor (Furse and Lybrand, 2003). Similar to other small-molecule binding G protein-coupled receptors, the putative ligand binding pocket is located within the top (extracellular) one-third of the transmembrane helical bundle (Fig. 6A). Ligand docking simulations yielded several different low energy conformations for indomethacin within the ligand-binding pocket. It is interesting that in one low-energy indomethacin-mCRTH2 complex, the acetate carboxylate of indomethacin forms a charge stabilized hydrogen bond with Lys209 in helix V (Fig. 6B), and the p-chlorobenzoyl moiety is positioned to form hydrophobic stacking interactions with the aromatic ring of Phe110 if helix III (Fig. 6C).

Discussion

Although indomethacin is predominantly known as a nonselective COX inhibitor, it has been shown to elicit a number of COX-independent effects, including activation of peroxisome proliferator-activated receptor γ, leading to adipocyte differentiation (Lehmann et al., 1997), inhibition of malignant transformation of embryonic fibroblasts (Zhang et al., 1999), and inhibition of the PGD2 11-ketoreductase AKR1C3 (Lovering et al., 2004). It has also been demonstrated to stimulate cofilinoship shape change, CD11b expression, and chemotaxis via direct activation of the CRTH2 receptor (Hirai et al., 2002; Stubbs et al., 2002). Hirai and others have reported that indomethacin, but not other NSAIDs such as aspirin and ibuprofen, is an agonist at the human CRTH2 receptor (Hirai et al., 2002; Stubbs et al., 2002), and we confirmed that the mouse CRTH2 ortholog shares a similar pharmacological profile (Hata et al., 2003). The reported binding affinity constants (K_i) for indomethacin at the human CRTH2 receptor vary from 25 nM (Sawyer et al., 2002) to 8 μM (Hirai et al., 2002). The reason for this discrepancy is unclear; however, the assay conditions used in the present binding studies support affinities for indomethacin at both human and mouse CRTH2 receptors in the low micromolar range. The K_i value for sulindac sulfide at hCRTH2 has also been reported (3.5 μM; Sawyer et al., 2002) and is similar to the value of 2.5 μM for mCRTH2.

Although the structure-activity relationships of alylactic acid NSAIDs as selective and nonselective COX inhibitors have been characterized (Kalugutkar et al., 2000b), little is known about the structural determinants necessary for CRTH2 binding. No binding was observed for compounds that included only the indole or indole-acetic acid core, suggesting that the alylactic acid core alone is not sufficient for binding. On the other hand, the presence of the p-substituted N-acyl moiety seemed to correlate with mCRTH2 binding affinity. For instance, both 5'-DMI and 2-DMI bind to mCRTH2 with micromolar affinity, whereas 5-HIAA, which

Fig. 4. mCRTH2-evoked inhibition of FSK-stimulated [cAMP], by alylactic acid NSAIDs and indomethacin derivatives. ER293/mCRTH2 cells were stimulated with 10 μM forskolin in the absence (control) or presence of NSAIDs or indomethacin derivatives for 10 min and [cAMP] levels were determined as described under Materials and Methods. A, NSAIDs (1 μM) exhibit differing potencies for inhibiting [cAMP]; indomethacin maximally inhibits [cAMP] to a similar degree as the endogenous ligand PGD2 (100 nM). B, 5'-DMI (circles) exhibits similar potency to indomethacin (squares) for inhibiting [cAMP], whereas 2-DMI (diamonds) was 10-fold less potent. C, zomepirac does not inhibit [cAMP] at concentrations up to 50 μM. D, zomepirac is a weak antagonist of mCRTH2-evoked inhibition of [cAMP], by indomethacin. ER293/mCRTH2 cells were incubated with varying concentrations of zomepirac in the presence of 0.01 μM indomethacin. The ability of 1 μM ramatroban, a known CRTH2 antagonist, to reverse the inhibition of [cAMP], by indomethacin is shown for comparison. For A, C, and D, each data point was determined in triplicate and the mean value from 3–8 independent experiments is shown (error bars represent S.E.M.). For B, each data point was determined in duplicate; data are representative of three independent experiments. * p < 0.05 compared with control (FSK-stimulated in absence of ligand).
differs principally in the absence of this moiety, showed no significant binding at concentrations tested. Zomepirac, which lacks the indole acetic acid core but possesses the identical N-(p-chlorobenzoyl) group to indomethacin, binds with similar affinity to indomethacin. Our molecular modeling studies suggest that this moiety could form hydrophobic interactions with the aromatic ring of Phe110 of helix III. Beyond the apparent requirement for the p-substituted N-acyl moiety, the ligand-binding pocket of mCRTH2 tolerates slight variations in substituent size at the para position as demonstrated by the fact that sulindac sulfide and PBI have similar binding affinities. Binding studies also revealed that the 2-methyl substituent of indomethacin plays a role, albeit less important, in mCRTH2 binding. 2-DMI possesses a 10-fold lower affinity for mCRTH2 compared with indomethacin, and a similar relative difference was observed in ability to inhibit FSK-stimulated [cAMP].

Interactions between the negatively charged carboxylate moiety of a ligand and positively charged residues within the ligand binding pocket have been implicated in ligand binding and activation of prostanoid (Negishi et al., 1995; Audoly and Breyer, 1997) and chemoattractant receptors (DeMartino et al., 1995; Mills et al., 2000). Neutralization of the indomethacin carboxylate moiety by derivatization to the methyl ester or primary amide form abolished mCRTH2 binding. By comparison, neutralization of the carboxylate moiety of indomethacin decreases COX-1 but slightly increases COX-2 inhibitory activity, leading to a substantial enhancement of COX-2 selectivity (Kalgutkar et al., 2000b). Although the

![Fig. 5. Arylacetic acid NSAIDs and indomethacin derivatives stimulate chemotaxis of ER293/mCRTH2 cells.](image)

Fig. 5. Arylacetic acid NSAIDs and indomethacin derivatives stimulate chemotaxis of ER293/mCRTH2 cells. Migration of ER293/mCRTH2 (solid columns) or vector control ER293/pEGSH cells (open columns) in response to PGD₂, arylacetic acid NSAIDs and indomethacin derivatives (100 nM) was assessed as described in Materials and Methods. Data are expressed as percentage of cells migrating in the absence of added ligand (control). The average number of migrated cells from five high-power fields was determined for each experimental condition, and the mean value from three to five independent experiments is shown; error bars represent S.E.M. *, p < 0.05 compared with vector-transfected cells.

![Fig. 6. Molecular modeling of mCRTH2-indomethacin complexes.](image)

Fig. 6. Molecular modeling of mCRTH2-indomethacin complexes. A, side (left) and extracellular (right) views of indomethacin (red spheres) docked in the putative ligand binding pocket of mCRTH2. B, acetic acid carboxylate of indomethacin (red) forms a charge stabilized hydrogen bond with Lys209 (red) of helix V (inset, close-up view showing position of the Lys209 hydrogen participating in hydrogen bond). C, position of the p-chlorobenzyl moiety of indomethacin (red) suggests a hydrophobic stacking interaction with the aromatic ring of Phe110 (blue) of helix III.
potential impact of subtle volume differences between the indomethacin free acid and neutral derivatives cannot be ignored, it is likely that the decrease in binding affinity is caused by the loss of the negative charge and potential disruption of an interaction with a positively charged residue side chain within the binding pocket. The primary structure of mCRTH2 reveals several positively charged residues that may be candidates for this interaction including Lys209 in helix V, which is analogous to Arg205 in the formyl peptide receptor and Arg206 in the C5a receptor, both of which have been demonstrated to interact with ligand (DeMartino et al., 1995; Mills et al., 2000). Our molecular modeling studies support this possibility and suggest the presence of a charge-stabilized hydrogen bond between Lys209 and the indomethacin carboxylate.

In most instances, the arylacetic acid NSAIDs and indomethacin derivatives functioned as full mCRTH2 agonists and inhibited [cAMP], and stimulated cell migration to a similar magnitude as the endogenous ligand PGD2. However, zomepirac failed to inhibit [cAMP], even at high ligand concentrations where >90% of the receptor would be expected to be occupied by ligand (i.e., 50 μM). On the other hand, zomepirac weakly antagonized the ability of indomethacin to inhibit [cAMP], at high concentrations, raising the possibility that this class of molecules may be exploited for the development of CRTH2 antagonists. Ramatroban, the only CRTH2 antagonist reported to date, shares structural similarity with indomethacin, including a negatively charged carboxylate and a p-substituted acyl moiety. It is worth noting that indomethacin and its desmethyl derivatives are capable of activating mCRTH2 at nanomolar concentrations despite having micromolar binding affinities (EC50/[cAMP] and Kᵢ for indomethacin are 2.5 nM and 1.5 μM, respectively), whereas the difference between EC50 and Kᵢ for PGD2 is much smaller (EC50/[cAMP] and Kᵢ = 0.9 and 38 nM, respectively; Hata et al., 2003). A similar phenomenon has been reported for the human CRTH2 ortholog whereby indomethacin possesses micromolar binding affinity but stimulates intracellular calcium mobilization at nanomolar concentrations and PGD2 possesses nanomolar binding affinity and stimulates intracellular calcium mobilization at nanomolar concentrations (Duggan et al., 1972; Vree et al., 1993). 5'-DMI, a minor metabolite that is present in plasma and urine (Duggan et al., 1972; Vree et al., 1993). 5'-DMI has been reported to lack anti-inflammatory activity (Shen, 1964; Van Dyke et al., 1982) suggesting that it has lost the ability to inhibit cyclooxygenase. We found that 5'-DMI has similar potency to indomethacin for activating mCRTH2, initially suggesting that 5'-DMI may be a selective arylacetic acid-class CRTH2 ligand. However, further in vitro studies directly examining the ability of 5'-DMI to function as a COX inhibitor revealed that it is comparable with indomethacin in its ability to inhibit COX-2 and only slightly less potent for COX-1.

Several NSAIDs, including indomethacin, have been reported to negatively impact neutrophil function and inhibit binding of the bacterial-derived peptide f-Met-Leu-Phe (fMLP) to the surface of neutrophils (Cost et al., 1981). This is especially intriguing in light of the present studies, because the CRTH2 receptor shares significant sequence identity with the formyl peptide receptor (Nagata et al., 1999a), which is responsible for mediating the neutrophil response to the fMLP. However, whether the effects of NSAIDs on these two related receptors are caused by similarities in receptor structure is unclear. The effect of NSAIDs on fMLP binding does not seem to involve competitive binding to the fMLP binding site within the formyl peptide receptor (Minta and Williams, 1985; Perianin et al., 1987), in contrast to the hypothesized interaction between indomethacin and CRTH2. This study demonstrates that the CRTH2 receptor is selective for arylacetic acid NSAIDs, whereas the inhibitory effect on fMLP binding to neutrophils is also observed for nonarylacetic acid NSAIDs such as ibuprofen (Sukbitz and Hammarschmidt, 1986; Shelly and Hoff, 1989).

Previous studies have suggested that the ability of the CRTH2 receptor to be activated by indomethacin is not shared by the DP receptor (Hirai et al., 2002). However, indomethacin has been reported to bind to the human and mouse DP receptors, albeit with an order of magnitude lower affinity than CRTH2 (Kᵢ ~ 10 μM; Sawyer et al., 2002; Hirai et al., 2003). Torisu and coworkers recently reported the generation of novel indomethacin derivatives as antagonists at the mouse DP receptor. Modification of the N-acyl moiety and transformation of the indole-3-acetic acid template to indole-4-acetic acid gave yielded substantial increases in binding affinity and antagonistic activity (Torisu et al., 2004a). Further optimization of the N-acyl moiety to N-methyl benzomorpholine derivatives yielded potent orally active DP receptor antagonists that inhibit ovalbumin-induced vascular permeability in guinea pigs (Torisu et al., 2004b,c). Binding affinities for the four EP receptor isoforms and the IP receptor were also reported, but the affinity for binding CRTH2 was not discussed. Although, the DP and CRTH2 receptors possess different pharmacological profiles (Hirai et al., 2001; Sawyer et al., 2002), the observation that indomethacin and indomethacin derivatives function as CRTH2 agonists/antagonists raises the possibility that these novel DP antagonists may possess activity as agonists or antagonists at CRTH2 as well.

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References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor which causes 50 per cent inhibition (50%) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.
Interaction of NSAIDs with the Mouse CRTH2 Receptor


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