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# Selective Inhibition of Cyclooxygenase-1 and -2 Using Intact Insect Cell Assays

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**ABSTRACT.** We have utilized the baculovirus expression system to develop an *in vitro* intact cell assay for screening nonsteroidal anti-inflammatory drug (NSAID) inhibition of the two isozymes of human cyclooxygenase (prostaglandin endoperoxidase synthase, EC 1.14.99.1). Infected *Spodoptera frugiperda* (sf9) cells expressing either human cyclooxygenase-1 (hCOX-1) or human cyclooxygenase-2 (hCOX-2) were harvested 24 hr postinfection, a time point where all cells are viable and hCOX-1 or hCOX-2 are correctly processed. Cells were distributed to a 96-well plate, preincubated with various NSAIDs, and challenged with 10 μM arachidonic acid; then cyclooxygenase activity was assessed indirectly by prostaglandin $E_2$-specific radioimmunoassay. The rank order of potency of NSAID-mediated inhibitions of hCOX-1 and hCOX-2 paralleled those that have been observed in other cell systems. This sf9 cell-based assay can be utilized for the identification of potent and selective inhibitors of hCOX-1 and/or hCOX-2. Compounds that preferentially inhibit hCOX-2 may provide novel NSAIDs that reduce inflammation while sparing the stomach and kidneys of toxic side-effects seen with current nonselective NSAIDs. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1777–1785, 1996.

**KEY WORDS.** baculoviruses; cyclooxygenase; NSAID; arachidonic acid; prostaglandins; drug inhibition

Prostaglandin endoperoxide synthase (PGHS, EC 1.14.99.1) or COX† is the first enzyme in the prostanoid biosynthetic pathway catalyzing the conversion of arachidonic acid to prostaglandin $H_2$ [1, 2]. The anti-inflammatory, antipyretic, and analgesic effects of NSAIDs result from the inhibition of the COX enzymes, thereby decreasing prostanoid production [2–5]. Two COX isozymes have been identified, the constitutively expressed COX-1 and the inducible COX-2 [6–8]. COX-2, which shares 61% protein identity as well as a conserved catalytic region with COX-1, is induced in various cell types in response to inflammatory and mitogenic stimuli, whereas COX-1 is expressed constitutively in most tissues [6, 8–12]. This differential expression of the two COXs has led to the hypothesis that a selective COX-2 inhibitor may not have the toxic side-effects, such as ulceration of the gastric mucosa, found with the current NSAIDs.

To identify a COX-2 specific inhibitor, selective screening assays must be developed. These can be *in vitro* assays utilizing microsomal membranes or purified enzyme preparations, and cell-based assays in which cells expressing one or the other isoforms of COX are used. There are advantages and disadvantages to both cell-free and cell-based assays, so it is necessary to develop complementary systems. Several studies have indicated that the potency and selectivity of inhibitors differ, depending on whether the values are determined using cell-free COX assays or cell based assays [5, 13–16]. For example, the reported $IC_{50}$ values for indomethacin vary from 0.0015 to 16 μM for COX-1 and 0.0069 to >1000 μM for COX-2 [17]. The assays used to characterize the COX inhibitors and their selectivity have been performed using enzymes from a variety of cell backgrounds, sometimes from different species and with various levels of constitutively expressed COX-1 and induced COX-2 present [17]. The sf9 insect cell expression of hCOX-1 and hCOX-2 has been reported previously [18–22]. In these reports, the two enzymes were characterized with respect to activity and inhibition by NSAIDs using either crude microsomal or purified preparations. Both hCOX-1 and hCOX-2 produced in sf9 cells are correctly glycosylated and trafficked to the microsomal fraction at time points prior to 36 hpi. The infected insect cells have intact membranes and are functionally viable. The cells and the COX produced within these cells then are amenable to being used in a cell-based assay system. This paper demonstrates that the baculovirus overexpression system can be utilized for *in vitro* cell-based assays in addition to cell-free assays.

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† *Abbreviations:* COX, cyclooxygenase; NSAID(s), nonsteroidal anti-inflammatory drug(s); sf9, *Spodoptera frugiperda* cells; hCOX-1, human cyclooxygenase-1; hCOX-2, human cyclooxygenase-2; $PGE_2$, prostaglandin $E_2$; MOI, multiplicity of infection; ECL, enhanced chemiluminescence; hpi, hours postinfection; RIA, radioimmunoassay; ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator; $cPLA_2$, cytosolic phospholipase $A_2$; MAP, mitogen-activated protein; 5-HPETE, (5S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid.

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## MATERIALS AND METHODS

### Cell Culture

sf9 (American Type Culture Collection, Rockville, MD, U.S.A.) cells were cultured in spinner flasks at 27° in Grace's supplemented medium (Gibco-BRL, Mississauga, Ontario, Canada) with 10% heat-inactivated fetal bovine serum (Gibco-BRL) following the protocol of Summers and Smith [23].

### Construction of Recombinant Baculovirus Transfer Vectors

The cDNA for hCOX-1 in pcDNA was a gift from Dr. C. Funk (Vanderbilt University, Nashville, TN, U.S.A.) [24]. The baculovirus expression vector used was pVL941 (Pharmingen, San Diego, CA, U.S.A.). The COX-1 cDNA sequence was released from the COX-1/pcDNA plasmid as a 2.2-kb *Sma*I-*Eco*RV fragment that was ligated into the blunted *Bam*HI site of the pVL941 transfer vector to create COX-1pVL941. The COX-1pVL941 plasmid was transfected into sf9 cells with linearized BaculoGold baculovirus DNA as described by the manufacturer (Pharmingen). Four recombinant baculoviruses containing the COX-1 cDNA sequence that also expressed active COX-1 were identified. One of these was chosen for all subsequent assay infections.

The cloning and construction of hCOX-2 cDNA for baculovirus expression has been described previously [18].

### Production of Recombinant Proteins

Baculovirus infection of 100-mL spinner cultures of sf9 cells was performed essentially as described by Summers and Smith [23]. Briefly, sf9 cells at a density of 1–2 × $10^6$ cells/mL were pelleted by centrifugation at 300 *g* for 5 min, the supernatant was removed, and the cells were resuspended at a density of 1 × $10^7$ cells/mL in the appropriate recombinant viral stock (MOI of 10). Following 1 hr at room temperature, medium was added to adjust the cell density back to ~1 × $10^6$ cells/mL and the cells were cultured in suspension at 27°. The cells used for the cell-based assay were collected at 24 hpi by centrifugation at 700 rpm (100 *g*) for 10 min, washed once in assay buffer, and Hanks' solution buffered with 15 mM HEPES, pH 7.4 (Sigma, St. Louis, MO, U.S.A.) for 10 min. The cells were resuspended gently in assay buffer and examined using a hemacytometer and microscope for cell density and viability by trypan blue exclusion. For comparative studies of the hCOX-1 and hCOX-2 expression in sf9 cells, 250-mL cultures infected with the COX-1 and COX-2 recombinant baculoviruses were used. At specific times post-infection, two aliquots were removed for analysis of protein expression by SDS–PAGE and western blot, one for whole cell analysis (1 mL) and the other for microsomal analysis (30 mL).

### Preparation of Microsomal Protein Fractions and Whole Cell Extracts from Infected sf9 Cells

At various times post-infection, 30 mL of sf9 cells that had been infected with recombinant virus for hCOX-1 or hCOX-2 were centrifuged at 300 *g* for 10 min and the pelleted cells were washed with cold PBS. The cell pellet was resuspended in 4 mL of cold 0.1 M Tris, pH 7.4, 5 mM EDTA and sonicated 3 × 10 sec on ice. Samples were centrifuged for 10 min at 500 *g* at 4° and the resulting supernatant was retained. The pellet was resuspended in 4 mL of cold Tris/EDTA buffer, sonicated, and recentrifuged as described above. The two supernatant fractions were pooled and centrifuged at 4° for 1 hr at 100,000 *g*. The microsomal pellet was resuspended by a 2-sec pulse sonication in 0. 1 M Tris, pH 7.4, 5 mM EDTA.

Whole cell extracts were prepared from 1-mL aliquots of hCOX-1 and hCOX-2 infected sf9 cells. Pelleted cells were washed with PBS (4°), resuspended in 50 μL of water, and disrupted by four cycles of freeze–thaw. Protein concentrations were determined by the Bradford assay with bovine serum albumin as the standard. Specific activity of the crude microsomal COX was measured by oxygen uptake as described previously [18].

### SDS–PAGE and Western Blot Analysis

Whole cell lysates and microsomal fractions (pellet or supernatant) were mixed with SDS sample buffer, heated to 95° for 3 min, and electrophoresed on 10% Tris-glycine acrylamide denaturing gels (Novex, San Diego, CA, U.S.A.). Proteins were transferred electrophoretically to nitrocellulose for 16 hr at 100 mA constant current using a Novex transfer apparatus. The nitrocellulose membranes were blocked for 1 hr at room temperature in 3% powdered milk in 20 mM Tris–HCl, pH 7.5, 500 mM NaCl followed by three washes in the Tris/NaCl buffer containing 0.1% (v/v) Tween 20. The blots were then incubated with a 1/7500 final dilution of either an anti-COX-1 [25] or an anti-COX-2 polyclonal antiserum [25] in Tris/NaCl buffer containing 1% powdered milk and 0.05% (v/v) Tween 20. Immunoreactive proteins were visualized by using donkey anti-rabbit horseradish peroxidase as the second antibody and development by ECL as described by the manufacturer (Amersham Life Science, Oakville, Ontario, Canada).

### Cell-Based Assay and NSAID Titrations

Twenty-four hours after infecting sf9 cells with either hCOX-1 or hCOX-2 recombinant baculovirus, the cells were collected and washed in Hanks' solution buffered with 15 mM HEPES, pH 7.4. The assays were performed as follows, with all pipetting being done on a Tomtec Quadra 96 programmed to mix gently after each addition. Two hundred microliters of Hanks' solution containing either 2 × $10^5$ COX-1 expressing cells or 2 × $10^4$ COX-2 expressing cells plus 1.8 × $10^5$ uninfected sf9 cells was dispensed per well of 96-well polypropyline plates. Inhibitor or DMSO vehicle (2 μL) was added to the appropriate well containing the cell suspension. Following a 15-min drug/DMSO preincubation at 37°, the cells were challenged with 10 μM arachidonic acid (Caymen Chemical Co., Ann Arbor, MI,

U.S.A.) in ethanol (2 μL) and incubated for 10 min. Reactions were terminated by the addition of 20 μL of 1 N HCl, neutralized with 20 μL of 1 N NaOH, the cells were pelleted for 10 min at 300 *g* and the levels of $PGE_2$ in the supernatant were determined by a $PGE_2$-specific RIA (Amersham, Oakville, Ontario, Canada). The concentration of $PGE_2$ was then determined by interpolation from a standard curve and inhibition calculated by comparison of the $PGE_2$ production by drug-treated cells with that of DMSO-treated cells.

NSAIDs studied included diclofenac [2-[(2,6-dichlorophenyl)amino]benzeneacetic acid]; piroxicam [4-hydroxy-2-methyl-3-(pyrid-2-yl-carbamoyl)-2*H*-1,2-benzothiazine 1,1-dioxide]; tolmetin (1-methyl-5-[*p*-toluoyl]pyrrole-2-acetic acid); mefenamic acid (2-[(2,3-dimethylphenyl)amino]benzoic acid); nimesulide (*N*-[4-nitro-2-phenoxyphenyl]methanesulfonamide); tenoxicam (4-hydroxy-2-methyl-*N*-2-pyridinyl-2*H*-thieno[2,3-*e*]-1,2-thiazine-3-carboxamide 1,1-dioxide); ketoprofen (2-[3-benzoylphenyl]propionic acid); fenoprofen ([±]-2-[3-phenoxyphenyl]propionic acid); acetaminophen (4-acetamidophenol); isoxicam; niflumic acid; and flufenamic acid, all purchased from the Sigma Chemical Co. Indomethacin [1-[(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid]; ibuprofen [α-methyl-4-(2-methylpropyl)benzeneacetic acid]; aspirin (2-acetoxybenzoic acid); phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione); flurbiprofen ([±]-2-fluoro-α-methyl-4-biphenylacetic acid); and naproxen (6-methoxy-α-methyl-2-napthaleneacetic acid) were purchased from the Caymen Chemical Co. NS-398 (L-740,516); sulindac sulfide (L-612,835); fenclofenac (L-659,528); salicylate (L-190,090); flosulide (L-742,436); and thioflosulide (L-745,337) were obtained from the Merck sample collection.

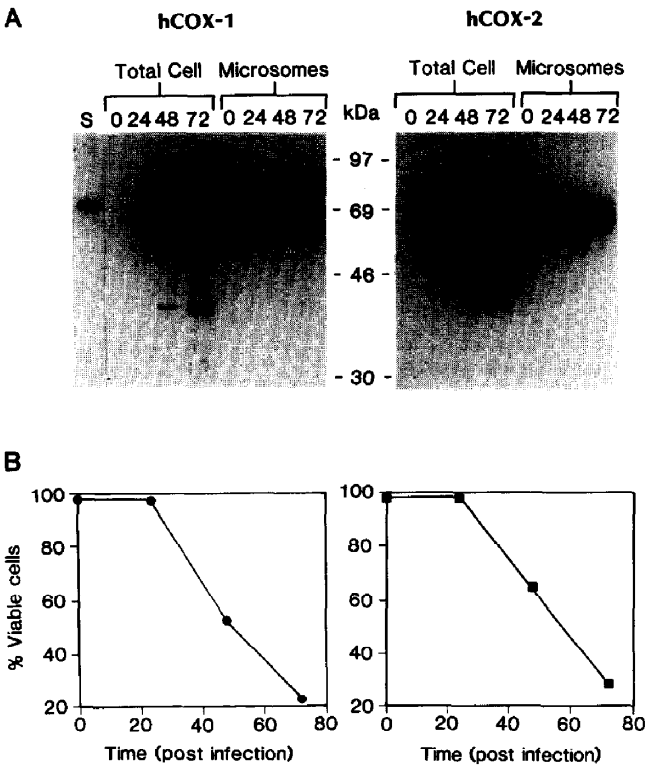


**FIG. 1. Western blot analysis of recombinant baculovirus expressed hCOX-1 and hCOX-2 in sf9 cells as a function of time in culture and cell viability. The sf9 cells (100 mL) at a cell density of 1 × $10^6$ cells/mL were infected with recombinant virus for either hCOX-1 or hCOX-2. At the designated times (0, 24, 48, and 72 hpi), cells were harvested and tested for cell viability (B), and then total cell lysates or microsomal fractions were prepared. Proteins (6.5 μg/lane) from total cell lysate or microsomal pellet were separated by SDS–PAGE, transferred to nitrocellulose, immunoblotted with anti-COX-1 or anti-COX-2 antiserum, and detected by chemiluminescence (A). Lane S, 20 ng of purified ovine COX-1 standard.**

## RESULTS

### *Expression of hCOX-1 and hCOX-2 Recombinant Protein*

Preliminary characterization of hCOX-1 and hCOX-2 expression in recombinant baculovirus infected sf9 cells was carried out to set up a cell-based assay for a selective COX inhibitor screen. A detailed description of hCOX-2 expressed in insect cells has already been published [18]. Figure 1 shows a comparison of the time course of hCOX-1 and hCOX-2 expression in infected sf9 cells. At various times post-infection (t = 0, 24, 48, 72 hpi), cells were harvested and a cell count in trypan blue was done to assess cell viability (Fig. 1B). Cyclooxygenase protein in total cell lysate and microsomes was then measured by western blot analysis (Fig. 1A). hCOX-1 and hCOX-2 were both detected as early as 24 hpi, where there was 97–98% cell viability. At this time point, fully glycosylated hCOX-1 and hCOX-2 were observed both in total cell lysates and more specifically in microsomal membranes. In addition, the 24 hpi time point had the highest proportion of expressed COX in the microsomal fraction. Furthermore, hCOX-1 appeared as a homogenous single species on SDS–PAGE, co-migrated with purified ram seminal vesicle COX-1 (Fig. 1A; lane S) and had a molecular mass of 72 kDa. hCOX-2 was detected as two closely migrating species of 72 and 74 kDa. This heterogeneity has been attributed to differential glycosylation at Asn 580 [26]. At 24 hpi, hCOX-1 and hCOX-2 were processed efficiently. Later than 48 hpi, cell viability decreased dramatically while the amount of immunoreactive COX species increased tremendously. At these later stages of infection, multiple glycosylation states of hCOX-1 and hCOX-2 were seen along with the completely unglycosylated forms of the two enzymes. Several COX immunoreactive bands of molecular mass less than 66 kDa were detected and presumably represent proteolytic breakdown products of hCOX-1 or hCOX-2.

The hCOX-1 and hCOX-2 enzyme activities in 24 hpi sf9 cells and microsomes are shown in Table 1. Intact uninfected sf9 cells have a basal level of COX activity as evidenced by the marginal increase in output of $PGE_2$ by the cells upon challenge with 10 μM arachidonic acid.

**TABLE 1. hCOX-1 and hCOX-2 enzyme activities at 24 hr post-infection in microsomes and in intact sf9 cells**

| | Specific activity | | |
|---|---|---|---|
| | sf9 | hCOX-1 | hCOX-2 |
| Microsomes (nmol $O_2$/min/mg) | | | |
| -AA | 0 | 0 | 0 |
| +AA | 0 | 0.39 | 4.7 |
| Intact cell (ng $PGE_2/10^6$ cells) | | | |
| -AA | 0.025 | 0.98 | 17.2 |
| +AA | 0.23 | 9.80 | 153.0 |

Microsomes were prepared from uninfected sf9 cells and sf9 cells infected with recombinant baculovirus for either hCOX-1 or hCOX-2 by high speed centrifugation as described in Materials and Methods. Specific activity was determined by oxygen uptake in the absence (-AA) or presence (+AA) of 100 μM arachidonic acid. Intact uninfected sf9 cells and sf9 cells infected with recombinant baculovirus for hCOX-1 or hCOX-2 were harvested by gentle centrifugation, washed with PBS, and resuspended in Hanks' buffer containing 15 mM HEPES, pH 7.4, at 37° as outlined in Materials and Methods. Specific activity was determined by RIA of the $PGE_2$ produced in the absence (-AA) or presence (+AA) of 10 μM arachidonic acid substrate.

Intact sf9 cells infected with either hCOX-1 ($2 \times 10^5$) or hCOX-2 ($2 \times 10^4$) recombinant baculovirus had detectable levels of COX activity in the absence of arachidonic acid. Addition of arachidonic acid to these cells resulted in about a 10-fold increase in the output of $PGE_2$ by both hCOX-1 and hCOX-2 with specific activities of 9.8 ng $PGE_2/10^6$ cells and 153 ng $PGE_2/10^6$ cells, respectively. Measurement of COX activity in both the microsomes and the intact cells, in the presence of arachidonic acid, indicated that the specific activity of hCOX-2 is 12- to 15-fold higher than that of hCOX-1.

Therefore at 24 hpi, the intact infected sf9 cells were highly viable, had significant levels of correctly processed COX protein, and were capable of converting exogenously added arachidonic acid to $PGE_2$, suggesting that they could be amenable to a cell-based assay.

### *Cell-Based Assay Development*

Further characterization of the COX insect cell-based assay at 24 hpi was carried out with respect to levels of $PGE_2$ production in response to arachidonic acid concentration and time of challenge. The number of cells was adjusted so as to have equal amount of hCOX-1 and hCOX-2 enzyme activity in the assays. Therefore, there were $2 \times 10^5$ COX-1 expressing cells/assay or $2 \times 10^4$ COX-2 expressing cells/assay, with the total cell number per assay ($2 \times 10^5$) being the same since the COX-2 assays were supplemented with $1.8 \times 10^5$ uninfected sf9 cells. When the time of substrate challenge was held constant at 10 min and the final arachidonic acid concentration was varied between 0 and 100 μM, the $PGE_2$ production by infected cells expressing hCOX-1 and hCOX-2 followed similar patterns. Figure 2 represents the typical $PGE_2$ output profiles. In the absence of exogenously added arachidonic acid, the production was very low, 0.5 and 1.8 ng $PGE_2$/mL for hCOX-1 and hCOX-2, respectively. This, however, increased 25-fold for hCOX-2 and 75-fold for hCOX-1 upon the addition of 0.1 μM final concentration arachidonic acid. Maximum levels over background (~40 ng $PGE_2$/mL) were achieved with 1–10 μM arachidonic acid for both hCOX-1 and hCOX-2. The extent of $PGE_2$ synthesis was at most 1.7-fold greater at 37° compared with room temperature incubated cells. It should be noted that uninfected sf9 cells do respond in a concentration-dependent manner to arachidonic acid challenge, although a maximum $PGE_2$ output of only 0.2 ng $PGE_2/10^6$ cells was attained with 10 μM arachidonic acid (data not shown).

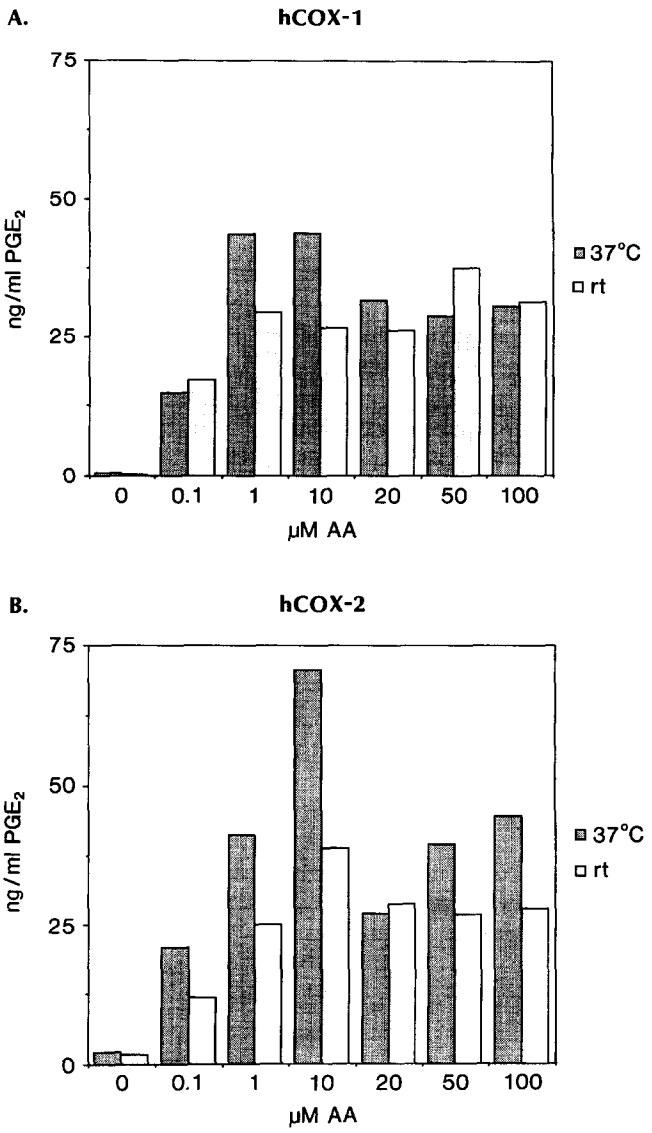


**FIG. 2. Typical $PGE_2$ production profiles of intact sf9 cells infected with recombinant hCOX-1 or hCOX-2 baculovirus as a function of arachidonic acid concentration. At 24 hpi, hCOX-1 (A) or hCOX-2 (B) infected cells ($1 \times 10^6$ cells/mL) were harvested, washed, and resuspended in Hanks'/HEPES buffer, pH 7.4, at either 37° (dark shaded bars) or room temperature (light shaded bars). The intact cells were challenged with 0, 0.1, 1, 10, 20, 50, and 100 μM arachidonic acid (AA) for 10 min. COX activity was determined indirectly by measuring the amount of $PGE_2$ synthesized, using a $PGE_2$-specific RIA.**

The time course of $PGE_2$ production from infected sf9 cells in the absence or presence of 10 μM arachidonic acid is shown in Fig. 3. Prostaglandin synthesis increased over the first 7 min, reaching a plateau by 10 min for both hCOX-1 and hCOX-2, at either 37° or room temperature. Incubations at 37° produced in the range of 1.5-fold more $PGE_2$ than incubations at room temperature. The infected sf9 cells expressing either hCOX-1 or hCOX-2 were also incubated for 1 hr at 37° or room temperature followed by a 10-min, 10 μM arachidonic acid challenge. Under these conditions there was a slight increase in the amount of $PGE_2$ produced at the 1-hr post-harvest time point as compared with that produced immediately following cell harvesting, indicating that the cells were viable and metabolically active (data not shown). The extent of $PGE_2$ synthesis at 10 min with 10 μM arachidonic acid was near maximal and was selected as a measure of COX activity in the cell.

The assay conditions used for all further experiments were as follows. The infected sf9 cells expressing either hCOX-1 or hCOX-2 were harvested 24 hpi and resuspended in HEPES-buffered Hanks' solution, pH 7.4, at 37°. The cells ($2 \times 10^5$ infected for hCOX-1 or $2 \times 10^4$ infected plus $1.8 \times 10^5$ uninfected for hCOX-2) were challenged for 10 min with 10 μM arachidonic acid (final concentration), and following termination/neutralization of the reaction, the levels of $PGE_2$ were determined by RIA.

### *Characterization of NSAID Inhibition*

The potency and selectivity of various NSAIDs at inhibiting COX activity in intact infected sf9 cells expressing comparable amounts of recombinant hCOX-1 or hCOX-2 were evaluated. Inhibitor time-dependency, which has been observed for many NSAIDs, was addressed by preincubating the drug with the infected sf9 cells for 15 min prior to the 10-min challenge with 10 μM arachidonic acid. The NSAIDs chosen for this study included potent anti-inflammatory agents currently available, which reportedly inhibit hCOX-1 more than hCOX-2, or hCOX-1 and hCOX-2 equally [17]. Also tested were three compounds that have been shown to inhibit hCOX-2 selectively in various other systems [16, 27–30]. Finally, acetaminophen and salicylate were included as they have analgesic, antipyretic and anti-inflammatory properties but do not seem to inhibit COX in *in vitro* assays [13, 17]. All of the NSAIDs assayed except acetaminophen and salicylate were effective inhibitors of baculovirus expressed hCOX-1 or hCOX-2. The tested compounds could be grouped into four categories based on their ability to inhibit $PGE_2$ production: equipotent, hCOX-1 selective, hCOX-2 selective, or non-COX inhibitors. Using the $IC_{50}$ ratio of COX-2 to COX-1, arbitrary cutoff ratios were chosen to classify the compounds into categories. Those compounds with a ratio of <5 were considered as equipotent against hCOX-1 and hCOX-2. A ratio of >10 was used to classify an inhibitor as hCOX-1 selective, while an index of 0.01 or less indicated that the compound was selectively inhibiting hCOX-2. Representative inhibition profiles, obtained with this cell-based assay system, illustrating the magnitude of the differences in $IC_{50}$ values for each group are shown in Fig. 4. Table 2 lists the $IC_{50}$ values (potency index) for each of the compounds on hCOX-1 and hCOX-2 as well as the COX-2/COX-1 ratio (selectivity index). Inhibitors of prostaglandin synthesis have come from a variety of structural classes [31]. In this study, it is interesting to note that compounds from the fenamic acid and enolic acid families, along with a few aryl

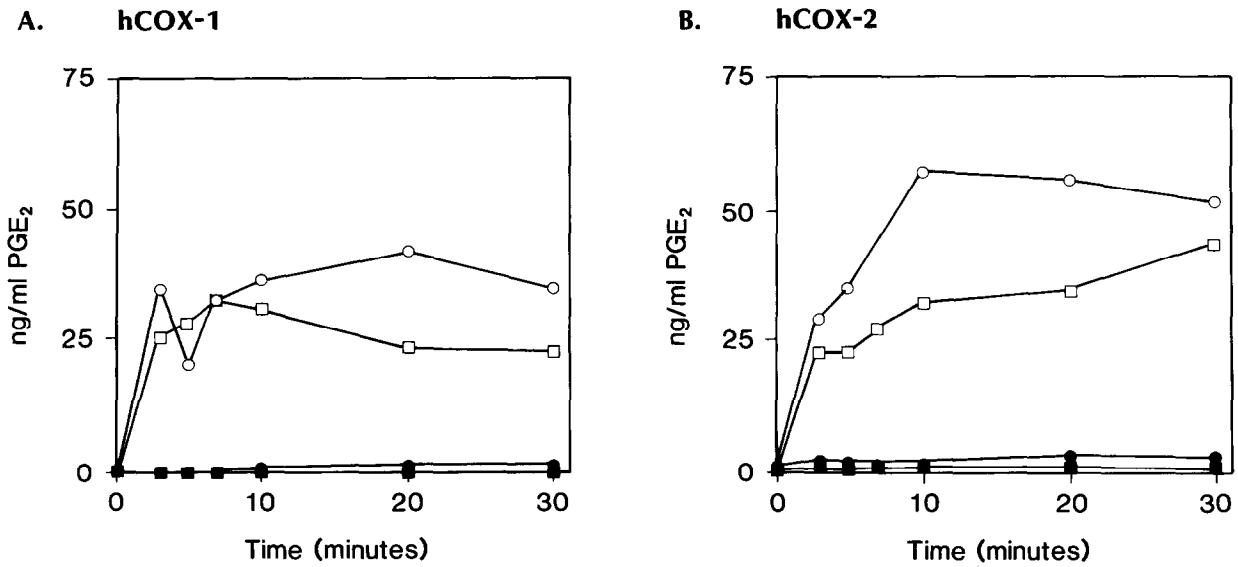


**FIG. 3. Time course of $PGE_2$ production by intact sf9 cells infected with recombinant baculovirus for hCOX-1 or hCOX-2. At 24 hpi, hCOX-1 (A) or hCOX-2 (B) infected cells ($1 \times 10^6$ cells/mL) were harvested by gentle centrifugation, washed, and resuspended in Hanks'/HEPES buffer, pH 7.4, at either 37° or room temperature. The amount of $PGE_2$ produced at various time points in the absence of arachidonic acid at room temperature (■) and 37° (●) or in the presence of 10 μM arachidonic acid at room temperature (□) and 37° (○) was measured by RIA.**

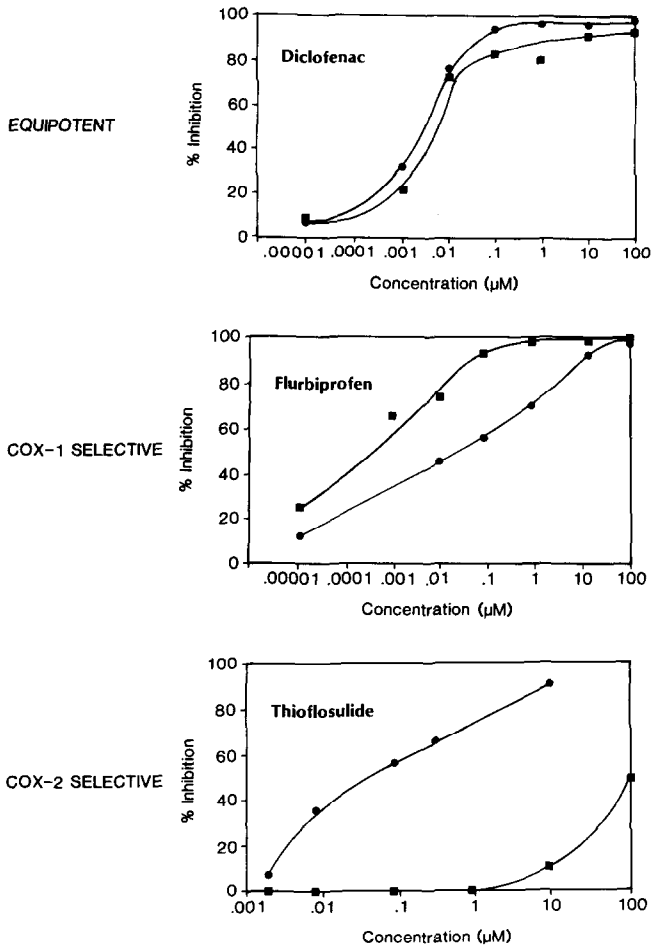


**FIG. 4. Representative inhibition profiles for hCOX-1 and hCOX-2 activities in intact sf9 cells by an equipotent, a COX-1 selective, and a COX-2 selective NSAID. $PGE_2$ synthesis by intact sf9 cells expressing hCOX-1 and hCOX-2 upon 10 μM arachidonic acid challenge was measured by $PGE_2$-specific RIA. Control synthesis levels were 20 ng/mL $PGE_2$ for both hCOX-1 and hCOX-2. Key: (■) hCOX-1 inhibition curves; and (●) hCOX-2 inhibition curves. The observed $IC_{50}$ values (in μM) were: diclofenac, 0.004 on hCOX-1 and 0.003 on hCOX-2; flurbiprofen, 0.0003 on hCOX-1 and 0.03 on hCOX-2; and thioflosulide, 100 on hCOX-1 and 0.05 on hCOX-2.**

acetic acid-related structures, fall into the equipotent group showing less than a 5-fold preference for either hCOX-1 or hCOX-2. hCOX-1 selective compounds were strictly aryl acetic acid-related structures, which inhibited hCOX-1 10- to 90-fold more potently than they inhibited hCOX-2. The third group consisted of three sulfonamides; NS-398, flosulide and thioflosulide, which were between 350- and 1925-fold better at inhibiting hCOX-2 than hCOX-1. The only sulfonamide that did not fall into this group was nimesulide, which was classified, using the arbitrary cutoff ratios, as equipotent. It, however, fell on the outer limits of equipotent, showing ~6-fold selectivity towards hCOX-2. The final group consisted of acetaminophen and salicylate, which even at concentrations as high as 100 μM had no detectable effects on $PGE_2$ output by hCOX-1 or hCOX-2 in this system. These results favorably agree with those published

**TABLE 2. $IC_{50}$ Values of various NSAIDs on hCOX-1 and hCOX-2 activity in sf9 cells at 24 hr postinfection**

| Inhibitor | $IC_{50}$ (μM)* hCOX-1 | hCOX-2 | COX-2/COX-1 |
|---|---|---|---|
| Equipotent | | | |
| Diclofenac | 0.003 | 0.006 | 2 |
| Sulindac sulfide† | 0.06 | 0.29 | 4.8 |
| Mefenamic acid | 0.15 | 0.28 | 1.8 |
| Tolmetin | 0.58 | 1.44 | 2.5 |
| Flufenamic acid | 2.8 | 5.3 | 1.9 |
| Aspirin | 4.2 | 15.9 | 3.8 |
| Nimesulide | 5.2 | 0.87 | 0.16 |
| Niflumic acid | 5.6 | 2.3 | 0.41 |
| Piroxicam | 0.8 | 0.53 | 0.66 |
| Tenoxicam | 17.8 | 36.9 | 2.1 |
| Phenylbutazone | 21 | 61 | 2.9 |
| Isoxicam | >50 | >75 | 1.5 |
| Fenclofenac | >100 | >78 | 0.79 |
| COX-1 selective | | | |
| Flurbiprofen | 0.0008 | 0.069 | 87 |
| Ketoprofen | 0.0015 | 0.12 | 82 |
| Indomethacin | 0.0019 | 0.052 | 27 |
| Naproxen | 0.45 | 6.3 | 14 |
| Fenoprofen | 1.96 | 26.3 | 13 |
| Ibuprofen | 2.9 | >50 | 17 |
| COX-2 selective | | | |
| NS-398 | 7.7 | 0.004 | 0.0005 |
| Flosulide | >18 | 0.02 | 0.001 |
| L-745,337‡ | >93 | 0.26 | 0.003 |
| Non-COX selective | | | |
| Acetaminophen | >100 | >100 | |
| Salicylate | >100 | >100 | |

The level of COX activity in intact sf9 cells infected with recombinant hCOX-1 or hCOX-2 baculovirus was determined by measuring $PGE_2$ synthesis at 37°. The sf9 cells (24 hpi) were washed and resuspended in Hanks/HEPES buffer, pH 7.4, preincubated with NSAID for 15 min, and then challenged for 10 min with 10 μM arachidonic acid. $PGE_2$ synthesis was determined by RIA. Using the COX-2/COX-1 ratio of $IC_{50}$ values as a selective index, the NSAIDs were classified as follows. Compounds with a ratio of <5 were considered as equipotent against hCOX-1 and hCOX-2. hCOX-1 selective NSAIDs had a selective index of >10, while hCOX-2 selective compounds showed COX-2/COX-1 ratios of <0.01.

* Values represent the average of three or more determinations.

† The active metabolite of sulindac.

‡ Thioflosulide.

for other cell-based assay systems. For example, diclofenac, indomethacin, ketoprofen, and flurbiprofen, which have been reported to inhibit COX potently in other cell-based assays [16, 27, 28], inhibited the human COXs expressed in sf9 insect cells with very low nanomolar inhibitor concentrations. The reported hCOX-2 specific compounds, NS-398, flosulide, and L-745,337 [16, 27, 28], were potent selective inhibitors of hCOX-2 expressed in this baculovirus system. Laneuville *et al.* [13] have expressed the hCOX isozymes in *cos*-1 cells and measured instantaneous inhibition by common NSAIDs. Somewhat different potencies were obtained as compared with this reported assay due to the issue of time-dependent inhibition exhibited by some compounds.

## DISCUSSION

Much interest has developed over the possibility of inhibiting the two COX isozymes potently and selectively. Drugs

that preferentially inhibit COX-2 may have a therapeutic advantage over current NSAIDs since they would have anti-inflammatory properties without renal and gastrointestinal toxicity. To develop such inhibitors, a supply of hCOX-1 and hCOX-2 is required. The baculovirus system, in particular, has been shown to provide sufficient recombinant cyclooxygenase to carry out enzyme characterization, such as mechanistic/kinetic studies and X-ray crystallography. The purified enzyme or the microsomes from infected sf9 cells expressing hCOX-1 or hCOX-2 can be used in *in vitro* screening assays. The results presented in this study have shown that in addition to these uses, the baculovirus expression system can be developed into a cell-based assay for use in COX-2 drug screening. Using intact sf9 cells, it is possible to detect potent and highly selective compounds that inhibit either hCOX-1, hCOX-2, or both enzymes.

The major use of the baculovirus expression system to date has been for high level production of proteins or enzymes of interest for purification and characterization. For this purpose, cells are usually harvested at time points later than 48 hpi. The ER secretion pathway of insect cells, however, is extremely compromised in the latter stages of the baculovirus infection cycle and, therefore, proteins that require extensive membrane trafficking are produced at much lower levels [32]. In addition, deleterious effects upon the integrity of the plasma membrane and the depolarization of microtubules, which may be essential for secretion, occur late in infection [33]. Earlier in the infective cycle, sf9 cells are very healthy and viable. Up to 36 hpi and prior to the distension of the ER secretory pathway, proper glycosylation and sorting occur when low levels of the protein of interest are being expressed. This window of ~24–36 hpi allows for the use of the infected sf9 cells in cell-based assays. Previous reports have shown that intact infected sf9 cells can be used to study various human proteins and enzymes. Intact infected sf9 cells expressing the CFTR were monitored by both patch clamping and radioiodide efflux to demonstrate a new cyclic AMP-stimulated anion permeability not present in uninfected cells [34]. In sf9 cells, we have been able to assay arachidonic acid release by expressed $cPLA_2$ as measured with GC-MS (data unpublished) and have observed MAP kinase phosphorylation of this same protein by western blot [35]. Dual infection of sf9 cells with recombinant baculoviruses for 5-lipoxygenase and 5-lipoxygenase-activating protein led to the production of 5-HPETE from arachidonic acid and the subsequent synthesis of leukotriene $A_4$ as measured by immunoblot of intact infected cell homogenates [36]. Recently, an intact cell system utilizing recombinant baculoviruses to study enzymes involved in drug and xenobiotic metabolism was described [37].

Prostaglandins have been extracted and measured in a variety of tissues from insects and, in particular, cells associated with the reproductive tract [38]. The sf9 insect cell line is derived from ovarian tissue of the fall armyworm *Spodoptera* [23]. The cellular environment and factors necessary for COX activity are therefore present in insect cells. The COX enzymes appears to be uniformly associated with the microsomal fraction of mammalian cells hCOX-1 and hCOX-2 being localized to the ER and nuclear envelope, respectively [26, 38–40]. In contrast, subcellular localization of prostaglandin biosynthetic activity in insect cells has been inconsistent, with activity being localized to 12,000 or 15,000 *g* pellets for some insect species [38, 41]. Overexpression of the hCOXs in sf9 insect cells shows that at 24 hpi a large proportion of the produced enzymes are properly targeted to the microsomal membrane fraction of the cells. At later time points, COX is found with the nuclear fraction. Since the two isozymes of COX can readily be expressed in sf9 cells, a whole cell assay was developed in which the enzymes could be characterized in the same cellular background with respect to NSAID-mediated inhibition. For a COX sf9 cell-based assay to be useful in screening for potential new NSAIDs, it was imperative to harvest healthy, viable cells prior to the onset of the deleterious effects associated with the later stages of the viral cycle. Therefore, infected sf9 cells were harvested at 24 hpi, a point at which the expressed COXs were fully glycosylated, properly targeted, and active. High throughput assaying was facilitated using a 96-well format and partially mechanizing with a Tomtec Quadra 96 pipetting device. The inhibition of the COX by NSAIDs was solely responsible for the reduction in $PGE_2$ measured. It is unknown whether insect cells contain a $PGE_2$ synthase; thus, the measured $PGE_2$ most likely represents a breakdown product of $PGH_2$ [42]. It is interesting to note that the same sf9 cellular background can be used to look at COXs from many species and compare the species-specific attributes of an inhibitor.

NSAIDs from different structural classes were profiled in this cell system with respect to intrinsic potency and isozyme selectivity. The majority of the twenty-four NSAIDs assayed were either equipotent against hCOX-1 and hCOX-2 or slightly hCOX-1 selective. Two well known NSAIDs on the market, acetaminophen and salicylate, did not inhibit either hCOX-1 or hCOX-2 in this cellular system. Finally, three NSAIDs were shown to be 350- to 1925-fold selective at inhibiting hCOX-2. In general, it is difficult to compare our results with other reported studies. This is due to the fact that many assay conditions have been used. The intrinsic potencies and selective ratios can be affected by factors such as the time of preincubation with enzyme, the substrate concentration, and duration of substrate challenge and by the method of determining COX activity. Many of the studies have used different sources of COX enzyme (recombinant, purified enzymes, microsomal fractions, broken cell extracts or whole cells in culture). Many times the source of COX isoforms came from different species. The studies have indicated that the potency and selectivity of inhibitors determined using intact cells expressing COX differ from values established using cell-free systems [3, 5, 17]. For example, indomethacin and aspirin are between 3- and 25-fold more potent at inhibiting

COX-1 from bovine aortic endothelial cells or COX-2 from endotoxin-activated J774.2 murine macrophage in an intact cell assay, as compared with their inhibition in a broken cell assay [5]. There are, however, published whole cell results for a limited number of compounds that we have tested. As with most other intact cell assays, we have nanomolar range $IC_{50}$ values, compared with micromolar results usually obtained for cell-free systems. There is a good correlation between our sf9 cell-based results and those of Prasit *et al.* [28], in which they inhibited endogenous hCOX-2 in osteosarcoma cells. The $IC_{50}$ values for hCOX-2 inhibition in sf9 cells versus osteosarcoma cells, respectively, were: 6 and 1 nM for diclofenac; 52 and 37 nM for indomethacin; 4 and 1 nM for NS-398; 20 and 50 nm for flosulide; and 290 and 100 nM for sulindac sulfide. Values for hCOX-1 from their study cannot be compared with ours, since they have challenged the hCOX-1 in U937 cells with 0.5 μM arachidonic acid while we have used 10 μM. In a review of the development of selective NSAIDs, Battistini *et al.* [17] reported inhibitors tested on human platelets, IL-1-treated rat mesangial cells, or hCOX-1 and hCOX-2 expressed in *cos*-7 cells. The $IC_{50}$ values on hCOX-1 and hCOX-2 for aspirin, indomethacin, naproxen, ibuprofen, and flosulide are essentially the same as those reported by us in this study.

A major advantage of producing a drug target protein in the baculovirus expression system is that only one expression system needs to be developed. The baculovirus system can supply recombinant protein for enzymatic characterization, X-ray crystallographic studies, *in vitro* purified enzyme and microsomal assays and, as reported here, for cell-based analysis of COX inhibition. There is no need to develop multiple expression systems to carry out through characterizations. The comparison of $IC_{50}$ values of COX inhibition for compounds from different NSAID classes, in the same cell background, should be very useful in clearly highlighting inhibitor structures that are important for interactions with each of the isozymes and hopefully lead to the synthesis of compounds with even greater selectivities.

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