Expression and enzyme activity determination of human cyclooxygenase-1 and -2 in a baculovirus-insect cell system

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KEY WORDS baculoviruses; prostaglandin-endoperoxide synthase; non-steroidal anti-inflammatory agents, prostaglandins; drug inhibition

ABSTRACT

AIM: To develop an *in vitro* intact cell-based assay for screening selective cyclooxygenase inhibitors. **METHODS:** Human cyclooxygenase-1 (hCOX-1) and cyclooxygenase-2 (hCOX-2) genes were cloned from human monocyte cell line THP-1 cells and expressed in *Spodoptera frugiperda* (sf9) insect cell line by Bac-to-Bac baculovirus expression systems. Infected sf9 cells were harvested 24 h post-infection (hpi), and distributed to a 24-well plate, preincubated with various nonsteroidal anti-inflammatory drugs, and challenged with 10 mmol/L arachidonic acid; the cyclooxygenase activity was assessed indirectly by prostaglandin E_2-specific radioimmunoassay. **RESULTS:** Polymerase chain reaction detection demonstrated that hCOX-1 and hCOX-2 were transposed to the bacmid. Western blot analysis showed that infected sf9 cells could express hCOX-1 and hCOX-2 proteins. Radioimmunoassay demonstrated that both recombinant proteins functioned well in sf9 cells. **CONCLUSION:** Human cyclooxygenase-1 and cyclooxygenase-2 were successfully expressed in sf9 insect cell line. It can be utilized for the identification of potent and selective inhibitors of hCOX-1 and/or hCOX-2.

INTRODUCTION

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) enzymes contain both cyclooxygenase and peroxidase activities. COX catalyzes the first step in the biosynthesis of prostaglandins (PGs), thromboxanes, and prostacyclins; the conversion of arachidonic acid to PGH_2[1,2]. The anti-inflammatory, antipyretic, and analgesic effects of nonsteroidal anti-inflammatory drugs (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]. COX-1 is the major enzyme form found in most tissues and seems to be responsible for housekeeping roles for normal physiological functions including maintenance of the integrity of the gastric mucosa and regulation of renal blood flow[7]. In contrast, COX-2 is induced by endotoxin, cytokines, and mitogens and has been associated with the elevated production of prostaglandins observed during inflamma-tion, pain, and pyretic responses[3,8,9]. It is currently hypothesized that the undesirable side-effects of NSAIDs are due to COX-1 inhibition, whereas the beneficial effects, such as the reduction of swelling and analgesia, are related to COX-2 inhibition[5,6].

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To identify a COX-2 specific inhibitor, selective screening assays must be developed. These can be *in vitro* assays utilizing microsomal membranes or purifed enzyme preparations, and cell-based assays in which cells expressing one or the other isoforms of COX are used[10-16]. In the present studies, we developed *in vitro* intact cell assays for screening COX-1 or COX-2 inhibitors under the same cell backgrounds. The COX-1 and COX-2 were cloned from human monocyte cell line THP-1, and expressed in sf9 insect cell line by using Bac-to-Bac system (GibcoBRL).

MATERIALS AND METHODS

Cell culture and expression system The human monocyte cell line THP-1 and insect cell line *Spodoptera frugiperda* (sf9) were obtained from the Institutes of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences. THP-1 cells were grown in RPMI-1640 medium containing 10 % fetal bovine serum (GibcoBRL). Sf9 cells were cultured in monolayer at 28 ºC in Grace's supplemented medium with 10 % heat- inactivated fetal bovine serum. The Gibco BRL Bac-to-Bac baculovirus expression system consists of the transposing vector pFastBac, CELLFECTIN reagent, and Max Efficiency DH10Bac competent cells which contain bacmid (baculorivus shuttle vector plasmid) and helper plasmid to be used to generate recombinant bacmids.

Cloning and characterization of human COX-1 and COX-2 Total RNA was extracted from THP-1 cells using TRIzol (Invitrogen, Inc) according to the manufacturer's protocol. The RNA (5 μg) was reverse transcribed into cDNA by using M-MLV reverse transcriptase (H^-, point mutant, Promega). The cDNA was synthesized, and primers were designed for polymerase chain reaction (PCR) amplification (Tab 1). PCR was performed with *Pyrobest* DNA polymerase (Takara Biotech), using 40 cycles of 95 ºC for 60 s, 65 ºC for 90 s, and 72 ºC for 5 min, with a final 15-min extension step at 72 ºC. The products were isolated, subcloned into pGEM-T vector (Promega Corp) and sequenced.

Production of recombinant baculovirus DNA Recombinant baculovirus DNA was produced using Bac-to-Bac baculovirus expression systems according to the manufacturer's protocol. Briefly, human COX-1 and COX-2 genes were subcloned into pFastBac donor plasmid and transformed into DH10Bac cells. Recombinant bacmid DNA was isolated and transfected into sf9 cells. After 72 h incubation the virus- containing supernatant was harvested.

Production of recombinant proteins Total 7×10^6 sf9 cells were seeded on a 75-cm^2 cell culture flask, and infected with the appropriate recombinant viral stock (MOI of 10). Following incubation for 1 h at room temperature, medium was added and the cells were cultured at 27 ºC. The cells used for the cell-based assay were collected at 24 h post-infection (hpi) by centrifugation at 500×*g* for 5 min, and washed once in HHBS (Hanks' solution buffered with 15 mmol/L HEPES, pH 7.4). The cells were resuspended gently in HHBS and examined using a hemacytometer and microscope for cell density and viability by trypan blue exclusion.

SDS-PAGE and Western blot analysis Sf9 cells were grown in 75-cm^2 cell culture flasks, and infected with recombinant baculovirus (MOI of 10). At the indicated time-points, pelleted cells were washed with PBS (4 ºC pH 7.4), resuspended in 500 μL ice-cold insect cell lysis buffer (Tris 10 mmol/L pH 7.5, NaCl 130 mmol/L, Triton X-100 1 %, NaF 10 mmol/L, NaPi 10 mmol/L, NaPPi 10 mmol/L) containing protease inhibitor cocktail (aprotinin 10 μg/mL, leupeptin 10 μg/mL, pepstatin A 10 μg/mL, PMSF 1 mmol/L), and lysed cells on ice for 45 min. The lysate was centrifuged at 40 000×*g* for 45 min. Protein concentrations were determined by BCA Protein Assay Kit (Pierce). Whole cell lysates were mixed with SDS sample buffer and heated to 100 ºC for 5 min. Samples, containing 20 μg protein, were electrophoresed in 10 % SDS-polyacrylamide gels and separated proteins were transferred on to a PVDF membrane. The membrane was blocked at 4-8 ºC for overnight with 5 % non-fat dried milk in Tris-buffered saline with Tween 20 (0.1 %) and incubated at room temperature for 1 h with goat polyclonal IgG anti-COX-1 or anti-COX-2 antibodies (Santa Cruz Biotechnology). The appropriate secondary horseradish peroxidase-conjugated antibody (DAKO) was added and complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Drug inhibition assays Cell-based assay was performed as described previously with modifications[15]. Briefly, 24 h after infecting sf9 cells with hCOX-1 or hCOX-2 recombinant baculovirus, the cells were collected and washed in HHBS. The assays were performed as follows. One milliliter of Hank's solution containing either 1×10^6 COX-1 expressing cells or 1×10^5

COX-2 expressing cells plus 9×10^5 uninfected sf9 cells was dispensed per well of 24-well polypropylene plates. Inhibitor or Me_2SO vehicle (10 μL) was added to the appropriate well containing the cell suspension. Following a 15-min drug or Me_2SO preincubation at 37 ºC, the cells were challenged with 10 μmol/L arachidonic acid (Sigma) in ethanol and incubated for 10 min. Reactions were terminated by the addition of 100 μL of 1 mol/L HCl, neutralized with 100 μL of 1 mol/L NaOH. The cells were pelleted for 10 min at 300×*g* and the levels of PGE_2 in the supernant were determined by a PGE_2-specific RIA (Beijing East Asia Institute of Immunology). 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 Me_2SO-treated cells.

Samples tested included diclofenac [2-[(2,6-dichlorophenyl)amino] benzeneacetic acid]; indomethacin [1-[(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid]; and NS-398, all purchased from the Sigma Chemical Co.

RESULTS

Identification of recombinant pFastBac-hCOX-1/hCOX-2 and Bacmid-hCOX-1/hCOX-2 The fragments of gene COX-1 and COX-2 were amplified by PCR using specific primers (Tab 1) and sequenced to ensure the correctness of the open reading frame (ORF). Restriction endonuclease digestion was performed to verify the correct insertion of the gene COX-1 and COX-2 in the recombinant pFasBac plasmid (Fig 1). The gel electrophoresis in 1 % agarose showed 1.8 kb of COX-1 and 2 kb of COX-2 respectively, and 4.8 kb of pFastBac donor plasmid (Fig 2).

The bacmid DNA is very large (>135 kb). Verification of the insertion of both genes in recombinant bacmid is difficult using classical restriction endonuclease digestion analysis. So PCR was used to confirm

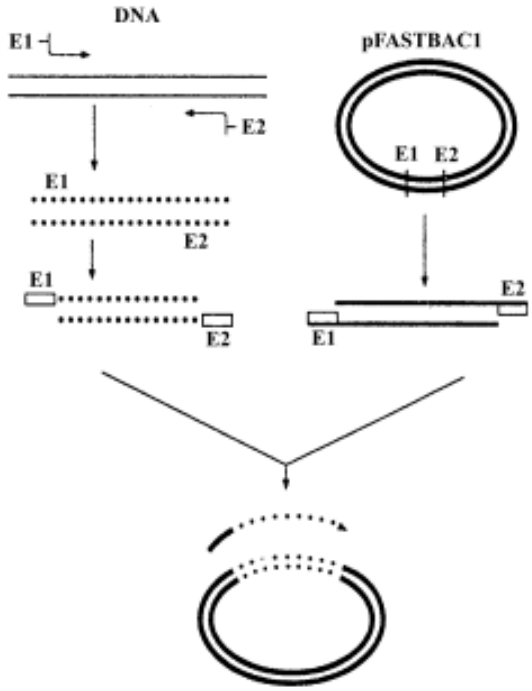

Fig 1. The genes of COX-1 and COX-2 were cloned from the human monocyte cell line THP-1 and then subcloned into pFastBac transposing vector.

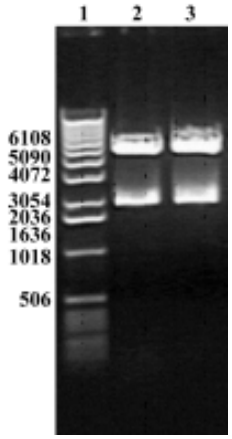

Fig 2. Restriction endonuclease digestion of recombinant transposing vector. Lane 1: 1 kb DNA Ladder; Lane 2: pFastBac-COX1/*BamH* I+*Hind* III; Lane 3: pFastBac-COX2/*Not* I+*Xba* I.

Tab 1. The specific primers for human COX-1 and COX-2.

Oligonucleotides designed for RT-PCR	Sequence
COX-1 sense primer	5'-CGCGGATCCACCATGAGCCGGAGTCTCTTG-3'
COX-1 anti-sense primer	5'-TGCTTTCAAGCTTCTCAGAGCTCTGTGGATGGT-3'
COX-2 sense primer	5'-TTGCGGCCGCCACCATGGTCGCCCGCGCCCTGCTGCTGTG-3'
COX-2 anti-sense primer	5'- GCTCTAGAGACTTCTACAGTTCAGTCGAACGT-3'

the recombinant bacmid-COX1 and bacmid-COX2. The pUC/M13 amplification primers are directed at sequences on either side of the mini-*att*Tn7 site with the *lac*Zα-complemention region of the bacmid. If transposition has occurred, the PCR product produced by these primers should be 2300 bp plus the size of the insert (Fig 3: Lane 2, 4). Alternatively, we amplified a product using one gene-specific primer and one pUC/M13 primer (Fig 3: Lane 3, 5), while amplification of the non-recombinant Bacmid plasmid generated a 300 bp band (Fig 3: lane 6).

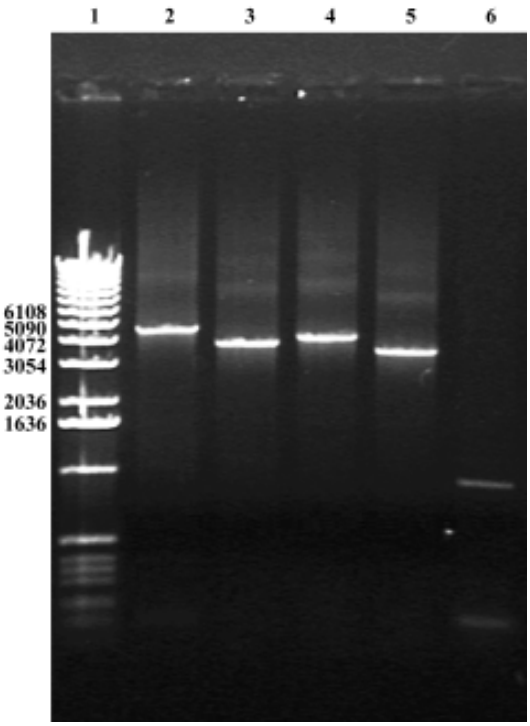

Fig 3. Electrophoresis of PCR product of recombinant baculovirus extracted from infected sf-9 cells. 1: 1 kb DNA Ladder; 2: M13/pUC primer (COX-2); 3: M13/pUC forward primer and COX-2 anti-sense primer; 4: M13/pUC primer (COX-1); 5: M13/pUC forward primer and COX-1 anti-sense primer: 6: M13/pUC primer (uninfected sf-9 cells).

Expression of hCOX-1 and hCOX-2 recombinant protein Recombinant bacmid was isolated and transfected into insect cell line sf9 with cellfectin reagents. Infected and uninfected sf9 cells can be distinguished by morphology. Uninfected cells continued to divide and form a confluent monolayer while infected cells stopped dividing and enlarged (Fig 4).

The transfected cells were collected and analyzed for recombinant protein expression by Western blot. Fig 5 showed a comparison of the time course of hCOX-1 and hCOX-2 expression in infected sf9 cells. At various time after infection (t=0, 24, 36, 48, 72, 96 h), cells were harvested and measured by Western blot analysis (Fig 5). hCOX-1 and hCOX-2 were both detected as early as 24 hpi and processed efficiently. Later than 48 hpi, 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. 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.

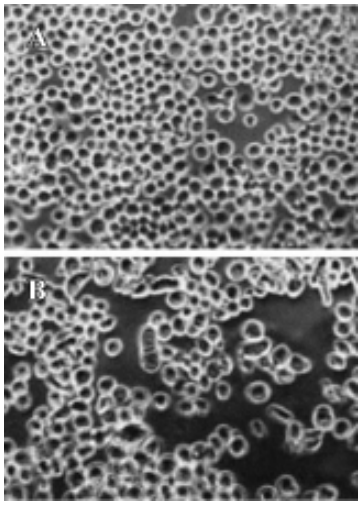

Fig 4. Observation of Sf9 cells under light microscope. A: Normal Sf9 cells; B: Recombinant bacmid transfected Sf9 cells.

The hCOX-1 and hCOX-2 enzyme activities in 24 hpi sf9 cells were shown in Tab 2. Intact uninfected sf9 cells had a basal level of COX activity as evidenced by the marginal increase in output of PGE_2 by the cells upon challenged with 10 μmol/L arachidonic acid. Intact sf9 cells infected with either hCOX-1 (1×10^6) or hCOX-2 (1×10^5) recombinant baculovirus had detectable levels of COX activity in the absence of arachi-

Tab 2. hCOX-1 and hCOX-2 enzyme activities at 24 h post-infection in intact sf9 cells.

	Enzyme activities (ng $PGE_2/1\times10^6$ cells)		
	sf9	hCOX-1	hCOX-2
-AA	0.099	0.52	44.6
+AA	0.49	15.94	383.4

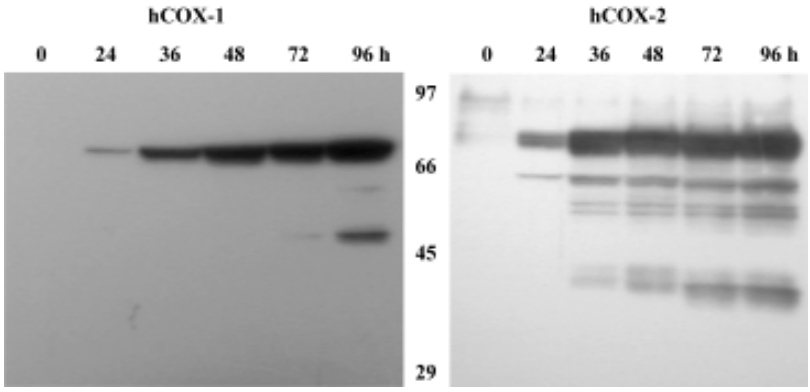

Fig 5. Western blot analysis of recombinant baculovirus expressed hCOX-1 and hCOX-2 in sf9 cells. The sf9 cells (2×10^6 cells) were infected with recombinant virus for either hCOX-1 or hCOX-2.

donic 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 activity of 15.94 ng $PGE_2/1\times10^6$ cells and 383.4 ng $PGE_2/1\times10^6$ cells.

Characterization of NSAID inhibition The potency and selectivity of various NSAIDs to inhibit COX activity in intact infected sf9 cells expressing comparable amounts of recombinant hCOX-1 or hCOX-2 was evaluated. The drugs were preincubated with the infected sf9 cells for 15 min prior to the 10-min challenge with 10 mmol/L arachidonic acid. The NSAIDs chosen for this study included potent anti-inflammatory agents currently available: indomethacin (preferentially COX-1 inhibitor); NS-398 (COX-2 selective inhibitor); and diclofenac (non-selective COX inhibitor). Inhibition profiles, obtained with this cell-based assay system, illustrating the magnitude of the differences in IC_{50} values for three drugs were shown in Fig 6.

DISCUSSION

Soon after the two isoforms of COX had been sequenced, numerous assays were developed to test putative selective inhibitors in the hope that drugs which preferentially inhibit COX-2 may have a therapeutic advantage over current NSAIDs. These included cell-based assays, which utilize either cells that express only one of the two isoforms of COX[5], or cells that have been transfected or infected with one of the human COX genes[16,17]. More recently, human whole-blood assays have been used to screen compounds for their selectivity for COX-2 versus COX-1[18]. In present studies, Bac-to-Bac baculovirus expression system was used which allows rapid and efficient generation of recombinant baculovirus. With this system, recombinant virus DNA isolated from selected colonies was not mixed with parental viruses which eliminated the need for multiple rounds of plaque purification. High-titer viruses were also produced from the initial transfection when this system was used. These features reduced the time to identify and purify recombinant virus from 4 to 6 weeks to 7 to 9 d. In the present study, the cDNAs of the hCOX-1 were subcloned into pFastBac donor vector at the *BamH* I and *Hind* III sites, and the cDNAs of the hCOX-2 were subcloned into pFastBac donor vector at the *Not* I and *Xba* I sites. The recombinant bacmid was constructed by transposing a mini-Tn7 element from a pFastBac donor plasmid to the mini-*att*Tn7 attachment site on the bacmid when the Tn7 transposition functions were provided in *trans* by a helper plasmid in DH10Bac competent cells. After transfecting the recombinant bacmid to Sf9 cells, we successfully expressed the recombinant protein COX-1 and COX-2.

In this paper, the Western blot analysis and drug inhibition assay showed that recombinant human cyclooxygenase expressed correctly and actively in sf9 cells. The COX enzymes are localized to the endoplasmic reticulum (ER) and nuclear envelope, respectively [19,20]. Earlier in the infective cycle, sf9 cells are very healthy and viable, while deleterious effects upon the integrity of the plasma membrane and the depolarization of microtubules occur late in infection[21]. Up to 36 hpi, proper glycosylation and sorting occur when low levels of the protein of interest are being expressed. Overexpression of the hCOXs in sf9 insect cells showed that at 24 hpi a large proportion of the produced enzymes were properly targeted the microsomal membrane fraction of the cells[15]. For a COX sf9 cell-based

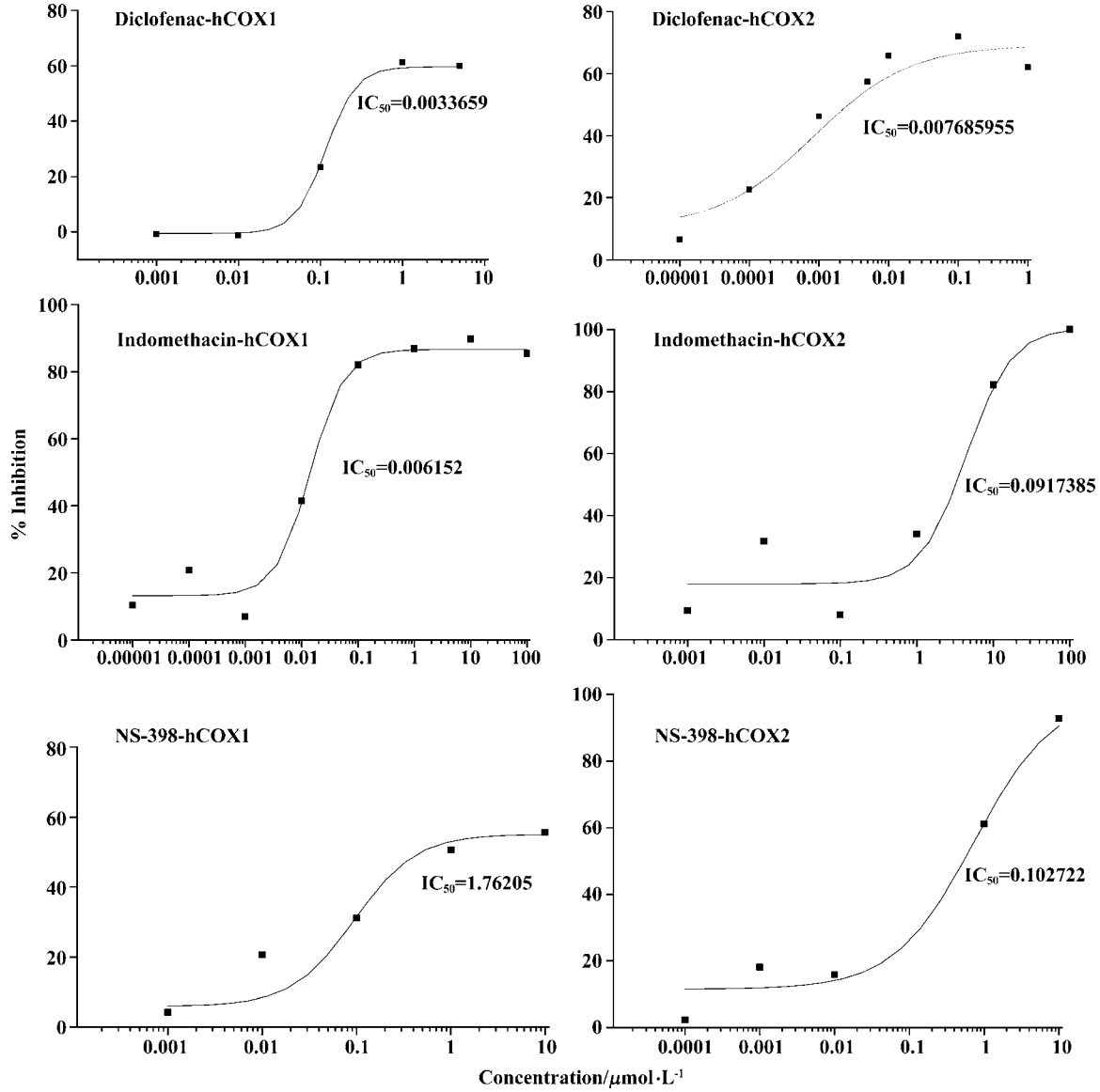

Fig 6. Representative inhibition profiles for hCOX-1 and hCOX-2 activities in intact sf9 cells by NSAIDs. PGE_2 synthesis by intact sf9 cells expressing hCOX-1 and hCOX-2 upon 10 μmol/L arachidonic acid challenge was measured by PGE_2-specific RIA.

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.

The other major advantage of producing a drug target protein in the baculovirus expression system is that only one expression system needs to be developed. 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 search of compounds with even greater selectivity.

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