—Regular Articles—

# Screening Method for Nonsteroidal Antiinflammatory Drugs Based on the Cyclooxygenase 2 Pathway Activated by Serum-Free Stimulation in A549 Cells

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Cyclooxygenase 2 (COX-2) pathway inhibitors were regarded as promising nonsteroidal antiinflammatory drugs (NSAIDs). We discovered that the COX-2 pathway in A549 cells, a human lung cancer cell line, was activated by serum-free stimulation, and a drug screening model for NSAIDs was established based on this principle with simple performance and sufficient reliability. The COX-2 pathway was activated by treating with serum-free medium for 12 h. The activated cells were incubated with NS398 (selective COX-2 inhibitor), SC560 (selective COX-1 inhibitor), acetyl salicylic acid (ASA) (nonselective COX inhibitor) at 37°C for 15 min. Then the cells were incubated with 10 $\mu$M of arachidonic acid (AA) for another 30 min prostaglandin $E_2$ and 6-keto-prostaglandin $F_{1\alpha}$ were assayed in an enzyme immunoassay (EIA). The results showed that the COX-2 pathway was dominant in A549 cells whether activated by serum-free medium or not, and the COX-1 pathway could be ignored. The model accepted the positive inhibition threshold as NS398 2 $\mu$M; if a compound (10 $\mu$M) inhibited COX-2 pathway more than NS398 (2 $\mu$M), it was regarded as a hit. The COX-2 pathway inhibition experiment showed that the Z`-factor of the screening model was 0.62, which suggests that the model is suitable for COX-2 pathway inhibitor screening.

**Key words**—nonsteroidal antiinflammatory drugs; prostaglandin $E_2$; enzyme immunoassay; A549; drug screening

## INTRODUCTION

According to the results of research in recent decades, there are at least three types of cyclooxygenase (COX), COX-1, COX-2 and COX-3, involved in arachidonic acid (AA) metabolism.[1,2] COX-1 was recognized as a constructive enzyme to maintain basic function; the activity of the COX-1 pathway can be measured by its downstream product 6-keto-$PGF_{1\alpha}$.[3] COX-3 is presented in the central nervous system (CNS) or neural cells.[2] Both COX-1 and COX-3 have only a slight relationship with peripheral inflammation. However, COX-2 can be induced by various inflammatory stimuli, such as lipopolysaccharide (LPS),[4] phorbol 12-myristate 13-acetate (PMA),[5] and cytokines.[6] Prostaglandin $E_2$ ($PGE_2$), a metabolite of AA through the COX-2 pathway was thought to be one of the key molecules involved in the inflammatory process.[7] In the COX-2 pathway, there are two steps in $PGE_2$ synthesis from AA: COX-2 catalyzes AA to PGH2 and then couples to $PGE_2$ synthesis *via* PGE synthase (PGES), mainly *via* PGES-2.[8,9] PGES inhibition was also regarded as a good strategy against inflammation.[10] The classic nonselective COX inhibitors and the newly developed COX-2-selective inhibitors are effective agents to quench, or help to quench inflammation through the pathway, although the former have common side effects assumed to be caused by their nonselective inhibition of COX-1.[11]

Selective COX-2 inhibitors have overcome some inherent disadvantages of classic nonsteroidal antiinflammatory drugs (NSAIDs),[11,12] but bring some new problems.[8,12] Further research suggested that selective COX-2 inhibitors are promising agents against osteoporosis,[13] and some are helpful in certain cancer therapy.[8] Therefore it is still interesting to discover powerful new NSAIDs through COX-2 pathway inhibition. So far, most COX inhibitors were discovered in animal-, cell-, or molecular-based models. Since the screening model is the basic foundation of drug discovery, we developed an improved cell-based model to screen NSAIDs through COX-2 pathway inhibition.

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## MATERIALS AND METHODS

**Materials** The A549 cell line was from the American Type Culture Collection (ATCC). NS398 (selective COX-2 inhibitor),[14] SC560 (selective COX-1 inhibitor),[15] AA, acetyl salicylic acid (ASA, nonselective COX inhibitor),[16] 96-well plates coated with goat polyclonal anti-mouse immunoglobulin G (IgG), mouse anti-$PGE_2$ antibody, acetylcholinesterase linked to $PGE_2$, mouse anti-6-keto-$PGF_{1\alpha}$, acetylcholinesterase linked to 6-keto-$PGF_{1\alpha}$, standard $PGE_2$, EIA buffer, EIA wash buffer, and Ellman's reagent were purchased from Cayman Chemical Company, USA. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich Corporation, USA. The Bio-Rad plate Reader was manufactured by Bio-Rad Company, USA. Ham's F12 culture media was produced by Hyclone Company, USA. Fetal bovine serum (FBS) was manufactured by Hangzhou Sijiqing Bio-material Co., Ltd., China. Other reagents used were of analytic purity and made in China.

**Cell Culture** A549 cells were incubated in F12 medium with L-glutamine 2 mM, sodium bicarbonate 1.5 g/l, and 10% FBS, at 37℃, in an atmosphere of 95% air and 5% $CO_2$.[17] When the cells were about to cover 80% of the flask area, they were disrupted and seeded on 96-well plates ($5\times10^4$ per ml, 180 $\mu$l per well) to establish the model.

**$PGE_2$ and 6-keto-$PGF_{1\alpha}$ Assay** After seeded cells was treated with LPS (10 $\mu$g/ml) or serum-free medium for 12 h, the culture medium was emptied and rinsed gently with 200 $\mu$l of phosphate buffered saline (PBS) (2.68 mM KCl, 1.47 mM $KH_2PO_4$, 5.81 mM $Na_2HPO_4$, 136.9 mM NaCl) once. The cells were incubated in PBS 200 $\mu$l for another 15 min, then incubated with AA (10 $\mu$g/ml) for an additional 30 min. The supernatant in every well was collected as sample for $PGE_2$ and 6-keto-$PGF_{1\alpha}$ enzyme immunoassay (EIA).

In an other experiment, A549 cells were incubated without serum in 96-well plates for 12 h, then incubated in PBS for 15 min with different COX inhibitors (NS398, SC560, and ASA). The dosages of NS398, SC560, and ASA were based on their $IC_{50}$ values in the COX-1 (SC560), or COX-2 (NS398 and ASA) pathway shown in Table 1. Then the cells were exposed to AA (10 $\mu$M) for 30 min. The supernatant was simultaneously transferred to another 96-well plate as sample. $PGE_2$ in the supernatant was determined in an EIA.

Table 1. $IC_{50}$ Values of the Three COX Pathway Inhibitors

| | COX-1 | COX-2 | Remarks | Ref. |
|---|---|---|---|---|
| NS398 | 75 $\mu$M (human) | 1.77 $\mu$M (human) | Selective COX-2 inhibitor | 14 |
| SC560 | 9 nM (human) | 6.3 $\mu$M (human) | Selective COX-1 inhibitor | 15 |
| ASA | 0.75 mM (ovine) | 1.25 mM (ovine) | Nonselective COX inhibitor | 16 |

* Information was supplied by Cayman Company.

For the $PGE_2$ assay, 100 $\mu$l of EIA buffer, 50 $\mu$l of sample or standard $PGE_2$, 50 $\mu$l of mouse anti-$PGE_2$ antibody, and 50 $\mu$l of acetylcholinesterase linked to $PGE_2$ were added to a plate coated with goat polyclonal anti-mouse IgG and incubate them on an orbital shaker (200 rpm) at ambient temperature for 60 min. The plates were rinsed five times with wash buffer and developed with Ellman's reagent. The developed plates were read with the Bio-Rad reader at 410 nm.

For the 6-keto-$PGF_{1\alpha}$ assay, 100 $\mu$l of EIA buffer, 50 $\mu$l of sample or standard 6-keto-$PGF_{1\alpha}$, 50 $\mu$l of acetylcholinesterase linked to 6-keto-$PGF_{1\alpha}$, and 50 $\mu$l of mouse anti-6-keto-$PGF_{1\alpha}$ antibody were added to a plate coated with goat polyclonal anti-mouse IgG and incubated at 4℃ for 18 h. The plates were rinsed five times with wash buffer and developed with Ellman's reagent. The developed plates were read with the Bio-Rad plate reader at 410 nm.

**Statistical Analyses** Values are expressed as mean±S.D. One-way analysis of variance (ANOVA) was performed, and Hochberg's GT2 method of the *post hoc* test was performed to compare the means with the control group. Statistically significant differences were accepted at $p<0.05$.

The Z`-factor, an index to measure the stability of a drug screening model, was calculated using the Eq. (1), where $\sigma$ is the standard deviation (S.D.) of the positive or negative control, and $\mu$ is its mean.[18]

$$Z` = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|} \quad (1)$$

**Hit Identification** COX-2 pathway inhibition was determined in the $PGE_2$ assay, and hit identification was made based on the hypothesis that if the inhibition of a compound (10 $\mu$M) was greater than that of NS398 (2 $\mu$M), the compound was accepted as a hit.

## RESULTS

**Yield of $PGE_2$ or 6-keto-$PGF_{1\alpha}$ in A549 Cells Induced by Different Stimulation** Without inducement, A549 cells were able to synthesize more $PGE_2$ than 6-keto-$PGF_{1\alpha}$; with LPS (10 $\mu$g/ml) stimulation, $PGE_2$ increased, and increased even more with serum-free stimulation. However, compared with $PGE_2$, 6-keto-$PGF_{1\alpha}$ always remained at a lower level whether stimulated or not (Table 2). The data suggests that COX-2 pathway is dominant ($\geq$97%) in A549 cells.

**$PGE_2$ Synthesis Affected by Different COX Pathway Inhibitors** NS398 and ASA can inhibit $PGE_2$ yield, while SC560 has less ability to do so whether measured by $PGE_2$ yield or EIA absorption (Table 3). The results supported the assumption that there was little influence on the COX-2 pathway due to COX-1 inhibition. The inhibition caused by NS398 (2 $\mu$M) was near its reported $IC_{50}$ value (Student's *t*-test with that in Table 1, $p>0.05$). The relationship between $PGE_2$ yield and COX-2 pathway inhibition by NS398, SC560, or ASA agreed with the results in Table 1.

Table 2. $PGE_2$ and 6-keto-$PGF_{1\alpha}$ in A549 Cells with Different Treatments (Mean$\pm$SD, $n$=3)

| Group | (1) PGE2 (pg/ml) | (2) 6-keto-$PGF_{1\alpha}$ (pg/ml) | (3) $\frac{(1)}{(1)+(2)}$ (%) |
|---|---|---|---|
| Ctrl | 268.3$\pm$36.20 | 6.66$\pm$0.55 | 2.4$\pm$0.2 |
| Sam1 | 395.2$\pm$29.02[a)] | 6.69$\pm$1.55 | 1.7$\pm$0.4 |
| Sam2 | 793.2$\pm$28.05[a),b)] | 16.9$\pm$7.52 | 2.1$\pm$0.9 |

Ctrl: control group, Sam1: treated with LPS 10 $\mu$g/ml, Sam2: treated with serum-free medium, $PGF_{1\alpha}$: 6-keto-$PGF_{1\alpha}$. *a)* $p<0.05$, compared with control group, *b)* $p<0.05$, Sam2 compared with Sam1. The molecular weight of $PGE_2$ is 352.47, and that of 6-keto-$PGF_{1\alpha}$ is 370.48.

**Relationship between $PGE_2$ and EIA Absorption** Based on the standard curve of $PGE_2$, there was a good negative relationship between $PGE_2$ and EIA absorbance, and it was fitted by linear or nonlinear regression as shown in Fig. 1. The negative relationship could be used as a direct calibrate for drug screening.

**Z`-factor Calculation** According to the data in Table 3, if the positive threshold was accepted as the

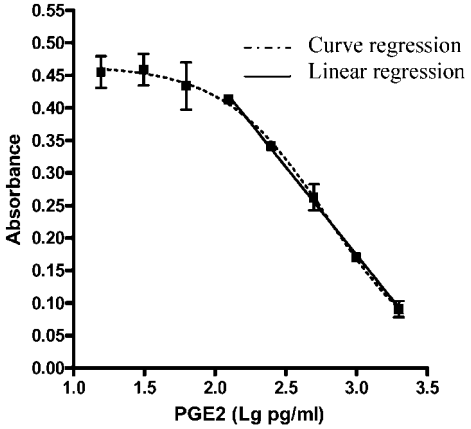


Fig. 1. Negative Relationship between $PGE_2$ and EIA Absorbance (Mean$\pm$SD, $n$=3).

The full curve was fitted with the curve equation: $Y=\frac{0.466}{1+10^{-1.193(2.792-LgX)}}-0.001$, X=Lg ($PGE_2$), $R^2$=0.9987; from 125 to 2000 pg/ml (LgX: from 2.0969 to 3.3010), the curve was fitted with linear equation: Y=$-$0.2711 X +0.9871, X=Lg ($PGE_2$), $R^2$=0.9983.

Table 3. $PGE_2$ Yield Inhibition by Three COX Inhibitors (Mean$\pm$SD, $n$=3)

| Group | Treatment | Dose | $PGE_2$ (pg/ml) | Absorbance | COX-2 pathway inhibition (%) |
|---|---|---|---|---|---|
| Ctrl | PBS | 20 ul | 793.2$\pm$28.1 | 0.201$\pm$0.004 | — |
| Low | NS398 | 0.2 $\mu$M | 589.5$\pm$21.9[a)] | 0.236$\pm$0.004[a)] | 25.7$\pm$1.0 |
| | SC560 | 1.0 nM | 735.4$\pm$25.6 | 0.210$\pm$0.004 | 7.3$\pm$0.3 |
| | ASA | 0.1 mM | 449.1$\pm$33.2[a)] | 0.268$\pm$0.009[a)] | 43.4$\pm$3.2 |
| Medium | NS398 | 2.0 $\mu$M | 432.1$\pm$17.3[a)] | 0.273$\pm$0.005[a)] | 45.5$\pm$1.8 |
| | SC560 | 10.0 nM | 718.9$\pm$43.6 | 0.213$\pm$0.007 | 9.4$\pm$0.6 |
| | ASA | 1.0 mM | 337.3$\pm$27.9[a)] | 0.302$\pm$0.010[a)] | 57.5$\pm$4.7 |
| High | NS398 | 20 $\mu$M | 257.9$\pm$26.3[a)] | 0.334$\pm$0.012[a)] | 67.5$\pm$6.9 |
| | SC560 | 100 nM | 554.0$\pm$25.0[a)] | 0.244$\pm$0.005[a)] | 30.1$\pm$1.4 |
| | ASA | 10 mM | 268.1$\pm$22.4[a)] | 0.329$\pm$0.009[a)] | 66.2$\pm$5.5 |

NS398 and ASA decreased $PGE_2$ significantly, while the ability of SC560 was much less. *a)* $p<0.05$, compared with Ctrl group.

effect of NS398 (2 $\mu$M, medium dose) in inhibiting the COX-2 pathway (Table 3), the value of the Z`-factor was 0.62.

## DISCUSSION

With inflammatory stimuli, a large quantity of endogenous AA is released from membrane phospholipids, the expression of COX-2 and PGE synthase (PGES) is upregulated,[10] and most AA steps in the COX-2 pathway to generate $PGE_2$ are activated. COX-2 pathway inhibition was regarded as a good strategy against inflammation.[3] COX-2 inhibitors and PGES inhibitors could inhibit the COX-2 pathway effectively, and both are promising drugs against inflammation.

There are many excellent animal models[19—23] reported for screening COX-2 inhibitors. Animal models are indispensable in drug development research, but they are time-consuming, required numerous animals to be killed, and are not suitable for drug screening in early drug discovery research with high throughput.

COX-2 pathway inhibitor screening models based on the COX-2 enzyme were established. Huss et al.[24] established a model using scintillation proximity assay (SPA) to screen compounds, but there was an underlying radiation hazard. Since the stereochemical structure of COX-2 and PGES-2[9] were established, some virtual screening models have been developed,[25,26] but the results must be confirmed by ''real'' screening. Kulmacz and Lands[27] developed a biochemical model based on the oxidizing reaction catalyzed by COX-2. The original purpose of their model was to analyze COX activity; according to the screening principle, false-positive and false-negative results would appear frequently. The *in vitro* state is so different from that *in vivo* that the screening models based on purified COX-2 enzyme *in vitro* only tells a part of the whole antiinflammatory story. In the inflammatory theory of the COX-2 pathway, it is important that the product, $PGE_2$, should be eliminated during inflammation. Therefore only cell-based models could tell the real inflammatory story *via* COX-2 pathway inhibition in drug screening with relative high throughput.

Shitashige et al.[28] established a COX-2 pathway inhibition model based on NIH 3T3 cells, which was similar to the present model. However, NIH 3T3 cells are embryonic mouse fibroblasts and the COX-1 pathway could not be neglected. To limit its effect, NIH 3T3 cells should be treated with ASA to inhibit COX-1 pathway completely and the treated cells rinsed several times to eliminate the effect of ASA, then treated with LPS or PMA to activate the COX-2 pathway to establish the model.[29] The procedure of the model is relatively tedious. The activity of the COX-2 pathway in macrophages is strong, especially when activated with LPS,[3] and Hu and Cheng[30] established a similar screening model based on those cells. Usually, microphages are obtained from noninfection induced peritonitis in the mouse or rat. Because the activity of primary cultured macrophages could vary occasionally, the model would not be sufficiently table between different experiments.

The present model was established based on A549 cells, and COX-2 in these cells has been confirmed to be the main COX.[31] The present study firstly discovered that the COX-2 pathway in A549 cells was able to be activated more potently when with serum-free stimulation, and the effect was even greater than that with LPS (10 $\mu$g/ml) simulation. The model took advantage of this and simplified the procedure, which can be done without LPS or ASA pretreatment and without frequent rinsing, and the model established based on A549 cells could overcome the disadvantages easily.

A Z`-factor greater than 0.5 is a well-accepted threshold for a drug screening model.[18] The Z`-factor in the present model was 0.62, which suggests that the model is suitable for drug screening. With the help of an automatic sampling system, the model could be applied to high-throughput screening.

According to general opinion in drug screening, if its $IC_{50}$ value is less than 3 $\mu$M, a compound can be regarded as a strong enzyme inhibitor.[32] In the case of false negatives and referring to the report by Duan et al.[33], we suggest the $IC_{50}$ threshold for candidate compounds should be less than 10 $\mu$M. To detect hits directly, the standard curve need not be established each time, and the hits can be detected from the original data (EIA absorbance) by observing whether the absorbance caused by a compound (10 $\mu$M) is less than that by the positive control (NS398 2 $\mu$M). Because there was a good relationship between the $PGE_2$ concentration and EIA absorbance (Fig. 1), the stability of our model *via* absorbance is as the same as *via* $PGE_2$ concentration, which can be deduced from Eq. (1).

In the COX-2 pathway theory, a hit from the present model may inhibit COX-2, PGES, or both. It is necessary to clarify which of them the hit would inhibit in further studies. Nevertheless, it does not prevent NSAID discovery using the present model.

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