© 2002, Acta Pharmacologica Sinica
ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Inhibitory effect of 3,4-diaryl-3-pyrrolin-2-one derivatives on cyclooxygenase 1 and 2 in murine peritoneal macrophages

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KEY WORDS cyclooxygenase 1; cyclooxygenase 2; peritoneal macrophages; rofecoxib; indomethacin

ABSTRACT

AIM: To develop a whole-cell assay based on murine peritoneal macrophages and evaluate the inhibitory effect of candidate compounds on cyclooxygenase-1 (COX-1) and COX-2. **METHODS:** Macrophages were stimulated with calcimycin or lipopolysaccharide (LPS) for various periods. Their abilities to convert endogenous arachidonic acid to 6-keto-$PGF_{1\alpha}$ or PGE_2 were examined by radioimmunoassay (RIA). RNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and COX-1/2 was detected by reverse transcription polymerase chain reaction (RT-PCR) using specific primers. **RESULTS:** Rofecoxib selectively inhibited LPS-induced, COX-2-derived PGE_2 synthesis with an IC_{50} value of (4.7±0.5) nmol/L compared with maximum inhibitory ratio of 17.3 % for the inhibition of calcimycin induced, COX-1-derived 6-keto-$PGF_{1\alpha}$ synthesis. Indomethacin exhibited dual inhibitory effects on COX-1 and COX-2 with IC_{50} of (4.7±1.1) nmol/L and (7.1±1.2) nmol/L, respectively. Two series of 17 compounds were tested. Most of compounds in series II showed comparable inhibitory activities to rofecoxib on COX-2. The relative position of the sulfonylphenyl group to the lactam carbonyl group has important effects on COX-2 inhibitory activity. **CONCLUSION:** The established whole cell assay is appropriate for drug-design oriented *in vitro* assay. 3,4-Diaryl-3-pyrrolin-2-one derivatives were proved to be prospective new type of COX-2 selective inhibitors.

INTRODUCTION

Prostanoids are important mediators of a wide variety of physiological processes. Cyclooxygenase (COX) plays a important role in prostaglandin synthesis[1]. Two different cyclooxygenase isozymes have been identified in mammals: COX-1 (EC 1.14.99.1) and COX-2[2]. COX-1 is expressed constitutively in stomach, kidney collecting tubules, platelets, vascular endothelial cells, and macrophages, exerting certain physiologic housekeeping functions including normal renal function, gastric mucosal integrity, and hemostasis. On the other hand, COX-2 is virtually undetectable in most tissues under physiological conditions, but it may

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Received 2001-09-25 Accepted 2002-05-31

be dramatically induced by a variety of stimulus. COX-2 is postulated to be involved in the generation of prostanoids in certain stages of cell proliferation and differentiation[1]. Recently, many research show that COX-2 is also over-expressed in colon cancer, which lead to further studies on COX-2 functions in cancer and development[3].

Nonsteroidal antiinflammatory drugs (NSAID) are known to mitigate pain and inflammation by blocking COX isozymes. Classical NSAID such as aspirin and indomethacin nonspecifically inhibit COX-1/2 at standard antiinflammatory doses. The benefits of anti-inflammation occur through inhibition of COX-2, but the gastrointestinal toxicity occurs as a result of concurrent inhibition of COX-1[4]. Selective COX-2 inhibitors such as celecoxib (Celebrex, Searle & Pfizer)[5] and rofecoxib (Vioxx, Merck)[6] are now widely accepted as promising agents to treat inflammations without side-effects associated with classical NSAID[7].

Macrophages are known to release prostanoids in two kinetically distinct patterns: the immediate and delayed phase[8]. In the immediate phase, 6-keto-$PGF_{1\alpha}$ and TXB_2 were the major arachidonic acid metabolites in response to Ca^{2+} stimulation. In the delayed phase, the PGE_2 production in response to lipopolysaccharide (LPS) over long-term culture is dependent on induced COX-2. In this study, we tried to develop a whole-cell assay based on murine peritoneal macrophages and evaluate the inhibitory effect of candidate compounds on COX-1/2.

MATERIALS AND METHODS

LPS (*E coli* 055:B5) and calcimycin (A23187) were from Sigma. Brewer thioglycollate medium was from Difco. RPMI-1640, M-MLV reverse transcriptase, and Trizol reagent were from GIBCO-BRL. Random hexamers were from Promega. dNTPs mixture and TaKaRa *Tag* were from TaKaRa. Newborn calf serum was from HyClone. 6-keto-$PGF_{1\alpha}$ and PGE_2 RIA kit were from PLA General Hospital.

Cell culture Adherent macrophages were harvested from the peritoneal cells of male mice (C57BL-6J, Grade II, from Experimental Animal Center, Academy of Military Medical Science) 3 d after the injection (ip) of brewer thioglycollate medium (50 mL/kg body weight) as described previously[9]. Shortly, peritoneal cells obtained from 3–4 mice were mixed and seeded in 48-well cell culture cluster (Costar) at a cell density of 1×10^9/L in RPMI-1640 supplemented with 5 % (v/v) newborn calf serum, penicillin 100 kU/L, and streptomycin 100 g/L. After settlement for 2–3 h, non-adherent cells were washed by *D*-Hanks' balanced salt solution. Then macrophages were cultured in RPMI-1640 without serum. Almost all of adherent cells were macrophages as assessed by Giemsa staining. Cell viability was examined by Trypan blue dye exclusion. All incubation procedures were performed with 5 % CO_2 in humidified air at 37 ¡æ

COX-1 assay Macrophages were incubated with test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with calcimycin 1 μmol/L for 1 h. The amount of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) in supernatant was measured by radioimmunoassay (RIA) according to manufacturer's guide. The inhibitory ratio (IR) was calculated as

$$\mathrm{IR} = \frac{(C_{\text{calcimycin}} - C_{\text{test compound}})}{(C_{\text{calcimycin}} - C_{\text{control}})}$$

C refer to 6-keto-$PGF_{1\alpha}$ concentration in supernatants of calcimycin, test compound, and control groups, respectively.

COX-2 assay Macrophages were incubated with test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with LPS 1 mg/L for 9 h. The amount of PGE_2 in supernatants was measured by RIA. The inhibitory ratio was calculated using the same formula as in COX-1 assay section. *C* refer to PGE_2 concentration in supernatants of LPS, test compound, and control groups, respectively.

RT-PCR The GAPDH primer used as 5'-GAGGGGCCATCCACAGTCTTC-3' and 5'-CATCACCATCTTCCAGGAGCG-3'. The COX-1 primer used as 5'-AGTGCGGTCCAACCTTATCC-3' and 5'-CCGCAGGTGATACTGTCGTT-3'. The COX-2 primer used as 5'-GGGAAGCCTTCTCCAACC-3' and

5'-GAACCCAGGTCCTCGCTT-3'. Total RNA was extracted from macrophages using Trizol reagent. First strain cDNA was synthesized from 2 μg of total RNA with M-MLV reverse transcriptase and random hexamer. Equal amount of RT product (1 μL) were amplified by PCR with TaKaRa *Tag* for 28 cycles consist of 94 ¡æ for 30 s, 55 ¡æfor 30 s, and 72 ¡æfor 45 s. The amplified cDNA were solved on 2 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide.

Inhibitors Rofecoxib, indomethacin, and all other compounds used in this study were synthesized by our group. Structures of compounds are shown (Fig 1, Tab 1). All tested samples were prepared in stock solution 0.01 mol/L with Me_2SO and stored at –20 ¡æ Before use, stock solutions were diluted to appropriate concentrations in RPMI-1640.

Statistical analysis Data were expressed as mean±SD of more than three independent experiments. Dose-inhibitory effect curves were fit through "uphill dose response curves, variable slope" using Prism, GraphPad version 3.00.

Tab 1. Substitution of 3,4-diaryl-3-pyrrolin-2-ones.

	R_1	R_2	R_3
I-1	CH_3	CH_3	CH_3
I-2	CH_3	*p*-F	$CH_2CH_2CH_3$
I-3	CH_3	m-Cl	CH_3
I-4	CH_3	*m*-Cl	$CH_2CH_2CH_3$
I-5	NH_2	*m*-Br	$CH_2CH_2CH_3$
I-6	NH_2	*m*-Cl	$CH_2CH_2CH_3$
I-7	NH_2	*m*-F	$CH_2CH_2CH_3$
I-8	NH_2	*m*-Cl	Cyclopropyl
II-1	CH_3	H	CH_3
II-2	CH_3	H	$CH_2CH_2CH_3$
II-3	NH_2	*p*-CH_3	Cyclopropyl
II-4	CH_3	*p*-Cl	CH_3
II-5	CH_3	*p*-F	$CH_2CH_2CH_3$
II-6	CH_3	*p*-Cl	$CH_2CH_2CH_3$
II-7	CH_3	*p*-CH_3	Cyclopropyl
II-8	CH_3	*p*-Cl	Cyclohexyl
II-9	CH_3	*p*-Br	CH_3

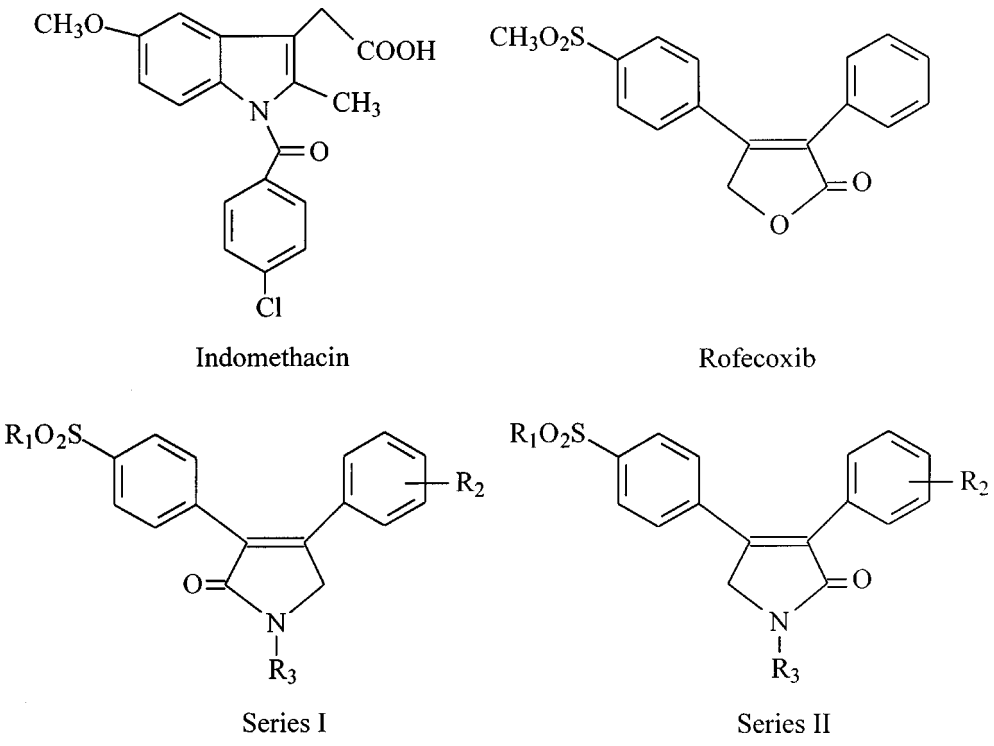

Fig 1. Structure of rofecoxib, indomethacin, and 3,4-diaryl-3-pyrrolin-2-ones.

RESULTS

Conversion of endogenous arachidonic acid to 6-keto-$PGF_{1\alpha}$ immediately after calcimycin stimulation The ability of macrophages to metabolize endogenous arachidonic acid to 6-keto-$PGF_{1\alpha}$ was dependent on calcimycin dosage. When cells were incubated with calcimycin 0, 0.125, 0.25, 0.5, 1, 2, or 4 μmol/L for 1 h, the concentrations of 6-keto-$PGF_{1\alpha}$ in supernatant reached (125±21), (119±33), (107±26), (447±116), (1423±244), (1748±152), or (2497±255) μg/L, respectively (n=5) (Fig 2).

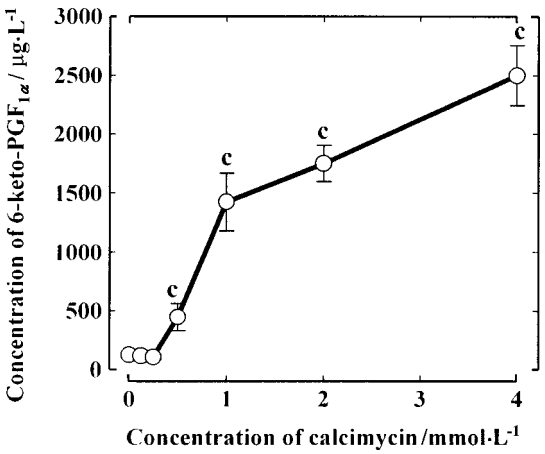

Fig 2. Efficacy of calcimycin on 6-keto-$PGF_{1\alpha}$ production in macrophages utilizing endogenous arachidonic acid. n=5 independent experiments. Mean±SD. ^cP<0.01 *vs* control group (Me_2SO).

Conversion of endogenous arachidonic acid to PGE_2 after long-term culture with LPS stimulation The inducible production of PGE_2 in macrophages utilizing endogenous arachidonic acid was dependent on both LPS dosage and time. When cells were incubated with LPS 0, 0.0625, 0.125, 0.25, 0.5, 1, or 2 mg/L for 9 h, the concentrations of PGE_2 in supernatant reached (2.6±0.3), (4.6±0.8), (5.1±0.7), (7.1±0.7), (9.6±1.2), (11.1±0.8), or (12.7±1.6) μg/L, respectively (n=5) (Fig 3). When cells were incubated with LPS 1 mg/L for 3, 6, 9, or 12 h, the concentration of PGE_2 in supernatant reached (2.4±0.9), (22±3), (73±4), or (75±4) μg/L, respectively (n=5). There was a latent period for 9 h. The PGE_2 production increased about 28-fold after 9 h culture with LPS 1 mg/L stimulation (Fig 3).

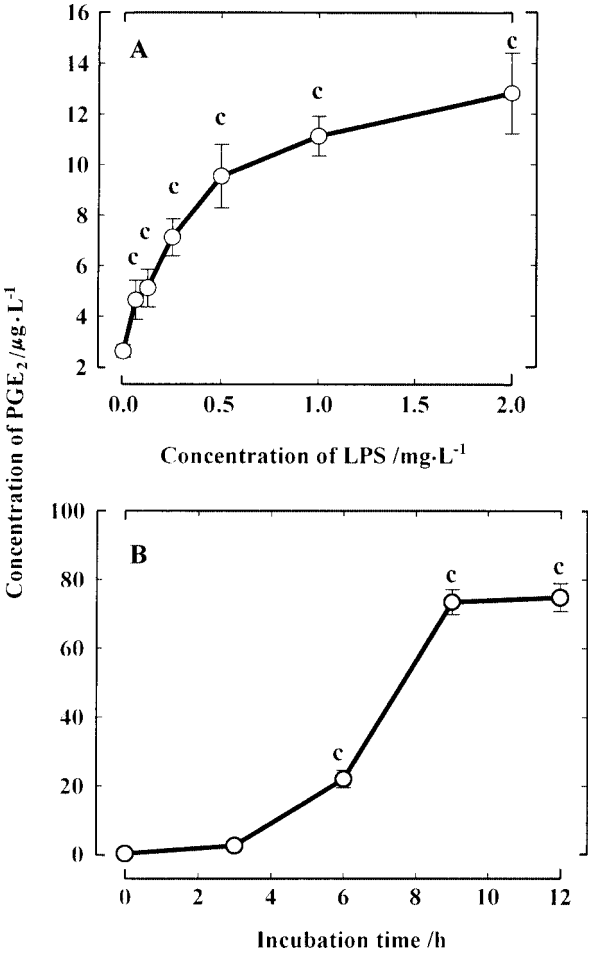

Fig 3. Efficacy and time course of LPS on PGE_2 production in macrophages utilizing endogenous arachidonic acid. Macrophages (5×10^5 cells per well) were incubated with LPS in different concentration for 9 h (A) or LPS 1 mg/L for different time (B). n=5 independent experiments. Mean±SD. ^cP<0.01 *vs* control group (Me_2SO).

The elevation of COX-2 mRNA level after LPS stimulation Macrophages were cultured in a medium containing LPS 1 mg/L. Total RNA was extracted from cells at different time. mRNA level of GAPDH and COX-1/2 was detected by RT-PCR using specific primers. After incubation for 0, 3, 6, 9, and 12 h, an accumulation of COX-2 mRNA was observed, while the mRNA level of COX-1 were almost not changed (Fig 4).

The inhibitory effect of rofecoxib and indomethacin on COX-1/2 The inhibitory ratio of rofecoxib on COX-1 at a concentration of 1 μmol/L was 17.3 % (n=5), while that of indomethacin at the

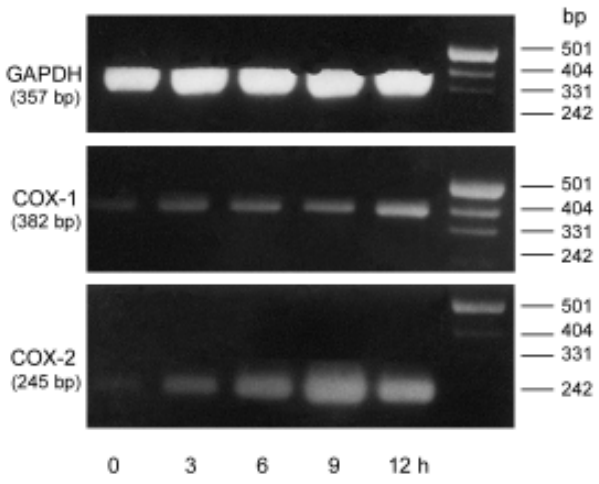

Fig 4. The mRNA level of GAPDH, COX-1, and COX-2 in macrophage with LPS stimulation.

same concentration was 104.2 % (n=5). The IC_{50} of indomethacin on COX-1 was (4.7±1.1) nmol/L. The IC_{50} of rofecoxib and indomethacin on COX-2 were (4.7±0.5) nmol/L and (7.1±1.2) nmol/L, respectively (Fig 5).

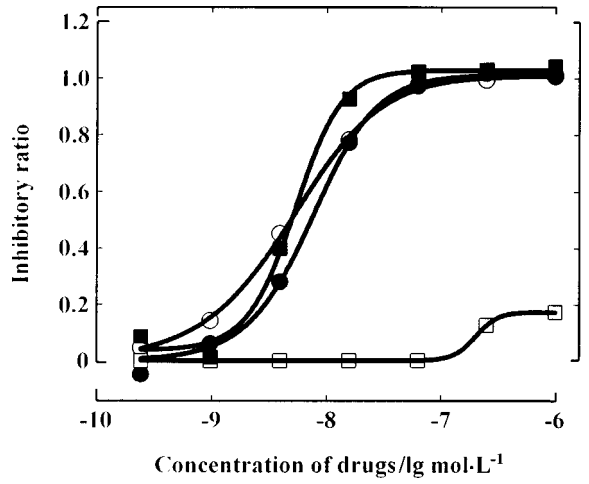

Fig 5. Does-response curve of rofecoxib and indomethacin on COX-1 and COX-2. The inhibitory ratios of indomethacin (¡ð), rofecoxib (¡ð) on COX-1 and rofecoxib (¡ð), indomethacin(¡ñ) on COX-2 at different concentrations were measured. *n*=4-6 repetitive wells in cell culture.

The inhibitory effect of substituted 3,4-diaryl-3-pyrrolin-2-ones on COX-1/2 Rofecoxib possesses two aryl rings in *cis*-position connecting with a third ring and one aromatic moiety is substituted by a sulfonyl group. To explore the effect of the position of sulfonylphenyl group relative to the lactam carbonyl group on activity and the influence of different substituents on nitrogen atom, 17 compounds in two series were designed and synthesized. The IC_{50} of candidate compounds on COX-1 and COX-2 were obtained. The selectivity of compounds was expressed as the $IC_{50,COX-1}/IC_{50,COX-2}$ (Tab 2). In our system, rofecoxib exhibits over 213-fold selectivity on COX-2 over COX-1, while indomethacin shows more selectivity on COX-1. Most of compounds in series II showed comparable inhibitory effect to rofecoxib on COX-2 and some compounds have high selectivity. In series I, the $IC_{50,COX-2}$ of compounds are 10-100 fold lower than that of compounds in series II. The induction of 6-keto-$PGF_{1\alpha}$ production by 0.1 % Me_2SO was also observed in our model (data not shown). Because of solubility, the IC_{50} of some compounds on COX-1 were not measured.

Tab 2. The inhibitory effect of substituted 3,4-diaryl-3-pyrrolin-2-ones on COX-1 and COX-2.

Compounds	IC_{50} /nmol·L^{-1}		$IC_{50,COX-1}$/ $IC_{50,COX-2}$
	COX-1	COX-2	
I-1	>1000	1950±685	>0.51
I-2	>1000	185±47	>5.4
I-3	>1000	305±89	>3.28
I-4	>1000	859±252	>1.16
I-5	>1000	933±306	>1.07
I-6	493±168	259±88	1.90
I-7	>1000	403±99	>2.48
I-8	>1000	1730±546	>0.58
II-1	933±256	16.7±2.3	55.9
II-2	149±52	24.7±7.9	6.03
II-3	>1000	18±4.2	>55.5
II-4	155±53	30.8±8.2	5.03
II-5	513±198	40.7±9.2	12.59
II-6	10.3±4.1	32.8±9.8	0.31
II-7	243±92	84.3±22	2.88
II-8	182±76	129±49	1.41
II-9	56.8±23	15±4	3.86
Rofecoxib	>1000	4.7±0.5	>213
Indomethacin	4.7±1.2	7.1±1.2	0.67

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