Imrecoxib: a novel and selective cyclooxygenase 2 inhibitor with anti-inflammatory effect[1]

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ABSTRACT

AIM: To investigate the inhibitory effect of imrecoxib, a synthetic compound of completely new structure, on cyclooxygenase 1 (COX-1) and 2 (COX-2) and its anti-inflammatory effect *in vivo*. **METHODS:** The inhibitory effects of imrecoxib on cyclooxygenase 1 and 2 were studied using whole cell assay with murine peritoneal macrophages induced by calcimycin and LPS. The inhibitory effects of imrecoxib on mRNA level of COX-1 and COX-2 in human macrophage cell line U937 were detected by reverse transcription polymerase chain reaction (RT-PCR) analysis. Effects of imrecoxib on acute and chronic inflammation were evaluated in rat carrageenan induced edema model and rat adjuvant-induced arthritis model, respectively. **RESULTS:** Imrecoxib was found to inhibit COX-1 and COX-2 with IC_{50} value of 115±28 nmol/L and 18±4 nmol/L, respectively. Imrecoxib was shown to selectively and dose-dependently inhibit COX-2 mRNA level. Imrecoxib effectively inhibited carrageenan-induced acute inflammation at the doses of 5, 10, and 20 $mg \cdot kg^{-1}$ ig and adjuvant-induced chronic inflammation at the doses of 10 and 20 $mg \cdot kg^{-1} \cdot d^{-1}$ ig. **CONCLUSION:** Imrecoxib is a novel and moderately selective COX-2 inhibitor that possesses anti-inflammatory effect by inhibition of COX-2 mRNA expression.

INTRODUCTION

Cyclooxygenase (COX) is a rate-limiting enzyme for prostaglandin synthesis. Three isotypes of COXs (COX-1, COX-2, and COX-3) have been identified[1-2], though COX-3 activity in human has not been confirmed[3]. COX-1 is constitutively involved in actions such as platelet activation, gastrointestinal protection and kidney function. COX-2 is primarily produced in response to tissue damage and is involved in inflammatory responses. Traditional non-steroidal anti-inflammatory drugs (NSAIDs) act primarily by inhibiting COXs[4]. Inhibition of COX-1 by NSAIDs leads to heavy gastrointestinal toxicity. Newly developed COX-2 selective inhibitors, such as celecoxib (Celebrex) and rofecoxib (Vioxx) potently inhibit COX-2[5]. This type of drugs is expected to be devoided of gastric toxicity mediated primarily by inhibition of COX-1, and retain high anti-inflammatory activity.

However, there is no clear-cut division between biological functions of COX-1 and COX-2. Experimental evidence indicates that a full inflammation response is likely sustained by prostanoids generated by both enzymes[6]. In this sense, drugs inhibiting both enzymes are theoretically more effective in inflamma-

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tory disease treatment. Moreover, an understanding of the physiologic features of COX-2 has led to the appreciation that COX-2 selective inhibitors may theoretically lead to problems in thrombosis, salt and water balance, and healing. The increased incidence of nongastrointestinal serious adverse events, with the COX-2 selective NSAIDs as compared with nonselective NSAIDs, in the Celecoxib Long-term Arthritis Safety Study (CLASS) and the Vioxx Gastrointestinal Outcomes Research (VIGOR) study remains a major concern[7]. With all these aspects considered, developing drugs that preferentially inhibit COX-2 with moderate selectivity may be more promising.

Imrecoxib [also known as BAP-909, 4-(4-methane-sulfonyl-phenyl)-1-propyl-3-p-tolyl-1,5-dihydropyrrol-2-one], is a synthetic compound of completely new structure (patent application No 00105899.1). In the present study, inhibitory effects of imrecoxib on cyclooxygenase 1 and 2 and its anti-inflammatory effect *in vivo* were described.

MATERIALS AND METHODS

Reagents LPS (*E coli* 055:B5) and calcimycin (A23187) were from Sigma; Brewer thioglycollate medium was from Difco; 6-keto-$PGF_{1\alpha}$ and PGE_2 radioimmunoassay (RIA) kit were from PLA General Hospital, Beijing, China; RPMI-1640, M-MLV reverse transcriptase, and Trizol reagent were from GIBCO-BRL; phorbol 12-myristate 13-acetate (PMA), carrageenan, and Bacillus Calmette Guerin (BCG) were all from Sigma.

Test compounds Imrecoxib (white powder), rofecoxib, and indomethacin were synthesized by our group. Purities of them were beyond 98.5 %. For *in vitro* studies, all test drugs were prepared in stock solution 0.1 mol/L with Me_2SO and stored at -20 ºC. Before using, the stock solution was diluted to appropriate concentrations in RPMI-1640. For *in vivo* studies, all drugs were ground into fine powder and diluted to appropriate concentrations with 5 % gum arabic just before use.

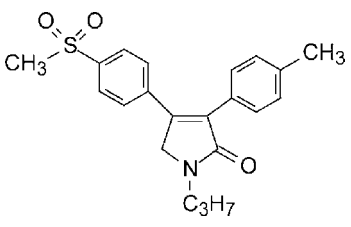

Fig 1. Structure of imrecoxib (M_r=369.3).

Animals Male C57BL-6J mice (17±1 g, 6-7 weeks) and Wistar rats (200±10 g, 5-6 weeks) were from the Experimental Animal Center, Institute of Experimental Animal, Chinese Academy of Medical Sciences & Peking Union Medical College (SPF, certificate No SCXK 11-00-0006). They were housed in groups under 12 : 12 h regime (lights on from 7:00 h to 19:00 h) at 23±2 ºC prior to the experiments, and were given standard laboratory chow and tap water *ad libitum*.

Whole cell assay with murine peritoneal macrophages induced by calcimycin and LPS The assay was done according to the method of Shen *et al*[8]. Briefly, peritoneal macrophages were harvested from male C57BL-6J mice 3 d after the injection (ip) of brewer thioglycollate medium (50 mL/kg body weight), washed once in D-Hanks' buffer and resuspended in RPMI-1640 supplemented with 5 % (v/v) newborn calf serum. For COX-1 assay, cells (5×10^5 cells in 500 μL) were incubated with the test compounds at different concentrations or Me_2SO vehicle 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 the supernatant was measured by radioimmunoassay. For COX-2 assay, macrophages (5×10^5 cells in 500 μL) were incubated with the test compounds at different concentrations or Me_2SO vehicle for 1 h and were stimulated with LPS of 1 mg/L for 9 h. The amount of PGE_2 in the supernatant was measured by radioimmunoassay.

RT-PCR analysis with human macrophage cell line U937 Human cell line U937 was from Cell Center, Chinese Academy of Medical Sciences & Peking Union Medical College. Cells were maintained in RPMI-1640 supplemented with 10 % (v/v) newborn calf serum in a humidified environment of 5 % CO_2 at 37 ºC and differentiated into macrophages by 10 nmol/L PMA according to the method of Miriam *et al*[9]. Macrophages were incubated with the test compounds at different concentrations or Me_2SO vehicle for 1 h and were stimulated with 100 μg/L LPS for 12 h. Then cells were harvested and total RNA was extracted with Trizol reagent. First strain cDNA was synthesized from equal amount of total RNA with M-MLV reverse transcriptase and random hexamer. The cDNA of GAPDH, COX-1 and COX-2 were separately amplified by PCR with TaKaRa *Tag* for 28 cycles consisting of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 45 s using GAPDH

primers of 5'-ACGGATTTGGTCGTATTGGG-3' and 5'-CGCTCCTGGAAGATGGTGAT-3', COX-1 primers of 5'-GCTCAGGAGGAAGTTCATACC-3' and 5'-AGGAAGCAGCCCAAACAC-3' primers, COX-2 primers of 5'-CCTGTGCCTGATGATTGC-3' and 5'-CGGTGAAACTCTGGCTAG-3'. PCR products were solved on 1 % (w/v) agarose gel and visualized by ethidium bromide.

Rat carrageenan-induced edema model Effect of imrecoxib on acute inflammation was evaluated in rat carrageenan-induced edema model as described previously[10]. Briefly, 0.1 mL of 1 % carrageenan in normal saline was intradermally injected into the left hindpad of male Wistar rats. Imrecoxib (5, 10, or 20 $mg \cdot kg^{-1}$, ig), rofecoxib (5 $mg \cdot kg^{-1}$, ig), and gum arabic vehicle were administrated 1 h before carrageenan injection. The degree of swelling was determined by measuring the circumference of the ankle joint with a ruler made of Scotch cellophane tape before and at 2, 3, 4, 5, and 6 h after the carrageenan injection. The percent degree at each time was determined by comparing with vehicle controls.

Rat adjuvant-induced arthritis (AIA) model Effects of imrecoxib on chronic inflammation was evaluated in AIA model as described previously[11]. Briefly, male Wistar rats were immunized on d 0 by intradermal injection of Freund's complete adjuvant containing 10 mg heat-inactivated BCG in 1 mL paraffin oil, into the left footpad in 0.1 mL for each rat. Imrecoxib (5, 10, or 20 $mg \cdot kg^{-1} \cdot d^{-1}$, ig), rofecoxib (5 $mg \cdot kg^{-1} \cdot d^{-1}$, ig), and gum arabic vehicle were started on d 7 after immunization and continued throughout the experiment. The degree of the secondary paw (noninjected paw) swelling was determined by measuring the circumference of the ankle joint with a ruler made of Scotch cellophane tape on d 14 and d 26 after immunization. The percent degree at each time was determined by comparing with vehicle controls. Rats were euthanized by carbon dioxide inhalation on d 26. The thymus and spleen of all rats were removed and weighed.

Statistical analysis Data were expressed as mean±SD of more than three independent experiments. Differences between groups were tested using one-way ANOVA, followed by multiple comparisons by Dunnett's test using SPSS 11.5 for Windows. A value of $P<0.05$ was considered statistically significant. IC_{50} was derived from dose-inhibitory effect curves which were fit through "uphill dose response curves, variable slope" using Prism, GraphPad version 3.00.

RESULTS

Selective inhibition of COX-2 by imrecoxib in whole cell assay The inhibitory effect of imrecoxib on calcimycin induced COX-1 activity was dose-dependent at the concentrations of 100-10000 nmol/L, with IC_{50} value of 115±28 nmol/L. The inhibitory effect of imrecoxib on LPS induced COX-2 activity was dose dependent at the concentrations of 10-1000 nmol/L, with IC_{50} value of 18±4 nmol/L. The selective ratio ($IC_{50,\ COX\text{-}1}/IC_{50,\ COX\text{-}2}$) of inhibition was 6.39, between that of indomethacin and rofecoxib (Tab 1).

Tab 1. Inhibitory effects of compounds on activity of COX-1 and COX-2.

Compounds	IC_{50} /nmol·L^{-1}		$IC_{50,COX\text{-}1}/IC_{50,COX\text{-}2}$
	COX-1	COX-2	
Imrecoxib	115±28	18±4	6.39
Indomethacin	4.7±1.1	7.1±1.2	0.67
Rofecoxib	>1000	4.7±0.5	>213

Selective decreasing of COX-2 mRNA level by imrecoxib COX-2 mRNA level in U937 cell was increased by PMA and PMA+LPS stimulation, while COX-1 mRNA level showed no change. Imrecoxib at concentrations of 0.10-10 μmol/L selectively and dose dependently decreased COX-2 mRNA level induced by PMA+LPS, but showed no effect on COX-1 mRNA level (Fig 2). In contrast, rofecoxib and indomethacin at concentrations of 0.10-10 μmol/L showed no effect on both COX-2 and COX-1 mRNA level.

Inhibitory effect of imrecoxib on carrageenan-induced paw edema The administration of imrecoxib and rofecoxib 1 h before injection of carrageenan significantly inhibited the edema response at 3, 4, 5, and 6 h, and the inhibitory effect was maximal at 4 h (Tab 2). Moreover, there is no significant difference between the inhibitory potency of imrecoxib at different doses and rofecoxib ($P>0.05$).

Inhibitory effect of imrecoxib on rat with AIA Inflammtory polyarthritis was induced in all immunized rats. The peak incidence occurred on d 14 after immunization. Treatment with imrecoxib (10 or 20 $mg \cdot kg^{-1} \cdot d^{-1}$) and rofecoxib diminished the secondary paw swelling on both d 14 and d 26 after immunization (Tab 3). Moreover, there is no significant difference between

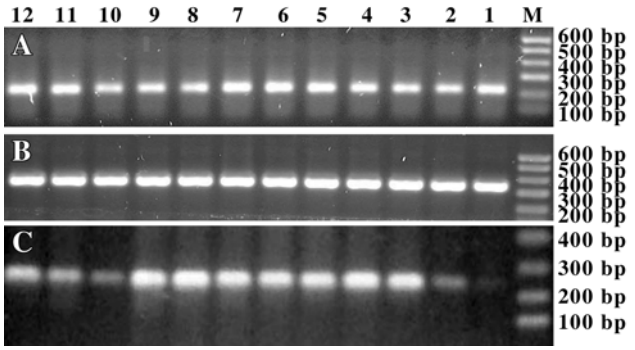

Fig 2. mRNA level of GAPDH, COX-1, COX-2 in U937 cell treated with PMA+LPS and inhibitory effect of compounds. PMA-differentiated macrophages were incubated with test compound at different concentrations or Me_2SO vehicle for 1 h and were stimulated with 100 μg/L LPS for 24 h. Total RNA was extracted with Trizol reagent and first strain cDNA was synthesized from equal amount of total RNA with M-MLV reverse transcriptase and random hexamer. mRNA level of GAPDH (A), COX-1 (B), and COX-2 (C) were detected by PCR using specific primers. The amplified cDNA were solved on 1 % (w/v) agarose gel and visualized by ethidium bromide. Lane M: DNA marker; 1: control; 2: PMA; 3: PMA+LPS; 4-6: rofecoxib of 10, 1, and 0.1 μmol/L; 7-9: indomethacin of 10, 1, and 0.1 μmol/L; 10-12: imrecoxib of 10, 1, and 0.1 μmol/L.

the inhibitory potency of imrecoxib at different doses and rofecoxib (P>0.05). The index of thymus and spleens were not significantly affected by both imurecoxib and rofecoxib (P>0.05, data not shown). At the end of the study, a general necropsy was performed, and abdominal, peritoneal, and thoracic cavities were normal in all rats.

DISCUSSION

Activated macrophages express COX-2 and produce excessive amounts of PGE_2, which plays a key role in the process of inflammation. The present study demonstrated that imrecoxib, a synthetic compound of completely new structure, was a selective inhibitor of COX-2 in two *in vitro* assays based on macrophages. In whole cell assay with murine peritoneal macrophages induced by calcimycin and LPS, imrecoxib selectively inhibited COX-2 activity with moderate selectivity, and the IC_{50} value of COX-2 inhibition by imrecoxib was comparable to that of indomethacin. This provided further consideration that imrecoxib might be a novel COX-2 inhibitor with moderate selectivity. RT-PCR analysis with human macrophage cell line U937 presents the difference between imrecoxib and other COX-2 inhibitors. Imrecoxib was shown to selectively and dose dependently inhibit COX-2 mRNA expression in the present study, and showed no effect on COX-1 mRNA expression. Indomethacin and rofecoxib, however, showed no such effect, which conforms to the result of Barrios-Rodiles *et al*[12]. We also investigated effect of imrecoxib on mRNA expression of other inflammatory mediators, such as interleukin-1, interleukin-6, interleukin-8, and MMP-9 in PMA-differentiated U937 cell. However, the compound did not affect their mRNA expression (data not shown).

To investigate anti-inflammatory effect of imrecoxib *in vivo*, we evaluated the inhibitory effects of imrecoxib on rat carrageenan-induced paw edema and rat adjuvant-induced arthritis, respectively. Imrecoxib at different doses were shown to effectively

Tab 2. Inhibitory effects of compounds on carrageenan-induced paw edema in rat at different time. n=10. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control.

Time/h		BAP-909/mg·kg^{-1}			Rofecoxib/mg·kg^{-1}	Control
		5	10	20	5	-
2	Paw edema/Δcm	0.46±0.05	0.51±0.04	0.51±0.04	0.49±0.06	0.51±0.06
	Inhibition/%	9.8	0	0	4.9	-
3	Paw edema/Δcm	0.54±0.05	0.51±0.08	0.48±0.07	0.51±0.06	0.65±0.12
	Inhibition/%	17.3^b	21.2^c	26.9^c	21.2^c	-
4	Paw edema/Δcm	0.63±0.07	0.54±0.05	0.54±0.07	0.59±0.06	0.75±0.12
	Inhibition/%	16.7^b	28.3^c	28.3^c	21.7^c	-
5	Paw edema/Δcm	0.65±0.09	0.65±0.08	0.56±0.07	0.64±0.05	0.75±0.05
	Inhibition/%	13.3^b	13.3^b	25.0^c	15.0^b	-
6	Paw edema/Δcm	0.64±0.07	0.66±0.07	0.63±0.05	0.63±0.05	0.75±0.05
	Inhibition/%	15.0^b	11.7^b	16.7^c	16.7^c	-

Tab 3. Inhibitory effects of compounds on secondary paw swelling in rats with AIA on d 14 and d 26. *n*=10. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control.

Group	Dose/ mg·kg^{-1}·d^{-1}	d 14		d 26	
		Paw edema/Δcm	Inhibition/%	Paw edema/Δcm	Inhibition/%
Imrecoxib	10	0.17±0.19	58.5[b]	0.23±0.09	66.7[b]
	20	0.17±0.08	58.5[b]	0.18±0.25	73.9[b]
Rofecoxib	5	0.09±0.09	78.0[c]	0.06±0.07	91.3[c]
Control	-	0.41±0.30	-	0.69±0.67	-

inhibit carrageenan-induced paw edema and adjuvant-induced secondary paw swelling. Moreover, there was no significant difference between inhibitory efficacy of imrecoxib at different doses and rofecoxib in two inflammation models. The results suggest that imrecoxib may effectively relieve acute and chronic inflammation in treatment of inflammatory disease.

In conclusion, the present study clearly demonstrated that imrecoxib was a novel and moderately selective COX-2 inhibitor that possessed anti-inflammatory effect by inhibiting COX-2 mRNA expression. It has potential therapeutic role for acute and chronic inflammatory disease.

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