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Original Article

Pharmacokinetic-pharmacodynamic modeling of diclofenac in normal and Freund's complete adjuvant-induced arthritic rats

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Aim: To characterize pharmacokinetic-pharmacodynamic modeling of diclofenac in Freund's complete adjuvant (FCA)-induced arthritic rats using prostaglandin E_2 (PGE_2) as a biomarker.
Methods: The pharmacokinetics of diclofenac was investigated using 20-day-old arthritic rats. PGE_2 level in the rats was measured using an enzyme immunoassay. A pharmacokinetic-pharmacodynamic (PK-PD) model was developed to illustrate the relationship between the plasma concentration of diclofenac and the inhibition of PGE_2 production. The inhibition of diclofenac on lipopolysaccharide (LPS)-induced PGE_2 production in blood cells was investigated *in vitro*.
Results: Similar pharmacokinetic behavior of diclofenac was found both in normal and FCA-induced arthritic rats. Diclofenac significantly decreased the plasma levels of PGE_2 in both normal and arthritic rats. The inhibitory effect on PGE_2 levels in the plasma was in proportion to the plasma concentration of diclofenac. No delay in the onset of inhibition was observed, suggesting that the effect compartment was located in the central compartment. An inhibitory effect sigmoid I_{max} model was selected to characterize the relationship between the plasma concentration of diclofenac and the inhibition of PGE_2 production *in vivo*. The I_{max} model was also used to illustrate the inhibition of diclofenac on LPS-induced PGE_2 production in blood cells *in vitro*.
Conclusion: Arthritis induced by FCA does not alter the pharmacokinetic behaviors of diclofenac in rats, but the pharmacodynamics of diclofenac is slightly affected. A PK-PD model characterizing an inhibitory effect sigmoid I_{max} can be used to fit the relationship between the plasma PGE_2 and diclofenac levels in both normal rats and FCA-induced arthritic rats.

Keywords: pharmacokinetic-pharmacodynamic modeling; diclofenac; prostaglandin E_2 (PGE_2); Freund's complete adjuvant (FCA)-induced arthritis; lipopolysaccharide; blood cells

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Introduction

Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) that is widely prescribed and used for relieving the pain and edema associated with inflammatory conditions, such as osteoarthritis and rheumatoid arthritis[1–3]. It is commonly known that diclofenac acts by potent cyclo-oxygenase (COX) inhibition, which decreases the formation of proinflammatory mediators, such as prostaglandins (PGs)[4]. The prediction of safety and long-term efficacy has become the main challenge in the evaluation of NSAIDs for the treatment of pain in chronic inflammatory conditions. Pharmacokinetic-pharmacodynamic (PK-PD) modeling represents a powerful tool to quantitatively describe the pharmacokinetic, pharmacodynamic and system-related processes[5]. Several attempts have been made to illustrate the relationship between the plasma concentration-time profiles of NSAIDs and the pharmacological activity of these drugs using two types of traditional pharmacological endpoints: (i) those exploring the inflammatory response and having a mechanistic interest, such as central and local hyperthermia (body and skin temperature), hyperalgesia (pain score) and edema (paw volume) and (ii) the hybrid endpoints that have direct clinical relevance and reflect both the pain and functional impairments[2, 6–11]. However, these studies have disregarded the direct relationship between the drug concentration and the pharmacological response to COX inhibition. In addition, the lack of direct correlation between the plasma drug concentrations and the analgesic or adverse effects in chronic inflammatory conditions has made it difficult to predict the appropriate dosing regimen for the treatment of chronic inflammatory pain[12].

Prostaglandin E_2 (PGE_2) is associated with acute and chronic inflammatory pain, and NSAIDs exert analgesic effects via

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the inhibition of COX-2 activity. As a biomarker of COX activity, it is well known that PGE_2 can reflect a downstream process on the causal pathway between target occupancy and analgesic response. This indicates that PGE_2 can be used as a specific biomarker to explain and understand the variability in the therapeutic of these drugs[12]. Recently, efforts have been made to establish the relationship between biomarkers, pain measurement and safety[13–16]. However, information on the integrated pharmacokinetic-pharmacodynamic profiles of these drugs under normal and chronic inflammatory conditions is still limited.

The aim of this study was to characterize the PK-PD profiles of diclofenac in normal and Freund's complete adjuvant (FCA)-induced arthritic rats using PGE_2 as a biomarker. The inhibitory effect of diclofenac on PGE_2 release induced by lipopolysaccharide (LPS) was also measured *in vitro* in the blood cells of normal and FCA-induced arthritic rats.

Materials and methods

Chemicals

Diclofenac sodium and Bacillus Calmette Guerin (BCG) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), pentobarbital and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co (St Louis, MO, USA), and the ELISA kits for PGE_2 were from Cayman Chemical Co (Ann Arbor, MI, USA). All other reagents were of analytical grade and were commercially available.

Animals

Male Sprague Dawley rats (110–120 g) were purchased from B&K Universal Group Ltd (Shanghai, China). The rats were maintained in air-conditioned animal quarters at a temperature of 22±2 °C with a relative humidity of 50%±10% and a 12-h light/dark cycle. The rats received a standard diet (laboratory rodent chow; Nanjing, China) and water *ad libitum*. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

Induction of Freund's complete adjuvant (FCA)-induced arthritic rats

FCA-induced arthritic rats were developed according to a previously described method[17]. Rats were acclimated for 1 week before the experiments and randomly divided into two groups. Freund's complete adjuvant (FCA) was prepared by grinding 60 mg of heat-killed BCG in a mortar and adding a mixture of liquid paraffin and lanolin (2:1, *v*/*v*) so that the final concentration of BCG was 10 mg/mL. Rats received a single intradermal injection of 0.1 mL of FCA in the right hindpaw by inserting a 25-gauge, 0.5-inch needle between the second and third digits into the dorsum of the hind paw on d 0[18]. For clinical evaluation of FCA-induced arthritis, the polyarthritis severity was graded on a scale of 0–4[19]: 0, no swelling; 1, isolated phalanx joint involvement; 2, involvement of the phalanx joint and digits; 3, involvement of the entire region to the ankle; and 4, involvement of the entire paw including the ankle. Scores were given for the left hindpaw and both forepaws for each rat, yielding a maximum possible score of 12 on d 20 after treatment with adjuvant. The total score was defined as the secondary inflammation index (arthritic index). Body weight and food intake were monitored every day. A sensitized animal was considered to have arthritis when at least one non-injected paw was inflamed, and these rats were then used for the following experiments.

In vivo experiments

On d 20, after FCA injection, the FCA-induced arthritic rats and age-matched normal rats were fasted overnight and given a dose of diclofenac sodium intravenously (10 mg/kg) in the tail vein. Blood samples (approximately 200 μL) were collected under light ether anesthesia via the oculi chorioideae vein before dosing, as well as at 5, 15, 30, 60, 120, 180, 240, and 360 min after dosing. The blood sample taken prior to dosing was used for measuring the basal levels of PGE_2. The plasma samples were obtained by centrifugation at 5000 rounds per minute for 10 min and stored at -80 °C for assaying diclofenac and PGE_2 levels.

In vitro experiments in whole blood

For the *in vitro* experiments, the blood samples of FCA-induced arthritic rats and normal rats were collected under anesthesia via the abdominal aorta with intraperitoneal administration of pentobarbital (60 mg/kg). The PGE_2 released by LPS in the blood samples was documented according to a previously described method[20]. A 200-μL blood sample was added to a tube containing different levels of diclofenac sodium, heparin (0.3%) and aspirin (10 μg/mL). After adding LPS (final levels 10 μg/mL), the blood samples were incubated for 24 h at 37 °C in a gently stirring water bath. The plasma was separated by centrifugation at 5000 rounds per minute for 10 min and was stored at -80 °C for assessing PGE_2 levels.

HPLC analysis of diclofenac

The concentration of diclofenac in the plasma was analyzed by a validated HPLC procedure using ultraviolet (UV) detection that has been previously described[21]. Briefly, plasma samples were spiked with 10 μL of internal standard (50 μg/mL naproxen in methanol). Then, 200 μL acetonitrile was added, and the samples were vortex-mixed. After centrifugation at 15000 rounds per minute for 10 min, the organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen gas in a water bath at 45 °C. The residue was reconstituted in 100 μL of mobile phase and centrifuged (1000 rounds per minute, 10 min). Next, 20 μL of the supernatant was injected into an HPLC system equipped with an LC-10AD pump, a CTO-10ASvp column oven, and a SPD-10A UV absorbance detector (Shimadzu, Kyoto, Japan) set to a wavelength at 276 nm. Chromatography was performed on a Diamonsil C_{18} 5-μm column (150 mm×4.6 mm, Dikma, Technologies, Beijing, China). The mobile phase consisted of 0.03% phosphoric acid and acetonitrile (45:55, *v*/*v*) with a flow rate of 1.0 mL/min. The linear range of diclofenac in plasma was

0.078–10 μg/mL, and the limit of quantification for diclofenac was 0.078 μg/mL.

Analysis of PGE_2

The PGE_2 levels were measured with an enzyme immunoassay (EIA) using the Cayman EIA kits according to the manufacturer's protocol. Briefly, samples were diluted in EIA buffer, and a 50-μL aliquot was transferred into a coated well plate. After the addition of 50 μL of the corresponding antibody and 50 μL of acetylcholinesterase conjugate, samples were incubated at 4 °C for 18 h, washed five times and incubated for 60 min in an orbital shaker after 200 μL substrate was added. The absorbance was measured in a plate reader at 412 nm.

Pharmacokinetic-pharmacodynamic modeling

A combined pharmacokinetic and pharmacodynamic (PK-PD) model was used to describe the intensity of the effect as a function of time. This model was made on a computer running Windows XP with WinNonlin Professional software (Version 6.1, Pharsight Corporation, Mountain View, CA, USA).

The diclofenac concentration-time data for plasma were individually fitted using one-, two-, and three-compartment models. Goodness-of-fit was assessed by the objective functions and visual inspection of various diagnostic plots. Based on model selection criteria such as parameter correlations and Akaike Information Criterion (AIC), good fittings were observed using a two-compartmental model.

The obtained pharmacokinetic parameters were then used to characterize the relationship between diclofenac concentrations and PGE_2 levels. Several models, including I_{max} and indirect response models, were tried to fit pharmacodynamic data (PGE_2 levels), and the results showed that the sigmoid I_{max} model yielded the best fit. Therefore, a PK-PD model characterizing an inhibitory effect sigmoid I_{max} was introduced to illustrate the relationship between diclofenac concentrations and PGE_2 levels in plasma, which is described in Figure 1.

The relationship between diclofenac concentrations (C) and the PGE_2 levels (E) was expressed as follows.

$$E=E_0\cdot[1-I_{max}\cdot C^{\gamma}/(C^{\gamma}+IC^{\gamma}_{50})] \quad (1)$$

where E_0 is the basal level of PGE_2, I_{max} represents the maximum inhibitory fraction to diclofenac, IC_{50} is the drug concentration required to produce 50% of the maximum inhibition and γ is a slope factor, which determines the steepness of the curve. The IC_{80} value was also estimated according to the equation described by Huntjens *et al* [16].

$$IC_{80}=IC_{50}\cdot\sqrt[\gamma]{4} \quad (2)$$

Statistical analysis

The data were expressed as the mean±standard error (SEM) (CV%). The statistical analysis was performed using a *t*-test. Differences were considered significant when $P<0.05$.

Results

Development of FCA-induced arthritic rats

The development of the FCA-induced arthritic rats (Figure 2) was observed. The rats that were treated with FCA showed a decreased rate in body weight gain. The body weight of the arthritic rats (219.5±7.5 g) was significantly lower than that of normal rats (263.4±5.5 g, $P<0.01$) on d 20. The incidence of the arthritis was approximately 28%, which is agreement with a previous study[22]. The FCA-induced arthritic rats showed typical arthritic symptoms, including involvement of the phalanx joint and digits, involvement of the entire region to the ankle, or involvement of entire paw including the ankle. The arthritic index of the non-injected paws of the FCA-induced arthritic rats gradually rose with time. The arthritic index was 6.5±0.7 on d 20 after the FCA injection. Only FCA-induced arthritic rats were chosen for following experiments.

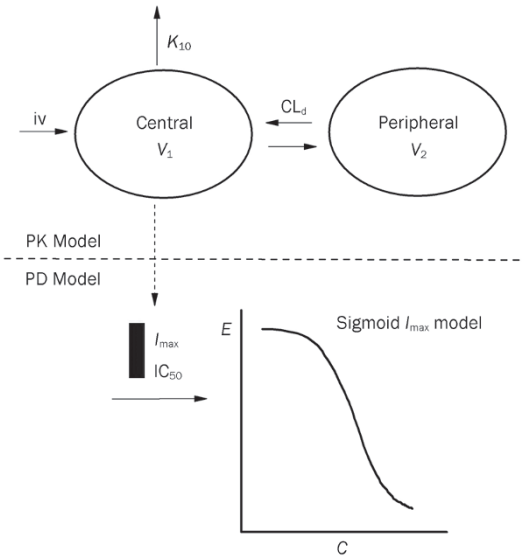

Figure 1. The pharmacokinetic-pharmacodynamic model characterizing an inhibitory effect sigmoid I_{max} that illustrates the relationship between diclofenac concentration and PGE_2 production *in vivo*.

Pharmacokinetics of diclofenac in normal and FCA-induced arthritic rats

The plasma concentrations of diclofenac in normal and FCA-induced arthritic rats were measured following an iv (intravenously) injection of 10 mg/kg diclofenac (Figure 3). The plasma concentration-time data were fitted by a two-compartment model, and the corresponding pharmacokinetic parameters were estimated (Table 1). The results showed that arthritis induced by FCA did not alter the pharmacokinetic behaviors of diclofenac.

Pharmacodynamics of diclofenac in normal and FCA-induced arthritic rats

PGE_2 served as a biomarker to represent the pharmacodynamic index of diclofenac in rats. The results showed that FCA-induced arthritic rats had higher basal levels of plasma PGE_2 compared with those of normal rats, although no significance was found (Figure 4). The PGE_2 level-time data were assayed (Figure 4A) following an iv injection of 10 mg/kg

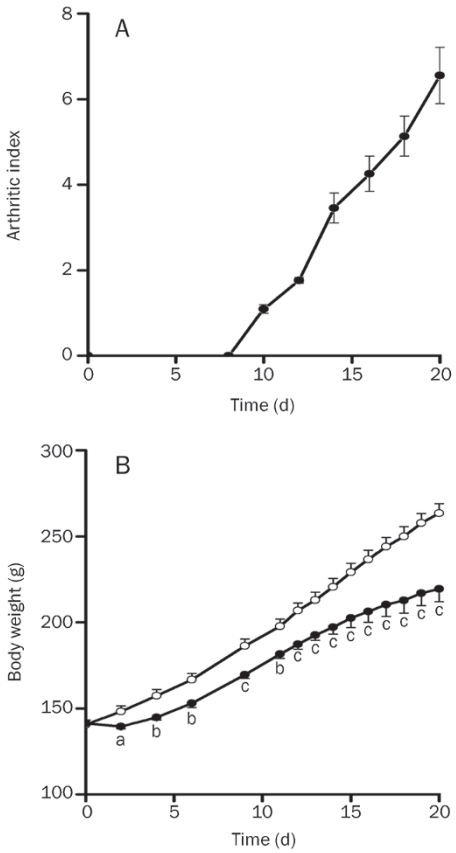

Figure 2. Arthritic index (A) and body weight (B) of normal (○, *n*=20) and FCA-induced rats (●, *n*=11) *vs* time (d). Values are the mean±SEM. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* normal group.

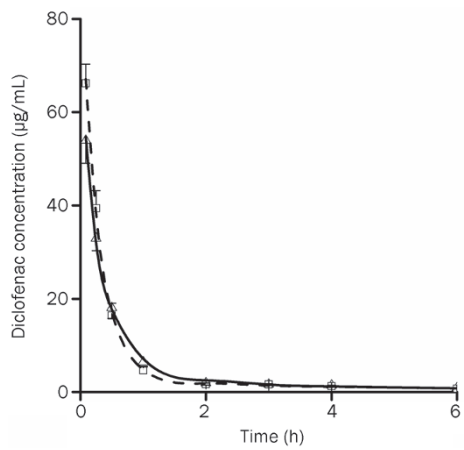

Figure 3. Observed (point) and fitted (line) diclofenac plasma concentration-time data after iv injection (10 mg/kg) of diclofenac in normal (□) and FCA-induced arthritic rats (Δ). The data are represented as the mean±SEM (*n*=6).

diclofenac. As expected, the administration of diclofenac significantly decreased the levels of PGE_2 in the plasma of both normal and FCA-induced arthritic rats, although a large degree of variability was observed. The maximum inhibition occurred 5 min after dosing. No delay in the onset of inhibition was observed, revealing that the effect compartment was located in the central compartment. This result agrees with a previous report that described the use of naproxen[14, 23].

The relationship between PGE_2 levels and diclofenac levels

Table 1. Pharmacokinetic parameters of diclofenac in normal and FCA-induced arthritis rats following iv 10 mg/kg diclofenac (mean±SEM).

Parameter	Normal	Arthritis
α (h^{-1})	3.6±0.2	4.4±1.7
β (h^{-1})	0.2±0.1	0.3±0.1
A (μg/mL)	86.9±3.1	75.5±15.9
B (μg/mL)	2.9±0.6	6.4±3.9
CL_1 [L/(kg·h)]	0.3±0.02	0.2±0.03
AUC (μg·h)/mL	40.0±2.9	52.0±11.9
$C_{5\ min}$ (μg/mL)	66.2±4.1	53.9±4.9
Beta $t_{1/2}$ (h)	3.3±0.8	3.2±0.9
V_1 (L)	0.11±0.005	0.14±0.02

CL_1, apparent plasma clearance of the central compartment; V_1, apparent volumes of distribution of the central compartment.

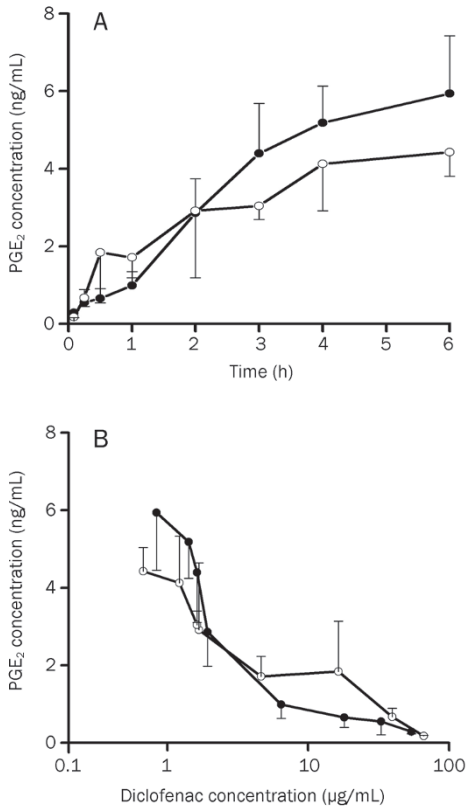

Figure 4. (A) Observed PGE_2 concentration-time data and (B) PGE_2 concentration-diclofenac concentration data after iv injection (10 mg/kg) of diclofenac in the plasma of normal rats (○) and FCA-induced arthritic rats (●). Data are represented as the mean±SEM (*n*=6).

in the plasma were fitted by an inhibitory effect sigmoid I_{max} model, and the pharmacodynamic parameters were estimated (Table 2). The potency of diclofenac was expressed as IC_{50} and IC_{80} values. Compared with normal rats, higher IC_{50} and IC_{80} values for PGE_2 production in FCA-induced arthritic rats suggested that the inhibitory effects of diclofenac on PGE_2 production in FCA-induced arthritic rats were less profound than that in normal rats, although a higher I_{max} value was found. The observed and fitted data for PGE_2 concentration (ng/mL) *vs* time (h) *in vivo* after iv (10 mg/kg) for normal (A) and FCA-induced rats (B) are shown in Figure 5.

Table 2. Pharmacodynamic parameter estimates for inhibition on PGE_2 production by diclofenac *in vitro* and *in vivo* using inhibitory effect sigmoid I_{max} model.

Model parameter estimates		
	Normal	
Parameter	*In vivo*	*In vitro* whole blood
IC_{50} (μg/mL)	0.5±0.2	1.3±0.7
IC_{80} (μg/mL)	2.8±1.4	2.3±1.3
E_0 (ng/mL)	11.7±1.3	63.9±17.6
I_{max} (ng/mL)	11.4±1.5	54.8±16.1
γ	1.1±0.3	2.0±0.4
	Arthritis	
Parameter	*In vivo*	*In vitro* whole blood
IC_{50} (μg/mL)	0.7±0.3	0.4±0.2
IC_{80} (μg/mL)	10.9±7.1	1.4±0.5
E_0 (ng/mL)	21.0±5.9	54.5±12.5
I_{max} (ng/mL)	22.1±6.0	40.7±5.3
γ	1.5±0.7	1.3±0.4

Inhibition of PGE_2 production by diclofenac in whole blood

The effect of diclofenac on PGE_2 production induced by LPS *in vitro* in whole blood was also investigated (Figure 6). The basal levels of PGE_2 induced by LPS *in vitro* in whole blood from normal and FCA-induced arthritic rats were measured to be 63.9±17.6 and 54.5±12.5 ng/mL, respectively. Diclofenac decreased PGE_2 levels in a concentration-dependent manner. The data from PGE_2-diclofenac levels *in vitro* in whole blood were also fitted using the inhibitory effect sigmoid I_{max} model (Table 2). The percent of maximum inhibition (I_{max}·100%) by diclofenac *in vitro* in whole blood (85.8% in normal rats and 74.6% in FCA-induced arthritic rats) was less than the maximum inhibition (98%) found *in vivo*. The estimated IC_{50} value *in vitro* in the whole blood of normal rats was higher than that *in vivo* (1.3±0.7 μg/mL *in vitro* whole blood *vs* 0.5±0.2 μg/mL *in vivo*), but the estimated IC_{50} value *in vitro* in the whole blood of FCA-induced arthritic rats was less than that *in vivo* (0.4±0.2 μg/mL *in vitro* whole blood *vs* 0.7±0.3 μg/mL *in vivo*). The IC_{50} and IC_{80} values *in vitro* in the whole blood of FCA-induced arthritic rats were less than those in normal rats. The observed and fitted data for PGE_2 concentration (ng/mL) *vs* diclofenac concentration (μg/mL) *in vitro* in the whole blood in normal (A) and FCA-induced rats (B) are shown in Figure 7.

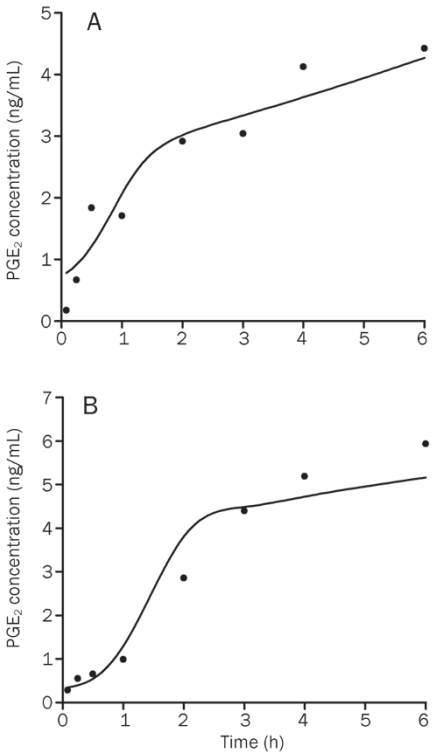

Figure 5. Observed and fitted PGE_2 concentration (ng/mL) *vs* time (h) *in vivo* after iv (10 mg/kg) in normal (A) and FCA-induced rats (B) (*n*=6, respectively). The symbols represent the mean raw data, and the lines represent the predictions from the model.

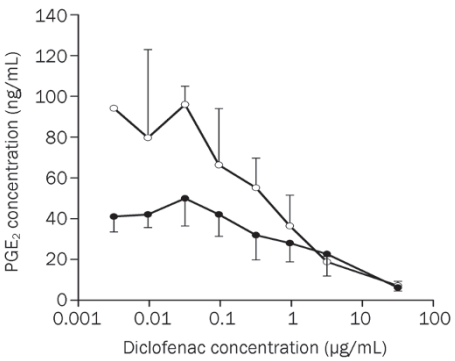

Figure 6. Observed PGE_2 concentration *vs* diclofenac concentration data *in vitro* in the whole blood of normal rats (○) and FCA-induced arthritic rats (●). Data are represented as the mean±SEM (*n*=4).

Discussion

It has been established that PGE_2 is released after inflammatory stimuli and the inhibition of PGE_2 can largely account for the therapeutic effects of NSAIDs[4]. PGE_2 levels are highly correlated with pain and inflammation, which indicates that PGE_2 can serve as a biomarker to bridge an intermediate step

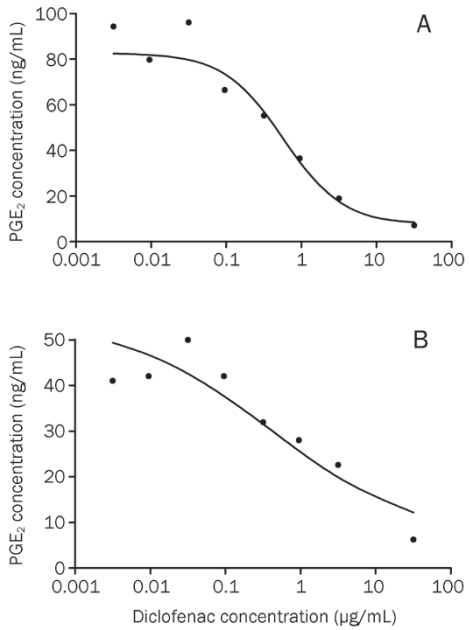

Figure 7. Observed and fitted PGE_2 concentration (ng/mL) *vs* diclofenac concentration (μg/mL) *in vitro* in the whole blood in normal (A) and FCA-induced rats (B) (n=4, respectively). The symbols represent the individual raw data, and the lines represent the predictions from the model.

between drug exposure and response. Due to the possibility of repeated measurements and increased reproducibility and sensitivity, PGE_2 could be a suitable alternative endpoint for investigations and comparisons of the time-course and potency of various drug candidates[23]. Several reports have shown the integrated PK-PD modeling of NSAIDs using hyperthermia, hyperalgesia, edema or PGE_2 as pharmacological endpoints[5, 11, 23], but little attention has been paid to the impact of the disease status on drug effects[16]. The aim of this study was to characterize the PK-PD modeling of diclofenac in normal and FCA-induced arthritic rats using PGE_2 as a pharmacological endpoint. Our results clearly demonstrate that arthritis induced by FCA does not alter the pharmacokinetic behaviors of diclofenac in rats. However, some reports have shown that acute inflammation may alter the pharmacokinetics of NSAIDs[24, 25]. This discrepancy may come from the oscillations of activities of CYP enzymes over period of inflammation and drugs[25]. The levels of diclofenac and the corresponding biomarker PGE_2 were measured after the administration of diclofenac. It was found that FCA-induced arthritic rats had higher basal levels of PGE_2 in the plasma, although no significance was found because of large inter-individual variability. A large degree of inter-individual variability for PGE_2 has also been shown in other reports[16, 26]. Similarly, such variability is often observed in levels of other endogenous compounds[27, 28]. It is generally accepted that cyclo-oxygenase-2 (COX-2) is responsible for PGE_2 production, which indicates that the increased biomarker levels are due to COX-2 activity during inflammation[12]. Diclofenac significantly inhibited PGE_2 production, and the maximum inhibition occurred 5 min after dosing. Then, the inhibitory effects gradually fell with the decline in diclofenac levels, which indicates that the inhibition of PGE_2 by diclofenac is reversible. The relationship between PGE_2 levels and diclofenac levels in plasma was successfully illustrated using an inhibitory effect sigmoid I_{max} model. Estimated IC_{50} and IC_{80} values for PGE_2 production in FCA-induced arthritic rats were higher than those in normal rats.

The effect of diclofenac on PGE_2 production induced by LPS *in vitro* in whole blood was investigated. In contrast to the *in vivo* results, the basal PGE_2 levels induced by LPS *in vitro* in the whole blood of FCA-induced arthritic rats was lower than that in normal rats, which indicates that the sensitivity of the whole blood of the FCA-induced arthritic rats to LPS was less than that of the whole blood of the normal rats. In addition, the percent of maximum inhibition (I_{max}·100%) by diclofenac *in vitro* in whole blood was less than that *in vivo*. This discrepancy might have resulted from COX-1 inhibition. The *in vitro* whole blood experiment was performed in the presence of aspirin (10 μg/mL), which irreversibly inactivates platelet COX-1. It has been reported that rat blood might also produce copious amounts of PGE_2 via the actions of the COX-1 enzyme that is constitutively present in platelets[29]. The inhibition of COX-1 by aspirin might explain the discrepancies between the *in vivo* studies and the *in vitro* whole blood studies for PGE_2 inhibition.

In summary, these results support the conclusion that arthritis induced by FCA did not alter the pharmacokinetic behaviors of diclofenac in rats, but the pharmacodynamics of diclofenac were slightly affected. A pharmacokinetic-pharmacodynamic model characterizing an inhibitory effect sigmoid I_{max} was developed to illustrate the relationship between the plasma concentration of diclofenac and the inhibition of PGE_2 production both in normal and FCA-induced arthritic rats.

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Author contribution

Jing ZHANG and Xiao-dong LIU designed the experiments and analyzed the data; Jing ZHANG wrote the paper; Xiao-dong LIU and Li LIU revised the paper; Jing ZHANG, Pei LI, and Hai-fang GUO performed the research.

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