Design, Synthesis, and *in-Vivo* Evaluation of 4,5-Diaryloxazole as Novel Nonsteroidal Anti-inflammatory Drug

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A series of 4,5-diaryloxazole analogs were designed and the interaction between oxaprozin and cyclooxygenase-2 studied by the docking method to improve the biological activity and reduce the gastrointestinal side effects of oxaprozin. Finally, 3-(4-(4-fluorophenyl)-5-(4-aminosulfonyl-3-fluorophenyl)-oxazole-2-yl) propanoic acid (NC-2142), the best candidate, was selected for synthesis and bioassay based on the screening result. NC-2142 could lower the tumefaction rates of back metatarsus in rats, as well as reduce the writhing times in mice. NC-2142 produced fewer gastric lesions than oxaprozin. After the aminosulfonyl group was introduced into the benzene ring of oxaprozin, its analgesic and anti-inflammatory activities remained unchanged, and it reduced the number of gastric lesions. This provided a feasible method for further structure modification and optimization of oxaprozin.

Key words 4,5-diaryloxazole; analgesic activity; anti-inflammatory activity; oxaprozin analogue

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used agents for the treatment of pain, inflammation, and fever. Long-term or high-dose therapy with NSAIDs is associated with increased risk of damage to the gastrointestinal (GI) tract^{1,2)} and renal side effects.^{3–5)} This is because of the blocking of prostaglandin (PG) production in inflammatory cells, and interference of the homeostasis of PG production in the GI tract. Finding novel agents that can maintain the representation of physiological PGs and reduce the production of pathological PGs is a challenge.

Oxaprozin (Fig. 1) possesses a longer-term effect and lower GI toxicity than other NSAIDs,^{6,7)} but its poor selectivity, high dose, and long half-life greatly limit its clinical use.^{8,9)} Puig *et al.* have studied a series of 3,4-diaryloxazole derivatives, the results show that when the benzene ring has the substituent of 4-methylsulfonyl or 4-aminosulfonyl, the derivatives possess anti-inflammatory and analgesic activities, at the same time, the fluoro substitutes on the benzene ring, the potency has increased 3 times compared with the non-substituted derivatives.¹⁰⁾ In the study of cyclooxygenase-2 (COX-2) inhibitors, a series of isoxazole derivatives have also been developed. The structure–activity relationship research shows that when introducing aminosulfonyl group into benzene ring which is directly connected with isoxazole, its aminosulfonyl group can be deep into the COX-2 hydrophobic side pocket, thus enhancing the selectivity of COX-2 inhibition activity, showing good analgesic and anti-inflammatory activities.^{11–13)} Accordingly, we try to introduce methylsulfonyl or aminosulfonyl as well as fluoro substitution into oxaprozin. A novel class of 4,5-diaryloxazole derivatives were designed to elevate the biological activity

Fig. 1. Structure of Oxaprozin

and reduce the GI toxicity of oxaprozin (Fig. 2). This design was according to the structure–activity relationship of the diarylheterocycle class of COX-2 inhibitors¹⁴⁾ and the mechanism of NSAIDs.¹⁵⁾ Docking was introduced to evaluate the antagonistic potency of these analogs towards COX-2 in silico, and the best hit was selected for synthesis and bioassay.

MATERIALS AND METHODS

Materials Melting points were determined using a YRT-3 melting point apparatus (Tianjin Optical Instruments Factory, China). IR spectra were obtained with a Nicolet Instruments Impact 400 Fourier-Transform Infrared Spectrophotometer using potassium bromide plates. ¹H- and ¹³C-NMR spectra were determined on a Bruker DPX-300 MHz spectrometer. Mass spectra were obtained with an Agilent-1100 spectrometer. Elemental analysis (EA) was carried out on a Perkin-Elmer 2400 C, H, N analyzer. Elemental microanalyses were carried out with a Flash EA-1112 apparatus. All reagents were purchased from the Reagent Number One Factory of Shanghai Chemical Reagent Company, Limited

Compd	R ¹	R ²	R ³	R ⁴
NC-2141	H	F	H	H
NC-2141a	F	H	H	H
NC-2141b	H	H	F	H
NC-2142	H	F	SO ₂ NH ₂	H
NC-2142a	H	SO ₂ NH ₂	H	F
NC-2142b	SO ₂ NH ₂	H	H	F
NC-2143	H	F	SO ₂ CH ₃	H
NC-2143a	H	SO ₂ CH ₃	H	F
NC-2143b	SO ₂ CH ₃	H	H	F

Fig. 2. Novel-Designed Oxaprozin Analogues

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KM Male and female mice (18–22 g) were used for the writhing test. F/M male Wistar rats (140–160 g) were used for the carrageenan-induced paw edema test and the ulcerogenicity test and determination experiment of PGE₂ level. Mice were housed individually at room temperature in a 12-h light/12-h dark cycle. All the experimental animals were provided by the Laboratory Animal Center of Jilin University. The protocol was performed in accordance with ethical guidelines for the use and care of animals. Oxaprozoin was obtained from Jiamusi Lulling Pharmaceutical Company Limited (Heilongjiang, China).

Molecular Modeling (Docking) Studies Docking studies were performed using Insight II software running on a SGL O38600 workstation. Coordinates for the X-ray crystal structure of the selective COX-2 inhibitor SC-558 bound to the electric ray COX-2 enzyme was from the RCSB Protein Data Bank (PDB ID:1CX2, resolution=3.0 Å). Ligand molecules were constructed using the Builder module and were energy optimized. 1CX2 were molecular dynamics-optimized using a consistent valence force field (CVFF). Energy-minimized ligands were superimposed on SC-558 in the PDB file 1CX2 after which SC-558 was deleted. The optimized ligand–enzyme complex was further subjected to molecular dynamic simulation using the affinity/docking module of Insight II software (Figs. 3, 4 and Table 1).

Synthesis of 3-[4-(4-Fluorophenyl)-5-(4-aminosulfonyl-3-fluorophenyl)oxazole-2-yl] Propanoic Acid (NC-2142) NC-2142 was synthesized from α -(3-fluorophenyl)-4-fluoroacetophenone *via* Chart 1. Intermediates **1** and **2** were prepared according to the reported method.¹⁶⁾ The intermediate **2** (15 mmol) in chlorosulfonic acid (15 ml) was stirred at room temperature for 96 h, and then immediately poured into ice-water. The product was filtered, dried, and recrystallized from acetone/petroleum ether; the yield was 45%. The solid in aqua ammonia (60 ml) was refluxed for 2.5 h, and pH adjusted to 1.0–2.0 using hydrochloric acid. The solid was filtered and recrystallized with tetrahydrofuran/*n*-hexane. mp 233–235 °C. IR (KBr) cm⁻¹: 3342, 3258, 2917, 1718, 1611, 1561, 1334. ¹H-NMR (DMSO-*d*₆) δ : 3.08 (2H, t, *J*=7.2 Hz), 2.80 (2H, t, *J*=7.2 Hz), 7.33 (2H, m), 7.45 (2H, m), 7.59 (2H, m), 7.72 (2H, d), 7.83 (1H, t), 12.40 (1H, br s). ¹³C-NMR (DMSO-*d*₆) δ : 173.2, 23.1, 30.2, 163.7, 136.4, 142.3, 128.0, 130.3, 116.3, 162.5, 116.1, 130.2, 134.0, 114.0, 158.4,

131.3, 129.6, 121.9. MS (EI) *m/z*: 409 (M⁺+1), 330. *Anal.* Calcd for C₁₈H₁₄F₂N₂O₅S: C, 52.94; H, 3.46; N, 6.86; S, 7.85. Found: C, 53.0; H, 3.54; N, 6.87; S, 7.81.

Pharmacology. Analgesic Activity Analgesic activity was evaluated by acetic acid-induced writhing response test in mice.¹⁷⁾ The mice were randomly divided into control group, oxaprizin (100 mg·kg⁻¹) group, NC-2142 25 mg·kg⁻¹, 50 mg·kg⁻¹ and 100 mg·kg⁻¹ three groups, 10 mice every group. The mice were administered NC-2142 and oxaprozoin by intragastric administration once daily for 3 d.

Fig. 3. Docking NC-2142 in the Active Site of COX-2

Fig. 4. Docking NC-2142 in the Active Site of COX-1

Table 1. Affinity Energy of Novel Designed Oxaprozoin Analogues and COX-2/COX-1

	Total (kcal·mol ⁻¹)		Tot NB ^{a)} (kcal·mol ⁻¹)		Ele ^{b)} (kcal·mol ⁻¹)		Vdw ^{c)} (kcal·mol ⁻¹)		ΔE^d (kcal·mol ⁻¹)	
	COX-2	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2	COX-1
Enzyme	649.86	765.41	15.16	15.16	-39.44	-24.46	54.60	50.24		
Oxaprozoin	635.48	751.78	21.36	14.17	-40.46	-35.34	61.82	65.73	-14.38	-13.63
NC-2141	640.05	761.58	18.17	6.69	-41.66	-31.89	59.84	60.01	-9.81	-3.83
NC-2141a	644.34	763.69	16.99	6.00	-40.74	-36.89	57.73	58.36	-5.52	-1.72
NC-2141b	642.15	762.63	17.11	8.32	-41.10	-35.48	58.22	58.45	-7.708	-2.78
NC-2142	570.79	723.07	31.00	22.04	-48.87	-38.76	79.87	70.23	-79.07	-42.34
NC-2142a	573.95	732.87	29.42	18.38	-46.26	-36.67	75.67	68.39	-75.90	-32.54
NC-2142b	575.13	741.50	28.20	16.69	-45.03	-33.35	73.22	67.18	-74.72	-23.91
NC-2143	581.52	742.84	27.03	14.56	-43.90	-32.98	70.93	63.76	-68.31	-22.57
NC-2143a	583.81	745.24	26.69	13.39	-43.22	-33.12	69.91	61.51	-66.04	-20.17
NC-2143b	584.63	747.03	25.16	11.26	-42.12	-31.78	67.29	61.22	-65.22	-18.38

a) Tot NB is total non-bond energy; b) Ele is electrostatic energy; c) Vdw is Van der Waals energy; d) ΔE is binding energy.

The same volume of 0.5% carboxymethyl cellulose as vehicle was administered to the control group. One hour after the last treatment, the total number of writhings after intraperitoneal administration of 1.0% acetic acid (v/v, 0.1 ml/10 g body weight) was recorded for 15 min after the acetic acid injection. The mean writhing scores in the control group were calculated (Table 2).

Anti-inflammatory Activity Anti-inflammatory activity was evaluated by carrageenan-induced paw edema test in rats.¹⁸⁾ The mice were randomly divided into control group, oxaprizin ($70 \text{ mg} \cdot \text{kg}^{-1}$) group, NC-2142 $20 \text{ mg} \cdot \text{kg}^{-1}$, $40 \text{ mg} \cdot \text{g}^{-1}$ and $80 \text{ mg} \cdot \text{kg}^{-1}$ three groups, 10 mice every group.

Chart 1. Synthesis of NC-2142

Reagents and conditions: (a) NBS, 25°C , 5 h; (b) $\text{CH}_3\text{COCH}_3/\text{H}_2\text{O}$, NaHCO_3 , 50°C , 5 h; (c) succinic anhydride, pyridine, $90\text{--}95^\circ\text{C}$, 1.5 h; (d) ammonium acetate, glacial acetic acid, $90\text{--}95^\circ\text{C}$, 2.5 h; (e) HSO_3Cl , 25°C , 96 h; (f) NH_4OH , reflux, 2.5 h.

Table 2. Effect of NC-2142 on Acetic Acid-Induced Writhing Response Test in Mice

Compound	Dose (mg/kg, i.g.)	Writhing times	Inhibition (%)
Control	0	33.3 ± 14.9	0
NC-2142	25	21.9 ± 19.4	34.2
	50	$16.5 \pm 15.3^{**}$	50.5
	100	$15.3 \pm 9.6^{***}$	54.0
Oxaprozoin	100	$11.6 \pm 7.0^{***}$	65.2

The results given are mean $\pm$ S.D. ($n=10$), $^{**}p<0.01$, $^{***}p<0.001$ compared with control group.

Table 3. Effects of NC-2142 on Carrageenan-Induced Paw Edema Test in Rats

Compound	Dose (mg $\cdot$ kg $^{-1}$, i.g.)	Normal paw volume (ml)	Paw volume 3 h after inflammation (ml)	Anti-inflammatory activity (%) ^{a)}	<i>p</i> value
NC-2142	20	1.07 ± 0.07	1.78 ± 0.25	13.4	>0.05
NC-2142	40	1.02 ± 0.05	1.66 ± 0.27	22.0	<0.05
NC-2142	80	1.03 ± 0.08	1.59 ± 0.26	31.7	<0.01
Oxaprozoin	70	1.08 ± 0.09	1.51 ± 0.32	47.6	<0.05
Control	—	1.05 ± 0.07	1.87 ± 0.32	—	—

The results given are mean $\pm$ S.D. ($n=10$). ^{a)} Anti-inflammatory activity (%) = $(1 - A/B) \times 100$, where *A* is the percentage difference in paw volume after NC-2142 was administered to the rats; *B* is the percentage difference of volume in control groups.

NC-2142 and oxaprozoin were given to rats by intragastric administration once daily for 3 d. The same volume of 0.5% carboxymethyl cellulose used as vehicle was given to the control group. One hour after the last treatment, carrageenan 0.1 ml (1%, w/v) solution in distilled water was subcutaneously injected into the plantar surface of the right front paw of all rats. Paw volume until the knee joint was measured by plethysmometer before injection of carrageenan solution. Carrageenan-induced paw edema was measured at 1 h intervals for 3 h. Anti-inflammatory activities of NC-2142 and oxaprozoin were determined by comparing the results of the experimental group with those of the control group (Table 3).

Induced inflammatory of carrageenan in rats 3 h later, draw blood from femoral artery, centrifugate, take serum and preserve it at 4°C . Then execute the rats, cut the inflammation swelling paw and weigh, mince, steep in physiological saline, centrifugate, get the supernatant and preserve it at 4°C .

Prostaglandin E_2 Determination According to the method by Hibino *et al.*¹⁹⁾ to determinate the PGE_2 level of inflammatory paw tissue and serum in rats. Get serum and supernate 0.1 ml respectively, put in $0.5 \text{ mol} \cdot \text{ml}^{-1}$ KOH-methanol solution 2 ml, isomerization 20 min at 50°C , then use methanol to dilute 5 ml, and determinate the *A* at wavelength 278 nm. Use serum (*A*/ml) and paw tissue (*A*/g) to demonstrate the PGE_2 level (Table 4).

Evaluation of Gastric Lesions F/M wistar rats were divided into six groups, fasted overnight, and allowed to have water. Rats were intragastric administrated the test compounds. After administration, the rats were returned to their cages without food. Rats were killed using CO_2 16 h after dosing. The stomach was removed and dissected along the greater curvature. The mucosal lesion area was determined by the method of Higuchi and Osada²⁰⁾ (Table 5).

Statistical Analysis Statistical analysis of the biological activity of the synthesized NC-2142 on animals was evaluated using Student's *t*-test. Data are presented as mean $\pm$ standard deviation (S.D.).

RESULTS AND DISCUSSION

In the drug and the enzyme docking process, the lower the binding energy (ΔE) of the drug and the enzyme is, the more stable the complex formed will be, and the drug is located in the active site of the enzyme with a stable and low-energy state. Table 1 shows that the binding energy of Oxaprozoin analogues with COX-2 are lower than that with COX-1, which indicate that oxaprozoin analogues with COX-

Table 4. Effects of NC-2142 on PGE₂ from Paw Tissue and Blood Serum in Rats

Compound	Dose (mg/kg, i.g.)	PGE ₂	
		Serum (A/ml)	Paw tissue (A/g)
NC-2142	20	0.183±0.022	0.153±0.018*
	40	0.178±0.024*	0.121±0.015*
	80	0.165±0.018**	0.109±0.026**
Oxaprozin	70	0.177±0.025**	0.119±0.030**
Control	—	0.268±0.032	0.184±0.013

* $p<0.05$, ** $p<0.01$ compared with control. The results given are mean $\pm$ S.D. ($n=10$).

Table 5. Ulcerogenicities of NC-2142 and Oxaprozin in Rats (16 h)

Compound	Dose (mg/kg, i.g.)	Mucosal lesion area (mm ²)	p value
NC-2142	200	0.04±0.03	>0.05
	400	0.13±0.05	>0.05
	800	1.04±0.47	<0.01
Oxaprozin	200	0.17±0.04	<0.05
	400	1.76±0.55	<0.01
	800	10.34±1.24	<0.05

Each value is the mean $\pm$ S.D. ($n=10$).

2 complexes are more stable than that with COX-1 complexes. Namely, in the presence of oxaprozin analogues, COX-2 changes its conformation, making the analogues adsorbed on the COX-2 firmly. The binding energy when only one fluorine atom is introduced into the benzene ring of oxaprozin analogues is higher than that introduced fluoro and aminosulfonyl group at the same time, that is, the introduction of aminosulfonyl group is beneficial to the binding between ligands and enzyme, making the ligands exist stably in the active site of the enzyme with a stable low-energy state. The binding energy between NC-2142 and COX-2 is lowest of all, and nearly 2 fold lower than that with COX-1 (COX-1: $\Delta E = -42.34 \text{ kcal} \cdot \text{mol}^{-1}$, COX-2: $\Delta E = -79.07 \text{ kcal} \cdot \text{mol}^{-1}$). The affinity energy between drug and enzyme is mainly non-bond energy, comprising van der Waals energy, electrostatic energy and hydrogen bonding energy, of which the hydrogen bonding energy is the most important. Figures 3 and 4 showed the docking results of NC-2142 with COX-2 and COX-1 respectively. Figure 3 showed that NC-2142 could be accurately docked into the active site of COX-2, and that the SO₂NH₂ group of NC-2142 had multiple interactions with Leu352, Arg513, Phe518, and Val523 by selective insertion into the side pocket of the COX-2 active site. There are 5 couples of hydrogen bonding between the oxygen, nitrogen and sulfur atoms of NC-2142 and amino acid residues existing in active site, such as Phe-A518, Arg-A120, Arg-A513. Figure 4 showed 4 couples of hydrogen bonding between NC-2142 and amino acid residues (His-A388, Asn-A382) of the COX-1 active site. The system formed by NC-2142 and COX-2, electrostatic energy $-48.87 \text{ kcal} \cdot \text{mol}^{-1}$, has the lowest energy of all oxaprozin analogues and enzyme systems. The smaller the electrostatic energy is, the stronger the electrostatic interaction is, the more stable the complex formed is. According to the results above, after introducing a fluoro and an aminosulfonyl group into benzene ring of

oxaprozin analogues, the complex formed with COX-2 was more stable than that with COX-1. These results also indicated that the oxaprozin analogs possessing a sulfonyl substituent in one of the phenyl rings can selectively inhibit the activity of COX-2, of which NC-2142 is the most notable. NC-2142, the best candidate, was selected for synthesis and bioassay based on the screening result.

We have designed the synthetic route of NC-2142 and investigate its synthesis technology, and determine the optimization of synthetic route. After the bromination product of α -(3-fluorophenyl)-4-fluoroacetophenone hydrolyzed, there was no need to separate and purify the hydrolysis, directly added ammonium acetate and glacial acetic acid, the yield was 76%. Then after sulfonylchlorination, together with ammonia solution reaction, obtained target compound NC-2142, NC-2142 was confirmed by IR, NMR, MS and elemental analysis.

Table 2 shows the analgesic effect of NC-2142 on the acetic acid-induced writhing response test in mice. After 1.0% acetic acid was injected, NC-2142 showed an inhibitory value of up to 54.0% at 100 mg/kg over 15 min. The writhing times of all experimental groups were significantly different from those of the control group ($p<0.01$ or $p<0.001$). The anti-inflammatory activity of NC-2142 was evaluated by carrageenan-induced paw edema test in rats. Carrageenan-induced edema is commonly used as an experimental model of acute inflammation in animals and is believed to be biphasic; the early or first phase is mediated by the release of histamine and 5-hydroxytryptamine (5-HT), followed by kinin release, followed by PG release in the later or second phase.²¹⁾ It is also known that COX and lipoxygenase also have a crucial role in the formation of carrageenan-induced edema.²²⁾ The effects of NC-2142 and the reference drug oxaprozin on carrageenan-induced paw edema in rats are shown in Table 3. NC-2142 showed anti-inflammatory activity up to 31.7% at 80 mg·kg⁻¹ after 3 h of drug treatment in carrageenan-induced paw edema.

The mechanism of inflammation is correspondingly complicated, it's generally considered that exterior stimulus results in initial injuries, thereby release many mediators of inflammation which induce inflammation. PGE₂ is one of the important mediators of inflammation, the generation of PGE₂ is closely correlated with COX, and COX-2 can induce generous pathological PGE₂ which promotes inflammatory reaction and tissue damage.^{23,24)} We have studied the effects of NC-2142 on PGE₂ level of inflammatory parts and serum in rats, the results show that NC-2142 can inhibit the product of PGE₂ of inflammatory tissue and serum (Table 4). Therefore, we may conclude that NC-2142 can alleviate inflammatory reaction by inhibiting the synthesis of PGE₂.

Analgesic and anti-inflammatory bioactivity remained effective when the aminosulfonyl group was introduced into the benzene ring of oxaprozin. Gastric lesions (Table 5) induced by NC-2142 and oxaprozin indicate that oxaprozin had a significant effect on the gastric mucosa in a dose-related manner. The results were consistent with those of Sheiver *et al.*²⁵⁾ NC-2142 produced fewer gastric lesions than oxaprozin, with statistical significance at 800 mg/kg. Gastric lesions may correlate with the binding energy with the COX-2 receptor.

In summary, the compounds formed after introduction of

the aminosulfonyl group into the benzene ring of oxaprozin not only retained the analgesic activity and anti-inflammatory activity of oxaprozin, but also significantly reduced the number of gastric lesions. The present study is promising for further structure modification and optimization of oxaprozin, as well as for developing novel anti-inflammation therapies.

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