Leflunomide Analogues as Potential Antiinflammatory Agents

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A series of leflunomide (1a) analogues were examined for antiinflammatory activity using the carrageenan-induced paw edema assay. Some of the compounds were significantly more potent than leflunomide, particularly those with electron-donating or negative inductive groups situated in the phenyl rings. In contrast, all the non-substituted compounds or with further chain-extension in the 4-position of the rings led to a decrease in activity. The LD_{50} values of the most active compounds (1d, g—j) in male ICR mice were significantly greater than those of either 1a or its active metabolite 2 and therefore merit further study.

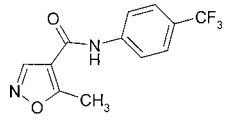
Key words antiinflammatory agent; leflunomide; carrageenan-induced paw edema assay

Leflunomide (**1a**) is an isoxazole-containing heterocyclic compound recently approved for the treatment of active rheumatoid arthritis in the U.S.A.[1] Being a prodrug, upon absorption leflunomide quickly converts to its ring-opened isomer malononitrilamide **2**[2—4] as the active therapeutic agent, which in turn confers immunomodulating activity through the dual mechanisms of selective inhibition of dihydroorotate dehydrogenase[5] and tyrosine kinase[6] and thus suppresses T cell proliferation.

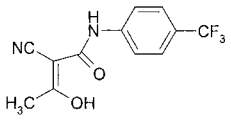
Leflunomide is clinically efficacious, but nevertheless is not free from adverse effects. Its sluggish clearance, caused by the very long plasma half-life (18 d) of **2**,[6] and severe liver function impairment have resulted in unusual dose regimens or drug monitoring protocols. These clinical difficulties have prompted intensive efforts to investigate leflunomide analogues, mainly by structural optimization in the phenyl ring, as novel, potent immunomodulating agents with fewer and less severe adverse effects. We report herein their antiinflammatory activity and structure–activity relationships (SARs).

Chemistry Compounds **1a**—**f** were prepared as the patent describes,[7] and compounds **1g**—**k** by our previously method reported.[8] Compound **1l** was obtained by hydrolysis of **1k**. The active metabolite **2** used as a positive control in the antiinflammatory test was obtained by hydrolysis of **1a**.[9,10] The structures of unpublished **1i** (mp 152—154 °C), **1k** (mp 252—254 °C) and **1l** (mp 184—186 °C) were established by spectroscopy and elemental analysis.

Even though **2** exhibited two conformations in a solvent-dependent manner, the (*Z*)-isomer is dominant due to marked stabilization by forming a strong intramolecular hydrogen bond, which was considered crucial in the biological activity.[9] The presence of an intramolecular hydrogen bond of **2** was supported by ^{1}H-NMR studies in which the 2-hydroxy proton signal appeared as a singlet in the downfield region of the spectrum (δ 12.38, DMSO-d_6). This was also confirmed by X-ray crystallography[9,11] and a molecular modeling study[12] in the literature.



1a, Leflunomide



2, Active metabolite

Biological Evaluations The carrageenan-induced paw edema assay[13] in male Sprague–Dawley rats (250±35 g) was used to evaluate the *in vivo* antiinflammatory activity. The results, expressed as percent inhibition of the response to carrageenan-induced inflammation, are presented in Tables 1 and 2. For comparison, dexamethasone and ibuprofen were used. Table 1 shows that leflunomide (**1a**) demonstrates more activity than ibuprofen, and both exhibit dose-dependent efficacy. The active metabolite **2**, as expected, is superior to its parent compound **1a** at 10 mg/kg. From Table 2, it is noteworthy that, compared with leflunomide (**1a**), nearly all the analogues show higher or similar intraperitoneal activity with the exceptions of nonsubstituted **1b**, 2-chloro-substituted **1c** and glutamic acid derivatives **1k** and **1l**.

Our preliminary results show that in the structure modification of leflunomide (**1a**), introduction of electron-donating substituents such as 4-OCH_3 (**1h**), 3,4,5-$(OCH_3)_3$ (**1i**), or negative inductive substituents with a flanking steric hindrance such as 3-Cl (**1d**), 3-CF_3 (**1f**), and 2,4-$(Cl)_2$ (**1g**) in the aromatic moiety significantly increases the antiinflammatory activity. The nature of the substituents in the 4-position does not seem to influence the activity noticeably, as shown in **1e** (4-Cl), **1h** (4-OCH_3), and **1j** (4-COOH). The unsubstituted

Table 1. Antiinflammatory Activities of Dexamethasone, Ibuprofen, **1a**, and **2**

Compounds	Dose (mg/kg)	Inhibition (%)* after carrageenan administration (h)[a,b]		
		1	3	5
Dexamethasone	1.5	73.6±0.7	65.8±1.5	66.4±0.4
Ibuprofen	10	9.1±1.1	45.9±0.1	38.0±0.4
	20	20.8±1.8	55.7±1.1	51.6±1.6
	30	65.4±0.2	79.7±0.2	53.0±4.2
Leflunomide (**1a**)	5	3.8±0.9	41.0±0.7	38.3±1.2
	10	28.4±1.2	55.0±2.4	46.1±1.4
	20	45.7±0.8	60.4±1.2	57.4±0.9
Malononitrilamide **2**[c]	10	26.9±1.5	63.5±0.5	62.5±1.3

a) Edema rate (*E* %) was calculated as follows: $E\,\% = (V_t - V_0)/V_0 \times 100$; V_0: volume of hind paw before 1% carrageenan administration; V_t: volume of hind paw after 1% carrageenan administration at *t* h. Percentage of inhibition (*I* %) was determined as follows: $I\,\% = (E_c - E_t)/E_c \times 100$; E_c: edema rate of control group; E_t: edema rate of test compound at *t* h. Student's *t*-test was performed. Each value represents the mean±S.D. of 5 animals. Each statistically significant difference from control was expressed as $*p<0.05$. *b*) Volume of control group was 3.18±0.15 (0 h), 3.90±0.19 (1 h), 4.94±0.24 (3 h), 5.23±0.35 (5 h) ml; injection volume of normal saline is 0.1 ml at 0 h. *c*) Malononitrilamide is the active metabolite of leflunomide.

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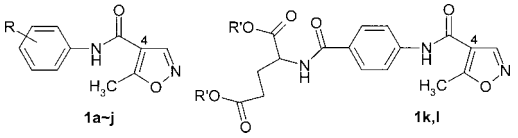
Table 2. Antiinflammatory Activities of Leflunomide Analogues at the Dose of 10 mg/kg



Compound	R (**1a**—**j**) and R′ (**1k**—**l**)	Inhibition (%) after carrageenan administration (h)[a]		
		1	3	5
Leflunomide (**1a**)	4-CF_3	28.4±1.2	55.0±2.4	46.1±1.4
1b**	H	6.7±0.8	27.7±2.1	27.8±2.2
1c**	2-Cl	8.2±0.4	16.6±1.0	17.2±2.1
1d*	3-Cl	46.2±0.5	69.1±0.3	67.1±1.8
1e*	4-Cl	32.2±1.1	42.9±2.0	42.3±2.0
1f*	3-CF_3	38.9±2.0	40.7±0.4	30.7±1.4
1g*	2,4-$(Cl)_2$	40.9±0.6	63.3±0.3	63.7±0.3
1h*	4-OCH_3	31.3±0.1	66.5±0.2	66.2±0.6
1i*	3,4,5-$(OCH_3)_3$	53.4±0.8	73.9±1.1	66.2±0.5
1j*	4-COOH	46.6±1.4	40.9±2.7	41.4±3.1
1k*	C_2H_5	34.1±0.8	25.5±1.8	28.2±2.1
1l**	H	3.8±1.4	22.1±2.7	25.1±2.4

a) Percentage of inhibition was determined as described in the Table 1 footnote. Student's *t*-test was performed. Each value represents the mean±S.D. of 5 animals. Each statistically significant difference from control was expressed as $*p<0.05$ or $**p<0.1$.

compound **1b** and 2-chloro-substituted **1c**, on the other hand, were virtually inactive. However, a carboxylic acid group appended in the 4-position of the aromatic ring made **1j** as potent as leflunomide. This indicates that the COOH group in **1j** ionized in the physiological environment might play a vital role in the requisite drug–receptor complexation through electrostatic interaction. Interestingly, increasing the bulk and the length of the chain of the substituents in the 4-position by extending the carboxylic acid group as part of glutamic acid **1l** or its ester **1k**, mimicking methotrexate, drastically reduces activity, which might be interpreted as a steric requirement for activity.

Determination of LD_{50} Values in Male Mice Leflunomide (**1a**) and its metabolite **2** and the most potent products (**1d**, **g**—**j**) were chosen to determine the LD_{50} values in male ICR mice (20±2 g) after intraperitoneal administration of the compounds.[14] The values measured for **1d**, **1g**, **1h**, **1i**, and **1j** were all 400 mg/kg, which was significantly higher than that of leflunomide (250 mg/kg) and **2** (200 mg/kg). The acute symptoms observed before death included agitation, lacrimation, crawling, trembling, and reduced motility. Cyanosis and deep necrosis were noticed at death in mice that received **1a**, **2**, and **1i** between 1 and 12 h after dosing. This may suggest that structural modification in the phenyl ring of leflunomide might lead to a reduction in toxicity, while the cyanopropenamide moiety in the ring-opened form of the active metabolite **2** might contribute to toxicity. If this is the case, the analogues might also have improved the stability of isoxazole ring. Currently, we are making further modifications in position 3 of the isoxazole ring of leflunomide on the basis of bioisosterism to produce 5-methylisoxazole-3-carboxamides with to study their SARs and stability in the physiological environment further. This investigation is in progress and will be reported shortly.

In conclusion, we have shown that optimization of the phenyl ring of leflunomide offers a method to improve pharmacological profiles and thus suggests approaches to develop new drugs useful in the treatment of inflammatory or immunologic disorders.

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