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Design and synthesis of furoxan-based nitric oxide-releasing glucocorticoid derivatives with potent anti-inflammatory activity and improved safety

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Abstract—A series of furoxan-based nitric oxide-releasing glucocorticoid derivatives was synthesized. The pharmacological assays indicated that three compounds, including I_4, I_5, and I_6, had anti-inflammatory activity. Furthermore compared with the leading compound hydrocortisone the safety of I_6 was greatly improved. Due to releasing NO in vivo the side effects of glucocorticoids, including hypertension and osteoporosis, were effectively avoided.
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Glucocorticoids (GC) have been widely used for their potent anti-inflammatory and immunosuppressive actions since the 1950s. However, the side effects, which depend on the dose used and duration of treatment, greatly limit the application of GC.[1] The long-term treatment with GC leads to an array of unwanted effects ranging from alteration of the endocrine homeostasis to a number of complications such as hypertension, osteoporosis, gastrointestinal damage, etc. Therefore, the development of potent GC derivatives with improved safety is crucial.

Nitric Oxide (NO), which is naturally generated from L-arginine by the action of NO synthase (NOS), is a key signaling molecule involved in the regulation of many physiological processes including vascular relaxation, neurotransmission, and events of the immune system. Dysfunction of NO formation has been implicated in the pathogenesis of a number of disorders. Exogenous NO sources constitute a powerful way to supplement NO when the body cannot generate enough for normal biological functions.[2,3] NO-releasing agents, such as glyceryl trinitrate, have been used to treat cardiovascular diseases for several decades, though its mechanism of action was not elucidated until the 1980s when the biology of NO was identified. Moreover, a great number of encouraging research results for NO-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) have demonstrated that NO could exert strong anti-inflammatory effects as well as reduce gastrointestinal damage.[4] Recently, it was found that NO could both inhibit bone resorption and increase bone formation,[5] which will be very beneficial to osteoporosis sufferers.

In our study, we describe a group of NO-releasing derivatives of hydrocortisone that act as a new class of improved GC-related agents. Benzenesulfonyl-substituted furoxans, a kind of NO donors which can donate two molecules of NO in vivo under the action of thiol cofactors,[6] had been incorporated to the 21 position of hydrocortisone through various spacers (as shown in Table 1). In 2004, Pier and his co-workers reported a group of NO-releasing derivatives of GC, which have proved that the structure modification of the 21 position does not interfere with the molecular recognition of GC.[7] In our design, furoxans were substituted for organic nitrates acting as NO donors, because furoxans are supposed to be able to release higher concentrations of NO in vivo and can also effectively avoid "the nitrate tolerance". By releasing NO in vivo, we hope to enhance the anti-inflammatory activity of these derivatives and particularly to minimize the side effects.

Keywords: Glucocorticoids; Hydrocortisone; Furoxan; NO-donors; Anti-inflammatory activity; Safety.

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Table 1. The structures of the target compounds and spacers

Compound	X	$Spacers_{2-8}$
I_1	/	/
I_2	$-O(CH_2)_2O-$	$HO(CH_2)_2OH$
I_3	$-NH(CH_2)_2O-$	$H_2N(CH_2)_2OH$
I_4	$-O(CH_2)_4O-$	$HO(CH_2)_4OH$
I_5	$-OCH(CH_3)CH_2CH_2O-$	$HOCH(CH_3)CH_2CH_2OH$
I_6	$-O(CH_2)_2O(CH_2)_2O-$	$HO(CH_2)_2O(CH_2)_2OH$
I_7	$-OCH_2-C_6H_4-O-$	$HOCH_2-C_6H_4-OH$
I_8	$-O(H_2C)_2-C_6H_4-O-$	$HO(H_2C)_2-C_6H_4-OH$

Hydrocortisone (**1**, Hyc) was reacted with succinic anhydride in refluxing pyridine to give ester (**2**) in 94% yield. The substituted furoxans were prepared in a four-step sequence. The starting material benzenethiol (**3**) was converted to 2-(phenylthio)acetic acid (**4**) by treatment with chloroacetic acid. Compound **4** was oxidized by 30% H_2O_2 aqueous solution to give 2-(phenylsulfonyl)acetic acid (**5**), followed by treatment with fuming HNO_3 to offer diphenylsulfonylfuroxan (**6**). It was then converted to various mono-phenylsulfonylfuroxans (7_{1-8}) by treatment with corresponding diol, or amino substituted alcohol ($spacers_{2-8}$). Finally, the resulting furoxans were reacted with **2** to give target compounds.[8] The synthetic route is shown in Scheme 1.

Scheme 1. The synthetic route of the target compounds I_{1-8}.

In preparing compound 7_7 and compound 7_8, we assumed two isomers existed, because OH functions in both phenol and alcohol reportedly react with the diphenylsulfonylfuroxans (**6**).[9] However, only one product was gained. Its ^1H NMR spectrum showed no phenol –OH signal[10] and when added to $FeCl_3$ solution, no color change was observed. Thus, we could confirm only that phenol –OH reacted with the furoxans. The reason is probably that under the basic conditions the phenol –OH could be converted to relatively more stable ph–O^- anion than alcohol –O^- anion, and then the ph–O^- anion subsequently substitutes for the phenylsulfonyl group of the furoxans to form the products (Scheme 2).

The anti-inflammatory activity of the target compounds was at first evaluated using xylene-induced mouse ear swelling as a model.

Three of the compounds, including I_4, I_5, and I_6, show anti-inflammatory activity (Fig. 1). Particularly, the activity of I_6 was comparable to that of the control Hyc. The different results of the tested compounds may be due to the different spacers. Derivatives with spacers of 5–7 atoms are more active than the compounds with shorter or longer spacers. Moreover, the aliphatic spacers were found to be better than the aromatic ones. The activity of the tested compounds is supposed to depend on the release of Hyc and NO in vivo.

Scheme 2. The mechanism of the reaction of preparing compound 7_7.

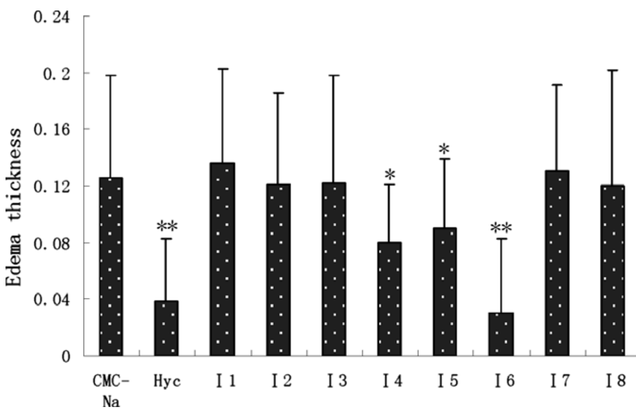

Figure 1. Effects of tested compounds on xylene-induced ear edema in mice ($n = 10$, $\bar{x} \pm s$). $^*P < 0.05$, $^{**}P < 0.01$ versus CMC-Na.

Different length and steric conformation of the spacers can affect both the hydrolysis of the 21 position ester bond, which needs catalysis by enzymes, and interaction with thiol cofactors for releasing NO out of the furoxan.

Furthermore, I_6 was selected to be evaluated for anti-acute-inflammatory activity by carrageenan-induced rat paw edema model (Table 2) and anti-subacute-inflammatory activity by cotton pellet-induced granular tissue formation model (Fig. 2). And the anti-immune inflammatory activity of I_6 was also tested in the arthritis model induced by complete Freund's adjuvant (Fig. 3).

The results revealed that I_6 significantly inhibited carrageenan-induced hind paw edema in acute inflammatory models, and exhibited pronounced dose-related inhibitory activity in the formation of granuloma in the subacute inflammatory model. The anti-inflammatory effects of an equimolar dose of I_6 were comparable to Hyc and no difference among the groups was observed. Both Hyc and an equimolar dose of I_6 inhibited the arthritis induced by complete Freund's adjuvant significantly. In the early phase (at the 17th day) Hyc had a similar effect on the secondary arthritis, whereas in the later phase (at the 21th and the 24th day) I_6 had a more potent effect than Hyc.

Osteoporosis (OP) is one of the primary adverse effects of GC. NO is supposed to prevent OP by both inhibiting bone resorption and promoting bone growth.[11] Thus to determine the bone formation activity of I_6, osteoblasts were cultured from the parietal bone of newborn SD mice in vitro[12] using a similar method to that described by Garett.[13] Then the osteoblasts were treated with I_6. The numbers of osteoblasts (Fig. 4) as well as the activity of alkaline phosphatase (ALP) (Table 3) were measured.

In addition, in order to assess the bone resorption activity of I_6 osteoclasts were also obtained from the extreme bones of newborn rats in vitro.[14] After the treatment of I_6, the numbers of osteoclasts (Fig. 5) and tartrate-resistant acid phosphatase cells (TRAP) (Fig. 6) were measured.

The osteoblast assays demonstrated that there was no significant difference among I_6 and Hyc group in the proliferation of osteoblasts. Both I_6 and Hyc could stim-

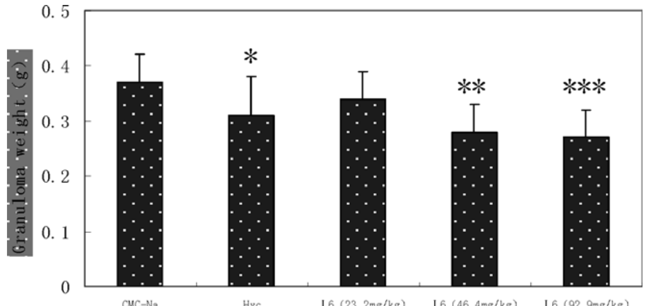

Figure 2. Effects of I_6 on cotton pellet-induced granular tissue formation ($n = 8$, $\bar{x} \pm s$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus CMC-Na.

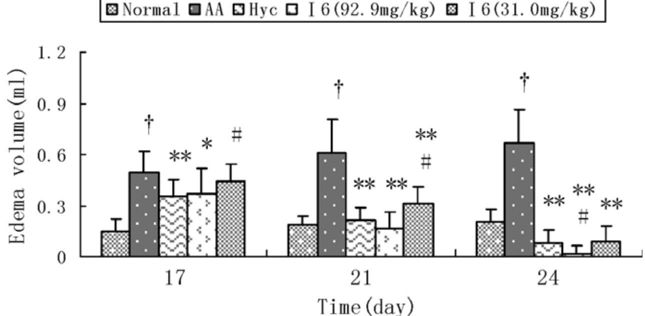

Figure 3. Effect of I_6 on paw swelling in AA rats ($n = 10$, $\bar{x} \pm s$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus AA model group; $^{\#}P < 0.05$ versus Hyc group; $^{\dagger}P < 0.01$ versus normal group.

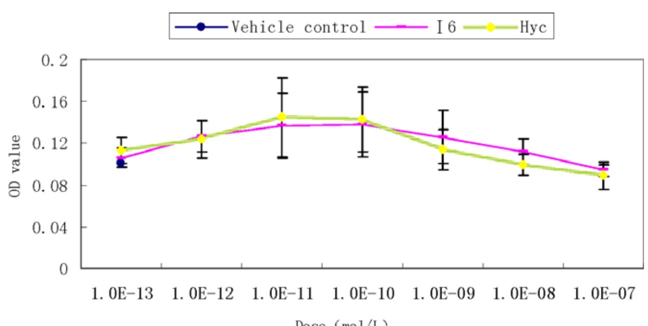

Figure 4. Effects of I_6 and Hyc on proliferation of osteoblasts ($n = 6$, $\bar{x} \pm s$).

Table 2. Effects of I_6 on carrageenan-induced rat paw edema ($n = 8$, $\bar{x} \pm s$)

Group		CMC-Na	Hyc	I_6	I_6	I_6
Dose (mg/kg)		—	21.7	23.2	46.4	92.9
Edema volume (ml)	1 h	0.24 ± 0.04	0.15 ± 0.07**	0.10 ± 0.10**	0.13 ± 0.06**	0.12 ± 0.06***
	2 h	0.62 ± 0.13	0.25 ± 0.10***	0.39 ± 0.25*	0.24 ± 0.10***	0.22 ± 0.13***
	3 h	0.93 ± 0.13	0.54 ± 0.19***	0.74 ± 0.32	0.56 ± 0.11***	0.41 ± 0.21***
	4 h	1.14 ± 0.14	0.85 ± 0.21**	0.99 ± 0.21	0.94 ± 0.13*	0.65 ± 0.19***
	5 h	1.09 ± 0.25	0.98 ± 0.20	1.12 ± 0.17	0.95 ± 0.17	0.80 ± 0.21*
	6 h	1.04 ± 0.17	1.02 ± 0.23	1.07 ± 0.17	0.92 ± 0.18	0.81 ± 0.19*

$^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$ versus CMC-Na.

Table 3. Effects of I_6 and Hyc on the activity of ALP in osteoblasts ($n = 4$, $\bar{x} \pm s$)

Group (mol/L)	King's U/100 ml		
	I_6	Hyc	DMSO
—			2.13 ± 0.26
10^{-13}	1.89 ± 0.08#	2.07 ± 0.06	
10^{-12}	2.24 ± 0.26#	2.69 ± 0.10**	
10^{-11}	2.30 ± 0.08#	2.68 ± 0.19*	
10^{-10}	1.70 ± 0.97#	3.16 ± 0.51*	
10^{-9}	2.97 ± 0.23**	2.66 ± 0.43	
10^{-8}	2.23 ± 0.19	2.50 ±0.58	
10^{-7}	1.54 ± 0.28*	2.26 ± 0.19	

* $P < 0.05$. ** $P < 0.01$ versus DMSO. # $P < 0.05$ versus Hyc.

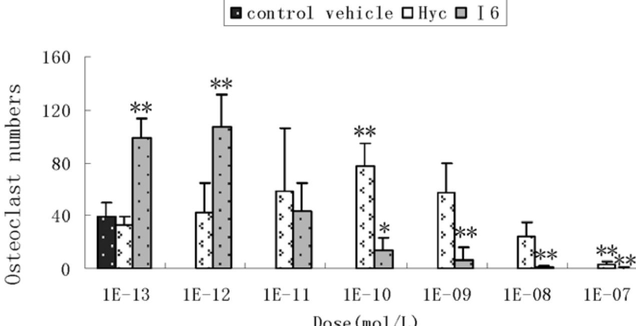

Figure 5. Effect of control vehicle, Hyc and I_6 at different concentrations on numbers of TRAP (+) cell ($n = 4$, $\bar{x} \pm s$). *$P < 0.05$, **$P < 0.01$ versus control.

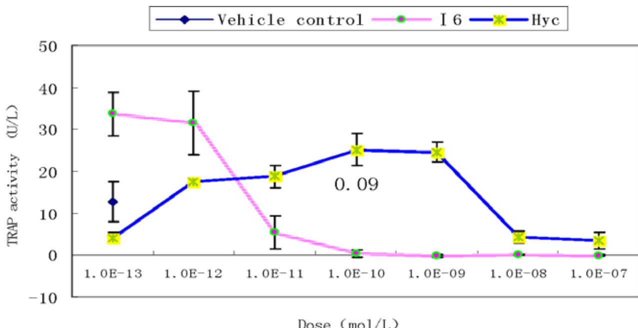

Figure 6. Effect of control vehicle, Hyc, and I_6 at different concentrations on TRAP activity in osteoclasts ($n = 4$, $\bar{x} \pm s$).

ulate bone formation by increasing the proliferation of osteoblasts as well as the activity of ALP. The reason is perhaps that compared with osteoclasts, osteoblasts are reportedly less sensitive to the change of NO concentration, especially when the concentration of NO is not high.[15] However, in the osteoclast assays, Hyc appeared to have no obvious effect on osteoclasts. Only at 10^{-7} mol/L concentration could Hyc inhibit the survival rate of osteoclasts ($P < 0.05$), while I_6 affected osteoclasts potently. At 10^{-13}–10^{-11} mol/L concentrations, I_6 significantly increased the survival rate of osteoclast cells cultured in vitro. However, at 10^{-11}–10^{-7} mol/L concentration, I_6 significantly inhibited the survival rate of osteoclast cells cultured in vitro ($P < 0.01$). What's more, the higher the concentration, the stronger the inhibition. This result may be due to the release of NO and is consistent with the previous research of Wimalawansa.[16]

Hypertension is another adverse effect of GC. In our assays, I_6 (92.9 mg/kg and 31.0 mg/mg), Hyc, and CMC-Na were given to SD mice for 3 weeks. The blood pressure was measured by the BESN system of multi-channel tail-artery blood pressure measurement.[17] Then plasma samples were collected and serum NO was determined by nitrate reductase assay (Table 4).

Compared with the CMC-Na group, the blood pressure of the Hyc group was obviously stimulated to a higher level, while that of I_6 group did not vary significantly. Furthermore, in the high-concentration group (92.9 mg/kg) the blood pressure varied even less than that in the low-concentration group (31.0 mg/kg). We hypothesized that this activity of I_6 is due to the release of NO in vivo, so serum NO was measured. As the result indicated, serum NO of I_6 group was evidently higher than that of either the Hyc group or the CMC-Na group. And the higher the concentration of NO was, the more potent the anti-hypertension activity was, which was consistent with our hypothesis.

In summary, we have designed and synthesized a series of furoxan-based NO-releasing GC derivatives, which are of interest as a new class of improved GC-related agents. Three of the derivatives maintain the anti-inflammatory activity and meanwhile compared with GC the safety is greatly improved. By releasing NO in vivo, the tested compound could effectively avoid stimulating hypertension; moreover, it was also effective in preventing bone loss in the cell assays. Poor water solubility is one of the disadvantages of the tested compound and

Table 4. Effect of I_6 on blood pressure (BP) and heart rate (HR) in rats ($n = 10$, $\bar{x} \pm s$)

Dose (mg/kg)	CMC-Na	Hyc (43.4)	I_6 (92.9)	I_6 (31.0)
SAP(mmHg)	131.6 ± 10.5	156.7 ± 8.5**	137.8 ± 11.8##	144.5 ± 11.3*,#
DAP(mmHg)	89.5 ± 11.8	109.6 ± 7.4**	86.9 ± 7.3##	97.8 ± 14.5#
HR	363.0 ± 30.6	365.2 ± 24.9	379.3 ± 22.9	360.3 ± 17.8
Serum NO (μmol/L)	27.5 ± 5.4	28.0 ± 6.0	40.9 ± 6.7**,#	31.0 ± 2.7

* $P < 0.05$. ** $P < 0.01$ versus Normal group. # $P < 0.05$. ## $P < 0.01$ versus Hyc group.

further structural modification to improve the bioavailability of the compound is in progress.

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8. Data for $\mathbf{I_6}$. mp = 69–71°C. IR(ν, cm^{-1}): 2937vs, 1730vs, 1618vs, 1552vs. ESI-MS: *m/z* $[M+Na]^+ = 797$. ^1H NMR (400.1 MHz, $CDCl_3$): δ = 0.96 (s, 3H, C(19)–CH_3), 1.00–1.05 (m, 1H, C(9)–H), 1.10–1.26 (m, 2H, C(6)–H_b, C(15)–H_b), 1.44 (s, 3H, C(18)–CH_3), 1.47–1.61 (m, 4H, C(12)–H_b, C(14)–H, C(15)–H_a, C(16)–H_b), 1.66–1.81 (m, 2H, C(12)–H_a, C(1)–H_b), 1.83–1.95 (m, 3H, C(1)–H_a, C(1)–H, C(6)–H_a), 1.91–2.15 (m, 4H, C(7)–H, C(2)–H_b, C(2)–H_a, C(16)–H_a), 2.16–2.27 (m, 2H, C(5)–H_a, C(5)–H_b), 2.27–2.39 (m, 2H, C(24)–CH_2), 2.42–2.60 (m, 2H, C(23)–CH_2), 3.47–3.52 (m, 1H, C(11)–H), 3.80 (t, J = 4.5 Hz, 2H, C(28)–OCH_2), 3.92 (t, J = 4.5 Hz, 2H, C(29)–CH_2O), 4.30 (t, J = 4.5 Hz, 2H, C(27)–CH_2O), 4.46 (s, 1H, C(11)–OH), 4.57 (t, J = 4.5 Hz, 2H, C(26)–OCH_2), 4.85 (d, J = 9.0 Hz, 1H, C(21)–H_b), 5.06 (d, J = 9.0 Hz, 1H, C(21)–H_a), 5.68 (s, 1H, C(4)–H), 7.60–7.65 (m, 2H, C(3′)–H, C(5′)–H), 7.73–7.76 (m, 1H, C(4′)–H], 8.05–8.08 (m, 2H, C(2′)–H, C(6′)–H). Anal. Calcd for ($C_{37}H_{46}N_2O_{14}S$): C, H, N.
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12. Osteoblast assay: Parietal bones were obtained from 3-day-old SD mice and dissected free of adhering tissue. After the treatment with the trypsin the bones were cut off and cultured in DMEM supplemented with 10% fetal calf serum. The incubation was conducted at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. Half of the medium was changed once after 2 days. Proliferated osteoblasts were harvested and transferred to a new dish containing the culture medium. This was repeated three times. The third passage osteoblasts were used in the later measurement. To measure the proliferation of osteoblasts, the osteoblasts were removed from the dish to be incubated in 96-well plates at a density of 5×10^4 cells/ml for 24 h. Then I_6 and Hyc at the concentrations of from 10^{-13} to 10^{-7} mol/L were added to the medium; each concentration repeated in 6 wells. One vehicle control group was also performed. Forty-eight hours later 20 μl of 5 mg/ml MTT was added to the medium and the cells continued be incubated for another 4 h. Then the medium was removed and 100 μl DMSO was added to each well. Then OD was measured at λ = 490 nm. To measure the activity of ALP, the osteoblasts were incubated in 24-well plates at a density of 1×10^5 cells/ml for 24 h. I_6 and Hyc at concentrations of from 10^{-13} to 10^{-7} mol/L were added to the medium; each concentration repeated in 4 wells. One vehicle control group was also performed. 48 h later the medium was removed and 200 μL of 1% aqueous solution of Triton X-100 was supplemented. The cells were kept in a refrigerator at 4 °C for 1 h. Thus the activity of ALP was measured by the commercially available kits using the method described by the manufacturer.
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17. Blood pressure assay: 40 SD mice were divided into 4 groups and administered I_6 (92.9 mg/kg), I_6 (31.0 mg/kg), Hyc (43.4 mg/kg) and CMC-Na, respectively, for 3 weeks. Then after 1 h since the last treatment of the drugs, the blood pressure of each mouse was determined by the BESN system of multi-channel tail-artery blood pressure measurement. Plasma samples were then collected from the femoral artery and serum NO (μmol/L) was measured using a nitrate reductase assay.