ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Effects of *C*-nitropyrazoles and *C*-nitroazoles on ocular blood flow and retinal function recovery after ischemic insult

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KEY WORDS pyrazoles; azoles; ocular hypertension; electroretinography; ischemia

ABSTRACT

AIM: Effects of *C*-nitropyrazoles and *C*-nitroazoles on ocular blood flow and retinal function recovery after ischemia have been studied. **METHODS:** The compounds were tested on ocular blood flow of ocular hypertensive (40 mmHg) rabbit eyes with colored microsphere technique. They were also tested on the retinal function recovery after ischemia of rat eyes with electroretinography. **RESULTS:** All compounds (DC-1 through DC-17) showed significant increase in retinal function recovery after ischemia in the range of 26 % to 120 % ($P<0.05$). Among five compounds (DC-1 through DC-5) studied, four compounds (DC-2 through DC-5) increased the blood flow in choroid, iris, and ciliary body, but not in retina. DC-1 did not show significant increase of blood flow in any of these ocular tissues. **CONCLUSION:** *C*-Nitropyrazoles can facilitate significant retinal function recovery after ischemic insult through the increase of ocular blood flow. Since rabbit's retina is scarce in vasculature, it did not show significant change in blood flow by *C*-nitropyrazoles as expected. Among all 17 compounds, DC-5 seems to be the most potent compound.

INTRODUCTION

Ischemic retinopathy is a complicated vascular disease with more than a dozen of etiologies, including age related macular degeneration[1]. The number of patients suffering from the disease is very high at several million in the USA alone. However, none of the drugs tried show any reliable efficacies which put patients in a dark and terrible prognosis of becoming blind[1,2].

Numerous new agents, including both natural and synthetic organic compounds have been studied[3-14]. Among them, *N*-nitropyrazoles showed the most promising results[13,14]. Consequently, *C*-nitropyrazoles and other *C*-nitroazoles had been synthesized for studying their biological actions in the eyes. It is hoped to find that *C*-nitropyrazoles and some other *C*-nitroazoles are similar to *N*-nitropyrazoles to improve ocular blood flow and retinal function.

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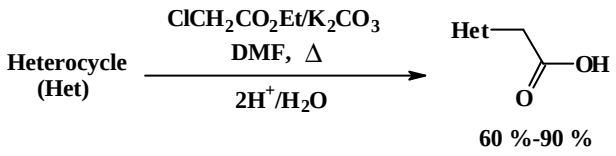
Received 2002-01-17 Accepted 2002-05-10

MATERIALS AND METHODS

Materials Compounds DC-12 and DC-13 were commercial products (Aldrich, Milwaukee, WI).

Compounds DC-1 was synthesized according to the published procedure of Huttel *et al*[15]; compound DC-2 was by method of Robins *et al*[16]; compound DC-3 was by method of Torf *et al*[17]; compound DC-4 was synthesized according to the procedure of Shevelev *et al*[18] and compound DC-17 was according to the method Kanishchev *et al*[19]. Compounds DC-5 (yield 88 %), DC-6 (yield 60 %) and DC-7 (yield 40 %) were obtained according to the published procedure[18].

Novel compounds DC-8–DC-11 and DC-14 – DC-16 were synthesized according to the following scheme:



General procedure The mixture of heterocycle (0.22 mol), 26.95 g $ClCH_2CO_2Et$ (0.24 mol) and 36.05 g K_2CO_3 (0.26 mol) in 200 mL DMF was refluxed for 5 h. The solvent was removed in vacuo. To a solution product in 80 mL H_2O was added to benzene (30 mL). Mixture was stirred for 15 min and organic layer was removed. To the aqueous layer H_2SO_4 was added until the pH was 2. The precipitate that formed was filtered off, washed with water and dried in air.

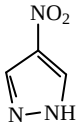
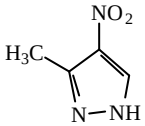
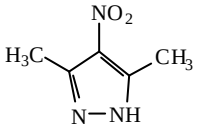
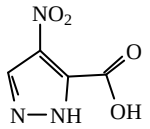
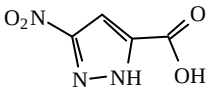
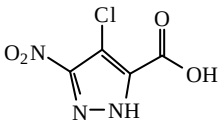
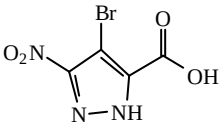
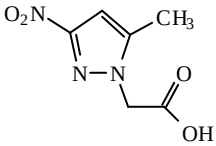
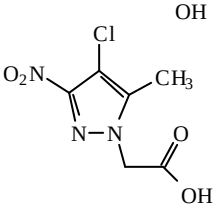
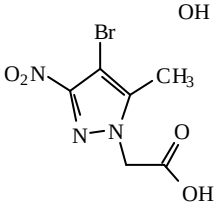
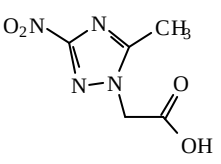
All compounds were recrystalized from appropriate solvents.

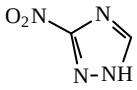
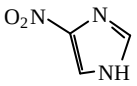
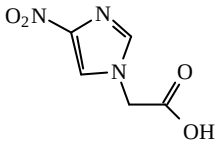
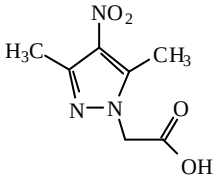
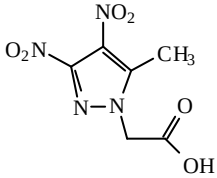
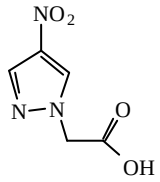
Measurement of ocular blood flow in ocular hypertensive rabbit eyes New Zealand white rabbits, weighing 2.5–3.0 kg, were anesthetized with ketamine 35 mg/kg and xylazine 5 mg/kg intramuscularly. Half of the initial dose was given hourly to maintain anesthesia. An ocular hypertensive model was created by raising the intraocular pressure of the left eye to 40 mmHg with saline manometer, which reduced the ocular blood flow to approximately 1/3 of the normal values[2]. The left ventricle was cannulated through the right carotid artery for the injection of microspheres and the femoral artery was cannulated for blood sampling. One percent drug solution (50 μL) or vehicle (50 μL) was instilled topically to the left eye and the ocular blood flow of the ocular hypertensive rabbits was measured with colored microspheres at 0, 30, 60, 120, and 180 min thereafter. At each time point, 2 million microspheres in 0.2 mL were injected as a reference and blood samples were taken from the femoral artery for exactly 1 min immediately following injection of the microspheres. The blood sample was collected in a heparinized tube and the volume was recorded. The rabbits were euthanized with an injection of pentobarbital sodium 100 mg/kg after the last blood sampling. The left eyes were enucleated and dissected into the retina, choroid, iris, and ciliary body. The tissue samples were weighed.

The details of sample processing and microsphere counting were provided by E-Z Trac. In brief, Hemolysis Reagent was added to the microfuge tubes with the blood sample, then vortexed and centrifuged for 30 min at 4000×*g*. The supernatant was removed and the Tissue/Blood Digest Reagents I and II were added. The tubes were capped, vortexed, and centrifuged for 30 min again. The supernatant was removed and the Counting Reagent was added, then vortexed and centrifuged for 15 min at the same revolutions as above. The supernatant was removed and the microspheres were resuspended in a precise volume of the Counting Reagent. The number of microspheres was counted with a hemocytometer.

The Tissue/Blood Digest Reagent I was added to the microfuge tubes with the tissue samples, sealed, and heated at 95 ℃ for 15 min. The tubes were vortexed for 30 s, then reheated and revortexed until all tissue samples were dissolved. The Tissue/Blood Digest Reagent II was added while the tissue samples were still hot, then the tubes were capped, vortexed, and centrifuged for 30 min. The protocol, thereafter, was the same as that used to process the blood sample and the microspheres were counted.

The blood flow of each tissue at a certain time point was calculated from the following equation: $Q_m = (C_m \times Q_r)/C_r$, where Q_m is the blood flow of a tissue in terms of $mL \cdot min^{-1} \cdot g^{-1}$, C_m is the microsphere count per mg of tissue, Q_r is the flow rate of blood sample in

Compound	Structure	Name	Mp (℃)	NMR-^{1}H, δ (Me_2SO-d_6)
DC-1		4-Nitro-1*H*-pyrazole	110	
DC-2		3-Methyl-4-nitro-1*H*-pyrazole	134	
DC-3		3,5-Dimethyl-4-nitro-1*H*-pyrazole	125	
DC-4		4-Nitro-1*H*-pyrazole-5-carboxylic acid	207	
DC-5		3-Nitro-1*H*-pyrazole-5-carboxylic acid	174	7.25 c (1H, H_4); 10.60 br s (1H, NH); 14.60 br s (1H, OH)
DC-6		4-Chloro-3-nitro-1*H*-pyrazole-5-carboxylic acid	178	C13: 108.10 C4; 134.62 C5; 151.45 C3; 158.68 C(O)
DC-7		4-Bromo-3-nitro-1*H*-pyrazole-5-carboxylic acid	194	C: 92.18 C4; 136.09 C5; 153.27 C3; 158.79 C(O)
DC-8		2-(5-Methyl-3-nitro-1*H*-pyrazol-1-yl)acetic acid	204	2.30 s (3H, CH_3); 5.01 s (2H, CH_2); 6.75 s (1H, H_4)
DC-9		2-(4-Chloro-5-methyl-3-nitro-1*H*-pyrazol-1-yl)acetic acid	222	2.28 s (3H, CH_3); 4.85 s (2H, CH_2)
DC-10		2-(4-Bromo-5-methyl-3-nitro-1*H*-pyrazol-1-yl)acetic acid	207	2.31 s (3H, CH_3); 5.12 s (2H, CH_2)
DC-11		2-(3-Nitro-1*H*-1,2,4-triazol-1-yl)acetic acid	190	5.26 s (2H, CH_2); 8.84 s (1H, H_5)

Compound	Structure	Name	Mp (℃)	NMR-^{1}H, δ (Me_2SO-d_6)
DC-12		3-Nitro-1*H*-1,2,4-triazole		
DC-13		4-Nitro-1*H*-imidazole		
DC-14		2-(4-Nitro-1*H*-imidazol-1-yl)acetic acid	193	4.94 s (2H, CH_2); 7.71 s (1H, H_5); 8.22 s (1H, H_3)
DC-15		2-(3,5-Dimethyl-4-nitro-1*H*-pyrazol-1-yl)acetic acid	182	2.41 s (3H, CH_3); 2.56 s (3H, CH_3); 4.90 s (2H, CH_2)
DC-16		2-(5-Methyl-3,4-dinitro-1*H*-pyrazol-1-yl)acetic acid	154	2.60 s (3H, CH_3); 5.17 s (2H, CH_2)
DC-17		2-(4-Nitro-1*H*-pyrazol-1-yl)acetic acid	160	5.06 c (2H, CH_2); 8.23 s (1H, H_5); 8.85 s (1H, H_3)

terms of mL·min^{-1}, and C_r is the total microsphere count in the referenced blood sample.

Measurement of retinal function recovery after ischemic insult in rat eyes Electroretinograms (ERG), were determined to provide assessment of the retinal function prior to and following ischemic insult. ERG were recorded by means of Ag/AgCl electrodes placed in contact with the cornea. One stainless steel needle was inserted sc between the two eyes as a reference electrode, and another needle was inserted sc to the neck as a ground electrode. A photostimulator (Grass PS22 Flash) was used to produce flashes of light five inches from the eye, and the ERG potentials were recorded with a polygraph system. The ERG machine was purchased from LKC Technologies, Inc (Gaithersburg, MD). A single flash (10 ms duration) white light stimuli was used to elicit ERG a- and b-waves. Peak b-wave amplitudes were measured from the trough of the a-wave to the peak of the b-wave.

Dark-adapted, female Long-Evans rats (200–50 g) were anesthetized with ketamine 35 mg/kg plus xylazine 5 mg/kg im. Half of the initial dose was given thereafter at one-hour intervals to maintain adequate anesthesia. The pupils were dilated with 1 % tropicamide plus 10 % phenylephrine (50 μL) for ERG experiments. Retinal ischemia was produced by occlusion of the central retina and posterior ciliary arteries by means of a ligature placed around the optic nerve and the posterior ciliary artery. The ligature was then tightly drawn for 30 min to occlude the retinal vessels. The retinal is-

chemia was confirmed by the extinction of the ERG waves. After 30 min of retinal ischemia, the ligature was released and the retinal arteries allowed to reperfuse. ERG were then measured at 0, 30, 60, 90, 120, 180, and 240 min thereafter.

All drugs and vehicles were administered ip. These drugs were administered immediately prior to occlusion of the central retinal arteries.

Statistical analysis All data were presented as mean±SD. Non-paired *t*-test was performed to analyze the significance between two means at a certain time point. The differences were considered significant if $P \leq 0.05$.

RESULTS

When the blood flow to rat's retina was blocked for 30 min, the b-wave of ERG disappeared. The b-wave returned gradually to approximately 30 %–40 % of the original amplitude of b-wave after the retina was reperfused with blood circulation (Tab 1). The b-wave recovery was significantly improved when the animal was treated with ip of *C*-nitropyrazoles 10 mg/kg (Tab 1). The facilitation of b-wave recovery ranged from 26.8 % to 120.6 % of the control recovery. DC-5 showed the most potent recovery of retinal function whereas DC-2 was the weakest. The overall average percentage of the facilitation of b-wave recovery of 17 compounds was 58.6 %

The effects of *C*-nitropyrazoles on ocular blood flow were tested with DC-1 through DC-5. None of these compounds showed statistically significant effect on rabbit's retina as this tissue contains very low vasculature (Tab 2). All compounds showed significant increase of the blood flow in choroid, with DC-1 at 120 min after drug administration; DC-2 at 30 min and 60 min thereafter; DC-3 at 60 min thereafter; DC-4 at 120 min thereafter; and DC-5 at all time points after drug instillation (Tab 3).

As for the blood flow in ciliary body, DC-1 did not effect the blood flow significantly, whereas DC-2 increased the blood flow in ciliary body significantly at 30 min and 60 min after drug instillation; DC-3 and DC-4 at 60 min and 180 min thereafter; and DC-5 at 30 min, 60 min, and 180 min after drug instillation (Tab 4).

In case of the blood flow in iris, DC-1 did not show significant change at any time point after drug administration, whereas DC-2 significantly increased the blood flow at 60 min after drug instillation; DC-3 at 60 min and 180 min thereafter; DC-4 at 60 min thereafter; and DC-5 at 60 min and 180 min after drug administration (Tab 5).

Tab 1. Effects of *C*-nitropyrazoles and some other *C*-nitroazoles on retinal function recovery after ischemic insult. *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control.

Compound (10 mg/kg, ip)	Control (C) (% recovery)	Treated (T) (% recovery)	Net increased (T–C)/C
DC-1	33±8	53±20^{b}	60.6
DC-2	42±5	53±5^{b}	26.8
DC-3	38±6	60±9^{b}	57.3
DC-4	38±6	62±17^{b}	62.8
DC-5	33±8	73±16^{b}	120.6
DC-6	33±8	48±5^{b}	45.1
DC-7	38±6	59±13^{b}	53.6
DC-8	33±8	58±16^{b}	76.0
DC-9	33±8	55±8^{b}	67.8
DC-10	42±5	62±10^{b}	67.5
DC-11	38±6	51±8^{b}	34.2
DC-12	22±6	35±10^{b}	61.2
DC-13	33±8	56±20^{b}	68.7
DC-14	38±6	52±9^{b}	36.3
DC-15	33±8	49±13^{b}	48.4
DC-16	38±6	60±18^{b}	57.0
DC-17	42±5	64±17^{b}	52.9

Tab 2. Effects of *C*-nitropyrazoles on retinal blood flow ($mL \times min^{-1} \times g^{-1}$). *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control (Me_2SO).

Compound	30 min	60 min	120 min	180 min
Me_2SO	0.25±0.12	0.15±0.08	0.16±0.10	0.11±0.07
DC-1	0.14±0.07	0.14±0.04	0.13±0.03	0.11±0.06
DC-2	0.28±0.17	0.23±0.10	0.16±0.07	0.14±0.04
DC-3	0.18±0.03	0.16±0.09	0.12±0.06	0.10±0.02
DC-4	0.17±0.04	0.11±0.05	0.11±0.07	0.15±0.17
DC-5	0.20±0.14	0.20±0.14	0.20±0.11	0.09±0.03

Tab 3. Effects of *C*-nitropyrazoles on choroid blood flow (mL^{x} min^{-1x} g^{-1}). *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control (Me_2SO).

Compound	30 min	60 min	120 min	180 min
Me_2SO	7±4	7.0±2.9	5.6±1.7	4.9±1.9
DC-1	8±5	7±3	10±5^{b}	6±4
DC-2	14±5^{b}	15±7^{b}	7±3	4.0±1.9
DC-3	9±3	12±5^{b}	6.6±2.3	6.2±2.3
DC-4	11±6	10±3	8.7±3.5^{b}	7±4
DC-5	12±4^{b}	12±4^{b}	10.1±2.4^{b}	11.0±2.8^{b}

Tab 4. Effects of *C*-nitropyrazoles on ciliary body blood flow (mL^{x} min^{-1x} g^{-1}). *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control (Me_2SO).

Compound	30 min	60 min	120 min	180 min
Me_2SO	3.8±1.2	2.6±1.2	2.7±1.8	1.5±0.8
DC-1	1.6±1.9	1.7±1.1	1.7±1.2	1.0±0.6
DC-2	6.8±1.1^{b}	8.4±2.8^{b}	2.4±0.9	2.8±1.4
DC-3	3.6±1.3	7.1±2.6^{b}	3.20±0.06	2.8±1.1^{b}
DC-4	3.8±0.8	4.9±1.9^{b}	4.3±1.6	3.2±1.7^{b}
DC-5	5.5±1.8^{b}	5.2±2.9^{b}	4.1±2.2	3.3±1.3^{b}

Tab 5. Effects of *C*-nitropyrazoles on iris blood flow (mL^{x} min^{-1x} g^{-1}). *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control (Me_2SO).

Compound	30 min	60 min	120 min	180 min
Me_2SO	3.8±1.7	2.2±0.9	2.0±0.8	1.3±0.6
DC-1	1.1±0.6	1.0±0.3	1.1±0.6	0.8±0.4
DC-2	4.8±2.1	6.6±2.5^{b}	2.5±1.0	2.0±1.2
DC-3	3.9±1.3	6.8±2.7^{b}	1.9±0.6	2.3±1.1^{b}
DC-4	3.7±1.9	4.6±1.4^{b}	2.9±1.3	2.2±1.2
DC-5	4.8±1.5	4.1±1.5^{b}	3.2±2.0	2.3±0.8^{b}

DISCUSSION

A large number of compounds, including natural[6,7,9-12] and synthetic[3-5,8,13,14] have been studied for the treatment/prevention of ischemic retinopathy. Among them, *N*-nitropyrazoles and *C*-nitropyrazoles have been found to be most promising.

In *N*-nitropyrazoles, those bearing carboxylic function or containing no other substituents except a halogen atom or methyl group at position 4 produced the most significant increase of blood flow in ciliary body, iris and choroid[14]. In addition, the compound with a methyl group at position 5 plus two nitro groups at positions 1 and 3 and no substituent at position 4 was good to increase blood flow in iris. As for retinal function recovery after ischemic insults, *N*-nitropyrazoles bearing carboxylic functions or compounds with two nitro groups at positions 1 and 3 or 1 and 4 produced the best result to facilitate b-wave recovery[14].

The examined *C*-nitropyrazoles and other nitroazoles do not yield to *N*-nitropyrazoles in effects on retinal function recovery after ischemic insult on the whole (Tab 1) comparing with data in previous references[13,14]. The following *C*-nitropyrazoles showed significant increase of retina function recovery (in the range of 45 % to 120 %): 4-nitro-1*H*-pyrazole (DC-1) and its intermediates (DC-3, DC-4, DC-16, and DC-17), except for 3-methyl-4-nitro-1*H*-pyrazole (DC-2), as wells as all 3(5)-nitro-1*H*-pyrazole intermediates with the carbonyl function (DC-5 – DC-10). 3-Nitro-1*H*-1,2,4-triazole (DC-12) and 4-nitro-1*H*-imidazole (DC-13) are rather effective, though should the acetic acid fragment be introduced in position 1, their efficiency declines dramatically (DC-11 and DC-14). At the same time, nitropyrazolyl-1-acetic acids (DC-8–DC-10, DC-16–DC-17) are specific of rather high efficiency, especially 3-nitro-pyrazolyl-1-acetic acids (DC-8–DC-10).

3-Nitro-1*H*-pyrazole-5-carboxylic acid (DC-5) is distinguished for the highest efficiency among the studied chemicals. It is not inferior to another carbonic acid DN-7[14] which is the best among *N*-nitropyrazoles. It should be pinpointed that isometric 4-nitro-1*H*-pyrazole-3-carboxylic acid (DC-4) is notably lower than DC-5 in efficiency and differs from DC-5 only in the *C*-nitro group position.

The effects of *C*-nitropyrazoles on ocular blood flow were tested only with five compounds (DC-1–DC-5). As mentioned above, none of these compounds showed significant effects on rabbit's retina (Tab 2),

whereas some *N*-nitropyrazoles increased the retina blood flow dramatically[14]. All of the examined *C*-nitropyrazoles bring about a dramatic increase of the choroid blood flow (Tab 3), most effective being 3-methyl-4-nitropyrazole (DC-2) and 5-nitropyrazole-3-carbonic acid (DC-5). Note that DC-5 exceeds considerably isomer DC-4 (4-nitropyrazole-3-carboxylic acid) in efficiency and differs from it only in the *C*-nitro group position. Each examined *C*-nitropyrazole but DC-1 (4-nitropyrazole) increases markedly the blood flow in ciliary body (Tab 4). Particularly effective are DC-2 (3-methyl-4-nitro-1*H*-pyrazole) and DC-3 (3,5-dimethyl-4-nitro-1*H*-pyrazole). Thus, the introduction of one (DC-2) or two (DC-3) methyl groups to the 4-nitropyrazole molecule (DC-1) rises in effectiveness sharply.

All the studied *C*-nitropyrazoles increase the blood flow in iris (Tab 5), for the exception of DC-1, though it may also significantly raise blood flow efficiency if one or two methyl groups (DC-2 and DC-3, respectively) are added to the 4-nitropyrazole molecule.

Hence, 4-nitropyrazole methyl intermediates (DC-2 and DC-3) and 5- and 4-nitropyrazole-3-carbonic acids (DC-5 and DC-4) increase significantly the blood flow in ocular tissues (except for rabbit's retina). DC-2 (3-methyl-4-nitropyrazole) is most effective in almost all the events, yet it exhibits low efficiency for retinal function recovery. DC-5 (3-nitropyrazole-5-carboxylic acid) should also be highlighted for its ability to effectively increase both the blood flow and, specifically, retinal function recovery.

An important issue of the mechanism of NO generation from *C*-nitroazoles, as affected by endogenous thiols, calls for another research reasoned by a much stronger *C*-NO_2 bond if compared with *N*-NO_2.

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C-硝基吡唑类和C-硝基吡咯类对局部缺血损伤后眼血流量和视网膜功能恢复的作用

关键词　吡唑类；吡咯类；眼高压；视网膜电描记术；局部缺血

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