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Synthesis and Biological Evaluation of Tetramethylpyrazine Nitrates as Potential Anti-ischemic Stroke Agent

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Ischemic stroke, resulting from the blockade of cerebral blood vessels, produces neuronal cell damage and death. [(3,5,6-Trimethylpyrazin-2-yl)methyl nitrate hydrochloride] (NT-1), which contains one nitrate group, was previously synthesized and shown good activity to inhibit platelet aggregation. However, NT-1' sability to release nitric oxide (NO) and dilate blood vessels was not reported. To increase the activity of dilating cerebral blood vessels and increasing blood supply, we designed and synthesized [(3,6-dimethylpyrazine-2,5-diyl)-*bis*(methylene)dinitrate] (NT-2), which contains two nitrate groups. Both NT-1 and NT-2 were able to release NO *in vitro* and dilate the isolated rat middle cerebral artery. In addition, they protected against glutamate-induced cortical neurons damage. NT-2 was more potent than NT-1 in dilating blood vessels.

Keywords: Tetramethylpyrazine nitrate, Nitric oxide, Ischemic stroke.

INTRODUCTION

Ischemic stroke is ranked as the leading cause of disability and death in some developing countries. The main cause of ischemic stroke is the blockage of cerebral blood vessels by thrombus. Once the supply of blood to brain tissues was interrupted, the energy generation of brain tissues was disturbed and therefore, the ischemic cascade leading to cerebral damage was rapidly initiated. Thus, the most import therapeutic intervention after ischemic stroke is to restore blood flow to salvage the neurons in ischemic penumbra.

Tetramethylpyrazine (TMP), the main active ingredient of *Ligusticum walichii* Franchat (Chuan Xiong), has been used for the treatment of ischemic stroke for centuries [1]. Increasing evidence demonstrated that tetramethylpyrazine is effective to reduce brain infarct size and edema in rat models of ischemic stroke [2-4]. Although the exact mechanism(s) of action has not been completely understood, a variety of mechanisms have been attributed to tetramethylpyrazine's beneficial effects in stroke patients. Tetramethylpyrazine was found to inhibit platelet aggregation [5,6], lyse blood clots [6], dilate blood vessels and improve microcirculation. In addition, tetramethylpyrazine has protective effects in cultured cells by acting as a free radical scavenger [7].

Nitric oxide (NO) modulates the physiological functions of the cardiovascular system, which includes relaxation of vascular smooth muscle and inhibition of platelet aggregation. Nitric oxide provides therapeutic benefits in the treatment of cardiovascular diseases, such as essential hypertension, stroke, coronary artery disease, platelet aggregation after percutaneous transluminal coronary angioplasty and ischemia/reperfusion injury. Nitric oxide-donating agents such as organic nitrates and sodium nitroprusside have been used as therapeutic agents. However, the clinical use of these NO donors is significantly limited by their severe side effect, *i.e.*, hypotension. The hypotensive effect of these NO-donating agents is due to their rapid systematic release of NO. Thus, it is necessary to develop new NO donors with better targeting and low side effects.

Tetramethylpyrazine's superior ability to enter the brain was documented as early as 500 years ago in the traditional Chinese medicine literature [8]. Tetramethylpyrazine can effectively penetrate blood-brain barrier and is extensively distributed in brain tissues. To find compounds that can effectively dilate the cerebrovascular blood vessels but don't systematically lower blood pressure, we designed and synthesized tetramethylpyrazine nitrates NT-1 and NT-2. NT-1 is tetramethylpyrazine conjugated with one nitrate group and NT-2 with two nitrate groups. NT-1 has been previously reported by Yan [9]. Like the parent tetramethylpyrazine, NT-1 inhibited platelet aggregation, however, its ability to release NO and dilate cerebral blood vessels was not reported. Herein, we report the synthesis of NT-1 and NT-2 and their activities

to release NO. Their effects on blood vessel dilation and neuroprotection were also reported.

EXPERIMENTAL

All the chemicals and solvents were analytical grade and, when necessary, purified and dried using standard methods. TLC analyses were carried out on silica gel GF/UV 254 (Yantai Chemical group, Yantai, China). All chemicals were purchased from the Tianjin Fuyu Chemical Factory (Tianjin, China). Melting points were measured on an XT3A micro-melting point apparatus and were uncorrected (Beijing Keyi Company, Beijing, China). ^{1}H NMR and ^{13}C NMR spectra were recorded on a BRUKER-AV400 instrument (Bruker, Ettlingen, Germany) in the indicated solvents (TMS as internal standard): the values of the chemical shifts expressed in δ values (ppm). Mass spectra were recorded on an HP 1100 LC/MSD spectrometer (HP, Palo Alto, USA). High resolution mass spectra were determined on an Agilent QTOF 6520 Accurate-Mass Q-TOF LC/MS (Agilent, Palo Alto, USA).The chromatograms were conducted on silica gel (200-300 mesh) and visualized under UV light at 254.The purities of the compounds were characterized by HPLC analysis. The target compounds were used for subsequent experiment with a purity of 98 % unless otherwise noted.

(3,5,6-Trimethylpyrazin-2-yl)methanol (1): Compound **1** was synthesized according to known method [10]. White solid, yield 44.8 %, m.p.: 72-74 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.59 (s, 2H), 2.41 (s, 6H), 2.33 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 149.46, 147.75, 147.40, 146.85, 61.04, 21.24, 21.11, 19.30. MS (ESI) *m/z*: 153 $[M+H]^+$.

(3,5,6-Trimethylpyrazin-2-yl)methyl nitrate (2): Compound **2** was synthesized according to a published method [9]. Light yellow liquid, yield 84.3 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.56 (s, 2H), 2.58 (s, 3H), 2.54 (s, 3H), 2.52 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 158.47, 149.80, 149.59, 145.00, 72.40, 22.58, 19.59, 19.09. MS (ESI) *m/z*: 198 $[M+H]^+$.

(3,5,6-Trimethylpyrazin-2-yl)methyl nitrate hydrochloride (NT-1): To compound **2** (2.0 g, 10.1 mmol) in 60 mL anhydrous CH_2Cl_2 at room temperature was added. Anhydrous HCl gas was introduced into the mixture and a white solid was precipitated. The solid was filtrated and washed with anhydrous CH_2Cl_2 to afford NT-1 hydrochloride as a white solid (2.1 g, 90.8 % yield), m.p.: 98-99 °C. ^{1}H NMR (400 MHz, D_2O) δ: 5.55 (s,2H), 2.58 (s, 3H), 2.53 (s, 3H), 2.52 (s, 3H). ^{13}C NMR (400 MHz, D_2O)δ: 158.51, 150.10, 149.95, 145.80, 72.39, 22.72, 19.59, 19.09. MS (ESI) *m/z*: 198 $[M+H]^+$.

3,6-Dimethylpyrazine-2,5-dicarbaldehyde (3): Compound **3** was synthesized according to a published method [11]. Yellow solid, yield 67.2 %, m.p.: 139-141 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.17 (s, 2H), 2.87 (s, 6H). ^{13}C NMR ($CDCl_3$) δ: 193.59, 152.00, 144.74, 21.39. MS (ESI) *m/z*: 165 $[M+H]^+$.

(3,6-Dimethylpyrazine-2,5-diyl)dimethanol (4): To compound **3** (6.0 g, 36.5 mmol) in 80 mL anhydrous THF at 0 °C was added $NaHB_4$ (1.38 g, 36.5 mmol) drop-wise. The reaction was allowed to continue for 2 h. Then, the solution was poured into 5 % hydrochloric acid (50 mL). The mixture was concentrated *in vacuo* and the residue was extracted with ethyl acetate (3 × 200 mL). The combined organic solutions were dried over anhydrous Na_2SO_4 and filtered. The solvent was removed *in vacuo*. Excessive CH_2Cl_2 was added to the residue and the mixture was ultrasonically oscillated. The mixture was filtered and was then concentrated. The residue was recrystallized from CH_2Cl_2, affording compound **4** as a light yellow solid (2.9 g, 47.1 % yield), m.p.: 118-119 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.70 (s, 4H), 2.44 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 149.14, 146.24, 61.07, 19.24. MS (ESI) *m/z*: 169 $[M+H]^+$.

(3,6-Dimethylpyrazine-2,5-diyl)*bis*(methylene)dinitrate (NT-2): To compound **4** (2 g, 11.9 mmol) in 80 mL anhydrous CH_2Cl_2 at 0 °C was added a solution of fuming HNO_3 and acetic anhydride (1:1.5 v:v, 8 mL). The solution was stirred for 0.5 h on an ice bath. The solution was poured into 200 mL ice water and extracted with CH_2Cl_2 (3 × 150 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography using petroleum and ethyl acetate (3:1) as eluent to afford NT-2 as a white solid (2.5 g, 80.4 % yield), m.p.: 63-65 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.59 (s, 4H), 2.61 (s, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 149.65, 145.22, 71.62, 20.32. HR-MS (ESI) *m/z*: 259.0671 $[M+H]^+$ (Calcd. for $C_8H_{10}N_4O_6$, 259.0673).

Pharmacology

NO releasing assay: The NO-releasing activities of the tetramethylpyrazine nitrates were assayed according to a known method [12]. Briefly, the Griess reagent was prepared by dissolving sulfanilamide (4.0 g) and *N*-napthylenediamine·2HCl (0.2 g) in a mixture of 85 % H_3PO_4 (10 mL) and distilled water (90 mL). Phosphate buffer (PBS) was prepared by dissolving KH_2PO_4 (0.2694 g) and K_2HPO_4 (1.8310 g) in distilled water (100 mL). L-cysteine (0, 121 and 303 mg) was dissolved in in 100 mL PBS to produce different concentrations of L-cysteine solution. Tetramethylpyrazine and isosorbide dinitrate were used as positive controls.

As previously reported that the NO-releasing ability of isosorbide dinitrate was L-cysteine dependent [13], we first tested the NO-releasing activity of isosorbide dinitrate in different concentrations of L-cysteine (0, 10 and 25 mM). We found that isosorbide dinitrate released a higher level of NO at a concentration of 25 mM L-cysteine. Therefore, isosorbide dinitrate at a concentration of 25 mM was used in the subsequent studies.

The test compound NT-1 or NT-2 stock solution (200 μL, the final concentration was 300 μM) was added to PBS with or without L-cysteine (1.8 mL, the final concentration was 25 mM). The solution was incubated at 37 °C in a water bath. To measure the levels of NO at different times, Griess reagent (500 μL) was added at 0, 0.5, 1, 2, 3, 4, 6 h, respectively. The solution was mixed and then kept at room temperature for 10 min. The absorbance at 540 nm was measured. Tetramethylpyrazine and isosorbide dinitrate with the same concentrations to NT-1or NT-2 were used as positive controls.

Blood vessel-dilating activity: The blood vessel-dilating activities of the tetramethylpyrazine nitrates were assayed according to a published method [14]. Briefly, Male Sprague-Dawley rats (250-350 g) were provided by the Guangdong Medical Laboratory Animal Center. Animal care and experi-

mental protocol were in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health. Rats were anaesthetized by CO_2 and decapitated. The brain was immediately removed and placed in a cold buffer solution with the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 5.5. Middle cerebral arteries were dissected free of adhering tissues and cut into ring segments. Rings were suspended between two stainless steel wires (40 μm in diameter) in a Multi Wire Myograph (Model 610 M; Danish Myo Technology A/S) and changes in isometric tension were recorded as described [15]. The optimal tension was set at 2 mN for middle cerebral arteries. Each bathing chamber contained 5 mL Krebs solution and was oxygenated continuously with a mixture of 95 % O_2 and 5 % CO_2 to maintain a pH of 7.4. The rings were allowed to equilibrate for 1 h before the experiments.

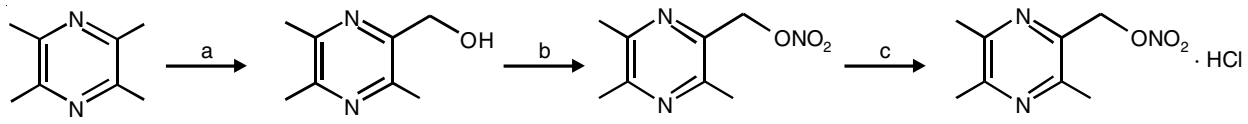
Protective effect on cultured cortical neurons: The neuroprotective activities of tetramethylpyrazine nitrates were investigated using primary cortical neurons insulted by glutamate. Cortical neurons were prepared and cultured according to a published method [16]. Cortical neurons were placed into 96-well plates and were cultured for 11 days at 37 °C under 5 % CO_2. Tetramethylpyrazine nitrates at different concentrations were added and the cells were incubated at 37 °C for 2 h. Glutamate was then added (the final concentration of glutamate was 200 μM) and the cells were incubated for 24 h at 37 °C under 5 % CO_2. A solution of 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl-tetrazoliun bromide was added and the cells were incubated for another 4 h before DMSO was added. After the crystals were completely dissolved, the absorbance was read at 570 nm with a spectrophotometer (Bio-Rad Model 680, Japan). The results were expressed as the percentage of the control group.

RESULTS AND DISCUSSION

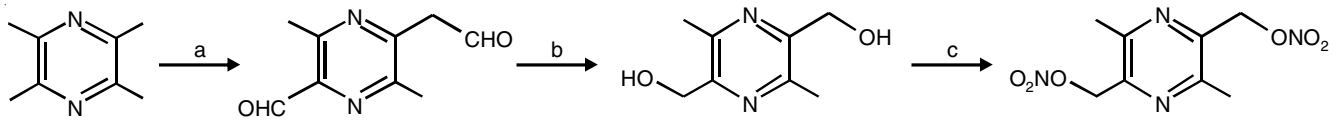
The synthesis of compound NT-1 was showed in **Scheme-I**. Compound **1** was prepared according to a known method [10]. Compound **1** was then converted to compound **2** in the presence of fuming HNO_3 and acetic anhydride (1:1.5/v:v). In order to increase compounds **2**'s water solubility, it was treated with anhydrous HCl to afford NT-1 as an HCl salt. The synthesis of NT-2 was described in **Scheme-II**. Tetramethylpyrazine was first oxidized by SeO_2 to afford compound **3** with a 67.2 % yield. Compound **3** was then reduced by $NaBH_4$ to produce compound **4**. The target NT-2 was obtained by treating compound **4** with fuming HNO_3 in the presence of acetic anhydride.

Nitric oxide-releasing assay: The NO-releasing activities of NT-1 and NT-2 were determined using Griess agent according to a published method [12]. Isosorbide dinitrate (ISDN), an organic nitrate, producing NO and dilating blood vessels and tetramethylpyrazine were used as positive controls. As shown in Fig. 1a, the *in vitro* NO-releasing activity of isosorbide dinitrate was L-cysteine (L-Cys) dependent. The level of NO released by isosorbide dinitrate was much higher in the presence of 25 mM L-cysteine than in 10 mM L-cysteine. This result was in consistence with previous findings [13]. Therefore, L-cysteine at a concentration of 25 mM was used in subsequent studies.

In order to investigate the NO-releasing activities of NT-1 and NT-2 and explore whether their NO-releasing activities were L-cysteine dependent, NO concentrations released by NT-1 and NT-2 were determined in the absence and in the presence of L-cysteine. As shown in Fig. 1b, in the absence of L-cysteine, NT-1 and the positive control drugs, isosorbide dinitrate and tetramethylpyrazine, showed no NO-releasing activities. This result indicated that the NO-releasing activity



Scheme-I: Synthesis of NT-1



Scheme-II: Synthesis of NT-2

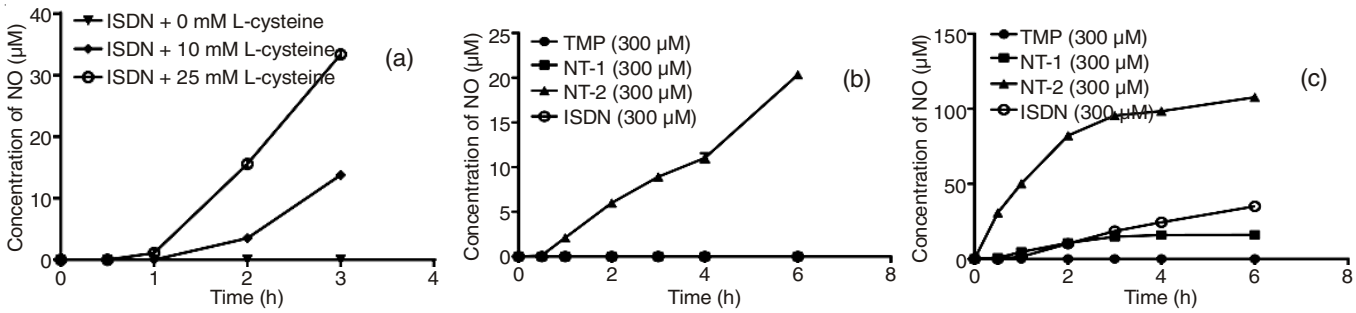


Fig. 1. Nitric oxide concentration *in vitro*

of NT-1 was L-cysteine dependent. In sharp contrast, NT-2 could release NO (Fig. 1b) and the release of NO can last for at least 6 h up to a concentration of 300 μM. The NO-releasing activity of NT-2 was not L-cysteine dependent. In the presence of L-cysteine, NT-1, NT-2 and isosorbide dinitrate displayed NO-releasing activities. As expected, tetramethylpyrazine, bearing no nitrate group, was not able to produce NO (Fig. 1c). As shown in Fig. 1c, the order of NO-releasing ability was NT-2 > ISDN > NT-1 > TMP. In addition, in the presence of L-cysteine, NT-2 produced higher levels of NO compared to the condition when L-cysteine was not present.

By analysis of the structure-activity relationship, we found that (1) the NO-releasing activity was directly related to the number of the nitrate groups; (2) The number of nitrate groups affected the NO-releasing activity in the absence of L-cysteine.

Blood vessel-dilating activity: Since NT-1 and NT-2 can release NO *in vitro*, we then further investigated their vessel-dilating activities on rat middle cerebral artery (MCA). As shown in Fig. 2, both NT-1 and NT-2 produced relaxation on rat middle cerebral artery (MCA) pre-contracted by KCl (60 mM) in a concentration dependent manner. The dilating-effect of NT-2 was much potent than that of NT-1. However, the positive control drug isosorbide dinitrate, bearing two nitrate groups, showed weaker dilating effect on middle cerebral artery, even weaker than that of NT-1, bearing one nitrate group.

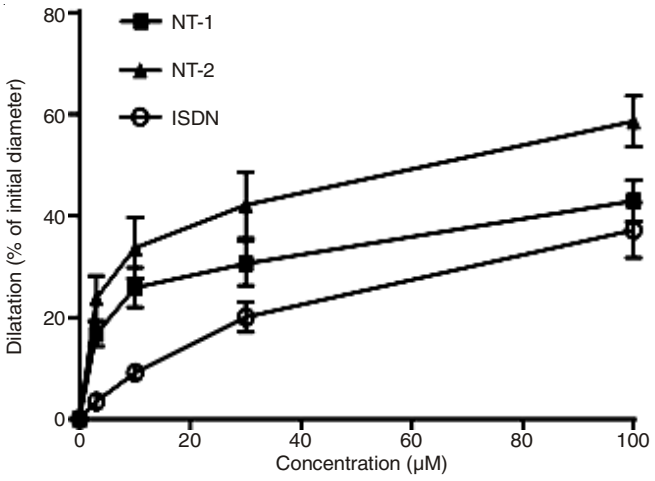


Fig. 2. Dilating activities of NT-1 and NT-2 on rat middle cerebral artery

Protective effect on cultured cortical neurons: Both tetramethylpyrazine and NO-donating agents have neuroprotective effect *in vitro* and produce therapeutic benefits in rat model of ischemic stroke [17-20]. In the present study, we tested the neuroprotective effects of NT-1 and NT-2 in cultured primary cortical neurons. As showed in Fig. 3, both NT-1 and NT-2 prevented cortical neurons from glutamate-induced damage in a concentration dependent manner. There was no significant difference between the neuroprotective effects of NT-1 and NT-2. In comparison, isosorbide dinitrate had a weaker protective effect than those of NT-1 and NT-2 at a concentration of 10 mM. In sharp contrast, tetramethylpyrazine showed no protective effect at the concentration of 300 μM.

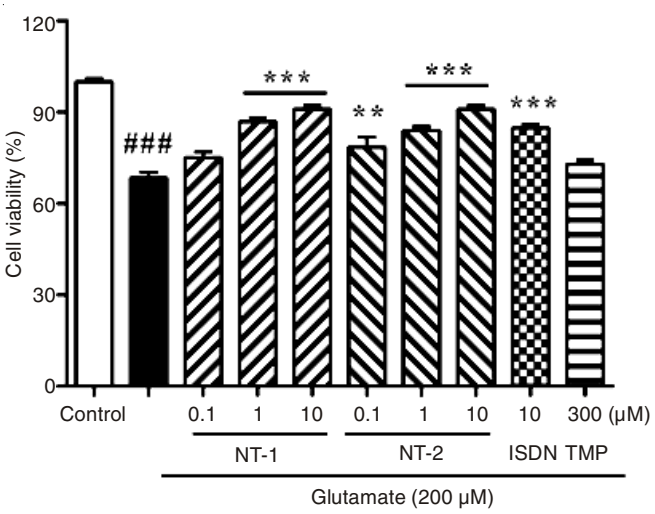


Fig. 3. Protective effect against glutamate-induced cell damage

Conclusion

In conclusion, a new tetramethylpyrazine nitrate derivative NT-2 was designed and synthesized. Both NT-1 and NT-2 released NO, exerted vessel-dilating activities and dis-played neuroprotective effects. Among these two compounds, NT-2 had higher activity in releasing NO and dilating vessels than NT-1. NT-2 may be valuable for the treatment of ischemic stroke. Further evaluations of the novel NT-2 are being conducted and the results will be reported in due course.

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