Ligustrazine Derivatives. Part 6: Design, Synthesis and Evaluation of Novel Ligustrazinyl Acylguanidine Derivatives as Potential Cardiovascular Agents

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Abstract: A series of novel Ligustrazinyl acylguanidines was designed, synthesized and evaluated for their protective effect on injured vascular endothelial cell (ECV-304). The preliminary results demonstrated that some compounds possessed more potent activities than that of Ligustrazine in stimulating replication of the injured endothelial cell. Among the active compounds, compounds **8b**, **8f** and **8l** displayed remarkable antioxidative activity with low EC_{50} values of 0.097, 0.059 and 0.094 mM, respectively. Structure-activity relationships were briefly discussed.

Keywords: Ligustrazine, Acylguanidines, Synthesis, Cardiovascular disease.

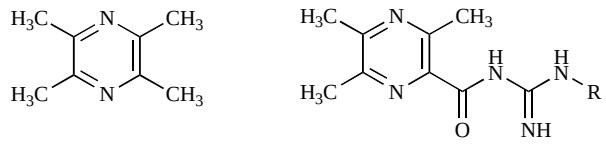
INTRODUCTION

Cardiovascular diseases (CVDs) seriously threaten peoples' health with 21% proportion of the death worldwide. Endothelial cells play an important role in maintaining the function of normal vessel and organ [1]. Much evidence proves that endothelial cell damage as an initial event causes the alteration of endothelial permeability barrier and vascular tone, eventually development of atherosclerosis and other CVDs. It is also demonstrated that oxidative stress, as a major cardiovascular risk factor, significantly contributes to endothelial injury during atherogenesis. Therefore, the protection of endothelial cells against oxidative stress is a crucial strategy in clinical CVDs therapy [1-3].

Ligustrazine (Lig; 2,3,5,6-tetramethylpyrazine, TMP, Fig. **1**), a major active component of the Chinese traditional medicine herb *Chuanxiong* (*Ligusticum wallichii Franchat*), possesses variety of cardiovascular activities, acting as platelet aggregation inhibitor, vasodilator, free radical scavenger, calcium channel antagonist, anti-thrombosis and anti-hypertension agent, and endothelial injury protector, which is now widely used in clinical for treatment of coronary atherosclerotic cardiovascular disease and ischemic cardiovascular disease [4-5]. However, pharmacokinetics studies showed that Ligustrazine was rapidly absorbed when taken orally, but quickly excreted in the urine, displaying a low bioavailability [2-3, 6]. As a result, accumulated toxicity often occurred in the patients by the frequent administration for keeping an plasma concentration. Therefore, it is necessary to develop new generations of Ligustrazine-based cardiovascular drugs through molecular modification.

In our previous work, we have designed and synthesized a series of novel Ligustrazine derivatives by incorporation of Ligustrazine with pharmacophores or drug-like groups from active cardiovascular agents [1-3, 7]. Through biological activity studies, several Ligustrazine derivatives were advanced into promising drug candidates that are now under preclinical evaluation.

Recently, acylguanidine derivatives have become of particular interest to medicinal chemists because of their broad spectrum of cardiovascular activities and potential pharmacological applications. For examples, some acylguanidine derivatives was reported to be active inhibitors of ADP-induced platelet aggregation [8], potent FXa inhibitors as anticoagulant agents [9], and Na^+/H^+ exchanger (NHE) inhibitor, e.g. amiloride, clinically for treatment of hypertension [10-11]. Inspired by structural characteristics of Ligustrazine and acylguanidine derivatives, we integrated Ligustrazine ring and acylguanidine fragment into the one molecule according to the principles of hybridization and bioisosteric replacement in medicinal chemistry, constructing a series of novel Ligustrazinyl acylguanidine derivatives (Fig. **1**). Herein, we reported the preparation of the novel Ligus-



Ligustrazine Ligustrazinyl acylguanidines

Fig. (1). Ligustrazine and Newly Designed Ligustrazinyl Acylguanidine Derivatives.

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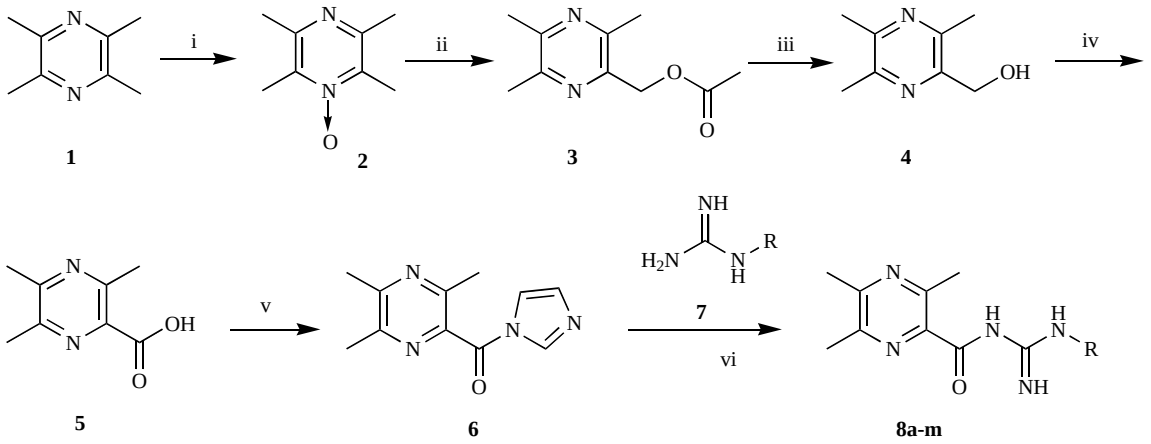
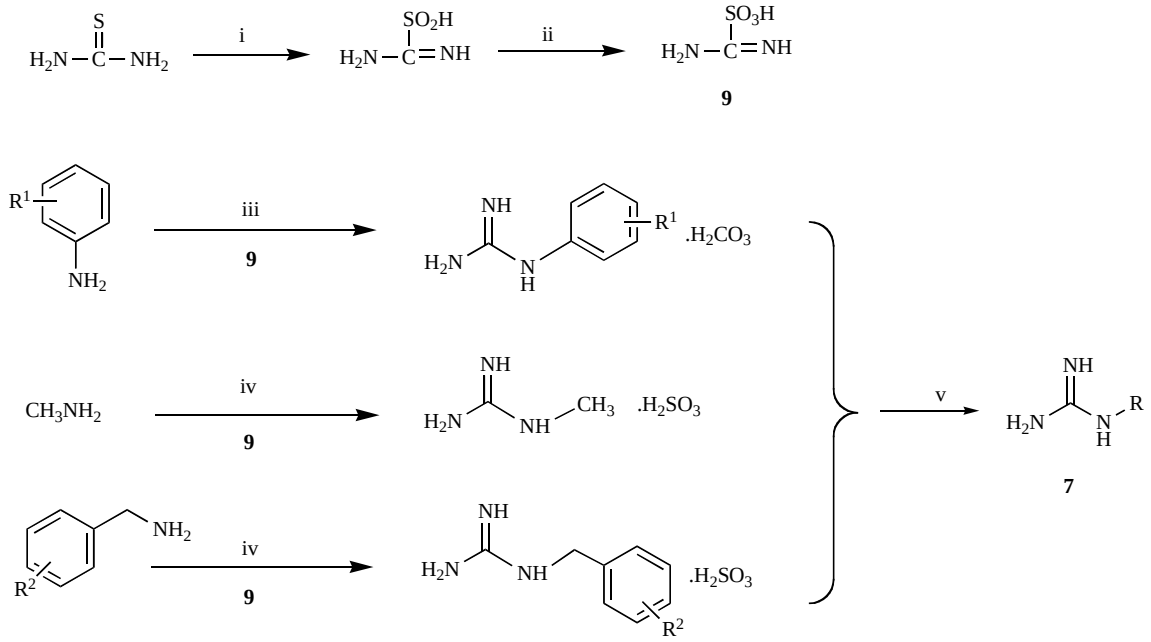


Scheme 1. Reagents: (**i**) 30% H_2O_2, AcOH; (**ii**) Ac_2O; (**iii**) 20% NaOH; (**iv**) $KMnO_4/H_2O$, Na_2SO_3; (**v**) CDI/DMF, Room Temperature; (**vi**) 5h, Room Temperature.



Scheme 2. Reagents: (**i**) 30% H_2O_2; (**ii**) peracetic acid or H^+/H_2O_2; (**iii**) K_2CO_3, EtOH; (**iv**) EtOH; (**v**) NaH.

trazinyl derivatives, as well as their biological evaluation for protective effects on injured ECV-304 cells.

RESULTS AND DISCUSSION

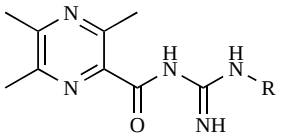
Synthesis of Compound 8a-m

The important intermediate (3,5,6-trimethylpyrazin-2-yl)methyl acetate (**3**) was prepared by the Boekelheide reaction starting from Ligustrazine (**1**) and directly hydrolyzed with sodium hydroxide water solution to obtain hydroxyl Ligustrazine (HTMP, **4**) [1]. This process was used one-pot reaction according to our previous publication with 64% of the total yield (Scheme **1**). The 3,5,6-trimethylpyrazine-2-carboxylic acid (**5**) was synthesized by the oxidation of **4** with potassium permanganate in the water. The target compounds 2-acylguanidino-3,5,6-trimethylpyrazine (**8**) were readily prepared in moderate yields by the reaction of various of substituted guanidines (**7**) with the key intermediate (1H-imidazol-1-yl)(3,5,6-trimethylpyrazin-2-yl)methanone (**6**), generated in situ from compound **5**. The substituents **7** were prepared by the reaction of fatty amine and arylamine with thiourea trioxide (**9**), which was synthesized through two steps oxidation of thiourea (Scheme **2**). The synthesized compounds **8a-m** were characterized by physicochemical and the MS, IR and NMR spectral data were found in agreement with the assigned molecular structures (see Experimental part).

Biological Evaluation

Setting Ligustrazine as the positive control drug, all the synthesized compounds were tested for their protective effects on the human umbilical vascular endothelial cells (ECV-304 cells) damaged by hydrogen peroxide [2-3]. The viability of normal and injured ECV-304 cells was assessed by MTT assay. The proliferation rate (P%) and 50% effective concentration for protecting damaged ECV-304 cells of newly synthesized compounds were shown in Table **1**.

Table 1. The Proliferation Rate (%) and EC_{50} Values for Protecting Damaged ECV-304 Cells of the Ligustrazinyl Acylguanidine Derivatives



No.	R	Proliferation Rate (%)				EC_{50}(mM) [a]
		50μM	100μM	200μM	400μM	
8a	H	1.93	10.54	14.24	32.34	0.769
8b	Methyl	70.57	47.17	15.01	4.29	0.097
8c	4-Methylphenyl	2.47	6.39	11.13	26.60	0.866
8d	4-Methoxylphenyl	0.84	36.12	58.25	102.92	0.147
8e	4-Fluorobenzyl	29.14	31.34	31.54	32.73	>100
8f	3-Chlorobenzyl	40.29	76.62	100.84	155.95	0.059
8g	4-Methoxybenzyl	-19.79	-65.98	-25.98	-66.19	-
8h	2-Fluorobenzyl	25.68	27.97	28.18	29.44	>100
8i	2-Chlorobenzyl	-45.15	-12.99	-8.87	8.66	>100
8j	Benzyl	25.95	28.14	31.54	42.91	0.564
8k	2,4-Dichlorobenzyl	1.75	25.34	30.99	49.90	0.391
8l	3,4-Dichlorobenzyl	4.09	57.70	93.76	-5.85	0.094
8m	3,5,6-Trimethylpyrazine-2-yl	-103.34	-61.51	-44.99	-24.96	-
TMP	-	11.03	14.64	26.23	43.08	0.549

[a]Concentration of compound required to achieve 50% protection of ECV-304 cell from H_2O_2 induced cytotoxicity, as determined by the MTT method.

In general, from the obtained data, it was observed that some of the Ligustrazinyl acylguanidine derivatives protected the proliferation of injured ECV-304 cells with effects comparable to or better than TMP (EC_{50}= 0.549 mM). Among all the Ligustrazinyl acylguanidine derivatives, **8b**, **8f** and **8l** displayed remarkable antioxidative activity (EC_{50}= 0.097, 0.059 and 0.094 mM, respectively). Particularly, the compound **8f** presented almost 10 times higher potency than TMP, and exhibited the greatest proliferation rate (P% = 155.95%) at 0.040 mM. Some other compounds **8d** and **8k** also exhibited reasonable antioxidant activities, similar to that of TMP.

The structure-activity relationships (SARs) analysis showed that the nature of the substituents greatly influenced the antioxidative activity of these compounds. When substituents were fatty group (**8a** and **8b**), methyl group was more active than hydrogen. In phenyl case, the activity order was **8d** > **8c**, illustrating that methoxy group was more needed for keeping high protective effect than methyl group.

In the benzyl series, the active sequence of derivatives was **8f** > **8l** > **8k** > **8j** >>**8e**, **8h**, **8i**, **8g**, which reflect the significance of substituents at the benzyl moiety. Chloro-substitution at the phenyl group seems more active than other substituents. Furthermore, when compared chloro-substituted compounds **8f**, **8i**, **8k** and **8l**, we can see that the position of the chlorine can largely affect the activities of the compounds. The active sequence of the substituents at the benzyl moiety was as follows: 3-Cl>3,4-2Cl>3,5-2Cl>>2-Cl.

Interestingly, between the compounds **8b** and **8g**, the activity order was **8b** >> **8g**, which might reflect the importance of carbon numbers between the phenyl group and guanidino group. No carbon between the phenyl group and guanidino group seems more favorable than one carbon. In addition, it also can be seen that the proliferation rates presented in the Table **1** were increased with the increasing of the concentration of the compounds. However, this rule is not suitable for compound **8b**.

CONCLUSION

In conclusion, a series of novel Ligustrazinyl acylguanidine derivatives were designed, synthesized and biologically evaluated for their protective effect on injured vascular endothelial cell (ECV-304). The results showed that some of the compounds displayed high activities, demonstrating that the Ligustrazinyl acylguanidines are potent cardiovascular agents. Among all the active compounds, compound **8f** displayed an excellent antioxidant activity with low EC_{50} value of 0.059 mM, which about 10 times higher than that of Ligustrazine, presenting a most promising lead for further investigation.

EXPERIMENTAL SECTION

Chemistry

The melting points of the compounds were metered on a micromelting point apparatus. Infrared spectra were recorded with a Nexus 470 FT-IR Spectrometer. ^{1}HNMR spectra were determined by a Bruker Avance (600 MHz) NMR-spectrometer in the indicated solvents. Chemical shifts are expressed in d units and TMS as internal reference. Mass spectra were recorded with an LC Autosampler Device: Standard G1313A instrument. TLC was performed on silica gel GF254 for TLC (Merck) and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Flash column chromatography was performed on column packed with silica gel 60 (230–400 mesh). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure. The yields were calculated by the last step reaction.

Preparation of (3,5,6-Trimethylpyrazin-2-yl)Methanol (4)

Compound **4** was prepared according to our previous reported method by using one-port reaction [1]. The crude product was purified by recrystallization with *n*-hexane to obtain yellow needles. Yield: 60%. M.p. 88-89 ºC (lit. 88–89 ºC) [1].

Preparation of 3,5,6-trimethylpyrazine-2-carboxylic Acid (5)

To a solution of (3,5,6-Trimethylpyrazin-2-yl)methanol (**4**) (10.268 g, 0.068 mol) in 60 ml water, potassium permanganate ($KMnO_4$) aqueous solution (14.4 g $KMnO_4$: 240 mL water) was added dropwise at room temperature for about 40 min. After finishing dropping, the mixture was stirred at room temperature for 30 min. Then saturated aqueous solution of sodium bisulfite was added to neutralize the remanent $KMnO_4$, filtration, and filter was washed with 400 mL water (90 ºC). The filtrate and washing liquor were merged, cooling to 0-5 ºC, and regulated pH to 2.0 with concentrated hydrochloric acid. Extraction was performed with ethyl acetate (100 mL × 5), and the organic phase was dried with anhydrous sodium sulfate. The solvent was removed by distillation under vacuum, and the residue was recrystallized with methyl ethyl ketone to yield light yellow solid 5.8g. Yield: 52.0%. M.p. 161-164 ºC.

Preparation of Amidine Agent Thiourea Trioxide (9)

A solution of thiourea 10g in 100 mL water, cooling to about 8 ºC in ice-water bath, 30% hydrogen peroxide was added dropwise to control the reaction temperature of about 15 °C. When the pH value descended to 3-5, another 10 g of thiourea was added and 30% hydrogen peroxide was added dropwise (66g was added in all). After finishing dropping, the mixture was stirred at room temperature for 3 h. Abundant precipitation seperated out (pH = 2-3), cooling to about 5 ºC, filtration, and washed with ethanol to get thiourea dioxide. The product was dried at 50 ºC under vacuum. Yield: 57.3%.

To a mixture of 0.26 mL concentrated sulfuric acid and 6.5 mL 30% hydrogen peroxide, 5.4g thiourea dioxide was added one spoon by one spoon at 55 ºC for 80 min. After finishing adding, the mixture was stirred for 15 min to generate a large number of colorless granular crystals. Then placed the mixture in the refrigerator overnight, and filtered to yield colorless crystalline thiourea trioxide monohydrate 4.5g. Yield: 83.3%.

General Procedure for the Preparation of Substituted Phenylguanidine

To a solution of substituted phenylamine 0.005 mol and anhydrous potassium carbonate (0.76 g, 0.0055 mol) in 8 mL ethanol-H_2O (ethanol : H_2O = 1:1) at 35-40 ºC water bath, thiourea trioxide (0.67 g, 0.0055 mol) was added one spoon by on spoon. After finishing adding, the mixture was stirred for 4h at at 35 ºC. Then placed the mixture for 24h at room temperature and the product was filtered off, washing with acetone. The crude product was recrystallized from water to get white solid. Yield: 45-48%.

General Procedure for the Preparation of Substituted Benzylguanidine and Adipic Guanidine

A solution of substituted benzylamine (methylguanidine was prepared by 25% methylamine) 0.01 mol in 15 mL ethanol at 35-40 ºC water bath, thiourea trioxide (1.33g, 0.011mol) was added to the solution. After finishing adding, the mixture was stirred for 4h at at 35 ºC. Then placed the mixture in the refrigerator overnight and the product was filtered off, washing with ethanol. The crude product was recrystallized from water to get white solid. Yield: 16-50%.

General Procedure for the Preparation of Compounds 8a-8m

To a mixture of NaH (0.2 g, 60%, 0.005 mol) and various substituted guanidine 0.005 mol in 10 ml dry DMF-dioxane (DMF-dioxane = 1:1), heated to 50 - 60 ºC for 20 min under the protection of nitrogen. Then sodium salt was filtered off to obtain the substituted guanidine. 3,5,6-Trimethylpyrazine-2-carboxylic acid (**5**) (0.83 g, 0.005 mol) and carbonyldiimidazole (CDI, 0.89 g, 0.0055 mol) was added to 10 ml dry DMF-dioxane (DMF-dioxane = 1:1), the mixture stirred for 2 h. Two kinds of reaction solution were mixed and stirred at room temperature for 5h. The solvent was removed by distillation under vacuum to get the crude product. The final product was purified by flash column chromatography [dichloromethane/methanol = 3:1 (**8a**); 5:1 (**8b**); 20:1 (**8c-8m**)] and recrystallized from methanol (**8a**) or water (**8b-8m**).

N-carbamimidoyl-3,5,6-trimethylpyrazine-2-carboxamide (8a)

Light yellow crystal. Yield: 38.9%. M.p. 220-223°C. IR (KBr, cm^{-1}): 3350.61 (NH), 1588.00, 1450.21, 1410.35 (C=N, C=C), 1671.04 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.31 (s, 1H, NH), 8.55 (br, 2H, NH_2), 2.62 (s, 3H, CH3), 2.57 (s, 3H, CH3), 2.48 (s, 3H, CH3); ESI-MS: *m*/*z* 208.3 [M+H]. $C_9H_{13}N_5O$ (207.23).

3,5,6-Trimethyl-N-(N-methylcarbamimidoyl)pyrazine-2-carboxamide (8b)

White solid. Yield: 14.5%. M.p. 86-88°C. IR (KBr, cm^{-1}): 3356.64 (NH), 1574.28, 1431.23, 1405,76 (C=N,C=C), 1672.55 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.32 (s, 1H, NH), 8.43 (s, 1H, NH), 3.63 (s, 3H, NCH_3), 2.59 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: *m*/*z* 222.3 [M+H]. $C_{10}H_{15}N_5O$ (221.26).

3,5,6-Trimethyl-N-(N-p-tolylcarbamimidoyl)pyrazine-2-carboxamide (8c)

Yellow solid. Yield: 24.0%. M.p. 168-171°C. IR (KBr, cm^{-1}): 3367.38 (NH), 1656.42, 1577.45, 1478.53 (C=N, C=C), 1686.37 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.35 (s, 1H, NH), 7.40 (s, 1H, NH), 7.30-7.37 (m, 4H, Ar-H), 4.34 (s, 2H, CH_2), 2.52 (s, 3H, Ph-CH_3), 2.37-2.50 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 298.5 [M+H]. $C_{16}H_{19}N_5O$ (297.35).

N-(N-(4-methoxyphenyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8d)

White solid. Yield: 29.3%. M.p. 160-164°C. IR (KBr, cm^{-1}): 3350.65 (NH), 1592.31, 1514.23, 1427.14 (C=N, C=C), 1632.49 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.29 (s, 1H, NH), 7.38 (s, 1H, NH), 7.28-7.38 (m, 4H, Ar-H), 4.30 (s, 1H, NH), 2.92 (s, 3H, O-CH_3), 2.32-2.49 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 314.3 [M+H]. $C_{16}H_{19}N_5O_2$ (313.35).

N-(N-(4-fluorobenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8e)

Lightyellow solid. Yield: 32.1%. M.p. 93-94°C. IR (KBr, cm^{-1}): 3331.56 (NH), 1516.13, 1423.95, 1338.51 (C=N,C=C), 1596.98 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.34 (s, 1H, NH), 7.14-7.39 (m, 5H, Ar-H and NH), 4.28 (s, 2H, CH_2), 2.38-2.44 (m, 9H, 3×CH_3), 2.14 (s, 1H, NH); ESI-MS: *m*/*z* 314.3 [M-H]. $C_{16}H_{18}FN_5O$ (315.35).

N-(N-(3-chlorobenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8f)

White solid. Yield: 26.3%. M.p. 95-100°C. IR (KBr, cm^{-1}): 6293.04 (NH), 1597.71, 1514.69, 1422.68 (C=N,C=C), 1743.37 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.35 (s, 1H, NH), 7.43 (s, 1H, NH), 6.92-7.35 (m, 4H, Ar-H), 4.43 (s, 2H, CH_2), 2.42-2.52 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 332.1 [M+H]. $C_{16}H_{18}ClN_5O$ (331.80).

N-(N-(4-methoxybenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8g)

White solid. Yield: 33.3%. M.p. 84-86°C. IR (KBr, cm^{-1}): 3319.22 (NH), 1513.55, 1422.22, 1399.13 (C=N,C=C), 1590.66 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.25 (s, 1H, NH), 6.88-7.29 (m, 5H, Ar-H and NH), 4.33 (s, 2H, CH_2), 2.93 (s, 3H, O-CH_3), 2.39-2.50 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 328.5 [M+H]. $C_{17}H_{21}N_5O_2$ (327.38).

N-(N-(2-fluorobenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8h)

White solid. Yield: 37.5%. M.p. 141-142°C. IR (KBr, cm^{-1}): 3372.21 (NH), 1601.72, 1490.80, 1424.43 (C=N, C=C), 1629.53 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.36 (s, 1H, NH), 7.48 (s, 1H, NH), 6.98-7.30 (m, 4H, Ar-H), 4.32 (s, 2H, CH_2), 2.37-2.49 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 316.3 [M+H]. $C_{16}H_{18}FN_5O$ (315.35).

N-(N-(2-chlorobenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8i)

White solid. Yield: 28.9%. M.p. 97-100°C. IR (KBr, cm^{-1}): 3367.70, 3245.99, 2923.85 (NH), 1600.34, 1514.26, 1474.32, 1425.18 (C=N,C=C), 1628.03 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.27 (s, 1H, NH), 7.39 (s, 1H, NH), 7.02-7.35 (m, 4H, Ar-H), 4.45 (s, 2H, CH_2), 2.42-2.51 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 332.4 [M+H]. $C_{16}H_{18}ClN_5O$ (331.80).

N-(N-benzylcarbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8j)

White solid. Yield: 27.7%. M.p. 146-149°C. IR (KBr, cm^{-1}): 3452.31, 3394.06, 3087.78 (NH), 1590.42, 1422.05 (C=N, C=C), 1669.39 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.36 (s, 1H, NH), 7.30-7.37 (m, 6H, Ar-H and NH), 4.42 (s, 2H, CH_2), 2.39-2.51 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 298.3 [M+H]. $C_{16}H_{19}N_5O$ (297.35).

N-(N-(2,4-dichlorobenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8k)

White solid. Yield: 64.3%. M.p. 117-120°C. IR (KBr, cm^{-1}): 3360.46 (NH), 1589.60, 1434.81 (C=N, C=C), 1621.47 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.30 (s, 1H, NH), 7.39 (s ,1H, NH), 6.99-7.38 (m, 3H, Ar-H), 4.42 (s, 2H, CH_2), 2.38-2.45 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 366.2 [M], 367.1 [M+H]. $C_{16}H_{17}Cl_2N_5O$ (366.25).

N-(N-(3,4-dichlorobenzyl)carbamimidoyl)-3,5,6-trimethylpyrazine-2-carboxamide (8l)

White solid. Yield: 59.5%. M.p. 98-101°C. IR (KBr, cm^{-1}): 3322.90 (NH), 1589.88, 1467.43, 1425.01 (C=N,C=C), 1635.78 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.23 (s, 1H, NH), 7.49 (s, 1H, NH), 7.18-7.40 (m, 3H, Ar-H), 4.41 (s, 2H, CH_2), 2.39-2.50 (m, 9H, 3×CH_3); ESI-MS: *m*/*z* 366.2 [M], 367.4 [M+H]. $C_{16}H_{17}Cl_2N_5O$ (366.25).

N,N'-(iminomethylene)bis(3,5,6-trimethylpyrazine-2-carboxamide) (8m)

White solid. Yield: 38.1%. M.p. 233-234°C. IR (KBr, cm^{-1}): 3393.97 (NH), 1598.32, 1577.66, 1439.46, 1409.51 (C=N,C=C), 1701.75 (C=O); ^{1}H-NMR (DMSO-d_6, ppm) δ: 9.36 (s, 1H, NH), 9.30 (s ,1H, NH), 2.34-2.55 (m, 18H, 6×CH_3); ESI-MS: *m*/*z* 357.2 [M+H]. $C_{17}H_{21}N_7O_2$ (355.39).

Protective Effect on Damaged ECV-304 Cells Assay [2, 12]

ECV-304 cells were seeded in a 24-well plate at a density of 6×10^3 /well and allowed to grow to the desired confluence. The cells were pretreated with various concentrations of Ligustrazinyl derivatives for 24 h, and then exposed to 150 μM H_2O_2 for another 12 h. Control cells were incubated with a media containing an equivalent solvent amount without the test materials. The plate was incubated at 37 °C in a humidified 5% CO_2 atmosphere. 12 hours later, 0.01 mL MTT solution (5 mg/mL) was added to each well and then incubated for 4 h. Ligustrazine derivatives were dissolved in dimethyl sulfoxide (DMSO) and added into the wells (the

final concentration of Ligustrazinyl derivatives was to 400, 200, 100, 50 μM, and the DMSO content should never exceed 0.05%) and were incubated with cells for 24 h before the addition of H_2O_2. The supernatant was removed carefully by pipetting from wells without disturbing the attached cells and formazan crystals were solubilized by adding 200 μL of DMSO to each well and shaken for 15 min. The absorbance at 570 nm was measured with a microplate reader, using wells without cells as control. The proliferation rates of damaged ECV-304 cells were calculated by [OD570 (Compound)-OD570 (H_2O_2)]/[OD570 (Control)- OD570 (H_2O_2)]×100%, which was then used to obtain EC_{50} values, according to the equation: $-pEC_{50} = \log C_{max} - 2\times(\Sigma P - 0.75 + 0.25P_{max} + 0.25P_{min})$, where C_{max} = maximum concentration, ΣP = sum of proliferation rates, P_{max} = maximum value of proliferation rate and P_{min} = minimum value of proliferation rate.

CONFLICT OF INTEREST

The author(s) declare that they have no competing interests.

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REFERENCES

[1] Liu, X.; Zhang, R.; Xu, W.; Li, C.; Zhao, Q.; Wang, X. Synthesis of the novel liqustrazine derivatives and their protective effect on injured vascular endothelial cell damaged by hydrogen peroxide. *Bioorg. Med. Chem. Lett.*, **2003**, *13*(13), 2123-6.

[2] Cheng, X.C.; Liu, X.Y.; Xu, W.F.; Guo, X.L.; Ou, Y. Design, synthesis, and biological activities of novel Ligustrazine derivatives. *Bioorg. Med. Chem.*, **2007**, *15*(10), 3315-20.

[3] Cheng, X.C.; Liu, X.Y.; Xu, W.F.; Guo, X.L.; Zhang, N.; Song, Y.N. Ligustrazine derivatives. Part 3: Design, synthesis and evaluation of novel acylpiperazinyl derivatives as potential cerebrocardiac vascular agents. *Bioorg. Med. Chem.*, **2009**, *17*(8), 3018-24.

[4] Ren, M.; Dong, J.; Xu, Y.; Wen, N.; Gong, G. Synthesis of novel ligustrazine derivatives as NA+/H+ exchange inhibitors. *Chem. Biodivers*, **2010**, *7*(11), 2727-36.

[5] Cheng, X.C.; Liu, X.Y.; Xu, W.F. Recent advances in the structural modification of ligustrazine: cerebro- and cardiovascular activity of ligustrazine derivatives. *Drugs of the Future*, **2005**, *30*(10), 1059-1065.

[6] Ju, X.D.; Deng, M.; Ao, Y.F.; Yu, C.L.; Wang, J.Q.; Yu, J.K.; Cui, G.Q.; Hu, Y.L. The protective effect of tetramethylpyrazine on cartilage explants and chondrocytes. *J. Ethnopharmacol*, **2010**, *132*(2), 414-20.

[7] Liu, X.Y.; Cheng, X.C.; Xu, W.F.; Guo, X.L.; Ou, Y. Design, synthesis, and biological activities of novel Ligustrazine derivatives. *Bioorg. Med. Chem.*, **2007**, *15*(10), 3315-3320.

[8] Thomas, E.W.; Nishizawa, E.E.; Zimmermann, D.C.; Williams, D.J. Synthesis of acylguanidine analogues: inhibitors of ADP-induced platelet aggregation. *J. Med. Chem.*, **1989**, *32*(1), 228-36.

[9] O'Connor, S.P.; Atwal, K.; Li, C.; Liu, E.C.; Seiler, S.M.; Shi, M.; Shi, Y.; Stein, P.D.; Wang, Y. Synthesis and evaluation of acylguanidine FXa inhibitors. *Bioorg Med Chem Lett*, **2008**, *18*(16), 4696-9.

[10] Zerbini, G.; Maestroni, A.; Mangili, R.; Pozza, G. Amiloride-insensitive Na+-H+ exchange: a candidate mediator of erythrocyte Na+-Li+ countertransport. *J. Am. Soc. Nephrol.*, **1998**, *9*(12), 2203-11.

[11] Pedersen, S.F.; King, S.A.; Nygaard, E.B.; Rigor, R.R.; Cala, P.M. NHE1 inhibition by amiloride- and benzoylguanidine-type compounds. Inhibitor binding loci deduced from chimeras of NHE1 homologues with endogenous differences in inhibitor sensitivity. *J. Biol. Chem.*, **2007**, *282*(27), 19716-27.

[12] Wang, Y.K.; Huang, Z.Q. Protective effects of icariin on human umbilical vein endothelial cell injury induced by H_2O_2 *in vitro*. *Pharmacol. Res.*, **2005**, *52*(2), 174-82.

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