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Design, synthesis, and biological activities of novel Ligustrazine derivatives

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Abstract—A series of novel Ligustrazine derivatives was designed, synthesized, and assayed for their protective effects on damaged ECV-304 cells and antiplatelet aggregation activities. The results showed that most Ligustrazine derivatives exhibited lower EC_{50} values for protective effects on the ECV-304 cells damaged by hydrogen peroxide in comparison with Ligustrazine. And some Ligustrazine derivatives presented better antiplatelet aggregation activities than Ligustrazine. The derivatives containing the bisphenylmethyl pharmacophore (**7a**–**c**) exhibited highest potency. Compound **7a** displayed most potential protective effects on the ECV-304 cells damaged by hydrogen peroxide, and compound **7c** was found to be the most active antiplatelet aggregation agent. Structure–activity relationships were briefly discussed.

1. Introduction

Ligustrazine (Lig; tetramethylpyrazine, TMP; see Fig. 1) is one major efficient component from Chinese traditional medicine herb *Chuanxiong(Ligusticum wallichii Franchat)*, which is currently widely used in China as a new kind of calcium channel antagonist for the treatment of coronary atherosclerotic cardiovascular disease and ischemic cerebrocardiac vascular disease.[1] Ligustrazine has been reported to inhibit the platelet aggregation,[2] to cause negative chronotropic and inotropic responses on isolated atria,[3] to inhibit vasoconstriction in isolated vascular strips,[4] and to act as a vasodilator, a free-radical scavenger, and anti-thrombosis and anti-hypertension agent.[1] More recently, it has been found to be more effective in protection against injured vascular endothelial cell.[5]

However, pharmacokinetics studies found that Ligustrazine presented low bioavailability and to be metabolized fast in vivo with short half-life of $T_{1/2} = 2.89$ h, so accumulated toxicity often appeared in the patients for keeping an effective plasma concentration by the frequent administration.[6] Therefore, it is necessary to develop new generation of the cerebrocardiac vascular drugs from molecular modification of Ligustrazine.

Structure–activity relationship studies indicated that pyrazine ring in the molecule of Ligustrazine might largely be the determinant of its pharmacodynamics, while the substituted groups might primarily govern its pharmacokinetics and toxicity.[7] So some drug-like groups and pharmacophores can be introduced to the methyl position of Ligustrazine, for acquiring the pharmacologically additive or synergetic effects to improve pharmacokinetic properties.

Calcium channel antagonists such as Cinnarizine, Flunarizine, and Lomerizine (see Fig. 1) are very important cerebrocardiac vascular drugs currently used in the clinic. As common characters in their molecular structures, piperazine ring acts as a linker to be considered as the functional group for keeping the drugs' potential.[8]

According to the principles of hybridization and bioisosteric replacement in medicinal chemistry, we designed a series of novel Ligustrazine derivatives by combination with a piperazine and some pharmacophores or drug-like groups, such as substituted benzyl, cinnamyl,

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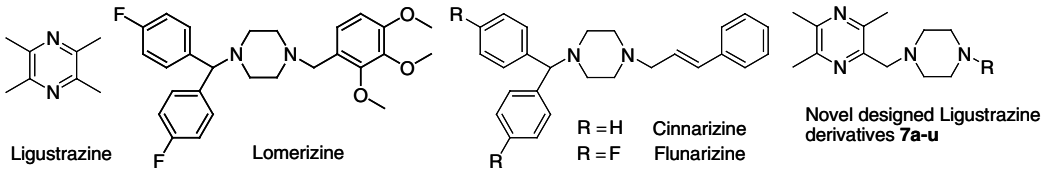
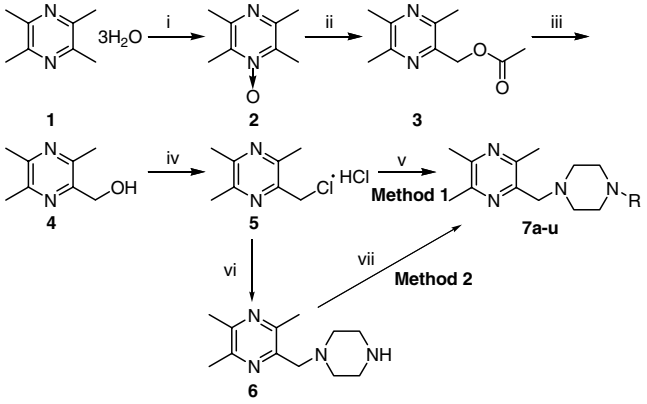


Figure 1. Structures of Ligustrazine, Lomerizine, Cinnarizine, Flunarizine, and newly synthesized Ligustrazine derivatives **7a**–**u**.

bisphenylmethyl, ligustrazinyl to form the new integrated structural pattern (see Fig. 1). Some other groups such as phenyl, β-phenylethyl were also introduced in order to further expand our exploration and better understand the structure–activity relationships of Ligustrazine derivatives.

2. Chemistry

In the synthesis, (3,5,6-trimethylpyrazin-2-yl)methanol (**4**) was prepared by the Boekelheide reaction starting from Ligustrazine trihydrate (**1**), but one-pot reaction was used according to our previous publication.[9] The important intermediate 2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**5**) was synthesized by the chlorination of **4** with $SOCl_2$ in anhydrous CH_2Cl_2 (see Scheme 1). Ligustrazine derivatives (**7a**–**u**) were synthesized by the following two methods. In Method 1, 2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**5**) was directly reacted with the mono-substituted *N*-alkylpiperazines to afford alkylpiperazinyl Ligustrazine derivatives (**7a**–**d** and **7t**, see Method 1 of Scheme 1). In Method 2, anhydrous piperazine was alkylated with compound **5** in chloroform to produce the intermediate 2,3,5-trimethyl-6-(piperazin-1-ylmethyl)pyrazine (**6**), which was reacted with various substituted benzyl halide to give the corresponding alkylpiperazinyl Ligustrazine derivatives (**7e**–**s** and **7u**, see Method 2 of Scheme 1). The chemical structures of the newly synthesized compounds were confirmed by IR, ^{1}H NMR, and ESI-MS.



Scheme 1. Reagents and conditions: (i) 30% H_2O_2/AcOH, 70 °C; (ii) Ac_2O/reflux for 2 h; (iii) 20% NaOH; (iv) $SOCl_2$/anhydrous CH_2Cl_2; (v) various N-mono-substituted piperazines, toluene, NaI, Et_3N/reflux for 10 h; (vi) piperazine; (vii) RX, toluene, NaI, Et_3N/reflux for 10 h.

3. Biological evaluation and discussion

Endothelial cells play a critical physiological role in maintaining normal vessel and organ function. Much evidence showed that vascular endothelial cell damage that causes the alteration of endothelial permeability barrier and vascular tone is a major promoter of atherogenesis, thrombosis, and consequently cardiovascular events. Oxidative stress is a cardiovascular risk factor and contributes significantly to endothelial injury during atherogenesis. Therefore, the protection of endothelial cells against damage caused by oxidative stress is a very important therapeutic strategy.[10]

The newly synthesized Ligustrazine derivatives were assayed for the protective effects on the human umbilical vascular endothelial cells (ECV-304 cells) damaged by hydrogen peroxide.[11] The results (Table 1) showed that Ligustrazine and its derivatives presented protective effects on the damaged ECV-304 cells and most of the Ligustrazine derivatives were more active (with lower EC_{50} values) than Ligustrazine (EC_{50} value at 452 μM).

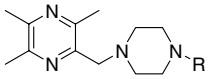
The derivatives containing bisphenylmethyl substituents **7a**–**c** exhibited high potency with EC_{50} values below 300 μM, among which **7a** was the most active one with the EC_{50} value at 218 μM. As expected, the introduction of bisphenylmethyl group, which is confirmed active in cerebrocardiac vascular profiles of calcium channel antagonists such as Cinnarizine, Flunarizine, and Lomerizine, produced the remarkable combinational effects. However, this principle did not work in **7d** (EC_{50} value at 428 μM), because introduction of cinnamyl pharmacophore only moderately favored the protective effects as compared with Ligustrazine.

In the benzyl series, non-substituted compound at the phenyl moiety (**7f**) exhibited highest potency and its EC_{50} values were revealed at 246 μM. 4-Nitro-substitution at the phenyl moiety (**7s**) moderately impaired the activity (EC_{50} value at 299 μM) and other substituted compound seriously impaired the activity. 3-Nitril substitution (**7q**) showed highest EC_{50} value (1114 μM), almost completely losing the activity.

The 2,2′-piperazinyl-diligustrazine (**7e**) showed a low EC_{50} value at 311 μM, reflecting the combinational effects of two ligustrazinyl groups. This compound may act as a promising prodrug with improved pharmacokinetics properties.

Among compounds **7f**, **7t**, and **7u**, the activity order was **7f > 7u > 7t**, which might reflect the importance of

Table 1. The structures, EC_{50} for protecting damaged ECV-304 cells, and platelet aggregation rate (*A*%) of Ligustrazine derivatives **6** and **7a–u**



Compound	R	Method	EC_{50} (μM)	*A*%[a] (200 μM)
6	H	2	481	11.36 ± 6.52
7a	Bisphenylmethyl	1	218	9.88 ± 4.26
7b	Bis(4-Fluorophenyl)methyl	1	223	6.32 ± 8.22
7c	(4-Chlorophenyl)(phenyl)methyl	1	253	5.19 ± 3.52
7d	(*E*)-Cinnamyl	1	428	8.36 ± 7.11
7e	Ligustrazinyl	2	311	19.36 ± 5.84
7f	Benzyl	2	246	9.06 ± 1.08
7g	4-Methylbenzyl	2	328	17.69 ± 2.64
7h	4-Ethylbenzyl	2	375	13.56 ± 4.07
7i	4-*tert*-Butylbenzyl	2	431	15.32 ± 3.30
7j	2-Chlorobenzyl	2	405	9.67 ± 3.02
7k	3-Chlorobenzyl	2	503	10.56 ± 7.06
7l	4-Chlorobenzyl	2	339	12.31 ± 3.22
7m	2,4-Dichlorobenzyl	2	424	5.70 ± 3.56
7n	2-Bromobenzyl	2	784	7.68 ± 4.66
7o	4-Bromobenzyl	2	845	13.26 ± 8.75
7p	2-Nitrilbenzyl	2	637	12.16 ± 3.45
7q	3-Nitrilbenzyl	2	1114	14.19 ± 5.22
7r	4-Nitrilbenzyl	2	417	11.37 ± 3.56
7s	4-Nitrobenzyl	2	299	14.22 ± 1.05
7t	Phenyl	1	665	21.43 ± 2.63
7u	β-Phenylethyl	2	542	9.77 ± 5.39
Lig			452	7.86 ± 4.17

[a] The *A*% value for blank is 0.73 ± 2.93, and for ADP is 39.77 ± 3.69.

carbon numbers between the phenyl group and piperazine ring. One carbon between the phenyl group and piperazine ring seems more favorable than other carbon numbers. This assumption can be further confirmed by the EC_{50} values of compounds **7a–c**.

The activity comparison between Ligustrazine and compound **6** indicated that introduction of a piperazine ring into Ligustrazine did not favor the protective effect of Ligustrazine. However, further N-alkylation at piperazine with R group produced compounds **7a–u**, some of which favored the activity of compound **6**. The pharmacophore-based compounds, such as **7a–c** showed lower EC_{50} values as compared with compound **6**. The structure–activity relationship studies above encouraged us to connect compound **6** with another series of pharmacophores containing acyl groups, such as acetylsalicyloyl, nicotinoyl, and gallic acyl groups, which would be reported later.

Ligustrazine has been reported to inhibit the platelet aggregation,[2] so these Ligustrazine derivatives were further tested for antiplatelet aggregation induced by ADP.[12] The preliminary antiplatelet aggregation results (Table 1) obtained from the experiments showed that Ligustrazine derivatives bearing pharmacophores or drug-like groups, such as **7b** (bis(4-fluorophenyl)methyl, 6.32 ± 18.22%), **7c** ((4-chlorophenyl)(phenyl)methyl, 5.19 ± 3.52%), **7m** (2,4-dichlorobenzyl, 5.70 ± 3.56%), **7n** (2-bromobenzyl, 7.68 ± 4.66%), exhibited the high potent activities in antiplatelet aggregation at the concentration of 200 μM, and with lower values than that of Ligustrazine (7.86 ± 4.17%). Compound **7c** was found to be the most active antiplatelet aggregation agent, presenting the pharmacologically additive or synergetic effects of Ligustrazine with pharmacophores.

In the benzyl-substituted series, 2-substituted or unsubstituted derivatives presented higher antiplatelet aggregation activities than 4-substituted derivatives, but with some exceptions of 2-, 3- or 4-nitril derivatives, which showed almost no changes in platelet aggregation rates. 2-Halogen substitution might favor the antiplatelet aggregation activities, while alkyl, nitril or nitro-substitution impaired antiplatelet aggregation activities. Benzyl or β-phenylethyl derivatives showed higher antiplatelet aggregation activities than the phenyl derivative, which might reflect the importance of methylene between the phenyl and piperazine ring.

4. Conclusions

In conclusion, a series of novel Ligustrazine derivatives was designed and synthesized. Most Ligustrazine derivatives exhibited lower EC_{50} values for protective effects on the ECV-304 cells damaged by hydrogen peroxide in comparison with Ligustrazine. And some Ligustrazine derivatives presented better antiplatelet aggregation activities than Ligustrazine. The derivatives containing the bisphenylmethyl pharmacophore (**7a–c**) exhibited highest potency. Compound **7a** displayed most potential protective effects on the ECV-304 cells damaged by hydrogen peroxide, and compound **7c** was found to be the most active antiplatelet aggregation agent. Structure–activity relationships were briefly discussed. Further bioassay of these compounds on cerebrocardiac vascular activity on animal models is underway.

5. Experimental

5.1. Synthetic methods and spectroscopic details

Infrared spectra were measured using a nicolet nexus 470 FT-IR spectrometer using smear KBr crystal or KBr plate. ^{1}H NMR spectra were recorded on a Bruker Avance (600 MHz) spectrometer; *J* values are in Hz. ^{13}C NMR spectra were also recorded on the Bruker Avance spectrometer. Mass spectra were recorded on an electrospray ionization mass spectrometer as the value *m*/*z*. Thin-layer chromatography (TLC) was performed on E. Merck silica gel 60-F-254 plates. Flash chromatography was performed using 300 mesh silica gel. The yields were calculated by the last step reaction.

5.1.1. (3,5,6-Trimethylpyrazin-2-yl)methanol (4). 2,3,5,6-Tetramethylpyrazine trihydrate (**1**) (30.40 g, 160 mmol) was heated with 30% hydrogen peroxide (18 ml, 160 mmol) in glacial acetic acid (40 ml) for 4 h at 70 °C. Further 30% hydrogen peroxide (18 ml, 160 mmol) was then added and the heating was continued for 4 h. The solution was made alkaline with 50% sodium hydroxide and extracted with chloroform. The combined extracts were dried and evaporated in vacuo; tetramethylpyrazine mono-*N*-oxide (**2**) was obtained. To this, acetic anhydride (15.1 ml, 160 mmol) was added and the mixture was refluxed for 3 h, checking for product formation via TLC. The excess of acetic anhydride was evaporated and (3,5,6-trimethylpyrazin-2-yl)methyl acetate (**3**) was obtained, which was directly saponified with 20% NaOH (155 ml) and extracted with chloroform. The combined extracts were dried and the solvent removed. The residual oil was recrystallized from *n*-hexane; (3,5,6-trimethylpyrazin-2-yl)methanol (**4**) was obtained as yellow needles (15.50 g, 64%); mp 88–89 °C.

5.1.2. 2-Chloromethyl-3,5,6-trimethylpyrazine hydrochloride (5). Thionyl chloride (7.41 ml, 102 mmol) was added dropwise to (3,5,6-trimethylpyrazin-2-yl)methanol (**4**) (15.50 g, 102 mmol) in anhydrous CH_2Cl_2 (300 ml) at 0 °C. The mixture was allowed to stand for 2.5 h, checking for product formation via TLC. The solvent was evaporated in vacuo and the crude product 2-chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**5**) was obtained as a yellow solid (21.11 g, 100%); mp 102–105 °C. Compound **5** was basified and then purified to give 2-chloromethyl-3,5,6-trimethylpyrazine as oil for spectral confirmation. IR (KBr, cm^{-1}): 2993 (CH), 2952 (CH), 2923 (CH), 2856 (CH), 1548 (C=N). NMR: δ_H (600 MHz, $CDCl_3$) 4.68 (s, 2H, CH_2), 2.63 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 2.52 (s, 3H, CH_3); δ_C (150 MHz, $CDCl_3$) 151.6 (C=N), 149.3 (C=N), 149.0 (C=N), 146.3 (C=N), 44.8 (CH_2), 21.6, 21.4, and 20.5 (3× CH_3). ESI-MS: 171 (M+1).

5.1.3. 2,3,5-Trimethyl-6-(piperazin-1-ylmethyl)pyrazine (6). 2-Chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**5**) (20.7 g, 100 mmol) in chloroform (100 ml) was added dropwise into anhydrous piperazine (49.88 g, 580 mmol) in chloroform (300 ml) at 0 °C. The solution was left at room temperature for 5 h, checking for product formation via TLC. The mixture solution was washed with aqueous ammonia (4 M), and the organic layers were dried. The solvent was evaporated in vacuo and the crude product was recrystallized from *n*-hexane to give 2,3,5-trimethyl-6-(piperazin-1-ylmethyl)pyrazine (**6**) as white crystals (11 g, 50%); mp 94 °C. IR (KBr, cm^{-1}): 3443, 3272 (NH), 2943 (CH), 1546 (C=N). NMR: δ_H (600 MHz, $CDCl_3$) 2.57–3.60 (m, 10H, CH_2), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 1.90 (s, 1H, NH); δ_C (150 MHz, $CDCl_3$) 153.6 (C=N), 150.4 (C=N), 148.7 (C=N), 147.2 (C=N), 56.6, 54.4, 54.1, 45.2, and 44.4 (5× CH_2), 21.4, 21.0, and 20.1 (3× CH_3). ESI-MS: 221 (M+1).

5.1.4. General procedure for the preparation of 2-(4-substituted-1-piperazinmethyl)-3,5,6-trimethylpyrazine (7a–d and 7t, Method 1 of Scheme 1). 2-Chloromethyl-3,5,6-trimethylpyrazine hydrochloride (**5**) (2.07 g, 10 mmol) and mono-substituted *N*-alkylpiperazines (10 mmol) were dissolved in toluene (70 ml). Triethylamine (3.46 ml, 25 mmol) and NaI (catalytic quantity) were added to the solution. The mixture solution was refluxed for 10 h until the reaction was complete (monitored by TLC). After cooling, the mixture was filtered and the filtrate was evaporated in vacuo. The final product was purified by flash column chromatography and recrystallization from *n*-hexane.

5.1.4.1. 2-(4-Biphenylmethyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (7a). Flash column chromatography: ethyl acetate/cyclohexane = 1:3; yield 56%; white crystal; mp 121–122 °C; IR (KBr, cm^{-1}): 2809.86 (CH), 1598.06 (C=C), 1585.41(C=N); ^{1}H NMR ($CDCl_3$, δ ppm): 7.41 (d, 4H, Ar–H, *J* = 7.75 Hz), 7.26 (t, 4H, Ar–H, *J* = 7.70 Hz), 7.16 (t, 2H, Ar–H,*J* = 7.46 Hz), 4.22 (1H, CH), 2.30–3.62 (m, 10H, CH_2), 2.56 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 387.5 (M+1).

5.1.4.2. 2-[4-(4,4′-Difluro)biphenylmethyl-1-piperazinmethyl]-3,5,6- trimethyl pyrazine (7b). Flash column chromatography: ethyl acetate/cyclohexane = 1:1; yield 56%; white crystal; mp 138–140 °C; IR(KBr, cm^{-1}): 2954.35 (CH), 1603.08 (C=C), 1505.49 (C=N); ^{1}H NMR ($CDCl_3$, δ ppm): 7.34 (q, 4H, Ar–H, *J* = 7.04 Hz), 6.97 (t, 4H, Ar–H, *J* = 8.65 Hz), 4.21 (s, 1H, CH), 3.61–3.62 (m, 10H, CH_2), 2.57 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 423.6 (M+1).

5.1.4.3. 2-[4-(4-Chlorophenyl)phenylmethyl-1-piperazinmethyl]-3,5,6-trimethyl pyrazine (7c). Flash column chromatography: ethyl acetate/cyclohexane = 1:2; yield 45%; white crystal; mp 112–114 °C; IR(KBr, cm^{-1}): 2950.77 (CH), 1599.71 (C=C), 1546.64 (C=N); ^{1}H NMR ($CDCl_3$, δ ppm): 7.35 (q, 4H, Ar–H), 7.27 (t, 2H, Ar–H), 7.24 (t, 2H, Ar –H), 7.18 (t, 1H, Ar–H, *J* = 7.27 Hz), 4.20 (1H, CH), 2.30–3.62 (m, 10H, CH_2), 2.56 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 421.5 (M+1).

5.1.4.4. 2-[4-[(*E*)-Cinnamyl]-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7d). Flash column chromatography: ethyl acetate/cyclohexane = 1:1; yield 43%; yellow crystal; mp 74–75 °C; IR (KBr, cm^{-1}): 2935.55 (CH), 1596.95 (C=N), 977.98 (=CH); ^{1}H NMR ($CDCl_3$, δ,

ppm): 7.36 (d, 2H, Ar–H, J = 7.49 Hz), 7.29 (q, 2H, Ar–H), 7.22 (t, 1H, Ar–H, J = 7.33 Hz), 6.51 (d, 1H, =CH, J = 15.85 Hz), 6.27 (m, 1H, =CH), 2.50–3.63 (m, 12H, CH_2), 2.57(s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 337.5 (M+1).

5.1.4.5. 2-(4-Phenyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (7t). Flash column chromatography: ethyl acetate; yield 40%; white crystal; mp 82–84 °C; IR (KBr, cm^{-1}): 2957.59 (CH), 1603.01 (C=C); ^1H NMR ($CDCl_3$, δ ppm): 7.26 (m, 2H, Ar–H), 6.92 (d, 2H, Ar–H, J = 7.99 Hz), 6.85 (t, 1H, Ar–H, J = 7.28 Hz), 2.67–3.70 (m, 10H, CH_2), 2.62 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.51 (s, 3H, CH_3); ESI-MS: 297.6 (M+1).

5.1.5. General procedure for the preparation of 2-(4-substituted-1-piperazinmethyl)-3,5,6-trimethylpyrazine (7e–s and 7u, Method 2 of Scheme 1). To a mixture of 2,3,5-trimethyl-6-(piperazin-1-ylmethyl)pyrazine (**6**) (2.2 g, 10 mmol) and Na_2CO_3 (3.18 g, 30 mmol) in anhydrous CH_2Cl_2 (100 ml) was added dropwise various substituted benzyl halide (10 mmol) in anhydrous CH_2Cl_2 (100 ml) at room temperature. The mixture was refluxed for 10 h (checked by TLC), and the solvent was evaporated in vacuo. The final product was purified by flash column chromatography and recrystallization from *n*-hexane.

5.1.5.1. 2-[4-[(3,5,6-Trimethyl)-2-pyrazinmethyl]-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7e). Flash column chromatography: ethyl acetate/cyclohexane = 1:1; yield 59%; yellow crystal; mp 162–164 °C; IR (KBr, cm^{-1}): 2937.71 (CH); ^1H NMR ($CDCl_3$, δ ppm): 3.65 (m, 12H, CH_2), 2.58 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 355.6 (M+1).

5.1.5.2. 2-(4-Benzyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (7f). Flash column chromatography: ethyl acetate; yield 44%; white crystal; mp 66 °C; IR (KBr, cm^{-1}): 2936.68 (CH); ^1H NMR ($CDCl_3$, δ ppm): 7.30 (m, 4H, Ar–H), 7.24 (m, 1H, Ar–H), 3.49–3.61 (m, 12H, CH_2), 2.56 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 311.5 (M+1).

5.1.5.3. 2-[4-(4-Methylbenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7g). Flash column chromatography: ethyl acetate; yield 42%; white crystal; mp 66 °C; IR (KBr, cm^{-1}): 2929.10 (CH), 1515.58 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.26 (m, 1H, Ar–H), 7.20 (d, 1H, Ar–H, J = 7.57 Hz), 7.14 (q, 2H, Ar–H), 3.44–3.62 (m, 12H, CH_2), 2.57 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 2.35 (s, 3H, Ar–CH_3); ESI-MS: 325.6 (M+1).

5.1.5.4. 2-[4-(4-Ethylbenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7h). Flash column chromatography: ethyl acetate; yield 32%; yellow oil; IR (KBr, cm^{-1}): 2932.21 (CH), 1512.87 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.27 (t, 1H, Ar–H, J = 5.92 Hz), 7.21 (d, 1H, Ar–H, J = 7.88 Hz), 7.18 (t, 1H, Ar–H), 7.11 (q, 1H, Ar–H), 2.58–4.60 (m, 14H, CH_2), 2.52 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 1.22 (3H, CH_2CH_3); ESI-MS: 339.5 (M+1).

5.1.5.5. 2-[4-(4-*tert*-Butylbenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7i). Flash column chromatography: ethyl acetate; yield 47%; yellow oil; IR (KBr, cm^{-1}): 2960.15 (CH), 1656.00 (C=C), 1513.44 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.32 (t, 2H, Ar–H, J = 6.61 Hz), 7.24 (d, 2H, Ar–H, J = 8.20 Hz), 3.48–3.62 (m, 12H, CH_2), 2.57 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 1.32 (s, 9H, $C(CH_3)_3$); ESI-MS: 367.4 (M+1).

5.1.5.6. 2-[4-(2-Chlorobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7j). Flash column chromatography: ethyl acetate; yield 41%; white crystal; mp 60 °C; IR (KBr, cm^{-1}): 2935.80 (CH), 1594.16 (C=C), 1571.32 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.49 (s, 1H, Ar–H), 7.35 (d, 1H, Ar–H, J = 7.83 Hz), 7.24 (t, 1H, Ar–H, J = 7.42 Hz), 7.19 (t, 1H, Ar–H, J = 7.39 Hz), 2.50–3.64 (m, 12H, CH_2), 2.59 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 2.49 (s, 3H, CH_3); ESI-MS: 345.4 (M+1).

5.1.5.7. 2-[4-(3-Chlorobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7k). Flash column chromatography: ethyl acetate; yield 31%; yellow oil; IR (KBr, cm^{-1}): 2936.45 (CH), 1596.95 (C=C), 1574.90 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.32 (s, 1H, Ar–H), 7.20 (m, 3H, Ar–H), 3.45–3.62 (m, 12H, CH_2), 2.57 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.46 (s, 3H, CH_3); ESI-MS: 345.4 (M+1).

5.1.5.8. 2-[4-(4-Chlorobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7l). Flash column chromatography: ethyl acetate; yield 44%; yellow crystal; mp 68 °C; IR (KBr, cm^{-1}): 2946.03 (CH), 1596.71 (C=C), 1575.99 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.35 (d, 2H, Ar–H, J = 8.24 Hz), 7.29 (d, 2H, Ar–H, J = 8.21 Hz), 2.30–3.52 (m, 12H, CH_2), 2.47 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 2.38 (s, 3H, CH_3); ESI-MS: 345.4 (M+1).

5.1.5.9. 2-[4-(2,4-Dichlorobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7m). Flash column chromatography: ethyl acetate; yield 34%; white crystal; mp 62–64 °C; IR (KBr, cm^{-1}): 2943.87 (CH), 1587.77 (C=C), 1561.51 (C=N); ^1H NMR ($CDCl_3$, δ ppm): 7.63 (s, 1H, Ar–H), 7.53 (q, 2H, Ar–H), 7.40 (t, 1H, Ar–H, J = 7.69 Hz), 2.40–3.62 (m, 12H, CH_2), 2.56 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 379.5 (M), 381.4 (M+2).

5.1.5.10. 2-[4-(2-Bromobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7n). Flash column chromatography: ethyl acetate; yield 33%; white crystal; mp 90 °C; IR (KBr, cm^{-1}): 2928.22 (CH), 761.48 ($\gamma_{\varphi H}$); ^1H NMR ($CDCl_3$, δ ppm): 7.51 (d, 1H, Ar–H, J = 7.88 Hz), 7.47 (d, 1H, Ar–H, J = 7.54 Hz), 7.26 (m, 1H, Ar–H), 7.08 (t, 1H, Ar–H, J = 7.89 Hz), 3.58–3.62 (m, 12H, CH_2), 2.57 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 389.3 (M+1).

5.1.5.11. 2-[4-(4-Bromobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7o). Flash column chromatography: ethyl acetate; yield 44%; yellow crystal; mp 64–66 °C; IR (KBr, cm^{-1}): 2925.61 (CH), 1592.58

(C=C); ^{1}H NMR ($CDCl_3$, δ ppm): 7.42 (d, 2H, Ar–H, J = 7.37 Hz), 7.19 (d, 2H, Ar–H, J = 7.81 Hz), 2.40–3.61 (m, 12H, CH_2), 2.56 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 391 (M+2).

5.1.5.12. 2-[4-(2-Nitrilbenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7p). Flash column chromatography: ethyl acetate; yield 45%; white crystal; mp 106–108 °C; IR (KBr, cm^{-1}): 2931.17 (CH), 2219.88 (C≡N), 1652.92 (C=C), 1599.30 (C=N); ^{1}H NMR ($CDCl_3$, δ ppm): 7.62 (d, 1H, Ar–H, J = 7.69 Hz), 7.54 (m, 2H, Ar–H), 7.33 (m, 1H, Ar–H), 2.50–3.69 (m, 12H, CH_2), 2.56 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 336.6 (M+1).

5.1.5.13. 2-[4-(3-Nitrilbenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7q). Flash column chromatography: ethyl acetate; yield 43%; yellow crystal; mp 116–118 °C; IR (KBr, cm^{-1}): 2925.96 (CH), 2229.33 (C≡N); ^{1}H NMR ($CDCl_3$, δ ppm): 7.64 (s, 1H, Ar–H), 7.54 (q, 2H, Ar–H), 7.40 (t, 1H, Ar–H, J = 7.70 Hz), 2.40–3.62 (m, 12H, CH_2), 2.56 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 2.47 (s, 3H, CH_3); ESI-MS: 336.6 (M+1).

5.1.5.14. 2-[4-(4-Nitrilbenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7r). Flash column chromatography: ethyl acetate; yield 34%; white crystal; mp 94–96 °C; IR (KBr, cm^{-1}): 2953.65 (CH), 2227.05 (C≡N), 1607.08 (C=N), 1503.74 (C=C); ^{1}H NMR ($CDCl_3$, δ ppm): 7.59 (d, 2H, Ar–H, J = 8.15 Hz), 7.44 (d, 2H, Ar–H, J = 8.05 Hz), 2.40–3.63 (m, 12H, CH_2), 2.57 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 336.6 (M+1).

5.1.5.15. 2-[4-(4-Nitrobenzyl)-1-piperazinmethyl]-3,5,6-trimethylpyrazine (7s). Flash column chromatography: ethyl acetate; yield 39%; white crystal; mp 82–83 °C; IR (KBr, cm^{-1}): 2929.48 (CH), 1605.75 (C=C), 1522.47 (NO_2), 1343.16 (NO_2); ^{1}H NMR ($CDCl_3$, δ ppm): 8.17 (d, 2H, Ar–H, J = 8.49 Hz), 7.51 (d, 2H, Ar–H, J = 7.62 Hz), 3.61–3.62 (m, 12H, CH_2), 2.58 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 356.5 (M+1).

5.1.5.16. 2-(4-β-Phenylethyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (7u). Flash column chromatography: chloroform/acetone, 1:1; yield 40%; white crystal; mp 108 °C; IR (KBr, cm^{-1}): 2929.26 (CH), 1601.92 (C=C); ^{1}H NMR ($CDCl_3$, δ ppm): 7.30 (d, 2H, Ar–H, J = 7.93 Hz), 7.21 (d, 3H, Ar–H, J = 7.29 Hz), 3.60–3.65 (m, 14H, CH_2), 2.58 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 2.48 (s, 3H, CH_3); ESI-MS: 325.6 (M+1).

5.2. Biological evaluation

5.2.1. Protective effects on ECV-304 cells damaged by hydrogen peroxide. The ECV-304 cells were plated and grown for 24 h in cultured medium, then were switched to fresh medium in the presence of 25, 50, 100, 200, 400 μM Ligustrazine and its derivatives. After 0.5-h incubation, 150 μM hydrogen peroxide was added and the cells were incubated for an additional 6 h. The results were expressed as the values of absorbance at 570 nm. The data are means ± SEM (n = 6). The proliferation rates of damaged cells were calculated by [OD570 (Compd) − OD570 (H_2O_2)]/[OD570 (Control) − OD570 (H_2O_2)] × 100%, which was then used to obtain EC_{50} values (see Table 1), according to the equation: $-\mathrm{pEC}_{50} = \log \mathrm{Cmax} - \log 2 \times (\sum P - 0.75 + 0.25\mathrm{Pmax} + 0.25\mathrm{Pmin})$, where Cmax, maximum concentration; $\sum P$, sum of proliferation rates; Pmax, maximum value of proliferation rate; and Pmin, minimum value of proliferation rate.

5.2.2. Antiplatelet aggregation. Citric acid trisodium (3.8%, 0.3 ml), rabbit blood (2.7 ml), ADP, and Ligustrazine derivatives (200 μM) were added to cuvette in sequence, another two cuvette using as blank and ADP control. The blood samples were centrifuged (270 g/5 min) and the superstratum was extracted as platelet-abundant plasma. The remnant blood samples were centrifuged again (1000 g/10 min) and the superstratum was extracted as platelet penurious plasma (PPP), which was measured by aggregometer. The results are listed in Table 1 and expressed as the mean value of the platelet aggregation rate (A%, n = 6).

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