Synthesis of Novel Ligustrazine Derivatives as Na^+/H^+ Exchange Inhibitors

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A novel series of 3,5,6-trimethylpyrazine-2-methoxy (or methylamino) substituted benzoylguanidine derivatives were designed and synthesized as Na^+/H^+ exchange (NHE) inhibitors. In this study, compounds with electron-withdrawing substituents on the benzene ring seemed to improve NHE-1 inhibitory activities. Compounds **6d**, **6k**, and **6l** were found to be potent inhibitors of NHE-1 ($IC_{50} = 3.0 \pm 1.6$, 3.0 ± 1.4, and 1.6 ± 0.4 nmol/l, resp.). Furthermore, they showed a remarkable reduction of infarct size in the rat myocardial infarction model *in vivo*.

Introduction. – The Na^+/H^+ exchangers (NHEs) comprise a family of membrane proteins [1]. To date, nine NHE isoforms (NHE-1–NHE-9) have been identified in various organs in the human body [2]. NHE-1 is ubiquitously distributed in tissue, and it is the predominant NHE isoform in the myocardial cell [3][4]. It is rapidly activated during ischemia by intracellular H^+ accumulation that is exchanged for Na^+. The high intracellular Na^+ level leads to an increase in intracellular Ca^{2+} *via* Na^+/Ca^{2+} exchange [3–5]. At the cardiac level, this cellular Ca^{2+} overload is involved in ischemic and reperfusion injuries such as myocardial stunning and infarction, and tissue necrosis [5][6].

Although the results of clinical studies of the NHE-1 inhibitors cariporide (*Fig.*) and eniporide are quite disappointing, some of these studies indicated that these NHE-1 inhibitors can indeed provide the protection of the human myocardium from injury during ischemia and reperfusion in the appropriate setting [7]. These somewhat contradictory results may be due to the design of their trials or their modest potency [7][8].

While the improvement of the myocardial protective effects by replacing the acylguanidine moiety with its bioisostere such as aminoguanidine and 2-aminoimidazole was quite limited [9–11], the studies that focused on searching for diverse templates bearing an acylguanidine moiety have provided promising results [12–15][1]). Because the use of natural product templates is known as a viable source of new drug candidates [17], in our efforts to discover more potent and safe NHE-1 inhibitors, we designed our target compounds through combining a natural product possessing good cardioprotective effects with a benzoylguanidine moiety according to a hybrid approach.

[1]) A preliminary account of this work has been published: [16].

Ligustrazine (2,3,5,6-tetramethylpyrazine (TMP); *Fig.*), a major active component of the Chinese traditional medicine Chuanxiong (*Ligusticum wallichii* FRANCH.), was selected as the 'natural product' part of the desired molecules. It has been introduced on the market for the treatment of coronary atherosclerotic disease and ischemic cerebrovascular disease in China since the 1970s [18]. It also shows good protective effect on myocardial injury during ischemia and reperfusion [19]. Here, the synthesis and pharmacological properties of ligustrazine derivatives as NHE-1 inhibitors are described.

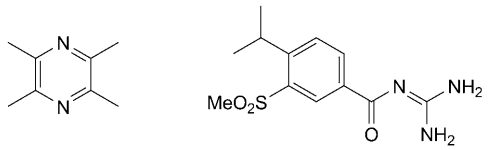
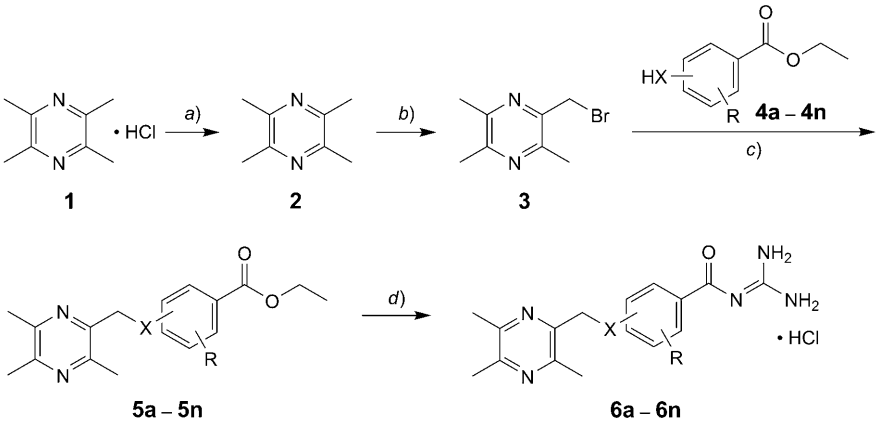


Figure. *Structures of ligustrazine and cariporide*

Results and Discussion. – *Synthesis.* As depicted in the *Scheme*, compound **3** was synthesized from 2,3,5,6-tetramethylpyrazine hydrochloride (**1**), which was deprotonated with aqueous NaOH to give **2**, followed by bromination with *N*-bromosuccinimide (NBS) in CCl_4, leading to **3**. The reaction temperature is very important in the bromination reaction. To reduce the yield of polybrominated by-products, the solution of **2** was stirred at 65°, and the product could be used in the next reaction step without further purification. The intermediates **5a**–**5n**, obtained by the reaction of **3** and ethyl benzoate or methyl benzoate derivatives **4a**–**4n**, were treated with guanidine in anhydrous ^{i}PrOH for 0.5–1 h, followed by formation of the hydrochloride salt with

Scheme

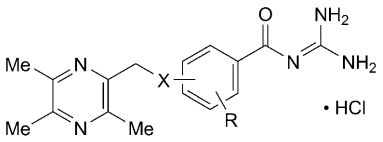


a) 1. NaOH, H_2O; 2. benzene. *b*) NBS, CCl_4, *hv*, reflux. *c*) Na_2CO_3, acetone or DMF, reflux. *d*) 1. Guanidine, ^{i}PrOH, reflux; 2. sat. HCl (g).

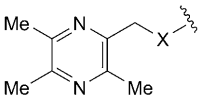
HCl gas, to afford our target compounds. In this reaction, using anhydrous ^{i}PrOH rather than anhydrous THF as solvent gave better yields.

Biological Activities. The NHE-1 inhibitory activities of 14 target compounds and cariporide were evaluated by rat platelet swelling assay (PSA). The experimental procedure was similar to that described in [20][21], with minor modifications. The PSA results showed that all tested compounds inhibited rat platelet NHE-1 in a concentration-dependent manner. Compounds **6b**–**6g**, **6k**, **6l**, and **6n** (see *Table 1*) are superior to cariporide in NHE inhibition. The IC_{50} values of compounds **6d**, **6k**, and **6l** were 3.0 ± 1.6, 3.0 ± 1.4, and 1.6 ± 0.4 nM, respectively, rendering them 20, 20, and 40 times more potent than cariporide (65.0 ± 8.6 nM). However, ligustrazine showed no effect on NHE-1 under these experimental conditions, suggesting that its protective effect on myocardial injury during ischemia and reperfusion was not regulated *via* the NHE-1 system (*Table 2*).

Table 1. *Chemical Structures and NHE-1 Inhibitory Activities of Compounds* **6a**–**6n**



6a – 6n

Compound	R	X	Position of 	IC_{50}[a]) [nM]
Cariporide				65.0 ± 8.6
6a	H	O	4	204 ± 20
6b	3-MeO	O	4	32.8 ± 6.7
6c	3-Cl	O	4	14.7 ± 3.8
6d	3-Br	O	4	3.0 ± 1.6
6e	3-NO_2	O	4	5.7 ± 2.0
6f	H	O	2	6.6 ± 2.3
6g	4-CF_3	O	2	11.7 ± 2.5
6h	H	NH	4	78.0 ± 5.3
6i	3-Me	NH	4	86.9 ± 7.8
6j	3-Cl	NH	4	95.7 ± 5.7
6k	3-Br	NH	4	3.0 ± 1.4
6l	3-NO_2	NH	4	1.6 ± 0.4
6m	H	NH	2	134 ± 9
6n	4-Me	NH	3	54.7 ± 4.9

[a]) Drug concentration to achieve half-maximal inhibition of acid-induced swelling in rat platelets.

Starting from **6a**, the structure–activity relationship (SAR) was described as follows. Replacing a benzene H-atom in **6a** with a MeO group (*i.e.*, compound **6b**) resulted in a six-times improvement in potency. The 3-Cl, 3-Br, and 3-NO_2 analogs (*i.e.*,

Table 2. *Cardioprotective Activity of Compounds* **6c**, **6d**, **6e**, **6f**, **6g**, **6k**, *and* **6l** *against Ischemic-Reperfusion*. Cariporide and the tested compounds were injected intravenously 5 min before LAD (left anterior descending) occlusion (0.01 mmol/kg).

	Dosage [mmol/kg]	CK[a]) [U/ml]	Infarct size[b]) [%]
Ischemia/reperfusion	–	67.3 ± 4.8	66.7 ± 4.3
Cariporide	0.01	35.6 ± 3.5**[c])	42.7 ± 3.2**
6c	0.01	47.5 ± 6.6**	52.7 ± 4.6*
6d	0.01	31.2 ± 2.9**	33.3 ± 2.7**
6e	0.01	42.3 ± 4.8**	43.3 ± 5.2**
6f	0.01	36.5 ± 4.3**	39.6 ± 2.5**
6g	0.01	45.7 ± 5.2**	49.8 ± 3.4**
6k	0.01	32.3 ± 3.6**	36.6 ± 4.1**
6l	0.01	34.4 ± 5.4**	38.4 ± 2.2**

[a]) Serum CK activity was expressed as U/ml. Values are means ± SD, $n = 6$ or higher. [b]) Infarct size was expressed as the ratio of myocardial infarct weight to weight of ventricle at risk. Values are means ± SD, $n = 6$ or higher. [c]) *: $p < 0.05$, **: $p < 0.01$ compared with ischemia-reperfusion group.

6c–**6e**, resp.) showed more significant improvement in potency relative to the starting compound **6a**, which suggested that introducing electron-withdrawing substituents to benzene ring improve the NHE-1 inhibitory potency. A similar SAR was observed for the aminobenzene analogs (**6j**–**6l**). In addition, although inhibition by compound **6h** was dramatically lower than that of **6a**, compound **6d** and **6k** showed similar potencies, suggesting that the influence of the atom X (O/N) at the benzene ring on NHE-1 inhibitory activity was limited.

The compounds showing good NHE-1 inhibitory activity were further investigated for their cardioprotective effects against ischemia-reperfusion injury in *Sprague-Dawley* (*SD*) rat hearts [22]. The infarct size and the creatine kinase (CK) level of cariporide were 42.7 ± 3.2 and 35.6 ± 3.5 U/ml, respectively. Most compounds with a good NHE-1 inhibitory activity exhibited a good cardioprotective efficacy both *in vitro* and *in vivo*. The infarct size and the CK level of compound **6d**, **6k**, and **6l** were better than those of cariporide at the same dose. Especially, compound **6d** significantly reduced the infarct size to 33.3 ± 2.7%.

Conclusions. – A series of novel derivatives of ligustrazine linked with substituted benzoylguanidine were synthesized and evaluated for their NHE-1 inhibitory activity and cardioprotective efficacy against ischemia-reperfusion injury. Compound **6d**, **6k**, and **6l** showed remarkably improved NHE-1 inhibitory activity and reduced the infarct size and the CK level in the rat model of ischemic heart *in vivo*. Further pharmacological studies are in progress.

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Experimental Part

General. M.p.: *RDCSY-I* apparatus; uncorrected. IR Spectra: *BRUKER Tensor 27* IR spectrophotometer, in KBr; in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: *BRUKER AM-300* spectrometer; δ in ppm rel. to Me_4Si, J in Hz. ESI-MS: *HP1100* mass spectrometer; in m/z (rel. %). Elemental analyses: *Elementar Vario EL III* instrument.

Syntheses. 2,3,5,6-Tetramethylpyrazine (**2**). A soln. of NaOH (13.9 g, 0.348 mol) in 100 ml of H_2O was poured into a soln. of 2,3,5,6-tetramethylpyrazine hydrochloride (**1**; 60.0 g, mol) in 250 ml of H_2O while stirring. The white precipitate formed was filtered and washed with H_2O until the filtrate was pH-neutral. The precipitate was dried with benzene through an azeotropic process to give **2** (36.0 g, 76.1%). White solid. M.p. 83–86°.

2-Bromomethyl-3,5,6-trimethylpyrazine (**3**). N-*Bromosuccinimide* (NBS; 12.8 g, 0.072 mol) and benzoyl peroxide (cat. amount) were added to a soln. of **2** (10.0 g, 0.072 mol) in anh. CCl_4. The mixture was stirred at 65° for 10 h under light (60-W tungsten-halogen lamp), then cooled to 5°, and filtered to remove succinimide. The filtrate was concentrated to give a yellow oil, which was purified by vacuum distillation to give **3** (9.2 g, 58.3%). Colorless oil. B.p. 127–131°/10 mm ([23]: b.p. 99–100°/2 mm).

General Procedure for the Synthesis of Compounds **5a**, **5c**, *and* **5e**. A mixture of **3** (2 g, 0.01 mol), **4a** (**4c** or **4e**; 0.01 mol), anh. Na_2CO_3 (0.5 g, 0.0047 mol), and triethyl(benzyl)ammonium chloride (TEBA; cat. amount) in anh. acetone (50 ml) was heated to reflux for 24 h and then filtered. The filtrate was adjusted to pH 3 with HCl (g), filtered, and washed with H_2O to give a yellowish solid product. A soln. of this yellowish solid in MeOH was adjusted with MeONa to pH 7, filtered and concentrated to give a solid.

Ethyl 4-[(3,5,6-Trimethylpyrazin-2-yl)methoxy]benzoate (**5a**). Yield: 1.4 g (46.7%). Yellowish solid. M.p. 62–64°. IR (KBr): 3454, 2949, 1713, 1604, 1552, 1510, 1435, 1416, 1359, 1280, 1251, 1172, 1103, 1010, 993, 848, 774, 767. ^1H-NMR (500 MHz, $CDCl_3$): 1.37 (*t*, $J = 7.1$, Me); 2.51 (*s*, 2 Me); 2.57 (*s*, Me); 4.34 (*q*, $J = 7.1$, MeCH_2O); 5.20 (*s*, CH_2O); 7.02 (*dd*, $J = 6.9$, 2.0, 2 arom. H); 7.99 (*dd*, $J = 2$, 6.9, 2 arom. H). ^{13}C-NMR (75 MHz, $CDCl_3$): 20.43; 21.20; 21.52; 51.67; 60.46; 69.81; 114.29 (2 arom. C); 131.36 (2 arom. C); 144.86; 148.51; 149.78; 151.38; 162.09; 166.56. ESI-MS: 301.1 ($[M+H]^+$). Anal. calc. for $C_{17}H_{20}N_2O_3$: C 67.98, H 6.71, N 9.33; found: C 67.66, H 6.85, N 9.34.

Ethyl 3-Chloro-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**5c**). Yield: 1.1 g (34.1%). White solid. M.p. 146–148°. ^1H-NMR (300 MHz, $CDCl_3$): 1.37 (*t*, $J = 7.1$, Me); 2.51 (*s*, Me); 2.52 (*s*, Me); 2.63 (*s*, Me); 4.34 (*q*, $J = 7.1$, MeCH_2O); 5.30 (*s*, CH_2O); 7.16 (*d*, $J = 8.6$, 1 arom. H); 7.90 (*dd*, $J = 2.1$, 8.6, 1 arom. H); 8.04 (*d*, $J = 2.1$, 1 arom. H).

Ethyl 3-Nitro-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**5e**). Yield: 1.1 g (31.2%). Yellow solid. M.p. 156–157°. ^1H-NMR (300 MHz, $CDCl_3$): 1.38 (*t*, $J = 7.1$, Me); 2.51 (*s*, Me); 2.52 (*s*, Me); 2.62 (*s*, Me); 4.37 (*q*, $J = 7.1$, MeCH_2O); 5.38 (*s*, CH_2O); 7.39 (*d*, $J = 8.9$, 1 arom. H); 8.17 (*dd*, $J = 2.1$, 8.8, 1 arom. H); 8.45 (*d*, $J = 2.1$, 1 arom. H).

General Procedure for the Synthesis of Compounds **5b** *and* **5d**. A mixture of **3** (1.1 g, 0.005 mol), **4b** (or **4d**; 0.01 mol), and anh. Na_2CO_3 (0.53 g, 0.005 mol) in anh. DMF (5 ml) was heated to 120° while stirring for 2 h. After cooling, the mixture was poured into ice-H_2O (50 ml), while stirring, to yield a solid, which was then filtered and dried to give the title intermediate.

Ethyl 3-Methoxy-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**5b**). Yield: 1.1 g (65.2%). White solid. M.p. 98–101°. ^1H-NMR (300 MHz, $CDCl_3$): 1.40 (*t*, $J = 7.1$, Me); 2.53 (*s*, 2 Me); 2.63 (*s*, Me); 3.91 (*s*, MeO); 4.37 (*q*, $J = 7.1$, MeCH_2O); 5.28 (*s*, CH_2O); 7.08 (*d*, $J = 8.4$, 1 arom. H); 7.57 (*d*, $J = 2.0$, 1 arom. H); 7.66 (*dd*, $J = 2.0$, 8.4, 1 arom. H).

Ethyl 3-Bromo-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**5d**). Yield: 1.4 g (73.7%). Yellow solid. M.p. 160°. ^1H-NMR (300 MHz, $CDCl_3$): 1.37 (*t*, $J = 7.1$, Me); 2.51 (*s*, Me); 2.52 (*s*, Me); 2.64 (*s*, Me); 4.34 (*q*, $J = 7.1$, MeCH_2O); 5.30 (*s*, CH_2O); 7.13 (*d*, $J = 8.7$, 1 arom. H); 7.95 (*dd*, $J = 2.1$, 8.7, 1 arom. H); 8.21 (*d*, $J = 2.1$, 1 arom. H).

General Procedure for the Synthesis of Compounds **5f** *and* **5g**. A mixture of **3** (2.6 g, 0.013 mol), **4f** (or **4g**; 0.012 mol), and anh. Na_2CO_3 (1.2 g, 0.011 mol) in anh. DMF (10 ml) was heated to 120° while stirring for 2 h. After cooling, the mixture was poured into ice-H_2O (50 ml), while stirring, and then extracted with $CHCl_3$ (3×20 ml). The combined org. layers were washed with sat. NaCl soln., dried

(Na_2SO_4), filtered, and concentrated. The residue was purified by column chromatography (CC; PE/AcOEt 4:1) to afford the title intermediate.

Ethyl 2-[(3,5,6-Trimethylpyrazin-2-yl)methoxy]benzoate (**5f**). Yield: 1.8 g (50.0%). White solid. M.p. 61–64°. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.27 (*t*, *J* = 7.1, Me); 2.51 (*s*, 2 Me); 2.63 (*s*, Me); 4.29 (*q*, *J* = 7.1, MeCH_2O); 5.24 (*s*, CH_2O); 6.98–7.01 (*m*, 1 arom. H); 7.13–7.16 (*m*, 1 arom. H); 7.39–7.42 (*m*, 1 arom. H); 7.76 (*dd*, *J* = 1.8, 7.7, 1 arom. H).

Ethyl 4-(Trifluoromethyl)-2-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**5g**). Yield: 3.0 g (68.7%). Colorless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.30 (*t*, *J* = 7.1, Me); 2.51 (*s*, 2 Me); 2.64 (*s*, Me); 4.33 (*q*, *J* = 7.1, MeCH_2O); 5.32 (*s*, CH_2O); 7.22 (*d*, *J* = 8.0, 1 arom. H); 7.55 (*s*, 1 arom. H); 7.81 (*d*, *J* = 8.0, 1 arom. H).

General Procedure for the Synthesis of Compounds **5h**, **5i**, *and* **5k**. A mixture of **3** (1 g, 0.0047 mol), **4h** (**4i** or **4k**; 0.0048 mol), and anh. Na_2CO_3 (0.5 g, 0.0048 mol) in anh. acetone (30 ml) was refluxed for 24 h. Then, the mixture was filtered to remove Na_2CO_3. After cooling, the precipitate formed from the filtrate was collected by filtration and dried to give the title intermediate.

Ethyl 4-{[(3,5,6-Trimethylpyrazin-2-yl)methyl]amino}benzoate (**5h**). Yield: 0.21 g (15.0%). White solid. M.p. 138–141°. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.36 (*t*, *J* = 7.1, Me); 2.53 (*s*, 2 Me); 2.55 (*s*, Me); 4.30–4.37 (*m*, MeCH_2O, CH_2N); 5.85 (br. *s*, NH); 6.73 (*d*, *J* = 8.7, 2 arom. H); 7.93 (*d*, *J* = 8.7, 2 arom. H).

Methyl 3-Methyl-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzoate (**5i**). Yield: 0.46 g (32.7%). Yellowish solid. M.p. 168–170°. ^{1}H-NMR (300 MHz, $CDCl_3$): 2.29 (*s*, Me); 2.54–2.55 (*m*, 3 Me); 3.86 (*s*, MeO); 4.38 (*s*, CH_2N); 5.90 (br. *s*, NH); 6.66 (*d*, *J* = 8.4, 1 arom. H); 7.79 (*d*, *J* = 1.2, 1 arom. H); 7.88 (*dd*, *J* = 1.9, 8.4, 1 arom. H).

Ethyl 3-Bromo-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzoate (**5k**). Yield: 0.75 g (42.3%). Yellow solid. M.p. 182–183°. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.38 (*t*, *J* = 7.1, Me); 2.53 (*s*, 2 Me); 2.57 (*s*, Me); 4.33 (*q*, *J* = 4.3, MeCH_2O); 4.39 (*s*, CH_2N); 6.69 (*d*, *J* = 8.6, 1 arom. H); 7.92 (*dd*, *J* = 2.0, 8.6, 1 arom. H); 8.16 (*d*, *J* = 2.0, 1 arom. H).

General Procedure for the Synthesis of Compounds **5j**, **5m**, *and* **5n**. A mixture of **3** (2.0 g, 0.009 mol), **4j** (**4m** or **4n**; 0.0085 mol), and anh. Na_2CO_3 (1.2 g, 0.011 mol) in anh. DMF (10 ml) was heated to 120° while stirring for 2 h. After cooling, the mixture was poured into ice-H_2O (50 ml) while stirring to yield a yellow-brown syrup-like substance, which was extracted with $CHCl_3$ (3 × 30 ml). The combined org. layers were washed with sat. NaCl soln. (3 × 90 ml), dried (Na_2SO_4), filtered, and concentrated. The residue was purified by CC (PE/AcOEt 4:1) to give the title intermediate.

Ethyl 3-Chloro-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzoate (**5j**). Yield: 1.5 g (53.2%). Yellowish solid. M.p. 155–158°. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.38 (*t*, *J* = 7.1, Me); 2.53–2.56 (*m*, 3 Me); 4.33 (*q*, *J* = 7.1, MeCH_2O); 4.40 (*s*, CH_2N); 6.61 (*s*, NH); 6.73 (*d*, *J* = 8.5, 1 arom. H); 7.89 (*dd*, *J* = 1.8, 8.5, 1 arom. H); 8.00 (*d*, *J* = 1.8, 1 arom. H).

Ethyl 2-{[(3,5,6-Trimethylpyrazin-2-yl)methyl]amino}benzoate (**5m**). Yield: 1.0 g (39.3%). Yellowish solid. M.p. 113–115°. IR (KBr): 3441, 3328, 2978, 2913, 1682, 1608, 1571, 1518, 1477, 1455, 1415, 1371, 1256, 1227, 1183, 1153, 1127, 1094, 1074, 990, 853, 792, 752. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.38 (*t*, *J* = 7.1, Me); 2.51 (*s*, Me); 2.54 (*s*, Me); 2.57 (*s*, Me); 4.35 (*q*, *J* = 7.1, MeCH_2O); 4.44 (*d*, *J* = 4.6, CH_2N), 6.62–6.65 (*m*, 1 arom. H); 6.82 (*d*, *J* = 8.4, 1 arom. H); 7.37–7.40 (*m*, 1 arom. H); 7.96 (*dd*, *J* = 1.7, 8.0, 1 arom. H); 8.63 (*s*, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): 14.38; 20.33; 21.41; 21.58; 45.83; 60.21; 111.06; 111.60; 114.90; 131.73; 134.42; 146.47; 147.78; 148.37; 149.62; 150.32; 168.42. ESI-MS: 300.2 ([*M*+H]$^+$). Anal. calc. for $C_{17}H_{21}N_3O_2$: C 68.20, H 7.07, N 14.04; found: C 67.90, H 7.08, N 13.81.

Ethyl 4-Methyl-3-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzoate (**5n**). Yield: 1.2 g (46.6%). White solid. M.p. 104–105°. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.39 (*t*, *J* = 7.1, Me); 2.45 (*s*, Me); 2.52 (*s*, Me); 2.53 (*s*, Me); 2.55 (*s*, Me); 4.34–4.38 (*m*, MeCH_2O, CH_2N); 5.57 (*s*, NH); 6.84 (*dd*, *J* = 1.5, 7.7, 1 arom. H); 7.16 (*dd*, *J* = 1.6, 7.8, 1 arom. H); 7.18–7.21 (*m*, 1 arom. H).

Synthesis of Ethyl 3-Nitro-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzoate (**5l**). A soln. of **4l** (0.5 g, 0.0024 mol) in 2 ml of anh. DMF was added to a mixture of NaH (0.05 g. 0.002 mol) suspended in 1 ml of anh. DMF while stirring. Then, the mixture was stirred under N_2 for 1 h at r.t., a soln. of **3** (0.5 g, 0.0024 mol) in 2 ml of anh. DMF was added, and the mixture was stirred for 4 h. The yellow precipitate formed was filtered, 5 ml of AcOEt was added into the filtrate, and the yellow precipitate formed was filtered. All precipitates were dissolved in $CHCl_3$, filtered, and the filtrate was concentrated to give **5l**.

Yield: 0.31 g (38.0%). Yellow solid. M.p. 206–210°. ^{1}H-NMR (500 MHz, $CDCl_3$): 1.41 (*t*, *J* = 7.2, Me); 2.70 (*s*, Me); 2.75 (*s*, Me); 2.77 (*s*, Me); 4.38 (*q*, *J* = 7.2, MeCH_2O); 4.64 (*d*, *J* = 2.7, CH_2N); 6.98 (*d*, *J* = 8.9, 1 arom. H); 8.15 (*dd*, *J* = 2.0, 8.9, 1 arom. H); 8.95 (*d*, *J* = 2.0, 1 arom. H); 9.73 (*s*, NH).

General Procedure for Synthesis of Compounds **6**. Guanidine (2.00 g, 0.0035 mol) and 3 ml of anh. ^{i}PrOH were mixed, and the mixture was then heated to 70° while stirring. A soln. of **5** (0.0017 mol) in 3 ml of anh. ^{i}PrOH was added, and the mixture was refluxed for 30 min. After cooling, the mixture was adjusted to pH 3 with HCl (g) in AcOEt, filtered, and concentrated under reduced pressure. The residue was purified by CC ($CHCl_3$/MeOH 10:1) to afford a solid product.

N-*(Diaminomethylidene)-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6a**). Yield: 0.31 g (51.7%). White solid. M.p. 102–106°. IR (KBr): 3435, 3352, 3129, 2952, 2924, 2853, 1701 (C=O), 1666 (C=N), 1606, 1543, 1463 (C=C), 1261 (C–O–C), 1174, 991, 809, 762. ^{1}H-NMR (500 MHz, (D_6)DMSO): 2.44 (*s*, Me); 2.45 (*s*, Me); 2.49 (*s*, Me); 5.23 (*s*, CH_2O); 7.10–7.13 (*m*, 2 arom. H); 7.88–7.90 (*m*, 2 arom. H); 12.61 (br. *s*, NH). ESI-MS: 314.1 ([*M*+H]$^+$). Anal. calc. for $C_{16}H_{19}N_5O_2\cdot HCl\cdot H_2O$: C 52.25, H 6.03, N 19.04; found: C 51.81, H 6.18, N 18.70.

N-*(Diaminomethylidene)-3-methoxy-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6b**). Yield: 0.37 g (57.7%). White solid. M.p. 197°. IR (KBr): 3329, 3186, 2950, 1695 (C=O), 1625, 1597, 1521, 1462 (C=C), 1271, 1218, 1174, 1135, 1027 (C–O–C), 991, 825, 756. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.44 (*s*, Me); 2.46 (*s*, Me); 2.49 (*s*, Me); 3.84 (*s*, MeO); 5.24 (*s*, CH_2O); 7.27 (*d*, *J* = 8.3, 1 arom. H); 7.79–7.80 (*m*, 2 arom. H); 8.70 (br. *s*, 4 NH); 12.10 (br. *s*, NH). ^{13}C-NMR (75 MHz, $CDCl_3$): 20.08; 20.92; 21.23; 56.01; 70.02; 111.67; 112.78; 122.52; 144.81; 148.32; 148.75; 149.47; 151.20; 152.22; 153.71; 156.27; 167.59. ESI-MS: 344.1 ([*M*+H]$^+$). Anal. calc. for $C_{17}H_{21}N_5O_3\cdot HCl\cdot 1.5\ H_2O$: C 50.18, H 6.19, N 17.21; found: C 49.80, H 6.22, N 17.38.

3-Chloro-N-*(diaminomethylidene)-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6c**). Yield: 0.25 g (38.4%). White solid. M.p. 162–164°. IR (KBr): 3371, 3130, 2945, 2929, 2872, 1705 (C=O), 1659 (C=N), 1599, 1520, 1440 (C=C), 1267 (C–O–C), 1175, 989, 813, 755. ^{1}H-NMR (500 MHz, (D_6)DMSO): 2.45 (*s*, Me); 2.47 (*s*, Me); 2.52 (*s*, Me); 5.35 (*s*, CH_2O); 7.22 (br. *s*, 2 NH); 7.40 (*d*, *J* = 8.6, 1 arom. H); 7.88–7.91 (*m*, 2 arom. H); 12.9 (br. *s*, 3 NH). ESI-MS: 348.1 ([*M*+H]$^+$). Anal. calc. for $C_{16}H_{18}ClN_5O_2\cdot HCl\cdot H_2O$: C 47.77, H 5.26, N 17.41; found: C 47.59, H 5.31, N 17.27.

3-Bromo-N-*(diaminomethylidene)-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6d**). Yield: 0.38 g (52.1%). Yellowish solid. M.p. 175–176°. IR (KBr): 3380, 3189, 2954, 2921, 2877, 2850, 1706 (C=O), 1657 (C=N), 1595, 1551, 1494, 1455 (C=C), 1359, 1265, 1176, 1148, 1043 (C–O–C), 1043, 990, 811, 786, 755. ^{1}H-NMR (500 MHz, (D_6)DMSO): 2.50 (*s*, 2 Me); 2.59 (*s*, Me); 5.29 (*s*, CH_2O); 7.20 (*d*, *J* = 8.8, 1 arom. H); 7.65 (br. *s*, 2 NH); 8.06 (*dd*, *J* = 2.0, 8.8, 1 arom. H); 8.32 (*d*, *J* = 2.0, 1 arom. H). ESI-MS: 394.0 ([*M*+H]$^+$). Anal. calc. for $C_{16}H_{18}BrN_5O_2\cdot HCl\cdot H_2O$: C 43.02, H 4.74, N 15.68; found: C 42.87, H 4.82, N 15.57.

N-*(Diaminomethylidene)-3-nitro-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6e**). Yield: 0.33 g (48.9%). White solid. M.p. 127–130°. IR (KBr): 3429, 3314, 2923, 2853, 1710 (C=O), 1681 (C=N), 1618, 1534, 1462, 1414 (C=C), 1383 (C–NO_2), 1274, 1150, 1089 (C–O–C), 983, 755, 708. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.44 (*s*, Me); 2.46 (*s*, Me); 2.49 (*s*, Me); 5.41 (*s*, CH_2O); 7.55 (*d*, *J* = 8.9, 1 arom. H); 7.88 (br. *s*, 2 NH); 8.27 (*dd*, *J* = 2.1, 8.8, 1 arom. H); 8.49 (*d*, *J* = 2.1, 1 arom. H). ESI-MS: 359.1 ([*M*+H]$^+$), 381.1 ([*M*+Na]$^+$). Anal. calc. for $C_{16}H_{18}N_6O_4\cdot HCl\cdot 0.5\ H_2O$: C 47.59, H 4.99, N 20.81; found: C 47.48, H 5.07, N 20.50.

N-*(Diaminomethylidene)-2-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6f**). Yield: 0.26 g (43.4%). Yellowish solid. M.p. 185–187°. IR (KBr): 3356, 3271, 3126, 3028, 2754, 1701 (C=O), 1687 (C=N), 1596, 1558, 1458 (C=C), 1278, 1240, 1211, 1170, 1014 (C–O–C), 979, 752. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.46 (*s*, Me); 2.52 (*s*, Me); 2.60 (*s*, Me); 5.52 (*s*, CH_2O); 7.08–7.13 (*m*, 1 arom. H); 7.39 (*d*, *J* = 8.4, 1 arom. H); 7.55–7.61 (*m*, 1 arom. H); 7.70 (*dd*, *J* = 1.4, 7.7, 1 arom. H); 8.78 (br. *s*, 4 NH); 11.66 (br. *s*, NH). ESI-MS: 314.2 ([*M*+H]). Anal. calc. for $C_{16}H_{19}N_5O_2\cdot HCl\cdot 0.5\ H_2O$: C 53.56, H 5.90, N 19.52; found: C 53.27, H 5.97, N 19.49.

N-*(Diaminomethylidene)-4-(trifluoromethyl)-2-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzamide Hydrochloride* (**6g**). Yield: 0.32 g (44.9%). White solid. M.p. 193–195°. IR (KBr): 3356, 3281, 3047, 2927, 1697 (C=O), 1679 (C=N), 1622, 1585, 1562, 1500 (C=C), 1440, 1325 (C–F), 1211, 1170, 1132, 979, 719, 696. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.42 (*s*, Me); 2.44 (*s*, Me); 2.51 (*s*, Me); 5.45 (*s*, CH_2O); 7.41 (*d*,

$J = 7.9$, 1 arom. H); 7.74–7.76 (*m*, 2 arom. H); 8.24 (br. *s*, 4 NH); 11.78 (br. *s*, NH). ^{1}H-NMR (500 MHz, MeOD): 2.48 (*s*, Me); 2.50 (*s*, Me); 2.58 (*s*, Me); 5.55 (*s*, CH_2O); 7.40 (*dd*, $J = 0.8, 8.0$, 1 arom. H); 7.70 (*s*, 1 arom. H); 7.89 (*d*, $J = 8.0$, 1 arom. H). ESI-MS: 382.1 ($[M+H]^+$). Anal. calc. for $C_{17}H_{18}N_5O_2 \cdot HCl$: C 48.87, H 4.58, N 16.76; found: C 49.17, H 4.61, N 16.57.

N-*(Diaminomethylidene)-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6h**). Yield: 0.25 g (42.3%). White solid. M.p. 251–255°. IR (KBr): 3361, 3297, 3125, 2987, 2918, 1691 (C=O), 1605, 1563, 1540, 1482 (C=C), 1272, 1258, 1191, 1116, 827, 687. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.43 (*s*, Me); 2.44 (*s*, Me); 2.48 (*s*, Me); 4.43 (*d*, $J = 5.3$, CH_2N); 6.79 (*d*, $J = 8.9$, 2 arom. H); 7.19 (br. *s*, CNH); 7.91 (*d*, $J = 8.9$, 2 arom. H); 8.53 (br. *s*, 4 NH); 11.39 (br. *s*, NH). ESI-MS: 313.2 ($[M+H]^+$). Anal. calc. for $C_{16}H_{20}N_6O \cdot HCl$: C 55.09, H 6.07, N 24.09; found: C 54.98, H 6.24, N 23.66.

N-*(Diaminomethylidene)-3-methyl-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6i**). Yield: 0.34 g (54.8%). Yellowish solid. M.p. 267°. IR (KBr): 3368, 3153, 2993, 1689 (C=O), 1606, 1564, 1450 (C=C), 1261, 1151, 1113, 990, 810, 760. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.22 (*s*, Me); 2.43 (*s*, Me); 2.45 (*s*, Me); 2.49 (*s*, Me); 4.50 (*s*, CH_2N); 6.77 (*d*, $J = 8.7$, 1 arom. H); 7.81 (*s*, 1 arom. H); 7.86 (*dd*, $J = 2.1, 8.6$, 1 arom. H); 8.50 (br. *s*, 4 NH). ESI-MS: 327.2 ($[M+H]^+$). Anal. calc. for $C_{17}H_{22}N_6O \cdot HCl \cdot H_2O$: C 53.61, H 6.62, N 22.07; found: C 53.77, H 6.58, N 22.29.

3-Chloro-N-*(diaminomethylidene)-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6j**). Yield: 0.45 g (69.3%). White solid. M.p. 207–210°. IR (KBr): 3356, 3281, 3045, 2926, 1697 (C=O), 1680, 1622, 1579, 1562, 1500 (C=C), 1325, 1209, 1170, 1132, 1002, 979, 719, 696. ^{1}H-NMR (500 MHz, (D_6)DMSO): 2.44 (*s*, Me); 2.47 (*s*, Me); 2.48 (*s*, Me); 4.46 (*s*, CH_2N); 6.48 (br. *s*, NH); 6.84 (*d*, $J = 8.6$, 1 arom. H); 7.65 (br. *s*, 4 NH); 7.85 (*dd*, $J = 1.8, 8.5$, 1 arom. H); 8.02 (*d*, $J = 1.8$, 1 arom. H). ESI-MS: 347.1 ($[M+H]^+$). Anal. calc. for $C_{16}H_{19}ClN_6O \cdot HCl \cdot H_2O$: C 47.89, H 5.53, N 20.94; found: C 48.02, H 5.47, N 21.14.

3-Bromo-N-*(diaminomethylidene)-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6k**). Yield: 0.53 g (72.7%). Yellow solid. M.p. 218–220°. IR (KBr): 3407, 2923, 2770, 1692 (C=O), 1632, 1596, 1542, 1461 (C=C), 1267, 1229, 1092, 755, 701. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.48 (*s*, Me); 2.52 (*s*, 2 Me); 4.56 (*s*, CH_2N); 6.94 (*d*, $J = 8.6$, 1 arom. H); 7.10 (br. *s*, CNH); 8.20 (*d*, $J = 8.3$, 1 arom. H); 8.34 (*d*, $J = 1.1$, 1 arom. H); 8.55 (br. *s*, 2 NH); 8.87 (br. *s*, 2 NH); 11.96 (br. *s*, NH). ESI-MS: 391.1 ($[M+H]^+$). Anal. calc. for $C_{16}H_{19}BrN_6O \cdot HCl \cdot H_2O$: C 43.11, H 4.97, N 18.85; found: C 43.46, H 4.82, N 19.10.

N-*(Diaminomethylidene)-3-nitro-4-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6l**). Yield: 0.23 g (34.2%). Yellow solid. M.p. 207–210°. IR (KBr): 3406, 1697 (C=O), 1627 (C=C), 1566, 1421, 1359 (C–NO_2), 1296, 1186, 1093 (C–N), 993, 831, 790. ^{1}H-NMR (500 MHz, (D_6)DMSO): 2.55 (*s*, Me); 2.56 (*s*, Me); 2.59 (*s*, Me); 3.16 (*s*, CH_2N); 7.18 (br. *s*, CNH); 7.90 (*d*, $J = 8.6$, 1 arom. H); 8.21 (*d*, $J = 8.6$, 1 arom. H); 8.54 (*s*, 1 arom. H); 8.79 (br. *s*, 2 NH); 8.92 (br. *s*, 2 NH); 12.33 (br. *s*, NH). ESI-MS: 358.1 ($[M+H]^+$). Anal. calc. for $C_{16}H_{19}N_7O_3 \cdot HCl \cdot 0.5\ H_2O$: C 47.70, H 5.25, N 24.34; found: C 47.56, H 5.29, N 24.11.

N-*(Diaminomethylidene)-2-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6m**). Yield: 0.17 g (28.7%). White solid. M.p. 247–250°. IR (KBr): 3330, 3227, 2991, 2952, 2920, 2856, 1695 (C=O), 1672, 1612, 1562, 1515 (C=C), 1357, 1271, 1161, 1091, 894, 750. ^{1}H-NMR (300 MHz, (D_6)DMSO): 2.40 (*s*, Me); 2.45 (*s*, Me); 2.47 (*s*, Me); 4.43 (*s*, CH_2N); 6.49–6.54 (*m*, 1 arom. H); 6.76 (*d*, $J = 8.0$, 1 arom. H); 6.90 (br. *s*, CNH); 7.23 (br. *s*, 1 arom. H); 7.96 (*d*, $J = 6.8$, 1 arom. H); 7.70 (br. *s*, 2 NH); 9.75 (br. *s*, 2 NH). ESI-MS: 313.1 ($[M+H]^+$). Anal. calc. for $C_{16}H_{20}N_6O \cdot HCl$: C 55.09, H 6.07, N 24.09; found: C 54.88, H 6.12, N 23.87.

N-*(Diaminomethylidene)-4-methyl-3-{[(3,5,6-trimethylpyrazin-2-yl)methyl]amino}benzamide Hydrochloride* (**6n**). Yield: 0.42 g (67.6%). White solid. M.p. 226–229°. IR (KBr): 3396, 3340, 3140, 2991, 2920, 2856, 2810, 1691 (C=O), 1585, 1504 (C=C), 1357, 1261, 1145, 1092, 989, 831, 783, 628. ^{1}H-NMR (500 MHz, (D_6)DMSO): 2.23 (*s*, Me); 2.44 (*s*, Me); 2.47 (*s*, Me); 2.50 (*s*, Me); 4.41 (*d*, $J = 4.6$, CH_2N); 6.55 (br. *s*, CNH); 6.83–6.88 (*m*, 2 arom. H); 7.11–7.14 (*m*, 1 arom. H); 8.37 (br. *s*, 4 NH); 11.80 (br. *s*, NH). ESI-MS: 327.1 ($[M+H]^+$). Anal. calc. for $C_{17}H_{22}N_6O \cdot HCl \cdot H_2O$: C 53.61, H 6.62, N 22.06; found: C 53.59, H 6.72, N 21.99.

3. *Bioassays. Animals. Sprague–Dawley* (*SD*) rats were provided by *Shanghai BK Biology Co.* The rats were acclimatized for a week before use. *Rodent* laboratory chow and tap H_2O were provided *ad*

libitum, and the animals were maintained under controlled conditions at a temp. of $24 \pm 1°$ and $50 \pm 10\%$ humidity, using a 12-h light/12-h dark cycle. All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals and with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institute of Health.

Rat Platelet Swelling Assay (*PSA*). *Sprague–Dawley* rats (380–420 g) were anesthetized with Et_2O, and blood was collected from their eyeholes with 25% (*v*/*v*) acid-citrate-dextrose (ACD; sodium citrate, 2.23 g; citric acid, 0.86 g; and glucose, 2.47 g in 100 ml of distilled H_2O). Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 1300*g*/min for 10 min at r.t. The upper two-thirds of the supernatants were used for the further measurements and stored at r.t. until used. All measurements were performed within 4–5 h.

All compounds were dissolved in DMSO and diluted with propionate medium (pH 7.4). A soln. of the tested compound (25 µl) was added to 175 µl of propionate buffer (in mM; sodium propionate, 140 mM; HEPES, 20 qs; glucose, 10 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1 mM; pH 6.7) contained in a spectrophotometer cuvette. Then, 50 µl of PRP prewarmed to 37° was added. The suspension was stirred, and the change in optical density (*OD*) was recorded each 7.5 s for 2 min at 550 nm (*Thermo Multiscan Spectrum*). The decrease of *OD* corresponded to a monoexponential curve following the equation $OD_{(t)} = OD_{t=0}$ e where *t* is the time (in s) corresponding to the recorded OD, and *k* is the decrease rate constant. For each compound, the concentrations were plotted against their corresponding *k* values. The maximum platelet swelling was measured in absence of any drug. The minimum swelling was observed in presence of cariporide (10 mol/l) and is the result of complete NHE-1 inhibition. Sigmoidal curves were drawn by non-linear regression analysis (Graphpad Prism software). The IC_{50} value was calculated according to the regression analysis. Each measurement was performed in triplicate for all molecules.

Cardioprotective Effects in Rat Model of Ischemic Heart. Adult male and female *SD* rats (280–300 g) were anesthetized with sodium pentobarbital (60 mg/kg, ip, *Shanghai Chemicals Reagent Co., Ltd.*, Shanghai, P. R. China). Coronary artery occlusion was produced by ligating the left anterior descending (LAD) coronary artery for 1 h. After that, the coronary artery was reperfused by loosing the ligature. After 2 h of reperfusion, the coronary artery was reoccluded, and 1 ml of a 2% *Evans* blue was injected *via* tail vein. Then, the heart was removed, and blood sample was collected.

The left ventricle of the removed heart was dissected free from other structures and sliced transversely into 1-mm thick sections. The sections were then incubated in 0.5% triphenyltetrazolium chloride (*Shanghai Chemicals Reagent Co., Ltd.*, Shanghai, P. R. China) for 10 min at 37° and then fixed for 1–2 h in a 10% formalin soln. to determine the infarct size. The infarct size was calculated by the formula as follow:

Myocardial infarct size [%] = (the weight of undyed myocardium/the weight of left ventricle) × 100

The blood samples were centrifuged at 3000*g* for 10 min. The supernatant serum was removed and stored in liquid N_2 until the biochemical analysis was performed. Creatine kinase (CK) in serum were measured by *722* grating photospectrometer (*Shanghai Precision & Scientific Instrument Co., LTD.*, Shanghai, P. R. China) using commercial kits (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, P. R. China).

Target compounds **6c**, **6e**, **6e**, **6d**, **6f**, **6g**, **6k**, and **6l**, and cariporide were intravenously given 5 min before reperfusion.

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