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Scaffold evaluation of liguzinediol analogs as novel cardiotonic agents

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Liguzinediol (LZDO) could mediate the positive inotropic effects through sarcoplasmic reticulum Ca^{2+} ATPase-dependent mechanism without the risk of arrhythmia. However, the pharmacophore of LZDO contributed to the activities was not clear. The aim of this work was to explore the relationship between positive inotropic effect and scaffold of LZDO as well as to check whether the pharmacophore of LZDO on anti-heart failure activity was located at the pyrazine ring. A series of LZDO analogs (**3a-b**, **4a-b**, **9–19**) were designed and synthesised, and their activities were evaluated on isolated heart contractility by Langendorff perfusion. The results showed that the efficacy of LZDO was reduced when the hydroxyl, carboxyl or ester moieties at the side chain position of LZDO were induced, and the *para*-dihydroxy in LZDO was necessary for its activity. Thus, the pharmacophore of the positive inotropic effect might be located at the whole scaffold of LZDO, but not at the pyrazine ring. The finding may provide an important clue of the pharmacophore for the development of novel cardiotonic agents.

1. Introduction

Cardiotonic agents have received considerable attention for their positive inotropic effect on the myocardium. They are either cAMP-dependent or cAMP-independent based upon their mechanism of action. cAMP-dependent drugs include β-receptor agonists, phosphodiesterase 3 inhibitors (Yan et al. 2007), and adenylate cyclase agonists. cAMP-independent drugs include Na^+/K^+-ATP enzyme inhibitors (Pederse et al. 2006; Rocchetti et al. 2003) and calcium sensitisers (Pollesello et al. 2007; Qin et al. 2008; Stawsky et al. 2000; Cohn et al. 1997; Cleland et al. 2005). But this mechanism of action is inherently responsible for the cardiac side effects observed in patients undergoing long-term treatment. The most common effects associated with cardiotonic agents therapy are high mortality (Lubsen et al. 1996; Cohn et al. 1998; Hampton et al. 1997; Van Veldhuisen et al. 1993; Oliva et al. 1999), myocardial ischemia aggravation (Singh et al. 2000; Packer et al. 1984), arrhythmia, hypotension (Moiseyev et al. 2002), and increased heart rate (Antila et al. 2004).

Liguzinediol (LZDO, **1a**), a *para*-dihydroxy derivative of ligustrazine (one active ingredient of Szechwan Lovage Rhizome, which is more used in China as a calcium channel antagonist for the treatment of coronary atherosclerotic cardiovascular disease and ischemic cerebrocardiac vascular disease rather than as a cardiotonic agent (Ren et al. 2012)), mediates positive inotropic effects in isolated rat heart by sarcoplasmic reticulum Ca^{2+} ATPase-dependent mechanism without the risk of arrhythmia (Chen et al. 2012). LZDO is metabolized mainly by oxidation, sulfation, glycine conjugation and glucuronidation (Shan et al. 2012) and has low toxicity (Wen et al. 2011).

LZDO may offer a novel target for inducing positive inotropic effects (Lipskaia et al. 2010) based on its Chinese patent (ZL200810157140.4), and U.S. Patent (US8,158,630B2). However, the scaffold of **1a** was different from the structure of cardiotonic agents in the market, the pharmacophore of LZDO contribute to the activities was not clear. Moreover, structure-activity relationship (SAR) studies of ligustrazine indicated that the pharmacophore might be located at the pyrazine ring while the substituted groups might primarily contribute to its pharmacokinetics and toxicity (Jiang et al. 1996).

In this study, we aimed to explore the relationship between positive inotropic effect and scaffold of LZDO as well as to check whether the pharmacophore of LZDO on anti-heart failure activity was located at the pyrazine ring. Therefore, a series of LZDO analogs (the introduction of hydroxyl, carboxyl and ester moieties at the side-chain position of LZDO, Fig. 1) were designed, and synthesised by modifying the substituted groups of LZDO. Additionally, their inotropic effects on the myocardium in normal isolated rat hearts evaluated.

2. Investigations and results

2.1. Chemistry

The designed compounds **3a-b, 4a-b, 9–13** were synthesised as summarised in Schemes 1–5. The other compounds **14–19** were prepared as previously reported (Liu 2006; Qin et al. 2010).

In the synthesis of LZDO esters **3a-b**, **4a-b**, oxidation of ligustrazine (**1**) with H_2O_2 afforded the key intermediates *N*,*N*'-dioxotetramethylpyrazine (**2**). Treatment of **2** with a variety of acid anhydrides gave **3a-b.** Next, **4a-b** was synthesised by the treatment of **2** with various substituted benzoyl chlorides. (Scheme 1).

In the synthesis of LZDO acid ester **9**, condensation of butanedione (**5**) with *o*-phenylene diamine (**6**) to afford **7**, followed by oxidation with $KMnO_4$ led to **8**, which after treatment with

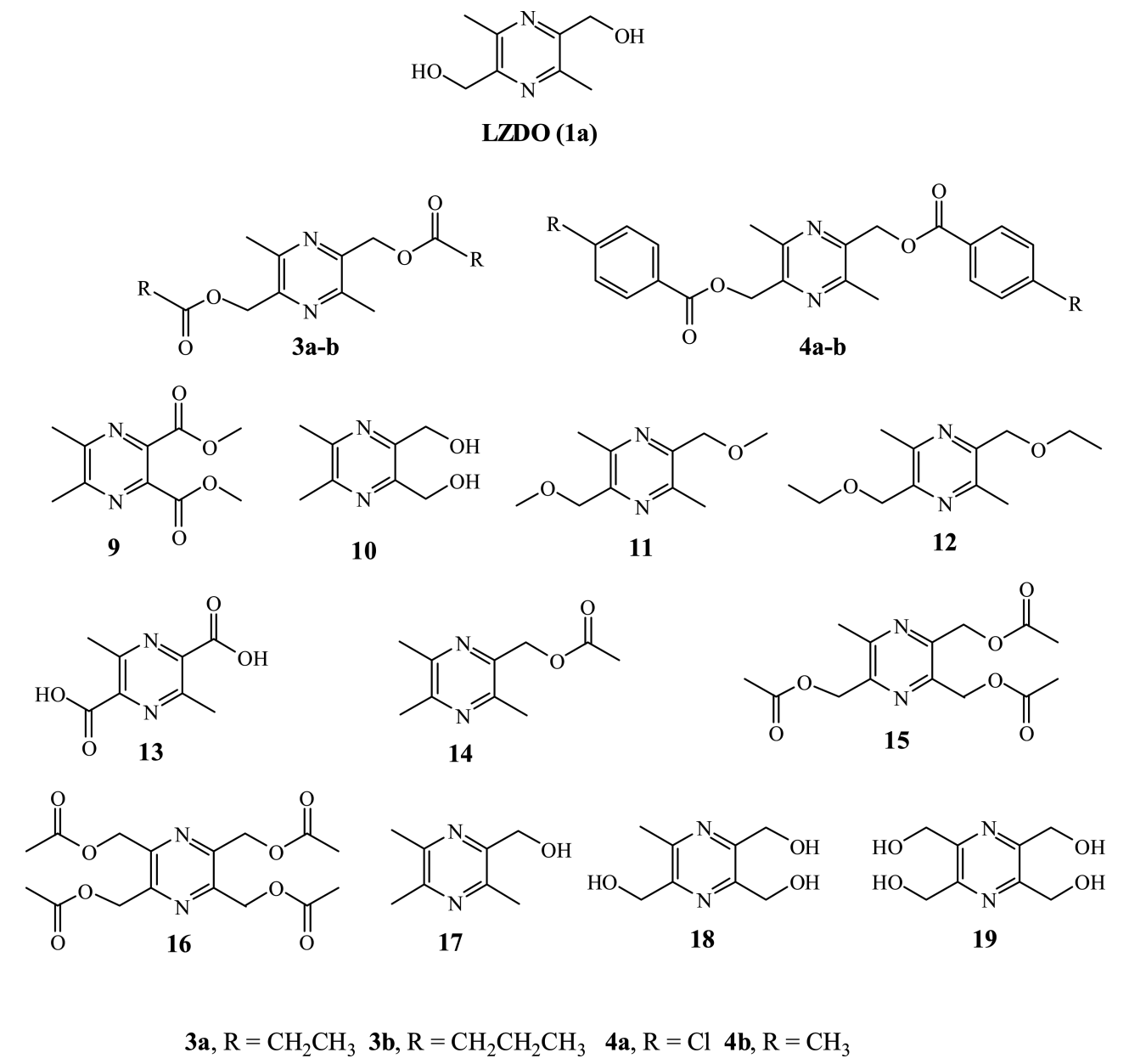


Fig. 1: Chemical structures of LZDO (**1a**) and LZDO analogs.

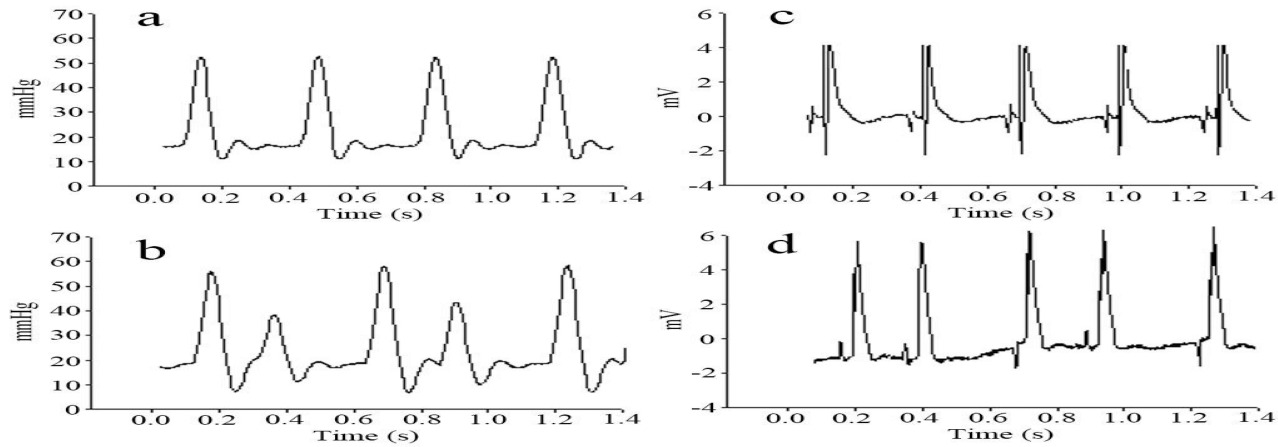
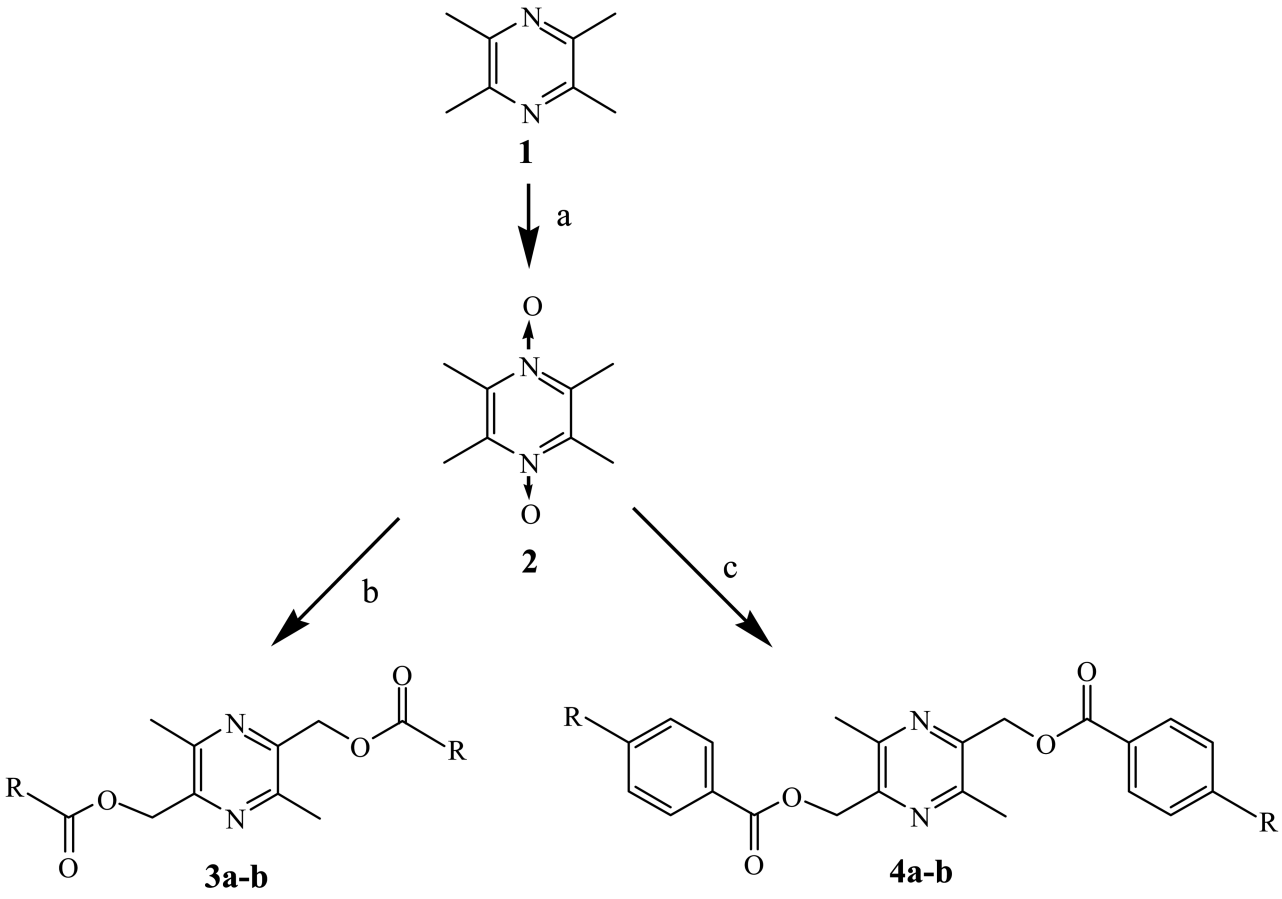


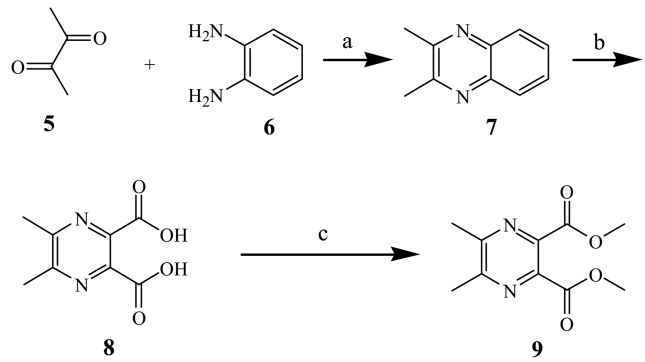
Fig. 2: Effects of 2,5-dimethoxymethyl-3,6-dimethylpyrazine (1 μM) on left ventricular pressure and ECG in normal isolated rat hearts. a, left ventricular pressure without drug; b, left ventricular pressure with 1 μM; c, ECG without drug; d, ECG with 1 μM.



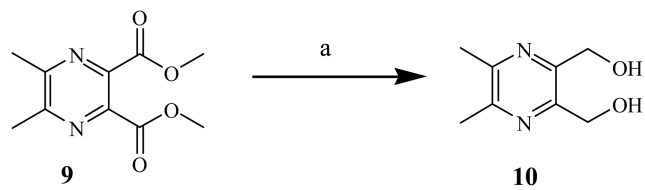
3a, R = CH_2CH_3 **3b**, R = $CH_2CH_2CH_3$ **4a**, R = Cl **4b**, R = CH_3

Scheme 1: Synthesis of LZDO esters **3a-b**, **4a-b**. Reagents and conditions: (a) 30% H_2O_2, glacial acetic acid, 98 °C, 12 h; (b) acid anhydride, 100 °C, 4 h; (c) para-chlorobenzoyl chloride, 4-methylbenzoyl chloride, 25 °C, 11 h; acetic anhydride, reflux for 4 h.

methanol afforded desired product **9** (More et al. 2006; Yoshizumi et al. 2003; Jones et al. 1963) (Scheme 2).
Ligustrazine alcohol, **10**, was obtained from **9** through reduction with sodium borohydride (Scheme 3).
In the synthesis of LZDO ether derivatives **11**, **12**, etherification of **1a** with sodium methoxide and ethoxide yielded **11** and **12**, respectively (Scheme 4). Oxidation of **1a** with saturated sodium hypochlorite solution furnished LZDO acid **13** (Scheme 5).
All of these derivatives were obtained in moderate to good isolated yields (17.8–91.0%), and their structures were confirmed by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. All compounds were analysed by HPLC, and their purity was confirmed to be greater than 98.0%.



Scheme 2: Synthesis of LZDO acid ester **9**. Reagents and conditions: (a) ceric ammonium nitrate (CAN), H_2O, rt, 25 °C, 1 h; (b) $KMnO_4$, H_2O, 90 °C; (c) H_2SO_4, CH_3OH, reflux for 20 h.



Scheme 3: Synthesis of ligustrazine alcohols **10**. Reagents and conditions: (a) sodium borohydride, dehydrated ethanol, reflux for 19 h.

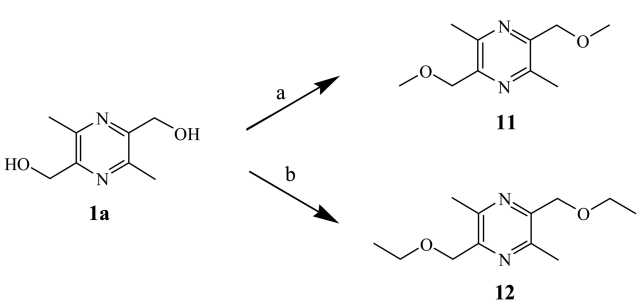
2.2. Cardiac function screening

The cardiac contractility and safety of LZDO derivatives were evaluated on the myocardium in normal isolated rat hearts with LZDO (**1a**) as a positive control. "Control" corresponded to healthy rat cardiac performance without any drug (Table).
Compared to control, LZDO derivatives **3a-b**, **4a-b**, **13**, **15**, **16**, and **19** exhibited better positive inotropic effects and diastolic function on the myocardium without the risk of arrhythmia. The increased rate in left ventricular developed pressure (LVDP, accepted as contractile force), the maximum rate of increase of left ventricular pressure (+dP/dt_{max}, used as an index of contractility), the maximum rate of pressure decrease of the left ventricle (-dP/dt_{min}, used as an index of diastolic function) and the heart rate (HR, used as an index of heart muscle oxygen consumption) of these compounds vs. control are listed in the Table. Compounds **10–12**, **17**, and **18** were found to induce severe arrhythmia (Fig. 2), while **14** had no significant effect on

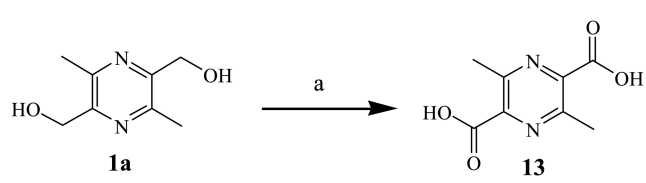
Table 1: Effects of LZDO derivatives on cardiac performance in isolated Langendorff-perfused rat hearts (mean ± SD, n = 6).

Compd.	Group (μM)	LVDP (mmHg)	Rate of change %	+ dP/dt_{max} (mmHg/s)	Rate of change %	-dP/dt_{max} (mmHg/s)	Rate of change %	HR (beat·min^{-1})
1a	control	50 ± 2.8		1,685 ± 147		−1,058 ± 81		183 ± 22
	1	60 ± 5[b]	19.4 ± 11.0	1,836 ± 121	9.4 ± 8.1	−1,175 ± 125	11.1 ± 10.0	186 ± 29
	10	73 ± 4[bd]	45.6 ± 9.0	2,125 ± 242[bc]	26.5 ± 13.4	−1,371 ± 186[bc]	30.3 ± 0.3	209 ± 21
	100	88 ± 3[bdf]	76.3 ± 12.3	2,702 ± 287[bdf]	60.9 ± 17.3	−1,911 ± 200[bdf]	82.0 ± 27.9	193 ± 15
3a	control	49 ± 2.6		1,602 ± 113		−1,021 ± 32		197 ± 17
	1	53 ± 3[b]	8.8 ± 7.3	1,787 ± 270	11.9 ± 17.9	−1,047 ± 41	2.8 ± 5.8	203 ± 13
	10	60 ± 1.6[bd]	21.8 ± 7.0	1,972 ± 271[b]	24.0 ± 21.7	−1,351 ± 140[bd]	32.7 ± 16.1	213 ± 11
	100	67 ± 1.3[bdf]	35.8 ± 8.0	1,960 ± 69[b]	22.9 ± 11.6	−1,297 ± 46[bd]	27.2 ± 5.8	210 ± 21
3b	control	52 ± 1.0		1,737 ± 75		−1,230 ± 93		202 ± 12
	1	55 ± 0.8[b]	5.9 ± 2.2	1,889 ± 14[a]	8.9 ± 4.6	−1,409 ± 60[b]	15.0 ± 7.6	197 ± 8
	10	58 ± 2.1[bd]	12.4 ± 4.3	2,021 ± 130[bc]	16.5 ± 9.7	−1,491 ± 41[b]	21.8 ± 10.0	203 ± 15
	100	67 ± 2.1[bdf]	29.8 ± 4.4	2,230 ± 151[bdf]	28.6 ± 10.2	−1,633 ± 114[bdf]	33.3 ± 13.4	189 ± 32
4a	control	54 ± 2.8		1,749 ± 93		−975 ± 38		203 ± 23
	1	59 ± 3.3[a]	8.3 ± 7.6	1,807 ± 131	3.3 ± 3.5	−1,152 ± 41[b]	18.3 ± 5.4	204 ± 26
	10	63 ± 2.2[bc]	11.8 ± 8.1	1,824 ± 131	3.7 ± 4.1	−1,267 ± 76[bd]	22.4 ± 9.6	198 ± 33
	100	68 ± 4[bde]	15.5 ± 10.7	1,972 ± 122[bce]	6.2 ± 6.1	−1,327 ± 82[bd]	25.7 ± 11.7	206 ± 49
4b	control	53 ± 1.4		1,636 ± 22		−1,064 ± 128		183 ± 15
	1	57 ± 2.6[a]	6.2 ± 2.7	1,735 ± 91[a]	6.0 ± 5.6	−1,106 ± 100	4.3 ± 4.1	186 ± 10
	10	62 ± 2.5[bd]	15.7 ± 2.9	1,818 ± 69[b]	11.2 ± 5.3	−1,202 ± 85[a]	13.7 ± 8.2	189 ± 18
	100	69 ± 2.0[bdf]	28.8 ± 4.6	1,960 ± 76 [bdf]	19.8 ± 5.2	−1,275 ± 71[bd]	21.3 ± 6.1	179 ± 19
13	control	54 ± 0.5		1,650 ± 31		−1,020 ± 38		198 ± 13
	1	57 ± 0.8[b]	5.7 ± 0.9	1,683 ± 39	2.0 ± 0.6	−1,100 ± 71[a]	7.8 ± 5.2	202 ± 16
	10	62 ± 0.9[bd]	15.3 ± 0.9	1,788 ± 21[bd]	8.4 ± 1.0	−1,182 ± 73[bc]	15.8 ± 3.9	199 ± 16
	100	66 ± 0.8[bdf]	23.4 ± 1.0	1,847 ± 41[bdf]	12.0 ± 2.0	−1,256 ± 59[bd]	23.1 ± 3.4	205 ± 13
15	control	51 ± 0.7		1,857 ± 225		−1,123 ± 86		222 ± 22
	1	55 ± 1.5[b]	9.1 ± 3.3	2,039 ± 102	10.9 ± 11.1	−1,153 ± 51	2.9 ± 3.7	212 ± 32
	10	58 ± 1.9[bd]	15.3 ± 4.4	2,029 ± 187	10.0 ± 10.7	−1,360 ± 72[bd]	21.7 ± 11.5	221 ± 34
	100	62 ± 1.0[bdf]	22.9 ± 3.5	2,097 ± 96[a]	14.2 ± 13.2	−1,375 ± 29[bd]	23.0 ± 8.4	219 ± 41
16	control	51 ± 2.3		1,898 ± 271		−1,140 ± 149		222 ± 16
	1	57 ± 2.1[b]	13.4 ± 8.0	2,015 ± 241	6.6 ± 5.9	−1,279 ± 162	12.5 ± 9.6	217 ± 49
	10	61 ± 3[bd]	21.9 ± 10.9	2,067 ± 108	10.9 ± 17.9	−1,356 ± 75[a]	20.5 ± 15.8	217 ± 33
	100	65 ± 2.3[bde]	29.5 ± 6.6	2,195 ± 206[a]	18.3 ± 24.7	−1,410 ± 142[b]	26.3 ± 25.8	215 ± 15
19	control	50 ± 0.7		2,045 ± 118		−1,192 ± 109		215 ± 22
	1	55 ± 0.8[b]	9.2 ± 1.9	2,144 ± 183	4.7 ± 4.2	−1,260 ± 115	5.8 ± 3.1	202 ± 29
	10	63 ± 1.2[bd]	25.0 ± 3.2	2,239 ± 108[a]	9.8 ± 7.7	−1,433 ± 61[bd]	21.2 ± 11.7	225 ± 23
	100	67 ± 1.1[bdf]	33.6 ± 2.6	2,543 ± 39[bdf]	24.7 ± 8.0	−1,550 ± 31[bde]	31.1 ± 14.1	213 ± 11

Each value is mean ± SD of 6 experiments. [a]$P < 0.05$, [b]$P < 0.01$ significantly different from control; [c]$P < 0.05$, [d]$P < 0.01$ significantly different from 1 μM; [e]$P < 0.05$, [f]$P < 0.01$ significantly different from 10 μM.



Scheme 4: Synthesis of LZDO ether derivatives **11**, **12**. Reagents and conditions: (a) thionyl chloride, CH_2Cl_2, sodium methoxide, 0 °C, 2.5 h; (b) thionyl chloride, CH_2Cl_2, sodium ethoxide, 0 °C, 2.5 h.



Scheme 5: Synthesis of LZDO derivative **13.** Reagents and conditions: (a) saturated sodium hypochlorite solution, 60 °C, 5 h.

cardiac performance at various concentrations (1, 10, 100 μM) (data not shown).

As shown in the Table, all the compounds had no significant effect on HR. However, the results showed that the positive control LZDO (**1a**) was the most potent compound concerning positive inotropic effect on the myocardium. It significantly increased LVDP increased + dP/dt_{max} and increased -dP/dt_{max}.

In the series of ester derivatives, compounds **3a-b** were more potent than compounds **4a-b.** Specifically, **3a** was the most potent for a positive inotropic effect on the myocardium.

SAR studies showed that as the size of the acyl side chain increased, the positive inotropic activity decreased. The positive inotropic effect was reduced when LZDO was esterified with a series of acyl groups, such as acetyl, propionyl, butyl and benzoyl. Introduction of an aliphatic carbon chain, however, seemed to be more favourable than an aromatic carbon chain. The fact that the increased cardiac performance of **4a** (100 μM) *vs.* control was reduced to 15.5 ± 10.7 %, 6.2 ± 6.1 %, and 25.7 ± 11.7 % for LVDP, + dP/dt_{max}, and -dP/dt_{min}, respectively, further confirmed that introduction of an electron-withdrawing group (-Cl) in the benzene ring reduced the activity, while introduction of an electron-donating group (-CH_3) in the benzene ring did not significantly affect activity.

The ether derivatives **11** and **12** were found to induce severe arrhythmia, while the acid derivative **13** increased LVDP, increased + dP/dt_{max} and, increased -dP/dt_{max} (Table). This showed that the hydroxyl group in LZDO was very important for positive inotropic activity.

In the hydroxyl substituted series, activity of the *holo*-substituted derivative **19** was lower than that of the *para*-dihydroxy derivative (Table) (**1a**). On the other hand, the *ortho*-dihydroxy substituted derivative **10**, the *mono*-substituted derivative **17** and the *tri*-substituted derivative **18** all induced severe arrhythmia. The results suggested that introduction of *para*-dihydroxy and *tetra*-hydroxy groups provided positive inotropic effects on the myocardium, and introduction of *para*-dihydroxy groups in LZDO seemed to be more favourable than *tetra*-hydroxy substituted derivatives.

3. Discussion

The preliminary structure activity relationships of LZDO for positive inotropic effects showed that the *para*-dihydroxy in LZDO was necessary for its activity, and the pharmacophore of positive inotropic effect might be located at the whole scaffold of LZDO, but not the pyrazine ring. Moreover, the scaffold of **1a** was different from the structure of cardiotonic agents in the market, we surmised that the whole scaffold of LZDO may provide an important clue of the pharmacophore for the development of novel cardiotonic agents. This study could improve the scientific process towards the development of used cardiotonic agents.

4. Experimental

4.1. General procedures

The purity of LZDO was more than 99.9%. All commercial reagents and solvents were used without further purification unless otherwise specified. Melting points were determined using model BUCHI B-450. ESI-MS was recorded on a TOF mass spectrometer (Applies-Biosystem Mariner). UV spectra were recorded on a Beckman DU640 spectrophotometer. IR spectra were acquired by using a THERMO IR spectrometer Model Nicolet-100. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance 300 (300 MHz ^{1}H, 75 MHz ^{13}C) or a Bruker Advance 500 (500 MHz ^{1}H, 125 MHz ^{13}C) spectrophotometer. Mass spectra were recorded on a ZAB-HS mass spectrometer as the value m/z. HPLC analysis was conducted on a Waters 2695 HPLC instrument equipped with a UV detector (model 2489). All compounds were analysed by HPLC, and their purity was confirmed to be greater than 98.0%. The positive inotropic effect on the myocardium of LZDO derivatives *in vitro* was evaluated on Langendorff-perfused hearts isolated from normal SD rats.

4.2. Chemistry

*4.2.1. 2,5-Dipropionyloxymethyl-3,6-dimethylpyrazine (**3a**).*

Compound **2** (16.8 g, 0.10 mol) was treated with propionic anhydride (200 mL, 0.32 mol) at 120 °C for 8 h. Reaction progress was monitored by TLC. After reaction completion, the excess propionic anhydride was evaporated. The residue was purified by silica gel column chromatography (petroleum: ethyl acetate = 4: 1 v/v) to give **3a** as a yellowish oil (5.18 g, 18.5% yield, HPLC > 98%). ESI-MS (M + H$^+$) calcd for $C_{14}H_{21}N_2O_4$ 281.1499, found 281.1500; UV max (methanol): 212, 277 nm; IR (KBr) cm^{-1}: 2968 (υ_{CH}), 1743 ($\upsilon_{C=O}$), 1454, 1367, 1273 (δ_{CH}), 1080 (υ_{C-O}); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 5.28 (s, 4H, 2 × CH_2-O), 2.56 (s, 6H, 2 × CH_3), 2.39 (q, 4H, J = 9.7 Hz, 2 × CH_2-C = O), 1.17 (t, 6H, J = 9.7 Hz, 2 × CH_2-C$\underline{H}_3$) ppm; ^{13}C NMR (DMSO-D6, 125 MHz) δ: 173.9 (C = O), 149.4, 147.4 (C = N), 64.6 (C-O), 27.4 (C-C = O), 20.5 (CH_3), 8.99 (CH_2-CH_3) ppm; EI-MS *m/z* (%): 280.2 (1.07, M), 223.2 (35.98, M-O = C-CH_2-CH_3), 167.1 (100, M + H -2 × O = C$^+$-CH_2-CH_3), 57.0 (85.57, O = C$^+$-CH_2-CH_3).

*4.2.2. 2,5-Dibutyryloxymethyl-3,6-dimethylpyrazine (**3b**).*

Compound **2** (16.8 g, 0.10 mol) was treated with butyric anhydride (200 mL, 0.32 mol) at 150 °C for 7 h. Reaction progress was monitored by TLC. After reaction completion, the excess propionic anhydride was evaporated. The residue was purified by silica gel column chromatography (petroleum: ethyl acetate = 4: 1 v/v) to give **3b** as a yellowish oil (5.48 g, 17.8% yield, HPLC > 98%). ESI-MS (M + H$^+$) calcd for $C_{16}H_{25}N_2O_4$ 309.1808, found 309.1873; UV max (methanol): 279 nm; IR (KBr) cm^{-1}: 2968 (υ_{CH}), 1714 ($\upsilon_{C=O}$), 1097 (υ_{C-O}), 1461, 1417, 1295 (δ_{CH}); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 5.22 (s, 4H, 2 × CH_2-O), 2.69 (s, 6H, 2 × CH_3), 2.35 (t, 4H, J = 8.1 Hz, 2 × CH_2-C = O), 1.68 (m, 4H, 2 × C$\underline{H}_2$-CH_3), 0.97 (t, 6H, J = 7.4 Hz, 2 × CH_2-C$\underline{H}_3$) ppm; ^{13}C NMR (DMSO-D6, 125 MHz) δ: 179.2 (C = O), 149.8, 139.9 (C = N), 64.5 (C-O), 35.8 (C-C = O), 20.5 (CH_3), 13.6 (CH_2-CH_3) ppm; EI-MS *m/z* (%): 308.3 (0.72, M), 237.2 (36.34, M-O = C-CH_2-CH_2-CH_3), 167.1 (100, M + H -2 × O = C-CH_2-CH_2-CH_3), 71.1 (51.58, O = $^+$C-CH_2-CH_2-CH_3).

*4.2.3. 2,5-Di-p-chlorobenzoyloxymethyl-3,6-dimethylpyrazine (**4a**).*

p-Chlorobenzoyl chloride (200 mL) was slowly added into **2** (16.8 g, 0.10 mol) stirred at 60 °C for 5 h. Reaction progress was monitored by TLC. After completion, the reaction mixture was washed with saturated sodium bicarbonate solution (3 × 100 mL), extracted with ethyl acetate, dried with anhydrous sodium sulphate, filtered, and concentrated under vacuum. The residue was purified by silica gel column chromatography (petroleum: ethyl acetate = 3: 1 v/v), affording **4b** as white needle crystals (13.12 g, 29.5% yield, HPLC > 98%). mp 178–180 °C; ESI-MS (M + H$^+$) $C_{22}H_{19}Cl_2N_2O_4$ 445.6897, found 445.9312; UV max (methanol): 276 nm; IR (KBr) cm^{-1}: 3047, 2970, 2947, 2858 (υ_{CH}), 1724 ($\upsilon_{O=C}$), 1590 ($\upsilon_{C=C}$), 1429 (δ_{CH}), 1208 (υ_{C-N}), 1133 (υ_{C-C}), 887, 825 ($\delta_{=CH}$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.96 (d, 4H, J = 6.4 Hz, 2 × Ph-H-2,6), 7.61 (d, 4H, J = 6.4 Hz, 2 × Ph-H-3,5), 5.63 (s, 4H, 2 × CH_2-O), 2.75 (s, 6H, 2 × CH_3) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 166.0 (C = O), 149.7, 147.5, 138.7 (C = N), 128.8 (Ph-C-1), 131.3 (Ph-C-2,6), 128.7 (Ph-C-3,5), 64.1 (C-O), 19.8 (CH_3) ppm; EI-MS *m/z*: 445 (M), 306 (M + H$^+$-Cl-Ph-C = O), 168 (M + 2H$^+$-2 × Cl-Ph-C = O), 140 (Cl-Ph-C = O).

*4.2.4. 2,5-Di-p-methylbenzoyloxymethyl-3,6-dimethylpyrazine (**4b**).*

p-Methylbenzoyl chloride (200 mL) was slowly added to **2** (16.8 g, 0.10 mol) and stirred at 80 °C for 4 h. Reaction progress was monitored by TLC. After completion, the reaction mixture was washed with saturated sodium bicarbonate solution, extracted with ethyl acetate, dried with anhydrous sodium sulphate, filtered and concentrated under vacuum. The residue was purified by silica gel column chromatography (petroleum: ethyl acetate = 3: 1 v/v) to afford **4c** as white needle crystals (14.46 g, 35.8% yield, HPLC > 98%). mp 175–177 °C; ESI-MS (M + H$^+$) calcd for $C_{24}H_{25}N_2O_4$ 405.1409, found 405.1366; UV max (methanol): 298 nm; IR (KBr) cm^{-1}: 2906, 2903 (υ_{CH}), 1733 ($\upsilon_{O=C}$), 1664 ($\upsilon_{C=C}$), 1430 (δ_{CH}), 1290 (υ_{C-N}), 1208 (υ_{C-O}), 1133 (υ_{C-C}), 888, 840 ($\delta_{=CH}$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.93 (d, 4H, J = 7.4 Hz, 2 × Ph-H-2,6), 7.34 (d, 4H, J = 7.4 Hz, 2 × Ph-H-3,5), 5.63 (s, 4H, 2 × CH_2-O), 2.75 (s, 6H, 2 × CH_3), 2.35 (s, 6H, 2 × CH_3) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 166.0 (C = O), 149.8, 147.5, 142.5, 127.1 (C = N), 129.8 (Ph-C-2,6), 128.9 (Ph-C-3,5), 64.1 (C-O), 21.3 (Ph-CH_3), 19.8 (Py-CH_3) ppm; EI-MS *m/z*: 404 (M), 286 (M + H$^+$-CH_3-Ph-C = O), 168 (M + 2H$^+$-2 × CH_3-Ph-C = O), 119 (CH_3-Ph-C = O).

*4.2.5. 2,3-Dimethylquinoxaline (**7**).*

Butanedione (**5,**1.72 g, 20 mmol) was dissolved in H_2O (20 mL). To this solution, *o*-phenylene diamine (**6,**2.38 g, 22 mmol) and CAN (0.434 g, 1 mmol) were added and a white precipitate formed immediately. The reaction mixture was then stirred for 1 h at room temperature. Reaction progress was monitored by TLC. After completion, the reaction was filtered and the residue washed with H_2O (3 × 20 mL) to afford **7** as a white solid (2.9 g, 91.0% yield, HPLC > 98%). mp 106–107 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.96 (s, 2H), 7.85 (s, 2H), 2.71 (s, 6H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 153.1 (Py-C-2,5), 140.7 (Py-C-3,6), 128.5, 128.0 (C = C), 22.9 (CH_3) ppm.

*4.2.6. 2,3-Dicarboxyl-5,6-dimethylpyrazine (**8**).*

Compound **7** (1.58 g, 10 mmol) was dissolved in H_2O (40 mL) at 90 °C and a saturated $KMnO_4$ solution (9.48 g of $KMnO_4$ in 150 mL H_2O) was added dropwise over 1.5–2.0 h. The reaction was filtered while hot and the filtrate was concentrated under vacuum and treated with concentrated hydrochloric acid. The acid solution was then evaporated completely under high vacuum, followed by the addition of isopropanol. The mixture was finally filtered and concentrated. The residue was purified by silica gel column chromatography in petroleum/ethyl acetate (1: 3), affording **8** as a white powder (0.75 g, 38% yield, HPLC > 98%). mp 173–174 °C. ESI-MS (M-H$^+$) *m/z*: 195.17. ^{1}H NMR (CD_3OD, 300 MHz) δ: 5.09 (s, 2H), 2.24 (s, 6H) ppm. ^{13}C NMR (CD_3OD, 75 MHz) δ: 168.0 (C = O), 156.0, 142.9 (C = N), 21.9 (CH_3) ppm.

*4.2.7. 5,6-Dimethylpyrazine-2,3-diformate (**9**).*

Concentrated sulphuric acid (3 mL) was added dropwise into a solution of **8** (2.00 g, 10 mmol) in methanol (30 mL) at 0 °C and then refluxed for 20 h. Reaction progress was monitored by TLC. After completion, the solution was cooled to room temperature, evaporated under vacuum, adjusted to pH 8 with saturated sodium bicarbonate, and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and evaporated under vacuum. The residue was purified by silica gel column chromatography in petroleum/ethyl acetate (4: 1) to afford **9** as a white solid (0.97 g, 43% yeild, HPLC purity of > 98%). mp 55–58 °C. ESI-MS ($M + Na^+$) *m/z*: 247.25. ^{1}H NMR ($(CD_3)_2CO$, 300 MHz) δ: 3.94 (s, 6H), 2.06 (s, 6H) ppm. ^{13}C NMR ($(CD_3)_2CO$, 75 MHz) δ: 166.9 (C = O), 156.7, 143.1 (C = N), 54.1 (C-O), 23.0 (CH_3) ppm.

*4.2.8. 2,3-Dihydroxymethyl-5,6-dimethylpyrazine (**10**).*

Sodium borohydride (0.45 g, 12 mmol) was added into a solution of **15** (0.45 g, 2 mmol) in dehydrated ethanol (20 mL) at 0 °C. After completion of the addition, the mixture was refluxed for 19 h. Reaction progress was monitored by TLC. After completion, the solution was cooled and evaporated under vacuum. The residue was purified by silica gel column chromatography in CH_2Cl_2/CH_3OH (3: 1), affording **19** as a yellowish solid (0.15 g, 44% yeild, HPLC purity of > 98%). mp 119–120 °C. ESI-MS ($M + H^+$) *m/z*: 169.17. UV max (methanol): 279.3 nm. IR (KBr) cm^{-1}: 3221 (OH), 2918, 2847 (CH_3), 1420 (C = N), 1177 (C-O). ^{1}H NMR (CD_3OD, 300 MHz) δ: 4.71 (s, 4H), 2.48 (s, 6H) ppm. ^{13}C NMR (CD_3OD, 75 MHz) δ: 151.5, 150.7 (C = N), 63.1 (C-O), 21.3 (CH_3) ppm.

*4.2.9. 2,5-Dimethoxymethyl-3,6-dimethylpyrazine (**11**).*

Compound **1a** (16.8 g, 0.10 mol) was treated with thionyl chloride (15 mL, 0.20 mol) in anhydrous CH_2Cl_2 (300 mL) at 0 °C for 2.5 h. Reaction progress was monitored by TLC. After completion, the solvent was evaporated under vacuum and the crude product was dissolved in anhydrous diethyl ether (10 mL). To this, sodium methoxide (20 mL) was added dropwise with stirring. After completion of the addition, the mixture was stirred for an additional 1 h. Water (30 mL) was added into the reaction mixture and extracted with diethyl ether (3 × 50 mL). The combined extracts were washed with water and sodium chloride solution, dried over anhydrous sodium sulphate, filtered, and concentrated under vacuum to give **11** as yellow needle crystals (11.5 g, 58.7%). mp 112–113 °C. ESI-MS ($M + H^+$) calcd for $C_{10}H_{17}N_2O_2$ 197.1285, found 197.1310. UV max (methanol): 278 nm. IR (KBr) cm^{-1}: 2953 (ν_{CH}), 1628, 1454 ($\nu_{C=C}$), 1102 ($\nu_{C\text{-}O}$). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 4.58 (s, 4H, 2 × CH_2-O), 3.44 (s, 6H, 2 × O-CH_3), 2.59 (s, 6H, 2 × CH_3) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 149.7, 147.4 (C = N), 73.9 (CH_2-O), 58.9 (O-CH_3), 20.1 (CH_3) ppm. EI-MS *m/z* (%): 196.1 (2.52, M), 182.1 (36.34, $M + H^+$-CH_3), 168.1 (100, $M + 2H^+$-2 × CH_3).

*4.2.10. 2,5-Diethyloxymethyl-3,6-dimethylpyrazine (**12**).*

Compound **1a** (16.8 g, 0.10 mol) was treated with thionyl chloride (15 mL, 0.20 mol) in anhydrous CH_2Cl_2 (300 mL) at 0 °C for 2.5 h. Reaction progress was monitored by TLC. After completion, the solvent was evaporated under vacuum and the crude product was dissolved in anhydrous diethyl ether (10 mL). To this, sodium ethoxide (20 mL) was added dropwise with stirring. After completion, water (30 mL) was added and the mixture was extracted with ethyl ether (3 × 50 mL). The extract was washed with water and sodium chloride solution, dried over anhydrous sodium sulphate, filtered, and concentrated under vacuum to give **12** as yellow needle crystals (13.5 g, 60.1%). mp 115–116 °C. ESI-MS ($M + Na^+$) calcd for $C_{12}H_{20}N_2O_2{\cdot}Na^+$ 247.1498, found 247.1452. UV max (methanol): 278 nm. IR (KBr) cm^{-1}: 2926 (ν_{CH}), 1642, 1451 ($\nu_{C=C}$), 1101 ($\nu_{C\text{-}O}$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 4.61 (s, 4H, 2 × CH_2-O), 3.60 (q, 4H, J = 7.0 Hz, 2 × $C\underline{H}_2$-CH_3), 2.59 (s, 6H, 2 × CH_3), 1.24 (t, 6H, J = 7.0 Hz, 2 × CH_2-$C\underline{H}_3$) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 149.7, 147.4 (C = N), 71.4 (C-O), 65.5 (CH_2), 20.1 (CH_3), 15.2 (CH_2-CH_3) ppm. EI-MS *m/z* (%): 224.2 (0.72, M) 196.2 (26.87, $M + H^+$-CH_2-CH_3), 168.2 ($M + 2H^+$-2 × CH_2-CH_3).

*4.2.11. 3,6-Dimethylpyrazine-2,5-dicarboxylic acid (**13**).*

Compound **1a** (16.8 g, 0.10 mol) was treated with saturated sodium hypochlorite solution (150 mL) at 60 °C for 5 h. Reaction progress was monitored by TLC. Methanol was added to the reaction mixture, filtered, and the filtrate was concentrated under vacuum. The residue was recrystallised from ethanol-water to obtain **13** as white needle crystals (16.64 g, 84.9%). mp 124–126 °C. ESI-MS ($M + H^+$) calcd for $C_8H_9N_2O_4$ 197.172, found 197.1318. UV max (methanol): 223, 279 nm. IR (KBr) cm^{-1}: 3586 (υ_{OH}). 2931, 2813 ($\upsilon_{C\text{-}H}$). 1425 (δ_{CH}). 1223 ($\upsilon_{C\text{-}O}$). 1103 ($\upsilon_{C\text{-}N}$). 920, 884 (δ_{CH}). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 12.20 (s, 2H, 2 × COOH), 2.27 (s, 6H, 2 × CH_3) ppm. ^{13}CNMR ($CDCl_3$, 75 MHz) δ: 168.3 (COOH), 154.9, 145.9 (C = N), 22.8 (CH_3) ppm. EI-MS *m/z*: 196 (M), 168.1 (M-OH), 151.1 (M-2 × OH), 108.1 (M-2 × COOH).

4.3. Ex vivo Langendorff-perfused rat heart

Sprague-Dawley adult male rats weighing 260–320 g were anaesthetised by intraperitoneal administration of 20% urethane (1.2 g•kg^{-1}). The hearts were quickly removed and placed into an ice-cold modified buffer until contractions ceased. The heart was cleaned of surrounding tissues, mounted on the aortic cannula of the Langendorff perfusion system apparatus *(Yang et al. 1993),* and perfused with oxygenated buffer (mM): NaCl 117, KCl 5.7, $CaCl_2$ 1.8, $MgCl_2$ 1.7, $NaHCO_3$ 4.4, NaH_2PO_4 1.5, HEPES 20 and glucose 11. The buffer was equilibrated with 95% O_2 and 5% CO_2 and adjusted to pH 7.4 with NaOH before it entered the heart. An epicardial electrogram was registered using two stainless steel electrodes, one attached to the apex of the heart and the other to the aortic cannula. To obtain an isovolumetrically beating preparation, a latex balloon filled with water and connected by a catheter to a transducer was inserted through the left atrium into the left ventricle and inflated to provide an end-diastolic pressure between 8 and 12 mmHg. Before each experimental protocol was initiated, the isolated hearts were set at a mean arterial pressure of 60–80 mmHg and allowed to stabilise at 37 °C for 40–60 min. The isolated rat hearts were then perfused in sequence with 1, 10, and 100 μM of the LZDO derivatives in each group. A RM6240B/C four channel physiological recording instrument was used for continuous recording of LVSP, LVEDP, HR (derived from electrogram), + dP/dt_{max} and -dP/dt_{max} throughout the experiments. The criteria for established stability were LVDP > 50 mmHg, + dP/dt_{max} > 1,600 mmHg s^{-1}, heart rate > 180 beats min^{-1} and normal sinus rhythm. The hearts were not studied if they had weak contractility (+dP/dt_{max} < 1,600 mmHg s^{-1}) and severe arrhythmia. Diastolic balloon pressure was maintained at 8 mmHg. LVDP was calculated as the difference between the systolic and diastolic pressures. The results are expressed as the mean value of hemodynamic variables (n = 6).

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