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Design, synthesis and evaluation of new ligustrazine derivatives as potential plasma-stable neuroprotective agents†‡

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A series of ligustrazine–phenolic acid esters which exhibited promising neuroprotective activities have previously been reported. Nevertheless, we found that these ester compounds (like T-VA) were not stable in plasma by further *in vivo* studies. To investigate plasma-stable neuroprotective agents, a series of new ligustrazine derivatives were synthesized by conjoining ligustrazine and phenols with ester, ether and amide bonds. Most of the compounds exhibited higher protective effects against CoCl₂-induced neurotoxicity in differentiated PC12 cells than ligustrazine. Structure–activity relationships were also briefly discussed. We found that compound 2c (2-((2-methoxy-4-(((3,5,6-trimethylpyrazin-2-yl)methoxy) methyl)phenoxy)-methyl)-3,5,6-trimethylpyrazine) displayed the highest protective effect on the PC12 cells damaged by CoCl₂ (EC₅₀ = 1.07 μM). Preliminary stability investigation in rat plasma was verified *in vitro* and better plasma stability was observed with 2c in comparison to T-VA.

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Introduction

Stroke is one of the most devastating diseases and a main cause of mortality in China as well as worldwide.¹ Most strokes are ischemic and the disease relates to both the cerebrovascular system and cranial nerves.² Despite the remarkable progress achieved in the therapy of stroke during the last two decades, there are no effective chemotherapy for cerebral ischemic stroke and neuroprotective agents that could attenuate lots of the clinical problems of ischemic stroke.^{3,4} This may explain the growing interest in discovering drugs led by traditional medicine.⁵

Ligustrazine, also known as 2,3,5,6-tetramethylpyrazine (TMP), is one of the most important active ingredients of the traditional Chinese medicinal herb *Ligusticum wallichii*.^{6,7} Previous studies have shown various pharmacological activities of this compound, such as calcium antagonism, free radical scavenging and antioxidant activity.^{8–11} To further improve TMP's neuroprotective properties, a series of novel ligustrazine–phenolic acid derivatives have been designed and synthesized based on several neuroprotective ingredients

from Chinese traditional medicinal herbs as starting materials.^{12–14} The results showed that the ligustrazine derivatives presented neuroprotective effects on injured differentiated PC12 cells, of which T-VA (C₂₄H₂₈N₄O₄) (Fig. 1) displayed promising protective effects (EC₅₀ = 4.25 μM).¹² In our previous study, T-VA also exhibited neuroprotective effects on a rat model of ischemic stroke with concomitant improvement of motor functions.¹⁵

However, because of carboxyl groups in phenolic acids and cinnamic acids, all these ligustrazine derivatives have ester bonds.¹⁶ To enrich the types of ligustrazine derivatives, twenty-one compounds were synthesized by conjoining ligustrazine and phenols with ester, ether and amide bonds. Their neuroprotective activities were evaluated on injured PC12 cells by thiazole blue (MTT) assay, and 2c (Fig. 1) displayed the highest protective effect. The structure–activity relationships (SARs) of these novel compounds are also briefly discussed.

Compounds with ester or amide bonds like T-VA are liable to enzymatic hydrolysis *in vivo* such as the action of plasma esterases.^{17–19} Hence, plasma stability is a very important issue in drug development. It has a direct impact on drug

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Fig. 1 The structures of TMP, T-VA and 2c.

performance *in vivo* and serves as the major limiting factor for drug candidate screening at an early stage of preclinical studies.^{20,21} Therefore, to verify the potential druggability of 2c, compared with T-VA, its plasma stability in rat plasma was preliminarily evaluated *in vitro*.

Results and discussion

Chemistry

As important intermediates, 2-(chloromethyl)-3,5,6-trimethylpyrazine (1) and 3,5,6-trimethylpyrazine-2-carboxylic acid (2) were prepared according to Schemes 1 and 2 (details shown in the ESI†). All the designed derivatives were synthesized *via* the routes outlined in Schemes 3–5.

As shown in Scheme 3, compounds 1a–1c and 2a–2c were synthesized by alkylation of 4-hydroxybenzyl alcohol, 4-hydroxyphenethyl alcohol and 4-hydroxy-3-methoxybenzyl alcohol with intermediate 1, respectively. The single-step coupling reaction between intermediate 2 and phenol amines was performed using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole (HOBT) and *N,N*-diisopropylethylamine (DIPEA) in anhydrous *N,N*-dimethylformamide (DMF), and then we obtained the ligustrazine derivatives (3a–3c, as shown in route i of Scheme 4). Subsequently, typical synthetic procedures for 4a–4c involved the combination of 3a–3c and chloro-TMP through the formation of ether bonds under alkaline conditions. Compounds 3a–3c were reacted with intermediate 2 under catalysis by EDCI and DMAP, respectively; we thus successfully obtained compounds 5a–5c (route iii of Scheme 4). As shown in Scheme 5, 4-hydroxybenzyl alcohol, 4-hydroxyphenethyl alcohol and 4-hydroxy-3-methoxybenzyl alcohol were treated with different proportions of intermediate 2 and catalyzed by EDCI and DMAP in CH₂Cl₂ to afford the corresponding ligustrazine derivatives (6a–6c, 7a–7c). The structures of the entire target compounds (Table 1) were confirmed by ¹H-NMR, ¹³C-NMR and high resolution mass spectrometry (HRMS). All of the compounds were new compounds and none of the compounds' neuroprotective activities have been explored.

Protective effect on injured neuronal-like PC12 cells

PC12 is a cell line derived from a rat adrenal medulla pheochromocytoma. Differentiated PC12 cells induced by nerve growth factor (NGF) have the typical characteristics of neurons in form and function; therefore, they are widely used as a model for *in vitro* neuron research.²² Cobalt chloride

Scheme 1 Synthesis of 2-(chloromethyl)-3,5,6-trimethylpyrazine. Reagents and conditions: (i) AcOH, H₂O₂ (30%), 90 °C, 4 h; (ii) Ac₂O, reflux, 105 °C, 2.5 h; (iii) NaOH, THF:MeOH:H₂O, r.t., 1 h; (iv) SOCl₂, CH₂Cl₂, 0 °C, 2.5 h; KOH, C₂H₅OH.

Scheme 2 Synthesis of 3,5,6-trimethylpyrazine-2-carboxylic acid. Reagents and conditions: (i) KMnO₄, 55 °C, 24 h.

(CoCl₂), a water-soluble compound, was used in this investigation because it is one of the best-known chemical inducers of PC12 injury; CoCl₂-induced neurotoxicity in differentiated PC12 cells is commonly used to screen new candidates for the intervention of stroke.^{23–25} All the synthesized compounds were tested for their protective effects on neuronal-like PC12 cells damaged by CoCl₂, and TMP was used as the positive control drug. This revealed the proliferation rates (%) at different concentrations and 50% effective concentrations (EC₅₀) for protecting damaged PC12 cells of the ligustrazine–phenol derivatives shown in Fig. 2.

The results showed that TMP and its derivatives presented protective effects on injured differentiated PC12 cells and most of the ligustrazine derivatives were more active (with lower EC₅₀ values) than TMP (EC₅₀ = 64.46 μM).

To enrich the types of ligustrazine derivatives, 21 new ligustrazine derivatives were synthesized by conjoining ligustrazine and phenols with ester, ether and amide bonds. Interestingly, derivatives containing ether bonds like 1a–1c and 2a–2c exhibited high potency, with EC₅₀ values below 15 μM; among them, 2c was the most active compound, with an EC₅₀ value of 1.07 μM. Furthermore, we found that these ether-conjoined derivatives containing bis-ligustrazine substituents showed better neuroprotective activities than the single-ligustrazine compounds, such as 2a > 1a, 2b > 1b, and 2c > 1c. Based on the above evidence, the additive methoxy moiety in 1c and 2c might enhance the protective activity, such as 1c > 1a > 1b and 2c > 2a > 2b. This structure–activity relationship analysis was in agreement with previous studies that the ether derivatives showed better protective effects than the ester derivatives.^{12,13}

However, most of the ligustrazine–phenol ester and amide derivatives (3b–3c, 4a–4c, 5a–5c, 6b, and 7a–7c) showed lower neuroprotective activity than the ligustrazine–phenol ether derivatives. Meanwhile, it was observed that amide-joined

Scheme 3 Synthetic routes to ligustrazine derivatives 1a–1c and 2a–2c. Reagents and conditions: (i) DMF, K₂CO₃, 85 °C, 4 h; (ii) DMF, NaH, 80 °C, 4 h.

Scheme 4 Synthetic routes to ligustrazine derivatives **3a–3c**, **4a–4c** and **5a–5c**. Reagents and conditions: (i) DMF, EDCI, HOBT, DIPEA, r.t., 12 h; (ii) DMF, K₂CO₃, 85 °C, 4 h; (iii) DMF, EDCI, DMAP, r.t., 12 h.

Scheme 5 Synthetic route to ligustrazine derivatives **6a–6c** and **7a–7c**. Reagents and conditions: (i) CH₂Cl₂, EDCI, DMAP, r.t., 12 h.

Table 1 The structures of ligustrazine derivatives

Compounds	R	n
1a, 2a, 3a, 4a, 5a, 6a, 7a	H	1
1b, 2b, 3b, 4b, 5b, 6b, 7b	H	2
1c, 2c, 3c, 4c, 5c, 6c, 7c	OCH ₃	1

Fig. 2 EC₅₀ values of ligustrazine derivatives. The results showed that ether bonds may contribute to the enhancement of the efficacy of such new ligustrazine derivatives.

single-ligustrazine derivatives' protective effects were better than those of the bis-ligustrazine substituent ones, as exemplified by the comparison of the respective pairs of compounds such as **3a** > **4a** > **5a**, **3b** > **4b** > **5a**, and **3c** > **4c** > **5c**. In addition, as for the ligustrazine–phenol ester derivatives, the additive ester bonds weakened the protective activity (**6a** > **7a**, **6b** > **7b**, **6c** > **7c**). These findings may provide a

new framework for the design of new ligustrazine derivatives as neuroprotective drugs for treating cerebral ischemic stroke.

Effect of 2c on CoCl₂-induced cell injury

Under optical microscopy, as shown in Fig. 3-A, we found that undifferentiated PC12 cells maintained under normal conditions were small and proliferated to form clone-like cell clusters without neural characteristics. By exposure to NGF, normal differentiated PC12 cells showed round cell bodies with fine dendritic networks similar to those of nerve cells, and the cell edges were intact and clear (Fig. 3-B).

Moreover, the mean value expressed as the percentage of neurite-bearing cells in NGF-treated cells was 58.5% (Fig. 4).

In contrast, the incubation of cells with 200 mM CoCl₂ for 12 h induced shrinkage of the cell bodies, disappearance of cell reticular formation, and disruption of the dendritic networks (Fig. 3-C); the mean value of neurite-bearing cells (18.9%, Fig. 4) showed a significant decrease. Pretreatment with 2c (30 µM) dramatically alleviated the morphological manifestations of cell damage and led to an increase (77.4%, Fig. 4) in neurite-bearing cells compared to model cells (Fig. 3-D).

Plasma stability of 2c *in vitro*

Plasma stability is an important issue in drug development and serves as the major limiting factor for drug candidate screening at an early stage of preclinical studies. Although 2c exhibits beneficial effects on CoCl₂-induced cell injury, its stability in biological matrices has not been studied. In addition, our studies showed that T-VA was liable to enzymatic hydrolysis *in vivo* such as the action of plasma esterases. Since this structural modification may result in different

Fig. 3 Protective effects of compound 2c against CoCl₂-induced injury in differentiated PC12 cells (×200). The most representative fields were shown. A: Undifferentiated PC12 cells. B: Differentiated PC12 cells by NGF. C: CoCl₂-induced neurotoxicity of differentiated PC12 cells. D: CoCl₂-induced neurotoxicity + 2c (30 µM).

Fig. 4 Protective effects of compound 2c (30 μ M) against CoCl_2 -induced injury in differentiated PC12 cells. The neurite-bearing ratios are shown as mean $\pm$ S.D. of at least three independent microscopic fields. ## means $p < 0.01$ compared with the NGF group; ** means $p < 0.01$ compared with the CoCl_2 group.

Fig. 5 The HPLC analysis for 2c and T-VA degradation and residual percentages after 240 min of incubation in rat plasma. A: The chromatogram for 2c degradation in rat plasma at different points in time; B: the chromatogram for T-VA degradation in rat plasma at different points in time; C: time profiles of 2c and T-VA concentrations in rat plasma. Each point represents the mean $\pm$ S.D. of three independent determinations.

stabilities, 2c may have some advantages over T-VA. Therefore, to verify the potential druggability of 2c, compared with T-VA, its plasma stability in rat plasma was preliminarily evaluated by high performance liquid chromatography (HPLC).²⁶

Fig. 5 indicates the residual 2c and T-VA percentages of the initial concentration (t_0) after 240 min of incubation in rat plasma. After incubation at 37 $^{\circ}\text{C}$ for 240 min, the residual percentages of 2c (t_{240}) were greater than 90%. There was practically no degradation for 240 min at 37 $^{\circ}\text{C}$. In contrast, the curve of residual T-VA indicates that more than 50% of the substrate was degraded within 60 min. These results indicated that T-VA degradation in rat plasma may be caused by enzymatic hydrolysis, and structural modification can improve the plasma stability. This study provides useful information for future design of analogous ligustrazine derivatives and pharmacokinetic studies of 2c.

Conclusions

In conclusion, 21 novel ligustrazine derivatives were designed and synthesized through forming ester, ether and amide bonds between bioactive phenols and ligustrazine. Their neuroprotective activity was evaluated on CoCl_2 -induced neurotoxicity in differentiated PC12 cells by the standard MTT assay. The preliminary biological results have demonstrated that most of the ligustrazine-phenol derivatives exhibited good protective effects in comparison with TMP. Among the active compounds, 2c was the most active congener with $\text{EC}_{50} = 1.07 \mu\text{M}$, which is much higher than those of TMP and compounds synthesized in our previous study (T-VA, $\text{EC}_{50} = 4.25 \mu\text{M}$),¹² presenting a most promising lead for further investigation. Therefore, to verify the potential druggability of 2c, compared with T-VA, its plasma stability in rat plasma was preliminarily evaluated by HPLC. These results indicated that ligustrazine-phenolic acid ester (like T-VA) degradation in rat plasma may be caused by enzymatic hydrolysis, and structural modification can observably improve the plasma stability. This study provides useful information for future design of analogous ligustrazine derivatives and metabolic studies of 2c. Further studies of the mechanisms of 2c's neuroprotective effects are currently underway. The results suggest that the attempt to apply structure combination to discover more efficient and plasma-stable lead compounds from natural products is viable.

Conflicts of interest

The authors declare no conflict of interest.

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