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Metabolic investigation on ZL006 for the discovery of a potent prodrug for the treatment of cerebral ischemia

Dongyin Chen, Ting Zhao, Kaidong Ni, Peng Dai, Lei Yang, Yi Xu, Fei Li *

Department of Medicinal Chemistry, School of Pharmacy, Nanjing Medical University, Nanjing 211166, PR China

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ABSTRACT

4-((3,5-Dichloro-2-hydroxybenzyl)amino)-2-hydroxybenzoic acid (ZL006, **1**) is a small-molecular inhibitor of the nNOS/PSD-95 interaction, that is under preclinical evaluation stage for cerebral ischemia. However, the fast metabolism and low permeability across the blood brain barrier (BBB) have restricted its further use. In this manuscript, the mass spectroscopy analysis showed that ZL006 mainly combined with glucuronic acid in mice plasma, which accelerated its metabolism and elimination. Hence, six ZL006 analogs were designed according to the probable metabolism sites of ZL006, and featured the alkylation at phenolic hydroxyl, secondary amine and carboxyl groups. These compounds were synthesized in moderate to good yields, and fully characterized with ^{1}H NMR and MS. Further metabolism investigation of ZL006 analogs showed that phenolic hydroxyl group of aromatic ring A was the major conjugation site with glucuronic acid, and ZL006 cyclohexyl ester (**6**) had a better permeability across BBB, which was a potent prodrug for cerebral ischemia.

Postsynaptic density protein-95 (PSD-95) is a key scaffolding protein that links neuronal nitric oxide synthase (nNOS) with the *N*-methyl-D-aspartic acid receptor (NMDAR), and mediates a specific coupling between NMDAR activation and NO production in the central nervous system (CNS).[1] As a signaling macromolecular, the NMDAR/PSD-95/nNOS complex has been implicated in several neurological disorders including chronic pain,[2] ischemic brain injury,[3] depression[4] and Parkinson's disease.[5] In recent years, the NMDAR/PSD-95/nNOS complex has emerged as a promising target for treating these CNS disorders; some peptide fragments and small-molecule inhibitors have achieved the disruption of the NMDAR/PSD-95/nNOS interaction.[2–4] Among them, 4-((3,5-dichloro-2-hydroxybenzyl)amino)-2-hydroxybenzoic acid (ZL006 (**1**), Fig. 1) attracted considerable attention, which selectively blocked the ischemia-induced PSD-95/nNOS coupling, exhibited remarkable neuroprotective activity and ameliorated focal cerebral ischemic damage, without effect on aggressive behavior and spatial memory.[3a] So far, ZL006 (**1**) has served as a potential candidate for cerebral ischemia. However, the fast metabolism and low permeability across the blood brain barrier (BBB) have restricted its further use. In addition, it is crucial to prolong therapeutic drug level and increase its permeability across BBB for the recovery process after ischemic brain injury.[6] In this manuscript, we designed six ZL006 analogs and carried out metabolic studies on these compounds, which identified the phenolic hydroxyl group of aromatic ring A was its major conjugation site with glucuronic acid. Moreover, compared with ZL006 (**1**), the compounds that were esterified at carboxyl group of aromatic ring B were observed having significantly higher permeability across BBB and longer duration time.

Initial mass spectroscopy analysis of urine samples from the mice that were collected after intravenous injections (i.v.) of 20 mg/kg of ZL006 (**1**) revealed the presence of major peak (m/z = 502.1) (see Supporting information). The same metabolite was detected in mice plasma and brain tissue. Then, tandem mass spectroscopy analysis of the major metabolite showed the features of mass spec fragmentation patterns of ZL006 (m/z = 326.0, 152.0). Glucuronidation serves as an integral step in drug disposition and metabolism, which transforms lipophilic substrates into hydrophilic glucuronides, facilitating the transport to excretory organs and subsequent elimination through the bile and urine.[7] Therefore, we were sure that the major metabolite was the glucuronide conjugates of ZL006 (**1**). Furthermore, we measured concentrations of ZL006 (20 mg/kg, i.v.) in mice plasma and brain tissue at 15, 30 and 60 min after the dosing. As shown in Figure 2, ZL006 (**1**) exhibited fast rate of elimination from plasma and low permeability across BBB. Accordingly, we hypothesized that the limited metabolic stability of ZL006 (**1**) may involve a relatively rapid metabolism of phenolic hydroxyl, secondary amine and

* Corresponding author. Tel.: +86 25 86868485.
E-mail address: kldlf@njmu.edu.cn (F. Li).

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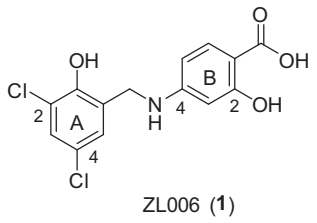


ZL006 (**1**)

Figure 1. Structure of ZL006 (**1**).

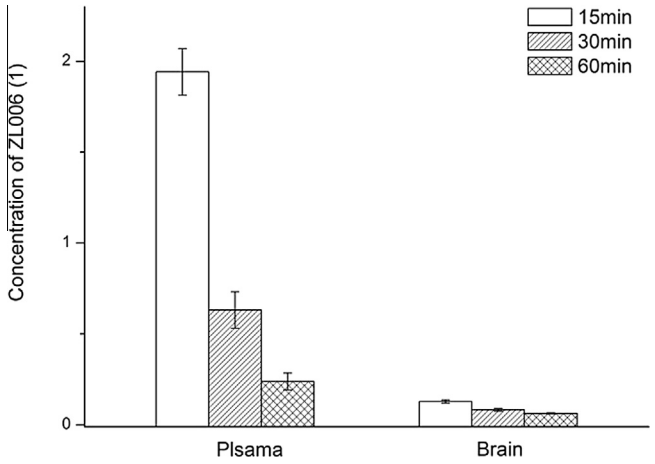
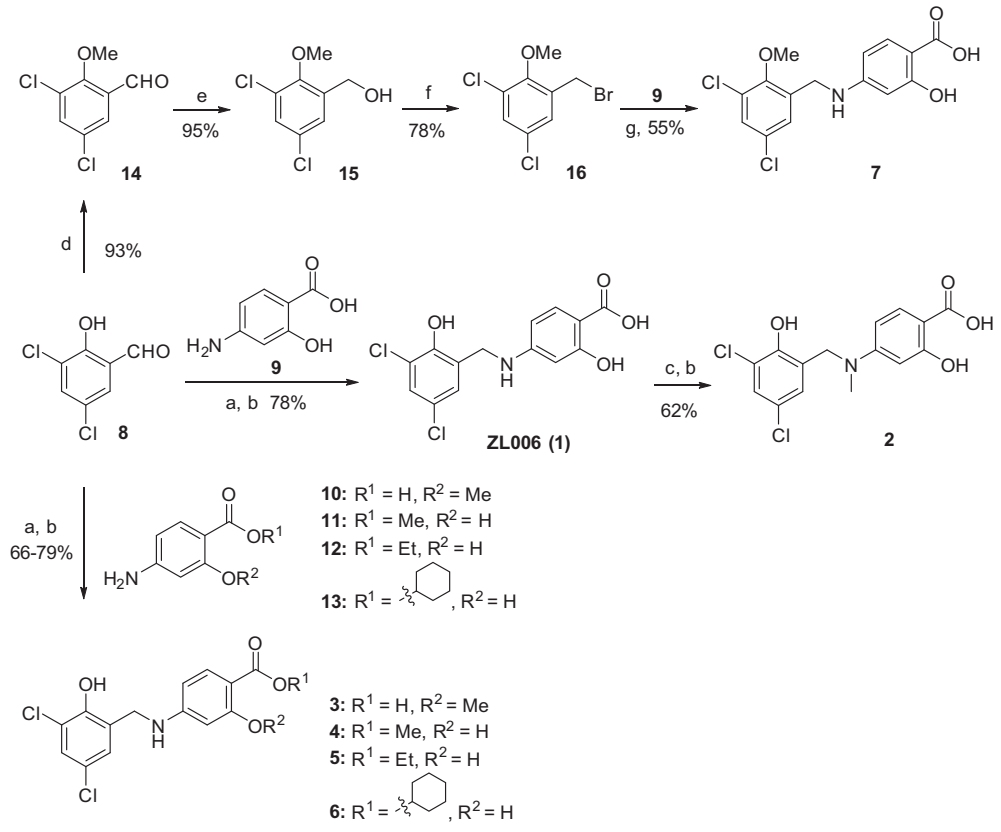


Figure 2. Concentrations of ZL006 in mice plasma and brain tissue at 15, 30 and 60 min after the dosing (μg/mL for plasma, μg/g for brain tissue). ZL006 (20 mg/kg) were administered intravenously. Values are means ± SEM, $n = 6$.

carboxyl groups, to form the corresponding glucuronides assisted by some glucuronosyltransferases.

Based on these results as well as the characteristics of glucuronidation of many endogenous substrates and clinically relevant drugs, we surmised that four possible conjugation sites of ZL006 with glucuronic acid existed, including phenolic hydroxyl groups of aromatic ring A and B, carboxyl group of aromatic ring B and secondary amine group. So, six ZL006 analogs **2**–**7** were designed and synthesized, which featured the alkylation at phenolic hydroxyl, carboxyl and secondary amine groups, to screen for the major conjugation site with glucuronic acid. The synthetic route of compounds **1**–**7** is shown in Scheme 1. ZL006 (**1**) was synthesized in 78% yield from reductive amination of 3,5-dichloro-2-hydroxybenzaldehyde (**8**) with 4-amino-2-hydroxybenzoic acid (**9**). Then, ZL006 (**1**) was converted into compound **2** in 62% yield by subsequent reductive amination with formaldehyde. Following the procedure described for preparation of **1**, compounds **3**–**6** were prepared in 66–79% yield from compounds **10**–**13** by reductive amination with compound **8**. According to reported procedures,[8–10] compounds **11**–**13** were prepared from the esterification of 4-nitro-2-hydroxybenzoic acid with alcohols, followed by reduction with iron powder/hydrochloric acid. The intermediate **16** for the preparation of compound **7** was readily obtained from the methylation of compound **8** with iodomethane in the presence of potassium carbonate,[11] followed by reduction by sodium borohydride and bromination by phosphorus tribromide. Finally, compound **7** was obtained in 55% yield from the N-alkylation of compounds **9** with **16**. All these synthesized compounds were fully characterized with ^{1}H NMR and MS. They afforded MS spectra with the m/z values corresponding to $[M-H]^-$. Their NMR spectra were also in full agreement with the given structures (see Supporting information). Their purity was judged from ^{1}H NMR and TLC.



Scheme 1. Synthetic route for compounds **1**–**7**. Reagents and conditions: (a) EtOH, rt or reflux; (b) $NaBH_4$, EtOH, HCl; (c) HCHO, rt; (d) MeI, K_2CO_3, DMF; (e) $NaBH_4$, EtOH; (f) PBr_3, THF; (g) acetone, reflux.