

Preclinical evaluation of ZL006-05, a new antistroke drug with fast-onset antidepressant and anxiolytic effects

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ABSTRACT

Background Poststroke depression and anxiety, independent predictor of poor functional outcomes, are common in the acute phase of stroke. Up to now, there is no fast-onset antidepressant and anxiolytic agents suitable for the management of acute stroke. ZL006-05, a dual-target analgesic we developed, dissociates nitric oxide synthase from postsynaptic density-95 while potentiates $\alpha 2$ -containing γ -aminobutyric acid type A receptor. This study aims to determine whether ZL006-05 can be used as an antistroke agent with fast-onset antidepressant and anxiolytic effects.

Methods Photothrombotic stroke and transient middle cerebral artery occlusion were induced in rats and mice. Infarct size was measured by TTC(2,3,5-Triphenyltetrazolium chloride) staining or Nissl staining. Neurological defects were assessed by four-point scale neurological score or modified Neurological Severity Scores. Grid-walking, cylinder and modified adhesive removal tasks were conducted to assess sensorimotor functions. Spatial learning was assessed using Morris water maze task. Depression and anxiety were induced by unpredictable chronic mild stress. Depressive behaviours were assessed by tail suspension, forced swim and sucrose preference tests. Anxiety behaviours were assessed by novelty-suppressed feeding and elevated plus maze tests. Pharmacokinetics, toxicokinetics and long-term toxicity studies were performed in rats.

Results Administration of ZL006-05 in the acute phase of stroke attenuated transient and permanent ischaemic injury and ameliorated long-term functional impairments significantly, with a treatment window of 12 hours after ischemia, and reduced plasminogen activator-induced haemorrhagic transformation. ZL006-05 produced fast-onset antidepressant and anxiolytic effects with onset latency of 1 hour in the normal and CMS mice, had antidepressant and anxiolytic effects in stroke mice. ZL006-05 crossed the blood–brain barrier and distributed into the brain rapidly, and had a high safety profile in toxicokinetics and long-term toxicological studies.

Conclusion ZL006-05 is a new neuroprotectant with fast-onset antidepressant and anxiolytic effects and has translational properties in terms of efficacy, safety and targeting of clinical issues.

INTRODUCTION

To date, stroke remained the second-leading cause of death and one of the leading causes of

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Poststroke depression and anxiety are common in the acute phase of stroke. Up to now, there is no fast-onset antidepressant and anxiolytic agents suitable for the management of acute stroke.

WHAT THIS STUDY ADDS

⇒ ZL006-05, a new drug we developed in clinical trials (I/II phase), attenuated transient and permanent ischaemic injury, ameliorated long-term functional impairments significantly, and had fast-onset antidepressant and anxiolytic effects.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ ZL006-05 is a new neuroprotectant with fast-onset antidepressant and anxiolytic effects and has translational properties in terms of efficacy, safety and targeting of clinical issues.

disability in much of the world.¹ In particular, China faces the greatest challenge from stroke in the world.² Current treatments for acute ischaemic stroke are based solely on intravenous thrombolysis and mechanical thrombectomy.³ Moreover, poststroke depression (PSD) and anxiety (PSA) are very common, and constitute an independent predictor of poor functional outcomes and quality of life during both the acute and chronic phases.^{4–9} Unfortunately, high-quality clinical evidence shows that selective serotonin reuptake inhibitors (SSRIs), first-line options for pharmacological management of depression and anxiety disorders, do not reduce disability after stroke and cause a slightly increased risk of seizures and fractures, although they reduce the risk of future depression by about a quarter.¹⁰ Additionally, because of slow onset,¹¹ SSRIs are not suitable for the management of depression and anxiety symptoms in the acute phase of stroke. Therefore, there is a need to develop new neuroprotective agents with fast-onset antidepressant and anxiolytic effects for acute stroke treatment.

Decline of blood perfusion after acute stroke produces an infarct core surrounded by penumbra, a rescueable area that is functionally impaired but metabolically active. Overactivation of N-methyl-D-aspartate receptors (NMDARs) induces excitotoxicity, mainly by increasing interaction of postsynaptic density-95 (PSD-95) with nitric oxide synthase (nNOS), leads to secondary neuronal death in the penumbra.^{12 13} In the acute phase of stroke, surface expression of γ -Aminobutyric acid type A receptors (GABA_ARs) is substantially decreased due to their rapid nanoscale rearrangement,^{14 15} which contributes to the lethal excitotoxicity.^{14–16} Thus, ischaemic neuronal death can be attenuated by positive allosteric modulation of GABA_ARs.^{17 18} Moreover, GABA_ARs play crucial role in the modulation of depression and anxiety.^{19 20} Positive allosteric modulators of GABA_ARs are medications used in the management and treatment of depression and anxiety disorders.^{21 22} Benzodiazepines target GABA_ARs containing α 1, α 2, α 3 or α 5 subunits non-selectively, in which, α 2-containing GABA_AR is the anxiolytic and antidepressant subtype.¹⁹ Selectively targeting α 2-containing GABA_AR can avoid sedation, motor impairment and tolerance development.²³ However, because of the reduced surface expression of GABA_ARs after stroke,^{14 15} effects of GABA_AR agonists alone may be limited. ZL006-05, a dual-target analgesic we developed, blocks nNOS-PSD-95 interaction and potentiates α 2-containing GABA_AR, without analgesic tolerance and unwanted side effects.²⁴ We here show that ZL006-05 prevents permanent ischaemic and ischaemic/reperfusion injuries and promotes functional recovery in experimental stroke models, and produces fast-onset antidepressant and anxiolytic effects. Moreover, ZL006-05 readily crosses the blood–brain barrier (BBB) and has a very high safety profile and has been approved by China Food and Drug Administration (CFDA) for phase I/II clinical trial of acute stroke.

METHODS

Animals

Adult Sprague Dawley rats and C57BL/6 mice were used in this study. Details appear in online supplemental material.

Inclusion and exclusion criteria

For stroke experiments, the criteria were based on the degree of neurological deficits assessed by four-point scale neurological score.²⁵ Details appear in online supplemental material and table S1.

Drugs

ZL006-05 injection, edaravone dexborneol (ED) injection and recombinant human tissue plasminogen activator (tPA) were used in this study. Details appear in online supplemental material and table S1.

Stroke models and the measurements of infarct, injury and haemorrhage sizes

Photothrombotic stroke and transient middle cerebral artery occlusion (tMCAO) were performed as described.^{13 26} Infarct size was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining for tMCAO model and by Nissl staining for Photothrombotic model, respectively, as described.^{13 26} Volumes of haemorrhage and injury were measured as described.²⁷

Behavioural assessments

Neurological defects were assessed by four-point scale neurological score or modified Neurological Severity Scores (mNSS) as described.^{25 28} Grid-walking, cylinder and modified adhesive removal tasks were conducted to assess sensorimotor functions as described.^{29 30} Spatial learning was assessed using Morris water maze (MWM) task. Depression and anxiety were induced by unpredictable chronic mild stress (UCMS) as we described previously.³¹ Depressive behaviours were assessed by tail suspension, forced swim and sucrose preference tests (SPTs).^{31 32} Anxiety behaviours were assessed by novelty-suppressed feeding (NSF) and elevated plus maze (EPM) tests.^{33 34} Details appear in online supplemental material.

Coimmunoprecipitation and Western blot analysis

Coimmunoprecipitation and Western blot analysis were performed as described.³⁵

Electrophysiology, pharmacokinetics, toxicokinetics and long-term toxicity

Detailed methods on electrophysiology, pharmacokinetics, toxicokinetics and long-term toxicity are available in online supplemental material.

Statistical analyses

Data are presented as mean \pm SD. Stata V.9.0 software and SPSS Statistics V.22 software were used for statistical analysis. The threshold level of significance was set at $p < 0.05$. Details appear in online supplemental material.

RESULTS

ZL006-05 prevents stroke damage after permanent and transient cerebral ischaemia

In the stroke mice, ZL006-05 significantly reduced stroke-induced nNOS-PSD-95 interaction in the peri-infarct cortex and increased miniature inhibitory postsynaptic currents amplitude (online supplemental figure S1), suggesting an increased inhibitory synaptic transmission. Based on the above data and our previous study,²⁴ we hypothesised that ZL006-05 may prevent stroke damage in the acute phase (figure 1A). To test this, we treated the rats subjected to tMCAO with ZL006-05 or positive control ED that showed good functional outcomes in phase III clinical trial.³⁶ Drugs were intravenously injected at 60 min after reperfusion. Both in male and female rats, ZL006-05 treatment significantly reduced infarct volume and neurological score dose-dependently

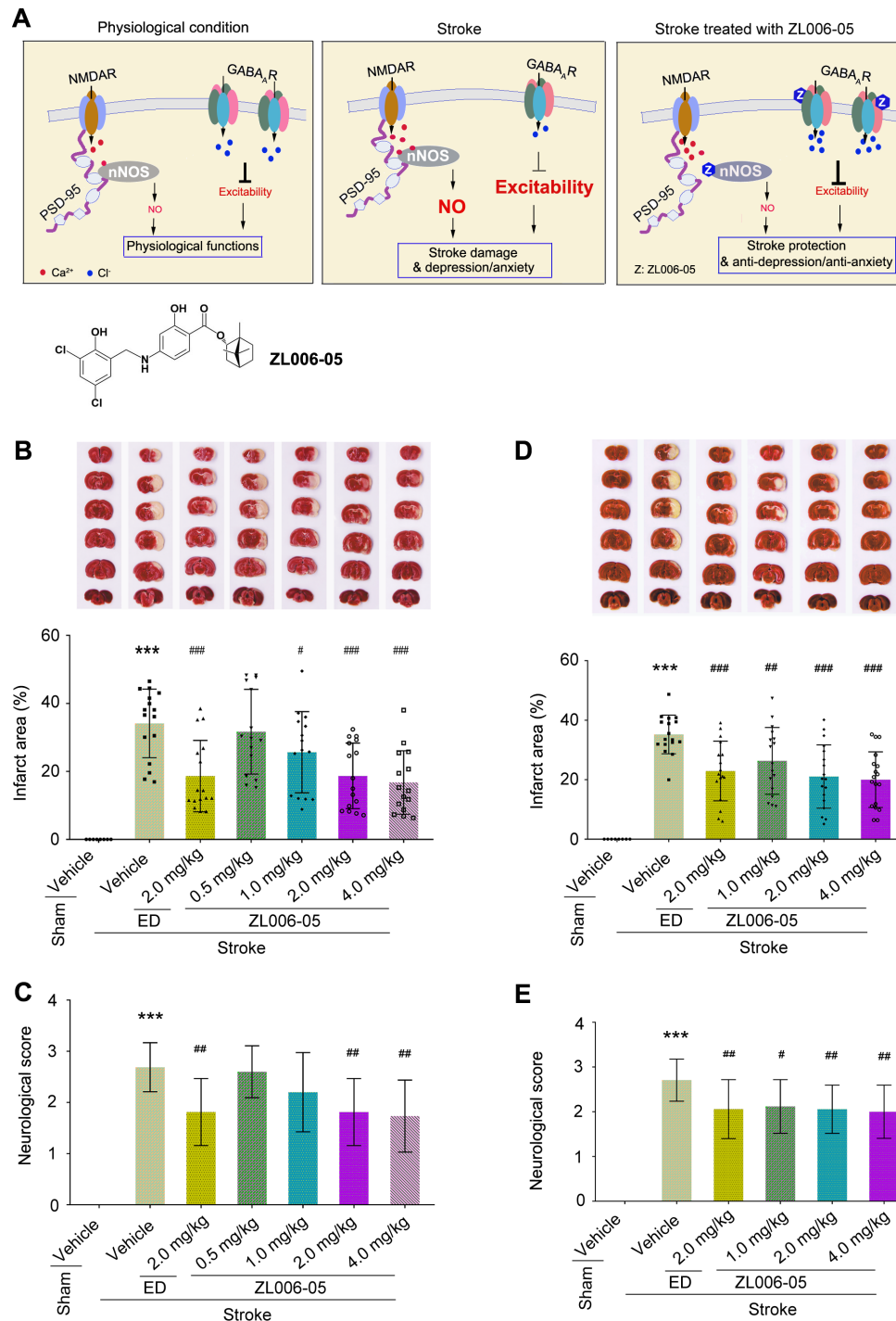


Figure 1 Protective effects of ZL006-05 after cerebral ischaemia/reperfusion. (A) (Upper) The hypothesis: in the acute phase of stroke, NO overproduction caused by increased nNOS-PSD-95 interaction, and GABAARs dysfunction lead to ischaemic damage and depression and/or anxiety. ZL006-05 prevents the ischaemic damage and produces fast-onset antidepressant and/or anxiolytic effects by (1) reducing NO production via uncoupling nNOS-PSD-95 and (2) potentiating $\alpha 2$ -containing GABAAR. (Lower) Chemical structure of ZL006-05. (B, C) Effects of ZL006-05 in male rats. $n=8$ for sham, $n=15-16$ for other groups. (B) Representative cerebral infarct images stained using TTC in coronal sections of rat brains (upper) and infarct volume presented as a percentage of the intact hemisphere (lower) (one-way ANOVA, $F_{6,94}=13.494$. $***p<0.001$, vs sham; $###p<0.001$, $\#p=0.024$, vs stroke/vehicle). (C) Neurological scores (Mann-Whitney U test, $***p<0.001$, vs sham; $##p<0.01$, vs vehicle/stroke). (D, E) Effects of ZL006-05 in female rats. $n=8$ for sham, $n=17-18$ for other groups. (D) Representative cerebral infarct images in coronal sections of rat brains (upper) and infarct volume presented as a percentage of the intact hemisphere (lower) (one-way ANOVA, $F_{5,89}=16.580$. $***p<0.001$, vs sham; $###p<0.001$, $\#p=0.006$, vs stroke/vehicle). (E) Neurological scores (Mann-Whitney U test, $***p<0.001$, vs sham; $##p<0.01$, $\#p=0.011$, vs vehicle/stroke). ANOVA, analysis of variance; ED, edaravone dextroborate; GABAAR, γ -Aminobutyric acid type A receptor; NMDAR, N-methyl-D-aspartate receptors; nNOS, nitric oxide synthase; TTC, 2,3,5-Triphenyltetrazolium chloride.

at 48 hours after tMCAO (figure 1B–E). The effective dose of ZL006-05 was 1 mg/kg. The effects of ZL006-05 and ED at dose of 2 mg/kg were comparable. Moreover, ZL006-05 (2 mg/kg, intravenously) significantly reduced infarct volume, foot faults and neurological score on day 7 after tMCAO in male rats (online supplemental figure S2). Therefore, ZL006-05 can prevent stroke damage caused by ischaemia/reperfusion.

So far, only a small percentage of stroke patients are able to receive reperfusion therapy, permanent ischaemia without reperfusion remains a huge challenge after stroke. We, thus, observed the effects of ZL006-05 on permanent ischaemia using photothrombotic stroke model in mice. Two doses of ZL006-05 or ED (2 mg/kg, intravenously) were respectively given at 2 and 26 hours after ischaemia, and motor functions and infarct volume were measured on day 7 after ischaemia. In male mice, foot faults of contralateral forelimb in the stroke mice were significantly higher than that in the sham mice ($p < 0.001$), and ZL006-05-treated or ED-treated stroke mice displayed significantly reduced foot faults, compared with vehicle-treated stroke mice in the grid-walking test (figure 2A,B). In the cylinder test, as shown in figure 2C, stroke mice had significantly higher asymmetry index than sham mice did, and ZL006-05- or ED-treated stroke mice had significantly reduced asymmetry index compared with vehicle-treated stroke mice. Both ZL006-05 and ED significantly reduced infarct volume (figure 2D,E). Similarly, ZL006-05 significantly improved motor functions and reduced infarct volume on day 7 after ischaemia in female mice (online supplemental figure S3). Moreover, we observed effect of ZL006-05 in male rats subjected to photothrombotic stroke and found that it significantly improved sensorimotor functions and reduced infarct volume at 48 hours after ischaemia (online supplemental figure S4). Thus, ZL006-05 can prevent stroke damage caused by permanent ischaemia. Haemorrhagic transformation often affects the outcome of ischaemic stroke, especially after thrombolytic therapy. We found that ZL006-05 significantly reduced tPA-induced haemorrhagic transformation and deterioration of brain injury (online supplemental figure S5).

Given that only a small percentage of stroke patients can arrive at the hospital shortly, we thus investigated whether delayed treatment with ZL006-05 could reduce stroke damage caused by photothrombotic stroke model in male rats. The first dose of ZL006-05 (2 mg/kg, intravenously) was given at 6 or 12 hours after permanent ischaemia, and the second dose was given at 24 hours after the first dose. On day 7 after ischaemia, motor function was assessed using the grid-walking test, somatosensory function was assessed using modified adhesive removal (sticky-tape) test (MST), and infarct volume were measured using Nissl staining. As shown in figure 2F–J, stroke rats displayed significantly increased foot faults of contralateral forelimb and significantly reduced ratio of left to right performance on the MST, compared with sham rats, indicating impaired somatosensory and motor functions.

Treatment with ZL006-05 starting at 6 hours or 12 hours after ischaemia significantly ameliorated the stroke-induced somatosensory and motor functions impairment, and significantly reduced infarct volume. Thus, ZL006-05 can reduce stroke damage even when treatment is started 12 hours after permanent ischaemia.

In the clinical trial of acute ischaemic stroke, functional independence at 90 days after stroke is often served as the primary efficacy end point. For clinical translation, we observed long-term effect of ZL006-05. ZL006-05 (2 mg/kg/day, $\times 3$ or $\times 7$ days, intravenously) was used in the male rats subjected to photothrombotic ischaemia, and ED (2 mg/kg/day, $\times 7$ days, intravenously) as a positive control. We assessed somatosensory function using MST on day 28 and motor function using the grid-walking test on day 30, and measured infarct volume using Nissl staining on day 31 after stroke (figure 3A). Stroke rats displayed significantly increased foot faults of contralateral forelimb in the grid-walking test and significantly reduced ratio of left to right performance on the MST, compared with sham rats ($p < 0.001$), and ZL006-05-treated or ED-treated stroke rats displayed significantly reduced foot faults and increased ratio of left to right performance on the MST, compared with vehicle-treated stroke rats (figure 3B–D). Moreover, ZL006-05 and ED reduced infarct volume significantly (figure 3E). These data suggest long-term beneficial effect of ZL006-05.

To further test the long-term effect of ZL006-05, we treated the male rats subjected to tMCAO with ZL006-05 (intravenously, 2 mg/kg/day) for 3 or 7 consecutive days. We assessed neurological deficit using mNSS and motor function using the grid-walking test on day 28 and examined spatial learning ability using MWM task during days 30–35 after stroke (figure 3F). ZL006-05-treated or ED-treated stroke rats had significantly decreased mNSS and foot faults, compared with vehicle-treated stroke rats (figure 3G–I), suggesting improved neurological deficit and motor function. In the MWM task (figure 3J,K), the latency (time to reach the platform) of tMCAO rats was significantly more than that in the sham mice ($p < 0.001$), suggesting an impaired spatial learning, and ZL006-05-treated or ED-treated stroke rats had significantly decreased latency compared with vehicle-treated tMCAO rats, although similar swimming speeds were similar between groups. Together, ZL006-05 administrated in the acute phase of permanent or transient cerebral ischaemia can improve long-term functions, including neurological deficit, sensorimotor functions and spatial learning.

ZL006-05 has fast-onset antidepressive and anxiolytic effects

As nNOS-PSD-95 interaction has been implicated in emotional regulation and $\alpha 2$ -containing GABA_AR is an anxiolytic and antidepressant subtype,^{19 24 37} we hypothesised that ZL006-05 may have antidepressant and anxiolytic effects (figure 1A). To test this, ZL006-05 was intravenously injected into the male mice. At 60 min after the injection, we measured the time in the open arms in the EPM test and latency to feed in the NSF test. As shown in

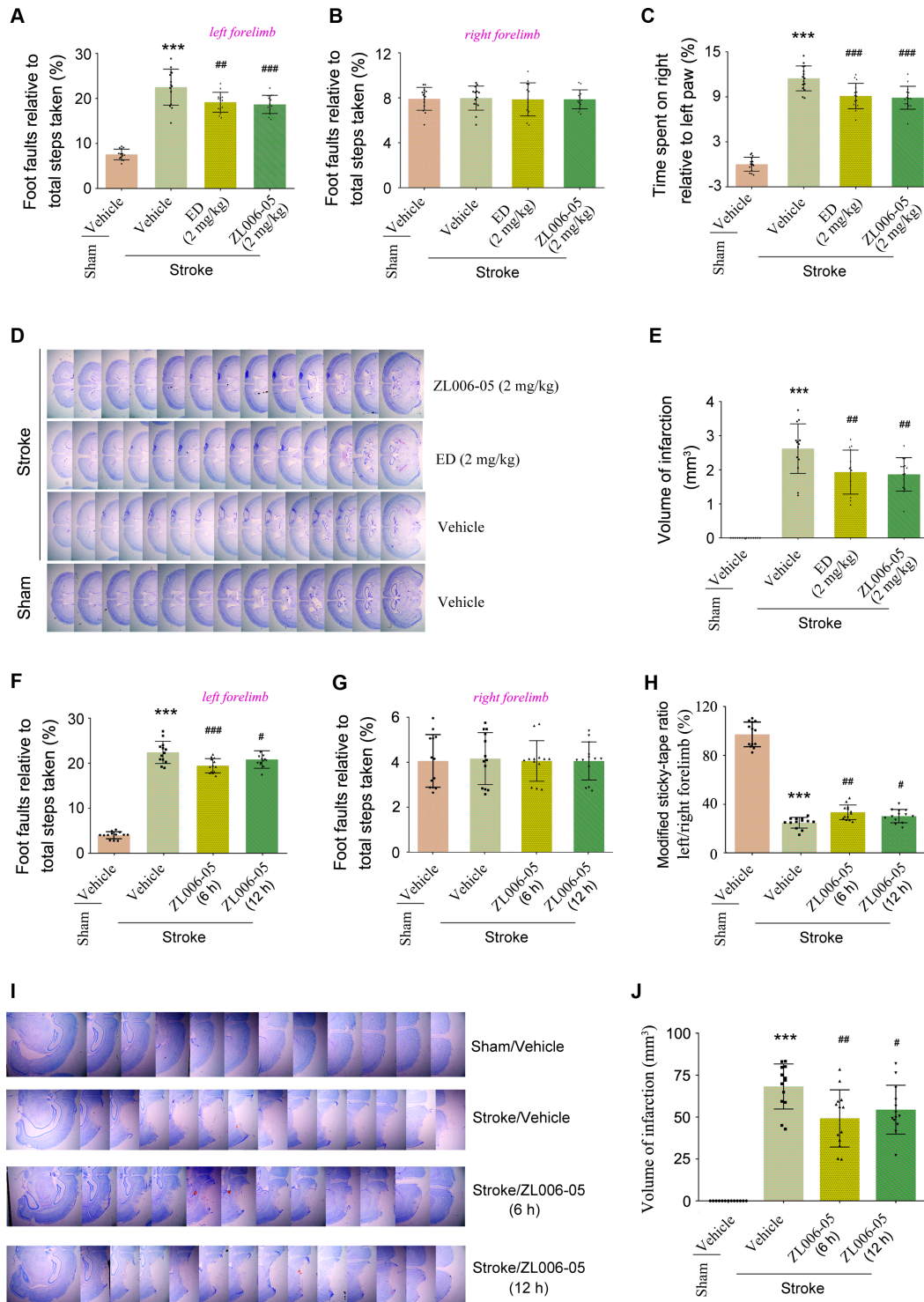


Figure 2 Therapeutic time window of ZL006-05 after permanent focal cerebral ischaemia. (A–E) Effects of ZL006-05 in the mice subjected to photothrombotic stroke. $n=14$. (A) Foot faults of the left forelimb (one-way ANOVA, $F_{3,52}=89.690$. $***P < 0.001$, vs sham; $##p < 0.01$, $###p < 0.001$, vs vehicle/stroke) and (B) foot faults of the right forelimb (one-way ANOVA, $F_{3,52}=0.031$, $p=0.993$) in the grid-walking task. (C) Forelimb symmetry in the cylinder task (one-way ANOVA, $F_{3,52}=149.963$. $***p < 0.001$, vs sham; $###p < 0.001$, vs vehicle/stroke). (D) Representative cerebral infarct images stained using Nissl and (E) Bar graph showing infarct size at 11 d after stroke (one-way ANOVA, $F_{3,52}=59.533$. $***p < 0.001$, vs sham; $##p < 0.01$, vs vehicle/stroke). (F–J) Effects of ZL006-05 in the rats subjected to photothrombotic stroke. $n=13$. (F) Foot faults of the left forelimb (one-way ANOVA, $F_{3,48}=291.84$. $***p < 0.001$, vs sham; $###p < 0.001$, $#p=0.028$, vs vehicle/stroke) and (G) foot faults of the right forelimb (one-way ANOVA, $F_{3,48}=0.034$, $p=0.991$) in the grid-walking task. (H) The ratio of left to right performance on the modified sticky-tape test (one-way ANOVA, $F_{3,48}=320.54$. $***p < 0.001$, vs sham; $##p=0.003$, $#p=0.049$, vs vehicle/stroke). (I) Representative cerebral infarct images stained using Nissl and (J) bar graph showing infarct size on d seven after stroke (one-way ANOVA, $F_{3,48}=67.34$. $***p < 0.001$, vs sham; $##p=0.001$, $#p=0.010$, vs vehicle/stroke). ANOVA, analysis of variance; ED, edaravone dextroborneol.

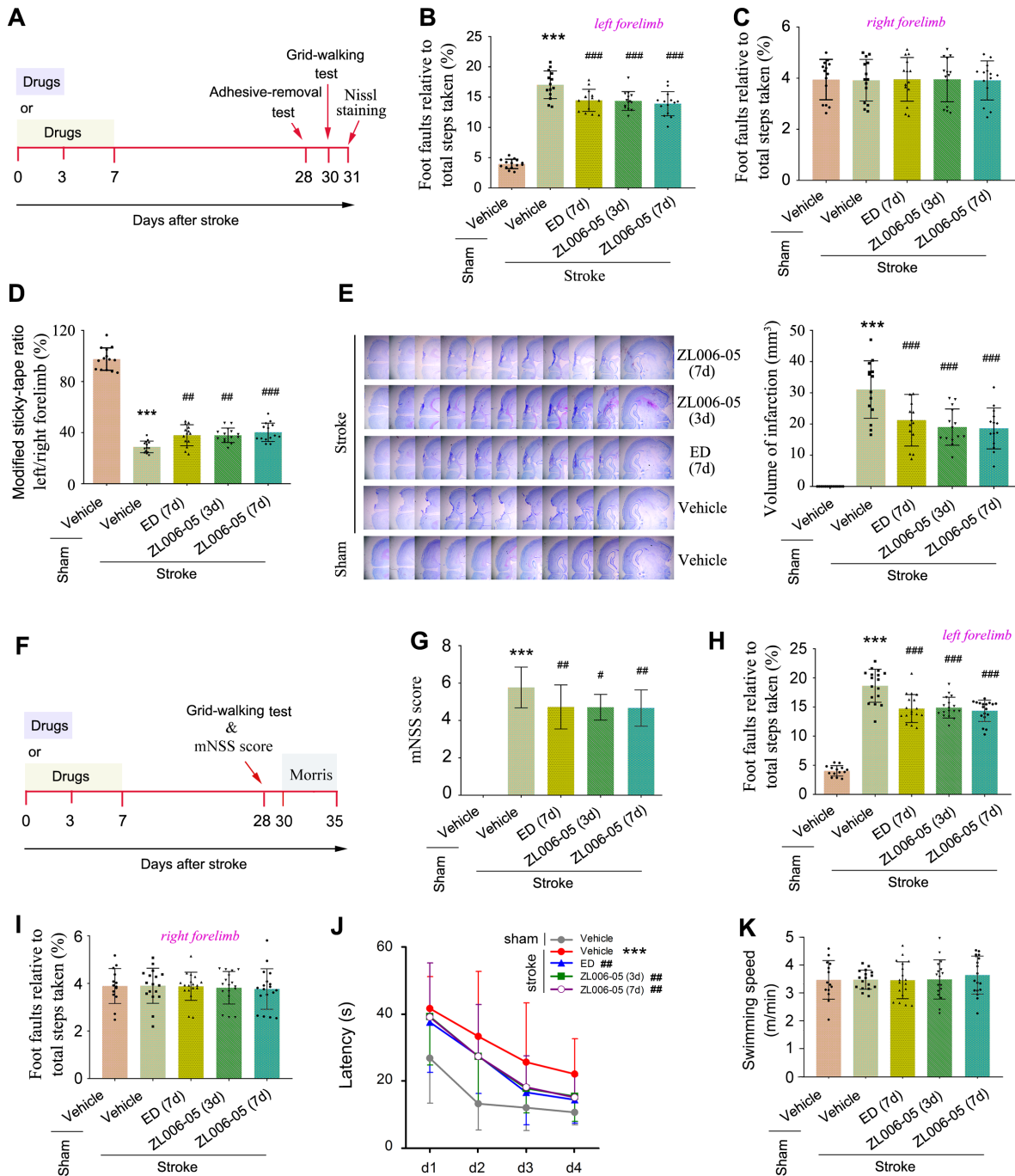


Figure 3 Long-term effects of ZL006-05 after stroke. (A–E) Effects of ZL006-05 in the rats subjected to photothrombotic stroke, in which, 3d and 7d are consecutive days of drugs administration after stroke. $n=14$. (A) Experimental design for B–E) (B) Foot faults of the left forelimb (one-way ANOVA, $F_{4,65}=115.161$. *** $p<0.001$, vs sham; ### $p<0.001$, vs vehicle/stroke) and (C) foot faults of the right forelimb (one-way ANOVA, $F_{4,65}=0.008$, $p=1.000$) in the grid-walking task. (D) The ratio of left to right performance on the modified sticky-tape test (one-way ANOVA, $F_{4,65}=216.323$. *** $P<0.001$, vs sham; ### $p<0.01$, ### $p<0.001$, vs vehicle/stroke). (E) Representative cerebral infarct images stained using Nissl (left) and bar graph showing infarct size at 31 d after stroke (right) (one-way ANOVA, $F_{4,65}=38.474$. *** $p<0.001$, vs sham; ### $p<0.001$, vs vehicle/stroke). (F–J) Effects of ZL006-05 in the rats subjected to MCAO/reperfusion, in which, 3d and 7d are consecutive days of drugs administration after cerebral ischaemia. $n=13-14$ for sham, $n=16-18$ for other groups. (F) Experimental design for (G–J) (G) Modified neurological severity score (Mann-Whitney U test, *** $p<0.001$, vs sham; for ## $p<0.01$, # $p=0.030$, vs vehicle/stroke). (H) Foot faults of the left forelimb (one-way ANOVA, $F_{4,79}=101.549$. *** $p<0.001$, vs sham; ### $p<0.001$, vs vehicle/stroke) and (I) foot faults of the right forelimb (one-way ANOVA, $F_{4,79}=0.111$, $p=0.978$) in the grid-walking task. (J) Escape latency in Morris water maze task (two-way repeated-measures ANOVA, $F_{7,315}=29.620$. *** $p<0.001$, Vehicle/sham vs vehicle/stroke; ## $p=0.0015$, ED/stroke vs vehicle/stroke; ## $p=0.007$, ZL006-05 (3d)/stroke vs vehicle/stroke; ## $p=0.0058$, ZL006-05 (7d)/stroke vs vehicle/stroke). (K) Swimming speed in Morris water maze task ($F_{4,79}=0.259$, $p=0.903$). ANOVA, analysis of variance; ED, edaravone dextroisomer; MCAO, middle cerebral artery occlusion.

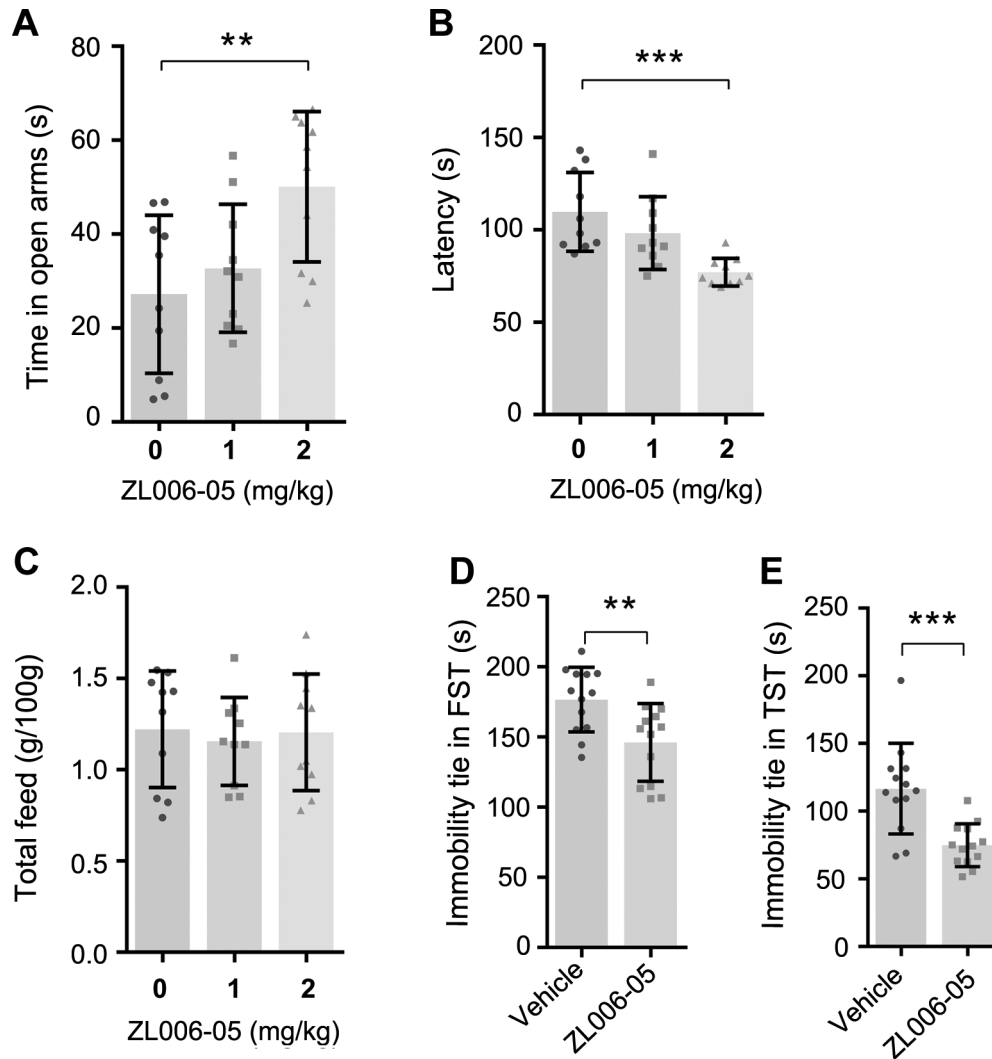


Figure 4 ZL006-05 produces fast-onset anxiolytic-like and antidepressant-like effects. (A–E) Effects of ZL006-05 in mice. (A–C), $n=10$; (D, E), $n=13$. (A) Time spent in open arms in EPM test (one-way ANOVA, $F_{2,27}=275.899$, $^{**}P = 0.003$), (B) Latency in the NSF test (one-way ANOVA, $F_{2,27}=279.206$, $^{***}P < 0.001$). (C) Food consumption in the NSF test (one-way ANOVA, $F_{2,27}=270.134$, $p=0.875$). (D) Immobility time in the FST test ($^{**}p=0.005$, two-tailed t-test). (E) Immobility time in the TST test ($^{***}p<0.001$, two-tailed t-test). ANOVA, analysis of variance; EPM, elevated plus maze; NSF, novelty-suppressed feeding; TST, tail suspension test.

figure 4A,B, ZL006-05 at dose of 2 mg/kg caused the mice to spend more time in the open arms in the EPM test and to display significantly decreased latency to feed in the NSF test, compared with vehicle. The drug did not affect the amount of food consumed by mice (figure 4C). These data suggest that the drug has a fast-onset anxiolytic-like effect at dose of 2 mg/kg. Accordingly, we next investigated the effect of ZL006-05 at dose of 2 mg/kg (intravenously) on depression behaviours. At 60 min after injection, the mice displayed significantly reduced immobility time in the tail suspension test (TST) and forced swimming test (FST), two common and classic depression behaviour tests, suggesting a fast-onset antidepressant-like effect (figure 4D,E).

ZL006-05 is a chemical molecule formed by linking ZL006, a nNOS-PSD-95 blocker,¹³ and (+)-borneol, a positive allosteric modulator of $\alpha 2$ -containing and

$\alpha 3$ -containing GABA_ARs.²⁴ To test whether targeting GABA_AR alone has fast-onset anxiolytic-like and antidepressant-like effects, we treated mice with (+)-borneol at dose of 1, 3 or 9 mg/kg (i.p.). We measured the time spent in the open arms in the EPM test, latency to feed in the NSF test, and immobility time in the TST and FST at 60 min after drug injection. As shown in online supplemental figures S6A–E, although (+)-borneol at dose 1 or 3 mg/kg caused the mice to spend more time in the open arms in the EPM test, the drug did not affect their latency to feed in the NSF test and their immobility time in the TST and FST, suggesting a fast-onset anxiolytic-like but not antidepressant-like effect. Next, we treated mice with ZL006 at a dose of 40 mg/kg/day (i.p.) for 7 consecutive days and measured behavioural modifications at 60 min after injection on days 1, 3, 5 and 7 of dosing, and found that the treatment did not change the time

spent in the open arms in the EPM test and immobility time in the TST (online supplemental figures S6F,G). However, when treated with 40 mg/kg of ZL006 (i.p.) for 11 consecutive days, the mice displayed significantly increased time spent in the open arms in the EPM test and reduced immobility time in the TST (online supplemental figures S6H,I), suggesting that ZL006 has delayed-onset antidepressant-like and anxiolytic-like effects. Thus, dissociating nNOS-PSD-95 or activating GABA_ARs alone has no fast-onset antianxiety and antidepressant effect.

Next, we thus treated male mice with (UCMS), an unpredictable chronic stress condition leading to anxiety and depression behaviours.^{31,38} Adult mice were exposed to UCMS for 28 days and treated with ZL006-05 (1 mg/kg or 2 mg/kg, intravenously) during 2–12 hours after the end of UCMS, and anxiety-related and depression-related behaviours were measured at 60 min after drug injection (figure 5A, schedule 1). As shown in figures 5B–H, 2 mg/kg of ZL006-05 reversed UCMS-induced behavioural modifications, as indicated by significantly increased time spent in the open arms in the EPM test, decreased latency to feed in the NSF test, reduced immobility time in the FST and TST, and increased sucrose water preference in the SPT, compared with vehicle-treated UCMS mice. Moreover, UCMS exposure or ZL006-05 treatment did not affect locomotor activity (figure 5C) and the amount of food consumption (figure 5E).

Finally, we investigated whether ZL006-05 had anxiolytic and antidepressant effects in tMCAO mice. ZL006-05 (intravenously, 2 mg/kg/day) was given for 7 consecutive days after stroke, and SPT and NSF tests were performed on 7 and 10 days after stroke, respectively (figure 5A, schedule 2). ZL006-05 prevented stroke-induced behavioural modifications, as indicated by significantly increased sucrose water preference in the SPT and decreased latency to feed in the NSF test, compared with vehicle (figure 5I,J), and did not affect the amount of food consumption (figure 5K). These data suggest that ZL006-05 administered in the acute phase can prevent tMCAO-induced anxiety and depression. However, the permanent ischaemia of motor cortex caused by photothrombotic model did not produce depressive behaviour (online supplemental figure S7).

Pharmacokinetics and long-term toxicity of ZL006-05

As a drug for stroke and depression and anxiety disorders, whether it can cross the BBB and enter the brain parenchyma is very important. We, thus, investigated pharmacokinetics of ZL006-05 in the blood and brain of male tMCAO rats. Because parent drug is main metabolite,²⁴ we only measured the concentration of ZL006-05. After injecting a single dose of ZL006-05 (2 mg/kg, intravenously), concentration-time profiles in the blood and brain and the brain-to-plasma ratio of ZL006-05 were shown in figure 6A. The drug could cross BBB and distribute to brain tissue rapidly, with brain-to-plasma ratios increasing from approximately 1.5%–432% over time. As shown in figure 6B, the T_{max} of ZL006-05 in the

brain was same as that in the plasma, suggesting a very rapid distribution into the brain. The increase in brain-to-plasma ratios over time and the high area under concentration-time curve (AUC) in the brain were driven by that the elimination half-life of the drug in the brain was much longer than that in the blood. Moreover, similar pharmacokinetics profiles were observed in the photothrombotic stroke and sham rats (online supplemental figure S8).

Next, we investigated the long-term toxicity of ZL006-05, including body weight, food consumption, haematology, coagulation, serum biochemistry, organ weight and organ weight to terminal body weight ratio, gross anatomy and histopathological examination of major organs. ZL006-05 at dose of 10, 20 or 40 mg/kg/day was intravenously injected into male and female rats for 28 consecutive days. Except for a slightly decreased food intake in male rats in the 40 mg/kg/day group on day 3 after dosing and slightly decreased RBC and HGB in the female rats in the 40 mg/kg/day group on d 1 after the end of dosing, no other significant changes were observed (online supplemental table S2–S7 and figure S9), suggesting a high safety profile.

Moreover, we investigated the toxicokinetics of ZL006-05 in rats after a single dose or 28-day repeat dose. ZL006-05 at dose of 10, 20 or 40 mg/kg/day was intravenously injected. As shown in online supplemental figure S10 and table S8–S9, the levels of systemic exposure (AUC and C_{max}) of ZL006-05 after single dose or repeat dose were dose-dependently increased, no gender difference and accumulation were observed.

DISCUSSION

Excessive stimulation of NMDARs and the resulting nNOS activation are crucial for stroke damage. However, directly inhibiting NMDARs or nNOS can cause severe side effects.¹³ Disrupting nNOS-PSD-95 interaction can prevent stroke damage without the major side effects caused by directly inhibiting NMDARs or nNOS.¹³ Substantially decreased surface expression of GABA_ARs contributes to the lethal excitotoxicity in the acute phase of stroke.^{14–16} Positive allosteric modulation of GABA_ARs can attenuate ischaemic neuronal death.^{17,18} Thus, potentiation of GABA_AR while uncoupling nNOS-PSD-95 may synergistically prevent ischaemic injury in the acute phase of stroke. We found that, in permanent ischaemia and ischaemia/reperfusion stroke models, animals treated with ZL006-05 during the acute phase of stroke had significantly reduced infarct volume, neurological scores and substantially improved motor function (figures 1 and 2), and displayed long-term functional improvements as indicated by significantly reduced neurological deficit, sensorimotor functional and spatial learning impairments (figure 3). More interestingly, ZL006-05 had a treatment window of up to 12 hours after permanent ischaemia (figure 2). Given that only a small percentage of stroke patients arrive at the hospital within hours,

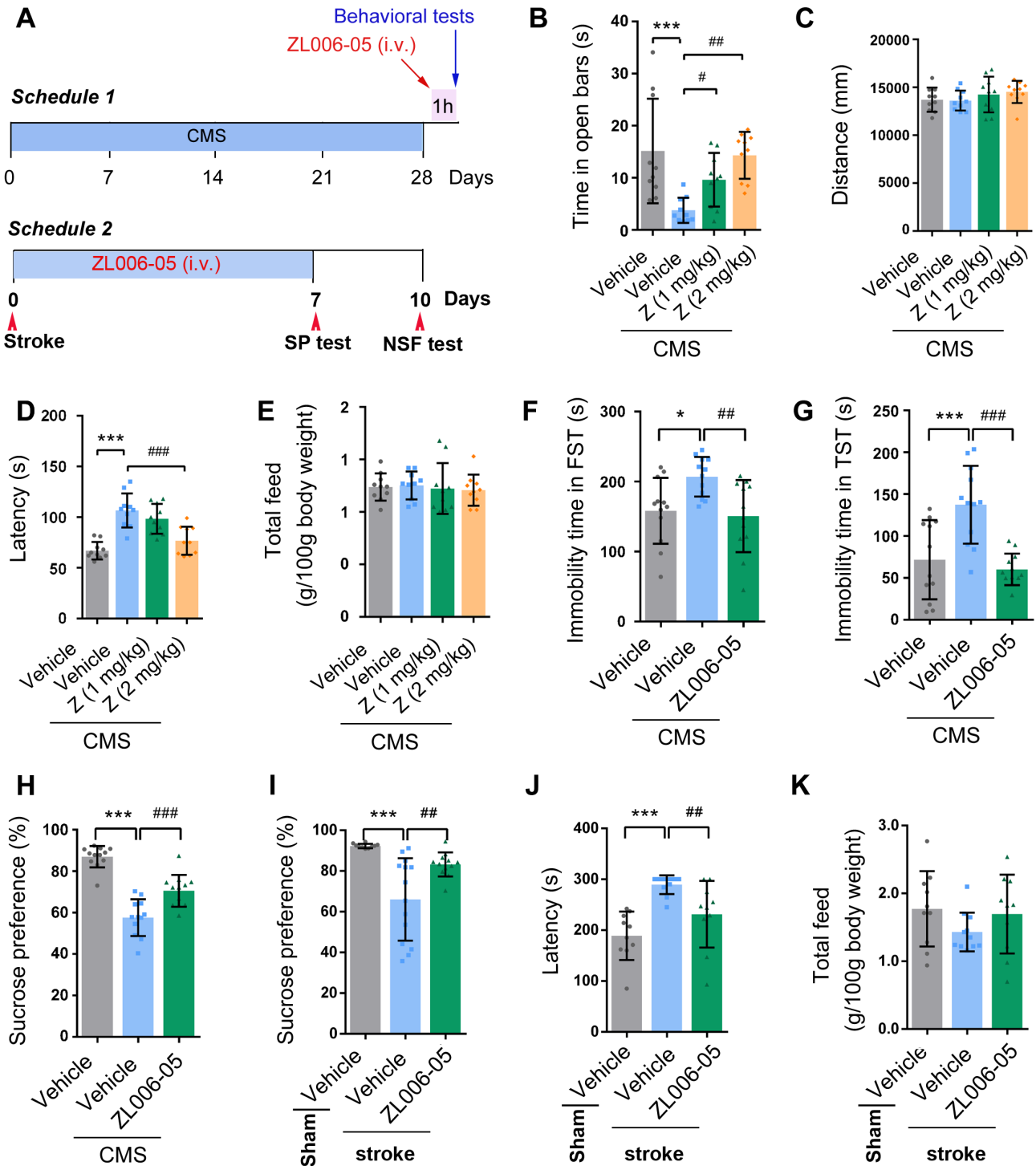
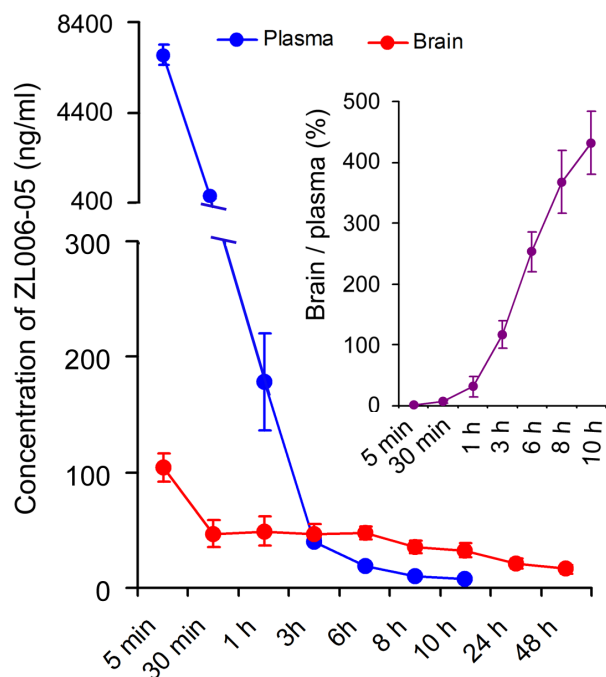


Figure 5 ZL006-05 reverses UCMS-induced anxiety and depression in a fast acting manner. (A) Experimental designs for (B–H) (upper) and (I–K) (lower). (B–E), n=10; (F–H), n=12. (B) Time spent in open arms in EPM test (one-way ANOVA, $F_{3,36}=367.122$, $***p<0.001$, $^{\#}p=0.042$, $^{\#\#}p=0.001$). (C) Locomotor activities in the open field test (one-way ANOVA, $F_{3,36}=360.997$, $p>0.05$, between groups). (D) Latency in the NSF test (one-way ANOVA, $F_{3,36}=3617.782$, $***p<0.001$, $^{\#\#\#}p<0.001$). (E) Food consumption in the NSF test (one-way ANOVA, $F_{3,36}=360.133$, $p>0.05$, between groups). (F) Immobility time in the FST test (one-way ANOVA, $F_{2,33}=5.92$, $*p=0.01$, $^{\#\#}p=0.003$). (G) Immobility time in the TST test (one-way ANOVA, $F_{2,33}=13.173$, $***p<0.001$, $^{\#\#\#}p<0.001$). (H) Sucrose preference in the SPT test (one-way ANOVA, $F_{2,33}=47.186$, $***p<0.001$, $^{\#\#\#}p<0.001$). (I) Sucrose preference in the SPT test (one-way ANOVA, $F_{2,33}=12.481$, $***p<0.001$, $^{\#\#}p=0.020$; n=10, for sham; n=14, for tMCAO+vehicle; n=12, for tMCAO+ZL006-05). (J) Latency in the NSF test (one-way ANOVA, $F_{2,28}=2811.982$, $***p<0.001$, $^{\#\#}p=0.009$; n=10, for sham and tMCAO+ZL006-05; n=11, for tMCAO+vehicle). (K) Food consumption in the NSF test (one-way ANOVA, $F_{2,28}=281.436$, $p>0.05$, between groups, n=10, for sham and tMCAO+ZL006-05; n=11, for tMCAO+vehicle). ANOVA, analysis of variance; EPM, elevated plus maze; NSF, novelty-suppressed feeding; SPT, sucrose preference test; tMCAO, transient middle cerebral artery occlusion; TST, tail suspension test; UCMS, unpredictable chronic mild stress.

A



B

PK parameters of ZL006-05
after a single 2 mg/kg intravenous dose

PK Parameters	unit	plasma	brain	B/P ratio
K_{el}	1/h	0.2492	0.0348	14%
$t_{1/2}$	h	2.78	19.89	715%
T_{max}	h	0.08	0.08	100%
C_{max}	ng/mL	6947	104	1.5%
AUC_{0-t}	h·ng/mL	2900	936	32%
AUC_{0-inf}	h·ng/mL	2930	1398	48%
$AUMC_{0-inf}$	h.h.gn/mL	1750	37844	2163%
V_{ss}	mL/kg	408	38749	9497%
CL	mL/h/kg	683	1431	210%
MRT_{0-t}	h	0.46	11.46	2500%
MRT_{0-inf}	h	0.597	27.08	4536%

Figure 6 Time-concentration profiles of ZL006-05 in plasma and brain tissue and PK parameters after a single 2 mg/kg intravenous dose in male rats. (A) Concentrations of ZL006-05 in plasma and brain tissue and the ratio of brain/plasma. $n=3$, each time point. (B) Mean pharmacokinetics parameters of ZL006-05 in brain tissue and plasma. ZL006-05 was intravenously injected immediately after reperfusion. AUC, area under curve; B, brain; P, plasma; PK, pharmacokinetics.

this large therapeutic time window has attractive clinical translational prospects.

PSD and PSA in the acute phase of stroke are independent predictors of poor functional outcomes.⁴⁻⁹ Because of slow onset,¹¹ SSRIs are not suitable for the management of depression and anxiety symptoms in the acute phase of stroke. Positive allosteric modulators of GABA_ARs have been used in the management of depression and anxiety disorders.²¹⁻²² Alpha2-containing GABA_AR is the anxiolytic and antidepressant subtype.¹⁹ We show here that (+)-borneol, a positive allosteric modulator of $\alpha 2/3$ -containing GABA_ARs,²⁴ had a fast-onset anxiolytic-like but not antidepressant-like effect (online supplemental figure S2). It has been reported that ZL006, a nNOS-PSD-95 inhibitor,¹³ has antidepressant-like effect in rodents.³⁷ However, our data showed that ZL006 did not show anxiolytic-like and antidepressant-like effects until after treating for 11 consecutive days (online supplemental figure S2), indicating a slow onset. Interestingly, ZL006-05, a dual-target drug inhibiting nNOS-PSD-95 and potentiating $\alpha 2$ -containing GABA_AR (online supplemental figure S1),²⁴ produced fast-onset anxiolytic and antidepressant effects (figures 4 and 5). Increased nNOS activity contributes to depression and anxiety.³¹⁻³³ nNOS activity in neurons depends on nNOS-PSD-95 interaction.¹³ Moreover, the activity of nNOS controls surface expression of GABA_ARs by mediating S-nitrosylation of gephyrin,³⁹ a scaffold protein responsible for postsynaptic

traffic and clustering of GABA_ARs.¹⁵⁻¹⁶ Thus, the dual targets may explain the fast-onset effect of ZL006-05. In view of this, the combination of ZL006 and (+)-borneol may also produce fast-onset antidepressant effect, which needs to be investigated in the future.

We found that ZL006-05 crossed BBB and distributed to brain tissue rapidly in sham, photothrombotic and tMCAO rats (figure 6, online supplemental figure S8). However, brain/plasma ratios of ZL006-05 in both sham and photothrombotic rats were slightly lower than that in tMCAO rats, suggesting that reperfusion may affect brain exposure of ZL006-05 slightly. The different brain exposure of ZL006-05 in different model may be caused by their different $t_{1/2}$ in the brain. The toxicokinetics study showed that the C_{max} and AUC of ZL006-05 after single dose or repeat dose were dose-dependent, without gender difference and accumulation (online supplemental tables S8 and S9). These pharmacokinetic and toxicokinetic profiles suggest that the drug has the potential for clinical translation. Long-term toxicity is often a critical factor limiting the clinical use of drugs. We found that 28-day repeat dose of ZL006-05 (40 mg/kg/day, 20–40fold of effective dose) did not affect weight gain, haematological, coagulation and biochemical indexes, organ weight and organ weight to terminal body weight ratio, excepting a transient decrease in food intake of male rats (online supplemental tables S2–S7), and did not cause gross pathological and histopathological changes

(online supplemental figure S3), suggesting a desired safety. Dissociating nNOS-PSD-95 can avoid undesirable effects caused by directly blocking NMDARs or inhibiting nNOS.¹³ It is known that α 1-containing GABA_AR in the brain mediates benzodiazepines-associated side effects, while α 2-containing GABA_AR in the brain contributes to anxiolysis and antidepressant.^{19–23} ZL006-05 inhibits nNOS-PSD-95 and selectively potentiates α 2-containing GABA_AR,²⁴ which may explain the high safety of the drug.

In sum, ZL006-05 attenuates permanent and reperfusion ischaemic injuries and promotes long-term functional recovery, with a therapeutic time window of up to 12 hours after permanent ischaemia, and produces fast-onset antidepressant and anxiolytic effects, has a good pharmacokinetic profile and high safety, indicating transformable properties.

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REFERENCES

- Feigin VL, Stark BA, Johnson CO, *et al*. Global, regional, and national burden of stroke and its risk factors, 1990–2019: a systematic analysis for the global burden of disease study 2019. *The Lancet Neurology* 2021;20:795–820.
- Ma Q, Li R, Wang L, *et al*. Temporal trend and attributable risk factors of stroke burden in China, 1990–2019: an analysis for the global burden of disease study 2019. *The Lancet Public Health* 2021;6:e897–906.
- Liaw N, Liebeskind D. Emerging therapies in acute ischemic stroke. *F1000Res* 2020;9:F1000 Faculty Rev-546.
- Kouwenhoven SE, Kirkevel M, Engedal K, *et al*. Depression in acute stroke: prevalence, dominant symptoms and associated factors. A systematic literature review. *Disabil Rehabil* 2011;33:539–56.
- Schöttke H, Gerke L, Düsing R, *et al*. Post-Stroke depression and functional impairments—a 3-year prospective study. *Compr Psychiatry* 2020;99:152171.
- Isuru A, Hapangama A, Ediriweera D, *et al*. Prevalence and predictors of new onset depression in the acute phase of stroke. *Asian J Psychiatr* 2021;59:102636.
- Rafsten L, Danielsson A, Sunnerhagen KS. Anxiety after stroke: a systematic review and meta-analysis. *J Rehabil Med* 2018;50:769–78.
- Meng G, Ma X, Li L, *et al*. Predictors of early-onset post-ischemic stroke depression: a cross-sectional study. *BMC Neurol* 2017;17:199.
- He L, Wang J, Zhang L, *et al*. Admission heart rate variability is associated with poststroke depression in patients with acute mild-moderate ischemic stroke. *Front Psychiatry* 2020;11:696.
- Legg LA, Tilney R, Hsieh C-F, *et al*. n.d. Selective serotonin reuptake inhibitors (SSRIs) for stroke recovery. *Cochrane Database of Systematic Reviews*;2021 11:CD009286.
- Li YF. A hypothesis of monoamine (5-HT) - glutamate/GABA long neural circuit: aiming for fast-onset antidepressant discovery. *Pharmacol Ther* 2020;208:107494.
- Lo EH. A new penumbra: transitioning from injury into repair after stroke. *Nat Med* 2008;14:497–500.
- Zhou L, Li F, Xu H-B, *et al*. Treatment of cerebral ischemia by disrupting ischemia-induced interaction of nNOS with PSD-95. *Nat Med* 2010;16:1439–43.
- Mele M, Aspromonte MC, Duarte CB. Downregulation of GABA_A receptor recycling mediated by Hap1 contributes to neuronal death in vitro brain ischemia. *Mol Neurobiol* 2017;54:45–57.
- Garcia JD, Gookin SE, Crosby KC, *et al*. Stepwise disassembly of GABAergic synapses during pathogenic excitotoxicity. *Cell Rep* 2021;37:110142.
- Costa JT, Mele M, Baptista MS, *et al*. Gephyrin cleavage in vitro brain ischemia decreases GABA_A receptor clustering and contributes to neuronal death. *Mol Neurobiol* 2016;53:3513–27.
- Lamtahri R, Hazime M, Gowing EK, *et al*. The gliopeptide ODN, a ligand for the benzodiazepine site of GABA_A receptors, boosts functional recovery after stroke. *J Neurosci* 2021;41:7148–59.
- Zhou C, Li C, Yu HM, *et al*. Neuroprotection of gamma-aminobutyric acid receptor agonists via enhancing neuronal nitric oxide synthase (Ser847) phosphorylation through increased neuronal nitric oxide synthase and Psd95 interaction and inhibited protein phosphatase activity in cerebral ischemia. *J Neurosci Res* 2008;86:2973–83.
- Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABA_A receptor subtypes. *Nat Rev Drug Discov* 2011;10:685–97.
- Han W, Shepard RD, Lu W. Regulation of gabaars by transmembrane accessory proteins. *Trends in Neurosciences* 2021;44:152–65.
- Deligiannidis KM, Meltzer-Brody S, Gunduz-Bruce H, *et al*. Effect of zuranolone vs placebo in postpartum depression: a randomized clinical trial. *JAMA Psychiatry* 2021;78:951–9.
- Edwards Z, Preuss CV. GABA receptor positive allosteric modulators. In: *StatPearls*. Treasure Island (FL): StatPearls Publishing, 2022.
- Ralvenius WT, Benke D, Acuña MA, *et al*. Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABA_A receptor subtype. *Nat Commun* 2015;6:6803.
- Li J, Zhang L, Xu C, *et al*. A pain killer without analgesic tolerance designed by co-targeting PSD-95-nnos interaction and α 2-containing GABA_ARs. *Theranostics* 2021;11:5970–85.



- 25 Spescha RD, Klohs J, Semerano A, *et al.* Post-Ischaemic silencing of p66Shc reduces ischaemia/reperfusion brain injury and its expression correlates to clinical outcome in stroke. *Eur Heart J* 2015;36:1590–600.
- 26 Ni H-Y, Song Y-X, Lin Y-H, *et al.* Dissociating nNOS (neuronal NO synthase) -CAPON (carboxy-terminal postsynaptic density-95/discs large/zona occludens-1 ligand of nNOS) interaction promotes functional recovery after stroke via enhanced structural neuroplasticity. *Stroke* 2019;50:728–37.
- 27 Torrente D, Su EJ, Fredriksson L, *et al.* Compartmentalized actions of the plasminogen activator inhibitors, PAI-1 and NSP, in ischemic stroke. *Transl Stroke Res* 2022;13:801–15.
- 28 Chen J, Sanberg PR, Li Y, *et al.* Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* 2001;32:2682–8.
- 29 Lin Y-H, Dong J, Tang Y, *et al.* Opening a new time window for treatment of stroke by targeting HDAC2. *J Neurosci* 2017;37:6712–28.
- 30 Komotar RJ, Kim GH, Sughrie ME, *et al.* Neurologic assessment of somatosensory dysfunction following an experimental rodent model of cerebral ischemia. *Nat Protoc* 2007;2:2345–7.
- 31 Zhou Q-G, Zhu L-J, Chen C, *et al.* Hippocampal neuronal nitric oxide synthase mediates the stress-related depressive behaviors of glucocorticoids by downregulating glucocorticoid receptor. *J Neurosci* 2011;31:7579–90.
- 32 Liu M-Y, Yin C-Y, Zhu L-J, *et al.* Sucrose preference test for measurement of stress-induced anhedonia in mice. *Nat Protoc* 2018;13:1686–98.
- 33 Zhang J, Huang X-Y, Ye M-L, *et al.* Neuronal nitric oxide synthase alteration accounts for the role of 5-HT1A receptor in modulating anxiety-related behaviors. *J Neurosci* 2010;30:2433–41.
- 34 Liang H-Y, Chen Z-J, Xiao H, *et al.* NNOS-expressing neurons in the vmPFC transform ppvt-derived chronic pain signals into anxiety behaviors. *Nat Commun* 2020;11:2501.
- 35 Luo C-X, Lin Y-H, Qian X-D, *et al.* Interaction of nNOS with PSD-95 negatively controls regenerative repair after stroke. *J Neurosci* 2014;34:13535–48.
- 36 Xu J, Wang A, Meng X, *et al.* Edaravone dextroamphetamine versus edaravone alone for the treatment of acute ischemic stroke: a phase III, randomized, double-blind, comparative trial. *Stroke* 2021;52:772–80.
- 37 Doucet MV, Levine H, Dev KK, *et al.* Small-Molecule inhibitors at the PSD-95/nNOS interface have antidepressant-like properties in mice. *Neuropsychopharmacology* 2013;38:1575–84.
- 38 Zhu L-J, Li T-Y, Luo C-X, *et al.* CAPON-nNOS coupling can serve as a target for developing new anxiolytics. *Nat Med* 2014;20:1050–4.
- 39 Dejanovic B, Schwarz G. Neuronal nitric oxide synthase-dependent S-nitrosylation of gephyrin regulates gephyrin clustering at GABAergic synapses. *J Neurosci* 2014;34:7763–8.