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Review Article

A Review of Anfibatide Studies in Animal Models with Ischemic Stroke and Cerebral Ischemia-Reperfusion Injury and in Healthy Human

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Abstract

Anfibatide is a synthetic antiplatelet thrombolysin derived from snake venom and is proposed to treat ischemic stroke and cerebral ischemia-reperfusion injury. Current standard treatments for ischemic stroke include the administration of rt-PA, a thrombolytic agent, or endovascular removal of thrombi. However, the post-treatments are often associated with the occlusion of vessels due to a lack of antiplatelet and unrecovered vessel injury. Anfibatide, as a GPIbα antagonist that interrupts the initiation of platelet aggregation caused by GPIbα-vWF binding, becomes a potential novel candidate for treating ischemic stroke due to its antiplatelet and antithrombosis effects. Key findings show that Anfibatide can significantly reduce microthrombus formation in cerebral vessels and reduce neuron apoptosis due to the release of pro-inflammatory mediators caused by ischemia or Ischemia-Reperfusion (I/R) injury. Significant improvement in neurological scores and reversal of brain structure alterations are observed in the Anfibatide-treated ischemic animal models. To facilitate the clinical development of Anfibatide, this paper aims to summarize the completed preclinical studies and the Phase I clinical trial results of anfibatide to evaluate the risks and therapeutic potentials of Anfibatide in ischemic patients.

Keywords: Anfibatide; Ischemic stroke; Cerebral ischemia-reperfusion injury; Antiplatelet; GPIbα-vWF binding; Antithrombosis

Abbreviation: rt-PA: Recombinant Tissue Plasminogen Activator; MT: Mechanical Thrombectomy; DALY: Disability-Adjusted Life Years; GP: Glycoprotein; NS: Normal Saline; FCA: Freund's Complete Adjuvant; ICH: Intracerebral Hemorrhage; β-TG: β-thromboglobulin; H&E staining: Hematoxylin and Eosin staining; SOD: Serum superoxide dismutase; GSH-Px: Serum Gutathione Peroxidase; MDA: Malonaldehyde; LDH: Lactate Dehydrogenase; NO: Nitrogen Oxides; I/R injury: Ischemia-Reperfusion Injury; EI: Edaravone-Injection; CRI: Constant Rate Infusion; PRP: Platelet-Rich Plasma; PT: Prothrombin Time; TT: Thrombin Time; aPTT: Activated Thromboplastin Time; INR: International Normalized Ratio

Introduction

In China, according to the China Stroke Statistics 2020, the number of stroke death in 2018 accounted for almost 2 million people and the number of people aged 40 or above who currently suffer or suffered from stroke accounted for 17 million [1]. The national burden of stroke is exponentially growing.

Among the subtypes of stroke, ischemic stroke is the most common subtype accounting for nearly 80% of the total stroke case [2]. Ischemic stroke is a manifestation of cerebrovascular disease. It occurs when the blood supply to the brain is blocked via thrombosis, embolism, or stenosis [3]. The core principle of treating ischemic stroke is to restore the blood flow of the affected brain region as soon as possible. Current major treatments for ischemic stroke include injection of Recombinant tissue Plasminogen Activator (rt-PA) and endovascular therapies, such as balloon angioplasty and Mechanical Thrombectomy (MT).

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However, both treatments have their limitations. The therapeutic time window of rt-PA treatment is short; and less than 3% of patients with ischemic stroke benefit. Both rt-PA and endovascular treatments have poor prognoses and high Disability-Adjusted Life Years (DALY). The mortality rate of patients after rt-PA treatment 3-6 months is high as 17.9% and around two-thirds of the patients have different levels of disability. While the DALY of post-MT treatment ranges from 29% to 58%. In addition, vascular re-occlusion is a common post-treatment complication in patients who underwent endovascular therapies. The endothelial injury that is left untreated after endovascular treatment recruits and activates blood platelets. Aggregation of blood platelets narrows the blood vessels and slows the blood flow, causing re-occlusion. Insufficient anti-platelet agents can cause re-occlusion in both post-MT and -angioplasty treatment [4,5].

To enhance the recanalization rate of treatments, anti-platelet drugs are commonly used. These drugs act on Glycoprotein (GP), a key mediator of platelet aggregation. Anfibatide, a GPIb antagonist, is an anti-platelet thrombolysin derived from the snake venom of *Agkistrodon acutus* [6]. It has anticoagulation, fibrinolysis, thrombolysis, and anti-platelet effects. It prevents GPIb from binding vWF, which facilitates platelet adhesion to the exposed collagens in the damaged subendothelial matrix. Anfibatide does not interact with any anti-coagulation factors and thus has no impact on normal blood coagulation in patients.

Several preclinical studies on the mechanism, efficacy, and safety of Anfibatide have been done on various types of animal models and show significant anti-platelet effects and good safety results. In this paper, the preclinical study results and Anfibatide-related publications are summarized and analyzed to assess the therapeutic potential of Anfibatide in treating ischemic stroke patients and its clinical development potential.

Preclinical Studies on Safety and Toxicology

Irritation Responses

Tests for intradermal injection irritation, primary skin irritation, and Draize (eye irritation) were performed on rabbits to evaluate the potential irritative characteristics of Anfibatide at different dosage levels (900U/ml and 300U/ml) in comparison with 3% ethanol and Normal Saline (NS) as positive and control groups [7].

Results from the intradermal injection irritation test (n=6) showed no observable inflammation signs, such as skin red spots and swelling at injected or surrounding tissues of the injected region, from Anfibatide-treated rabbits compared to ethanol-treated rabbits. Primary skin irritation test (n=12), in which Anfibatide and control were applied to scratched wounds of rabbits directly, showed that the Average Primary Irritation Index values in groups were zero, indicating no irritating substances in Anfibatide. In the Draize test (n=8), in which solutions were applied to conjunctional sacs of rabbit eyes as eyedrops, stimulus-responses of the eye such as tearing, hyperemia, and presence of eye secretes were parameters of eye irritation scoring and all rabbits showed zero scores. No signs of cornea turbidity or damage; hyperemia, swelling, or secretion in rabbit conjunctiva. Rabbit irises were normal. A high dose of Anfibatide did not provoke irritation responses through intradermal injection, scratched wounds, and eyedrops in the rabbits. The potential of causing acute irritation responses in humans will be low. Anfibatide is regarded as safe in terms of irritation responses.

Biocompatibility

Antihypertensive substance screening in cats, and pyrogen and hemolysis tests in rabbit models were done to evaluate the biocompatibility of Anfibatide.

In the antihypertensive substance screening test (n=9) [7], the antihypertensive reaction rate of cats at 900U/kg, 90U/kg, and 9U/kg Anfibatide intravenous injection doses were compared with histamine standard aqueous solution (0.05, 0.1, 0.15 μg/kg) as a control. Average blood pressure reduction at different concentrations of Anfibatide was lower than 2 kPa, which was lower than the control range (3-12 kPa), indicating that Anfibatide had no significant impact on the blood pressure of cats.

In the rabbit pyrogen test (n=9) [7], rabbit body temperature changes at 60, 120, and 180 min after treatment at 900U/kg and 225U/kg of Anfibatide doses were compared with subjects treated with an equivalent volume of NS [7]. The body temperature changes did not exceed 1℃ in all experimental groups, indicating that Anfibatide had no significant impact on body temperature.

In vitro rabbit hemolysis test [7] was done in comparison of 2ml of 26.25U/ml, 0.0016U/ml of Anfibatide, and an equivalent volume of NS (positive) or water (negative). 2% rabbit blood serum was directly mixed with the solutions and a spectrophotometer at 545nm was applied to determine the absorbance of the mixture. Calculation of the hemolytic rate of samples was based on the absorbance of samples, positive and negative control. The standard hemolytic rate was 5% and the hemolytic rate of Anfibatide in all testing groups was within 0-5%. Hence, Anfibatide had no significant hemolytic impact on rabbit blood.

In terms of antihypertensive, pyrogenic, and hemolytic characteristics, Anfibatide at different dosage levels showed no significant bioincompatible signs in the cat and rabbit models. There is a high chance of translating similar results onto humans.

Immunotoxicity

Acute and delayed allergic responses to Anfibatide were tested on various animal models, including guinea pig (n=32), SD rat (n=32), and KM mouse (n=60) models. Key findings from the acute allergic response test from the guinea pig model [7] showed that injection of Anfibatide with the addition of Freund's Complete Adjuvant (FCA) increased the incidence of allergic reactions and death rate. In the high-dose (99.9U/kg) Anfibatide group with FCA, the subjects showed immediate behavioral responses, such as shrugging, and ear and face scratching, followed by shivering and rapid breathing. All guinea pigs died after 5 min of injections. Without FCA, subjects showed similar immediate behavioral responses. 3/8 of the subjects showed obvious muscle spasms through the body and lying-sided. Subjects died after 7 min of injections. For the low-dose (11.1U/kg) group, subjects showed light allergic signs at around 10 min. Only one subject died after 20 min; while other subjects fully recovered from the allergic effect of Anfibatide within 10h. No severe allergy symptoms were observed in the testing group. As per the systemic allergic intensity grading standard, the score of the high-dose group with FCA was 4; the high-dose group without FCA was 2.25; and the low-dose group was 1.375. The minimum lethal dosage to the model was 11.1U/kg. these findings showed that Anfibatide had a dose-dependent allergic effect on the guinea pigs and that the addition of FCA would enhance the acute allergic response screening accuracy

and effectiveness.

In rat models [7], 4/8 of testing rats treated with high-dose (99.9U/kg) Anfibatide died 1h after injection and all rats showed different signs and levels of acute allergic reactions, such as cyanosis, deep and rapid breathing, systemic muscle impotence, and paralysis. In the pathological examination of these rats, hyperemia of the intestine was observed but no significant bleeding in the abdominal wall and systemic muscles. For the low-dose (11.1U/kg) group subjects only showed slight excitation, restlessness, and difficulty in breathing, but no subjects died. The IC50 dosage was 60U/kg (1/128 LD50); and the minimum lethal dosage was 33.3U/kg (1/230 LD50), which is 100 times the clinical dosage for humans according to weight. Though incidences of death and allergic reactions were frequent in the high-dose testing groups, allergic responses in rats were relatively mild and less likely to cause severe allergic reactions in the low-dose group which meets the clinical dosage requirement in humans.

For the mouse model [7], the allergic symptoms and death in the testing groups showed positive dose dependency and the death-to-dose ratio were 0/10, 2/10, 5/10, 5/10, and 10/10 for 0.3, 7, 11.1, 33.3, 99.9, 299.7 U/kg. The IC50 dosage was 47U/kg (1/85 LD50); whereas the minimum lethal dosage was 11.1U/kg (1/360 LD50, which was 33 times of dosage correspondence to human weight)

In regard to the delayed allergic response test (n=4) done on the guinea pig model [7] did not show allergic symptoms with a response rate of zero; while the response rate in the positive group (DNCB) was 70%. Anfibatide had no significant delayed allergic effect and similar results would be assumed in humans.

In terms of immunotoxicity, Anfibatide exerted more significant acute allergy reactions in different animal models. However, the allergy-causing dosages of Anfibatide tested in animal models far exceeded the clinical dosage for humans. Based on the findings, allergy responses would be mild and not lethal at human clinical dosage. But it is recommended that the addition of FCA would enhance immunotoxicity testing accuracy.

Hemorrhage

Intracerebral Hemorrhage (ICH) can be a risk of thrombolysis or mechanical removal of thrombus in the treatment of cerebral ischemia due to vessel damage caused by a sudden burst of blood flow after recanalization. In the mouse whole blood spectrophotometric assay [8], treatments with Anfibatide 4 μg/kg and tirofiban 0.5 μg/kg, which is a fibrinogen-dependent platelet aggregation inhibitor for acute coronary syndromes, caused an increase in hemorrhage volumes compared to positive group (middle cerebral artery occlusion) mice. But the Anfibatide-induced hemorrhage volumes (less than 10 μL) were about 50% less severe than that of tirofiban (over 15 μL), $P<0.05$, and tirofiban-treated mice (4 out of 8 mice) had much higher bleeding complications than that of Anfibatide-treated mice (1 out of 8 mice). The finding suggests that Anfibatide can cause ICH, but its risk of ICH is less than the broadly used tirofiban.

Preclinical Studies on the Effect

Antiplatelet Aggregation

Anfibatide was found to have a positive impact on reducing platelet aggregation mediators. The *in vitro* anti-platelet aggregation effect of Anfibatide was compared with that of Ticlopidine, Aspirin, and Dipyridamole in rat models. ADP, collagen, blood coagulant, and arachidonic acid were used as inducing agents. Results showed that Anfibatide suppressed platelet aggregation in a dose-dependent manner [9]. The effect of Anfibatide was comparable to Ticlopidine and stronger than Aspirin and Dipyridamole. *In vivo* rat model also showed the positive antiplatelet effect that Anfibatide reduced the amount of arachidonic acid-induced TXA2 released by active blood platelet; and increased carotid artery wall PGI2 expression level [9]. Of note, TXA2 is a platelet activator and vasoconstrictor [10] while PGI2 is an effective vasodilator and platelet activation inhibitor [11]. It is also found that a low dosage of Anfibatide (1 $\mu g \cdot kg^{-1}$) can significantly reduce the expression of fibrin (ogen) in the MCAO mouse model ($P<0.05$). The significant reduction of GPIbα ($P<0.01$) and vWF ($P<0.01$) in a cerebral ischemic mouse model after Anfibatide treatment proves the presence of Anfibatide and its inhibition on GPIα and vWF interaction reduced platelet aggregation and related mediators are due to the.

In addition, neutrophil accumulation in MCAO mice was inhibited in Anfibatide treatment [12]. A high level of MAC-1 (macrophage-1antigen) was found in the MCAO mouse brains in comparison to normal mice and leukocytes were observed infiltrated into cerebral parenchyma. However, the Anfibatide group showed a significant reduction in Mac-1 positive cells ($P<0.05$, $P<0.01$), indicating that Anfibatide inhibited the recruitment of neutrophils to the ischemic brain regions.

Immunohistochemical staining of the MCAO mice also showed a significant reduction of immunopositivity for P-selectin, a cell adhesion molecule that facilitates cell adhesion to injured sites, after Anfibatide-treatment [12,13]. This may explain the inhibition of neutrophil recruitment which is due to the reduction of P-selectin expression caused by Anfibatide administration. The findings also suggest a potential mechanism-of-action of Anfibatide in antiplatelet aggregation beyond the inhibition of GPIbα and vWF interaction.

Antithrombosis in Various Cerebral Ischemic Models

Antithrombotic effects of Anfibatide were tested *ex vivo* and *in vivo* with different mice models, including FeCl3-induced mesenteric arteriole thrombosis model, laser-induced cremaster arteriole thrombosis model, pulmonary microthrombi model, and $vWF^{-/-}$ mice models [9].

The whole blood thrombus formation assay in an *ex vivo* perfusion chamber inhibited the platelet adhesion, aggregation, and thrombus formation to a collagen-coated surface of a microcapillary glass at high shear (1,800 s^{-1}) and a low shear (300s^{-1}) rates ($P<0.01$ and $P<0.05$ respectively); only a few platelets were observed [14]. FeCl3-induced mesenteric arteriole thrombosis model results showed doubled time required for the first thrombus formation with over 20μm in diameter at the injured site ($P<0.01$) and delayed complete vessel occlusion time ($P<0.01$) with Anfibatide treatment [14]. Laser-induced cremaster arteriole thrombosis model showed a sustained low level of platelet mean fluorescence intensity (less than 1,000,000) compared to that of the control wild-type mice, which was higher than 1,000,000 throughout the experiment (240 seconds) and even peaked at 4,000,000 [14]. Study on the $vWF^{-/-}$ mice model revealed unexpected findings that Anfibatide further inhibited laser-induced thrombosis in vWF knock-out mice ($P<0.05$) than wild-type mice, suggesting antithrombotic function other than GPIb-vWF interaction [14].

Effects on Cerebral Tissues/Cells

Reperfusion injuries often occur after thrombolysis and mechanical recanalization in ischemic stroke subjects. The injuries trigger the expression of inflammatory mediators which

can cause destruction/apoptosis in brain cells and neurological deficits. Anfibatide treatment has shown improvement in the subject neurological functions and prevention of cerebral infarction in several studies. Cerebral infarction volume of I/R models treated with/without Anfibatide revealed the effectiveness of Anfibatide in preventing cerebral neurodegeneration. Studies from Jia, D. W. *et al*. [15] (cerebral focal ischemia-reperfusion injury model on the rat), Li, T. T. *et al*. [8] (focal cerebral ischemia model on MCAO mouse), Luo, S. Y. *et al*. [16] (focal cerebral ischemia tMCAO model on the rat) and Chen, C., *et al* [12] (cerebral I/R MCAO model on mice) showed consistent outcomes that Anfibatide-treated rats had a significant reduction in cerebral infarct size in comparison to the model groups, Edaravone, and Tirofiban. In Chen's study, the infarct volumes after 24h ischemic reperfusion of Anfibatide-treated (1, 2, 4 ug/kg) groups were 21.2±3.3%, 17.6±2.3%, and 11.4±3.0% respectively, while infarct volume of model MCAO mice was high as 36.5±4.4% [12]. Lesion volumes were much smaller in Anfibatide-treated MCAO mice than that in saline-treated MCAO mice [12]. Prophylactic administration of Anfibatide also reduced cerebral infarct size, and cerebral edema, and improved symptoms of neurological deficits [9].

In addition, Anfibatide-treated I/R models showed a decrease in cerebral water content and observable improvement in brain/neuron cell conditions. In Luo, S. Y. *et al*. [17] study, the cerebral water content in MCAO model mice was 82.31±4.38%, while in Anfibatide-treated mice (0.02 mg/kg and 0.01 mg/kg) was 75.84±5.69% ($P<0.01$) and 77.56±28% ($P<0.05$). Compared to model and edaravone-treated mice, Anfibatide-treated mice showed increased size in neuronal cells in the hippocampal region; only a few cells, i.e. glial cells shrank. Li, T. T. *et al*. study [8] also revealed that 2 μg/kg of Anfibatide attenuated brain damage in MCAO mice to varying degrees, such as reducing neuronal necrosis and intercellular edema. Immunohistochemical staining results of in *vitro* cultured neuron cells [18] showed that Anfibatide-treated neuron cells could reduce neuronal cell damage observed in the model group, such as an unclear nucleus, reduced neurite protrusions, edema, and incomplete cell membranes.

The above-described improvements in cerebral conditions well explained the reduction of neuron apoptosis rate and increase in neuron survival rates obtained from the studies. TUNNEL staining results [15] of cerebral focal I/R injury model rat brain tissues showed that the apoptotic cell counts were decreased by half in Anfibatide-treated rats (35.21±4.90 and 38.26±5.35 at high- and low-dose; $P<0.01$) compared to the model group rat. In OCG/Rep focal cerebral ischemia rat model [18], Anfibatide high- (73.94±7.54; $P<0.01$), middle- (68.69±6.35; $P<0.01$), and low-dose (63.58±7.49; $P<0.05$) group rats had a significant increase in neuronal cell survival rates compared to the model group rat (54.73±5.83; $P<0.01$).

Expression of Inflammatory/Apoptotic Regulators

TLR4-MyD88 Pathway Regulators

About a 5-fold of increase in TLR4 expression and a 3-fold increase in JNK and Bax expression were observed in cerebral ischemic rats compared to normal rats [19]. The binding of TLR4 with MyD88 protein activates the c-Jun (JNK) proteins that trigger a series of downstream apoptotic reactions, i.e. induction of NFκB, activation of caspase-3, and stimulation on expression of Bax, Bcl-2, and more apoptosis regulators [20]. Luo, S. Y. *et al*. (2018) study [19] revealed that Anfibatide treatment significantly reduced TLR4, JNK, and Bax expression by 30-40%, $P<0.01$; Luo, S. Y. *et al*. study [17] showed 30-40% of reduction in JNK activation, Bax and Bcl-2 expression, around 20% reduction in caspase-3 and NFκB expression. These findings revealed the inhibitory effect of Anfibatide on TLR4-MyD88 pathway regulator expression and its mechanism against neurodegeneration.

RhoA/ROCK Pathway

RhoA/ROCK pathway is triggered by the binding of TLR4 whose increased expression is correlated with cerebral ischemia as described above. RhoA triggered by TLR4 activates ROCK proteins, mainly ROCK1 and ROCK2, which stimulates downstream mediators that cause vasoconstrictions, lowering cerebral blood flow, and more pathological changes [21].

These changes increase damage to the cerebral ischemic region and neurological deficits due to neuronal loss. Studies show that Anfibatide treatment can significantly lower the levels of expression in RhoA and ROCK1/2 proteins in rat primary cortical neurons during oxygen and glucose deprivation/reperfusion injury (OGD/Rep) [18] and focal cerebral ischemia-reperfusion injury model on TLR4-siRNA rat [16]. In the OGD/Rep rat model, the ROCK1/2 expression levels in the model group were 0.91±0.13 and 0.85±0.11 respectively; while that in Anfibatide-treated rat were only 0.66±0.12, 0.61±0.10 in the middle-dose group (20 μg/mL), and 0.64±0.10, 0.59±0.13 in the high-dose group (40 μg/mL) respectively. In the TLR4-siRNA model, the Anfibatide-treated rat did not show a reduction in RhoA/ROCK expression, indicating that Anfibatide did not act directly on the RhoA/ROCK expression but indirectly via suppression of TLR4.

Pro-Inflammatory Mediators

An increase in pro-inflammatory mediators like IL-1β, IL-6, and TNFα is expected in cerebral I/R injury. In a focal cerebral ischemia rat model [16], serum IL-1β, IL-6, and TNFα levels were significantly high in the ischemic rat without Anfibatide treatment. Anfibatide treatment (2 μg/kg and 4 μg/kg) significantly decrease each expression by 20-30%. The finding shows a positive anti-inflammatory effect of Anfibatide in the ischemia model.

Other Effects

Anti-Atherosclerosis

Fat or blood clot deposition inside the cerebral vessels causes narrowing, thickening, and hardening of the arteries, which is defined as atherosclerosis. It is associated with cerebral infarction or stroke and coronary heart disease [22]. In crane quail and rabbit diet-induced atherosclerosis models [9], Anfibatide dose-dependently reduced blood serum total cholesterol and low-density lipopolysaccharides levels.

The atherosclerotic plaque was shrunken and endothelial injuries in blood vessels were also reduced. The effective dose of Anfibatide was found to be 10 mg/kg and its anti-atherosclerosis effect was better than Sodium diacetate alginate. Anfibatide could potentially treat cerebral atherosclerotic chronic ischemia patients.

Anti-hypertension

In a myocardial hypertrophy rat model [9], Anfibatide was found dose-dependently suppress myocardial hypertrophy induced by abdominal aortic ligation. Its anti-hypertension effect was found more significant than Enalapril Maleate.

Preclinical Studies on the Efficacy

Antiplatelet and Antithrombosis

Anfibatide antiplatelet efficacy was tested in the MCAO mouse model [13] for the reduction in β-thromboglobulin (β-TG), which is a chemokine secreted by activated platelets for the recruitment of platelets. The results showed that at 4 μg/kg and 2 μg/kg dose Anfibatide group, mouse serum β-TG significantly dropped by over 50% (both $P<0.01$) compared to the MCAO mice without Anfibatide treatment. The level of β-TG in Anfibatide-treated mice was also lower than that of the normal mice, indicating the high antiplatelet efficacy of Anfibatide.

The antithrombosis efficacy of Anfibatide was revealed in the histopathological assessment of MCAO mice with/without Anfibatide or TRF by Hematoxylin and Eosin (H&E) staining [12]. The number of microthrombus in the MCAO mice was over 15. With the Anfibatide treatment (2, 4 μg/kg), the number of microthrombus reduced to the range 5-10 ($P<0.01$); while microthrombus count in TRF treatment did reduce but was still over 10, indicating that Anfibatide has a higher efficacy in antithrombin formation than TRF.

Neurological Scores

Different neurological scoring systems, including Zea Longa [23], modified Bederson, and modified Neurological Severity Scores (NSS) [24], were applied to evaluate Anfibatide efficacy in neuroprotection by recording abnormal neurobehaviors. A lower neurological score indicates better neurological function.

Zea Longa score of MCAO rats with/without Anfibatide was evaluated in both Jia, D. W. *et al*. [15] and Luo, S. Y. *et al*. [17] studies. In both studies, the Zea Longa scores after treatment of Anfibatide were all lower than that of the model MCAO rats with $P<0.05$.

Bederson neurological (4-point) scale method was applied in the Li, T. T. *et al*. [8] and Luo, S. Y. *et al*. [16] studies, in which Anfibatide was tested on MCAO rats in comparison to TRF, TAK242, and Edaravone. In the Li, T. T. *et al*. study [8], Anfibatide at 4 and 2 μg/kg doses significantly reduced the score from 3 in MCAO rats to within 1-2 ($P<0.01$); while MCAO rats treated with TRF only scored within the range of 2-3. In the Luo, S. Y. *et al*. study [16], Anfibatide also reduced the Bederson score (2.25±0.71 at 2 μg/kg Anfibatide; 2.13±0.64 at 4 μg/kg) compared to the model MCAO rats (scored 3.00±0.53). It was revealed that 6 ml/kg Edaravone and 7 mg/kg TAK242 had similar effects in reducing the neurological score as Anfibatide.

In the Chen, C., et al. study [12], the neurological function of MCAO rats was evaluated with the mNSS test and the scores were consistent with previously described outcomes that Anfibatide dose-dependently reduced the mNSS score with $P<0.01$. Anfibatide 2 and 4 μg/kg performed better in the score reduction than TRF. Besides the observation of neurobehavioral difference, Nissl staining for morphological change in neuron cells was done in Chen C, et al. study [12] to evaluate improvements in cultured murine neuron injury or loss with/without Anfibatide. MCAO rats without Anfibatide treatment showed nuclear pyknosis, shrunken cell size but intercellular space enlarged, and severe neuronal loss with intact cell numbers below 50 in the cortex ipsilateral. Intact cell numbers of 2 and 4 μg/kg Anfibatide-treated rats were 100 or above ($P<0.01$), which were 4-folds of the MCAO rats; and were better than TRF-treated rats that had less than 100 intact cells. Anfibatide-treated murine neurons have higher survivability. In a TUNNEL assay [16] assessing neuronal apoptosis in the ischemic hippocampus, findings showed the number of TUNNEL-positive (apoptotic) neurons was reduced to 6.73±1.62 and 6.23±1.70 when treated with 2 and 4 μg/kg of Anfibatide respectively ($P<0.05$), compared to the model group (11.38±2.27). This finding also supported that Anfibatide is efficacious in suppressing apoptosis and reducing neuron loss.

Inflammation Markers

Serum Superoxide Dismutase (SOD), serum Glutathione Peroxidase (GSH-Px), Malonaldehyde (MDA), Lactate Dehydrogenase (LDH), and Nitrogen Oxides (NO) levels in cerebral contents are markers for oxidative stress indicating the severity of inflammation caused by cerebral ischemia or I/R injury [25]. In Jia, D. W. *et al*. study [15], blood serum SOD and GSH-Px activities in MCAO rats without treatment were 123.88±27.16 and 58.63±17.37 respectively, which were only 50% of the sham rat group. While Anfibatide dose-dependently increased the activities of serum SOD (0.02 mg/kg Anfibatide: 159.50±20.94, $P<0.05$; 0.01 mg/kg Anfibatide: 165.25±29.13, $P<0.05$) and GSH-Px (0.02 mg/kg Anfibatide: 84.63±20.69, $P<0.05$; 0.01 mg/kg Anfibatide: 80.00±20.14, $P<0.05$). In contrast, the expression level of MDA ($P<0.01$) and NO ($P<0.05$) decreased in Anfibatide treatment also in a dose-dependent manner and the efficacy of 0.02 μmol/g was comparable to that of Edaravone-Injection (EI) treatment. Jia Dewu & Luo Shenyong [18] further studied the changes in MDA and LDH expression levels with/without Anfibatide. The study findings showed similar outcomes as Jia, D. W. *et al* [15]. Compared to the model rat neuron cell with 46.89±7.88 nmol/mL MDA level ($P<0.01$) and 2748.63±482.83U/L LDH level, Anfibatide-treated neuron cells showed dose-dependent reductions in the expression levels of MDA (below 40 nmol/mL) and LDH (2400 U/L) expression level. Increased antioxidant activities and decreased oxidant expression indicated a reduction in oxidative stress and suggested efficacious suppression of cerebral inflammation by Anfibatide.

Histopathological/Structural Changes in Cerebral Ischemia

Under cerebral ischemia, neuron cells often displayed abnormal structures, such as a disordered arrangement with loosened cytoplasm, anachromasis vacuolar neuronal degeneration, swelling, pyknosis of nuclei, shrunken cell size, unclear membrane outline, karyorrhexis increased lysosomes [16]. it was found that 2 μg/kg and 4 μg/kg Anfibatide could reverse the described alterations and efficiently attenuate the extent of brain damage to neurons and tissue structures. Similar effects were observed in TAK242 and edaravone, proving Anfibatide efficacy in protecting brain structures.

Phase I Clinical Trial

94 healthy human volunteers were enrolled in a prospective, randomized, open-label Phase I clinical trial (NCT01588132), entitled First Assessment of the Glycoprotein Ib-IV-V Complex Antagonist Anfibatide in Healthy Human Volunteers, to evaluate the safety and efficacy of purified Anfibatide. The healthy volunteers were administered with either single dose bolus (dose groups include 0.33, 0.66, 1, 1.5, 2, 3, 4, and 5μg/60kg body weight) or multiple dose boluses (dose groups include 3 and 5μg/60kg body weight) of Anfibatide intravenously followed by 0.12 μg/60kg/h Constant Rate Infusion (CRI) for 24 hours. Before and after Anfibatide treatment, whole blood samples were obtained from the volunteers and infused with ristocetin

(ex vivo) for induction of platelet aggregation in human Platelet-Rich Plasma (PRP) [26].

In terms of anti-platelet efficacy, inhibition of platelet aggregation occurred immediately after infusion and was time- and dose-dependent based on the aggregometry results. The inhibition in single-dose groups gradually dropped from a level ≥80% to zero for 6 to 8h, while the inhibition in the multiple-dose groups (3.0μg/60kg + CRI and 5.0μg/60kg + CRI) sustained at more than 60% for 25h. The maximal effect (E_{max}) on platelet aggregation inhibition was generally over 80% for all groups. Anfibatide demonstrated rapid and strong inhibition of platelet aggregation in whole blood samples, and it was observed that there was no significant change in the platelet count in all the samples when comparing the before and 24h after Anfibatide administration. There were no significant changes or variations in Prothrombin Time (PT), Thrombin Time (TT), Activated Thromboplastin Time (aPTT), International Normalized Ratio (INR), bleeding time, and circulating D-dimers. The results support that Anfibatide does not affect coagulation, fibrinolysis, or prolonged bleeding time. In terms of safety, Anfibatide was well-tolerated in the healthy volunteers. There was no occurrence of serious adverse events or detection of anti-anfibatide antibodies [26].

The phase I trial results were consistent with the preclinical results that Anfibatide is safe and tolerable and has high efficacy in inhibiting platelet aggregation without affecting the normal coagulation and fibrinolysis mechanism.

Discussion

Comprehensive preclinical studies on aspects of safety, effects (and mechanism), and efficacy have been done in Anfibatide research. Next is to discuss whether these findings may provide insights/support to the in-human clinical trials of Anfibatide.

In terms of preclinical safety, Anfibatide has no irritation effects on rabbit eyes or skin. It has relatively high biocompatibility with no significant impact on blood pressure and pyrogenic changes were induced in Anfibatide treatment. The hemolytic rate of Anfibatide was under the acceptable range, below 5%. However, Anfibatide exerts acute allergenic characteristics that the number of deaths among Anfibatide-treated guinea pigs, rats, and mice was high at the doses of 99.9U/kg and 33.U/kg and the rats showed different levels of allergic responses such as paralysis, muscle spasm, cyanosis, increasing breathing rate, shrugging, face scratching, etc. The death rate was even higher with the addition of FCA. But 99.9U/kg and 33.3U/kg were far higher than the clinical dosage for humans according to weight and no animal subject died at low-dose Anfibatide (11.1U/kg). Most low-dose treated animals only showed mild to light allergic responses. Hence, if a human is treated with Anfibatide at the clinical dose, it is less likely to cause severe or lethal allergic reactions. But when testing the immunotoxicity of Anfibatide in humans, it is recommended to test with the presence of FCA to enhance the testing accuracy. Apart from immunotoxicity risk, ICH could be a concern for Anfibatide safety in humans since bleeding from the injured vessel may not be ceased due to the antiplatelet aggregation mechanism of Anfibatide. Findings showed that the hemorrhage volumes and bleeding complications were both less severe than tirofiban, a standard anticoagulant for the prevention of blood clotting. In comparison to tirofiban, the ICH risk of Anfibatide in humans is expected to be lower or at least as safe as tirofiban.

In terms of preclinical effects, Anfibatide has shown significant *in vivo, ex vivo,* and *in vitro* effects in antiplatelet, antithrombosis, neuroprotection, reduction of cerebral infarction, cerebral water contents, and pro-inflammatory mediators. Anfibatide showed therapeutic effects beyond treating cerebral ischemia and I/R injury, also anti-atherosclerosis, and anti-myocardial hypertrophy. Testing of Anfibatide effects in comparison to different current commonly used drugs such as edaravone, TAK242, tirofiban, etc., showed either similar or better performance with the drugs. Hence, the translatability of these positive Anfibatide effects on humans is expected to be high. Moreover, the mechanism of action of Anfibatide is revealed through its effects. Via inhibition of GPIbα and vWF interaction [9], and potential inhibition on the expression of P-selectin, which facilitates platelet and neutrophil adhesion [12,14], platelet aggregation and neutrophil recruitment to the ischemic region and injured vessels were significantly reduced. It leads to antithrombosis outcomes that help recanalization and reduces cerebral infarct volume. I/R injury and cerebral ischemia were also found associated with the increased expression of TLR4 which activates downstream MyD88 and RhoA/ROCK pro-inflammatory, vasoconstriction, and apoptosis activities, leading to neurodegeneration and brain structure alterations. Anfibatide significantly reduces the expression of TLR4, Bax, Bcl-2, caspase-3, NFkB, JNK, RhoA, ROCK1/2 proteins, IL-6, IL-1β, and TNF-α [17]. The reduction of apoptotic and pro-inflammatory substance expressions prevents neuron loss and minimizes cerebral damage caused by ischemia or I/R injury. With insights into the mechanism of action and preclinical evidence to support the therapeutic effects of Anfibatide, proof of concept of an early-stage clinical trial can be held with more confidence.

In terms of preclinical efficacy, 1) antiplatelet/antithrombosis, 2) neurological improvements i.e. neurological score, neuron loss, and neuron survivability 3) inflammation markers 4) brain structure alterations can be used as parameters to evaluate the therapeutic potential of Anfibatide in treating ischemia and I/R injury. Preclinical results of different studies showed consistent and significant efficacy of Anfibatide in these parameters:

For 1) antiplatelet/antithrombosis efficacy, Xu, Z. H. *et al*. [13] and Chen, C., *et al* [12] studies demonstrated that Anfibatide treatment could significantly reduce serum chemokines (β-TG) and microthrombus formation. The antithrombosis efficacy was better than that of TRF.

For 2) neurological improvements, different neurological scale methods (Zea Longa, Bederson, modified NSS) were applied to assess the neurobehavioral changes in the subjects treated with Anfibatide versus those treated without Anfibatide (treated with TRF, TAK242, Edaravone). Anfibatide was found to be more efficacious in lowering neurological scores than TRF. 2 and 4 μg/kg Anfibatide exerted similar neuroprotection effects as 7 μg/kg TAK242 and 6 μg/kg Edaravone but were effective at a lower dose. The TUNNEL assay [12] also showed more than a 40% reduction in apoptotic neuron number in Anfibatide-treated ischemic hippocampus compared to the control MCAO mice.

For 3) inflammation markers, reduction of oxidative stress and increase of antioxidants are the signs of improvement in inflammation. Studies had shown an increase in serum SOD and GSH-PX; and a decrease in MDA, LDH, and NO levels in the MCAO rat brain with statistical significance. 0.02 μmol/g Anfibatide was efficacious in decreasing MDA and LDH levels, and it was comparable with Edaravone-injection.

For 4) brain structure alterations, 2 μg/kg, and 4 μg/kg Anfibatide could reverse brain alterations and attenuate the damage to neurons. Similar effects were observed in TAK242 and Edaravone. Based on the comparison between Anfibatide and standard drugs such as Edaravone, TAK242, and TRF, the translatability of these preclinical data is largely enhanced.

The preclinical results of Anfibatide are comprehensive regarding the variety of animal models and assays. Anfibatide prevents ischemia caused by thrombus formation and prevents neuron apoptosis due to pro-inflammatory mediators induced by ischemia-reperfusion injury, which is a common complication of post-rt-PA or post-endovascular treatment. Anfibatide exerts efficacious antiplatelet and antithrombosis effects with stronger neuroprotection than TRF and a lower effective dose than edaravone and TAK242. It is recommended to take good note of acute allergy and ICH risk management during in-human safety assessment. But regarding the results of the Phase I trial, Anfibatide appeared to be safe and highly tolerable as there were no serious adverse events caused. And Anfibatide possessed a high inhibitory ability on ristocetin-induced platelet aggregation and did not impact coagulation or fibrinolysis. However, one problem raised from the Phase I trial is that the active ingredient of Anfibatide purified from the snake venom directly remains uncertain. Thus, studying recombinant Anfibatide would be a better option to evaluate the exact therapeutic efficacy. Based on the comprehensive preclinical study results and references from the phase I trial, Anfibatide is a promising drug candidate. Clinical development of Anfibatide has great potential to fulfill the unmet medical needs in ischemia stroke treatment and facilitate the antithrombotic therapy development.

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