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The failure of animal models of neuroprotection in acute ischemic stroke to translate to clinical efficacy

Sui-yi Xu
Su-yue Pan

Department of Neurology, Nanfang Hospital, Southern Medical University, GuangZhou, China

Author's address: Su-yue Pan, e-mail: suyuepan@yahoo.com.cn
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Summary

The discrepancy in results regarding neuroprotective agents in animal experiments compared to clinical trials is a major problem. While many neuroprotective agents have been proven effective in a variety of animal ischemic stroke models, none have been shown to work in phase III clinical trials. This review retrospectively summarizes the neuroprotectants selected for human randomized controlled trials (RCT) and explores the reasons behind the clinical translational failure of these agents. Here, we suggest that there are many factors (model selection, anesthetic choice, physiological monitoring, model success criteria, embolus property, reperfusion damage, infarction area, therapeutic time window, drug penetration, blood concentration, gender difference, and outcome evaluation) responsible for this phenomenon. Ultra-early treatment using a "home run" drug and multi-target therapy may be the most promising for future consideration.

key words: clinical translation | ischemic stroke | neuroprotectants | target

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Background

Scholars around the world have identified many targets and mechanisms for acute ischemic stroke. Although some of these targets may be derived from reasoning, almost all targets have studies of corresponding neuroprotective agents. The drugs used for the treatment of acute ischemic stroke can be divided into 2 categories: 1 to reduce ischemic damage by improving hemodynamics (eg, thrombolytics, antiplatelet drugs, anticoagulants, fibrinogen depleting agents, etc.) and 1 for targeting the harmful molecular targets that contribute to acute ischemic injury pathophysiology, known as "neuroprotective agents." There are numerous failed clinical trials for neuroprotectants in ischemic stroke in which the initial animal studies or even the early stage "proof of concept" trials showed promising results, but further phase II or III clinical trials were striking failures. Here, we review the representative registered neuroprotective agents (Internet Stroke Center, 2011) (Table 1) and investigate the reasons behind their clinical translational failure.

Partial Current Neuroprotectants

Calcium antagonist: Nimodipine, Quercetin

Nimodipine is a 1,4-dihydropyridine calcium antagonist. It can dilate cerebral blood vessels and reduce vasospasm after a subarachnoid hemorrhage without causing a significant expansion of peripheral blood vessels. Thus, it has a reduced impact on blood pressure. In the VENUS trial [1], 454 patients were randomly assigned to the oral nimodipine or placebo group. Treatment began within 6 hours and lasted for 10 days. There was no difference between the treatment groups. Intracellular calcium overload is facilitated by ASIC1a activation during ischemic injury. ASIC1a can be inhibited by certain flavonoids, especially quercetin [2]. Quercetin can also inhibit the acid-mediated intracellular calcium overload of synaptosomes *in vitro*.

Glutamate antagonist

The excitatory amino acid glutamate can interact with many receptors, including N-methyl-D-aspartic acid (NMDA) and á-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptors.

Non-competitive NMDA antagonists: Dizocilpine (MK-801), Dextromethorphan, and Aptiganel (CNS-1102, Cerestat)

Dizocilpine (MK-801) binds tightly to the NMDA receptor, resulting in persistent channel blockade. Dextromethorphan is a type of codeine dextral body, and its metabolites are high affinity non-competitive NMDA channel blockers. The clinical application of the above 2 drugs ended in the pilot phase. The adverse effects of these compounds include nausea, vomiting, drowsiness, hallucinations, irritability, hypotension, stupor, and even respiratory arrest. For Aptiganel (CNS-1102, Cerestat), the maximum dose of an intravenous injection is ~30 µg/kg (>15 min) in humans, and this dose is less than the effective threshold reported in many animal models. Higher doses can lead to hypertension, excessive sedation or excitement, euphoria, hallucinations, psychomotor inhibition, paranoia, and catatonia.

Competitive NMDA antagonist: CGS19755 (Selfotel)

With 2 mg/kg or less, the adverse effects include confusion, hallucination, irritability, paranoia, and insanity. This dose is much lower than the proven effective dose in pre-clinical trials. This NMDA antagonist may have a neuroprotective effect on focal ischemia, but it is also harmful because it will damage the endogenous NMDA receptor-mediated mechanism of neurons [3].

Free radical scavengers/Antioxidants: Ebselen, Tirilazad (U-74006F), Edaravone (MCI-186), Tamoxifen, Pyruvate, NXY-059

Ebselen is a type of selenium compound with glutathione peroxidase activity and can react with peroxynitrite. Tirilazad is an iron-dependent lipid non-glucocorticoid 21-amino-steroid inhibitor with only weak blood-brain barrier (BBB) penetration. Edaravone (MCI-186) is a free radical scavenger and lipid peroxidation blocker. Tamoxifen has a neuroprotective effect on middle cerebral artery occlusion (MCAO) in rats [4]. This neuroprotective effect is not due to estrogen receptor activation, but is instead a result of the anti-oxidation effect of this compound. Pyruvate is the substrate of mitochondrial oxidation in the Krebs cycle and the end product of glycolysis. It scavenges free radicals and acts as an anti-inflammatory and antioxidant. Pyruvate shows neuroprotective effects in a variety of cerebral ischemia models, but the drug administration time window is so narrow that it limits further study. The neuroprotection is transient within 3 hours after ischemic stroke [5]. NXY-059 is the neuroprotective agent that best conforms to the stroke therapy academic industry roundtable (STAIR) criteria. Human plasma concentrations can reach or even exceed the effective concentration in animal models [6], but the drug ultimately failed in human trials. There are many reasons for this phenomenon: (1) it is a polar molecule and highly water-soluble rather than fat-soluble, thus the BBB penetration and redox are poor; (2) the therapeutic time window is too long in both SAINT I and II (6 hours); and (3) NXY-059 can lead to severe hypokalemia in patients [7].

Nitric oxide inhibitor: Lubeluzole

A meta-analysis found that mortality and dependency were unaltered by this drug, but that the occurrence of cardiac

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Table 1. The registered neuroprotective agents (Internet Stroke Center, 2011).

Class/drugs	Ongoing	Completed	Trail	Phase	Total
1. Calcium channel blocker					
Nimodipine	0	23	23	III	45
Flunarizine	0	22	22	III	
2. Calcium chelator					
DP-b99 (DP-BAPTA)	1	3	4	II	4
3. Free radical scavenger/Antioxidant					
Ebselen	1	1	2	III	9
Tirilazad (U-74006F)	0	7	7	II	
4. GABA agonist					
Clomethiazole	0	2	2	III	2
5. Glutamate antagonist AMPA antagonist					
YM872 (zonampanel)	0	2	2	II	21
ZK200775 (MPQX)	0	1	1	II	
Competitive NMDA antagonist					
CGS19755 (selfotel)	0	2	2	III	
NMDA channel blocker					
Aptiganel(CNS-1102, Cerestat)	0	1	1	III	
CP-101,606 (Traxoprodil)	0	0	0	III	
Magnesium	2	6	8	III	
NPS1506	0	1	1	I	
Glycine site antagonist					
ACEA1021 (Licostinel)	0	0	0	I	
GV150526 (Gavestinel)	0	5	5	III	
Polyamine site antagonist					
Eliprodil (SL 82-0715)	0	1	1	III	
6. Growth factor					
Fibroblast growth factor (bFGF)	0	2	2	III	2
7. Leukocyte adhesion inhibitor					
Anti-ICAM antibody (Enlimomab)	0	1	1	III	2
Hu23F2G (Rovelizumab)	0	1	1	III	
8. Nitric oxide inhibitor					
Lubeluzole	0	3	3	III	3
9. Opioid antagonist					
Nalmefene (Cervene)	0	2	2	III	2

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Table 1 continued. The registered neuroprotective agents (Internet Stroke Center, 2011).

Class/drugs	Ongoing	Completed	Trail	Phase	Total
10. Phosphatidylcholine precursor					
Citicoline (CDP-choline)	2	7	9	III	9
11. Serotonin agonist					
Bayx3702 (Repinotan)	0	2	2	III	2
12. Sodium channel blocker					
Fosphenytion	0	1	1	III	4
Lubeluzole	0	3	3	III	
13. Potassium channel opener					
BMS-204352	0	1	1	III	1
14. Mechanism unknown or uncertain					
Piracetam	1	3	4	III	4

The numbers shown in sections of "ongoing," "completed," and "trial" represent the number of RCT for the corresponding drugs on the left.

conduction disorders (Q-T prolongation) increased significantly [8].

Serotonin agonists: Repinotan (BAY x3072), Piclozotan (SUN N4057)

Repinotan (BAY x3072) is a 5-HT_{1A} receptor agonist. It can inhibit the ischemia-mediated glutamate release and make the cell hyperpolarized to reduce hypoxia-induced cell depolarization. Drug administration is effective even after 5 hours of blockage in both transient MCAO (tMCAO) and permanent MCAO (pMCAO) models [9]. Piclozotan (SUN N4057) is another 5-HT_{1A} agonist, and it is effective in tMCAO models after immediate delivery.

Potassium channel openers: BMS-204352, α-linolenic acid, and Riluzole

BMS-204352 is a neuronal potassium channel activator. α-Linolenic acid and riluzole are agonists of the TREK/TRAAK K-channel family, and they are thought to be neuroprotective [10].

Unknown targets: Piracetam, Gangliosides, Melanocortin

Piracetam is thought to increase membrane fluidity, to enhance cognition and microcirculation and to act as a neuroprotective agent and anticonvulsant. GM1 ganglioside is a type of monosialoganglioside. A meta-analysis found that the beneficial evidence for GM1 on stroke is inadequate but that the incidence of Guillain Barré Syndrome (GBS) following GM1 treatment cannot be ignored [11]. Melanocortin can produce continuous protection in gerbil tMCAO models. This effect is related to the activation of the melanocortin MC4 receptor [12].

Calcium overload and excitatory amino acid toxicity are important factors in cell death. Accordingly, calcium channel blockers and glutamate antagonists are the most studied potential therapies. For decades, scientists around the world have attempted to identify the terminal target in nerve cell death. Unfortunately, this single-target neuroprotective strategy has not been successful. This suggests that the ischemic injury cascade pathway is not a series, but rather several processes running in parallel. Thus, attention is now focused on multi-target neuroprotective agents.

Multi-targets: Statin, Progesterone, Magnesium, Albumin, EPO and derivatives

The protective effect of simvastatin is dependent on its lipophilicity in the guinea pig model of MCAO [13]. It has been shown that simvastatin, lovastatin, and pravastatin can be detected in the rat cortex [14]. Atorvastatin and simvastatin acid can be esterified *in vivo*, increasing their ability to penetrate the BBB. Lipophilic statins can easily pass the BBB, but whether hydrophilic statins, such as pravastatin, can pass the BBB is unclear [15]. Whether the effect of these compounds is protective or toxic depends on the statin concentration [16,17]. Even when the concentration is within the protective range, statin therapies in stroke patients still need to be monitored. Blindly applying high-dose intensive lipid-lowering therapy will elevate cerebral microbleeds, liver enzymes, and even rhabdomyolysis.

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Progesterone, referred to as a neural steroid, can easily pass through the BBB. Neurons and glia synthesize it from cholesterol. Progesterone stimulates axon myelination and improves neuronal activity [18,19], and it is a potential modulator of $GABA_A$ receptors [20]. However, small structural changes in this compound can result in very different pharmacological effects. For instance, medroxyprogesterone acetate (MPA) has a toxic effect on neurons [19]. A systematic meta-analysis confirmed that progesterone can reduce infarct size after cerebral ischemia [21] in both tMCAO and pMCAO models [22].

Magnesium may play a protective role in a variety of ways, including the closure of the NMDA receptors, the inhibition of excitatory neurotransmitter release and the blockade of calcium channels [23]. Serum magnesium levels of 3 mmol/L or less can be well tolerated, but higher levels will inhibit heart function [24]. No severe adverse effects were reported for magnesium in a FASTMAG trial in which a 4-g loading dose of magnesium sulfate was given within 12 hours of the stroke onset followed by 16 g in the following 24 hours [25]. Magnesium sulfate does not affect tPA-mediated fibrinolytic activity. This finding supports the potential of a combination therapy using this compound for acute stroke [26].

Albumin acts on multiple targets and is considered to be neuroprotective for several reasons [27]: (1) it is the major plasma antioxidant and can fight endogenous and exogenous oxidative stress products; (2) it can mediate hemodilution at pharmacological doses; (3) it reacts with nitric oxide to form a stable S-nitroso-sulfur anhydride, which is the endothelial cell-derived relaxing factor; and (4) it decreases the deposition of red blood cells and improves microvascular blood flow in the ischemic cortex [28]. The major adverse effect of albumin therapy is pulmonary edema, which can be treated symptomatically. The prognosis when using albumin in combination with tPA was 3 times better in a high-dose albumin group compared to a low-dose albumin group, suggesting a synergistic effect [29].

The brain has a number of hematopoietic growth factors. Erythropoietin (EPO) plays a major role in neuroprotection. EPO and the EPO receptor, EPOR, can be found in the rodent and primate cerebral cortex, caudate nucleus, hippocampus, cerebellum, and hypothalamus. Astrocytes are the major source of EPO in the brain [30]. The expression of EPO and EPOR will increase in the brain following cerebral ischemia. Endogenous EPO (derived from the kidney and liver) has little effect on brain EPO utilization. A high dose of EPO can penetrate the BBB. EPO can indirectly affect the expression of brain-derived neurotrophic factor (BDNF) [31]. The long-term use of EPO can lead to serious adverse effects, including an increase in blood cell volume and viscosity, leading to ischemic stroke. The plasma half-life of asialo-EPO is quite short (1.4 min) compared with EPO. Due to its rapid degradation in plasma, Asialo-EPO will not activate the RBC. However, once it enters the BBB, the degradation rate immediately slows. It can combine with EPOR, thus exerting a neuroprotective effect [32]. Carbamoyl-EPO (CEPO) is similar to asialo-EPO in that there is no effect on RBC, but CEPO shows the same neuroprotection as EPO [33].

The Reasons Behind Clinical Translational Failure

In addition to the attributes of the individual neuroprotective agents, the translation from the experimental phase to clinical trials may have weaknesses. These weaknesses may explain why drugs are effective in animals but not in humans.

Model selection

The hypoxia tolerance and neural regeneration ability of different species are different. When comparing the genetic sequence of humans and rats, approximately 90% of the gene sequence is conserved. Although less than 1% of the genes are different, the protein expression of these genes should not be overlooked [34], meaning that hypoxia tolerance and reaction to neuroprotective agents, in addition to the processes of transcription and metabolism, are probably different between humans and rats. The rats used in stroke models are commonly young and healthy. However, stroke patients are usually aged and often present with multiple diseases. Therefore, an animal stroke model must simulate the human stroke-related situation. Aged primate animal models with complications are more in line with the human clinical situation. The factors that need to be simulated include age, hypertension, diabetes, hyperlipidemia, smoking, and drinking.

Anesthetic choice

Many anesthetics have a certain neuroprotective effect [35]. The anesthesia exposure time of the neuroprotection group tends to be longer than that of the control group. This means that the dose of anesthetic received is higher in the former group. Dexmedetomidine is an α_2 adrenergic receptor agonist that has a potential neuroprotective effect. It can reduce the number of coma days in mechanically ventilated patients compared with midazolam [36,37]. Some inhaled anesthetics (e.g., xenon, a type of NMDA receptor antagonist) show neuroprotective effects both *in vivo* and *in vitro*. Therefore, to avoid potential anesthetic interference, improved handling of the drug screening and statistical analysis of confounding factors are needed.

Physiological monitoring

Blood pressure dictates the perfusion pressure, and the latter determines cerebral blood flow, and local drug concentration.

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The brain temperature will drop after a large number of liquid administrations. Hypothermia is neuroprotective. The liquid will also dilute blood and change blood pH. These are all confounding factors for the assessment of neuroprotective agents. Therefore, the STAIR criteria suggest the monitoring of animal physiological changes. Unfortunately, most trials fail to meet these criteria.

Model success criteria

Using rat models as an example, most MCAO models require the insertion of a thread into X artery at X length for X hours without the detection of cerebral blood flow (such as laser Doppler). Are the models really successful? Is an infarct actually formed? Is the middle cerebral artery completely blocked? There are no objective evaluation criteria to address these questions. Therefore, dynamic monitoring of the MCA cerebral blood flow is needed.

Embolus property

The homogeneity of the MCAO model is fairly good. For example, in the TOAST classification, the human cerebral infarction is relatively complex and the embolus property is diverse (e.g., fat embolism, cartilage embolism, bacterial embolism, gas embolism, tumor embolism, etc.) Therefore, neuroprotective agents that have been shown to be effective on cerebral thrombosis are not necessarily effective on cerebral embolisms.

Reperfusion damage

Decreased cerebral blood flow is the initial cause of brain damage. The ischemia is usually reversible in animal reperfusion models, but this is only occasionally the case in clinical trial subjects. Few pre-clinical trials are able follow the relevant STAIR criteria – the application of neuroprotective agents in both the pMCAO model and the tMCAO model simultaneously. To satisfy the criteria, the agents should work in both models. However, the permanent model will occur in a certain degree of reperfusion subjects because the blood clot can only be partly dissolved. Many neuroprotective agents are more effective in the tMCAO model compared with the pMCAO model, so researchers tend to prefer the former.

Infarction area

The infarction is mostly confined to the cortex in rat models, while human ischemic strokes are often located in the white matter. Whether the ischemic area is located in gray or white matter impacts the outcome. An ischemic lesion in the deep white matter means a poor outcome due to lack of blood supply. The neuroprotection of white matter may require the repair of the axon myelin sheath, while neuroprotection in gray matter may be more focused on the protection of neurons themselves. The patients in the SAINT trials are diverse, and 25% of the infarctions contain subcortical or white matter ischemic stroke. The patients who do not meet the requirement of homogeneity should be removed. For example, NXY-059 is not effective on white matter in pre-clinical trials [38]. Thus, neuroprotective agents should be effective for both cortical and subcortical infarctions in order to meet STAIR criteria. The targeted selection of neuroprotective agents according to different infarction locations may be beneficial.

Therapeutic time window

The drug is often administered immediately after the blockade of the vessel, or even before stroke onset in some animal experiments. This is incompatible with the clinical progression of the disease. There is no evidence that a neuroprotective agent can be effective on acute ischemic stroke beyond 6 hours, and the function is likely to be negatively correlated with the administration time. Many of the animal models demonstrated optimal benefit at a timeframe that was not practical for clinical stroke intervention (Figure 1) such as either pre-treatment or within 90 minutes of the infarction. Because the time needed to diagnose patients in a clinical trial and obtain an MRI in acute stroke is relatively long, most subjects can only begin intervention at the end of the time window. The ischemic penumbra is key, while dead neurons mean nothing. Thus, the time window for clinical trials should be as long as possible and be consistent with animal experiments.

Drug penetration

Does a neuroprotective agent really reach the target organ? The best way to answer this question is to detect the labeled drug via PET or to measure the drug concentration in the CSF after intravascular administration. However, in the rat, which is the typical stroke model, it is hard to collect CSF. Drug penetration is not mentioned in most neuroprotection studies. Some studies have noted that the multi-drug resistant transporter protein can prevent drugs from penetrating the BBB [39]. Some adverse effects of the drugs suggest that they do get through the BBB (e.g., drowsiness, hallucinations, sedation, and irritableness in glutamate antagonists). Conscious patients often cannot tolerate these adverse effects and stop taking the neuroprotective agent. However, if the patients are unconscious, can the drug be reconsidered?

Blood concentration

The effective dose of a neuroprotective agent in an animal model is quite large when it is converted to a human dose. Furthermore, the adverse effects will increase accordingly, and the drug may even be fatal. One way to address this is to change

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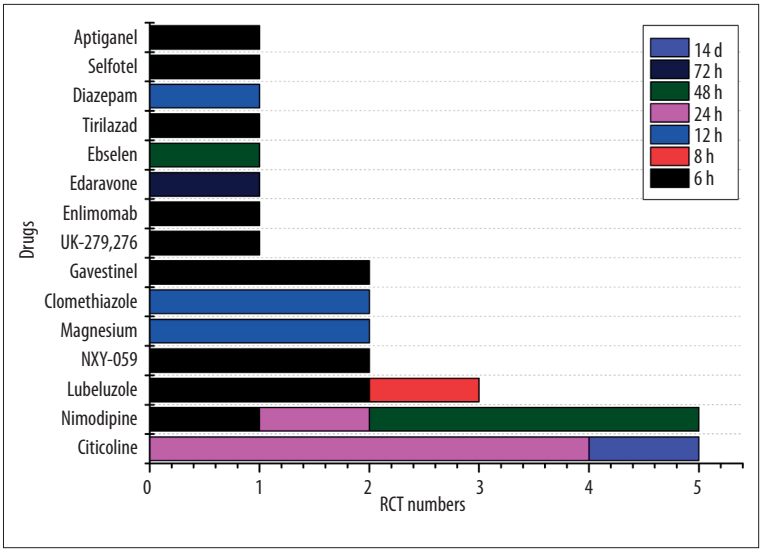

Figure 1. The time window of partial neuroprotectants (Internet Stroke Center, 2011).

the route of drug administration from the peripheral vein to a more selective intra-arterial local administration. Thus, the local drug concentration can be greatly increased and the peripheral drug concentration will be greatly reduced so that a number of peripheral adverse effects (such as heart and liver function) will also decrease accordingly. As a result, the neuroprotective agents that cannot be tolerated due to their peripheral adverse effects may be reconsidered as viable treatments.

Sex difference

To avoid the interference of the animal hormone cycle, most studies use male animals. However, Du et al. found that cultured male neurons are more sensitive to NMDA and nitric oxide, while female neurons are more sensitive to the inhibition of caspase 3. Male OGD neurons are more likely to release apoptosis-inducing factor (AIF) from mitochondria into the nucleus, while female neurons are more likely to release cytochrome C from the mitochondria and activate caspase 3. The AIF-knockout Harlequin male rat was able to tolerate ischemic injury, while the female was not [40]. Male neurons are more vulnerable than female neurons to the phagocytosis caused by nutrition deficiency [41]. The protective effect of dextromethorphan on ischemia is stronger in male rats, but EPO is stronger in females [42,43]. Animal experiments often use only male animals, while clinical trials incorporate either male or female patients. If males and females differ in their cellular pathways during stroke damage, does it suggest that a distinction should be made between the treatment strategies? To address this, clinical studies should include layered subgroups.

Outcome evaluation

The cerebral infarction area is an important means of assessment in animal experiments. In contrast, neurological function and quality of life are more important in humans. If the ischemic penumbra is used as the assessment, patients with large ischemic penumbras should be chosen. The mismatch between DWI and PWI is by far the more practical means of evaluation [44]. However, if it is a progressing stroke, the development of an ischemic penumbra or infarct volume will weaken the neuroprotection [45]. Animals are often evaluated shortly after drug intervention. On the other hand, long-term follow-up visits to assess the treatment outcome seem more important than short-term relief in humans. Thus, the follow-up of clinical trials should be of a sufficient length.

Conclusions

Intravenous tPA thrombolysis is the standard treatment for an ischemic stroke in which symptoms begin within 3~4.5 hours. Neuroprotective agents have a very short time window of effectiveness after an ischemic stroke. The use of ultra-early "home run" drugs may be helpful in pre-hospital therapy. However, they must be combined with thrombolytic agents, as are some large-scale experimental designs. However, such a combination may not always result in synergy. For instance, the combination of NXY-059 and tPA had only a limited benefit [46]. In SAINT I and SAINT II trials, the patients receiving this combination showed even worse outcomes than the ones treated with tPA alone. Do the two drugs offset one another? Does the thrombolytic agent take up the time window of the neuroprotective agent? There is very little research data regarding the drug chemical structure and potential interactions. Some test results may be negative, but the research data were never released and were reported as "unfinished" instead [47].

Neuron protection *per se* is not enough. Astrocyte maintenance may be the basis of normal neurovascular function. When

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assessing the neurovascular unit at the macroscopic level, early joint thrombolysis multi-target neuroprotection, intravascular hypothermia, and oxygen therapy may be the future direction. We also need to pay attention to endogenous repair mechanisms following a stroke [48]. The most efficient neuroprotectant may not be a single compound. Rather, similar to Chinese medicine, it may involve multi-target therapy. According to the different basic complications, it may be necessary to adjust the prescription. This requires an in-depth study of the ischemia cascade damage pathophysiology. We should be familiar with the various targets and the relationship between the upstream and downstream components to fully explore the best approach for neuroprotection. Will the components interact in the prescription? Should the appropriate compounds be administered together or sequentially? The Bayesian method may help us screen neuroprotective agents [49]. Ultra-early treatment using a "home run" drug and multi-target therapy may be most promising for future consideration. There is still a long way to go.

Conflicts of interest statement

None declared.

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