

# Reperfusion and cytoprotective agents are a mutually beneficial pair in ischaemic stroke therapy: an overview of pathophysiology, pharmacological targets and candidate drugs focusing on excitotoxicity and free radical

Xiumei Xu, Mingyu Chen, Dongya Zhu 

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School of Pharmacy, Nanjing Medical University, Nanjing, Jiangsu, China

## Correspondence to

Dr Dongya Zhu;  
dyzhu@njmu.edu.cn

## ABSTRACT

Stroke is the second-leading cause of death and the leading cause of disability in much of the world. In particular, China faces the greatest challenge from stroke, since the population is aged quickly. In decades of clinical trials, no neuroprotectant has had reproducible efficacy on primary clinical end points, because reperfusion is probably a necessity for neuroprotection to be clinically beneficial. Fortunately, the success of thrombolysis and endovascular thrombectomy has taken us into a reperfusion era of acute ischaemic stroke (AIS) therapy. Brain cytoprotective agents can prevent detrimental effects of ischaemia, and therefore 'freeze' ischaemic penumbra before reperfusion, extend the time window for reperfusion therapy. Because reperfusion often leads to reperfusion injury, including haemorrhagic transformation, brain oedema, infarct progression and neurological worsening, cytoprotective agents will enhance the efficacy and safety of reperfusion therapy by preventing or reducing reperfusion injuries. Therefore, reperfusion and cytoprotective agents are a mutually beneficial pair in AIS therapy. In this review, we outline critical pathophysiological events causing cell death within the penumbra after ischaemia or ischaemia/reperfusion in the acute phase of AIS, focusing on excitotoxicity and free radicals. We discuss key pharmacological targets for cytoprotective therapy and evaluate the recent advances of cytoprotective agents going through clinical trials, highlighting multitarget cytoprotective agents that intervene at multiple levels of the ischaemic and reperfusion cascade.

## INTRODUCTION

To date, stroke remains the second-leading cause of death and the leading cause of disability in much of the world with many people left with impaired motor function. In particular, China faces the greatest challenge from stroke in the world, with 3.94 million new stroke cases in 2019, since the population is aged quickly.<sup>1</sup> Stroke therapeutics based on modern medical theory began in the 1980s. Two critical mechanistic discoveries,

the ischaemic penumbra and the excitotoxic hypothesis,<sup>2</sup> are the landmark for stroke treatment. Since then, the gradual elucidation of the molecular mechanisms underlying ischaemia and ischaemia/reperfusion (I/R) has led many pharmaceutical enterprises to carry out rational design of antistroke drugs. Based on the pathophysiology of acute ischaemic stroke (AIS), directly targeting the ischaemic cascade in the ischaemic region can theoretically rescue the ischaemic injury. Unfortunately, despite targeting different components of the ischaemic cascade and showing promising effects in animal models of stroke, no cytoprotective agent has a clear and reproducible efficacy on primary clinical end points in clinical trials.<sup>3</sup> More than 40 years of translational failure makes the search for new treatments imperative.

Recently, clinical trials demonstrated that intravenous thrombolysis and endovascular thrombectomy in the acute time window to rescue ischaemic penumbra can significantly improve the outcome in patients with AIS due to large vessel occlusion,<sup>4</sup> suggesting that reperfusion is crucial for the good outcome of ischaemic stroke. However, there are many limitations to thrombolysis or endovascular thrombectomy.<sup>5</sup> So far, only <50% of the patients treated with recombinant tissue plasminogen activator (tPA) within 4.5 hours time window have achieved a complete reperfusion by 24 hours, and up to 40% of the patients who had a stroke remain severely disabled or died. Endovascular embolectomy can extend the time window to 24 hours after AIS. Even so, only ~3% of patients are qualified for the thrombectomy, and among the patients receiving thrombectomy, many of them still experience some degree of

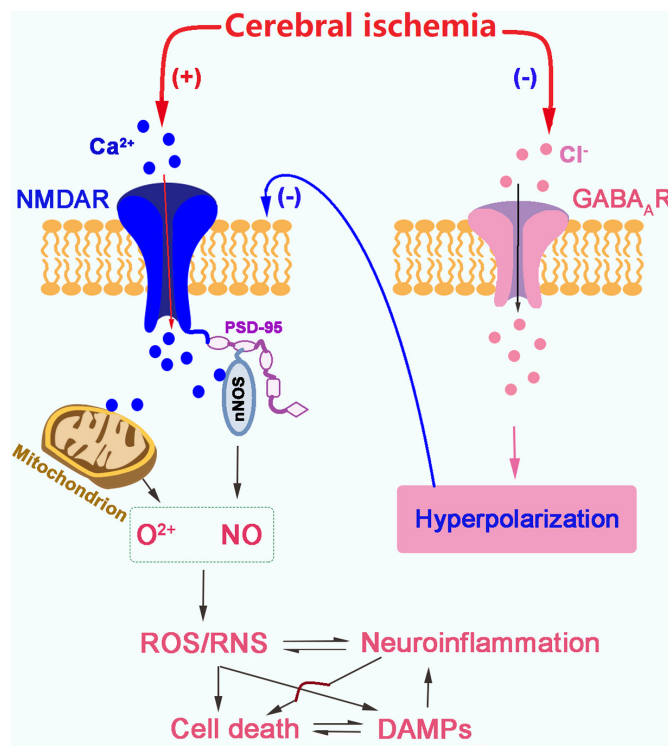
neurological deficits following the therapy. Moreover, reperfusion of the ischaemic tissue is not risk-free, often causes haemorrhagic transformation (HT) and blood–brain barrier (BBB) disruption.<sup>6,7</sup> The effect of thrombolysis and thrombectomy on functional outcome largely depends on the degree of recanalisation. The ischaemic penumbra, severely hypoperfused, electrically silent, at risk but not yet infarcted tissue, is an important mediator between functional outcome and recanalisation degree.<sup>8</sup> Cytoprotective agents might benefit stroke outcome either by preventing detrimental effects of ischaemia and thereby ‘freezing’ ischaemic penumbra before reperfusion, or by preventing reperfusion injuries, including but not limited to BBB disruption and HT after endovascular therapy.<sup>3</sup>

In the following sections, we will discuss the pathophysiological events occurred in the acute phase of AIS and review the research progress of key therapeutic targets and representative cytoprotective agents for preventing I/R injury in the acute phase, as well as for promoting stroke recovery in the subacute and repair phase. Due to the complex pathophysiology of AIS, we will focus on excitotoxicity and free radicals rather than a systematical discussion.

### Pathophysiology of stroke

In the infarcted core after AIS, cell necrosis and tissue loss occur rapidly, while the ischaemic penumbra can survive for several hours, or even days,<sup>9</sup> providing a time window for the treatment of AIS. Several key pathophysiological events causing ischaemic cell death within the penumbra have been identified, including excitotoxicity, oxidative and nitrosative stress and neuroinflammation (figure 1). Excitotoxicity caused by the N-methyl-D-aspartate receptor (NMDAR) overactivation-mediated excessive  $\text{Ca}^{2+}$  influx and the resulting interaction between neuronal nitric oxide synthase (nNOS) and postsynaptic density 95 (PSD-95) is a critical mechanism underlying ischaemia-induced neuronal death, because ischaemia-induced nNOS-PSD-95 interaction leads to a large amount of nitric oxide (NO) production within minutes after ischaemic stroke.<sup>10,11</sup> Although high concentrations of NO are toxic to brain tissue, the intra-vessel NO generated by the activation of the endothelial NOS may have protective effects in acute stroke. The activation of  $\gamma$ -aminobutyric acid A receptor ( $\text{GABA}_A\text{R}$ ) causes hyperpolarisation, and then blocks the depolarisation-gated  $\text{Ca}^{2+}$  influx,<sup>12</sup> therefore, inhibiting excitotoxicity. Unfortunately, however, acute excitotoxic insults lead to  $\text{GABA}_A\text{R}$ s loss from the synapse, resulting in drastically reduced GABAergic inhibition.<sup>12,13</sup>  $\text{GABA}_A\text{R}$  agonists have been shown to reduce infarct size and improve functional outcome in animal models of stroke,<sup>14</sup> and drugs can protect against ischaemic neuron death by preserving  $\text{GABA}_A\text{R}$  function.<sup>15</sup>

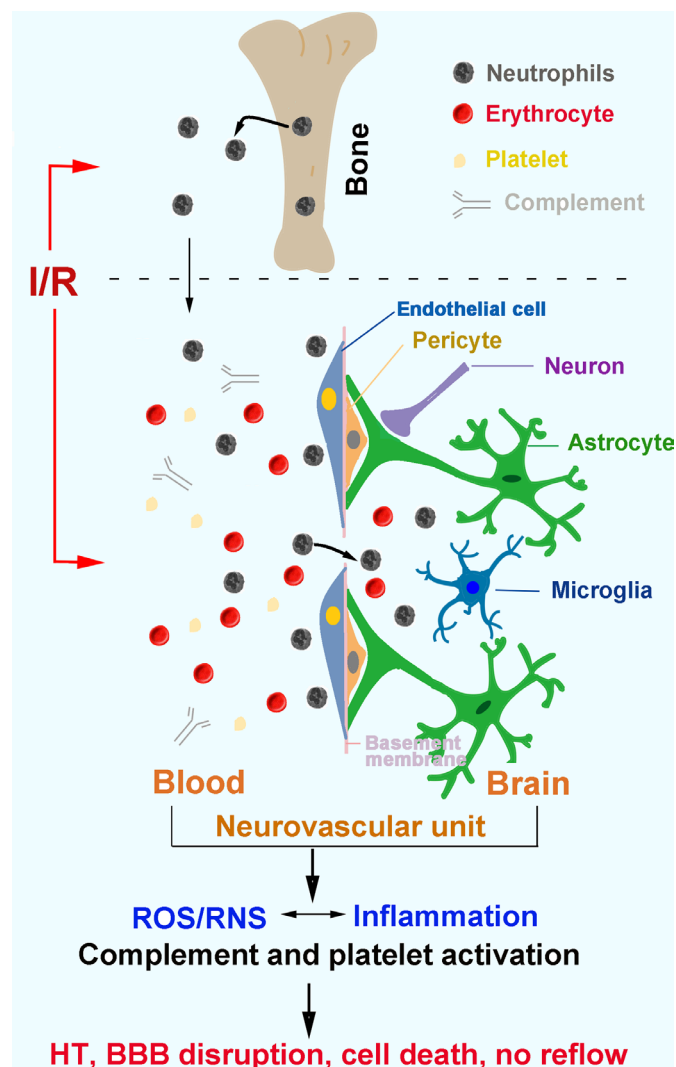
Cortical spreading depression (CSD), a slowly propagating wave of altered brain activity that involves coordinated neuronal and glial depolarisation in a regenerative manner across the brain tissue, increases neuronal



**Figure 1** Schematic representation of key mechanisms underlying ischaemic injuries after stroke. DAMPs, damage-associated molecular pattern molecules;  $\text{GABA}_A\text{R}$ ,  $\gamma$ -aminobutyric acid A receptor; NMDAR, N-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PSD-95, postsynaptic density 95; RNS, reactive nitrogen species; ROS, reactive oxygen species. (+) activation, (-) inhibition.

excitability and facilitates synchronisation of neuronal discharges, contributing to the enlargement of infarct volume.<sup>16,17</sup> In AIS patients, CSD-like events occur repetitively for many hours and days after stroke onset.<sup>18</sup> CSD and related events are significant sources of neuronal  $\text{Zn}^{2+}$  release from nerve terminals.<sup>16</sup>  $\text{Zn}^{2+}$  is released together with glutamate into the synaptic cleft and its concentration can be increased up to 100  $\mu\text{M}$  following ischaemic brain insult.<sup>19</sup> The activation of NMDAR by glutamate serves as the crucial link that allows CSD initiation and propagation. GABA-mediated inhibition can prevent this process.<sup>17</sup> In contrast,  $\text{Zn}^{2+}$  facilitates this process, because it nullifies the GABA response by non-competitively plugging the extracellular end of the pore to block chloride conductance,<sup>20</sup> and influences  $\text{GABA}_A\text{R}$ -mediated synaptic transmission.

The NMDAR overactivation and/or  $\text{GABA}_A\text{R}$  dysfunction leads to the activation of a series of intracellular signalling cascades, resulting in mitochondrial dysfunction, energy failure and excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS and RNS induce the release of damage-associated molecular pattern (DAMP) molecules, and then, the DAMP molecules together with ROS and RNS trigger inflammatory responses through activation of



**Figure 2** Schematic representation of key mechanisms underlying reperfusion-induced injuries after stroke. BBB, blood–brain barrier; HT, haemorrhagic transformation; I/R, ischaemia/reperfusion; RNS, reactive nitrogen species; ROS, reactive oxygen species.

microglia, BBB damage and peripheral immune cell infiltration, resulting in secondary progression of infarction.<sup>21</sup> Therefore, in the acute phase, in addition to NMDAR, GABA<sub>A</sub>R is another important target for inhibiting excitotoxicity and CSD.

Recanalisation of occluded vessels through thrombolysis and/or thrombectomy can restore blood flow to ischaemic tissue, providing oxygen via red blood cells, thus facilitating functional recovery from stroke.<sup>4</sup> Despite successfully removing clot in large vessel occlusion stroke, ~50% of patients have an unfavourable clinical outcome,<sup>5</sup> because reperfusion may result in reperfusion injury. I/R can trigger pathophysiological events different from only ischaemic insults (figure 2). Because of lower concentration of antioxidative agents in ischaemic cells, reperfusion causes the overgeneration of ROS and RNS, resulting in endothelial dysfunction, DNA damage, local inflammatory responses and cell death.<sup>22</sup> Reperfusion also results

in complement and platelet activation, neutrophil recruitment and activation, BBB disruption and HT.<sup>6 7 23–25</sup> In addition to BBB disruption and HT, tissue no reflow is another adverse event after clot removal and vessel recanalisation.<sup>24</sup> No reflow after recanalisation affects not only tissue survival but also cytoprotective drugs delivery to tissues. Moreover, reperfusion also causes NMDAR overactivation. In a canine model, ischaemia was induced by 10 min cardiac arrest, followed by restoration of spontaneous circulation, and investigators found that the binding of [<sup>3</sup>H] glutamate to NMDAR increased 50%–200% at 0.5–4 hours after the start of reperfusion, suggesting sharply increased NMDAR activity.<sup>26</sup> I/R induces large, rapid and sustained increases in the tyrosine phosphorylation of NMDAR GluN2 subunits,<sup>27</sup> which may explain reperfusion-induced NMDAR activation. I/R also induces PSD-95-nNOS interaction, a critical signalling mediating excitotoxicity.<sup>10</sup>

### Key pharmacological targets

#### NMDARs

Excitotoxicity mediated by NMDARs has been at the centre stage of stroke research. NMDARs and  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPA) are two main subtypes of ionotropic glutamate receptors. At the resting state, the channel pores of NMDARs are normally blocked by extracellular Mg<sup>2+</sup>. Glutamate released from presynaptic sites activates AMPARs, leading to a partial depolarisation in the post-synaptic membrane sufficient to remove the Mg<sup>2+</sup> block from NMDARs, and therefore, resulting in NMDARs activation. Although the NMDARs activation is critical for the normal physiological processes in neurons, overactivation of NMDARs plays a major role in initiating ischaemic cell death, called as excitotoxicity.<sup>10</sup> NMDAR antagonists are divided into three categories according to different target sites: non-competitive antagonists blocking ion channels, competitive antagonists blocking glutamate recognition site and glycine antagonists blocking the glycine recognition site. Seven genes encode the NMDAR subunits: a single *GRIN1* gene encodes GluN1, four *GRIN2* genes encode GluN2A–D and two *GRIN3* genes encode GluN3A–B.<sup>28</sup> Structurally, NMDAR is a tetrameric complex composed of two glycine-binding GluN1 and two glutamate-binding GluN2 subunits. GluN2A-containing NMDAR highly expressed at the synapse and GluN2B-containing NMDAR enriched in the extrasynaptic sites are the two predominant types of NMDARs in the adult forebrain, but they play different roles in response to ischaemic insults, the activation of former can prevent ischaemic injury, while the activation of later leads to neuronal apoptosis.<sup>29</sup>

#### $\gamma$ -aminobutyric acid A receptor

GABA<sub>A</sub>Rs are heteropentamers ion channels made up from 19 known subunits, including  $\alpha$ 1–6,  $\beta$ 1–3,  $\gamma$ 1–3,  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$  and  $\rho$ 1–3.<sup>30</sup> Most of GABA<sub>A</sub>Rs in the brain are composed of two  $\alpha$ , two  $\beta$  and one  $\gamma$ 2 subunit. It has been



known that the sedative, anterograde amnesic, addictive properties and in part the anticonvulsant actions of benzodiazepines (BDZs) are mediated by  $\alpha$ 1-containing GABA<sub>A</sub>R,  $\alpha$ 2-containing GABA<sub>A</sub>R is an anxiolytic subtype,  $\alpha$ 3- and  $\alpha$ 5-containing GABA<sub>A</sub>Rs mediate the myorelaxant action,  $\alpha$ 2-containing and  $\alpha$ 3-containing GABA<sub>A</sub>Rs are critical components of pain control, and the development of tolerance to the sedative action of BDZs has been linked to  $\alpha$ 5-containing GABA<sub>A</sub>R.<sup>30–32</sup> Therefore, from a safety perspective,  $\alpha$ 2-containing GABA<sub>A</sub>R may be the best target for stroke treatment among GABA<sub>A</sub>Rs.

### Postsynaptic density 95

PSD protein (PSD) is a disc-shaped protein complex located in the postsynaptic membrane specialised for postsynaptic signal transduction and processing.<sup>10 11</sup> Four PSD family members are identified according to their molecular weight, including PSD-95, PSD-93, synaptic-associated proteins 97kDa (SAP-97) and 102kDa (SAP-102). The PSD-95 contains a Src-homology-3 domain, a guanylate kinase domain and three PSD95/Disks large/Zona occludens-1 (PDZ) domains. The carboxyl terminal of GluN2A or GluN2B contains a PDZ motif (–ESDV) that binds PDZ1 or PDZ2 of PSD-95, and the PDZ2 of PSD-95 also interacts with an internal PDZ motif of nNOS ( $\beta$ -finger) at residues 108–111 (–ETTF).<sup>33 34</sup> The GluN2B constitutively bind to PSD-95 under physiological conditions, while the increased nNOS-PSD-95 interaction is caused by ischaemia-induced translocation of nNOS from the cytoplasm to the membrane. Moreover, the nNOS-PSD-95 interaction leads to NO production only, whereas GluN2B-PSD-95 interaction may affect multiple signal pathways due to that the coupling protein of PSD-95 is not limited to nNOS.<sup>10 33 34</sup> Thus, for the treatment of ischaemic stroke, nNOS-PSD-95 may be a more selective target compared with GluN2B-PSD-95.

### Nicotinamide adenine dinucleotide phosphate oxidase

ROS activates microglia and triggers a series of inflammatory reactions. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) is a group of seven ROS-generating enzymes, including NOX1 to 5 and dual oxidase 1 and 2, and its main activity is to catalyse the production of ROS by transferring electrons from NADPH to oxygen. NOX4 is specifically involved in cerebral ischaemia but not in cardiovascular and peripheral vascular ischaemia. Endothelial NOX4 can destroy the BBB, whereas neuronal NOX4 can directly lead to auto-toxicity and apoptosis. NOX2 is also involved in ischaemic stroke and its inhibition or deficiency induces a neuroprotective effect over various stages of cerebral ischaemia reperfusion. Although the role of NOX1-produced ROS is reportedly found in an increasing number of diseases, the exact role of NOX1 in I/R injury is still unclear. Since NOX5 does not exist in rodents, the study of NOX5 in rodent disease models has been limited. Recently, the mice expressing human NOX5 display increased ROS production and lead to BBB leakage and impaired

neurological function after cerebral I/R,<sup>21 35</sup> indicating that NOX5 may be a valuable target for preventing I/R injury in AIS patients.

### Nuclear factor E2-related factor 2

Nuclear factor E2-related factor 2 (Nrf2), a member of the cap'n'collar family of transcription factors, regulates the expression of a great number of antioxidant and/or defence proteins. Nrf2 can be activated by the classical Kelch-like ECH-associated protein 1 (Keap1)-dependent and Keap1-independent pathway. Elevated oxidants (ie, H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>•−</sup>) after I/R activate Nrf2, and the upregulation of Nrf2 activity promotes the expression of antioxidant response element-regulated genes involved in the control of free radical levels, such as heme oxygenase-1 and superoxide dismutase, thereby reduces oxidative stress, neuroinflammation and BBB disruption.<sup>36</sup> Therefore, targeting Nrf2 can evoke robust neuroprotection against ischaemic stroke. Nevertheless, the clinical translation of Nrf2 activators (more precisely termed Keap1 inhibitors as their molecular target is in fact Keap1) faces challenges, as the strategy for activating Nrf2 relies on a transcription process that takes a long time to take effect, and the treatment of stroke has a strict time window.<sup>37</sup>

### Myeloperoxidase

Myeloperoxidase (MPO), a critical inflammatory factor, is produced by neutrophils, microglia and monocytes after stroke and damages the BBB directly and indirectly.<sup>38</sup> The elevated MPO activity has been found in both experimental stroke animal models and ischaemic stroke patients. Reperfusion can aggravate the activation of MPO and the penumbra area has higher MPO expression than the core. MPO interacts with H<sub>2</sub>O<sub>2</sub> to generate hypochlorite (OCl<sup>−</sup>) and radicalised oxygen species (O<sub>2</sub><sup>•−</sup>, ONOO<sup>−</sup>), and thereby damage the BBB. MPO inhibition can decrease inflammatory cell recruitment, ROS/RNS-mediated inflammation and neuronal damage, and improve functional outcome after stroke.<sup>38 39</sup>

### Others

Matrix metalloproteinases (MMPs):<sup>40 41</sup> MMPs are a group of endopeptidases capable of cleaving extracellular matrix proteins. There are 23 types of MMPs expressed in humans. ROS are able to activate MMPs via oxidation of the thiol moiety within the cysteine switch and ONOO<sup>−</sup> activates several MMPs by cysteinyl S-glutathiolation and/or S-nitrosylation. Although increased levels of MMP-2, MMP-3 and MMP-9 are demonstrated after stroke, growing evidence in animals and clinical studies indicates that HT after tPA treatment and mechanical recanalisation of AIS is related with MMP-9.

DAMPs:<sup>42</sup> Stroke triggers a robust inflammatory response because that injury or necrosis tissues release DAMPs, including heat shock proteins, adenosine, high-mobility group box 1 (HMGB1), interleukin-33 and peroxiredoxin. Neutrophil extracellular traps (NETs):<sup>23 43</sup> NETs, web-like chromatin structures that are composed

of cytosolic and granule proteins, play a primary role in inflammatory disease pathogenesis through constitutive activation, dysregulation of suppressive mechanisms and overproduction. NETs formation in the acute phase of stroke is mainly caused by increased platelet surface-expressed HMGB1, NETs release from neutrophils contributes to tPA-induced BBB breakdown and HT, tPA resistance and impairs vascular remodelling during stroke recovery. *Dynamin-related protein 1 (Drp1)*:<sup>44</sup> Drp1 plays a key role in regulating fission, fusion, mitophagy and even motility of mitochondria. The activation of Drp1 contributes to ischaemic cerebral injury, the administration of a small-molecule inhibitor of Drp1, mdivi-1, before ischaemic insult ameliorates brain injury in rodent stroke models.

### Candidate drugs and their clinical translation

#### NA-1

Excitotoxicity is the most critical pathophysiological mechanism event underlying ischaemic cell death within the penumbra in the acute phase of stroke. Clinical trials of glutamate receptors antagonists and GABA<sub>A</sub>AR agonists in patients who had a stroke failed, although excitotoxicity is principally caused by glutamate receptors overactivation and GABA<sub>A</sub>Rs dysfunction.<sup>10–13</sup> NA-1 (nerinetide; TATNR2B9c), a peptide disrupting the NMDAR-PSD-95 interaction, prevents excitotoxic cell death without blocking normal synaptic function of NMDARs.<sup>11</sup> In rodent and non-human primate models of ischaemic stroke, NA-1 can reduce infarct size along with improved neurological deficits. In 2012, NA-1 completed a phase 2 clinical trial called ENACT to evaluate neuroprotection in aneurysm coiling therapy and found that the NA-1-treated group exhibited reduced lesion and improved clinical outcomes.<sup>21</sup> Recently, the efficacy and safety of NA-1 were evaluated in a phase 3 clinical trial called ESCAPE (Efficacy and safety of nerinetide for the treatment of acute ischaemic stroke). The findings show that 61.4% of patients with NA-1 and 59.2% of patients with placebo achieved an mRS score of 0–2 at 90 days after stroke. Investigators concluded that NA-1 does not improve the proportion of patients achieving good clinical outcomes after endovascular thrombectomy compared with patients receiving placebo. In this study, however, investigators observed treatment effect modification resulting in inhibition of treatment effect in patients receiving tPA.<sup>45</sup> Further studies with this cerebroprotective agent are necessary to extensively explore its effects in patients who had a stroke.

#### ZL006-05

Because of complex and varied detrimental cascades following ischaemic reperfusion, the single-target approach to stroke therapy may not be as successful as expected. The combination of glutamate antagonists and GABA agonists or assembled ‘cocktails’ appears promising in experimental models. ZL006, a small molecular nNOS-PSD-95 blocker we developed, can prevent cerebral

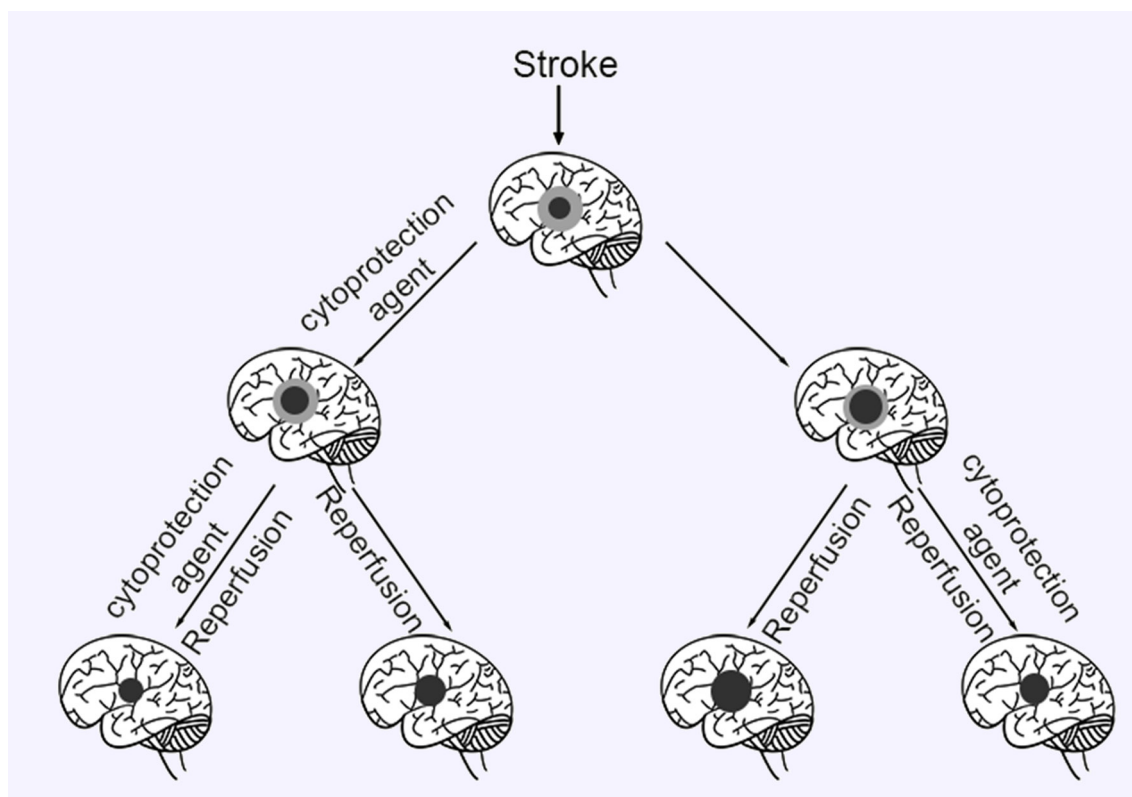
I/R injury, promote regenerative repair and functional recovery from stroke, without the major side effects caused by directly inhibiting NMDARs or nNOS.<sup>10</sup> The beneficial effect of ZL006 is further demonstrated in a humanised ischaemic stroke model. (+)-Borneol is a bicyclic monoterpene alcohol present in the essential oils of numerous medicinal plants. Our study indicates that (+)-borneol is a positive allosteric modulation of  $\alpha 2/\alpha 3$ -containing GABA<sub>A</sub>Rs and can attenuate ischaemic neuronal death. To obtain a dual-target neuroprotectant blocking nNOS-PSD-95 while potentiating  $\alpha 2$ -containing GABA<sub>A</sub>Rs, we designed and synthesised a compound ZL006-05 (Y3) that linked ZL006 and (+)-borneol through an ester bond.<sup>31</sup> ZL006-05 attenuates transient and permanent ischaemic injury and ameliorates long-term functional impairments with a treatment window of 12 hours after stroke, and reduces tPA-induced HT, readily crosses the BBB, has a very high safety profile in toxicokinetics and long-term toxicological studies, and it is well tolerated in normal volunteers following administration of a single or multiple doses in phase 1 clinical trial. Poststroke depression and anxiety are very common, and constitute an independent predictor of poor functional outcomes. ZL006-05 produces fast-onset antidepressant and anxiolytic effects in the normal mice and the mice exposed to unpredictable chronic mild stress or subjected to stroke. Now, the drug is going through a phase 2 clinical trial.<sup>32</sup>

#### Edaravone dextroborneol

Edaravone, a free radical scavenger, can reduce infarct volume and improve neurological function in rodent models of AIS. However, its efficacy in long-term outcome has not been reported. A meta-analysis of randomised controlled trials with limited studies concludes that edaravone can improve neurological impairment with a definite effect at 3-month follow-up. However, several adverse side effects of edaravone have also been reported in stroke clinical trials. Edaravone scavenges  $\bullet\text{OH}$ ,  $\text{NO}\bullet$  and  $\text{ONOO}^-$ , (+)-borneol inhibits ischaemia-induced inflammatory factors expression, and the combination of edaravone and borneol has a synergistic effect on ischaemic stroke in rats. Recently, we found that (+)-borneol is a positive allosteric modulator of  $\alpha 2/\alpha 3$ -containing GABA<sub>A</sub>Rs.<sup>31</sup> Interestingly, in a multicentre, randomised, double-blind, comparative, phase 3 clinical trial, when the combination of edaravone and borneol or edaravone alone is administered within 48 hours after AIS, 90-day good functional outcomes favour the combination group, again suggesting the advantages of multiple targets for treating AIS.<sup>46</sup>

#### Nelonemdaz

Nelonemdaz, previously known as Neu2000, is a novel synthetic derivative of aspirin and sulfasalazine that targets both NMDAR-mediated excitotoxicity and oxidative stress. Nelonemdaz strongly scavenges ROS, electively inhibits the NR2B-containing NMDAR and prevents the BBB disruption. A randomised phase 2 trial showed that



**Figure 3** Reperfusion and cytoprotective agents are a mutually beneficial pair for the outcomes of acute ischaemic stroke. Reperfusion not only improve the outcome of ischaemic stroke but also provides a physiological environment for cytoprotective agents to function. Cytoprotective agents extend the time window for reperfusion therapy by ‘freezing’ ischaemic penumbra when used before reperfusion, and reduce reperfusion injuries when used after reperfusion.

there is no significant difference in the proportion of patients with large-artery occlusion in the anterior circulation achieving mRS scores of 0–2 at 12 weeks between groups, but nelonemdaz-treated patients display a favourable tendency towards achieving these scores, without serious adverse effects. A double-blinded phase III trial of Nelonemdaz in patients with hyperacute ischaemic stroke and endovascular thrombectomy has been registered.<sup>47</sup>

#### GKT137831 and GKT136901

GKT137831 and GKT136901 are specific dual NOX1/NOX4 inhibitors and can effectively scavenge peroxynitrite and  $H_2O_2$ . They have excellent pharmacokinetic characteristics and oral bioavailability in vivo. It is demonstrated that GKT136901 has neuroprotective effects and can improve BBB stability. Interestingly, combining ineffective doses of GKT136901 and NO synthase inhibitor L-NAME display significant neuroprotection in three different brain ischaemia models, rat organotypic hippocampal culture, transient and permanent occlusion of the middle cerebral artery in mice, and human brain microvascular cells as a BBB model, showing a synergistic effect.<sup>48</sup> At present, GKT137831 is the only NOX inhibitor that has passed preclinical development to enter phase 3 clinical trials of diabetic nephropathy.<sup>35</sup> NOX inhibitors may become the most promising clinical candidate for treating stroke.

#### Mitiperstat (formerly AZD4831)

AZD4831 is a novel, potent and selective MPO inhibitor, with an in vitro  $IC_{50}$  of 0.7 nM for human MPO. The safety, tolerability, pharmacokinetics and pharmacodynamics of AZD4831 in a randomised, single-blind, placebo-controlled study in healthy subjects support further clinical development of AZD4831. Following phase 1 studies in healthy subjects and a phase 2a study in patients with heart failure with preserved ejection fraction, a phase 2b/3 efficacy study of AZD4831 in patients with heart failure started in 2021. Furthermore, inhibiting arterial MPO activity converts unstable into stable atherosclerotic lesions in a preclinical model of carotid artery plaque instability.<sup>49–51</sup> MPO is largely produced by neutrophils, microglia and monocytes after stroke, highlighting the potential therapeutic potency of AZD4831 for the development of novel protective strategies against AIS.

#### Antioxidant deferoxamine mesylate

Ferroptosis, a new form of programmed cell death attributed to an overwhelming lipidic peroxidation due to excessive free iron and ROS, has been recently reported to drive the acute neurodegeneration observed in ischaemic and haemorrhagic stroke. Iron chelation with deferoxamine mesylate (DFO) is neuroprotective in experimental stroke models. A multicentre, randomised, double-blind, placebo-controlled, dose-finding phase II



clinical trial demonstrated that DFO in a bolus followed by doses of 40–60 mg/Kg/day is safe and well tolerated in AIS patients, reduces systemic iron over 1–3 days and might provide long-term benefit to AIS patients.<sup>52</sup>

#### S44819

The  $\alpha 5$ -containing GABA<sub>A</sub>Rs are located extrasynaptically and play a crucial role in regulating the excitability of pyramidal cells through tonic inhibition. In the chronic stage of ischaemic stroke, hypoexcitability in the peri-infarct cortex occurs due to increased tonic inhibition by overactivation of  $\alpha 5$ -GABAARs. Pharmacological blockade of  $\alpha 5$ -GABAARs in the chronic state of stroke enhances functional recovery after stroke through reducing tonic inhibition. S44819 is a potent, competitive and selective antagonist of  $\alpha 5$ -GABAARs, can enhance motor-coordination recovery, spatial memory and perilesional neuroplasticity, reduce very delayed neuronal injury and brain atrophy when treatment starting at 3 days after stroke. Oral administration of S44819 increases cortical excitability in a phase 1 crossover, transcranial magnetic stimulation study done in healthy volunteers. However, a multicentre, randomised, double-blind, parallel-group, placebo-controlled phase 2 trial showed that there is no evidence that S44819 improves clinical outcome in patients after ischaemic stroke.<sup>53</sup>

#### Glyceryl trinitrate

NO has multiple effects that may be beneficial in acute stroke, including lowering blood pressure and promoting reperfusion. Glyceryl trinitrate (GTN) is a vasodilator approved by the FDA which acts as an NO donor.<sup>54</sup> A cochrane systemic review based on high-quality evidence reported that, in people with AIS, GTN reduces blood pressure, increases heart rate and headache, but does not alter clinical outcome. Recently, a single-centre, prospective randomised controlled trial to test the safety and efficacy of intra-catheter GTN in AIS patients after endovascular therapy has been registered, and the results will give insight for future GTN studies and new neuroprotective strategies for future AIS treatment strategies.<sup>55</sup>

#### Others

Natalizumab<sup>21 56</sup> is a humanised antibody against the cell adhesion glycoprotein  $\alpha 4$  integrin. The efficacy endpoint of a clinical trial (ACTION) on the safety and efficacy of a single dose of natalizumab (300 mg) is negative. A second clinical trial (ACTION 2) evaluating the effect of two doses of natalizumab administered  $\leq 24$  hours after AIS on functional outcomes again failed to meet the efficacy endpoint. DM199<sup>21 57</sup> is a recombinant form of tissue kallikrein (KLK1), a serine proteinase that initiates the release of bradykinin-related peptides from low-molecular weight kininogen. A form of human KLK1 isolated from human urine has been approved in by China State Food and Drug Administration for the treatment of mild to moderate stroke. DM199 has shown great potential in treating stroke with fewer side

effects. Phase 1 clinical trial of DM199 showed positive results on the safety and pharmacokinetic profile. 3K3A-activated protein C (APC)<sup>21 58</sup> is an analogue of APC where three lysine residues (residues 191–193) are replaced by three alanine residues, and has vasculoprotective, neuroprotective and anti-inflammatory activities after stroke, with significantly reduced anticoagulant activity compared with APC. A multi-centre, phase 2 trial for AIS shows that the maximally tolerated dose of 3K3A-APC in AIS patients is 540  $\mu$ g/kg, with an estimated toxicity rate of 7%, and there is a trend towards lower haemorrhage rate when compared with placebo-treated patients. SMTP-7,<sup>59</sup> a small molecule from the fungus *Stachybotrys microspore*, is a new thrombolytic agent. In phase 1 clinical study, the plasma SMPT-7 level is observed to be a linear function of the dose and no serious adverse events are observed. A phase 2 study of SMPT-7 in patients with AIS was initiated in 2017 to evaluate the safety and efficacy of a single-dose intravenous infusion. ApTOLL (unmodified single-stranded DNA aptamer)<sup>60</sup> is an antagonist of Toll-like receptor 4. A multicentre, double-blind, randomised, placebo-controlled, phase 1b/2a clinical study to evaluate tolerability, safety, pharmacokinetics and biological effect of ApTOLL in patients with AIS is currently being conducted. Citicoline (cytidine-5'-diphosphocholine)<sup>61</sup> is approved in some countries for the treatment of AIS. However, a recent meta-analysis reported that citicoline is not effective in improving neurological function, functional recovery and independence in daily living activities in patients with acute stroke. Choline alphoscerate, however, is effective. Citicoline and choline alphoscerate are both acetylcholine precursors and are considered to act as procholinergic nootropic agents. The differences in clinical efficacy of them are worth exploring in depth.

#### CONCLUSION AND FUTURE PERSPECTIVES

Reperfusion provides a physiological environment for cytoprotective agents to fully demonstrate to be clinically beneficial. At the same time, cytoprotective agents can extend the time window for reperfusion therapy, reduce reperfusion injuries and thereby enhance the effectiveness of reperfusion therapy. Therefore, reperfusion and cytoprotective agents are a mutually beneficial pair in AIS therapy (figure 3). For the development of effective cytoprotective agents, it is necessary to have a deep understanding of the pathophysiological mechanisms underlying ischaemia and I/R injuries. Excitotoxicity, and subsequent excessive production of ROS and RNS, as well as neuroinflammation, are the key events within the penumbra for ischaemic damage. For reperfusion-induced injuries, ROS and RNS, neutrophil infiltration, and complement and platelet activation are critical, although excitotoxicity may also be implicated. Many pharmacological targets for reducing ischaemia-induced or I/R-induced injuries and promoting stroke recovery have been identified. Of note, pharmacological targets involved in ischaemia and I/R are not entirely the same.

Priority should be given to targets that play key roles in both ischaemia and I/R.

To date, a large number of therapeutic agents have undergone clinical trials for evaluation of efficacy and safety in AIS. In spite of this extensive effort, the development of clinically effective cytoprotective agents is still elusive. Because of the simultaneous and sequential activation of multiple detrimental signalling cascades in both the ischaemic core and penumbra, therapeutic agents that target a single event may not be effective. Multitarget agents can intervene at multiple levels of the ischaemic cascade, with the hope of achieving a pleiotropic endpoint. Edaravone dextroboraneol can scavenge radicals, potentiate  $\alpha 2/\alpha 3$ -containing GABA<sub>A</sub>Rs, and inhibit neuroinflammation, and importantly, positive outcomes are obtained from phase 3 clinical trials, which is a good example of multitarget agents. Dual-target agent ZL006-05 that blocks nNOS-PSD-95 while potentiates  $\alpha 2$ -containing GABA<sub>A</sub>R now is going through clinical 2 trial. In addition to cytoprotective effect, ZL006-05 has rapid antidepressant and antianxiety effects, holding a promise for improving outcomes in AIS patients. Anyway, reperfusion provides a physiological environment that is more likely than ever to produce successful cytoprotective therapies for AIS. Furthermore, for brain cytoprotective agents, their ability to cross the BBB after systemic administration is a basic requirement. The limitation in clinical translation of many drugs is often their poor ability to penetrate the BBB. Intranasal or brain targeted biomaterial drug formulations are a possible approach to break through this limitation.

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#### ORCID iD

Dongya Zhu <http://orcid.org/0000-0002-2145-0225>

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