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Review

Understanding history, and not repeating it. Neuroprotection for acute ischemic stroke: From review to preview

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ABSTRACT

Background: Neuroprotection for ischemic stroke is a growing field, built upon the elucidation of the biochemical pathways of ischemia first studied in the 1970s. Beginning in the early 1990s, means by which to pharmacologically intervene and counteract these pathways have been sought, though with little clinical success. Through a comprehensive review of translations from laboratory to clinic, we aim to evaluate individual mechanisms of action, while highlighting potential barriers to success that will guide future research.

Methods: The MEDLINE database and The Internet Stroke Center clinical trials registry were queried for trials involving the use of neuroprotective agents in acute ischemic stroke in human subjects. For the purpose of the review, neuroprotective agents refer to medications used to preserve or protect the potentially ischemic tissue after an acute stroke, excluding treatments designed to re-establish perfusion. This excludes mechanical or pharmacological thrombolytics, anti-thrombic medications, or anti-platelet therapies.

Results: This review summarizes previously trialed neuroprotective agents, including but not limited to glutamate neurotransmission blockers, anti-oxidants, GABA agonists, leukocyte migration blockers, various small cation channel modulators, narcotic antagonists, and phospholipid membrane stabilizers. We outline key biochemical steps in ischemic injury that are the proposed areas of intervention. The agents, time to administration of therapeutic agent, follow-up, and trial results are reported.

Discussion: Stroke trials in humans are burdened with a marked heterogeneity of the patient population that is not seen in animal studies. Also, trials to date have included patients that are likely treated at a time outside of the window of efficacy for neuroprotective drugs, and have not effectively combined thrombolysis with neuroprotection. Through an evaluation of the accomplishments and failures in neuroprotection research, we propose new methodologies, agents, and techniques that may provide new routes for success.

Contents

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1. Introduction

Ischemic stroke is a major cause of morbidity and mortality worldwide. According to the American Heart Association and the National Institute of Neurological Disease and Stroke, an estimated 795,000 Americans suffer a stroke each year, with ~87% being ischemic [1]. Annually, stroke accounts for over 220,000 deaths, 4th overall behind cardiac disease, cancer, and chronic lower respiratory disease [2]. It is the leading cause of long-term disability in the US, with about one fourth of the survivors needing assistance with activities of daily living six months after their ictus [1]. Stroke's disastrous effects are the consequence of impaired blood flow to the brain, leading to inadequate influx of oxygen and nutrients and diminished clearance of metabolic toxins, with metabolic dysfunction leading ultimately to neuronal death. Most of our current strategies for treatment of ischemic stroke are based on re-establishing perfusion through the blocked blood vessels, using pharmacologic and mechanical thrombolysis. Conversely, neuroprotection targets biochemical pathways that lead to cell injury and death in ischemia in order to rescue salvageable nervous tissue. Despite encouraging data in experimental animal models, no clinical trials have demonstrated significant benefit in human stroke patients; to date, there is no standardly employed neuroprotective therapy. This review aims to discuss the biochemical cascades that lead to cell death in ischemia and describe pharmacologic agents targeting those mechanisms. We will postulate reasons for the failure of these trials thus far, and propose agents and methods that may provide routes for success.

In a normal human brain, cerebral blood flow is 50–55 mL/100 g tissue/minute [3]. Neurons can survive in a quiescent state at levels as low as 23 mL/100 g tissue/minute; below this ischemic cascades are set into motion that lead to neuron death. At 12 mL/100 g tissue/minute, cell death occurs rapidly and the insult is irreversible. It is this necessity for perfusion upon which current acute thrombolysis therapy is based. However, even in re-perfused state, mechanisms of ischemia remain active. As such, while the objective of using a neuroprotective agent used to be to rescue neurons that are in that intermediate zone, or ischemic penumbra, the ubiquitous use of acute thrombolysis paradigms has augmented its role. Neuroprotective agents may now hold promise to promote recovery and minimize injury when used in conjunction with thrombolysis. In order to understand why such an approach may be necessary, it is imperative to appreciate the mechanisms of ischemic injury in the brain.

2. Methods

The MEDLINE database and The Internet Stroke Center clinical trials registry were queried for trials involving the use of neuroprotective agents in acute ischemic stroke in human subjects. For the purpose of our discussion, neuroprotective agents refer to medications used to preserve or protect the ischemic penumbra after an acute stroke, excluding treatments designed to re-establish perfusion.

3. Excitotoxicity

Excitotoxicity is implicated in neuronal death in stroke. During ischemia, ATP depletion leads to failure of ion pumps that maintain membrane polarization. Subsequent depolarization caused by initial rise in cytosolic Ca^{2+} and Na^{+} caused by ion pump failure in addition to increased Ca^{2+} and Na^{+} influx due to NMDA and AMPA receptor activation by glutamate ultimately lead to increased cytosolic Ca^{2+} levels which appear to have a cytotoxic effect (Fig. 1). ATP depletion usually reverses within 15 min of reperfusion following ischemia [4]. Thus, thrombolysis may be successful in reversing the course of this mechanism for cell injury. One such cytotoxic effect is activation of Ca^{2+} dependent μ-calpain, a non-lysosomal neutral cysteine protease, which appears to be concentrated in the soma and dendrites of neurons and plays a role in neurite outgrowth, long-term potentiation, and synaptic remodeling in the brain. Pathologic activation of μ-calpain leads to degradation of certain proteins responsible for initiation of translation (eIF4G) and activation of apoptotic proteases like caspase 7 [4].

Many pharmacological agents have been studied to address these pathways, most aiming to block glutamate's excitatory activity at its receptor site. Dextromethorphan, and its active demethylated hepatic metabolite dextrorphan are non-competitive NMDA receptor antagonists that freely cross the blood–brain barrier. Pretreatment with dextrorphan has been shown to reduce neuronal death in an ischemia model in cultured mouse neocortex [5], and reduce infarct size in a rabbit model after 1 h of mechanical occlusion of the ICA and ACA [6]. It was deemed safe in humans at levels therapeutic to animals in phase I studies [7]. Studies using PO dextromethorphan vs placebo in humans with strokes between 6 and 24 h of ictus showed no benefit in terms of changes in NIH Stroke Scale (NIHSS) after 5 days of administration [8].

Magnesium is a blocker of voltage-gated calcium channels and the ion pore associated with the NMDA receptor [9]. Thus, it has also been investigated for its potential properties of limiting glutamate-mediated excitotoxicity. Studies in rats showed that intraperitoneal administration of magnesium chloride immediately after permanent surgical occlusion of the MCA significantly decreased the size of the resulting infarct [10]. The FAST-MAG pilot trial demonstrated feasibility of field administration of high dose IV magnesium by emergency medical personnel to patients deemed likely to be having a stroke [11]. The phase III portion of the FAST-Mag trial enrolled 1700 patients with mean time-to-treat of 48 min from symptom onset and primary endpoint of modified Rankin score determination of disability at 90 days. The authors found no difference in outcome between groups treated with 4 g IV loading dose of magnesium sulfate with subsequent maintenance infusion for 24 h versus saline placebo [12,13].

The Intravenous Magnesium Efficacy in Stroke trial (IMAGES) studied 2589 patients randomized to high dose intravenous magnesium vs placebo within 12 h of onset of ischemic stroke. This large study showed no significant benefit with regards to death or disability at 90 days from ictus, though the average time-to-treat was 7 h after stroke onset [14].

Lubeluzole is a drug that exerts indirect anti-glutamate activity, working by inhibiting glutamate-mediated synthesis of nitric oxide [15] and by preventing an increase in extracellular glutamate in the ischemic penumbra [16]. The neuroprotective effect of lubeluzole was initially demonstrated in a model using a glutamate trigger on cultured embryonic hippocampal neurons pretreated with this compound. A double blind placebo controlled phase III trial examining 1786 ischemic stroke patients randomized within 8 h of initial symptoms to a 5-day regimen of drug versus placebo failed to show clinical benefit of lubeluzole. Measured efficacy parameters included a three-tiered functional status category at 12 weeks from the initial event (independent vs. moderately dependent vs. vegetative), mortality at 12 weeks, modified Rankin score (mRS) and European Stroke Scale (ESS) score at 12 weeks, and survival time [17].

Cerestat/Aptiganel is a selective non-competitive NMDA receptor-associated ion pore blocker, found to be neuroprotective in experimental models of cerebral ischemia using MCA suture occlusion in rats [18]. Found to be safe and tolerable at concentrations that provided neuroprotection in rats [19], Cambridge

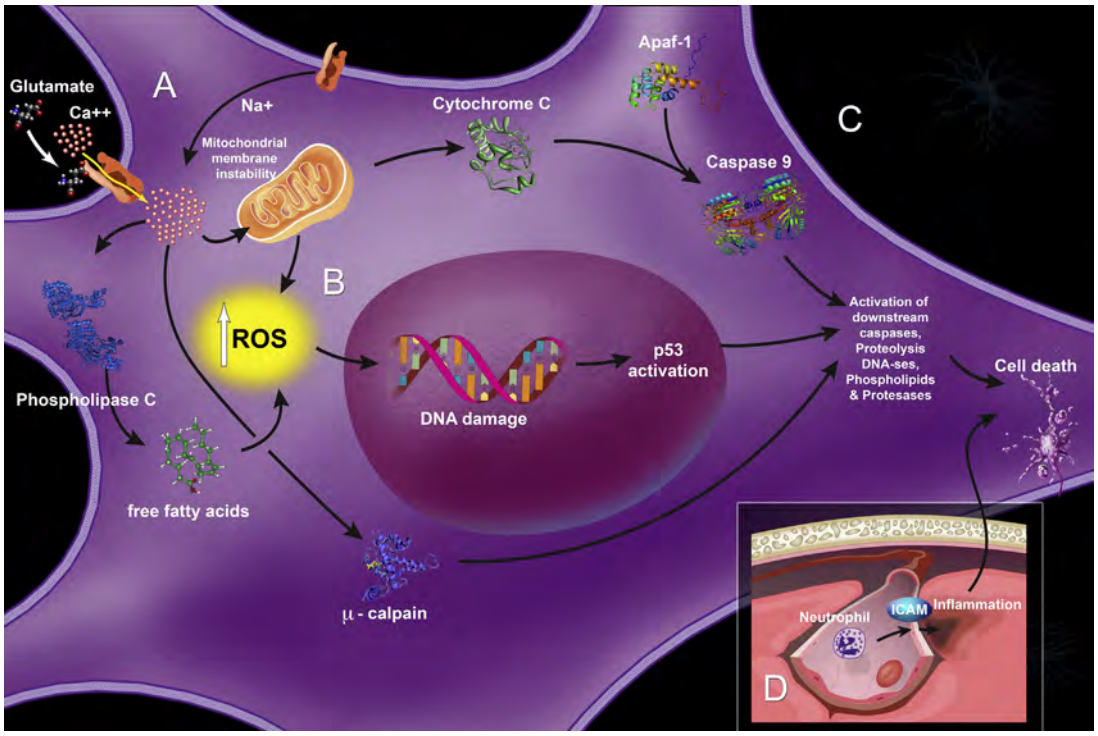

Fig. 1. Illustration of cell-signaling cascades and mechanisms of ischemia targeted by clinically evaluated neuroprotective agents. Glutamate-mediated sodium and calcium channel activation leads to depolarization and activation of cascades leading to apoptosis, targeted by inhibitors of ligand binding or direct blockade of the channels (A). Production of reactive oxidative species (ROS), (B), can lead to DNA damage and trigger downstream cell-death mechanisms. Administration of free radical scavengers has been attempted to thwart this. Administration of neurotrophic and vasoactive agents may modify the extracellular matrix (C) and has shown promise in the treatment of cerebral infarcts in rats. Finally, neutrophil-mediated inflammatory changes have been associated with brain injury in ischemia (D), and antibodies designed to target cell surface receptors on neutrophils and endothelial cells have been trialed in order to prevent infiltration of these cells into the ischemic bed and subsequent downstream oxidative damage.

NeuroSciences halted phase III trials after a planned interim analysis of 620 patients did not show clinical benefit [20].

Traxoprodil, also known as CP-101,606, is a postsynaptic NMDA antagonist selective for the NR2B subunit. Subunit selective NMDA blockers are a source of intense research for multiple different excitotoxicity-mediated neurological diseases due to their decreased propensity for adverse side effects, notably hypertension and hallucinations seen with non-selective NMDA blockers at high doses. Pretreatment with Traxoprodil decreased the size of infarct in cats with surgically occluded MCAs [21]. There are no published results in human ischemic stroke studies, but in one study 30 patients [20 TBI and 10 non-traumatic spontaneous IPH) were given infusions of Traxoprodil at varying dosages within 12 h of initial insult. Low doses yielded similar Glasgow Outcome Scale (GOS) results compared to historical controls at the study's institution, while higher doses showed significantly better outcomes [22]. Early trials to determine safety and efficacy of traxoprodil in human stroke patients showed no significant difference in lesion volume on MRI at 48 h, no difference in mortality, and no difference in NIHSS, GOS, or mRS at 90 days [23].

Remacemide hydrochloride (and its active metabolite desglycinyl-remacemide) is a low-affinity non-competitive NMDA receptor channel blocker, as well as a sodium fast-channel blocker shown to be protective in in vitro rat cortical neuron preparations exposed to glutamate receptor agonists, as well as in MCA-occlusion cat models with infusion of drug at the time of occlusion [24,25]. It was found to be tolerable in dosages predicted to be neuroprotective in animal models [26]. While there is currently no data showing benefit in humans in the setting of acute ischemic stroke, Remacemide did show clinical benefit in a 171 patient double-blind placebo-controlled trial examining drug administration prior to coronary artery bypass grafting, a procedure which often is complicated by clinically significant cerebral microembolism [27]. Because of its NMDA receptor and sodium channel blocking properties, remacemide has also been the subject of intense investigation in the treatment of other neurological disorders, such as epilepsy, Huntington's disease, and Parkinson's disease [28]. Unfortunately, proprietary rights for remacemide was sold by its initial developing pharmaceutical company Fisons (now-defunct), and has since changed hands several times. Remacemide's ultimate owner, the pharmaceutical company AstraZeneca, announced in July 2001 that development of remacemide had been discontinued; further development of this drug is on hold indefinitely [29].

Selfotel is a selective NMDA receptor antagonist that was shown to significantly decrease the size of infarct in rat brains when given within 5 min of permanent MCA occlusion [30]. Doses up to 1.5 mg/kg were found to be safe in preliminary tests in humans [31], however the phase III trial looking at 567 patients with acute (<6 h) ischemic stroke, given Selfotel vs placebo had to be suspended due to an increase in early mortality in the Selfotel treated group [32].

In an attempt to find an NMDA receptor blocker with a better safety/side effect profile than "pure" glutamate antagonists, Licostinel and Gavestinel have been evaluated for their potentially neuroprotective properties. Both work as NMDA receptor antagonists by preventing the binding of glycine to the receptor, a step required for activation. Agents that block the glutamate site of the NMDA receptor are associated with side effects including agitation, confusion, hallucinations, sedation, and cardiovascular effects, and the hope of studying glycine-site receptors was that the neuroprotective effects of NMDA receptor blockers seen in animal studies could be replicated with a less severe adverse side effect

profile. Indeed, intraperitoneal Licostinel (ACEA-1021) was shown to decrease infarct volumes and improve functional outcomes in a rat MCA occlusion/reperfusion model when given at the time of occlusion [33]. It was found to be tolerable in 44 acute CVA patients (drug administered via IV infusion within 48 h of ictus) with no major psychomotor effects, though interval improvement in NIHSS was comparable to a 20 patient placebo group [34]. Further development was delayed by Novartis after crystals of Licostinel were discovered to be precipitating in the urine of some patients with potential deleterious effects. CoCensys discontinued research and development on Licostinel in 2005.

Gavestinel also significantly reduced infarct size in rat MCA occlusion models and inhibited depolarization of neurons by NMDA administration in in vitro studies [35]. In the multi-center GAIN trial (Glycine Agonist in Neuroprotection) of 1367 ischemic stroke patients, Gavestinel intravenous loading dose versus placebo was given on hospital admission (average time to treat was 5.2 h). This trial demonstrated no functional difference at 3 months from ictus, as measured by Barthel index, between Gavestinel and placebo [36]. There was no significant difference in the size of infarct by MRI measurement in subsequent data analysis [37].

While not directly interacting with glutamate receptors, Clomethiazole has been examined as a potential neuroprotective agent for its ability to theoretically protect against excitotoxicity. Clomethiazole is a modulator of $GABA_A$ receptors that enhances the activity of endogenous GABA, producing membrane hyperpolarization. It was shown to provide neuroprotection in rodent and primate animal models [38–40], and was tested in humans in the clomethiazole acute stroke study (CLASS) trial. In the initial study, an intravenous infusion of clomethiazole vs placebo was given within 12 h of stroke onset to 1356 patients. Primary outcome analysis of percentage of patients achieving functional independence (BI $\geq$60) at 90 days showed no statistically significant difference, though post hoc subgroup analysis of 545 patients that presented with total anterior circulation syndrome showed 40.8% functional independence in clomethiazole-treated vs. 29.8% in the placebo group [41]. It was postulated that this subgroup demonstrated these changes because they had larger penumbras with more potentially salvageable tissue. This subgroup was specifically examined in a later study, CLASS-I [42], in which 1198 patients with total anterior circulation syndrome were treated with 68 mg/kg IV clomethiazole vs placebo over a period of 24 h. Treatment was started within 12 h of clinical presentation, and once again the primary outcome was functional independence (BI $\geq$60) at 90 days. Scandinavian stroke scale, NIHSS, and mRS were also assessed along with BI at 7, 30, and 90 days. CT infarct volume was measured at 30 days as well. There was no statistically significant difference in drug vs control groups in percentage of patients with BI $\geq$60, mRS $\leq$2, NIHSS change from baseline, SSS change from baseline, or CT infarct size. Interestingly, the authors suggest that lack of drug availability to the ischemic tissue could be the reason for lack of efficacy in this study, and they proposed future studies with clomethiazole in combination with tPA.

Nalmefene/Cervene is an opioid receptor blocker that prevents kappa receptor-mediated excitotoxicity by endogenous opioids. Microdialysis pretreatment with nalmefene has been shown in a global ischemia rat model to inhibit glutamate release [43] and to reduce reperfusion injury [44]. A randomized placebo controlled trial of 368 ischemic stroke patients, randomized within 6 h of symptom onset, showed no significant difference in functional outcome as measured by Barthel Index and Glasgow Outcome Score at 12 weeks follow up [45].

Repinotan, also known as Bay X 3702, is a 5-HT_{1A} serotonin receptor agonist which has been shown to have neuroprotective properties when tested against cultured hippocampal neurons and in a rat model of focal cerebral ischemia [46]. The mechanism of action is thought to be hyperpolarization of neurons via the opening of inward-rectifying potassium channels associated with the G-protein coupled serotonin receptor [47]. The BRAINS study, a Phase II trial, evaluated 240 patients with acute cerebral ischemia and a NIHSS of 4–25; patients were randomized to placebo versus repinotan at .5, 1.25, or 2.5 mg/day for 3 days. Treatment was given via intravenous infusion within 6 h of symptom onset. Interval evaluations were made at 4 and 12 weeks. This trial effectively demonstrated safety and tolerability of repinotan, though no significant difference in outcomes was seen [48]. The Modified Randomized Exposure Control Trial (mRECT) examined 681 patients who presented with ischemic insult, and were subsequently stratified into patients who received tPA and those who did not. A continuous 72-h infusion of Repinotan vs. placebo was started within 4.5 h of symptom onset. The primary outcome measure was Barthel Index at 3 months follow up, which was not significantly different between treatment and placebo groups [49].

4. Extracellular matrix modulation

Trafermin/bFGF/Fiblast is a recombinant human basic fibroblast growth factor that has demonstrated functional benefit and statistically significant reduction of infarct size in animal MCA occlusion models [50,51]. A phase II/III trial studying intravenous administration of Trafermin vs placebo in acute (<6 h) stroke was halted in May 1998 after interim analysis of 286 enrolled patients yielded futility in determining a significant difference in outcome between treatment and placebo groups [52].

5. Free radical generation and downstream oxidative damage

Ischemia and reperfusion result in generation of large amounts of free radicals (Fig. 1), most notably reactive oxygen species (ROS). Free fatty acids (FFAs), especially arachidonic acid (AA), are released during ischemia via the action of phospholipase C and phospholipase A_2, which are activated by depolarization and increased Ca^{2+}, respectively. During reperfusion, metabolism of AA generates large amounts of O_2^- which can react with NO released in large amounts during ischemia to produce peroxynitrite—implicated as the lipid peroxidation-initiating radical species during reperfusion. Lipid peroxidation can be seen as early as 15 min following reperfusion and can persist for up to 72 h and results in membrane damage and subsequent failure of ion partitioning. Lipid peroxidation is exacerbated by the presence of free ferrous iron (liberated from glial cells and reduced by O_2^-) and iron chelators have shown to diminish this effect. The problem of lipid peroxidation is complicated by the fact that lipid repair enzymes are inhibited during reperfusion [4].

Citicoline is a precursor to phosphatidylcholine, a major constituent of cell membranes. It acts as a scavenger of free radicals and may stabilize neuron cell membranes. It has shown benefit in rat models of ischemic stroke [53], and was initially tested in humans in a multicenter trial with 259 patients divided into four groups, given 3 different doses of citicoline vs placebo. Treatment was started within 24 h of stroke and maintained for 6 weeks. NIHSS, Barthel Index, mRS, and MMSE were assessed as measures of cognitive function at 12 weeks. BI was significantly improved in 500 mg and 2000 mg/day groups [54]. In a follow up study of 394 patients, patients with acute (<24 h old) ischemic strokes were treated with 500 mg PO citicoline per day vs placebo for 6 weeks. BI and mortality at 90 days were compared and found to have no statistically significant difference, though post hoc analysis showed a greater chance for full recovery in patients with initial NIHSS $\geq$ 8 [55]. The following year a study was published looking at the evolution of

ischemic injury using DW-MRI at baseline, 1 week, and 12 weeks in patients receiving 6 weeks of 500 mg po citicoline vs placebo. There was a reduction in lesion volume growth over 90 days with citicoline treatment [56]. In a phase III trial of 899 patients treated with 2 g po citicoline per day vs. placebo for 6 weeks, no difference was seen in NIHSS or infarct volume at the 12 week follow up [57].

Though previous trials showed no significant improvement in outcome on the basis of the studies' primary efficacy variables, there were some promising results in post hoc analyses, so a meta-analysis of 1372 patients taking PO citicoline for acute ischmic stroke was performed [58]. Given the heterogenous mix of outcome performance standards used in previous studies, this meta-analysis standardized the individual patients' data and calculated a global measure of outcome based on the NIHSS, BI, GOS, and mRS in accordance to previous recommendations from the National Institute of Neurological Disorders and Stroke [59]. The study concluded that treatment with citicoline led to a statistically significant increase in the likelihood of a favorable outcome at 90 days. It was felt by the authors that differences in the magnitude (but importantly, not direction) of the treatment effect of citicoline in previous studies was a result of suboptimal trial design – inadequate sample size, suboptimal choice of primary endpoint variables, inclusion of populations likely to dilute drug effect, etc. To follow up this meta-analysis, the ICTUS study was a large prospective, randomized, placebo-controlled trial conducted across multiple European countries that enrolled 2298 patients with moderate to severe ischemic MCA strokes. One gram of citicoloine iv was administered within 24 h of symptom onset and given BiD for 3 days, followed by 1 g PO bid for 6 weeks. The primary endpoint was a global measure of recovery at 90 days per Barthel Index, modified Rankin Score, and NIH stroke scale, as in the aforementioned meta-analysis. Unfortunately, the benefits of citicoline seen in that meta-analysis were not re-demonstrated in this prospective trial. Theories as to why this was the case include a 10-year lapse between studies, with advancement of "best medical care" being sufficient to dilute any benefits patients may get from citicoline [60].

The SAINT I and II trials looked at the soluble free-radical-trapping nitrone NXY-059, a drug shown to reduce the size of infarcts and reduce neurologic deficits in rats after a 2-h surgical MCA occlusion [61]. The first study was a massive multicenter trial that assigned 1722 patients that had a stroke with limb weakness and NIHSS of 6 or greater within 6 h of treatment to receive a 3-day infusion of NXY-059 or placebo [62]. NIHSS, mRS, Barthel index, stroke impact scale, and European quality of life 5 dimensions (EQ 5D) were assessed at 1, 3, 7, 30, and 90 days. There was a statistically significant difference in disability reduction with NXY-059 treatment as measured by the 90 day mRS. 4.4% more patients had a score of 0, meaning they were asymptomatic, and 3.7% more had a score 0–3, meaning they could walk unassisted. The safety profile was the same between experimental drug and placebo groups. The study failed to meet clinical significance on prespecified secondary endpoints however, and a second, larger trial was undertaken. SAINT II enrolled 3306 patients with the same inclusion criteria, and failed to show a difference between the study drug and placebo thoughout all possible dichotomizations of the mRS or in any of the study's prespecified secondary end points [63]. Pooled data from these two trials were examined, totaling 4946 patients receiving treatment. Mean time to treat was 3 h and 47 min. There was no difference in the distribution of mRS scores between the groups, no difference in NIHSS, and no difference in mortality. Subgroup analysis also yielded neutral results, with no difference in symptomatic or asymptomatic hemorrhage in alteplase-treated patients with or without NXY-059 (n = 950 and 965, respectively)[64].

Tirilazad mesylate is an aminosteroid that acts as a potent free radical scavenger and membrane lipid peroxidase inhibitor that does not exhibit classic steroid hormonal activity in humans [65]. Intraperitoneal pretreatment with this drug was shown to be neuroprotective in a 3-h MCA-occlusion gerbil model of ischemic stroke [66]. It was later deemed safe and tolerable in human elderly stroke patients [67]. However, in a meta-analysis of 6 randomized controlled trials encompassing 1757 patients, tirilazad was found to not only be ineffective in ischemic stroke, but EBI and GOS outcomes at 3 months post-ictus were actually worse in patients taking tirilizad [68].

Enlimomab is a murine monoclonal anti ICAM-1 antibody that acts by blocking neutrophil adhesion and migration through the endothelium, with the aim of reducing inflammatory-mediated irreversible brain damage after an ischemic event. Pretreatment in rabbits 30 min prior to micro-sphere vessel occlusion was shown to be neuroprotective [69]. There was no significant risk of increased morbidity/mortality at 30 days in phase I trials [70]. Phase III trials were undertaken in 625 patients in the Enlimomab Acute Stroke Trial (EAST); patients were randomized to enlimomab vs placebo within 6 h of stroke onset. Outcomes were measured by modified Rankin scale, NIH stroke scale, Barthel Index, and survival at 90 days; the final results showed that patients treated with placebo did significantly better. The treatment group had significantly worse mRS, higher mortality, and significantly more adverse events [71].

Rovelizumab/LeukArrest is a monoclonal antibody against CD11/CD18 neutrophil CAM. The rationale for its use as a neuroprotective agent is similar to Enlimomab; it is designed to prevent neutrophil invasion of the ischemic bed and subsequent inflammatory injury. While it was initially developed for treatment of hemorrhagic shock with the aim of decreasing multiple organ failure [72], rovelizumab was also trialed in acute ischemic stroke patients. Preliminary studies in rabbits with mechanically occluded MCA, ACA, or ICA showed demonstrably less ischemic neuronal damage on histological slides after treatment with the drug 20 min after vessel occlusion [73]. After establishing safety in humans, a phase III trial (HALT) that treated acute ischemic stroke patients with placebo vs. 2 different doses of Hu23F2G was started. Patients were treated within 12 h of onset and could receive tPA as long as they made the NINDS protocol guidelines. This trial was terminated at its first interim assessment, citing no suggestion of benefit [74].

6. Prevention of apoptosis

During ischemia and reperfusion, ligand activation of so called "death receptors" leads to intracellular activation of caspases that subsequently lead to cell death (Fig. 1). FAS is one such receptor, and FAS ligands and the downstream transcription factor JUN are elevated following ischemia. NF-κB is a pro-survival transcription factor that can be activated via a complicated mechanism following activation of TNFR. NF-κB induces expression of cellular inhibitors of apoptosis 1 and 2 (c-IAP1 and c-IAP2), A20, and manganese superoxide dismutase. Apoptosis can also be activated via intracellular mechanisms. Under normal circumstances, pro-apoptotic proteins APAF-1, and cytochrome c and caspase 9 are found in the cytosol and mitochondrial intramembranous space, respectively. Disruption of the mitochondrial membrane by free radicals or Bax allows release of cytochrome c (and caspase 9) which leads to APAF-1 oligomerization and activation of caspase 9, which is an initiator caspase. Caspase 3 is an effector caspase that degrades DNA repair enzymes. Levels of caspase 3 are upregulated during ischemia/reperfusion. Bax and Bad are both pro-apoptotic proteins implicated in ischemia/reperfusion injury. Bad binds Bcl-2, an anti-apoptotic protein that binds and inactivates Bax. Bax inserts into ER and mitochondrial membranes and leads to Ca^{2+} release from ER and cytochrome c and caspase 9 release fro the mitochondria. During reperfusion, conditions favor apoptosis. One such mechanism is the depletion of dephosphorylated eIF2α that was mentioned

earlier in reference to protein synthesis inhibition. Dephosphorylated eIF2α down regulates Bax [4].

Flunarizine is an organic calcium channel blocker, chosen for study in acute ischemic stroke under the hypothesis that calcium channel blockade would prevent or attenuate downstream apoptotic pathways. Pretreatment with flunazarine in young rats with a unilateral carotid artery ligation led to significant decreases in infarct volume [75]. A double-blinded pilot trial in 1990 looked at 26 patients with acute (<24 h old) supratentorial ischemic strokes treated with flunarizine versus placebo. Findings were suggestive of clinical benefit with flunarizine use (32% more severely disabled patients in the placebo group), though this difference was not statistically significant and there was an uneven distribution of other prognostic variables [76]. The Flunarizine in Stroke Trial (FIST) looked at 331 patients treated in a double-blind fashion with either flunarizine or placebo. Mean time interval to treatment was 13.5 in treatment group, 12.3 h in the placebo group. Treatment was 50 mg IV for one week, 21 mg po for 1 week, then 7 mg po for 2 weeks. Patients were followed for 24 weeks, and primary outcome measures were percentage of patients who were dead or dependent (based on mRS), Orgogozo Stroke Scale, and modified Barthel Index at 24 weeks after CVA. No difference was seen between treatment and placebo groups [77].

Recently, sulfonylureas have been looked at as potential neuroprotective agents for their ability to bind to and block the sulonylurea receptor 1 (SUR1), a receptor that regulates the opening of a nonselective cation channel that has been shown to be expressed in ischemic astrocytes. A rodent model of stroke was shown to have newly expressed SUR1 protein and mRNA in ischemic tissues, and treatment with glyburide reduced mortality and decreased cerebral infarct size [78]. A review of 61 patients with diabetes mellitus, 33 previously taking sulfonylureas and 28 not, was performed; it found that a statistically significant number of patients on sulfonylureas had a reduction of their NIHSS by 4 points by discharge. The treatment group also more frequently achieved a favorable mRS by discharge compared to patients that were not taking a sulfonylurea [79]. A prospective trial of iv glyburide for ischemic stroke is now underway [80].

7. Discussion: lessons from failure and future directions

As mentioned previously, cerebral ischemia leads to neuronal death through a complex series of interrelated biochemical processes – for a more detailed review of these processes we point readers to the review by Drs. Brouns and De Deyn [81]. Neuroprotective agents targeting different pathways involved in ischemic cell injury have been investigated, with discouraging clinical results thus far (Table 1). However, a review of these agents and their associated research studies provide interesting insight into the approach and pitfalls in neuroprotection research. The notion that "everything works in animals, nothing works in humans" may be largely due to a failure of model translation. In most of the animal studies, the animals are either pre-treated with their neuroprotective agent, or treatment is started within a very short time frame from a surgical vessel occlusion. The time-of-infarct is distinct and the time-to-treat is easily measurable. This is not so in a heterogenous human population; the best measurement of the time of infarct is designated "Last Known Normal" (the last time point the patient was noted to be neurologically at baseline). If a patient wakes up with a deficit, the exact time of their deficit is impossible to record clinically. Delays in seeking/obtaining medical treatment lead to wide ranges of treatment onset time, and, given the extremely time-sensitive nature of ischemic injury, patients treated with the same neuroprotective agent may have widely varying outcomes. 'Last Known Normal' time has become a critical variable in determining candidacy for acute thrombolysis treatment, both intravenous and intra-arterial. With a catchphrase of 'Time is Brain,' our clinical approach to ischemic stroke has changed from one of just managing the aftermath to aggressively intervening from the moment of onset. In such a way, we should view neuroprotection as a potentially key element, but one that may require similar aggressive timeliness. The failure of previous studies may also be related to the late time of first administration (5.2–48 h). A more aggressive approach would better mirror the successful animal studies that have been used as a basis for clinical trials. It would also conform to the current philosophy in clinical management of ischemic stroke which emphasizes early intervention.

While animal studies typically involve infarction of one vascular territory through a controlled ligation or occlusion of a parent vessel, strokes in human subjects vary widely and will lead to intrinsically widely differing outcomes. Many of the existing studies include patients with both hemorrhagic and ischemic strokes, adding yet another level of heterogeneity. Hemorrhagic stroke must be kept separate from ischemic, as the mechanisms and pathways for damage are extremely different.

One other striking mechanistic difference between animal studies and subsequent human trials is the course of the affected parent vessel. In animal studies, typically the parent vessel is mechanically occluded for some length of time with a suture or a filament or by some other physical obstruction. After a predetermined and uniform period of time this obstruction is removed and the infarcted bed is reperfused. In these subjects, intraperitoneal or intravenously administered medicines may have more bioavailability to the ischemic bed than they would in a human stroke patient who continues to have a permanent occlusion. This point is touched on in an editorial comment regarding the CLASS-I trial [42] – it is clear that the GABA-promoting drug clomethiazole is penetrating the blood–brain barrier because patients receiving the drug were shown to have sedation after administration, a known effect of the drug on the CNS. What was unclear was the drug's bioavailability to the ischemic penumbra given the blockage of the vascular route to this area, and whether a lack of efficacy of clomethiazole in this study was due to an insufficient amount of the drug reaching its target region.

Finally, the animal studies reviewed typically use healthy animals of similar ages. The human patients are of widely varying ages and have a non-uniform medical comorbidity profile. This non-uniformity among patients complicates clinical studies, introducing confounding variables. Furthermore, small animal models may have higher tolerance for ischemia that the human brain.

Many explanations have been offered, analyzed, and debated in the literature as to why animal studies have not translated into clinically relevant therapies yet, and this has been addressed by the Stroke Treatment Academic Industry Roundtable (STAIR) in years past [82,83]. These meetings have produced recommendations for future avenues of stroke research to support therapies that have high-yield potential, and to guide trial design to show statistical significance. Suggestions include strategies to decrease "time to treat" with field administration of agent (as in the FAST-MAG study), to alter outcome measures in a way that may reveal subtle but meaningful differences in patient performance (such as mRS shift in the SAINT-I trial compared to prior categorical comparison of dichotomized mRS), and to re-evaluate previously trialed agents in combination with newer approved therapies; combining neuroprotection with currently practiced thrombolytic therapies. To this end, some previously-trialed drugs may be examined in the future in conjunction with thrombolytic therapy (see the discussion of tPA and clomethiazole in the CLASS-I trial)[42]. Also, biomaterials for drug delivery are being refined to help target therapeutic agents. Lipid-based biomaterials like liposomes, solid lipid nanoparticles,

Table 1
Summary of trials examining neuroprotective agent usage in human subjects.

Class	Agent	Reference	N	Time to Admin	F/U	Result
GLU Blockers	Dextremethorphan	Mousavi 2011	40	6–24 hours	3months	No difference in NIHSS in treated vs placebo groups
	Magnesium	Muir 2004	2589	up to 12 hours (7 avg)	3 months	No difference in death or disability in treatment vs placebo groups
	Lubeluzole	Diener 2000	1786	up to 8 hours	3 months	No difference in mortality, survival time, mRS, or ESS in treatment vs placebo groups
	Aptiganel	PRNewswire 1998	620			Trial halted after interim eval showed futility of treatment
	Traxoprodil	Bullock 1999	30	up to 12 hours	3 months	Trend toward better outcomes at f/u in high-dose group
		Saltarelli 2004	114	up to 8 hours	48 h, 3 months	No difference in lesion volume or functional outcomes in treated vs placebo groups
	Ramecemide	Arrowsmith 1998	171	pre-operatively	8 weeks after OR	No difference in neuropsyche battery performance, development for CVA on hold indefinitely
	Selfotel	Grotta 1995	24	up to 12 hours	mean 86 days	Acceptable safety profile in Phase I study
		Davis 2000	567	up to 6 hours	halted	Trial halted due to early mortality increase in treated group
	Licostinel	Albers 1999	64	up to 48 hours	halted	Further development halted due to untoward side effects
	Gavestinel	Sacco 2001	1367	avg 5.2 hours	3 months	No difference in BI in treated vs placebo groups
	Nalmefene	Clark 2000	368	up to 6 hours	3 months	No difference in GOS or BI in treated vs placebo groups
	Repinotan	Teal 2005	240	up to 6 hours	1 and 3 months	Acceptible safety profile, no difference clinically in treated vs placebo groups
		Teal 2009	681	up to 4.5 h	3 months	No difference in BI in treated vs placebo groups
GABA Potentiators	Clomethiazole	Wahlgren 1999	1360	up to 12 hours	3 months	No difference in patients achieving functional independence (BI $\geq$ 60)
		Lyden 2002	1198	up to 12 hours	3 months	No difference in patients achieving functional independence (BI $\geq$ 60)
EC matrix mod.	Trafermin	Bogousslavsky 2002	286	up to 6 hours	halted	Trial halted after interim eval showed futility of treatment
Free Radical	Citicoline	Clark 1997	259	up to 24 hours	3 months	BI significantly improved in 500 mg and 2 g treatment groups
scavengers		Clark 1999	394	up to 24 hours	3 months	No difference in mortality or BI, greater chance for full recovery seen in subset of treated group with NIHSS <9
		Warach 2000	100	up to 24 hours	1 and 12 weeks	Reduction of lesion volume on DW-MRI in treated group
		Clark 2001	899	up to 24 hours	3 months	No difference in infarct volume or NIHSS in treated vs placebo groups
	Nxy-059	Lees 2006	1722	up to 6 hours	3 months	Reduction in disability based on mRS, no difference in NHSS, BI
		Shuaib 2007	3306	up to 6 hours	3 months	No difference in treated vs placebo groups, no difference when results pooled with previous study
		Davalos 2012	2398	up to 24 hours	3 months	No differencein NIHSS, BI or mRS in treated vs placebo groups
	Tirilizad	Tirilizad Int'l Steer. Comm. 2002	1757	metanalysis, varied	3 months	Decreased EBI and GOS in treated groups
InflammationBlockers	Enlimomab	EAST trial investigators 2001	625	within 6 hours	3 months	Higher mortality, worse mRS, and more adverse events in treatment group
	Rovelizumab	Icos Press Release		within 12 hours	halted	Trial halted after interim eval showed futility of treatment
Ca Channel Blockers	Flunarizine	Limburg 1990	26	within 24 hours	6 months	Non-significant clinical improvement in treatment group
		Franke 1996	331	within 24 hours	6 months	No difference in mRS,mBI, Orgogozo stroke scale in treated vs placebo groups

and micelles are being developed with surface receptors and targeting species that can encapsulate and help deliver neuroprotective and neuroregenerative agents to the ischemic penumbra. Polymeric nanoparticles that neuoprotective agents can covalently link to or adsorb to may also be a way of directing our treatments in future studies [84].

Bridging the gap between bench research and translational clinical studies is also coming from the laboratory side of the

equation in the form of Multi-PART, the Multicentre Preclinical Animal Research Team project. This is collaborative effort of European centers looking to improve the translatability of neuroprotective drugs by designing experiments that are more generalizable, by using larger sample sizes, by avoiding negative publication bias (and other incentives which may increase the desire to produce positive results), and by utilizing a central randomization center. They aim to avoid future studies that are underpowered or poorly conducted or falsely positive that lead to costly, large-scale studies in humans which end up showing no efficacy and not agreeing with the lab research [85]. The NIH is currently evaluating the role and applicability of Multi-PART in the U.S.

Despite the difficulties with patient heterogeneity, varying time of presentation, and differences in large/small vessel occlusion, we feel that there is still potential for the use of neuroprotective agents in acute ischemic stroke, and taking into account the suggestions of the STAIR group we propose methodologies to optimize translation of data from animal studies, while maximizing potential benefit through early and direct intervention. One group of patients of greatest potential would be those with large vessel occlusions. Increasingly, these patients are treated with intra-arterial endovascular therapies in which a microcatheter is advanced under fluoroscopic guidance into the occluded vessel, and the vessel is reopened directly. Thus, this group would represent a collection of patients in whom one or a cocktail of potential neuroprotectants could be infused immediately following reperfusion. This would mirror laboratory studies more closely, would deliver the agents directly to the at-risk tissue through the superselective intra-arterial route (potentially limited systemic effects), and would combine reperfusion with neuroprotection. As such, these patients offer a more homogenous and controlled group for clinical evaluation of neuroprotective agents. Finally, the addition of drug infusion would mechanistically add almost no additional risk to the endovascular thrombolysis procedure. Therefore, this methodology provides a more controlled group of patients, in a narrower time-administration window, with more directed therapeutic administration.

In conclusion, decades of research in neuroprotective agents have yielded some promising results in well-matched animal models of ischemic stroke, but have failed to show benefit in human patients. It is time to examine our methods, our translation from lab to clinic, and the urgency with which we administer neuroprotective therapy.

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