Universidade de Lisboa

Faculdade de Farmácia

Novel Pharmacological Strategies for Neuroprotection in Stroke: From Bench to Bedside

Maria Gabriela Rachadell Pereira

Monografia orientada pelo Professor Doutor João Pedro Fidalgo Rocha,
Categoria Professor Auxiliar

Mestrado Integrado em Ciências Farmacêuticas

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

O acidente vascular cerebral (AVC) é uma das principais causas de mortalidade e morbilidade a longo prazo no mundo. Esta doença tem uma forte repercussão não apenas nos doentes, mas também nos seus cuidadores, visto que os sobreviventes se tornam frequentemente incapazes de realizar tarefas diárias básicas de forma independente. O AVC pode ser dividido em duas grandes categorias: isquémico ou hemorrágico, sendo que o AVC isquémico é responsável por cerca de 87% da totalidade de casos. O grande impacto negativo provocado por esta doença justifica a necessidade de desenvolver novas e melhores estratégias que permitam melhorar a qualidade de vida dos sobreviventes. Atualmente o "*gold standard*" para tratamento do AVC é a trombólise através da administração intravenosa de trombolíticos (tPA e derivados recombinantes). Quando administrado nas primeiras horas após o AVC, estes fármacos permitem aumentar a probabilidade de recuperação dos doentes. Infelizmente, muitos doentes não cumprem os critérios necessários para realizar este tratamento, o que justifica a necessidade de desenvolver novas estratégias. Nas últimas décadas, o conceito de neuroprotecção tem recebido muita atenção por parte dos investigadores, e inúmeros agentes têm sido testados em contexto pré-clínico, e alguns em ensaios clínicos. A melhor compreensão da cascata isquémica que leva ao dano neuronal permitiu a identificação de novos alvos para estratégias de neuroprotecção. Muitos dos ensaios pré-clínicos com estes compostos têm tido resultados encorajadores, mas que falham em demonstrar benefício na translação para contexto clínico. Alguns autores têm identificado razões para esta falha de translação. Apesar dos desafios que têm surgido neste campo, muitos compostos foram testados em ensaios clínicos nos últimos 5 anos, alguns dos quais ainda estão a decorrer. Independentemente das falhas que ocorreram no passado, a neuroprotecção tem um futuro promissor, e novas terapêuticas têm emergido como a adropina, a tirosina fosfatase STEP, o verapamilo e os microRNAs. A terapêutica combinada tem também um grande potencial, visto que poderia aumentar o número de doentes elegíveis para tratamento com trombolíticos. É muito provável que nos próximos anos surjam descobertas no tratamento do AVC, e os investigadores devem manter em mente os erros que foram cometidos no passado de forma a prevenir que se voltem a repetir no futuro.

Palavras-chave: AVC; Neuroprotecção; Translação

Abstract

Stroke is among the main causes of death worldwide and it is a serious cause of long-term disability. This disease has an important impact not only on patients, but also on care providers, since often stroke survivors become unable to perform basic daily tasks on their own. Stroke can be divided into two major categories: ischemic and hemorrhagic. Ischemic stroke is the most common, being responsible for around 87% of all stroke cases. The huge burden associated with this disease, justifies the need to develop new and better therapeutic strategies that can improve survivor's quality of life. Currently, the gold standard for treatment of stroke is recombinant tissue plasminogen activator (rtPA) administered intravenously. Given within the first hours of symptoms onset, rtPA can considerably increase the chances of stroke recovery. Unfortunately, a lot of patients do not meet the criteria to be eligible for this treatment, thus the need to develop new therapeutic strategies. In the last decades, a lot of attention has been drawn to the concept of neuroprotection and numerous agents were tested in preclinical studies and some of them in clinical trials. A better understanding of the ischemic cascade that leads to neuronal damage has enabled the development of novel therapeutic targets for neuroprotective strategies. Numerous compounds have undergone preclinical trials with exciting results but have failed to translate into clinical benefit. Some reasons behind the gap between bench and bedside have been identified by authors. Despite the challenges of translation, multiple compounds have been tested in clinical trials in the last 5 years, some of which are still ongoing. Regardless of the failures that occurred in the past, neuroprotection has a promising future with new emergent treatments such as adropin, tyrosine phosphatase STEP, verapamil, and microRNAs. Combination therapy holds great potential, since it could amplify the number of patients eligible for treatment with rtPA. In the next years, is likely that new discoveries arise in stroke research, and investigators must keep in mind the failures that happened in the past, to prevent them from happening in the future.

Keywords: Stroke; Neuroprotection; Translation.

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Por fim, obrigada a ti Diogo, pelo amor que partilhamos e a felicidade que trazes aos meus dias.

Abbreviatures

ADME- Absorption, distribution, metabolism, and excretion

AIF- Apoptosis inducing factor

AIS- Acute ischemic stroke

AHA- American Heart Association

ATP-Adenosine triphosphate

BBB- Blood-Brain Barrier

CNS- Central Nervous system

DL-NBP- DL-3-N-butylphalide

ENHO- Energy homeostasis gene

ERK1/2-Extrecellular signal-regulating kinase 1 and 2

ESO-European Stroke Organization

FADD- Fas associated domain

FDA- Food and Drug Administration

GLP-1R- Glucagon like peptide 1

ICD- International Classification of Diseases and related health problems

iNOS- Nitric oxide synthase

IVT- Intravenous thrombolysis

MCA- Middle cerebral artery

MCAO- Middle cerebral artery occlusion

miRNAs- MicroRNA

MMPs- Matrix metalloproteinases

MRI- Magnetic resonance imaging

NA-1- Nerinetide

NDMA- N-methyl-D-aspartate

NFkB- Nuclear factor Kappa B

NINDS- National Institutes of neurological disorders and stroke

nNOS-Nitric oxide synthase

NO- Nitric oxide

PARP- Poly (ADP-Ribose) polymerase

PAR1- Protease-activated receptor

PSD-95- Postsynaptic density-95 protein

RIC- Remote ischemic conditioning

ROS/RNS- Reactive oxygen/nitrogen species

STAIR- Stroke Therapy Academic Industry Roundtable

rtPA- Recombinant Tissue plasminogen Activator

tCDS- Transcranial direct stimulation

TIMP- Tissue inhibitor of matrix metalloproteinase

UA- Uric Acid

VPN- Vinpocetine

1-NBP- 1-3-N-Butylphtalide

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1 Introduction

Stroke is an umbrella term usually applied for focal neurological deficits and central nervous system injuries of vascular origin (1). It is a non-communicable disease associated with a large burden world-wide. Is characterized by disruption of blood flow, which leads to ischemic damage.

In 1955, cerebrovascular diseases were reclassified as a disease of the circulatory system in the International Classification of Diseases and Related Health problems (ICD). As a result of this inadequate categorization, clinical data from stroke patients were included as a part of the cardiovascular diseases category, leading to a missed interpretation of the real severity and burden of this disease (1). It also limited government funding for stroke patients and research since they did not benefit from the support that was directed towards neurological disease (2). In ICD-11, published in 2018, stroke is accurately classified as a Disease of the Nervous System. This modification has led to more accurate documentation of data and allows for more funding in stroke research ultimately resulting in improvements in acute healthcare (2).

Despite the amount of research that has been developed in this field, we have not seen many changes on treatment in the last years. Nevertheless, a well-established principle is that "Time is Brain" and that human nervous tissue is rapidly lost as stroke progresses, thus early evaluation, and appropriate therapy are extremely important.

Acute ischemic stroke (AIS) can be caused by atherosclerosis, aortocardioembolism, small vessel occlusion or other known and unknown causes. Hemorrhagic stroke is more commonly caused by hypertension; however, it can be a result of specific blood vessel abnormalities and other medical conditions (3).

AIS is responsible for about 87% of all strokes (4). Rapid restoration of the cerebral blood flow is the primary goal of ischemic stroke therapy as well as a prerequisite to neuroprotective therapies (5).

This dissertation will approach the concept of neuroprotection, the preclinical and clinical research status as well as the challenges of translation in this field. Lastly, it will cover some of the emergent neuroprotective strategies and the future perspective of this area of investigation. A literature search was performed for articles in English and Portuguese language in PubMed and Google Scholar, up to November 2020.

2 Objectives

The purpose of this monograph is to analyze the recent developments of neuroprotective strategies in stroke as well as exploring the challenges of translation from preclinical studies to clinical practice in this field of investigation.

Additionally, this monograph provides an overview on the disease, its epidemiology and the strategies currently recommended in guidelines.

3 Epidemiology

According to WHO Global Health Estimates, stroke is the 2nd cause of death, responsible for approximately 11% of total deaths. It is a leading cause of serious long-term disability and reduces mobility in more than half of stroke survivors aged 65 and over (6).

3.1 Europe

In the European Union, stroke is the second most frequent cause of death and a leading cause of adult disability. Around 1,1 million habitants are affected by this disease every year and it is responsible for 440000 deaths (7).

Large differences in incidence, prevalence and mortality have been identified between Eastern and Western Europe. This can be justified by greater levels of hypertension and other risk factors in Eastern Europe population (8).

In 2017, the cost related with stroke was estimated at 45 billion euros, including direct and indirect costs of care provision and productivity loss (9).

Figure 1 represents the annual age-standardized incidence of stroke in Europe, at the beginning of the 21st century. It ranged from 95 to 290/1000,000 *per year*. Stroke incidence is largely dependent on the population analyzed in terms of sex and age, therefore there is a need for standardization, to allow comparison between different studies (7).

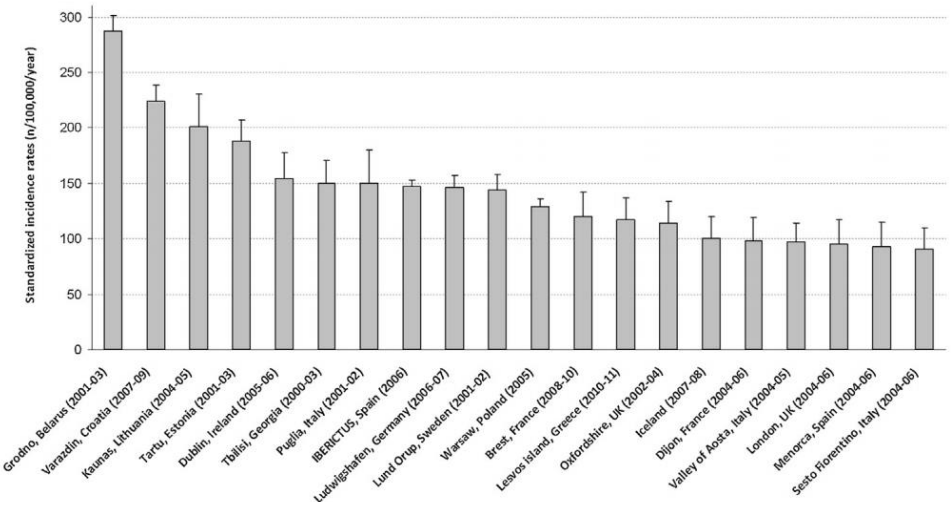

Figure 1: Annual age-standardized (to the European Population) stroke incidence rates in European population-based registries at the beginning of the 21st century (from reference (7))

Figure 2 (7) shows the association between the incidence of stroke and age of the population under analysis.

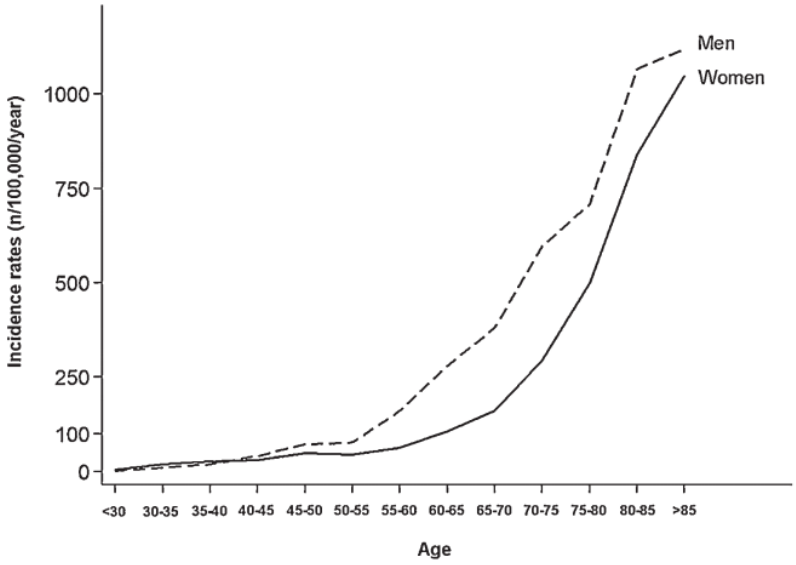

Figure 2: Crude annual incidence rates by sex according to age. Data from the Dijon Stroke Registry (from reference (7))

There is a clear correlation between age and the incidence of stroke, with and increased occurrence in older ages. Men have a higher age-specific incidence rate than women, which can be explained by the greater presence of traditional risk factors (7). Since women have a higher life expectancy, they are more likely to suffer a stroke event during their lifetime and less likely to recover (10).

Projection studies show that the burden of stroke in Europe will presumably increase in the next decades. As life expectancy continues to grow, an increase in the number of stroke events, the long-term sequelae, and associated costs is expected (9).

By 2047, the population size is likely going to remain stable, however there will be an additional 40000 stroke cases (3% increase) and 2.58 million prevalent cases (27% increase). This is mainly due to the projected changes in the population age structure and the increase of residents aged ≥ 70 years, in which stroke risk is the highest (9).

3.2 Portugal

Vascular diseases are the main cause of death in Portugal, being responsible for almost 40% of mortality in which around 45% are due to stroke (11).

The lethality rate at 28- and 30-days post stroke, has decreased in the last years, and this can be justified by a better control of risk factors. The disability rate has also diminished due to the quicker arrival at hospitals, through the "Via Verde do AVC" [1] and also due to the investment in healthcare campaigns, that help patients to better recognize early symptoms and call the emergency number more rapidly. Even so, there is still a large percentage of stroke survivors that become dependent on others for day to day activities (12).

In 2015, the "Direção Geral da Saúde" renewed its strategy on prevention and treatment of this disease. The main goals are reducing global cardiovascular death, reducing early mortality, decreasing global burden and morbidity, and promoting investigation on European Networks. The outlined strategy to achieve these goals involves improvement and consolidation of "Via Verde do AVC*"* strategies, monitoring essential activity and also resources and expenses (13)

*1-"*Via verde do AVC" is a specialized circuit that assures that when the patient arrives at the hospital, the specialized teams are ready to intervene rapidly, therefore preventing sequels of stroke and improving chances of recovery.

4 Pathophysiology of stroke

Understanding the pathophysiology of stroke, provides a better insight of potential neuroprotective targets.

Stroke occurs when blood flow to a certain area of the brain is disrupted, leading to a certain degree of neurological damage (14).

Ischemic stroke can be subdivided in categories according to its etiology. TOAST classification has been widely used in stroke research and in therapeutic studies (15). This method subdivides ischemic stroke in five categories:1) large-artery atherosclerosis, 2) cardioembolism, 3) small-vessel occlusion, 4) stroke of other determined etiology, and 5) stroke of undetermined etiology (16).

Brain injury after ischemic stroke emerges from a complex signaling cascade. Since the brain has a high demand of oxygen and glucose, a disturbance in circulation to the brain, rapidly leads to a depletion of substrates (17).

After a stroke, there are two major areas of injury: the penumbra and the infarct core. Penumbra refers to the area of the brain, in where blood flow is insufficient enough to cause hypoxia and compromise physiological function but not so complete to cause permanent damage. Ischemic penumbra is a dynamic process, and it offers a short time-window in which cells in this zone can be salvaged by reperfusion (18). Contrarily, cells in the infarct core die during the first minutes. For this reason, most treatment strategies focus on salvaging the penumbra (5).

The earlier stage of cerebral ischemia is fundamentally caused by an energetic problem. In the hypoxic area, adenosine triphosphate (ATP) continues to be used despite the insufficient production, leading to a decrease in total ATP levels and development of lactate acidosis. This is the first step of a series of events in a multimodal and multicell ischemic cascade (19).

These mechanisms are quite complex and will not be fully characterized in this dissertation since it is not the focus of this work. However, Figure 3 summarizes the events that occur in the ischemic cascade (20).

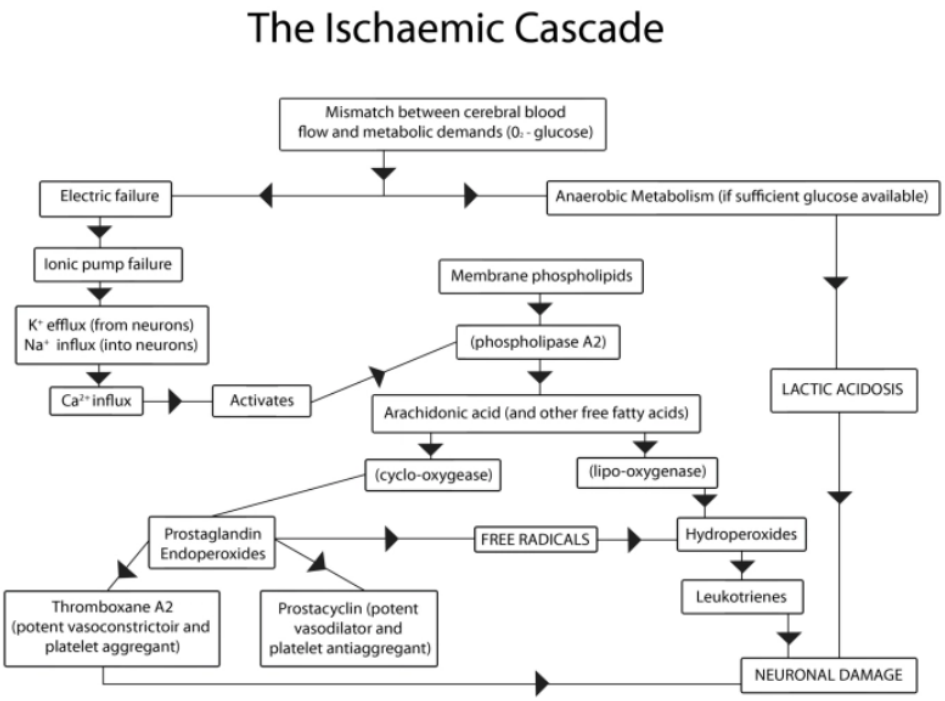

Figure 3: Illustration of the events that occur in the ischemic cascade
(from reference 20)

5 Current treatment strategies

Intravenous administration of tissue plasminogen activator is the gold standard in the treatment of AIS, regardless of its etiology if the patient is eligible. The notion that "Time is Brain" is still widely acknowledged as its estimated that 1.9 million neurons are lost with each minute of ischemia (21).

The 2008 European Stroke Organization (ESO) guidelines for management of ischemic stroke, recommend intravenous recombinant tissue plasminogen activator (rtPA) within 3 hours of onset of ischemic stroke (8). However, in 2021 the ESO released and updated guideline on intravenous thrombolysis for AIS. In the latest guideline, the ESO gathered data from randomized and observational studies that endorse a wider use of intravenous thrombolysis (IVT) (22).

The 2018 Guidelines from the American Heart Association (AHA), also recommends the use of IV alteplase in selected patients within a 3 hour time-window since the onset of stroke (23).

However, none of these guidelines currently recommend the use of neuroprotective agents in clinical practice.

3.12. Neuroprotective Agents

3.12. Neuroprotective Agents	COR	LOE	New, Revised, or Unchanged
1. At present, no pharmacological or non-pharmacological treatments with putative neuroprotective actions have demonstrated efficacy in improving outcomes after ischemic stroke, and therefore, other neuroprotective agents are not recommended.	**III: No Benefit**	**A**	Recommendation reworded for clarity from 2013 AIS Guidelines. LOE unchanged. COR amended to conform with ACC/AHA 2015 Recommendation Classification System. See Table LXXXIII in online Data Supplement 1 for original wording.
Recent trials of both pharmacological and nonpharmacological neuroprotective treatments in AIS have been negative. The FAST-MAG trial (Field Administration of Stroke Therapy–Magnesium) of hyperacute magnesium infusion was the first acute stroke neuroprotection drug trial to enroll participants during ambulance transport, but no differences were seen between the intervention group and placebo control subjects.[103] A recent Cochrane review of neuroprotection trials in AIS further confirms the recommendation of no benefit with previously studied interventions to date.[114]			See Table XLVIII in online Data Supplement 1.

Figure 4: Recommendations on the use of neuroprotective agents for AIS (AHA) (from reference 23)

6 The concept of neuroprotection

As stated before, IVT is the gold standard of stroke treatment. Despite the neurological improvement that this treatment provides, its benefits are limited by the short therapeutic time-window approved for its administration. Because of the severe inclusion criteria for the administration of rtPA, only a small percentage of the people that suffer a stroke are eligible for this therapy, leaving most patients out of the eligible group (24).

In the past decades, significant progress has been made in the comprehension of the mechanism of cell death in ischemia. This encouraged the emergence of new treatment approaches such as neuroprotection (25).

Cerebral neuroprotection is a therapy aimed at enhancing the brain's resilience to ischemia to improve the clinical outcome of patients. It is defined as "any strategy, or combination of strategies that antagonizes, interrupts, or slows the sequence of injurious biochemical and molecular events that, if left unchecked, would eventuate in irreversibly ischemic injury" (26).

According to this definition, neuroprotective strategies begin at the neuron itself (endogenous neuroprotection), as opposite to thrombolitycs, anthitrombotics, and antiplatelet drugs that target the cerebral vasculature (exogenous neuroprotection), and therefore are not classified as neuroprotective strategies. Naturally, these agents do protect the brain by restoring normal blood flow and preventing the formation of clots. However this is achieved by vascular-based mechanisms that do not target the brain parenchima itself (26).

While thrombolytic treatment breaks down the clot in order to restore blood flow, neuroprotection aims to prevent salvageable neurons located in the penumbra from dying (26). These strategies work by disrupting the neuronal cascades, blocking signaling pathways and minimizing the pathological processes that take place after stroke occurs (5).

Currently, thrombolysis is the best possible way to improve clinical outcome, by rapidly restoring oxygen and glucose supply to the brain. Nevertheless, strategies that directly target the brain parenchyma may be an interesting approach, especially in combination with IVT (26).

Even though most neuroprotective strategies seem promising in pre-clinical research, most of these agents have failed to show clinical benefit (27).

This gap between bench and bedside, raises the question of feasibility and practicability of neuroprotective strategies (27).

Feasibility is linked to biology and raises the question if it is possible to obtain neuroprotection in human brain, suggesting that although neuroprotection can be achieved in rats, it fails when attempted in the human brain. Recent studies with postsynaptic density-95 protein (PSD-95) inhibitors discard this hypothesis. The role of PSD-95 inhibitors is to uncouple PSD-95 from neurotoxic signaling pathways in central neurons. Some studies used cynomolgus macaques as animal models, that have genetic, anatomic, and behavioral similarities to humans and who were submitted to middle cerebral artery occlusion (MCAO). In summary, treatment with PSD-95 inhibitor, NA-1, reduced infarct volume as measured by magnetic resonance imaging (MRI) and histology, and significantly preserved the gene transcription capacity of ischemic cells and neurological function in neurobehavioral testing. These results prove that it is possible to achieve neuroprotection in high order brains even after a severe experimental stroke (27).

On the other hand, practicability refers to the capacity of neuroprotectants to be tested in a scenario that translates into clinical benefit for patients. This question remains unanswered since to this day, no neuroprotectant agent has been able to show clinical benefit (27).

7 Therapeutic targets for neuroprotection

Theoretically, neuroprotection could be achieved by drugs that target one or more key points of the ischemic cascade that leads to neuronal damage. There is a wide range of biochemical pathways that play a part in the ischemic cascade, and therefore are possible targets for neuroprotectants (27).

7.1 Inflammation

Inflammation is involved in ischemic brain injury and immune mechanisms are directly linked to the development of neuronal damage after stroke. Therefore, anti-inflammatory strategies have been developed in the last years, aiming for the inhibition of some cytokines, blocking intercellular adhesion molecules and blocking inflammatory cell trafficking (28).

Following a stroke event, the damaged neurons release proinflammatory cytokines and reactive oxygen species, that firstly activate the resting microglia that surrounds them and secondly lead to the infiltration of inflammatory cells such as neutrophils and macrophages, that in turn, also release their own secretory factors. Inflammatory processes also result in the production of free active oxygen species that cause oxidative stress and activation of matrix metalloproteinases (MMPs) resulting in disruption of the blood-brain barrier (BBB) and edema (29).

Tumor necrosis factor alpha (TNFα) is one of the first initiators of inflammatory processes making it an ideal target for neuroprotective compounds. These strategies aim to reduce or block the formation of TNFα (29). It is known that TNFα binds to its receptors, TNF-R1 and TNF-R2 forming a trimer on the plasma membrane surface (30). By binding to these receptors it mediates death signals via the Fas associated domain (FADD) and it modulates inflammation via the nuclear factor kappa-light-chain enhancer of activated B cells (NFkB) (29).

Table 1, summarizes some of the neuroprotective compounds that target the inflammation pathway (29).

Table 1: Examples of neuroprotective agents that interfere with TNFα related pathways (from reference (29))

Compound	Mechanism of action	Outcome
3'6-dithiothalidomide	Inhibition of TNFα synthesis	Reduced number of activated inflammatory cells and reduced extent of BBB disruption after ischemic stroke in mice
Caffeic Acid ester fraction	Inhibition of the production of TNFα, c, nitric oxide (NO) and IL-1β	Reduced infarct volume and improved performance on behavioral tests in rats submitted to MCAO
Decoy receptors	Binding to TNF receptor and passage through the BBB	Reduced infarct volume after tMCAO in mice
Honokiol	Suppression of the activation of NFkB and TNFα levels	Significant reduction of water content in ischemic mice
Rosmarinic acid	Blockage of activation of NFkB by TNFα	Reduced edema and tissue damage in diabetic rats submitted to tMCAO
Angiotensin	Suppression of NFkB activity	Reduced infarct volume, improved neurological deficits, and decreased oxidative stress in tMCAO rats
Kaempferol glycosides	Inhibition of NFkB activity and STAT3	Reduced infarct volume and neurological deficits in tMCAO rats

The first immune cells to respond are brain-intrinsic microglia, that when activated release mediators that attract neutrophils, monocytes, and lymphocytes (31). Limiting the activation of microglia can have neuroprotective effects, however these cells also have a beneficial role since they remove dead tissue from the injury (29).

Some compounds work by limiting microglia activation, for example, Ginseg metabolic compound K inhibits multiple upstream signaling molecules and therefore suppresses the activation of microglial and has neuroprotective effects in MCAO mice. On the other hand, Glibenclamide works in an opposite way, increasing the phagocytic ability of microglia, which resulted in reduced infarct volume, improved neurological outcomes, and enhanced neurogenesis in rats subjected to transient or permanent MCAO (29) .

7.2 Oxidative Stress

The generation of free radicals is a crucial step of the ischemic cascade. These molecules are highly reactive and damaging to multiple cellular components (32). When the production of reactive oxygen/nitrogen species (ROS/RNS) exceeds the body's antioxidative capacity, oxidative stress occurs (5).

The brain has several characteristics that makes it prone to free radical damage. It has a high index of polyunsaturated fatty acids, which are particularly susceptible to free radical induced peroxidation, and it also has a low content of antioxidant enzymes, such as catalase and glutathione peroxidase (33).

The reduction of oxidative stress can be achieved either by preventing the generation of free radicals or by enhancing the antioxidant capacity of the brain (32).

Nitric oxide is a normal molecule in the body with beneficial effects on stroke, however the increased activity of the induced nitric oxide synthase (iNOS) leads to aberrant signaling. When iNOS reacts with superoxide, it generates peroxynitrite (29). Peroxynitrite is a particularly interesting target since it doesn't have any known physiological functions, and it promotes lipid peroxidation (31). Nebivolol decreases the expression of iNOS and has proven to reduce histopathological changes in rat models (29).

On the other hand, there are compounds that aim to reinforce the body's capacity of free radical removal. In rats subjected to focal cerebral ischemia, hydrogen sulfide gas increased the activity of superoxide dismutase and glutathione peroxidase, resulting in decreased injury to neuronal mitochondria and diminished markers of apoptosis. Another example is the administration of hydrogen-rich saline in pMCAO rats, that also enhanced endogenous antioxidant activity consequently reducing the number of oxidative molecules (29).

The use of exogenous compounds with free radical scavenger activity has also been explored as a possible approach. A novel compound called MnTm4PyP showed reduction in infarct volume and neurological deficit after MCAO in mice, by mimicking the activity of endogenous manganese superoxide dismutase (29).

7.3 Blood-brain Barrier disruption

In a healthy brain, the BBB modulates the entry of cells and molecules into the brain which in conjugation with the central nervous system (CNS) microenvironment creates an area of immune privilege. During and after an AIS the BBB is disrupted. In the following hours after stroke, the BBB reversible opens, and in up following days the BBB suffers an irreversible disruption (34).

This disruption is associated with the action of two matrix metalloproteinases, MMP-2 and MMP-9. In a normal brain tissue, MMP-2 is expressed at low levels, but following stroke, the expression of this protein is increased, and its activity induces the cleavage and activation of MMP-9. In turn, MMP-9 degrades components of the basement membrane in vascular walls leading to BBB disruption. MMP activity is regulated by tissue inhibitor of matrix metalloproteinase (TIMP), hence treatments that stimulate it could be protective against brain damage after AIS (29).

Similarly to TIMP, compounds that inhibit the expression of MMPs can have neuroprotectant properties. Ethanol suppresses MMP-2 and MMP-9 expression in rat models after transient MCAO (tMCAO), and considerably reduces brain edema. *Apocynum venetum* leaf acts in the same way as ethanol, and it reduces the symptoms of BBB disruption (29).

7.4 Excitotoxicity

Excitotoxicity is a type of neurotoxicity mediated by glutamate and has received a lot of attention in stroke research. Glutamate is the main neurotransmitter in the adult CNS (35).

The lack of oxygen and energy levels in the brain during stroke, cause the release of toxic amounts of glutamate into extracellular space (29).The accumulation of this neurotransmitter activates its receptors leading to an aberrant influx of calcium and neuronal depolarization that over activate multiple downstream signaling pathways initiating processes such as necrosis, apoptosis and autophagy (31).

Glutamate post-synaptic receptors can be divided into ionotropic or metabotropic. In its turn, ionotropic receptors can be N-methyl-D-aspartate (NDMA) receptors and non-NMDA receptors (36).

Strategies that modulate glutamate excitotoxicity have been widely investigated and these include suppressing glutamate by inhibition of its release, decreasing glutamate levels by upregulating its uptake system and antagonizing glutamate receptors (5).

EGb761 is an extract of *Ginkgo biloba* and it has been demonstrated that it considerably decreases striatal glutamate levels in mice subjected to MCAO, resulting in diminished neurodegeneration and edema (37).

On the other hand, microRNA miR-223 works by blocking the action of glutamate receptors subunits GluR2 an NR2B in the brain, and it has shown to be neuroprotective against transient global ischemia. When activated, the transient receptor potential vanilloid 4 increases the activity of NMDA receptor. Therefore, HC-067047 a TRPV 4 antagonist, decreases the extension of the infarct after tMCAO in mice (29).

7.5 Apoptosis

Minutes after the occurrence of a focal ischemic stroke, the core of the infarct is irreversible injured and thus undergoes necrotic cell death. However, research has shown that in the ischemic penumbra, neurons may undergo apoptosis several hours or days after stroke, and therefore they are potentially salvageable (38).

ATP cell levels are key to distinguish between necrosis and apoptosis since apoptosis requires ATP and necrosis does not. For this reason, when ATP levels are insufficient, cells end up dying from necrosis. Apoptosis can be suppressed by several mechanisms and is therefore a potential target for neuroprotective strategies (29).

The mechanisms of apoptosis are highly complex and involve an energy dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (39).

Figure 5, represents the mechanisms of induction of apoptosis (29).

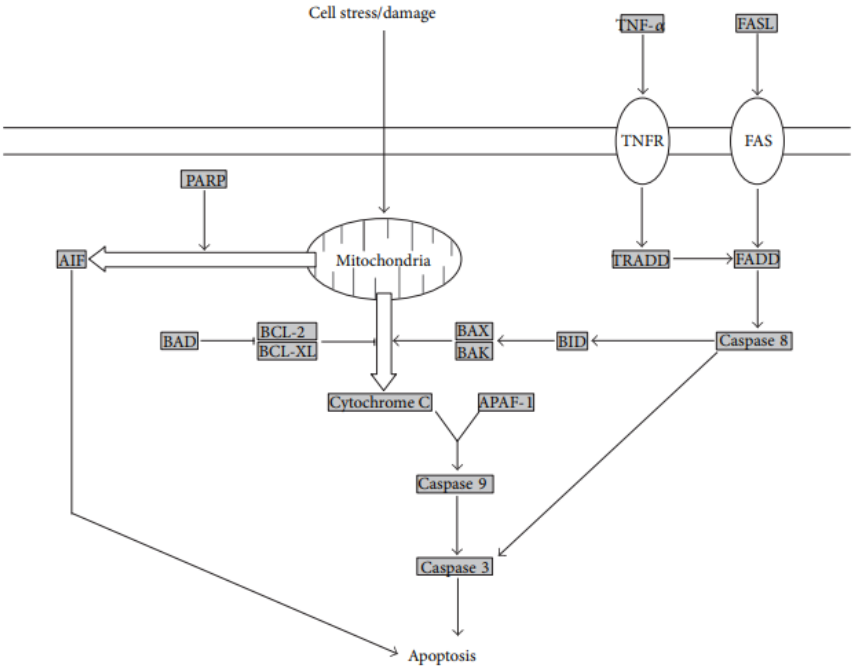

Figure 5: Mechanisms of induction of apoptosis (from reference (29))

In the caspase dependent pathway of mitochondrial apoptosis, the mitochondria releases Cytochrome C, resulting in the activation of caspase 9. Caspase 9 will then activate caspase 3 which in turn leads to a caspase cascade that terminates in degradation of cellular components and cell death (29).

The executioner caspase-3 activity is often used as a marker of apoptosis, for this reason many neuroprotective strategies aim at reducing caspase 3 activity. Some examples of drugs that target this pathway are Diallyl sulfide that reduces expression of this caspase and increases the expression of BCL-2, an endogenous antiapoptotic protein, in rats submitted to tMCAO, and Tanshinone IIA, that reduces caspase 3 in tMCAO rats, leading to a reduction in infarct volume, edema and neurological deficits (29).

In the caspase independent pathway, the activity of poly (ADP-Ribose) polymerase (PARP) stimulates the release of apoptosis inducing factor (AIF). This pathway does not require mitochondria; however, it does use caspase 3 as an effector, thus compounds that act on affect dependent pathway cascade can also work in the independent cascade (29).

Ethanol administration resulted in a decreased expression of caspase 3 and AIF after tMCAO in rats (40). Additionally, the nitric oxide donor (S)ZJM-289 stops the release

of both cytochrome C and AIF from mitochondria and considerably reduces injury in MCAO rats (41).

However, there are some compounds that target the caspase independent pathway specifically such as Ginsenoside-Rd, that inhibits PARP-1 activity and AIF release in rats subjected to MCAO (29).

7.6 Autophagy

Autophagy is an important mechanism that regulates multiple fundamental cellular processes that are involved with cellular growth and differentiation. It is responsible for the destruction of unviable proteins and organelles in a lysosome-dependent pathway maintaining homeostasis (42).

Besides the importance of autophagy, it appears to have a dual role in the response of cellular damage, being protective in some cases and causing cell death in others (29).

Figure 6 (29) illustrates the process of autophagy. It is mediated by the ATG family of proteins and regulated by mTOR. When autophagy is inducted, it may prevent cells from dying by apoptosis, thus it may be beneficial to induce it. Rapamycin inhibits mTOR and in a rat model of subarachnoid hemorrhage it inhibited autophagy avoiding cell death by apoptosis and neurological damage (43). Bexarotene is approved by the Food and Drug Administration (FDA) retinoid X receptor agonist used in cutaneous lymphoma. This drug can enhance autophagy and reduce infarct size in aged mice with thromboembolic stroke (44).

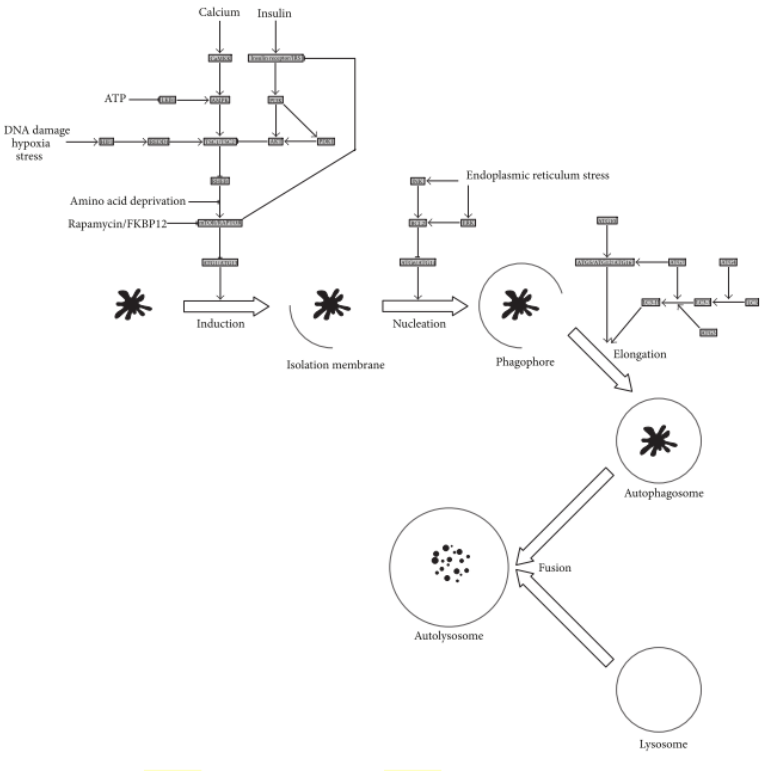

Figure 6: The process of autophagy and its regulation (from reference (29))

On the other hand, inhibiting autophagy can also be neuroprotective. Melatonin, works as an autophagy inhibitor and reduced infarct size and neurological deficits in MCAO rats (45). Ischemic postconditioning in pMCAO rats, can also reduce infarct size and edema trough the inhibition of autophagy (46).

8 Models for the study of neuroprotective strategies

To understand the underlying mechanism of novel compounds in neuroprotective strategies, along with its possible effects on physiological parameters, adequate stroke models are essential. While models of stroke will never fully mimic certain aspects of human stroke, they can lead to a better understanding of stroke pathophysiology and mechanisms of the novel compounds (32).

In vivo models of stroke are useful because they offer an improved understanding of the mechanism of potential new neuroprotective compounds and provide target validation at a molecular level. They allow demonstration of the impact that certain parameters have on the cells, such as privation of glucose and oxygen. This controlled environment enables to evaluate the causality in relation to modifications in specific variables. However, this tightly regulated models, are far from mimicking the complex interactions that occur in stroke in a human brain *in vivo* (32).

A substantial amount of research has been made using animal models. For this reason, different techniques for inducing stroke in animal models have been developed. It is important to understand each model since they have a different effect on the neuroprotective compounds. Most stroke models use rodents that are classified by the type of stroke they are intended to replicate (29).

The majority of the methods used to induce stroke in rodent models include the occlusion of one or more blood vessels. Middle cerebral artery occlusion is a method often used when trying to create focal ischemia models. On the other hand, for global ischemia models often more than one vessel is occluded, such as the common carotid arteries, or the vertebral artery. The models can also involve either permanent ischemia, or transient ischemia followed by reperfusion (29).

In most strokes, recanalization is very limited, making permanent ischemia models a more accurate representation of reality. In turn, transient models are more comparable to the effects of endovascular treatment, that is if the occlusion duration matches the onset time of recanalization observed in most stroke patients. These models also vary in the neuronal damage pathophysiology: while in permanent occlusion the loss of

neuronal damage is progressive, in the transient model there is a delayed-type insult (32).

The selection of stroke models should consider the preclinical research purposes. If the authors are aiming to test the efficacy of a new drug, the models used will not be the same as if they were looking to clarify a pathophysiological mechanism of the disease (32).

Embolic models produced trough the autologous clot method are more accurate models, but this technique is quite complex and has a high mortality rate. Some other methods can also be used to mimic embolic stroke, such as photochemical and microsphere-induced thrombus formation, together with some new methods involving the local injection of thrombin (32).

The quality and consistency of preclinical research in this field has been highly erratic. When designing a preclinical study, is important to include multiple species, and different strains since they have different characteristics that may affect the results, such as discrepancies in collateral circulation and behavioral responses (32).

9 Preclinical research

Over the past decades, preclinical stroke research has had a significantly low translational success rate, and the clinical need for novel neuroprotective therapeutics has not been achieved (47).

From 1957 to 2003, around 1026 stroke drugs have been identified, and this number has continued to increase in the last decades. Around two thirds of these studies reported benefit when compared to placebo groups (26). However, the efficacy of this treatments seems to be overstated by publication bias. Publication bias means that positive results are more likely to be published than negative data. Null results will probably remain unpublished, leading to an overstatement of efficacy (48).

Developing a new drug, requires the evaluation of candidate drugs first in vitro, followed by experimental animal models and lastly clinical trials. Preclinical research allows not only to access a drug efficacy but also to clarify the underlying mechanisms of action (26).

Using the correct stroke model is essential for translational applicability. Table 2, presents a summarized overview of the experimental models of cerebral ischemia commonly used for neuroprotective studies, as well as some advantages and disadvantages of each one (47).

Table 2: Overview of experimental models of cerebral ischemia (from reference (47))

Category of Model and Common Examples	Advantages and Disadvantages
In **vitro models** (cell cultures or organotypic slices): oxygen-glucose deprivation, metabolic-mitochondrial toxins, excitotoxicity (eg, glutamatergic agonists [NMDA])	Excellent for mechanistic investigations owing to the variety of tools that are available (eg, more amenable to knockdown/overexpression methods), reductionist in nature (enable study of cell-specific responses more conveniently than

	selective knockout animals), impossible to evaluate effects of physiology
In **vivo models** (global ischemia): 4-vessel occlusion (carotid and vertebral arteries), cardiac arrest, 2-vessel occlusion (carotid arteries) with hypotension	Very severe ischemia; allow examination of selective vulnerability in hippocampus, cortex, and cerebellum; replicate clinical cardiac arrest or strangulation rather than stroke
In **vivo models** (focal ischemia): intraluminal filament MCAO, direct MCA ligation/cauterization, photothrombosis, endothelin-1 injection, thromboembolism	Closest models of clinical stroke (particularly the permanent models), reliable and reproducible infarcts, transient or permanent, varying extent of surgical invasiveness (minimal for filament MCAO, more severe for direct approaches)

Improving preclinical research is paramount to closing the gap between bench and bedside. The Stroke Therapy Academic Industry Roundtable (STAIR) are guidelines that help investigators to follow the best practice when designing a preclinical study. The first STAIR criteria recommendations were released 17 years ago, and it focused on the importance of blinding, replicability in different species, accounting for sex differences, and clinical criteria for instance the route of administration used and therapeutic window. These guidelines have already been updated and although these concepts are now commonly accepted by investigators, translation of preclinical research is still challenging. Preclinical studies of rigorous quality could be obtained by maintaining consistency, reducing bias, and increasing reproducibility. However, there is a lack of funding for this type of high-quality research (32).

One way to overcome the lack of funding, would be to support large-scale collaborations between preclinical laboratories, and to obtain central funding for research. In 2018, the US National Institutes of Neurological Disorders and Stroke (NINDS) requested applications for participants in the Stroke Preclinical Assessment Network (SPAN) project. The NINDS opened applications for this project, and then proceeded to select six studies by competitive peer review. One coordination center was also selected. The SPAN project aims to achieve a better preclinical development by the implementation of technical innovations such as: central randomization, masking treatment assignment, power analysis and rational sample sizing, replication in multiple

laboratories, the use of models with different characteristics that impact the outcome like, hypertension, age, and sex. In this project, the preclinical investigation will follow rigorous measures that are normally used in clinical research, to achieve better results. One example is that all subjects of experiment will be demanded to have a permanent bar-coded ear tag. This will allow to identified drop out subjects and reflect a more accurate result. A central scheme will be used to achieve randomization, therefore reducing the probability of local investigators bias. The avoid bias the compounds were stored with identical packaging and shipped from a central pharmacy. The investigators were told to record and upload a video of the subjects performing the behavioral task to blind the behavioral assessments (32).

Working groups in both Europe and the USA, agree that these issued need to be considered in a coordinated method. It is unanimous that multisite investigation like designed in the SPAN project are needed and must include crucial factors that allow to avoid mistakes made in the past and overcome prior challenges (49).

10 Clinical Research

10.1 Recently completed clinical trials

Over the last 5 years there has been several clinical trials covering the topic of neuroprotection in stroke, and some are still ongoing. Some of the compounds addressed, underwent efficacy trials, while others were only tested on feasibility or safety trials. Many of these compounds already had experimental data available, including the mechanism of action, outcome in rodent models, and use at longer intervals after ischemic injury.

10.1.1 Uric Acid

Uric acid (UA) is the product of purine catabolism in humans, and a powerful antioxidant that scavenges reactive nitrogen and oxygen radicals and non-radicals. It showed potent neuroprotective effects in preclinical stroke models (50).

After an ischemic assault, the brain rises the production of uric acid, but as the infarct volume increases the antioxidant capacity of this compounds is gradually diminished (49).

This compound was the first one assessed in a randomized clinical trial. In this trial all the subjects received alteplase and some of them underwent rescue thrombectomy (51). In general, 39% of the patients receiving UA and 33% of the ones receiving placebo had a great outcome. In the placebo group, early ischemic worsening occurred more often. The results also showed a significant influence between treatment and sex. In women, who were submitted to UA therapy the effect of the placebo to acquire the primary outcome was duplicated (52). On the other hand, glucose levels also affected the results, as patients with hyperglycemic at the onset of stroke, had a three-fold increase in the primary outcome compared to placebo. In the group of patients that were submitted to rescue thrombectomy, there was a 19% increase in the good outcome, that was evaluated using the modified ranking scale (53). Although the results of treatment with uric acid were very promising, a confirmatory trial of its benefits is planned.

10.1.2 Nerinetide

Nerinetide (NA-1) is a cell permeable peptide, that disrupts the binding of the synaptic protein PSD-95 with NRB2 subunit of NMDA receptor and to the PDZ domain of

neuronal nitric oxide synthase (nNOS) (54). By interacting in this site it prevents cell death in acute ischemia and reduces stroke damage in animal models of ischemia/reperfusion including non-human primates (49).

The efficacy and safety of nerinetide for the treatment of acute ischemic stroke trial (ESCAPE-NA1) was a multi-center, double blind randomized, single dose, placebo-controlled clinical trial that enrolled patients with large vessel occlusion within a 12-hour treatment window in 48 acute care hospitals in eight countries (55).

The results showed that treatment with a single dose of NA-1 in patients submitted to endovascular thrombectomy, did not improve clinical outcome when compared to the patients that received endovascular thrombectomy along with placebo. However, in the subgroup of patients that received treatment only with NA-1 and were not subjected to endovascular thrombectomy there was a decreased in infarct volume, a lower mortality rate and improved functional outcome. The investigators speculated that in patients also receiving alteplase, this thrombolytic agent was activating proteases that were digesting the drug (55).

Currently, the FRONTIER (C) trial is investigating the safety and efficacy of prehospital intravenous NA-1 in the field for AIS in a 3-hour time-window after symptoms onset. A new trial is also planned to evaluate the benefit of nerinetide in patients that do not receive thrombolysis before thrombectomy (49).

10.1.3 Otaplimastat

Otaplimastat is a small molecule that improves neurological outcomes through various cytoprotective mechanism in multiple animal stroke models. It reduces edema and intracerebral hemorrhage induced by rtPA. The SAFE-TPA clinical trial evaluated the safety and efficacy of Otaplimast in patients with acute ischemic stroke requiring tPA (56). After starting the rtPA infusion, 80 patients received either Otaplimast or placebo. In the placebo group and the low-dose Otaplimast group, the incidence of parenchymal hemorrhage was 0%, contrarily, in the high dose Otaplimast group, the incidence was higher, at 4.7%. On the other hand, the low dose Otaplimast group was associated with a better proportion of good outcome when evaluated in the modified Rankin Scale. However, it was associated with the lowest rate of successful reperfusion (53% vs 74% placebo) (49).

8.1.1 9.1.4 Activated C protein (APC)

APC derives from the protein C zymogen produced by the liver, and it has anticoagulant and cytoprotective properties mediated via the protease-activated receptor 1 (PAR1) (49).

3K31-APC is a synthetic analogue of APC, and its safety was evaluated trough the RHAPSODY clinical trial. In this trial, 110 patients with one of four doses of the pleiotropic PAR1 agonist, 3K3A-APC or placebo after intravenous alteplase, thrombectomy or both. This trial showed that the drug is well tolerated in stroke patients and there was a trend towards lower hemorrhage rate when compared to placebo-treated patients (57).

10.1.4 Statins

Statins are the most effective drugs at lowering cholesterol levels and decreasing the mortality related to cardiovascular diseases. Recent studies, using animal models have demonstrated that statins can reduce infarct size when administered after stroke, whether with or without the use of thrombolytic agents (54).

NeuSTART, was a phase I clinical trial that determined that the use of lovastatin in stroke patients is effective and feasible. Following this trial NeuSTART2, assessed the efficacy of short-term administration of a high dose of levostatin (640 mg/day) for 3 days after stroke when compared with patients treated with the standard dose of levostatin (80 mg/day) before and after stroke. The outcome of this trial has not been published yet. The ASSORT trial, was a multicenter randomized controlled trial that recently finished. This trial revealed that statin therapy 7 days after stroke, was just as efficient as statin treatment administered in the early acute phase of ischemic stroke at alleviating physical disability 3 months after the insult (54).

10.1.5 Edavarone

Edavarone is a low-molecular weight free radical scavenger that can rapidly cross the BBB. Studies in rodent models of ischemic stroke have demonstrated that pre-treatment or post-treatment with edavarone can decrease infarct volume and ameliorate neurological function (54).

The safety and tolerability of edavarone has already been established by clinical trials, and this drug was well tolerated in normal volunteers following administration of a single or multiple doses (54).

The combination of edavarone with thrombolytics has been assessed in some clinical trials, and one of them showed that treatment with edavarone prior to intravenous administration of thrombolytics diminished intracerebral hemorrhage (58). These discoveries lead to the approval of edavarone for AIS treatment in Japan, although is not considered standard treatment globally (59).

10.1.6 Natalizumab

Natalizumab is a humanized antibody that targets the cell adhesion glycoprotein alpha-4 integrin (CD49d), that is expressed on the surface of lymphocytes and monocytes, and it eases their adhesion to the endothelium (54).

The ACTION trial evaluated the efficacy and safety of a single dose of natalizumab (300mg) administered within 9 hours of AIS, and it established that it was safe and feasible. Nonetheless, the efficacy endpoint was negative at this dose (60).

ACTION 2 trial followed based on the finding of ACTION. The goal was to assess the efficacy of a higher dose of natalizumab in stroke treatment. Unfortunately, the administration did not improve patient outcomes (61).

10.1.7 Vinpocetine

Vinpocetine (VPN) is a synthetic ethyl-ester derivate of the alkaloid apovincamine from Vinca minor leaves (61).

The neuroprotective effects of vinpocetine have been imputed to several mechanisms. It is extensively used in numerous European countries and in Asia for prevention of cerebrovascular diseases. It rapidly crosses the blood brain barrier whether it is administered orally or via IV (54).

Even though the safety and feasibility of the administration of vinpocetine in ischemic stroke patients has already been established, there is no evidence available that supports its routine use in these patients (54).

Most studies that reviewed the effects of vinpocetine were not designed correctly, and the two studies that had appropriate designs, showed no considerable differences between groups. These two studies also used small samples. Therefore, additional

clinical trials are necessary to determine if the administration of vinpocetine is benefic in ischemic stroke patients (54).

A phase II clinical trial (NCT02878772) was recently completed in where a small group of patients received treatment with vinpocetine in a 4.5h to 48h window after the onset of symptoms. However, the results of this trial are not available yet (54).

10.2 Ongoing clinical trials

As the knowledge on the stroke mechanism pathways continues to increase, more neuroprotectants are being tested in clinical trials. There are several ongoing randomized clinical trials that aim to assess the safety and benefit of using neuroprotectants in clinical practice.

10.2.1 Remote ischemic conditioning

Remote ischemic conditioning (RIC) triggers endogenous protective pathways in distant organs including the brain, thus being and interesting approach for neuroprotection (62).

Preclinical trials using rodent animal models have shown the efficacy of RIC in reducing infarct size and improving functional outcome. Combined treatment with intravenous thrombolysis has also shown to be effective. However, the exact mechanism by which this therapy works is still unknown (49).

The REVISE-1 (remote ischemic conditioning paired with endovascular treatment for acute ischemic stroke) trial is researching if RIC is safe and effective in patients with large vessel occlusion undergoing thrombectomy (49).

10.2.2 Hemodialysis

Excess of glutamate is toxic for the ischemic penumbra because it constantly activates postsynaptic receptors, that lead to a large influx of calcium, sodium, and water into neurons. After the blood vessel open, these levels rapidly return to normal (49).

Up to now, antiglutamatergic strategies have shown to be safe or effective in patients with acute ischemic stroke (49).

However, the DIAGLUICTUS2 (feasibility and safety study to evaluate the neuroprotective effect of hemodialysis in acute ischemic stroke) is a randomized controlled clinical trial where patients with complete reperfusion after thrombectomy

are randomized to receive two peritoneal dialysis sessions or to best medical treatment (49).

10.2.3 Transcranial direct stimulation

Transcranial direct stimulation (tCDS) is a non-invasive stimulation technique that can potentially modulate the brain cortical excitability with sustained effects (63).

The TESSERACT-BA (Transcranial direct current stimulation as a neuroprotection in acute stroke before and after thrombectomy) is a single-center, dose escalation clinical trial in which transcranial direct stimulation (tDCS) is given to the penumbra in patients with stroke undergoing thrombectomy (49).

10.2.4 Verapamil

Verapamil for neuroprotection in stroke is a small phase I clinical study that evaluates the safety of the administration of intraarterial verapamil in an 8-hour window after the onset of stroke symptoms (49).

10.2.5 Magnesium and Verapamil

The MAVARIC (magnesium and verapamil after recanalization in ischemia of the cerebrum: a clinical and translational study) trial is an ongoing phase I, blinded outcome, randomized, placebo-controlled investigations that is studying the safety and feasibility of the administrating verapamil with magnesium sulfate promptly after successful thrombectomy (49).

10.2.6 NEU2000

NEU2000 is a synthetic derivate of aspirin that targets NDMA receptors and free radicals functioning as a dual neuroprotectant (54).

The SONIC (safety and optimal neuroprotection of neu2000 in ischemic stroke with endovascular recanalization) trial investigates the benefit of Neu2000 and thrombectomy in patients with moderate-to-severe stroke secondary to a proximal vessel occlusion. The recruitment of patients was finished in July 2020, but the trial is waiting for final analysis (49).

10.2.7 OTR4132-MD

OTR4132-MD is an agent made of polymers designed to substitute degraded heparan sulfates and promote the restoration of the natural architecture of the extracellular

matrix. Even though this compound has not been highly explored in preclinical trials, the MATRISS (study to assess the safety of ReGeneraTing Agent (OTR4132) in patients with acute ischemic stroke) trial is investigating the value of a single intra-arterial injection of this agent if thrombectomy is successful within 6 hours of stroke onset (49).

10.2.8 Imatinib

Imatinib is a tyrosine kinase inhibitor approved by the FDA for the treatment of cancer (54).

The main goal of using imatinib in stroke is to improve the side effects of thrombolytic treatment with tPA and extend the time window of its administration. In preclinical studies post ischemic administration of imatinib along with a delayed tPA treatment significantly reduced hemorrhagic complications and infarct volume (64).

These findings encouraged the Imatinib in acute ischemic stroke trial, a phase III clinical trial where this compound was given within 8 hours of symptom onset and given for 6 days. The study is investigating if this approach improves functional outcome and reduces intracerebral hemorrhage and oedema in patients with stroke treated with alteplase and/or thrombectomy (54).

10.2.9 1-3-N-Butylphtalide

1-3-N-Butylphthalide (1-NBP) is a compound isolated from seeds of *Apium graveolens* (49). Both 1-3-NBP) and its racemic form DL-3-N-butylphalide (DL-NBP) have demonstrated considerable neuroprotective effects in stroke (65).

This compound has been studied in preclinical trials using rodent models of permanent transient ischemia, and at least two studies used hypertensive rats, to assess the efficacy of post-stroke 1-NBP treatment. In China, this compound has already been approved as an anti-ischemic drug, and its efficacy and safety has been demonstrated in several clinical trials. Moreover, NBP is currently in phase III/IV of clinical trials in China, to investigate its multitargeted effect in ischemic stroke (54).

Other clinical trials, such as EBCAS, AIS, BAST and NCT03394950 are currently studying the safety and efficacy of administration of NBP in patients with AIS who receive alteplase/and or thrombectomy (49).

10.2.10 Exenatide

Glucagon like peptide 1 (GLP-1R) is a gastrointestinal hormone, that induces the release of insulin from pancreatic beta cells. Exenatide is a synthetic form of this hormone (54).

Preclinical studies using diabetic and non-diabetic animal models, have demonstrated the neuroprotective effects exerted by the administration of exenatide. However, those effects are only visible when exenatide is administered up to 3 hours after stroke (54).

This compound has been widely studied in animal models; however clinical studies are limited.

A pilot study successfully proved the safety and feasibility of exenatide (54). This positive result encouraged the TEXAIS (trial of exenatide in acute ischemic stroke) phase II clinical trial, that compares exenatide to standard of care treatment given withing 9 hours after stroke onset (49).

10.2.11 JPI-289

PARP is a DNA repair enzyme that is upregulated in brain cells during ischemia, causing cellular injury by inducing cellular NAD+ and ATP depletion, mitochondrial dysfunction, reactive oxygen species generation, apoptosis inducing factor activation and inflammation (66).

JPI-289 also known as amelparib is a highly specific, water soluble PARP-1 inhibitor that reduces PARP activity, increases ATP and NAD+ levels and decreases apoptosis-associated molecules both in vitro and in culture cells. In animal studies, PARP-1 inhibition has limited cellular injury therefore improving the outcome after ischemic insult (54).

The safety and tolerability of JPI-289 was proved by a phase I randomized, double-blind and placebo-controlled trial. Currently, there is a clinical trial being conducted in Korea to evaluate the efficacy of amelparib in the treatment of ischemic stroke (49).

11. Discussion: From Bench to Bedside

In the last years, there has been a significant amount of research in new strategies to promote neuroprotection in stroke. Several compounds have shown positive and enthusiastic results in preclinical trials, but none have translated into clinical practice. This shows a clear gap between bench and bedside and there are some reasons that explain the translational failure observed in this field.

11.1 Outcome measures

Most preclinical studies use infarct volume for primary outcome and quantify it using a histological stain. Studies are considered successful if treatment can reduce infarct volume (26). However, human studies usually measure efficacy through neurological function, using tools such as the NIH Stroke Scale and the modified Rankin Scale. Translation from preclinical studies, to clinical trials is hard, since infarct volume correlates poorly to with funcitonal outcome. Small lesions in crucial locations can cause severe damage, and on the other way around large lesions can cause impercetible damage when it occurs at a silent area (67).

11.2 Functional Assessment

A variety of test have been developed to asses function in preclinical studies such as limb pacing, beam and grip walking, grip strength, T-maze retention test, Morris water maze results and radial arm maze performance (67). However behavorial test predominantly used, are not always representive of clinical outcome measures of disability or dependence after stroke (47). In clinical trials the NIH stroke scale, modified Rankin Scale and Barthel Index are the most frequently used of measuring endpoints. Nonetheless, none of these scales have been proven to correlate with infarct volume or any particular set of preclinical studies (67).

11.3 Selection of animal models

It is widely known that rodent population usually used does not mimic the standard AIS patient population (32). Preclinical research mainly uses young, male rodents of a similar strain, not representing the heterogeneity of stroke patients, who are commonly elderly and have comorbidities that impact outcome (47). Modifying this discrepancy

is a crucial aspect of translation, and it should not be overlooked, independently of the increased cost of animals with premorbid conditions (67).

11.4 Therapeutic Window

Most preclinical trials have a very short time window between the onset of symptoms and administration of therapy, however clinical trials often rely on a longer time window. Neuroprotective agent should show efficacy for at least 2 to 3 hours after artery occlusion. There are certain limits on what can be achieved in real world practice, and this should be reflected in preclinical studies. However, health systems should always aim to improve the workflow minimizing the delay of therapy administration (32).

11.5 Drug-dosing schedules

In animal models, there is a wider scope for dose optimization than in human studies. Often, clinical trials use considerably lower doses than those used in animal studies to avoid toxicity.

Additionally, in preclinical trials the drugs are often administered during a short period of time before or after the stroke insult. Contrarily, in clinical trials the drug dose regimens are very irregular and can vary between a single intravenous infusion to several oral doses during a 3-month period. To avoid this discrepancy, appropriate methods should be used to determine the right dose for humans based on the on the ones used in animal models (67).

11.6 Publication Bias

A crucial aspect of designing experimental studies, whether they are preclinical or clinical is the knowledge of the available literature. A good place to start is consulting systematic reviews, however reports suggest that only around 10% to 15% of all results published are invalid or negative results. There is a strong bias towards positive results and investigators are hesitant to publish null or negative results. This leads to an overstatement of efficacy of the novel compounds. This matter requires coordinated efforts from journals, institutions, and funding agencies (32).

The search of neuroprotectants in the last decades has been frustrating. Early success in preclinical studies, may have caused and early investment in clinical trials that did not turn out successful (67).

The image below describes some of the gaps between clinical and preclinical stroke as well as possible ways to minimize them (47).

Table 3: A Comparison of Clinical and Experimental Stroke, With Suggested Modifications to Increase Clinical Relevance of Animal Models (from reference (47))

Clinical Stroke	Preclinical Stroke	Potential Improvements	Caveats of Improving the Models
Typically >65 y	Typically young animals	Use of aged animals	Increased cost, time, mortality
Often extensively comorbid: hypertension, diabetes, hepatic disease, renal disease, cardiovascular disease	Typically healthy at time of stroke inducement	Use of comorbid animals: hypertensive rat strains, chemically induced diabetes	Increased cost, time, mortality; comorbidity models not necessarily accurate representations of clinical disorders
Great variation in site, duration, and extent of ischemia	Highly consistent areas of ischemia, targeting MCA in vast majority of experiments	Use of thromboembolic models, stroke-prone strains or transgenics (particularly in conjunction with comorbidities)	Current models are extremely well documented and widely used throughout the field, making conclusions more generalizable; targeting other arteries would be surgically more invasive.

Outcome measured in terms of mortality and functional impairment, on a chronic timescale	Outcome measured primarily in terms of histological or MRI changes (particularly in rodents), on an acute/subacute timescale	More extensive behavioral testing using clinically relevant tasks, longer survival periods following ischemia	Procedural confounders can complicate analysis: presence of neck and head wounds, ligation of arteries supplying cranial muscles, nerve damage
Dose and delivery optimization of putative neuroprotectants is limited; ethical concerns; patient availability	Wide scope for optimization of dose and delivery	Clinically relevant administration methods, based on ADME and toxicity data, need to be established preclinically.	Even if clinically inappropriate, experiments using large doses and pretreatment can provide insight into pathological processes and reveal further, potentially more accessible targets
Pretreatment is impossible (or, at best, very challenging and costly), except in very high-risk patient subsets	Pretreatment is widely used	Drug administration must be limited to clinically relevant time window (ie, ≥1 h from occlusion)	

Abbreviations:

ADME: absorption, distribution, metabolism, and excretion;

MCA: middle cerebral artery;

MRI: magnetic resonance imaging

12 Emergent neuroprotectants and future perspectives of neuroprotection in stroke

Aside from the disbelief that previous failures have caused around neuroprotection in stroke, as technology evolves new promising candidates emerge, rising hope in this field.

12.1 Adropin

Adropin, is a small polypeptide encoded by the Energy homeostasis gene (Enho), that is expressed in the several organs including the brain. New evidence shows that when the rat brain microvascular endothelial cells, when exposed to low glucose and oxygen levels, adropin is considerably downregulated. Accordingly, treatment with adropin increased endothelial permeability and can potentially be protective (54).

12.2 Tyrosine phosphatase STEP

The tyrosine phosphatase STEP is expressed, specifically in the central nervous system. It can dephosphorylate an inactive multiple crucial neuronal signaling proteins such as the extracellular signal-regulating kinase 1 and 2 (ERK1/2), stress activated protein kinases p38, the Src family tyrosine kinases Fyn and PYk2, NR2B-subunit of NMDARs and GluA2 and GluA3 subunit of AMPA receptors. During ischemic insult, these proteins are overly activated and are involved in excitotoxicity, oxidative stress, and inflammatory response. By inhibiting the activation of this proteins, STEP grants neuroprotection. In rat models, intravenous administration of STEP reduced stroke injury and functional deficits and additionally improved long-term recovery even when administered 6 hours after stroke insult (54).

12.3 Verapamil

Verapamil is a L-type calcium channel blocker, approved by the FDA for the treatment of hypertension, angina, and arrhythmia. Besides the mixed outcomes of earlier studies on the administration of verapamil for stroke treatment, more recent studies in which Verapamil was administer intra-arterially following recanalization in a mouse model of focal cortical ischemia showed significant reduction in infarct volume and improved function. Although the mechanisms of action of verapamil in stroke treatment are not

fully understood, authors hypothesize that by inhibiting L-type calcium channels this drug could reduce excitotoxic damage (54).

A phase I trial (SAVER-I) evaluated the safety and feasibility of intra-arterial administration of verapamil in human patients, and it demonstrated that this administration is safe and feasible when using an appropriate dose range (54).

12.4 MicroRNAs

Besides the conventional therapies, the identification of epigenetic factors that can possibly influence the etiology and progression of stroke have raised interest. MicroRNAs (miRNAs) bind to mRNA slowing or accelerating their degradation, and trough this they regulate its translation. In the context of stroke, miRNA seemingly regulate excitotoxicity, oxidative stress, inflammation, and BBB disfunction. MiRNAs with neuroprotective properties are typically downregulated whereas harmful miRNAs are often upregulated (54).

Technology advances, allow for the development of synthetic miRNAs, that can be agomirs, when they mimic the function of the biological miRNAs or antagomirs when they inhibit the function of the original miRNA. This progress raises the opportunity to regulate miRNA levels in stroke and evaluate how they influence neuroprotection (54).

The table below, presents an overview on the most promising miRNAs as possible neuroprotectants (54).

Table 4: Overview on the most promising miRNAs as possible neuroprotectants (from reference (54))

MicroRNAs	Mechanism of action
miRNA-29a	Preserves astrocyte glutamate transporter 1, leading to a reduction in excitotoxicity
miRNA-223	Reduces levels of NR2B-NMDAR and GluR2-AMPAR declining excitotoxicity
miRNA-424	Increases the expression of SOD, mnSOD and Nrf2 protecting the brain from oxidative stress

miRNA-99a	Alleviates oxidative stress injury and promotes neuronal surviving
miRNA-23a-3p	Reduces infarct volume and diminish oxidative stress
miRNA106b-5p (antagomir)	Decreases ischemic infarct volume and neurological deficts by reducing malondialdehyde content, restoring SOD activity, increasing the expression of Mcl-1, and decreases the expression of Bax
miRNA-124	Promotes anti-inflammatory M2 phenotype of microglial/macrophage consequently reducing inflammation
miRNA-let-7c-5p	Decreases infarct volume trough inhibition of microglial activation and attenuates neurological deficits
miRNA-181a (antagomir)	Reduces NF-ƙB and microglial activation, and leukocyte infiltration protecting the brain from oxidative stress and inflammatory response throughout ischemia
miRNA 15a/16-1 (antagomir)	Reduces infarct size, BBB leakage and decreases infiltration of peripheral immune cells

These recent breakthroughs on the functioning of microRNAs open a new and hopeful path for the development of neuroprotective strategies in the future (54).

The clinical research for neuroprotection in stroke is filled with discouraging results. In the future, is vital that researchers learn from past mistakes and studies are appropriately designed (26).

It is also essential that preclinical and clinical research communities cooperate in order to achieve a better methodological cohesion (47). Preclinical studies and preclinical trials should be complementary, enabling further investigation if translation is unsuccessful and allowing a better comparison of results (29).

Additionally, a more profound understanding of the ischemic death process can also be important to overturn this situation (49).

Despite all the challenges that have occurred in the past years, new technologies such as proteomics, metabolomics and RNA sequencing, the discovery of new molecular events involved in ischemic brain damage are still appearing (54).

An article published in the American Heart Association Journal (68) , envisions some ways to test neuroprotective agents in the future:

- "Extending the window of thrombolytic therapy", through testing of neuroprotective agents in combination with tPA.

- "Preventing or retarding infarct expansion in patients with large artery occlusion requiring endovascular treatment", using appropriate animal models such as dogs and nonhuman primate models of embolic cerebral artery occlusion.

- "Testing the additive effects of neuroprotection with intra-arterial therapy"

- "Intra-arterial delivery of neuroprotective agents, immediately after endovascular thrombectomy"

Several authors acknowledge combination therapy as a viable path for the future. Combination therapy would include both neuroprotective agents and rtPA administration. Development of such strategies holds potential, and in the next years is likely that new discoveries arise in stroke research (54).

13 Conclusion

Stroke remains as one of the mains cause of death in the world, and a leading cause of long-lasting disability. This disease causes a huge burden not only to patients but also to caregivers, since many stroke survivors become unable to perform basic daily task and therefore will have to rely on someone to take care of them.

Unfortunately, this scenery will not likely improve in the future. As population keeps ageing, risk factors accumulate increasing the odds of suffering a stroke during their lifetime.

There are many barriers to the development of new stroke therapies. For instance, the pathological mechanisms underlying stroke are very complex, and some targets may be harmful in an initial phase and then be beneficial for recovery. Researchers have established that effective drugs probably need to target multiple points in the ischemic cascade to show benefit, and this increases the complexity of research.

In the last decades Bench to Bedside translation has been challenging. But recognizing the gaps in previous investigations, allows for a better perspective on how to design preclinical studies that are translatable to clinical research. To achieve this, a joint effort must be made between investigators, journals, and research funders.

Besides this negative atmosphere that surrounds neuroprotection research, we now display a lot of novel opportunities as technology evolves and new therapies emerge. We must continue to invest in research in this field as this disease costs millions of lives every year and highly diminishes quality of life in its survivors.

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