Review

Current understanding of TRPM7 pharmacology and drug development for stroke

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The initial excitement and countless efforts to find a pharmacological agent that disrupts the excitotoxic pathway of ischemic neuronal death have only led to disappointing clinical trials. Currently, a thrombolytic agent called recombinant tissue plasminogen activator (rt-PA) is the only pharmacological treatment available for patients with acute ischemic stroke in most countries. Even though its efficacy has been confirmed repeatedly, rt-PA is considerably underused due to reasons including a short therapeutic window and repeated complications associated with its use. A search for alternative mechanisms that may operate dependently or independently with the well-established excitotoxic mechanism has led researchers to the discovery of newly described non-glutamate mechanisms. Among the latter, transient receptor potential melastatin 7 (TRPM7) is one of the important nonglutamate mechanisms in stroke, which has been evaluated in both in-vitro and in-vivo. In this review, we will discuss the current state of pharmacological treatments of ischemic stroke and provide evidence that TRPM7 is a promising therapeutic target of stroke.

Keywords: ion channels; TRPM7; cerebral ischemia; stroke; neuroprotection; recombinant tissue plasminogen activator (rt-PA); glutamate receptors

Introduction

Stroke is one of the most common causes of death and a leading cause of disability worldwide[1, 2]. The burden of stroke on stroke survivors, their family members and society as a whole is unquestionably immense. The mean lifetime cost of stroke per person in the United States is estimated to be $ 103,576 in 1990, which was an averaged estimate across all stroke subtypes[3]. The socioeconomic burden to meet the costs of managing stroke is expected to further increase largely due to our aging population. From 2005 to 2050, the direct and indirect costs of ischemic stroke alone in the United States is projected to exceed $2.2 trillion[4]. The most effective way of alleviating the burden of stroke is primary prevention. Hence, high-risk or stroke-prone individuals should aim to reduce modifiable risk factors of stroke, which are well documented in a guideline from the American Heart Association[5].

A stroke can be classified as either ischemic or hemorrhagic stroke. Ischemic stroke arises from an occlusion of a major cerebral artery (commonly the middle cerebral artery) by thrombosis or embolism, whereas hemorrhagic stroke arises from a rupturing of a blood vessel. The ischemic stroke is about 10-times more frequent than hemorrhagic stroke in Western countries[6].

Although compromised blood flow during ischemic stroke has detrimental effects on the entire neurovascular unit, neurons are particularly vulnerable to injury and even death due to their high metabolic demand and electrical excitability. With the use of quantitative neurostereology and stroke neuroimaging, Saver et al[6] estimated that a typical untreated large vessel ischemic stroke will result in losses of 120 million neurons, 830 billion synapses, and 714 km of myelinated fibers in each hour. These abrupt neuronal losses that occur during 1 h of untreated ischemia normally take 3.6 years to occur in a healthy aging brain. Such estimates highlight the importance of urgent stroke care once ischemia begins.

Over the past decades, much attention has been given to developing effective pharmacological treatments that can be administered immediately after acute ischemic insults to minimize cerebral damage. Contrary to initial expectations and the early promising results gained from animal stroke models, an overwhelming number of attempts to treat ischemia pharmacologically has failed to translate into clinical treatments. The only drug that is currently approved in most countries for clinical use is a recombinant tissue plasminogen activa-
tor (rt-PA, alteplase), which relieves occlusion by dissolving clots\cite{7, 8}. Although it has proven to be clinically effective, the usage is limited by several reasons that will be explained in the next section. Therefore, there is still a pressing need to develop new effective treatments for stroke. In this review, we will focus on the current situation with the acute pharmacological treatment of stroke. Then, for the remaining part of the review, we will discuss the potential clinical benefits that could be gained by targeting the TRPM7 (Transient Receptor Potential Melastatin 7) channel, a recently discovered non-glutamate mechanism shown to be involved in ischemic damage, during cerebral ischemia.

**Pharmacological approach**

Traditionally, pharmacological agents for treating ischemic stroke have been broadly classified into two groups: thrombolytics and neuroprotectants\cite{9-11}. Thrombolytics are compounds that act to restore blood flow by dissolving blood clots, while neuroprotectants are compounds that are designed to interfere with the biochemical events in the ischemic cascade and thereby lessenening neuronal damage.

**Thrombolytic agents**

At present, recombinant tissue plasminogen activator (rt-PA, alteplase), the thrombolytic agent, is the only drug approved by Food and Drug Administration (FDA) for clinical use, and proven to be the only effective intervention available for early management of ischemic stroke\cite{7, 8}. The original study done by the National Institute of Neurological Disorders and Stroke (NINDS)\cite{12} reported that when rt-PA is given intravenously within the first 3 h after the onset of symptoms, there was an absolute increase in favourable outcome by 11%–13% at 3 months as compared with placebo group.

However, rt-PA is not without shortcomings. Despite its proven efficacy, it is estimated that less than 2% patients receive rt-PA primarily due to inability to meet its short 3-h therapeutic window\cite{13}. Although the initial 3-h therapeutic window has been expanded to 4.5 h\cite{8, 14}, the earliest possible administration is still highly recommended to maximize the benefit, while minimizing adverse complications. Often, a poor understanding of stroke symptoms by stroke victims and/or their family members delay the time of hospital admission after onset\cite{15}. Upon hospital admission, administration of rt-PA is further delayed when a computed tomography scan is done to exclude patients with a brain hemorrhage or major infarction. This step inevitably limits more eligible patients from receiving the treatment. Rt-PA administration in patients with acute ischemic stroke is also more frequently associated with symptomatic intracranial hemorrhage though it is still 10 times more likely to benefit than to harm eligible patients with acute ischemic stroke\cite{16}. Unlike its beneficial effect in the intravascular space by acting as a thrombolytic enzyme, emerging evidence also suggests that rt-PA in the extravascular space lead to the development of edema and excitotoxic cell death. Both beneficial and deleterious effects of rt-PA are thoroughly reviewed by Yepes et al\cite{11}.

**Neuroprotectants**

Aside from thrombolytics that aid reperfusion of blood flow in an ischemic area, researchers have been seeking ways to screen and/or develop neuroprotectants that protect neurons by interfering with ischemic cascades triggered by acute ischemic episodes. With expanding knowledge of the molecular pathways leading to ischemic neuronal damage, more than 1000 experimental drugs have been evaluated for the neuroprotective effects over the years, but all failed to translate bench findings to the patient bed\cite{17}. Until recently, finding a way to disrupt the glutamatergic mechanism has been the key aim in neuroprotective research. Although the contribution of excitotoxic-cascade in ischemic injuries is well established and unquestionable, the repeated failure of clinical trials suggest that solely targeting excitotoxic cascade is not sufficient. Increasingly more attention has been given to finding other novel processes that may either work independently or jointly with glutamate pathways. Some of the channels involved in the non-glutamate mechanisms include (Figure 1A): transient receptor potential (TRP) channels\cite{18-22}, acid-sensing ion channels\cite{23-29}, volume-regulated anion channels\cite{26}, hemichannels\cite{27-29}, ATP-sensitive potassium channels\cite{30-32}, sodium-calcium exchangers\cite{33-35} and non-selective cation channels\cite{36}.

**Glutamate receptors: traditional, most sought-out therapeutic target**

The glutamate-mediated excitotoxic mechanism (Figure 1A) is the most extensively studied pathway in the context of ischemic neurodegeneration. Consequently, developing neuroprotectants that may interfere with glutamatergic signaling has been the focus of the early neuroprotection research. Following the early anoxic depolarization due to the failures of energy-dependent pumps, a massive release of glutamate in conjunction with the impairment (or reversal) of reuptake mechanisms result in accumulation of extracellular glutamate. This over-activates glutamate receptors, NMDAR (N-methyl-D-aspartic acid receptor) and AMPAR (DL-a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor), and causes excessive Ca$^{2+}$ overload, triggering Ca$^{2+}$ mediated harmful activation of enzymes, free radical formation and eventual cell death\cite{37, 38}. In order to interrupt this toxic Ca$^{2+}$ influx, various glutamate receptor antagonists were tested in rodents but failed to demonstrate efficacy in clinical trials\cite{10}.

One suggested explanation for repeated disappointing outcomes is that pathological glutamate release takes place too early in the ischemic cascade and antagonizing glutamate receptors in time may not be attainable in clinical settings. Most currently tested NMDAR antagonists have a very short therapeutic window following the onset of ischemia. For example, MK-801, which is a non-competitive antagonist for NMDARs, offers a neuroprotective effect only when given 30 min after the onset of ischemia\cite{39}, and dextrorphan, which is another noncompetitive NMDAR antagonist, also has a short therapeutic window of 2 h\cite{40}.

Furthermore, contrary to the simple traditional view that only focused on the destructive effects of NMDARs upon
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and these combine to produce peroxynitrite (ONOO$^-$). On the subcellular pools, activation of NMDARs could either be ischemic activation, it was recently revealed that depending on other non-glutamate dependent mechanisms. Glutamate dependent mechanisms include the traditional glutamate dependent mechanism and newly discovered non-glutamate dependent mechanisms. Glutamate dependent mechanism of cerebral ischemia is the most extensively studied mechanism and it is thought that energy failure due to ischemia leads to depolarization of presynaptic neurons, which leads to a massive release of glutamate. Increased glutamate in the extracellular space hyper-activates glutamate receptors, which consequently leads to excitotoxic Ca$^{2+}$ overload. Recently, other non-glutamate dependent mechanisms that may contribute to the overall ionic imbalance have been discovered and these are: TRPM7, ASICs, VRACs, hemichannels, $K_{\text{cat}}$, NCXs, and NSCCs. These pathways may even interact with each other suggesting complex molecular cascades upon ischemic insults. (B) Current working model of TRPM7 activation during cerebral Ischemia. Ischemic conditions are associated with decreases in extracellular pH (acidic) and concentrations of divalent ions (Ca$^{2+}$ and Mg$^{2+}$), and these changes may activate TRPM7 independent of other pathways. The initial influx of Ca$^{2+}$ via glutamate-dependent pathway stimulates productions of nitrogen oxide (NO) and superoxide (O$_2^-$), and these combine to produce peroxynitrite (ONOO$^-$). Reactive oxygen species and reactive nitrogen species (ROS and RNS) further promote sustained activation of TRPM7, which consequently causes a prolonged build-up of intracellular Ca$^{2+}$ leading to ischemic damages.

TRPM7: Newly discovered, promising therapeutic target

TRPM7 is the seventh member in the melastatin subfamily of TRP channels[47]. It is a ubiquitously expressed Ca$^{2+}$ permeable non-selective cation channel that is permeable to monovalent and most divalent cations with the selectivity profile in a sequence of $\text{Zn}^{2+} \approx \text{Ni}^{2+} \approx \text{Ba}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+} > \text{Mn}^{2+} > \text{Sr}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+}$. TRPM7 is unique in that it possesses the atypical serine/threonine protein kinase domain at the C-terminus[49]. Binding of Mg$^{2+}$-nucleotides to this kinase domain is suggested to be important for the tonic inhibition of constitutively active TRPM7[50]. Though this functional enzymatic domain is homologous to a family of alpha-kinases[49], there is a very limited understanding of its substrates and associated physiological functions. It has been proposed that TRPM7 kinase phosphorylates a conserved serine residue (Ser$^5$) in N-terminus of annexin I[51]. This phosphorylation may modulate the activity of annexin I as this region is important for interacting with other proteins and membranes, but further investigation is required to determine the functional significance of phosphorylating annexin I. TRPM7 kinase also seems to phosphorylate myosin II A, IIB, and IIC, which in turn affects actomyosin contractility and cell adhesion[52, 53]. However, no study has shown the involvement of the kinase domain in neuronal injury during stroke. Under physiological conditions, it has been repeatedly suggested that TRPM7 has a critical role in cell survival and proliferation[54-56]. Tight regulation of TRPM7 expression seems to be the key as both deletion[54, 55] and overexpression of the gene[57] lead to cell death. Although TRPM7 is constitutively active[48, 58], its activities seem to be modulated by various factors, including the ones that normally occur during ischemic stroke. For instance, TRPM7 activity is potentiated by oxidative stress[59] and reductions in extracellular pH

neuroprotectants that tend to antagonize all NMDARs, regardless of their subcellular localizations and respective downstream signaling cascades.

Another major setback that led to discontinuation of clinical trial for most glutamate antagonists, both competitive and noncompetitive, was a similar pattern of side effects that were presented in patients, regardless of the pharmacology of drugs[42]. These symptoms tended to be neurological in nature: changes in sensory perception, dysphoria, hypertension, disorientation, heightened agitation, paranoia, hallucination, and even catatonia at higher doses.

Hence, a new therapeutic approach targeting the glutamate excitotoxic pathway should aim to block only the NMDARs that are linked to neurotoxicity with minimal (or tolerable) side effects. This goal could also be met by developing drugs that can disturb downstream targets of NMDARs[43-45], or drugs that target the non-glutamate mechanisms. Blocking NMDARs may not have been sufficient in clinical situations because there seem to be other newly discovered non-glutamate mechanisms that contribute to ischemic neuronal cell death during stroke (see review[46]). Among many of these promising targets in ischemic neurodegeneration (Figure 1A), TRPM7 will be the focus of remaining parts.

Figure 1. (A) Schematic diagram of molecular pathways during ischemia including the traditional glutamate dependent mechanism and newly discovered non-glutamate dependent mechanisms. Glutamate dependent mechanism of cerebral ischemia is the most extensively studied mechanism and it is thought that energy failure due to ischemia leads to depolarization of presynaptic neurons, which leads to a massive release of glutamate. Increased glutamate in the extracellular space hyper-activates glutamate receptors, which consequently leads to excitotoxic Ca$^{2+}$ overload. Recently, other non-glutamate dependent mechanisms that may contribute to the overall ionic imbalance have been discovered and these are: TRPM7, ASICs, VRACs, hemichannels, $K_{\text{cat}}$, NCXs, and NSCCs. These pathways may even interact with each other suggesting complex molecular cascades upon ischemic insults. (B) Current working model of TRPM7 activation during cerebral Ischemia. Ischemic conditions are associated with decreases in extracellular pH (acidic) and concentrations of divalent ions (Ca$^{2+}$ and Mg$^{2+}$), and these changes may activate TRPM7 independent of other pathways. The initial influx of Ca$^{2+}$ via glutamate-dependent pathway stimulates productions of nitrogen oxide (NO) and superoxide (O$_2^-$), and these combine to produce peroxynitrite (ONOO$^-$). Reactive oxygen species and reactive nitrogen species (ROS and RNS) further promote sustained activation of TRPM7, which consequently causes a prolonged build-up of intracellular Ca$^{2+}$ leading to ischemic damages.
TRPM7. The initial rise in intracellular Ca\(^{2+}\) through NMDARs in mediating ischemic neuronal cell death\[21\]. When TRPM7 recently, getting TRPM7 may have a longer treatment window. More has an independent role in OGD-mediated cell death and tar-
calcium channels) of excitotoxicity, suggesting that TRPM7tors (eg glutamate NMDA and AMPA receptor and L-type glutamate antagonist, the intracellular Ca \(^{2+}\) accumulation during cerebral ischemia (Figure 1B), conditions associ-
eration by these non-specific blockers are not clear. Although

According to the current working model of TRPM7 activation during cerebral ischemia (Figure 1B), conditions associ-
ated with cerebral ischemia, such as reduction in extracellular Ca\(^{2+}\) and Mg\(^{2+}\) concentrations and decrease in pH, activate TRPM7. The initial rise in intracellular Ca\(^{2+}\) through NMDARs stimulates formation of reactive oxygen and nitrogen species (ROS and RNS), and these products feedback to TRPM7 and further activates TRPM7. The latter activation mechanism with ROS and RNS makes the role of TRPM7 more substantial and clinically relevant since it creates self-sustaining positive feedback loop. Consequently, even with glutamatergic channel antagonism, the intracellular Ca\(^{2+}\) accumulation during ischemia may be prolonged via TRPM7.

**Developing potential TRPM7 blocker**

Although more thorough investigations on molecular mech-
nisms of TRPM7 in the context of ischemic stroke should be done before validating it as a therapeutic target, one of the current issues with TRPM7 research is not having a selective pharmacological inhibitor\[47\]. Currently, some non-selective inhibitors are used to assist the understanding of its role in physiological and pathological conditions. These include: trivalent ions, such as Gd\(^{3+}\) ((IC\(_{50}\) ~1.4–2.5 μmol/L) and La\(^{3+}\) (IC\(_{50}\) ~17 μmol/L)\[22, 62\], 2-aminoethoxydiphenyl borate (2-APB) (IC\(_{50}\) ~50 μmol/L)\[80\], NDGA (nordihydroguaiaretic acid, IC\(_{50}\) ~6.3 μmol/L)\[64\], AA861 (IC\(_{50}\) ~6.0 μmol/L)\[64\], MK886 (IC\(_{50}\) ~8.6 μmol/L)\[64\] and carvacrol (IC\(_{50}\) ~307 μmol/L)\[69\]. Among these non-selective TRPM7 blockers, a group of 5-lipoxygenase inhibitors (5-LOX), NDGA, AA861, and MK886, showed potent effects on blocking TRPM7 whole-cell current in TRPM7 overexpressing HEK293 cells without affecting the TRPM7 protein expression\[64\]. These blockers also efficiently reduced the characteristic cell rounding that normally occurs with overexpressing TRPM7 in HEK293 providing another line of evidence that these blockers have an inhibitory effect on TRPM7. In contrast, other 5-LOX inhibitors, 5,6-dehydroarachidonic acid (5 μmol/L) did not affect the channel activity and was unable to prevent TRPM7-mediated cell rounding, suggesting that these inhibi-
tory effects of NDGA, AA861, and MK886 on TRPM7 are independent of their actions on 5-lipoxygenase. This independ-
cy was further confirmed when addition of 5-lipoxygenase products, leukotriene B4 and leukotriene D4, did not stimulate channel activities of TRPM7. Carvacrol, a predominant part of the essential oils found in herbs, suppressed constitutively active TRPM7 current in a concentration-dependent manner only in TRPM7-transfected HEK293 cells and not in mock transfected HEK293 cells. Furthermore, the action of carvacrol was also shown to be sufficient to block TRPM7-dependent transmitter release in CA3-CA1 hippocampal neurons over-
expressing TRPM7 channels, which is a functional role of TRPM7 that has previously been observed in cholinergic sympa-
thetic neurons\[66\]. In the same study\[65\], drosophila TRPL, which belongs to the TRPC subfamily, was also inhibited by carvacrol. Other than inhibiting TRPM7 and TRPC, carvacrol has shown to activate thermoTRPs, TRPV3, and TRPA1 in earlier study\[67\]. At present, the mechanisms of TRPM7 modu-
lation by these non-specific blockers are not clear. Although these are currently the best pharmacological tools available for studying TRPM7 functions, especially useful for the biophysical characterizations, finding and developing a specific pharma-
acamical blocker would be the first priority in the road of targeting TRPM7 as a novel therapeutic approach for stroke.

Noticing the pressing needs for discovering selective pharmacological blockers, Castillo et al\[68\] recently developed and optimized a Fura-2 based high-throughput screening (HTS) assay that can be used to narrow down the TRPM7 inhibitors. This bioassay utilizes HEK293 cells that have a tetracycline-inducible TRPM7 expression system. Compared to non-
duced cells, induced TRPM7-HEK293 cells exhibit TRPM7-
mediated Mn\(^{2+}\) entry, which can be monitored by measuring the fluorescent quench of Fura-2 by Mn\(^{2+}\). Hence, using this bioassay, potential TRPM7 blockers would block TRPM7-
mediated Mn\(^{2+}\) entry and subsequently increase Fura-2 fluo-
rescence. Instead of monitoring TRPM7-mediated Ca\(^{2+}\) entry, quenching of Fura-2 with the entry of Mn\(^{2+}\) was measured for
the following reasons: (1) Mn\(^{2+}\) provides the largest quench of Fura-2 in TRPM7 overexpressing HEK293 cells; (2) TRPM7 is more permeable to Mn\(^{2+}\) than other competing channels, such as calcium-release activated calcium (CRAC) channels; and (3) the assay can be done in physiological levels of Ca\(^{2+}\) and Mg\(^{2+}\), unlike measuring Ca\(^{2+}\) influx, which normally requires Ca\(^{2+}\)-deficient assay conditions for the optimal measurement. The assay conditions were carefully evaluated to achieve the most optimal, high reproducible 96-well plate HTS assay. For instance, when the optimized bioassay was used for two known non-selective TRPM7 inhibitors, LaCl\(_3\) and 2-APB, the potencies were comparable to the previous published data under the following conditions: (a) seeding cell density of 60 000 cells/well, (b) Fura-2-AM (dye) loading concentration of 2 µmol/L, (c) Fura-2-AM loading time of 60 min, (d) Mn\(^{2+}\) (Fura-2 quenching agent) concentration of 10 mmol/L, (e) MET (vehicle solvent) concentration of 1%. So far, no study using this HTS bioassay to screen potential TRPM7 channel inhibitors has been published. Along with the known non-selective inhibitors, newly screened candidates may even help us understand molecular and/or structural characteristics to design specific inhibitors de novo\(^{[79]}\).

Once potential blockers are carefully screened/designed, preclinical experiments should be designed to follow recommendations for preclinical stroke drug development established by the Stroke Academic Industry Roundtable (STAIR)\(^{[70, 71]}\). In response to striking discrepancy of the results between animal stroke models and human clinical trials, the STAIR group gathered in the late 1990s and published a set of criteria that should be followed before advancing candidates into clinical trials. Although meeting these criteria does not guarantee clinical success, close adherence to these guidelines will help us reduce the likelihood of failure and improve the chance for successful clinical trials.

Conclusions
Contrary to initial expectations formed based on the early animal stroke models, solely blocking glutamate receptors seems to be insufficient to preserve neurons during ischemic stroke. Considerable knowledge is gained about the non-glutamate dependent mechanisms of ischemic neuronal death and the idea of targeting these novel pathways offers a glimpse of hope in rather disappointing neuroprotection research. With in vitro and in vivo validation of the pathological effects of TRPM7 in cerebral ischemia, TRPM7 seems to be a promising target for the future generation of neuroprotective approach. Although there is no selective antagonist available for TRPM7, it may be possible to find or design a selective TRPM7 inhibitor with a thorough screening for potential compounds. Once found, extensive preclinical studies that fully adhere to recommendations from STAIR committee should be conducted before moving towards clinical trials.

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