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Human tissue kallikrein in the treatment of acute ischemic stroke

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Abstract: Acute ischemic stroke (AIS) remains a major cause of death and disability throughout the world. The most severe form of stroke results from large vessel occlusion of the major branches of the Circle of Willis. The treatment strategies currently available in western countries for large vessel occlusion involve rapid restoration of blood flow through removal of the offending blood clot using mechanical or pharmacological means (e.g. tissue plasma activator; tPA). This review assesses prospects for a novel pharmacological approach to enhance the availability of the natural enzyme tissue kallikrein (KLK1), an important regulator of local blood flow. KLK1 is responsible for the generation of kinins (bradykinin and kallidin), which promote local vasodilation and long-term vascularization. Moreover, KLK1 has been used clinically as a direct treatment for multiple diseases associated with impaired local blood flow including AIS. A form of human KLK1 isolated from human urine is approved in the People's Republic of China for subacute treatment of AIS. Here we review the rationale for using KLK1 as an additional pharmacological treatment for AIS by providing the biochemical mechanism as well as the human clinical data that support this approach.

Keywords: acute ischemic stroke, bradykinin, human tissue kallikrein, recombinant KLK1, vasodilation

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Introduction

Tissue kallikrein (KLK1) is a serine proteinase that plays a critical role in the regulation of microcirculation, blood pressure and blood flow (reviewed by Regoli and Gobeil).[1] Multiple lines of evidence indicate that KLK1 is important for a healthy cardiovascular system and that deficits in KLK1 are associated with cardiovascular and end-organ pathology. Importantly, KLK1 replenishment therapy is widely used in Asia, where multiple forms are used to treat diseases ranging from acute ischemic stroke (AIS) to complications of diabetes (retinopathy and kidney disease), all of which involve poor microcirculation or frank tissue ischemia. Furthermore, AIS pathogenesis includes a maladaptive immune system response and complex inflammation pathways (reviewed by Jin and colleagues)[2] both of which can be addressed by KLK1 treatment.[3] This review summarizes the mechanistic rationale for KLK1 replenishment therapy to treat patients with AIS and summarizes the preclinical and clinical data supporting the hypothesis that KLK1 treatments will improve outcomes after AIS.

Tissue kallikrein and the kallikrein–kinin system

Kallikrein proteins are generated from 15 genes found on human chromosome 19 and represent the largest protease gene cluster in the human genome.[4] These serine proteinases have substrate specificity similar to trypsin or chymotrypsin and are known to be involved in a variety of biochemical processes. Kallikrein proteins in this family are secreted as proenzymes that are activated by selective proteolysis, which releases the mature enzyme. Few details are known about the physiological or pathological

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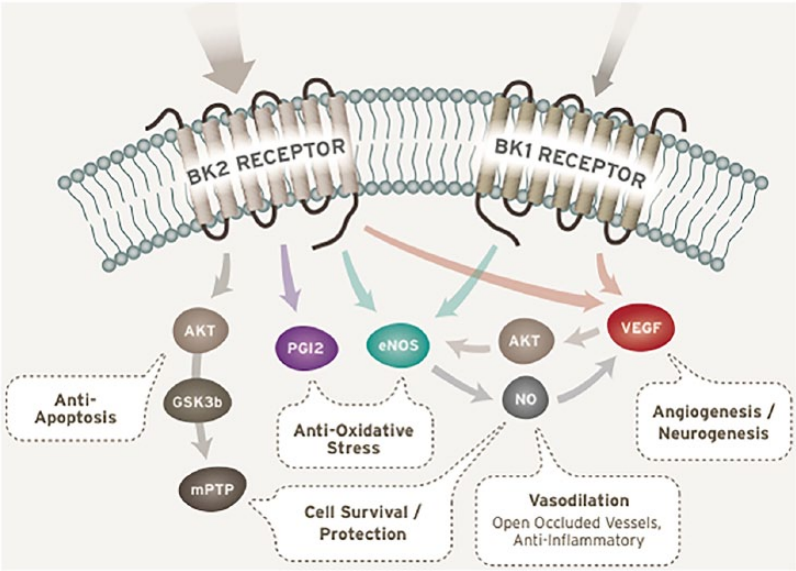

Figure 1. BK receptors: second messengers and physiological effects.
The main GPCR target of the KKS are bradykinin receptors, BK1 and BK2. Although, KLK1 is likely to have additional activities, the downstream activation of BK receptors by bradykinin triggers multiple second messenger pathways that may be beneficial in AIS. The physiological output of these systems directly maintains or improves blood flow and protects cells from damaging stressors often present during the hours and days following a stroke.
AIS, acute ischemic stroke; BK, bradykinin; GPCR, G-protein-coupled receptor; KLK1, kallikrein; KKS, kallikrein–kinin system.

triggers that release the active enzyme, but kallikrein family members can cleave their own proenzymes suggesting they are involved in a complex biochemical cascade.[5] At a minimum, it appears the proteinase action of KLK1 is controlled by multiple factors and conditions, highlighting its importance for maintaining homeostasis or responding to disease.

The lack of selective antagonists that block individual members of the kallikrein serine protease family has impeded complete understanding of their unique roles. However genetic manipulations of KLK1 suggests one of the specific primary roles is the cleavage of low molecular weight kininogen (LMWK) to release active bradykinin (BK). Although KLK1 is capable of cleaving a different kininogen substrate (high molecular weight kininogen), compelling evidence for the primary action on LMWK arises from characterization of genetically modified mice in which the KLK1 gene has been deleted.[6,7] Compared with wild type mice, KLK1 knockout (KO) mice were unable to properly generate BK and related peptides under normal conditions. These mice also showed impaired vascular responses to changes in blood flow and structural deficits of the heart occurring on the background of normal resting blood pressure. Similar results are also observed in humans having a mutation in the KLK1 gene that generates a partial loss-of-function in the KLK1 protein.[8] Individuals with this mutation showed a defect in the ability of the brachial artery to respond properly to changes in blood flow, supporting the hypothesis that KLK1 functions to regulate blood flow under normal conditions, and this regulation is at partly due to direct enzymatic cleavage of LMWK to generate BK. This entire mechanism is often referred to as the kallikrein–kinin system (KKS). BK and closely related kinin peptides exert both short- and long-term effects on end organs by directly stimulating two classes of receptors, BK1R and BK2R.[9,10] Both receptors belong to the family of G-protein-coupled receptors (GPCRs) that trigger a variety of intracellular second messenger systems through $G\alpha_q$ (and other $G\alpha$ variants) including activation of phospholipase C, mitogen-activated protein kinase and phospholipase A2[11–14] (Figure 1). Through these second messenger pathways, KLK1 and the KKS have been shown to exert critical regulation of multiple physiological functions including blood glucose uptake, glycogen synthesis, blood pressure regulation, insulin

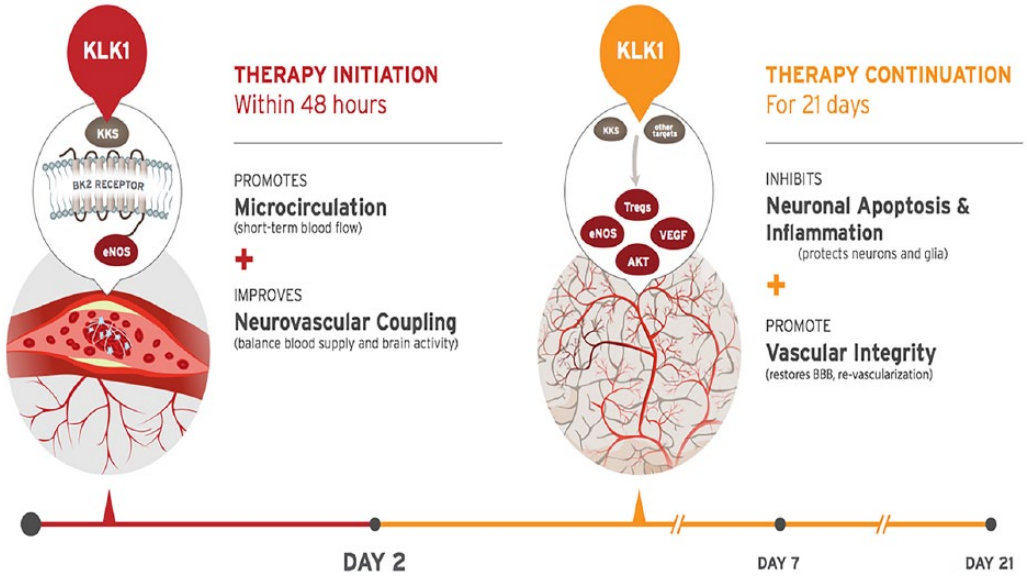

Figure 2. Short- and long-term activity: hypotheses that may explain the efficacy of KLK1 treatment in AIS. Treatment with human, kallikrein or recombinant KLK1 is hypothesized to have both immediate and long-lasting actions that may improve outcomes following AIS. Immediate actions are largely mediated by activation of the KKS to release nitric oxide and improve microcirculation in ischemic tissue as well as normalizing neurovascular coupling. In the days following a stroke, additional mechanisms may become important including restoration of the blood–brain barrier through increases in Tregs, dampening of apoptotic cells death through increases in AKT and VEGF-mediated control of revascularization.
AIS, acute ischemic stroke; AKT, protein kinase β, KLK1, kallikrein; KKS, kallikrein–kinin system; Tregs, regulatory T-cells; VEGF, vascular endothelial growth factor.

sensitization, *via* activation of protein kinase β (AKT), and antioxidative stress [$PG1_2$/endothelial nitric oxide synthase (eNOS)] all of which can promote cell protection following ischemia.

The KKS plays an important role in local regulation of blood flow in organs rich in KLK1.[1,15] KLK1 is located in cardiovascular tissue (including capillary endothelial cells), kidney, pancreas, salivary glands and brain.[16,17] KLK1 protein and mRNA are localized on endothelial and smooth muscle cells of large, medium and small blood vessels. In addition, immunohistochemical studies show a distribution of BK2R in arteries and arterioles in smooth muscle cells and endothelial arterioles.[18,19] Kinins and BK2Rs are among the most potent vascular endothelium activators, acting on endothelial cells to trigger the release of numerous signaling molecules to smooth muscle inhibit platelet aggregation and promotes fibrinolysis.[20] The KKS is not only the endogenous vasodilatory system in the mammalian system, but is considered the most active and efficient physiological mechanism that initiates and maintains vasodilation.[1] Finally, kinins inhibit norepinephrine release from vascular sympathetic nervous terminals, thus reducing the impact of sympathetic nervous system signals that trigger vasoconstriction.[21] Furthermore, the molecular actions of KLK1 and BK, especially following ischemia, suggest that KLK1 has both immediate and long-lasting actions that may improve outcomes following AIS (Figure 2).

BK receptors are widely distributed, with BK2R generally more abundant than BK1R.[22] Whereas BK2Rs are constitutively expressed, BK1Rs are found at relatively low levels under normal conditions but upregulated in proinflammatory conditions or in BK2R KO mice.[23–25] It is hypothesized that BK1Rs participate in chronic phases of diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes.[16] Based on pharmacological studies using antagonists selective for BK2R *versus* BK1R, it appears that many of the direct physiological actions of KLK1 are mainly mediated through BK2R receptors.[22] This may be due to the constitutively low expression of BK1R or the physical colocalization of KLK1, kininogen substrate, and receptors. Thus, it appears that KLK1

is functionally and physically positioned to release kinins (e.g. BK) to specifically enhance blood flow in inflammatory tissue *via* BK2R upregulation and activation.

Kinins appear to be liberated locally in the vicinity of BK receptors and quickly degraded. Free kinins are inactivated by several peptidases including kinases I, kinases II, aminopeptidase P, and kallistatin.[26] Kinases II or angiotensin converting enzyme (ACE) play a major role in controlling local kinin levels. In fact, ACE inhibitors (ACEi), a common treatment for high blood pressure, prevent the natural break down of endogenous kinins, which could be a key component or their therapeutic mechanism.[27] Interestingly, ACEi drugs have shown anti-inflammatory effects in a variety of diseases including AIS, which also could be related to ACEi-induced increase in kinin availability.[28,29] Furthermore, kinins should be thought of as autacoids acting locally rather than hormones, which act globally. There is heterogeneity among vascular territories in the capacity for kinin production or inactivation, which impacts regulation of local blood flow. For example, renal vessels have low ACE content, contributing to a higher degree of kinin regulation of renal blood flow.[30] Systems such as the KKS that control blood flow locally, dampen inflammation, and protect against ischemic reperfusion injury have implications for the physiology and pathology of the central nervous system and treatment strategies for AIS.

The exact physiological and pathological role of BK itself in the central nervous system continues to require further investigation. Multiple lines of evidence suggest that BK could exacerbate brain inflammation and neuronal damage. However, in many cases this may be due to an understandable confusion between the KLK1 system reviewed here and the plasma kallikrein system. The structurally unrelated protease, plasma kallikrein, is known to release active kinins primarily from the high molecular weight kininogen (HMWK) substrate.[31] Tissue kallikrein and plasma kallikrein are encoded by distinctive genes and differ in molecular weight, isoelectric point and amino acid sequence.[32] Moreover, KLK1 is present in many tissues and various body fluids[33] whereas plasma kallikrein is almost exclusively found in blood.[34] Plasma kallikrein is part of the contact activation system and is liberated from an inactive precursor under conditions of severe inflammation and tissue damage.[35] The actions of plasma kallikrein are widely associated with pathological states such as angioedema.[36] This system also may be involved in neuronal damage including retinopathy,[37] and traumatic brain injury.[38] Interestingly, kinins, such as BK, as well as the BK1R and BK2R are often proposed as the primary signaling mechanism for plasma kallikrein's pathological mechanisms, adding to the conundrum posed by these parallel and apparently unrelated systems. Few details of how these two systems interact have been reported, and further research is certainly warranted. Beyond the very likely possibility that BK release is not the sole output of either the plasma KKS or the tissue KKS, it is also very likely that numerous other factors differentiate these two pathways. For example, the exact location, regulation, and bulk amount of BK release is likely quite different. An added complication to this issue is the substantial technical challenge of accurately measuring BK or related kinins in tissues or body fluids.[39]

Additional activities of KLK1

In addition to the enzymatic action of liberating kinins from kininogen, KLK1 itself may also be capable of directly activating the BK2R. Early evidence showed that KLK1 directly and independently contracted smooth muscle tissue (rat uterine tissue) in the absence of kininogen and BK.[40] *In vitro* studies using cells expression recombinant BK2Rs demonstrated that the KLK1 activity was due to direct stimulation of BK2Rs without production of BK.[41,42] KLK1 activated BK2R more potently than a selection of related proteases such as trypsin or cathepsin C.[41] The effects of KLK1 treatment were blocked by the BK2R selective antagonist, icatibant. KLK1 also directly displaced radiolabeled BK from BK2Rs with a half maximal inhibitory concentration (IC_{50}) of 3 nM and induced the redistribution of the receptor in the plasma membrane.[43] Interestingly, KLK1 did not cross-desensitize the receptor for subsequent activation by BK suggesting that receptor activation by KLK1 may have different downstream effects from that of BK. *In vivo* studies confirmed and extended these findings by examining the cardioprotective effect of KLK1 in kininogen-deficient rats. KLK1 treatment reduced infarct size, cardiomyocyte apoptosis and intramyocardial inflammation through kinin BK2R activation and nitric oxide (NO) formation.[44]

Although these results indicate that the physiological role of KLK1 is not strictly confined to the

controlled release of BK, more work investigating the direct mechanisms of KLK1 needs to be conducted to fully understand the physiological relevance of this pathway in all tissues. For example, it appears that KLK1 may not compete for the BK2R in human umbilical cord tissue and that actions of KLK1 are dependent on available kininogen.[45] The presence of hybrid BK2R-angiotensin receptors[46] adds further complexity to a biochemical story that is still being delineated.

The serine protease activity of KLK1 also directly regulates multiple biochemical cascades and peptide signaling molecules independent of its role in the KKS. KLK1 directly binds to protease-activated receptors leading to cell migration and proliferation of keratinocytes.[47] KLK1 also appears to directly degrade active vascular endothelial growth factor$_{165}$ (VEGF) in the eye,[48] leading to the hypothesis that KLK1 treatment may benefit diabetic retinopathy and offer an alternative to intraocular injection of anti-VEGF antibodies for treating this condition.[49] KLK1 and other family members are known to cleave the proenzymes of forms of themselves, thus controlling the production of active enzymes in cascade fashion similar to that of the blood coagulation pathway.[5] The physiological roles of other kallikrein family members is not completely delineated, but they likely target a wide variety of substrates[50] and regulate numerous physiological and pathological functions.[51] It seems likely that KLK1 activity is both regulated and serves to control complex biochemical signaling critical for maintaining homeostasis and regulating tissue pathology. It is, therefore, not surprising that low KLK1 levels are associated with multiple disease states.

KLK1 levels and disease risk

Insufficient endogenous KLK1 correlates with a constellation of vascular diseases, further implicating the KKS as a crucial regulatory system for normal circulation, specifically in diseases that are considered risk factors for AIS. Two disorders that display low endogenous KLK1 levels are hypertension and diabetes, both of which are risk factors for AIS.[52] KLK1 deficits in plasma and urine arise from low enzyme concentrations or decreased enzymatic activity of KLK1. Patients with arterial hypertension[53] and essential hypertension[54] have lower levels of active KLK1 in their urine compared with healthy controls. Furthermore, epidemiological studies demonstrated an inverse correlation between KLK1 urine concentrations and blood pressure in infants, children and the parents.[54–56] Along with hypertension, evidence suggests endogenous levels of KLK1 are associated with diabetes and the progression of renal disease. Excretion of KLK1 in the urine was decreased in patients with both mild and severe renal disease.[57–59] Specifically, decreases in urinary KLK1 were correlated with impaired glomerular filtrations rate, a marker of kidney health.[59]

Deficiencies in KLK1 appear to have a genetic etiology in some patients. Specific alleles of the KLK1 promoter region may contribute significantly to the level of KLK1 gene expression, leading to variable KLK1 levels in a variety of diseases. For example, KLK1 gene polymorphisms are linked with blood pressure regulation in hypertensive rats[60,61] and rodent models of genetic hypertension show reduced urinary kallikrein excretion.[62,63] In humans, 10 polymorphic alleles of the KLK1 gene generate significantly different levels of expression.[64] Elevated KLK1 levels in the urine caused by a dominant allele in the KLK1 promoter may be associated with decreased risk for essential hypertension.[65] In a Chinese Han population with essential hypertension, abnormal polymorphisms (multiple substitutions and unusual length) in the regulatory region of the KLK1 gene were present,[66] suggesting a connection between hypertension and polymorphisms in the *KLK1* gene. In African American patients with hypertensive end stage renal disease, five KLK1 promoter alleles were identified with unusual overlapping substitutions and length.[67] The existence of natural genetic variations affecting KLK1 expression levels in patients with vascular disease further emphasize the important role played by the KLK1 enzyme in regulation of blood flow. Taken together, these data not only implicate KLK1 as a crucial regulatory of diseases related to circulatory and cardiovascular but suggest these deficits could be related to the risk of AIS since both hypertension and diabetes are major risk factors. If additional KLK1 could be supplied to the system, it might provide therapy to improve disease pathology or risk.

Activity of KLK1 in preclinical models of ischemic stroke

The therapeutic effect of KLK1 and the KKS has been investigated from a variety of perspectives using rodent models of AIS. Despite uncertainties about their predictive validity, these preclinical

models of AIS can provide supplemental support for clinical efficacy and often provide mechanistic insights about novel and approved drug therapies. Intravenous infusion of KLK1 (isolated from human urine) improved outcomes after experimental strokes in mice and rats when administered at a variety of times after the insult up to 24 h.[68–70] In these studies, KLK1 treatment was shown to reduce brain edema inflammation, protect cells from apoptosis, and promote both neurogenesis and angiogenesis. Tests involving genetic manipulation of KLK1 expression further support its critical role in maintaining and repairing brain damage caused by ischemia and reperfusion. Gene therapy studies of KLK1 show that injecting virus particles engineered to express KLK1 into the brains of mice after experimental cerebral ischemia also improve biochemical, physiological and functional endpoints.[71–74]

The underlying biochemical mechanisms of KLK1-based treatments are broadly consistent with the hypothesis that they arise from activation of BK receptors, specifically BK2R (reviewed in[75]). The effect of KLK1 gene therapy was blocked by the BK2R antagonist, icatibant,[73] and in other cases the constellation of second messenger molecules stimulated by KLK1 therapy were the same as those evoked by BK receptors activation in other systems. For example, signaling molecules such as AKT and NO were enhanced by gene therapy with KLK1.[74] These systems are known to be important targets for development of novel stroke treatments, especially the NO and the nitric oxide synthase (NOS) system. In eNOS KO mice, cerebral artery occlusion resulted in larger infarct size compared with wildtype mice[76] and impaired overall blood flow.[77]

Beyond the direct effects on blood flow, KLK1 treatment may also facilitate mechanisms that normalize the immune system response to AIS. It is becoming clear that immune system responses to stroke may be maladaptive, contributing to tissue damage. Regulatory T-cells (Tregs) that protect against autoimmune attack appear to drop significantly in the hours and days after a stroke[3,78] suggesting that immune system regulation is impaired after a stroke. However, the role of Treg expression following stroke is likely complex.[79] Some preclinical studies in mice with experimental stroke showed that Treg depletion is an important factor in post-infarct brain damage [79] and Treg replacement is beneficial at least partly through protection of the blood–brain barrier.[80] Other studies have demonstrated a detrimental effect of Treg expression following focal ischemic in a mouse, specifically in microvascular dysfunction.[81] Kallikreins and KLK1 in particular have been implicated in the regulation of autoimmunity in a number of situations[82] at least partly through the KKS. Notably, treatment of non-obese diabetic mice with recombinant human KLK1 has been shown to significantly increase Tregs and reduce autoimmune destruction of pancreatic beta cells.[83] This supports the hypothesis that KLK1 treatment in AIS may also involve therapeutic modulation of the immune system response to AIS, but more mechanistic research is certainly warranted.

KLK1 treatment can also improve the complex maladaptive inflammatory response following AIS. Various types of inflammatory responses occur following AIS, including increased proinflammatory immune cells and cytokines.[2] Previous preclinical research has demonstrated a protective role of KLK1 treatment in mouse models of ischemic/reperfusion injury by dampening the underlying inflammatory response. KLK1 gene transfer treatment resulted in a decrease in cerebral macrophage/microglial infiltration[74] as well as inhibition of proinflammatory Toll-like receptor (TLR)4 and nuclear factor (NF)-κB levels, and activation of the anti-inflammatory nuclear respiratory factor (NrF) pathway, likely contributing to the neuroprotective effects.[84] These results suggest reducing inflammation is another mechanisms whereby KLK1 treatment could be beneficial for stroke pathology.

Interestingly, studies that focus specifically on the role of BK receptors have generated contradictory results and exact function of these receptors following brain ischemia remains unclear. BKR2 KO mice have been shown to be both more susceptible[73] and protected from[85] brain damage caused by experimental ischemia. A similar dichotomy exists with respect to the effects of BK2R antagonists (compare Xia and colleagues[73] with various studies[85–87]). One hypothesis explaining this discrepancy is that the role of BK receptors depends heavily on the exact biochemical and physiological milieu present at the time of the insult. By contrast, the beneficial effects of the biochemically 'upstream' mechanisms of KLK1 are notably more consistent across animal

models. This suggests that the actions of KLK1 are more subtle and complex than direct activation of BK receptors. It is entirely possible that adding KLK1 to the system allows the regulated activation of BK receptors in a fashion that is both more physiological and beneficial. In general, the preponderance of preclinical evidence supporting KLK1-boosting approaches supports the continued to developing and testing of such therapies in AIS patients.

Therapeutic action of KLK1 treatment in human AIS

The most compelling body of evidence demonstrating that KLK1 therapy is beneficial in AIS comes from the extensive documented clinical experience in China. There, KLK1 isolated from human urine has been approved by the Chinese regulatory drug agency over 10 years ago and marketed for treatment of AIS. This product, called Kailikang® [urinary (u)KLK1] is administered once a day by 60-minute intravenous infusion for 21 days after a stroke. Approximately 400,000 AIS patients have been treated with uKLK1 over the past 10 years.[88] Extensive clinical literature documents the apparent efficacy of uKLK1 based on clinical research conducted in China. The regulatory approval in China was based, at least partly, upon a multicenter, randomized, double-blind, placebo-controlled trial of 446 AIS patients.[89] Patients who suffered a mild to moderate first time thrombotic stroke received injectable uKLK1 or placebo once a day as a 30-minute infusion for 21 days. Patients treated with uKLK1 showed significantly higher European Stroke Scale scores as well as significant improvement on Barthel Index (BI) scores at 90 days compared with the placebo group.[89] Only mild to moderate adverse events were reported, including palpitation, flush, dizziness and nausea, and two cases showed a sudden blood pressure drop which was attributed to the quick infusion speed in combination with the use of ACE inhibitors.[89] This study has been followed by numerous reports of additional clinical trials having similar design. A comprehensive systematic review covering 24 clinical studies through 2010 was recently published.[90] Only 2 of the 24 studies were fully compliant with standard practices for rigorous clinical testing while the others had some design deficiency, including lack of placebo treatment, single center trials or incomplete blinding procedures. In each case, the treatment groups received a 0.15 enzyme activity units (PNAU) dose of uKLK1, which is the dose approved in China for use in AIS, and the treatment period for all studies lasted between 7 and 21 days. The analysis showed that uKLK1 treatment was significantly associated with an improvement in neurological outcomes and a reduction in death and dependency at the 3-month follow up in the two well-controlled trials ($n = 459$). Similar effects were also evident in the less well-controlled trials. The incidence of adverse events ranged from 0.5–5 % with transient hypotension and nausea/vomiting being the most common events reported. The authors concluded that this collection of studies supported the use of uKLK1 treatment for AIS and that treatment was safe and well tolerated.

Since 2010, numerous additional clinical studies were conducted resulting in at least 40 publications, mostly in the Chinese medical literature. These studies invariably report positive efficacy for uKLK1, despite the variable quality of the trials and resulting publications. Most studies employ the standard dosing protocol (intravenous infusion once daily for 21 days) and most are placebo-controlled and blinded. Table 1 summarizes the neurological outcomes from seven studies deemed to be among the most reliable based on journal publication and study design control.

These studies included between 29 and 200 patients with AIS enrolled with an equal male–female distribution. Study participants were between 18 and 80 years of age, had an initial NIHSS between 4 and 25, and the time of onset of treatment following stroke was between 4 and 72 h. Among those studies, results showed that uKLK1 treatment improved outcomes over a variety of post-stroke times, including 6 months post-stroke.[92] Another study, involving 200 patients showed that uKLK1 could be safely and effectively given to patients who also had received tPA treatment.[95] Notably, the group treated with both tPA and uKLK1 showed significantly greater improvement at 90 days post-stroke on the National Institute of Stroke Scores (NIHSS) than those treated with tPA alone, suggesting KLK1 treatment can offer benefit beyond that of tPA treatment alone. The studies listed in Table 1 also showed significant improvements on other functional endpoints, including BI[92,98,99] and the Rankin Scale,[98] in the uKLK1 treated groups compared with the control group.

Table 1. Summary of uKLK1 clinical trials since 2010.

Study	Reference	Design	Total *N*	Functional endpoint	Effect size[a]	Significance between groups
1	Wang and colleagues[91]	Prospective randomized double blind	44	NIHSS, 6–72 h post treatment	0.011	0.858
				NIHSS, 14 days post treatment	−0.04	$p = 0.049$
				MBI, 30 days post treatment	0.1	$p = 0.032$
2	Song and colleagues[92]	Prospective randomized controlled	27	NIHSS, 6 mo. after treatment	1.40	$p < 0.05$
				BI, 6 mo. after treatment	1.45	$p < 0.05$
3	Chen and colleagues[93]	Controlled	127	NHISS after treatment	1.09	$p < 0.05$
				BI, after treatment	2.85	$p < 0.05$
4	Meng and colleagues[94]	Controlled	120	NDS	1.00	$p < 0.05$
5	Wang and colleagues[95]	Controlled	200	NIHSS, 7 days after treatment	2.70	$p = 0.045$
				NISSS, 90 days after treatment	0.47	$p = 0.041$
				BI, 90 days after treatment	0.98	$p = 0.012$
6	Li and colleagues[96]	Randomized controlled	110	NIHSS, after treatment	0.41	$p = 0.04$
7	Miao and colleagues[97]	Nonrandomized controlled	30	Change in NIHSS	0.85	$p = 0.04$

[a]Effect size calculated by Cohen's *d* statistic for differences between treatment and control group for each endpoint. Significance between groups was the *p* value reported in the cited paper.
BI, Barthel Index; MBI, Modified Barthel Index; mo, months; NDS, neurological deficit score; NIHSS, National Institute of Health Stroke Score; uKLK1, urinary KLK1.

Complementing the work focused on neurological functional endpoints, a number of studies have demonstrated positive effects of uKLK1 treatment on brain imaging and biomarker endpoints. Magnetic resonance imaging (MRI) studies show that uKLK1 treatment improved relative mean transit time (rMTT) in the penumbra 12 days after treatment compared with the control group. Mean transit time (MTT) is commonly used to predict the cerebral perfusion in AIS and the results show that uKLK1 enhances blood flow in at-risk brain tissue. Interestingly, patients who received uKLK1 treatment showed a more favorable outcome (72.4% of patients) compared with controls (42.3%), and these improved outcomes were correlated with rMTT scores. Furthermore, patients receiving uKLK1 showed increased levels of serum VEGF and apelin, biochemical markers associated with angiogenesis and blood flow.[100] A similar study showed improved cerebral blood

flow (CBF), cerebral blood volume (CBV) and MTT from day 1 to day 14 following a 7-day treatment of uKLK1 compared with the control group.[97] Increased CBF was present in the contralateral cortex from the stroke while improvements in CBV and MTT were evident in both contralateral and ipsilateral hemispheres. Regional brain activity measured by MRI also improved after uKLK1 treatment.[97] Other studies examined regional brain activity in response to uKLK1 treatment. One study observed greater sensorimotor cortex activation volume after 14 days of treatment compared with the control group[91] which corresponded to a significant improvement in NIHSS. At 6 months after treatment, patients receiving uKLK1 treatment showed greater activation in the ipsilateral motor cortex along with improved NIHSS and BI scores.[92] Taken together, these results suggest that KLK1 could increase the local concentration of oxygenated hemoglobin after brain ischemia, which could improve neurological function following ischemic stroke. In fact, some studies have demonstrated the earlier the activation of the affected brain region can occur, the better the recovery following stroke.[101,102]

Any one of the multiple biochemical processes activated by the KLK1 enzyme could presumably explain the observed efficacy of KLK1 treatment in AIS patients. However, actions on more global physiological functions may provide the most compelling rationale for wider use of KLK1 replenishment therapy. One hypothesis is that KLK1 treatment helps to normalize neurovascular coupling in the hours and days after a stroke. Neurovascular coupling refers to the process by which local brain activity influences local blood flow through direct communication between neuronal signaling molecules and dilation or constriction of blood vessels and capillaries. It is becoming increasingly apparent that maintaining a healthy balance between local brain activity and local brain blood flow is critical for proper neurological function.[103] Multiple lines of evidence support the concept that ischemic stroke can be associated with hemodynamic steal,[104] cortical spreading depression,[105] and inappropriate neurovascular coupling.[106] Particularly relevant to KLK1 action, increased NO appears to improve these deficits.[107] Notably, the hypothesis that KLK1 treatment can address impaired neurovascular coupling is quite testable with transcranial Doppler methods,[104] providing a relevant surrogate marker of drug action that can guide dosing in later-stage clinical studies.

Recombinant form of human KLK1

DM199 is a recombinant form of human KLK1 being developed to improve neurological function in AIS patients. DM199 has potential advantages over uKLK1 for several reasons. DM199 has improved bioavailability, a strong safety profile at proposed therapeutic doses and is more economical to produce. In a recently published phase I clinical trial investigating the safety and pharmacokinetic (PK) profile of DM199 showed positive results.[108] DM199 was safe and well tolerated when administered by either subcutaneous injection or intravenous infusion. This study successfully identified a dose of DM199 that generated plasma concentrations similar to that of the standard intravenous dose of uKLK1 used in China (0.15 PNAU[109]). The subcutaneous route of administration generated a sustained plasma concentration (measured by an enzyme-linked immunosorbent assay) over a prolonged period, which is a substantial improvement over the relatively brief increase in uKLK1 plasma concentration following an intravenous infusion.[109] Previous studies evaluating the PK profile of uKLK1 following intravenous administration,[109] an increase in uKLK1 was observed in two patients in the 0.02 PNAU groups but these levels were close to the lower limit of quantitation. However, the patients administered 0.04, 0.075 and 0.30 PNAU/body showed rapid evaluation of uKLK1 after dosing. Additionally, increases in plasma uKLK1 were dose proportional. Similar to DM199, when administration of uKLK1 was discontinued, plasma levels showed a rapid decrease. This should provide significant advantages in compliance and allow for greater efficacy due consistent exposure of the course of treatment when delivered subcutaneously. DM199 treatment had no effect on blood coagulation times, which suggests that it could also be safely administered to patients who have previously received tPA treatment.[108] DM199 has the same amino acid sequence, biochemical activity and pathophysiological effects as uKLK1 but requires no human tissue and is significantly more convenient to produce. Results from the DM199 clinical trial suggest a safer and more convenient administration profile for AIS patients in the hospital and at home given its superior PK profile. The possibility of sustained plasma exposure allows for

continuous enzyme replacement therapy not provided by uKLK1.

Conclusions and prospects

Evidence has been presented supporting the hypothesis that KLK1 treatment may prevent AIS pathogenesis and improve the recovery by augmenting penumbral blood flow, suppressing inflammation, restoring neurovascular coupling and regulating autoimmune reactions to the insult. Figure 2 summarizes the basic mechanisms that support the efficacy hypothesis for treatment with KLK1. The biochemical and physiological role of KLK1 as a critical homeostatic regulator of local blood flow along with the growing evidence that KLK1 levels appear to be depleted in patients with ischemic conditions shows that KLK1 functions to maintain proper tissue perfusion and health under a variety of conditions. These conditions also include hypertension, chronic kidney disease, and, interestingly, X-linked genetic diseases like Anderson–Fabry disease that shows central nervous system complications likely attributed to cerebral vasculopathy, impairments in endothelial function, and dysregulations of cerebral blood flow.[110,111] The experience with uKLK1 treatment in China shows documented improvement in neurological function that coincides with imaging-based measures of enhanced blood flow and brain activity. However, recombinant KLK1 treatment likely offers a better safety and PK profile in AIS patients. In fact, clinical data on PK and safety collected thus far significantly guide the development of the current clinical trial being conducted in AIS patients (ClinicalTrials.gov identifier: NCT03290560). The apparent efficacy of KLK1 treatment may be at least partly due to normalization of neurovascular coupling, an effect that be readily measured with widely available technologies. Together with the availability of a recombinant form of human KLK1, these results provide a strong rationale for continued clinical development of KLK1 treatment for AIS.

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Conflict of interest statement

Dr Alexander, Mr Pauls, and Dr Verdoorn are employees of DiaMedica Therapeutics, which funded the clinical trial cited using DM199. Dr Chao, Dr Bath and Dr Volpi are scientific advisors to DiaMedica Therapeutics, have received honoraria from the company and hold stock options.

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