The pharmacology and therapeutic potential of small molecule inhibitors of acid-sensing ion channels in stroke intervention

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In the nervous system, a decrease in extracellular pH is a common feature of various physiological and pathological processes, including synaptic transmission, cerebral ischemia, epilepsy, brain trauma, and tissue inflammation. Acid-sensing ion channels (ASICs) are proton-gated cation channels that are distributed throughout the central and peripheral nervous systems. Following the recent identification of ASICs as critical acid-sensing extracellular proton receptors, growing evidence has suggested that the activation of ASICs plays important roles in physiological processes such as nociception, mechanosensation, synaptic plasticity, learning and memory. However, the over-activation of ASICs is also linked to adverse outcomes for certain pathological processes, such as brain ischemia and multiple sclerosis. Based on the well-demonstrated role of ASIC1a activation in acidosis-mediated brain injury, small molecule inhibitors of ASIC1a may represent novel therapeutic agents for the treatment of neurological disorders, such as stroke.

Keywords: ion channels; acidosis; ASIC; proton; stroke; neurological disorders

Introduction

The maintenance of extracellular and intracellular pH levels within a physiological range is critical for normal cellular activity. Under physiological conditions, Na⁺/H⁺ and Cl⁻/HCO₃⁻ systems generally maintain the extracellular and intracellular pH at approximately 7.3 and 7.0, respectively[1, 2]. However, a local decrease in the extracellular pH may occur in certain micro-domains, such as the synaptic cleft. The synaptic vesicles are acidic, and thus their release during synaptic transmission can reduce the local pH of the synaptic cleft[3]. An extracellular pH reduction is also observed in hippocampal slices following electrical stimulation[4]. Thus, local pH fluctuations may occur under normal conditions[5], although the extent and the physiological significance of pH fluctuations remain poorly understood.

Extracellular acidosis is more commonly observed in pathological conditions, such as brain ischemia/stroke[6], seizure[7, 8], and brain trauma[9]. Moreover, the extent of the extracellular acidosis is closely related to the outcomes of these disorders. For instance, slight acidosis has been reported to reduce neuronal injury, partially due to the inhibition of N-methyl-D-aspartate (NMDA) receptor channels[10]. By contrast, severe acidosis during brain ischemia, which can reduce the extracellular pH levels to as low as 6.0, can cause deleterious brain damage[11, 12]. Although the underlying mechanism by which acidosis leads to adverse outcomes for certain neurological disorders has remained elusive, the discovery of acid-sensing ion channels (ASICs) has provided new potential explanations for this phenomenon.

ASICs are critical acid sensors in the nervous system. ASIC1a, for instance, can sense slight extracellular acidosis at pH levels of approximately 7.0[13]. Increasing evidence has demonstrated that ASICs play crucial physiological roles in processes such as nociception, learning and memory, and synaptic plasticity[14, 15]. More importantly, ASICs are implicated in neurological disorders such as stroke[16, 17], inflammatory pain[18-20], and epilepsy[21, 22]. In this review, we will discuss the pharmacological properties and therapeutic potential of small molecule inhibitors of ASICs for stroke intervention.

The molecular characterization and electrophysiological properties of ASICs

ASICs belong to the degenerin/epithelial Na⁺ channel (DEG/ENaC) superfamily, which also includes the FMRFamide-gated Na⁺ channel (FaNaC)[13,23]. ASICs possess short intracellular amino and carboxyl termini of 35–90 amino acids each...
and two hydrophobic transmembrane regions (approximately 20 amino acids each in size) that flank a large, cysteine-rich extracellular domain composed of approximately 370 amino acids[24]. In total, six ASIC isoforms have been described to date in mammals: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4; these isoforms are encoded by four genes[25, 26]. The recent crystal structure of chicken ASIC1 has revealed the trimeric assembly of this protein[27]. The high sequence identity (90%) between chicken and human ASIC1[27] not only implies that ASIC1 fulfills important and conserved functions in the chordate nervous system but also suggests that the three-dimensional structure of the protein may be similar in these two species; this information may prove invaluable for examining the function of ASIC1 and the design of potent and selective ASIC1 inhibitors.

ASICs are highly enriched in the nervous system[13, 15, 28]. The combined results of studies involving in situ hybridization, immunohistochemistry and electrophysiology demonstrate that ASIC1a, ASIC2a, and ASIC2b are widely distributed in both the central and peripheral nervous systems[13, 15, 17, 29]. By contrast, ASIC3 is preferentially expressed in dorsal root ganglion (DRG) neurons[30], suggesting its potential involvement in nociception in the peripheral nervous system.

ASICs are proton-gated, voltage-independent cation channels. Different ASIC isoforms have distinct sensitivities to reductions in extracellular pH. For instance, ASIC1a and ASIC3 are the subunits that are most sensitive to H⁺, as these proteins can respond to pH levels that drop below 7.0[13, 31, 32]. ASIC2a has the lowest sensitivity to reductions in extracellular pH; this protein has a pH₅₀ of approximately 4.4, and its channels remain closed even at a local pH of 6.0[29, 30]. Homomeric ASIC2b and ASIC4 are completely insensitive to extracellular pH reductions[29, 33, 34]. ASIC activation is typically characterized by a transient inward current. Of the ASIC proteins, the desensitization of ASIC1a occurs most rapidly, with a time constant of 1–2 s[13]. ASIC2a desensitizes more slowly than ASIC1a, whereas ASIC3 has a non-desensitized current component that persists during prolonged acidosis[30, 31]. It is worth noting that the properties of ASICs can be dramatically modulated by ischemia-related signaling molecules[35].

ASICs are generally highly permeable to Na⁺ and almost impermeable to Ca²⁺; however, the homomeric ASIC1a demonstrates Ca²⁺ conductance[13, 16, 25, 26]. The increase of Ca²⁺ concentration in the cell is critical for a number of physiological functions. However, Ca²⁺ overload can cause neuronal injury or death in the context of various neurological disorders, such as stroke[37–39]. The Ca²⁺ conductance of homomeric ASIC1a implies that this complex performs specific functions in Ca²⁺-related physiological and pathological processes.

Protons are the traditional agonists for ASICs; however, the existence of other endogenous activators cannot be excluded. Certain ASIC subunits, such as ASIC2b and ASIC4, form homomeric channels that cannot be gated by protons, and the potential role of ASICs in mechanoperception raises the possibility that ASIC ligands other than protons may exist[40–42]. This hypothesis is further supported by the recent identification of a small molecule non-proton ligand 2-guanidine-4-methylquinazoline that activates ASIC3 and a toxin from the Texas coral snake that activates ASIC1a in the absence of acid[19, 43].

The pathological significance of ASICs in stroke

The detailed physiological functions of ASICs have been discussed in previous publications[24, 41, 44]. In addition to fulfilling important physiological functions, ASICs are also well-known for their involvement in certain pathological conditions, most notably stroke/brain ischemia[16, 17, 45, 46]. Due to the conductance of Ca²⁺, ASIC1a channels have been shown to contribute to the Ca²⁺ overload and subsequent neuronal injury that occur in brain ischemia. The activation of homomeric ASIC1a channels may cause Ca²⁺ overload not only through the direct conductance of Ca²⁺ but also through the indirect activation of NMDA receptors, which occurs as a result of the depolarization of the neuronal membrane. Amiloride, a nonspecific ASIC inhibitor, and PcTX1, a specific homomeric ASIC1a channel inhibitor, have been demonstrated to significantly protect neurons against oxygen-glucose deprivation and acid-induced injury in vitro[16, 47]. Moreover, in mice and rats, these inhibitors dramatically reduced the volume of the infarcts that were induced by middle cerebral artery occlusion (MCAO). Additionally, ASIC1a knockout mice were resistant to MCAO-induced neuronal injury, further confirming that ASIC1a activation plays an important role in the pathophysiology of brain ischemia/stroke[16]. A particularly intriguing finding was that the ASIC1a inhibitor PcTX1 has a therapeutic time window of 5 h[46, 48], which is longer than the therapeutic time windows of 3 h and 1 h for tissue plasminogen activator and NMDA receptor antagonists, respectively[50, 51]. As the present strategy to treat stroke remains limited to the use of preventative measures, the restoration of blood supply to the affected regions, and the implementation of procedures to decrease patient metabolism, small molecule inhibitors of ASIC1a may represent promising novel neuroprotective agents for stroke intervention.

The pharmacological characterization and therapeutic potential of small molecule inhibitors of ASICs

The function of many ion channels, including ASICs, can often be modulated by metal ions, including Zn²⁺, Ca²⁺, Mg²⁺, and Cu²⁺, among others[55]. However, metal ions cannot be effectively used to treat ASIC-related diseases because of their diverse physiological functions and poor selectivity. In addition to metal ions, certain naturally occurring venom polypeptides could also modulate ASICs. For instance, PcTX1, a polypeptide purified from the venom of the south American tarantula Psalmopoeus cambridgei, can specifically and potently inhibit homomeric ASIC1a current[52] by promoting a desensitized state of ASIC1a[53], while APETx2, a sea anemone toxin, is a potent inhibitor of ASIC3 channels[54]. By contrast, the toxin of the Texas coral snake can activate ASIC1a at neutral pH[19]. Although all of these polypeptides constitute good
pharmacological tools for elucidating the functions of ASICs, they cannot be employed in clinical practice to treat neurological disorders such as stroke because their large molecular size limits their penetration across the blood-brain barrier (BBB)\[59\]. However, small molecule compounds can penetrate the BBB and have been used successfully in treating certain disorders of the CNS. Thus, small molecule inhibitors that target ASICs may represent promising therapeutic agents for the treatment of stroke.

**Amiloride**

Amiloride, which blocks ENaC, Na⁺/H⁺ exchanger, and Na⁺/Ca2⁺ exchanger, was once used as a diuretic agent\[55, 57\]. In addition, amiloride is a nonspecific blocker of ASICs that inhibits ASIC1a, ASIC1b and ASIC2a current with an IC₅₀ of approximately 10–20 µmol/L and inhibits the transient ASIC3 current with an IC₅₀ of approximately 60 µmol/L\[13, 30, 58, 59\]. However, the sustained ASIC3 current is completely resistant to amiloride\[30, 60\]. In fact, a study has reported that amiloride slightly potentiated the sustained ASIC3 current in cardiac sensory neurons\[61\]. Nevertheless, in the absence of more specific ASIC inhibitors or modulators, amiloride, in conjunction with molecular biology approaches, is a useful pharmacological tool for studying the functions of ASICs.

Consistent with its role as an ASIC inhibitor, amiloride has been shown to be capable of suppressing acid-induced pain in the peripheral sensory system and reducing acid-mediated ischemic neuronal injury and axonal degeneration in the CNS\[14, 62, 63\]. These effects support the potential of using amiloride for the treatment of certain neurological disorders. However, from a therapeutic point of view, the use of amiloride may be limited by its poor selectivity for ASICs and its interactions with the other ion channels/ion exchangers mentioned above. For instance, the Na⁺/Ca2⁺ exchanger is critical for normal neuronal function and survival because it maintains the stable homeostasis of intracellular Ca2⁺ concentrations. If the function of this exchanger is compromised by amiloride, a transient physiological Ca2⁺ influx may produce a lethal Ca2⁺ overload, a well-known pathological process that causes neuronal injury and death\[59\]. The inhibitory effect of amiloride on the Na⁺/Ca2⁺ exchanger may be partially responsible for the death of cultured cortical neurons that are incubated with amiloride in vitro for 5 h\[16, 55\]. This effect may also explain the fact that PcTX1 (a specific ASIC1a inhibitor) produces a greater neuroprotective effect than amiloride in in vivo studies of cerebral ischemia\[18\]. Although amiloride itself may not be an ideal agent for combating neurological disorders such as stroke, its use has provided important information regarding the structure and activity of ASICs. Further structural modifications of amiloride with the goal of obtaining a more selective and potent ASIC1a inhibitor may facilitate the identification of ideal agents for the treatment of stroke.

**A-317567**

A-317567 is a non-specific small molecule inhibitor of ASICs that is structurally unrelated to amiloride\[64\]. In contrast to amiloride, this compound can inhibit the sustained ASIC3 current\[64\], a current component closely associated with chronic pain sensations. The inhibitory effect of A-317567 on sustained ASIC3 current may partially explain the fact that A-317567 demonstrates greater efficacy than amiloride in reducing acid-induced pain\[64\].

A-317567 has no diuretic or natriuretic effect and has little influence on other members of the ENaC superfamily. Therefore, this molecule appears to have a higher selectivity for ASICs than amiloride; however, it is not yet known whether A-317567 can influence other channels or membrane receptors. In addition to relieving chronic pain, A-317567 might have the potential for treating ischemic stroke due to its potent inhibition on ASIC1a-like current (IC₅₀ ~2 µmol/L). However, there has been a lack of direct experimental evidence that confirms its neuroprotective activity either in vitro or in vivo. The poor penetration of A-317567 across the BBB may limit its use in the treatment of neurological disorders in the CNS\[64\], although the BBB may be compromised in certain conditions, such as ischemic stroke. The pursuit of further structural modifications to improve the lipophilic properties of A-317567 without decreasing its potency and selectivity may be a promising strategy for producing efficacious agents for stroke treatment.

**Non-steroidal anti-inflammatory (NSAIDs) drugs**

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical practice to relieve pain and treat inflammatory diseases. Furthermore, low dosages of aspirin are routinely used to prevent ischemic stroke by reducing patients’ risk of having blood clot\[65\]. The well-known mechanism underlying the analgesic effects is the inhibition of cyclooxygenase (COX) activity by NSAIDs. However, other mechanism(s) may exist, given the fact that NSAIDs appear to retain their analgesic activity even if COX is inhibited or knocked out\[66, 67\].

Recently, it has been demonstrated that some NSAIDs, ibuprofen and flurbiprofen for example, can inhibit ASIC3 current and ASIC3 expression in the peripheral nervous system at clinically relevant concentrations\[68\]. This observation may disclose a novel mechanism that mediates the analgesic effect of NSAIDs, as the activation of ASIC3 is associated with multiple painful sensations\[9, 19, 69\]. In addition to their effects on ASIC3, certain NSAIDs can also inhibit ASIC1a or ASIC1a-like current. For instance, aspirin can rapidly and reversibly inhibit 83.7% of the peak ASIC current in cultured rat cortical neurons at 3 mmol/L\[70\], a concentration that can be reached in clinical use. Ibuprofen and flurbiprofen can inhibit ASIC1a current with an IC₅₀ of approximately 350 µmol/L\[68\]. The inhibitory effects of NSAIDs on ASIC1a current imply that these molecules are potential therapeutic agents for the treatment of ischemic stroke. However, high dosages of NSAIDs may increase the incidence of intracerebral hemorrhages, which can produce adverse consequences in cases of stroke; this potential drawback of NSAIDs should therefore be considered in analy-
Diamidines
Aromatic diamidines are synthetic small molecules that bind to the minor groove of DNA[71]. These compounds have traditionally been used to treat leishmaniasis, trypanosomiasis, pneumocystis pneumonia and babesiosis[72]. Recently, Chen and colleagues performed a small-scale screen of aromatic diamidines to search for potent ASIC inhibitors. These researchers found that 4',6-diamidino-2-phenylindole (DAPI), diminazene, hydroxystilbamidine (HSB) and pentamidine potently inhibit the ASIC current in primary cultured hippocampal neurons with IC<sub>50</sub> values of 2.8±0.7 µmol/L, 0.29±0.11 µmol/L, 1.5±0.6 µmol/L and 38±11 µmol/L, respectively[73]. These compounds have more potent inhibitory effects on ASICs compared with amiloride, which has an IC<sub>50</sub> for ASIC current of 10–20 µmol/L and completely blocks neuronal ASIC currents at concentrations of 200–500 µmol/L[74,75]. The co-application of diminazene with acidic solution could also shorten the desensitization time constant of ASICs[73], suggesting that diminazene may promote ASIC closure. Interestingly, this effect appears to be specific to diminazene, as other aromatic diminazenes do not have effects on the desensitization rate of ASIC currents. Currently, diminazene is the most potent known small molecular inhibitor of ASICs. Its neuroprotective effects, however, have not been demonstrated directly, either in vitro or in vivo.

Although ASICs share considerable homology with ENaC proteins, even the high concentration of 100 µmol/L of diminazene has no effect on ENaCs[73], implying that diminazene has a higher selectivity for ASICs than amiloride. Furthermore, the long-term clinical use of diminazene for treating the diseases mentioned above suggests that this drug is relatively safe and has a low probability of causing intolerable side effects. However, diminazene still displays poor selectivity among the ASICs, and its highly hydrophilic nature may limit its penetration across the BBB[72]. Both of these concerns may limit the usefulness of diminazene in the treatment of stroke. DB829, the first aromatic diamidine derivative, can penetrate the BBB via an unknown transporter[72]. However, the toxicity and efficacy studies of DB829 have not been completed, and its effect on ASICs is unknown. It may be possible to construct a chimeric molecule that contains the diamidine functional group to inhibit ASIC1a channels and the critical group of DB829 to enhance penetration of the BBB.

Local anesthetics
Local anesthetics have various effects, including antiarrhythmia, antinociception, neuroprotection and analgesia[76]. The blocking of voltage-gated Na<sup>+</sup> channels is a well-known mechanism shared by many local anesthetics[77]. However, the multiple effects of these molecules cannot be explained solely in terms of the blocking of voltage-gated sodium channels. Other mechanisms must be involved in anesthetic effects, particularly during severe acidosis, as the activities of voltage-gated sodium channels, the primary target of local anesthetics, are significantly suppressed by protons[78,79].

Recently, we demonstrated that lidocaine could reversibly inhibit ASIC1a current without affecting ASIC2a current[76]. Similarly, tetracaine can inhibit ASIC1a current in a use-dependent manner[80]. Moreover, this anesthetic can inhibit both the transient and sustained ASIC3 currents without significantly affecting ASIC2a current[80]. By contrast, other amide-based local anesthetics, such as mepivacaine, bupivacaine, and ropivacaine, can block voltage-gated sodium channels but do not affect ASICs[79]. The concentration of lidocaine that is sufficient for the inhibition of ASIC1a can be achieved in clinical practice through direct local exposure[79]. Thus, lidocaine could be a potential candidate for stroke intervention. However, it has not yet been determined whether the potential side effects caused by the blockade of voltage-gated Na<sup>+</sup> channels would be minimal enough to allow lidocaine to be systemically used for the treatment of stroke. Undoubtedly, the triple blockade of voltage-gated Na<sup>+</sup> channels, ASIC1a channels and ASIC3 channels by tetracaine endows this molecule with the potential for being a potent painkiller. In addition, the use-dependent inhibition of ASIC1a by tetracaine makes it particularly effective at suppressing the higher-frequency activations of ASIC1a, which implies that this molecule will have a greater impact on abnormal ASIC1a activity than on the physiological functions of ASIC1a.

Conclusion
The lack of effective treatments for stroke damage emphasizes the urgent need for a better understanding of brain injury mechanisms and a wider range of neuroprotective agents. The targeting of ASICs by small molecule inhibitors may represent a promising strategy for combating ASIC-related neurodegenerative diseases, such as stroke. These neuroprotective agents should ideally not only display high selectivity and efficacy in the inhibition of ASIC1a channels but also possess the capability to effectively penetrate the BBB. The small molecule inhibitors of ASICs that are currently available display low selectivity for any specific ASIC subunit. Although the inhibition of Ca<sup>2+</sup>-permeable ASIC1a channels can provide neuroprotection, the blockade of other isoforms of ASICs may induce undesirable side effects. Moreover, a highly selective ASIC1a inhibitor may still produce side effects, as the complete inhibition of this channel may interfere with the important physiological functions of its channels; this lesson can be learned from the clinical failure of many putative NMDA receptor antagonists. Thus, a mild modulator that can attenuate the over-activation of ASICs but retain the physiological functions of these channels may be a good candidate for a stroke treatment drug. Finally, a large-scale structure-activity relationship study, based on the presently available structural data regarding ASIC1a inhibitors and the known crystal structure of ASIC1a, may expedite the search for a highly selective, potent and lipophilic small molecule that can effectively treat ischemic stroke.

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