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Salvaging brain ischemia by increasing neuroprotectant uptake via nanoagonist mediated blood brain barrier permeability enhancement

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ABSTRACT

Ischemic stroke is a leading cause of adult disability and cognitive impairment worldwide. Neuroprotective therapy aims to save neurons by impeding the deleterious ischemic insults. However, the low efficiency of the neuroprotectants crossing blood brain barrier (BBB) prevents their clinical translation. In this work, a nanoagonist (NA) was developed to enhance neuroprotectant uptake by specifically increasing BBB permeability in brain ischemia. This NA first targeted ischemic brain vasculatures, temporarily opened local BBB by activating adenosine 2A receptors, and up-regulated the neuroprotectant uptake in brain ischemia. This NA significantly increased the delivery of superoxide dismutase (SOD), a free radical scavenger, into mouse brain ischemia. The combined treatment of NA/SOD achieved a five-fold ischemic volume reduction rate compared to the animal models treated with SOD alone. Non-invasive magnetic resonance imaging (MRI) confirmed the ischemia targeted BBB opening, increased brain drug delivery efficiency and up-regulated therapeutic response during the combined NA/SOD treatment. Since the inefficient brain drug delivery is a general problem for the treatment of central nervous system (CNS) diseases, this work provides a novel strategy to deliver therapeutics by crossing BBB with high efficiency and targeting specificity.

1. Introduction

Stroke is the second most frequent cause of death and a leading cause of adult disability and cognitive impairment [1a,b]. Annually, 15 million people worldwide suffer stroke, in which 5 million die and another 5 millions are left permanently disabled, placing a heavy burden to family and community [2]. Ischemic stroke accounts for approximately 85% of all stroke cases and it is triggered by a transient or lasting reduction in cerebral blood flow (CBF) that arises from either in situ thrombosis or embolization of a clot from a proximal arterial or cardiac source. The vascular occlusion initiates a complex cascade of cellular events including depletion of energy compounds, excessive activation of glutamate receptors, influx of calcium cations, over-production of free radicals, abnormal recruitment of inflammatory cells and initiation of apoptosis that ultimately leads to irreversible tissue injury and infarction.

Thrombolytic therapy and neuroprotective therapy are two main strategies for ischemic stroke treatment, which aim to timely restore CBF and minimize deleterious ischemic damages on neurons respectively. As the only approved thrombolytic by FDA, recombinant tissue-type plasminogen activator (rt-PA) dredges the occlusive vasculatures and increases CBF in ischemic regions [3]. However, due to the short therapeutic time-window (within 4.5 h after stroke symptoms) and potential risk of brain hemorrhage [4], less than 5% patients benefits from the thrombolytic treatment [5]. As a promising therapeutic approach, neuroprotection aims to extend survival of neurons by impeding single or multiple above deleterious cascade evens. The neuroprotection can be applied in acute ischemic attack stage as well as the following rehabilitation stage, which benefits the patients missed the time-window for

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thrombolytic treatment. In the past two decades, more than 1000 neuroprotective agents such as glutamate antagonists, calcium channel blockers, free radical scavenges and anti-inflammatory agents had been developed [6]. Even though part of them efficiently reduced infarct volume and improved functional outcome in animal models [7], few of them demonstrated unequivocal efficacy in clinical trials that fulfilled regulatory requirements for approval [8]. Besides the inadequate preclinical testing and the animal model hardly simulating the stroke patients, the low brain uptake of the neuroprotectants is believed another key issue leading to their limited success [9]. For example, brain-derived neurotrophic factor (BDNF, MW: 28 kDa) demonstrated a pronounced protective effect, but its clinical trials failed owing to its poor intracerebral uptake [10]. Therefore, strategies to increase neuroprotectant uptake in brain ischemia will greatly improve the therapeutic efficiency and accelerate their clinical translation.

Blood brain barrier (BBB) is a unique physiological structure in brain vasculature, which precisely regulates the movement of molecules, ions, cells between the blood and brain and maintains a precisely controlled microenvironment for neuronal circuits, synaptic transmission and neurogenesis [11]. Even the BBB leakage caused by ischemic damage was intensively studied [12], the brain uptake of drugs, especially the macromolecular compounds is still too low to meet the threshold of therapeutic purpose. Adenosine receptors (ARs) are a class of G protein-coupled receptors (GPCRs) and have four subtypes, A_1, A_{2A}, A_{2B}, and A_3, which are actively involved in diseases such as inflammation, cancer, cardiovascular damage, and nervous system disorders [13]. Recent studies demonstrated that specific activation of adenosine A_{2A} receptor ($A_{2A}R$) on brain capillary endothelial cells (BCECs) leads to the enhanced BBB permeability by temporarily opening the tight junctions (TJs) [14], the key structure restricting the paracellular diffusion [15]. Our previous work developed a series of nanoagonists (NAs) that not only enhanced BBB permeability by efficiently activating $A_{2A}R$, but also tuned BBB opening time-window in a range of 0.5–2.0 h [16]. Compared to brain drug delivery strategies such as intracranial injection [17a,b], hypertonic agent [18] or focused ultrasound (FUS) [19a,b] induced BBB disruption, receptor/adsorption mediated transcytosis [20a,b], NA mediated BBB opening showed the advantages including the minimized physical/mechanic injury, controllable BBB opening time-windows and free of modifications on the therapeutics. Furthermore, by matching the BBB opening time-window with the pharmacokinetics of a therapeutics, it is also possible to maximize brain drug delivery but minimize the side effects induced by BBB leakage.

Due to $A_{2A}R$ distribution in whole brain [21a,b], how to specifically up-regulate BBB permeability in the ischemic but not the normal brain tissues is crucial for the success of NA-mediated brain drug delivery. Even though angiogenesis is a relatively late response of the brain to focal ischemia (7–9 days), up-regulation of $\alpha_V\beta_3$ integrin, a well-known angiogenesis marker, is indeed appeared as early as 1–2 h after stroke [22] as an initial ischemic response of microvessels [23]. Actually, $\alpha_V\beta_3$ integrin targeted radiotracer was used to visualize cerebral infarct with high target to background (T/B) ratio in pediatric patients [24]. With the reference of reported two-order targeted imaging strategy [25], a novel NA was developed to up-regulate brain uptake of neuroprotectant by specifically opening BBB in brain ischemia. As illustrated in Fig. 1, this NA is first injected to target $\alpha_V\beta_3$ integrin over-expressed in brain ischemic vessels. The increased local concentration of NA in the ischemic vasculatures triggers $A_{2A}R$ activation followed BBB permeability enhancement by temporarily opening the TJs. Neuroprotective drug is injected when the BBB permeability reaches its maximum to achieve the optimized therapeutic response.

Multi-parametric magnetic resonance imaging (MRI) technologies were applied to dynamically monitor the key events such as ischemia targeted BBB opening, brain drug delivery efficiency and therapeutic response during the NA mediated brain drug delivery strategy. For example, dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) evaluated the BBB permeability, T1-weighted MRI (T1W-MRI) quantified the targeted delivery of the therapeutics and T2-weighted MRI (T2W-MRI) detected the therapeutic response by delineating ischemic volume. The imaging feedbacks from anatomical, functional and pharmacodynamic aspects are also helpful to maximize the therapeutic efficiency but minimize the side-effects such as non-specific BBB opening by fine-tuning the labeling degree of targeting/$A_{2A}R$ activating groups on the NA.

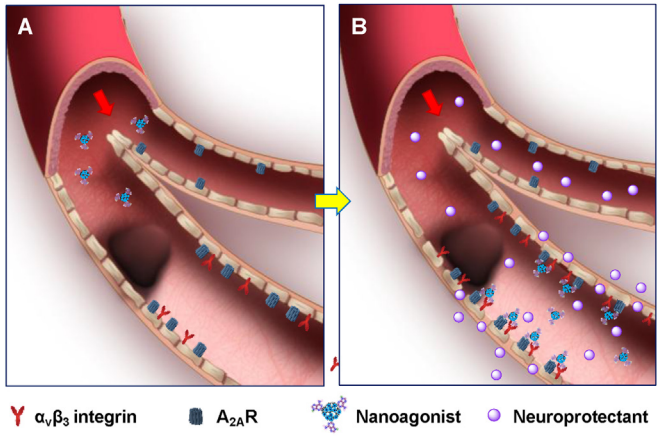

Fig. 1. NA mediated neuroprotective drug delivery in brain ischemia. (A) NAs first target the up-regulated $\alpha_v\beta_3$ integrin in the ischemic vessels. (B) The NAs with increased local concentration in ischemic vessels activate $A_{2A}R$ and trigger TJ opening. The neuroprotective drugs are injected to achieve the maximal uptake into brain ischemia.

2. Materials and methods

2.1. Materials

All chemical reagents were obtained from Aladdin Reagent (Shanghai, China) unless otherwise specified. The fourth generation (G4) poly(amidoamine) PAMAM dendrimer was purchased from Weihai CY Dendrimer Technology Co., Ltd (Weihai, China). $mPEG^{2K}$-NHS and Maleimide-PEG^{2K}-NHS were purchased from JenKem Technology Co. Ltd (Beijing, China). CGS21680 was purchased from Chengdu Novi biotechnology co. Ltd (Chengdu, China). c[RGDyK] was purchased from China Peptides Co. Ltd (Shanghai, China). Cell culture media, fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were purchased from Gibco (MA, USA). Rabbit anti-mouse CD31, active caspase-3, ZO-1, $A_{2A}R$ primary antibodies were purchased from Abcam (Cambridge, UK). Alexa Fluo488-labeled goat anti-rabbit secondary antibody and goat anti-rabbit lgG(H&L)-HRP were purchased from Cell Signaling Technology (Danvers, USA). Rhodamine phalloidin was obtained from Cytoskeleton Inc. (Denver, USA). TUNEL apoptosis assay kit was purchased from KeyGEN Bio Teck (Nanjing, China). NHS-Rhodamine was purchased from Thermo Scientific (MA, USA). Dihydroethidium (HEt) was purchased from AAT Bioquest Inc. (Sunnyvale, USA). Amicon ultra-15 centrifugal filter tubes (3000 and 10,000 MW cutoff) were from Millipore (Bedford, USA).

2.2. Synthesis

The synthesis of the NAs is described in Scheme 1. Briefly, treatment of CGS with *tert*-butyl (2-aminoethyl) carbamate in