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SMALL SYNTHETIC HYALURONAN DISACCHARIDES AFFORD NEUROPROTECTION IN BRAIN ISCHEMIA-RELATED MODELS

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Abstract—High molecular weight (HMW) glycosaminoglycanes of the extracellular matrix have been implicated in tissue repair. The aim of this study was to evaluate if small synthetic hyaluronan disaccharides with different degrees of sulfation (methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-*O*-sulfo-α-D-glucopyranoside, sodium salt (di0S), methyl 2-acetamido-2-deoxy-3-*O*-(β-d-glucopyranosyluronic acid)-6-di-*O*-sulfo-α-d-glucopyranoside, disodium salt (di6S) and methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4,6-di-*O*-sulfo-α-D-glucopyranoside, trisodium salt (di4,6S)) could improve cell survival in *in vitro* and *in vivo* brain ischemia-related models. Rat hippocampal slices subjected to oxygen and glucose deprivation and a photothrombotic stroke model in mice were used. The three hyaluran disaccharides, incubated during the oxygen and glucose deprivation (15 min) and re-oxygenation periods (120 min), reduced cell death of hippocampal slices measured as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction, being the most potent di4,6S; in contrast, high molecular hyaluronan was ineffective. The protective actions of di4,6S against oxygen and glucose deprivation were related to activation of the PI3K/Akt survival pathway, reduction of p65 translocation to the nucleus, inhibition of inducible nitric oxide oxidase induction and reactive oxygen species production, and to an increase in glutathione levels. Administered 1 h post-stroke, di4,6S reduced cerebral infarct size and improved motor activity in the beam walk test. In conclusion, di4,6S affords neuroprotection in *in vitro* and *in vivo* models of ischemic neuronal damage. Our results suggest that its neuroprotective effect could be exerted through its capability to reduce oxidative stress during ischemia. Its small molecular size makes it a more potential druggable drug to target the brain as compared with its HMW parent compound hyaluronan.

Key words: hyaluronan disaccharides, ischemia, neuroprotection, oxidative stress.

INTRODUCTION

Stroke is the third leading cause of death, behind cardiovascular diseases and cancer, and is a leading cause of serious, long-term disability. Although treatments for ischemic stroke have been rigorously investigated for two decades, up to now there is only one approved pharmacological treatment, the intravenous thrombolytic recombinant tissue plasminogen activator (rt-PA) (Jahan and Vinuela, 2009). Despite the fact that thrombolytic rt-PA has demonstrated efficacy in treating patients with thrombotic stroke, the short time window, the risk of hemorrhage and the requirement for computed tomography scan before the initiation of treatment reduces the number of patients (around 4%) actually receiving the drug; consequently, other approaches to treat stroke are urgently needed.

The use of neuroprotective agents to preserve neurons following an acute cerebral ischemic insult has provided so far disappointing results (Green, 2008). Despite the testing of over 1000 compounds with different chemical structures and mechanisms of action, several of which have proven to be efficacious in animal models of stroke, none has demonstrated efficacy in

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Abbreviations: BBB, blood–brain-barrier; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; DCFH, dichlorofluorescein; di0S, methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-*O*-sulfo-α-D-glucopyranoside, sodium salt; di4,6S, methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4,6-di-*O*-sulfo-α-D-glucopyranoside, trisodium salt; di6S, methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-6-di-*O*-sulfo-α-D-glucopyranoside, disodium salt; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GSH, glutathione; HA, hyaluronan or hyaluronic acid; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HMW, high molecular weight; iNOS, inducible nitric oxide synthase; LMW, low molecular weight; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PNN, perineuronal net; ROS, reactive oxygen species; rt-PA, recombinant tissue plasminogen activator; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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patients in over 100 clinical trials conducted (Green, 2008). This includes, NXY-059 (Shuaib et al., 2007), which was developed in accordance with the guidelines proposed by the academic-industry roundtable group (STAIR II 2001; Kahle and Bix, 2012). In spite of this rather obscure panorama, there is agreement that the search of new agents for effective treatment of stroke patients should continue with even greater effort. A potential target to develop new neuroprotective compounds is the extracellular matrix (ECM) and the perineuronal nets (PNNs).

In the adult brain, ECM is mainly present in the intercellular spaces between neurons and glial cells. Whereas most of this matrix is amorphous, there are specialized structures of dense organized matrix called PNNs around many neurons with holes at the sites of synaptic contacts (Hockfield and McKay, 1983; Celio et al., 1998). PNNs are composed of chondroitin sulfate proteoglycans (CSPGs), such as versican, brevican, neurocan, aggrecan, phosphocan, hyaluronan, tenascin-C, tenascin-R and linked proteins (Jaworski et al., 1994; Asher et al., 1995; Yamaguchi, 2000; Bekku et al., 2003; Carulli et al., 2006). There is evidence that these structures are involved in the regulation of neuronal plasticity (Hockfield et al., 1990; Pizzorusso et al., 2002), in neuroprotection (Bruckner et al., 1999; Morawski et al., 2004), and in the support of ion homeostasis around highly active neurons (Bruckner et al., 1993; Hartig et al., 1999).

Hyaluronan or hyaluronic acid (HA) is a straight chain glycosaminoglycan polymer composed of repeating units of the disaccharide [-D-glucuronic acid-β1–3-*N*-acetyl-D-glucosamine-β1–4-]. In contrast to other glucosaminoglicans found in nature, hyaluronan disaccharides are not sulfated. Hyaluronan is synthesized in mammals by at least three synthases with products of varying chain lengths. At the cellular level, it is degraded progressively by a series of enzymatic reactions that generate polymers of decreasing sizes. Despite their exceedingly simple primary structure, hyaluronan fragments have an extraordinarily wide-range and often opposing biological functions. There are large hyaluronan polymers that are space-filling, anti-angiogenic, immunosuppressive, and that impede differentiation, possibly by suppressing cell–cell interactions, or ligand access to cell surface receptors. High molecular weight (HMW) hyaluronan chains, which can reach 2×10^4 kDa in size, are involved in ovulation, embryogenesis, protection of epithelial layer integrity, wound repair, and regeneration. On the other hand, low molecular weight (LMW) fragments are pro-inflammatory, immuno-stimulatory and angiogenic. They can also compete with larger hyaluronan polymers for receptors. LMW polymers appear to function as endogenous ‘‘danger signals’’, while even smaller fragments can ameliorate these effects (Stern et al., 2006).

In stroke patients, the production of total hyaluronan and LMW 3–10 disaccharides of hyaluronan are increased in post-mortem tissue and in the serum of patients 1, 3, 7 and 17 days after ischemic stroke, as a consequence of up-regulation of HA synthases and hyaluronidases in inflammatory cells from both stroke and peri-infarcted regions (Al'Qteishat et al., 2006). Similar results have also been found in rats subjected to occlusion of the middle cerebral artery (Al Qteishat et al., 2006). However, there is still little and controversial information about which could be the actions of hyaluronan disaccharides during brain ischemia.

In nature, hyaluronan disaccharides are not sulfated, although in the case of some GAGs like chondroitin it has been observed that sulfation modulates its activity. In this study we have used synthetic hyaluronan disaccharides with different degrees of sulfation (methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-*O*-sulfo-α-D-glucopyranoside, sodium salt (di0S), methyl 2-acetamido-2-deoxy-3-*O*-(β-d-glucopyranosyluronic acid)-6-di-*O*-sulfo-α-d-glucopyranoside, disodium salt (di6S) and methyl 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4,6-di-*O*-sulfo-α-D-glucopyranoside, trisodium salt (di4,6S)) with the idea of exploring their capacity to improve cell survival in brain ischemia models. For this purpose we have tested their effects on rat hippocampal slices subjected to OGD and on an *in vivo* model of photothrombotic stroke. Our results indicate that all disaccharides were able to protect hippocampal slices from OGD although di4,6S was the most potent. In this model, the parent unsulfated HMW GAG, HA, was ineffective. Of interest is the observation that the neuroprotective action of di4,6S (Fig. 1) was also exhibited in an *in vivo* stroke model in mice when administered 1 h post-photothrombosis. The protective actions of di4,6S were related to its anti-inflammatory and antioxidative actions. Therefore, these small disaccharides with a smaller molecular weight, compared to hyaluran, could be of potential interest in brain ischemia conditions.

EXPERIMENTAL PROCEDURES

Materials

Di4,6S (Fig. 1), di6S and hyaluronic acid (M.W. 30.000 Da) (Fig. 1) were synthesized by Bioibérica SA,

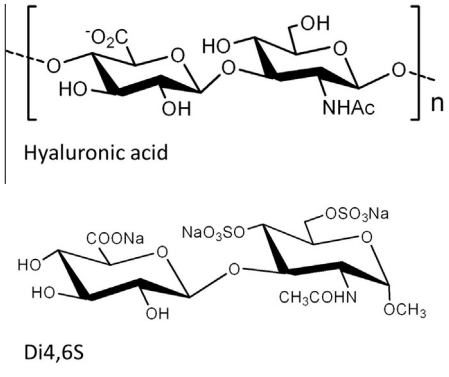

Fig. 1. Molecular structures of hyaluronan and its derivative disaccharide di4,6S.