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Biochemical and toxicological evaluation of nano-heparins in cell functional properties, proteasome activation and expression of key matrix molecules

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HIGHLIGHTS

- Nano-*Styela* compared to nano-mammalian analogue has higher inhibitory effect on cell proliferation, invasion and proteasome activity.
- Nano-*Styela* regulates cell apoptosis, expression of inflammatory molecules, and reduces the expression levels of extracellular matrix macromolecules.
- Nano-heparins and especially ascidian heparin are effective agents for heparin-induced effects in critical cancer cell functions.

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ABSTRACT

The glycosaminoglycan heparin and its derivatives act strongly on blood coagulation, controlling the activity of serine protease inhibitors in plasma. Nonetheless, there is accumulating evidence highlighting different anticancer activities of these molecules in numerous types of cancer. Nano-heparins may have great biological significance since they can inhibit cell proliferation and invasion as well as inhibiting proteasome activation. Moreover, they can cause alterations in the expression of major modulators of the tumor microenvironment, regulating cancer cell behavior. In the present study, we evaluated the effects of two nano-heparin formulations: one isolated from porcine intestine and the other from the sea squirt *Styela plicata*, on a breast cancer cell model. We determined whether these nano-heparins are able to affect cell proliferation, apoptosis and invasion, as well as proteasome activity and the expression of extracellular matrix molecules. Specifically, we observed that nano-*Styela* compared to nano-Mammalian analogue has higher inhibitory role on cell proliferation, invasion and proteasome activity. Moreover, nano-*Styela* regulates cell apoptosis, expression of inflammatory molecules, such as IL-6 and IL-8 and reduces the expression levels of extracellular matrix macromolecules, such as the proteolytic enzymes MT1-MMP, uPA and the cell surface proteoglycans syndecan-1 and -2, but not on syndecan-4. The observations reported in the present article indicate that nano-heparins and especially ascidian heparin are effective agents for heparin-induced effects in critical cancer cell functions, providing an important possibility in pharmacological targeting.

Abbreviations: ECM, extracellular matrix; GAG, glycosaminoglycan; PG, proteoglycan; HS, heparan sulfate; Hep, heparin; nM-Hep, nano-mammalian heparin; nS-Hep, nano-*Styela* heparin; CS, chondroitin sulfate; NPs, nanoparticles; FBS, fetal bovine serum; IL-6, interleukin-6; IL-8, interleukin-8; MMPs, metalloproteinases; uPA, urokinase plasminogen activator; Nrf2, nuclear erythroid factor 2; ROS, reactive oxygen species; HSPGs, heparan sulfate proteoglycancs; SDC, syndecan.

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1. Introduction

Glycosaminoglycans (GAGs) are linear, negatively charged, polysaccharides, comprised of disaccharide repeating units of hexosamines (*N*-acetyl-galactosamine or *N*-acetyl-glucosamine) and uronic acids (D-glucuronic acid or L-iduronic acid) sulfated at various positions. GAG chains, covalently linked to a specific core protein, constitute proteoglycans (PGs), representing one of the most investigated extracellular matrix (ECM) molecules that regulate various signaling pathways and as a result many normal and pathological processes (Iozzo and Karamanos, 2010; Iozzo and Schaefer, 2010). It is well established that GAGs are involved in interactions of PGs with cellular proteins, regulating cell behavior and signaling properties (Theocharis et al., 2010). They are essential key players in several pathological conditions and contribute in this direction in tissue development, remodeling, homeostasis and disease progression, refuting the old view that they were just the cellular glue, surrounding the cells (Afratis et al., 2012; Karamanos and Tzanakakis, 2012; Theocharis et al., 2015) and indicating that they can serve as potential pharmacological targets.

Modifications in GAGs structure seem to gain importance concerning their applications in the field of therapeutics. GAG-based anticancer therapy (Mizumoto and Sugahara, 2013) has been reported using heparan sulfate (HS), heparin (Hep) and their mimetics. In addition, clinical studies have proved significant antimetastatic properties of heparin and its derivatives, apart from their well-known anticoagulant activity (Borsig, 2010; Chalkiadaki et al., 2011a,b; Kozlowski and Pavao, 2011).

The cellular signaling properties of GAGs are strongly influenced by their structure; heparin has the highest negative charge density among all the GAGs. As a result, it interacts with various ECM molecules, such as PGs and proteins, modulating cell microenvironment. Heparin is the highly sulfated variant of HS, consisting of repeating disaccharide units of glucosamine (GlcN) and hexuronic acid residues [glucuronic acid (GlcA) and iduronic acid (IdoA)], where all hydroxyl groups are possible targets for sulfonylation (Karamanos et al., 1994). This GAG interacts with various growth factors and cytokines such as fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) and tumor necrosis factor-α (TNF-α), modulating cancer cell migration, adhesion and invasion, as well as angiogenesis and epithelial-to-mesenchymal transition (EMT), important processes for cancer initiation and progress (Afratis et al., 2012; Zittermann et al., 2010). Clinical studies have suggested heparin's anti-cancer activity; inhibiting haematogenous metastatic potential of cancer cells, by platelet deactivation and arrest of fibrin formation (Niers et al., 2007).

ECM macromolecules with their unique structural properties play key roles in supporting the dynamic extracellular matrix by generating complex structural networks with other macromolecules and regulating cellular phenotypes. The dynamic interplay between ECM macromolecules, such as PGs, and proteolytic enzymes is a crucial biological step that contributes to the pathophysiology of cancer and inflammation (Theocharis et al., 2014). A variety of synthetic molecules that mimic PGs structures, serve as functional and therapeutic replacements for natural PGs. Therefore, the control of GAG chains structure and as a consequence the design and construction of neo-GAGs is a promising tool providing a wide range of modulations in several signaling processes (Linhardt and Toida, 2004; Weyers and Linhardt, 2013). Undoubtedly, due to the unique properties of nanoparticles (NPs) involving their enormous mass to surface ratio, solubility, aggregation and encapsulation tendency (Nel et al., 2006), they are very attractive in drug delivery systems and in targeted therapy. Nano-GAG composites are nanoscale structures having attached GAG chains, where a nanomaterial serves as a substitute for core protein, with applications in tissue engineering and biomedical applications. Heparin-based hydrogels and nanoparticles have significant potential in a variety of biomedical applications (*e.g.*, biocompatibility and therapeutic efficacy) (Liang and Kiick, 2014). Heparin is of interest for use to cancer treatment as a drug delivery system, because it inhibits the angiogenesis and metastasis (Kemp and Linhardt, 2010). Moreover, several studies indicate that heparin has been referred as delivery of imaging agents by heparin nanoparticles. Specifically, heparin used as cover for imaging agents such as gold nanoparticles and quantum dots (QDs) in noninvasive biomedical imaging and also it has been reported the oral administration of semiconductor QDs loaded heparin nanoparticles (Nurunnabi et al., 2012). Recent studies have demonstrated that nano-encapsulated GAGs, such as mammalian heparin analogues and heparin isolated from the ascidian *Styela plicata* appear to have potent anti-inflammatory effects (Kozlowski et al., 2011). *In vivo* tests revealed significant anti-thrombotic effects, reduced intestinal inflammation in colitic animals, as well as low rates of epithelial apoptosis, reduced amount of collagen deposition and local production of inflammatory cytokines (Belmiro et al., 2009; Cardilo-Reis et al., 2006; Santos et al., 2007). Therefore, the potential therapeutic utilization of such analogues has been strongly supported (Belmiro et al., 2009; Cardilo-Reis et al., 2006; Santos et al., 2007).

Such nano-analogues are mammalian unfractionated heparin and *S. plicata*-isolated heparin. The ascidian heparin has a more modified structure, as it is composed mainly of the disaccharide $[\alpha\text{-L-IdoA}(2OSO_3)\text{-}1 \rightarrow 4\beta\text{-DGlcN}(OSO_3)(6OSO_3)\text{-}1]_n$, similarly to mammalian heparin, exhibiting differences in the degree of sulfation. About 25% of the disaccharide $[\alpha\text{-L-IdoA-}1 \rightarrow 4\beta\text{-D-GlcN}(OSO_3)(6OSO_3)\text{-}1]_n$ is also present. It is able to inhibit thrombin to the same extent as mammalian heparin. However, it has only 10% of the mammalian heparin anticoagulant activity, resulting in significantly reduced hemorrhagic effects *in vivo* (Cavalcante et al., 2000). This fact suggests a safer therapeutic action of *S. plicata* heparin in the treatment of thrombosis (Santos et al., 2007).

It is well established that cellular behavior is highly affected by specific regulatory mechanisms, such as ubiquitin–proteasome system (UPS). The 26S proteasome, which is located both in the cytoplasm and the nucleus, is responsible for the non-lysosomal degradation of a large number of key cellular proteins, playing a critical role in the maintenance of normal function in eukaryotic cells (Glickman and Adir, 2004; Reinstein and Ciechanover, 2006). The 26S proteasome is comprised of the 19S regulatory complex and the 20S catalytic complex, with three distinct subunits, the β5, β1 and β2, that are the catalytic centers of chymotrypsin-like, caspase-like/PGPH (peptidylgluatamyl-peptide hydrolyzing) and trypsin-like activities, respectively (Tanaka, 1998). The proteasome is a fruitful area for the targeted therapy, as it controls the removal of normal, misfolded and damaged proteins (Skandalis et al., 2012). As a result, it serves as cellular antioxidant. The oxidative stress through reactive oxygen species (ROS) is known to modulate cancer cells' invasion and metastasis (Nikitovic et al., 2013), altering the activity of significant proteolytic enzymes, such as metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), in order to facilitate metastasis and invasion (Gialeli et al., 2011). Furthermore, proteasome plays a critical role in cancer progression, influencing tumor microenvironment as well as the concentration and turnover of ECM macromolecules, including PGs.

In respect with the above, emerging data concerning the actions of nano-encapsulated heparins and the importance of ECM macromolecules in cancer progression, we evaluated the direct