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Synthesis, Radiolabeling with Fluorine-18 and Preliminary in vivo Evaluation of a Heparan Sulphate Mimetic as Potent Angiogenesis and Heparanase Inhibitor for Cancer Applications

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Abstract
Heparan Sulfate (HS) mimetics are able to block crucial interactions of the components of the extracellular matrix in angiogenic processes and as such, represent a valuable class of original candidates for cancer therapy. Here we first report the synthesis and in vitro angiogenic inhibition properties of a conjugated, novel and rationally-designed octasaccharide-based HS mimetic. We also herein report its labeling with fluorine-18 and present the preliminary in vivo Positron Emission Tomography imaging data in rats. This constitutes one of the rare examples of labeling and in vivo evaluation of a synthetic, polysaccharide-based, macromolecule.
Communication

Heparan sulfates (HS) are glycosaminoglycans that are ubiquitously expressed as proteoglycans on cell surfaces and throughout the extracellular matrix (ECM) in most multicellular animals. HS proteoglycans play an important role in the regulation of several aspects of cancer biology, tumor progression, metastasis but also angiogenesis, *i.e.* the growth of new blood vessels from those already surrounding a tumor (1, 2).

While it is now widely recognized that tumors are critically dependent on angiogenesis (3, 4), its inhibition constitute one of the major therapeutic strategy for cancer treatment (5). Several growth factors, their receptors, extracellular matrix molecules and enzymes bind to specific sites on the HS sugar chain to facilitate cellular and biochemical responses associated with angiogenesis. HS mimetics, by blocking these interactions and inhibiting crucial processes to tumor progression, could therefore be considered as a new class of cancer therapeutics (1, 6-8).

Positron Emission Tomography (PET) is a nuclear-based imaging technique that permits assessment and quantification of specific and fundamental biochemical and physiological processes at the molecular level *in vivo*, both in animals and humans. As such, PET represents a tremendous tool in drug development due to the possibility to access *in vivo* and non-invasively to the ADMET parameters of new drug candidates, once labeled with a positron-emitter (9). From a chemical point of view, PET imaging relies indeed on the preparation and administration of a positron-emitting radiolabeled probe also called a radiotracer. For this purpose, fluorine-18 (T\(_{1/2}\): 109.8 min) is one of the preferred radioisotope due to its adequate physical and nuclear characteristics (*i.e.* its short half-life and low positron energy) but also to the successful use in clinical oncology of 2-[\(^{18}\)F]fluoro-2-deoxy-D-glucose ([\(^{18}\)F]FDG), the currently most widely used PET-radiopharmaceutical and manifestly a motor behind the growing availability and interest for this positron emitter in radiopharmaceutical chemistry.

Fluorine-18 is also the radionuclide of choice when the labeling of high molecular weight compounds are concerned, such as HS mimetics, and this moreover when prosthetic labeling - one of the most efficient and reliable methodology to give access to fluorine-18-radiolabeled macromolecules - is envisaged. Prosthetic labeling consists in the preparation of a low molecular weight reagent bearing fluorine-18 followed by its conjugation with the desired macromolecule. This foreign-labeling strategy has the advantage of offering a flexibility in the choice of chemical routes, including those requiring drastic chemical conditions for the preparation of the labeled prosthetic reagent, while the conjugation of the latter with a macromolecule can then be done using milder
conditions needed to preserve the latter's integrity (10). Numerous prosthetic reagents have been designed among which $[^{18}\text{F}]$FPyME, a fluoropyridine-based maleimide reagent, that has demonstrated its efficiency for a selective conjugation with sulfhydryl functions borne by macromolecules (11, 12). Interestingly, during the timeframe of the present work, Ning et al. published the first radiolabeling of a maltohexaose derivative with fluorine-18 (13), based on a comparable two-step strategy, showing once more the efficacy of such prosthetic approaches.

With the aim of supporting the development of a novel class of HS mimetics as druggable entity in cancer therapy, we herein report the preparation of a fluoropyridinilated derivative of a rationally designed, fully synthetic, octasaccharide-based, lead candidate (Figure 1) and its in vitro preliminary characterization in a proliferation inhibition assay. We also describe its high yielding radiolabeling with fluorine-18 via a prosthetic approach using $[^{18}\text{F}]$FPyME and the in vivo preliminary results from PET-imaging studies in rats.

![Figure 1](image)

**Figure 1.** Chemical structure of the conjugated and labeled octasaccharide-based HS mimetic $[^{18/19}\text{F}]-14$.

The octasaccharide 8 (Scheme 1) is the key-intermediate for the preparation of 14. Compound 8 has been prepared by functionalization of 7, which has been assembled from 3 different disaccharide units (4, 5 and 6) (14). Over the past few years, several approaches for the preparation of oligosaccharides have been documented in the literature. These strategies mainly differ in the protecting group patterns of the building blocks, timing of the oxidation step to access the uronic acid and the glycosylation procedure. One of the major efforts in the synthesis of 8 is the preparation of the three different disaccharides (4, 5 and 6) that constitute respectively the reducing end of the chain, the repeating unit and the capping unit. The syntheses of these disaccharides, in sufficient quantities and with the appropriate protective groups (two mandatory requirements for the preparation of complex octasaccharide, e.g. 8), were performed in a similar manner as described previously by Petitou and co-workers (15) starting from three different monosaccharides (1, 2 and 3, Scheme 1) (14, 16-18).
Note that temporary protecting groups are required at the C4 position group of the building block 5 to allow for an easy deprotection in anticipation of chain elongation. Here, levulinoyl ester has been used (19, 20). Additionally, the hydroxyl groups that will be O-sulfated at a late stage and those that should remain free have to be protected differently. Commonly, acetate esters serve to mask hydroxyl groups to be orthogonally sulfated, while benzyl ethers mask hydroxyl groups that will not be modified. The amine group of D-glucosamine also requires the placement of different protecting groups. Azides have been used for these protections (21-23).

Scheme 1. Retrosynthetic scheme used in the preparation of the octasaccharide 8.

Compound 8 is expected to interfere with two major processes in tumor development: i) angiogenesis partly mediated via growth factors such as VEGF, FGF-2 and PDGF-β, or endothelial progenitor cells recruitment mediated via SDF-1α, and ii) metastatic potential mediated via heparanase activity. The heparanase is indeed able to cleave HS in the ECM thereby releasing the above mentioned growth factors involved in angiogenesis. The binding affinities of 8 have been determined using the BIAcore technology for the proteins VEGF-A, FGF-2, PDGF-β and SDF1-α, and the inhibition potential in a heparanase activity assay (14, 24, 25). The determined IC50 values (Table 1) showed a strong affinity for the growth factors (all in the nmolar range) as well as an efficient
inhibition of the heparanase activity. These in vitro characterizations demonstrate the potential of the octasaccharide 8 as inhibitor of angiogenesis processes.

Table 1. Growth factor binding and heparanase inhibition assays for compound 8.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>VEGF-A (nM)</th>
<th>FGF-2 (nM)</th>
<th>PDGF-β (nM)</th>
<th>SDF-1α (nM)</th>
<th>Heparanase (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>4.5±0.3</td>
<td>3.0±0.6</td>
<td>10.6±0.3</td>
<td>43±20</td>
<td>41±15</td>
</tr>
</tbody>
</table>

Derivatization of compound 8 and conjugation with 13 were carried out as illustrated in scheme 2. Briefly, copper-catalyzed Huisgen 1,3 dipolar cycloaddition of 8 (bearing an alkyne function at the anomeric-position of the reducing end of the octasaccharide) with 9 (bearing the azide counterpart) was thus performed leading to the formation of 10 in 92% yield. Deacetylation of the sulfhydryl moiety and subsequent reaction with the thiol-sensitive prosthetic reagent FPyME (13) afforded the target conjugated octasaccharide 14 in 63% yield. The thiol-containing oligosaccharide 12 was indeed produced and stored as its S-acylated form to avoid its dimerization as 11 by a disulfide bridge formation.

![Scheme 2](image)

Reagents and conditions : i) CuSO₄, ascorbic acid, t-BuOH/H₂O, rt, 1 h; ii) LiOH, H₂O, rt, 12 h; iii) TCEP, H₂O, rt, 10 min; iv) hydroxylamine (50 mM) in PBS (100 mM, pH 7.4), rt, 45 min; v) FPyME or [¹⁹/¹⁸F]FPyME in DMSO, PBS (100 mM, pH 7.4), rt, 15 min.

Scheme 2. Derivatization of compound 8 and subsequent conjugation with the prosthetic reagent [¹⁹/¹⁸F]FPyME, leading to the target octasaccharide [¹⁹/¹⁸F]-14.

The cycloaddition reaction between compounds 8 and 9 was conducted using standard conditions. A combination of tert-butanol and water, as reaction medium, was found optimal for the formation of
the triazole 10. Then, conjugation with FPyME was first carried out in non-radioactive conditions to obtain and characterize 14, which will also serve as authentic reference for the labeling with fluorine-18. For this, deprotection of the sulfhydryl function was performed according to two procedures. Firstly, treatment of compound 10 with lithium hydroxide in water over 12 h gave exclusively the dimer 11, which has been identified by HPLC (HPLC B, conditions B2 (see supporting information section): $R_t(10) = 13.4$ min, $R_t(11) = 14.1$ min) and characterized by ESI/MS (Table 2). This dimer was then dissociated using a TCEP-buffered aqueous solution to restore 12 and allow for the in situ conjugation with FPyME (13). Alternatively, compound 10 was treated with a buffered aqueous solution of hydroxylamine for 45 min to afford 12 to which compound 13, in solution in dimethyl sulfoxide (DMSO), was directly added. The deprotection of 10 was followed by HPLC analyses (HPLC B, conditions B2: $R_t(10) = 13.4$ min, $R_t(12) = 11.8$ min). Compound 14 has been purified by semi-preparative HPLC and characterized by MS (Table 2 and supporting information section).

Table 2. Mass spectrometry characterization of compounds 10, 11, 12 and 14.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated</td>
<td>3361.4121</td>
<td>6636.7874</td>
<td>3319.4015</td>
<td>3569.4769</td>
</tr>
<tr>
<td></td>
<td>C$<em>{121}$H$</em>{147}$N$<em>7$O$</em>{78}$S$_{13}$</td>
<td>C$<em>{238}$H$</em>{288}$N$<em>{14}$O$</em>{154}$S$_{26}$</td>
<td>C$<em>{119}$H$</em>{145}$N$<em>7$O$</em>{77}$S$_{13}$</td>
<td>C$<em>{131}$H$</em>{156}$FN$<em>9$O$</em>{80}$S$_{13}$</td>
</tr>
</tbody>
</table>

The biological activity of 14 has been evaluated in a FGF-2 induced NHDF proliferation assay and compared to the one of the parent octasaccharide 8. The results presented in figure 2 clearly demonstrated that 14 inhibits FGF-2-induced fibroblast proliferation at a concentration of 30 µM and this in a similar manner that 8. This also demonstrated the relevance of the prosthetic approach selected for the fluorine-18-labeling of these macromolecules.
Figure 2. Inhibition of FGF-2-induced NHDF cell proliferation by 8 and 14.

Fluorine-18-labeling of 14 was performed using $^{18}$F]-13, the radiofluorinated version of FPyME (Scheme 2). $^{18}$F]-FPyMe was prepared in high yields (28 to 37 % decay-corrected yield based on starting fluoride) according to already published procedures (11) (details are given in the supporting information section). In parallel to the preparation of $^{18}$F]-13, the $\beta$-acetyl protected octasaccharide 10 was treated with hydroxylamine for 45 min to obtain quantitatively compound 12 (as reported above). Conjugation of $^{18}$F]-13 with 12 was performed in mild conditions (DMSO/phosphate buffer saline) for a short reaction time of 15 min at room temperature. The yield of conjugation was evaluated to be $> 50\%$ according to radioTLC before purification. Rapid and efficient purification
using a commercially available pre-packed Sephadex NAP10™ cartridge allowed for a total separation of the conjugated fluorine-18-labeled octasaccharide \( ^{18}\text{F}}\)-14 and the non-reacted reagent \( ^{18}\text{F}}\)-13. Chemical purity and identity of \( ^{18}\text{F}}\)-14 were assessed by radioTLC and analytical radioHPLC. Co-injection of an aliquot of formulated \( ^{18}\text{F}}\)-14 with an authentic sample of 14 showed identical retention time as well as the absence of \( ^{18}\text{F}}\)-13 on the chromatogram (HPLC B, conditions B1 : Rt(10) = 16.5 min, Rt(12) = 15.8 min, Rt([\text{18}^F]-13) = 17.0 min and Rt([\text{18}^F]-14) = 18.4 min). The conjugation and purification steps lasted 20 to 25 min and allowed for the production of \( ^{18}\text{F}}\)-14 in 55 to 60% isolated and decay-corrected yields, based on \( ^{18}\text{F}}\)FPyME. Typically, starting from a 4.8 to 6.7 GBq \( ^{18}\text{F}}\)FPyME production batch, 2.4 to 4.0 GBq of \( ^{18}\text{F}}\)-14 was produced with an average decay-corrected specific radioactivity of 20 GBq/µmol. The entire process of preparation of \( ^{18}\text{F}}\)-14 (production of \( ^{18}\text{F}}\)-13 + conjugation with 12) lasted 130 to 135 min, a synthesis time that is compatible with the half-life of fluorine-18. The overall isolated and decay-corrected radiochemical yield of production of \( ^{18}\text{F}}\)-14 ranged from 15 to 24%, based on starting \( ^{18}\text{F}}\)fluoride.

Note that once again, combination of the high yielding radiosynthesis of the reagent and the efficient conjugation of it with sulphydryl containing macromolecules, would make it possible to prepare in parallel several oligosaccharide-based macromolecules starting from a unique batch of \( ^{18}\text{F}}\)FPyME (12).

Male Wister rats were injected with 15±2 MBq of \( ^{18}\text{F}}\)-14 and imaged using a high resolution HRRT (Siemens) PET scanner. Projection images (Figure 3) showed a rapid distribution of the radioactivity in vascular systems (vascular tree and heart), followed by an accumulation in kidneys and liver in a lesser extent while vascular radioactivity concentration remained elevated. Times Activity Curves (TACs, see supporting information section Figure S2 A) showed similar patterns for abdominal arteries, heart and lung indicating that the detected radioactivity is of vascular origin. Blood clearance was slow (T\(_{1/2}\) > 90 min). Urinary excretion is expected due to the presence of radioactivity in the bladder at late time points. Pharmacodistribution showed that the uptake of radioactivity in other organs (intestine, muscle, brain) remained low (see supporting information section Figure S2 B). \( ^{18}\text{F}}\)-14 displayed a long-lasting residence-time in the vascular system and a progressive accumulation in elimination organs (liver, kidneys, bladder) (see supporting information section Figure S3). These observations are consistent the biodistribution of M402, another rationally engineered HS mimetic, tagged with a fluorescent dye (26). These preliminary in vivo properties are favorable for the development of an HS mimetic drug candidate for cancer applications.
Figure 3. PET images (projection) following $^{18}$F-14 i.v. injection in rats at 30 sec, 15 min and 90 min.

In conclusion, the octasaccharide 14, as a conjugated analog of the HS mimetic inhibitor 8, has been synthesized and characterized in vitro. Fluorine-18-labeling has been achieved permitting in vivo preliminary PET-imaging evaluation in healthy rodents. Evaluation of the radiotracer in a rat tumor model will be the next step.

References
