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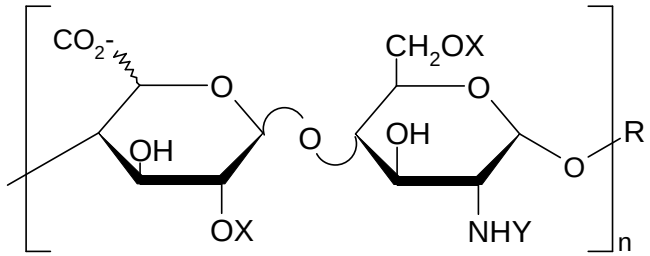
NRE

RE

U_{2X} A_{NY6X}

Major disaccharide repart unit of Heparin

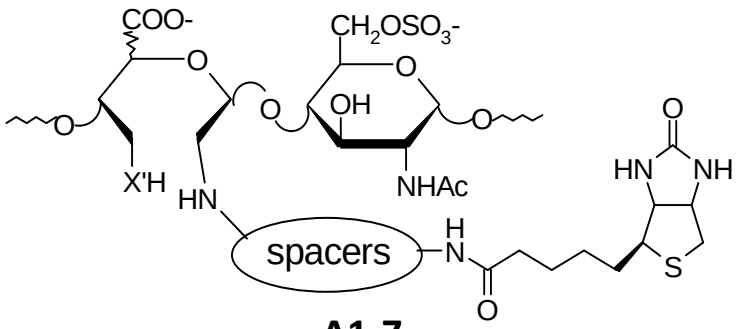


X=SO_3^- or H
Y= SO_3^- or CH_3CO^-
R= H or Polysaccharide chain

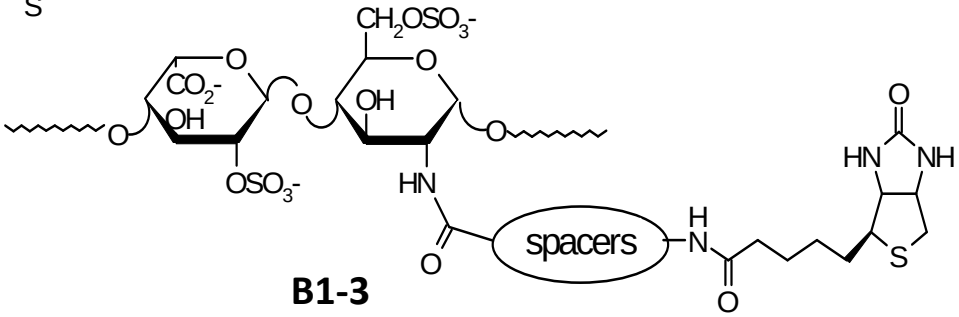
[A]

[B]

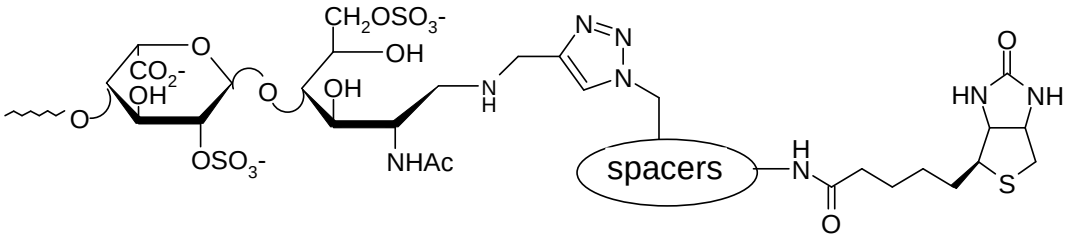
[C]



A1-7



B1-3



C1-3

Novel N-acetyl-Glycol-split heparin biotin-conjugates endowed with anti-heparanase activity

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KEYWORDS Heparanase inhibitors, Anticancer agents, Antimetastatic agents, Roneparstat, Biotinylated agents.

ABSTRACT Heparanase is regarded as a promising target for anticancer drugs and Ronepastat is one of the most promising heparanase inhibitors insert in clinical study for Multiple Myeloma Therapy. To improve its pharmacokinetic / pharmacodynamic profile, as well to have an

antidote able to neutralize its activity in case of over dosages or intolerance, a new class of its derivatives was obtained inserting non-carbohydrate moieties of different length between the polysaccharide chain and biotin or its derivatives. In vitro these novel derivatives maintain the anti-heparanase activity without induced toxicity. The newly synthesized compounds retained the ability to attenuate the growth of CAG myeloma tumors in mice with potency similar, or in one case even higher than that of the reference compound Roneparstat as well as inhibited metastatic dissemination (lung colonization) of murine B16-F10 melanoma cells in vivo.

INTRODUCTION

Roneparstat, N-acetylated glycol split (gs) heparin (also named SST0001)[1] is one of the most promising heparanase-inhibiting drugs,[2] possessing antitumor activity.[3,4] Heparanase appears to be involved in several human pathologies other than tumors such as chronic inflammation, diabetic nephropathy, bone osteolysis, thrombosis, atherosclerosis, as well as rare diseases.[5]

Roneparstat has been promisingly tested in a phase I clinical study [6,7] nevertheless it is considered useful to improve its pharmacokinetic / pharmacodynamic profile to reduce the number of daily subcutaneous administrations as well as to have an antidote able to neutralize its activity in case of over dosages or intolerance[8,9]. Therefore, new Roneparstat-like derivatives were prepared inserting non-carbohydrate moieties of different length between the polysaccharide chain and biotin or its derivatives. Exploiting different functional groups of the polysaccharide, our aim was to preserve the heparanase inhibitory activity, improve bioavailability and get ready biotin derivatives able to be neutralized by avidin endowed with a strong affinity for biotin[10]. In addition, studies have disclosed that folate receptors and more

specifically biotin receptor (BR) are overexpressed in a large number of cancer cells lines[11] and solid tumors, and hence may contribute to tumor-directed drug delivery[12]. Besides, biotin provides functional groups easily detectable by chemical-physical methods or interaction tests with avidin, thus allowing an easier monitoring of plasma concentrations of the drug.

Roneparstat has been prepared from heparin by periodate oxidation followed by borohydride reduction of fully N-desulfated and N-acetylated heparin[13] as represented in Figure. 1.

Figure 1

In this study biotinylation, through a linker of different functional groups of heparin derivatives, has been investigated by three distinct approaches (A, B, C) exploiting appropriate sequences of reactions, as schematized in Figure 2.

Figure 2

RESULTS AND DISCUSSION

In the first approach (route [A]) the intermediate (**I-3),** common to Roneparstat preparation obtained by periodate oxidation, was used to introduce a biotin-spacer through a reductive amination of the aldehyde groups, derived from the oxidized diol of uronic acid, with the amine group present on a biotin conjugated linker (abbreviated as biotin-spacer-NH_2). In the second approach (route [B]) starting from N-desulfated heparin, the first intermediate of Roneparstat synthesis (**I-1**), unsubstituted glucosamine residues, were N-acylated with biotinylated esters activated with N-hydroxysuccinimide (abbreviated as Biotin-spacer- NHS). The third method

(route [C]) consists of exploiting the aldehyde group of the reducing end (RE) residues of heparin chain to conjugate a Biotin-linker (abbreviated as Biotin-spacer-N_3) through "click "chemistry[14]. In particular, incorporation of biotin derivatives at the reducing terminus was achieved by a two-step procedure: (i) first, N-desulfated, N-acetylated heparin (**I-2**) was reductively aminated with an alkyne-amine such as propargylamine, and then subjected to glycol-splitting reaction to give end functionalized gs N-acetyl heparin (**I-6**). (ii) Second, reductively aminated heparin was linked with biotinylated spacers containing an azide group, using copper (I)-catalyzed azide-alkyne 1,3 dipolar-cycloaddition (CuAAC), so called "clik reaction".[15] To build-up a library of compounds, which differ not only in the position of the substituents, but also in their length and hydrophilicity, seven compounds have been used (Figure 3) classified into three types according to the functional group employed for the coupling with the polysaccharide. One of the items (**1**) is commercially available the others (**2**-**7**) were synthesized as described in the experimental section.

Figure 3

Biotinylated spacers **1, 4** and **6** contain only a simple 1,5 diamino-pentyl chain, as spacer arm between biotin moiety and heparin chain thus, moving away the biotin residue to reduce the steric hindrance that could interfere with biotin-binding assays. Differently, spacer **2** and **7** contain, in addition to the diamino pentyl moiety, a hydrophilic short chain of polyethylene glycol (PEG), incorporated in the final compound [Biotin-PEG-X]. The polypeptide spacer **3**, showing a length double than the biotin-Peg chain of **2** is constituted by the amino acidic sequence Gly-Gly-Gly-Ser-Gly-Gly-Gly-Ser-Gly-Gly-Gly. Such sequence is well known and

widely used in protein fusions, conferring a high flexibility and, due to the absence of proteases active on this sequence, is also quite stable[16].

Biotinylated-linker **2** [Biotin-PEG_4-NH_2] was synthesized, in presence of N,N'-dicyclohexylcarbodiimide (DCC), from the commercial products 5-(biotinamide)pentinylamine (**1)**, and 15-(tert-Butyloxycarbonyl)amino-4,7,10,13-tetra-oxa-pentanoic acid (Boc-NH-PEG_4-CO_2H), after activation with N-hydroxysuccinimide (NHS) followed by deprotection of t-butyloxycarbonyl group with trifluoroacetic acid. (Fig. S1, *Supporting Information*).

Biotinylated-linker **3** [Biotin-AA-NH_2] was prepared starting the commercial product **1**, which was coupled with protected linear precursor peptide **9** (Fig. S2, *Supporting Information*) using the conventional uronum-based reagent O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tretramethyluronium (HCTU)[17]]; the latter obtained, after cleavage from the resin, in solid phase synthesis MW-SPPS heating assisted.

Biotinylated-linker **4** [Biotin-N_3] was synthetized for direct diazo-transfer of the terminal primary amine present on biotinylated linker **1** to give the correspondent azide[18]. In the same way we prepared the glycine-azide (**17**) obtained from the corresponding commercial glycine (**16)**, subsequently linked with **1** through standard coupling conditions to obtain Biotinylated-linker **5** [Biotine-glycine-N_3]. (Fig. S3, *Supporting Information*)

Biotinylated-linker **7** [Biotin-PEG_4-NHS] was synthesized starting from commercial 6-biotinylamino-hexanoic acid **12** (Biotin-Ahx-OH) and 15-Amino-4,7,10,13-tetraoxa-pentadecanoic acid t-butyl ester **13** (tBuO-Peg_4-NH_2) through standard coupling conditions reactions (HCTU) and subsequently, after the deprotection of t-butyloxycarbonyl group with trifluoroacetic acid, activated with N-hydroxysuccinimide. (Fig. S4, *Supporting Information*).

Synthesis of Roneparstat-like derivatives

Preparation of novel biotinylated N-acetyl-glycol split heparins assembled with distinct synthetic strategy, involving the reacting groups of three different residues of the heparin chain is schematized in Fig. 2, and their chemical-physical properties and heparanase inhibition activity summarized in Table 1.

Conjugation on gs-uronic acid unit - Derivatives substituted at the uronic acid unit (D-glucuronic, GlcA, or L-iduronic, IdoA) were synthesized through derivatization of glycol split moiety. As an example, in Fig. 4 the scheme of reaction for compound **A3** is reported. N-acetylated heparin (**I2**) was oxidized with periodate obtaining the cleavage of C3-C2 bond of the pyranose ring of non-sulfated uronic acids. The resulting oxy-N acetylated heparin (**I-3**), instead of being stabilized immediately by borohydride reduction, leading to Ronepastat, was subjected to reductive amination reactions with different stoichiometric ratios of glycol-split residues and biotin-spacers (**1**, **2** or **3**) to obtain derivatives with different substitution degrees (SD), followed by $NaBH_4$ reduction of the unreacted aldehyde groups.

Table 1

Figure 4

Given the stoichiometric ratio between the reagents, the degree of substitution (DS), express as percentage of biotinylated disaccharide versus their total number, can be modulated (Tab. 1).

Considering that the glycol splitting can occur only at the level of non-sulfated *GlcA* and *IdoA* residues, which represent about 25 % of total uronic acid in a heparin chain, the biotinylation maximum DS obtainable is about 50% when all the gs generated aldehydes were substituted. As shown in Table 1 N-acetyl gs-heparin biotinylated derivatives show a wide range of DS values ranging from 3.0 - 41 %.

Conjugation on N-desulfated glucosamine units - Conjugation of biotinylated linkers (**4,5**) to N-desulfated glucosamine unit was achieved through their N-hydroxysuccinimide (NHS) esters. The synthetic process shown in Fig. 5 comprises five steps. N-desulfated heparin (**I-1**) was oxidized with periodate under controlled conditions to avoid concomitant glycol-splitting at level of glucosamine C2-C3 linkage bearing vicinal amino and hydroxyl group and significant depolymerization.[19,20] For this purpose pH was maintained at about 3.5 and the reaction time was reduced to 6 h. By this way, almost all the amino functions present on the heparin chains were preserved and available, after reduction of aldehyde groups, to be conjugate with the biotinylated linkers-succinimide derivative (**6** or **7**). Finally, the unreacted amine groups have been exhaustively N-acetylated in the presence of acetic anhydride to obtain the N-acetylated-N-biotinylated-glycol split heparins (B1-3). As in the case of uronic acids derivatization, the DS can be modulated as a function of the stoichiometric ratio between the reagents.

Figure 5

A first attempt to incorporate biotin-spacer **1** at the glucosamine units was carried out by acylation of the N-desulfated heparin intermediate (**I-1**), before the periodate oxidation and reduction with borohydride. Even though conjugates were obtained with good yields, this path was abandoned due to a partially periodate oxidation of the tetrahydrothiophene moiety (sulfinyl group SO) of biotin (data not shown)

Conjugation on chain reducing end. To conjugate the biotinylated spacer (**4, 5**) on the heparin terminal reducing-end, the N-acetylated heparin (**I-2**) was reductively aminated with propargylamine in order to introduce an alkyne function (**I-5**) (Fig. 6). The modification at the reducing end of **I-4** was confirmed by its ^{1}H-NMR spectrum that clearly revealed the presence of the characteristic acetylenic proton at 2.95 ppm. The alkyne intermediate was oxidized with periodate and reduced with borohydride in order to obtain intermediate compound **I-6**.

Figure 6

Click reaction between the intermediate N-acetylated-glycol split heparin alkyne aminated at RE (**I-6**) and biotinylated spacers functionalized with a terminal azide group (biotin-spacer-N_3,**4** or **5**) was carried out, at rt in a mixture of H_2O/tBuOH (2:1, v/v), in the presence of $CuSO_4$ and sodium ascorbate. The excess of spacer used to ensure the complete reaction with the alkyne amine function was easily removed by extensive dialysis (membrane with cut-off of 3500 Da).

In conclusion approaches [A] and [B] led to a random biotinylation along the heparin chains while the “clik reaction” [C] allowed derivatization at the RE units.

Characterization by NMR spectrometry

Purity and substitution degrees of all products, including intermediates, were mainly evaluated by one- and two-dimension NMR spectroscopy. The N-acetylation degree was determined by integration of ^{13}C-NMR signal at 60 and 56 ppm corresponding to the C-2 of *GlcNSO$_3$* and *GlcNAc*, respectively. (Fig. S5, *Supporting Information*). The percent of glycol-split residues (gs) present in all biotinylated compounds was calculated by using the HSQC-NMR spectroscopy approach previously developed for the structural characterization of unmodified heparins[21] *namely* from the H1/C1 signals in the anomeric region, typical for the glycol-split residues, at 4.94/106.9 ppm (gs*IdoA*, I) and 4.71/106.5 ppm (gs*GlcA*, G) versus the area of the anomeric signal of the 2-O sulfated *IdoA* at 5.2/102 ppm (I_{2s} B) as follows: gs*IdoA*= I/(I+G+ I_{2s}) x 100 and respectively for the glucuronic unit gs*GlcA*= G/(I+G+ I_{2s}) x 100.

Degree of biotinylation per disaccharide unit (DS) was determined by ^{1}H-NMR spectroscopy comparing the integrated peak area of the characteristic geminal diastereotopic proton 2.75 ppm (H-3´; α) of the tetrahydrothiophene ring present in all biotin-linkers versus that of methyl protons of the N-acetyl group of the polysaccharide, at 2.01 ppm (β), as follows: DS= α/(β/3) x 100. (Fig. S6 *Supporting Information*). For compounds bearing all glucosamine residues N-acetylated (pathway [A] and [C]) the N-acetyl signal represent all the disaccharide units. In the case of different N-derivatives of glucosamine (Compound B1-3), the formula has been changed as follows: DS= α/ (α + β/3) x 100 where the sum of the integrals of the protons (α + β/3) represents the sum of the disaccharide units as reported in Table 1.

The click reaction was confirmed by ^{1}H-NMR for the appearance of the aromatic proton singlet at δ 8.2-8.05 ppm typical of triazole ring.

For three derivatives (A7, B3 and C3) the molecular ratio of biotin incorporation was also determined applying the classical HABA (4'-hydroxyazobenzene-2-carboxylic acid)-Avidin assay and the results confirmed the degree of biotinylation measured by spectroscopy analysis (data not shown).

Heparanase inhibitory activity. To assess the inhibition of the heparanase catalytic activity, compounds were tested by an *in vitro* assay originally developed by Hammond and coworkers[22] based on heparanase-mediated cleavage of the synthetic heparin fragment, the pentasaccharide Fondaparinux (AGA*IA). The results for newly synthesized compounds, expressed as IC_{50} values generated from the dose-response curves, are reported in Table 1 with that of the reference compound Roneparstat.

According to their IC_{50} values, all the biotinylated derivatives were shown to inhibit the heparanase enzymatic activity with a potency (low nanomolar concentrations) similar to that of Roneparstat. Moreover, although a slight decrease of activity was observed for some derivatives (i.e. A3, B2, B3, C1 and C3) the results indicated that, independently of the chemical approach, these structural modifications did not significantly affect the inhibitory potency respect to the reference compound.

Evaluation of Biological Activities

Three new heparanase inhibitors, the biotinylated derivative A7, B3 and C3, representative of each type of derivatization and showing a similar degree of substitution, were further characterized *in vitro* by biological assays, with respect to the reference compound (Roneparstat), in order to evaluate their activity against selected human cancer cell lines.

Proliferation Assay. The selected compounds were tested *in vitro* by a cell proliferation assay to assess their effect on the growth of three human tumor cell lines expressing different levels of heparanase: HT1080 (human fibrosarcoma), U87MG (human glioblastoma astrocytoma) and U2OS (human osteosarcoma)[23]

[24,25]. Cells were treated for 72h with increasing concentrations (up to 100 µM) of each heparanase inhibitor, including Roneparstat. Results, expressed as percentage of proliferation with respect to vehicle-treated (control) cells, are summarized in Table 2.

Table 2

Overall, Ronepastat proved to be moderately active in inhibiting proliferation of two cell lines (U87MG and U2OS) out of the three and only at the highest concentration (100 µM) tested, and to be marginally or no active at lower concentrations. It is likely that this effect could be unrelated to heparanase inhibition due to the very high amount of compound needed to affect cell proliferation. Regarding the derivatives, only a very marginal antiproliferative activity was observed mainly at the highest concentration, indicating that the introduced substituents did not increased toxicity of compounds.

Invasion Assay. According to the pivotal role of heparanase in promoting cancer cell invasion and metastasis, we sought to assess the ability of derivatives to affect the invasive potential of HT1080, U87MG and U2OS human cell lines. To this aim, we used the Matrigel cell invasion assay. All the compounds were assessed at not toxic concentrations, below the relative IC_{10} of the anti-proliferative values measured for each cell line. Data are expressed in Table 3 as the percent invasion with respect to cell invasion in absence of compounds. Although in this assay Roneparstat, at least at the dose (10 μM) tested, failed to affect the invasive potential of the three cell lines, surprisingly, derivative B3 significantly blocked invasion of HT1080 cells (> 50% inhibition) and, at lesser extent (< 50% inhibition), also of U87MG cells. In contrast, the other two derivatives behaved as the reference compound and were ineffective in inhibiting cell invasion.

Table 3

Cell Adhesion Assay. [26] Cell surface heparan sulfate proteoglycans (HSPGs) appear to be involved in cell-extracellular matrix (ECM) adhesive interactions. Thus, to evaluate whether the new derivatives could interfere with adhesion properties of cancer cells, compounds were tested at not toxic concentrations on U87MG glioblastoma cells, by means of a classical cell adhesion assay, and the results (expressed as percent cell adhesion with respect to vehicle-treated cells) are presented in Table 4. All the compounds markedly inhibited adhesion of U87MG cells with potency similar to that of Roneparstat. Only compound C3 showed a minor potency when tested at the lowest dose.

Table 4

Expression of proangiogenic factors. Translocation of fraction of active heparanase into the cell nucleus and its involvement in controlling transcription of genes encoding for pro-angiogenic and pro-invasion genes, upon degradation of the nuclear heparan sulfate, has been reported.[27,28,29] Therefore, we wondered whether the new heparanase inhibitors could also affect transcription of genes encoding for proangiogenic factors in tumor cell lines. To this aim, HT1080 human fibrosarcoma cells were treated for 24 h with compound B3 compared to the reference compound Roneparstat, both at 10 µM. Then, the mRNA levels related to genes encoding for proangiogenic factors, such as FGF1/2, VEGF, MMP-9, and for HPSE-1 were measured by a quantitative Real-Time PCR. As shown in Figure 7, the biotinylated derivative B3 inhibited the expression of these genes to an extent comparable to Roneparstat.

Figure 7

Experimental metastasis model with B16-F10 mouse melanoma cells.[30] Roneparstat and the three biotinylated derivatives (A7, B3 and C3) were tested for their antimetastatic activity against murine B16-F10 melanoma cells. Mice were pre-treated with each compound (50 µg/mouse), administered 1h prior to the vein tail injection of the melanoma cells. The number of melanoma

colonies per lung (lung metastases) was determined after 18 days. Results are shown in Figure 8, and highlight a profound anti-metastatic activity of all derivatives, with potency comparable to that of the reference compound. Compound A7 appears somewhat better than Roneparstat ($p=0.06$, compound A7 vs Roneparstat), but at the concentration used (50 μg/mouse) the inhibition was >80% with all compounds making it difficult to assess minor differences.

Figure 8

Effect on growth of CAG myeloma. The heparanase inhibitor Roneparstat was previously shown to strongly inhibit the growth of myeloma tumors *in vivo* (subcutaneous tumors formed by CAG human myeloma cells) in part by targeting the tumor microenvironment, including a significant inhibition of tumor angiogenesis.[31] According to this data, we evaluated the growth inhibition of CAG myeloma cells, injected subcutaneously into NOD/SCID mice, upon treatment with three biotinylated compounds (C3; B3 and A7), using Roneparstat as reference. Results shown in Figure 9 indicate that all the biotinylated-derivatives inhibited CAG myeloma-tumor growth with potency similar to that of Roneparstat. The inhibitory effect of compound A-7 reached statistical significance ($p=0.05$).

Figure 9

CONCLUSIONS

Thirteen heparin derivatives, analogues of the well-studied heparanase inhibitor Roneparstat were prepared. All were conjugate with a non-carbohydrate molecule containing a biotin moiety and having different length and hydrophilicity. Independent of the nature and number of the heparin substituent (from 0.4 to 11 for chain) the anti-heparanase activity was conserved (IC_{50} in anomolar range (1.4 to 6.9 nM), similar to that of Roneparstat (Table 1).

The synthetic approaches preserved the glycol split (gs) residues able to interact with the active site of heparanase, as well as the sulfation of position 2 of iduronic acid and of position 6 of glucosamine residues, which are involved in enzyme recognition[32]. Moreover, it appears that, in this range of substitution, the substituents do not mask the negative charges able to interact with the heparan sulfate recognition sites of the protein.

In particular, the synthesis route A does not influence the degree of polymerization of the compound, leaving the inhibition capacity almost unchanged with respect to the Roneparstat, regardless of the substituent structure and the degree of substitution. On the contrary, synthesis route B shows a slight decrease in heparanase activity (up to 6.9 nM) which highlights the importance of the type of substitute of the amino function of glucosamine, which can be assumed to slightly interfere with binding to the protein. But it should be noted that the samples of the B series have a lower molecular weight than Roneparstat and of the samples of the other two series, this means that if the inhibition activity is expressed in weight and not in moles this observed difference disappears. On the other hand, the derivative B3 turned out to be able to inhibit the transcription of genes encoding for proangiogenic factors (MMP-9, VEGF, FGFs, HPSE) in tumor cells, likely due to inhibition of nuclear heparanase HS degrading activity.

Three derivatives (A7, B3, C3; SST0761NA1, SST0635NA1, SST0764NA1, Lab code respectively) with a nearly one substituent per chain and different chemical nature and length of the spacer were selected for further biological characterization. Briefly, A7 (SST0761NA1) is substituted on oxidized glucosamine residues, the linker between N-acetyl gs-heparin and biotin is a polypeptide chain made of a total of 33 atoms. B3 (SST0635NA1) is substituted on the amino group of glucosamine residue with a polyethylene glycol spacer made of a total of 17 atoms. C3 (SST0764NA1) is substituted only on the terminal reducing end residue with a glycine as spacer. These selected substituents did not induce significant effect on cell-proliferation as demonstrate with three human tumor cell lines. Notably, Roneparstat and its derivatives were endowed with significant anti-adhesion properties as clearly shown applying an in vitro adhesion assay and human U87MG glioblastoma cells. This effect may be attributed to an adjuvant effect of the PEG chain.

Importantly, the newly synthesized compounds retained the ability to attenuate the growth of CAG myeloma tumors in mice with potency similar, or even higher (in the case of derivative B3) than that of the reference compound Roneparstat. Furthermore, compound B3 inhibited cell invasion through a reconstituted basement membrane (Matrigel) in vitro and all three derivatives, as well as Roneparstat, profoundly inhibited metastatic dissemination (lung colonization) of murine B16-F10 melanoma cells in vivo.

It should be noted that the in vivo studies were performed only to gain initial information on the anti-tumor activity of the newly synthesized biotinylated derivatives. In-depth analysis of PK/PD properties will be the matter of subsequent studies, limited to the compound(s) that will be selected for further analyses.

In conclusion we have demonstrated that is possible to introduce even complex molecules without impair the anti-heparanase activity of the N-acetylated glycol-split heparin (Roneparstat) using sequence of reactions that maintain the structural feature needed for heparanase recognition. Only in-depth in vivo studies, which are not the subject of this study, will highlight differences due to the greater or lesser bioavailability of these samples. The present study supports the possibility of introducing biotin moiety in the substituent to allow the monitoring of pharmacokinetic and pharmacodynamic properties of Roneparstat, and to target specific folate receptors that may improve drug delivery into the tumor but, using the same approach the biotin moiety could be replaced by other active molecules to yield a synergistic inhibition of tumor growth.

EXPERIMENTAL SECTION

Materials:

Chemicals, reagent-grade obtained from Sigma Aldrich SRL (Milan, Italy) and Iris Biotech (GmbH, Germany), were used as received. Porcine heparin with an average Mw of 17 kDa was a preparation of porcine mucosal heparin sodium salt from Laboratori Derivati Organici, (L.D.O.),Trino Vercellese, Italy. N-desulfated, N-acetylated glycol split (gs) heparin, also named as Roneparstat taken as reference compound, was from Leadiant Bioscience S.A.; former Sigma-Tau Research, Switzerland.

NMR spectra were measured at 500 and/or 600 MHz on Bruker Avance spectrometers equipped with a 5-mm TCI cryoprobe at 298 K from D_2O solutions (15 -25 mg/0.5 mL of D_2O,

99.99% D). ^{1}H NMR spectra were recorded with 32 scans and were Fourier transformed after exponential multiplication (line broadening of 0.3 Hz) and zero filling (from 32 k to 128 k points), phased, baseline corrected and calibrated on the 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP) signal. The solvent residual peaks of MeOD d_4 or DMSO d_6 were used as internal standards, at 3.31 (298K) ppm and 2.48 ppm (298K) respectively. ^{13}C NMR spectra were recorded with a 10-mm multi-nuclear broad band probe, using a Bruker pulse program zgig. The spectra were acquired with relaxation delay 1 s, acquisition time 0.327 s, spectral width 299 ppm. at 303 K and the applied transient number was 20400. The transmitter frequency off-set was 90 ppm. The spectrum size used was128k with zero filling. Before Fourier transformation line broadening of 4 Hz was applied to decrease noise. To recorde HSQC-NMR spectra, heparin samples (20 mg) and its derivatives were dissolved in 0.6 mL of D_2O. Acquisition of the spectra was based on 24 scans, 32 dummy scans, 2s pulse delay, at 303 K (150 Hz1JC-H). Integration of peak volumes in the HSQC spectra was performed using standard Bruker TOPSPIN 3.0 software. Heparin signal assignment and the relative content of monosaccharide residues were calculated from the corresponding anomeric cross-peaks identified by HSQC according to published methods.[33]

Gel permeation chromatography (GPC) measurements, by high pressure size exclusion chromatography coupled with triple array detector (HP-SEC-TDA),[34] were employed to obtain the molecular weight parameters Mw, Mn, and polydispersion degree. A Viscotek (Houston, Texas) instrument equipped with a triple detector array302 with refraction index (RI), viscometer, and light-scattering (90°and 7°) detector was used. SEC was performed on G2500 and G3000 (7.8 mm x 30 cm TSK GMPWxl) Tosoh columns with 0.1 M $NaNO_3$ as eluent at a

flow-rate 0.6 mL/min, by injecting 100 µL of 5 mg/mL solution of each samples. Peak integration and data processing were performed using OMNISEC 4.1 software.

Desalting of biotinylated derivatives and intermediates was carried out by dialysis against H_2O through 3,5 kDa cut/off tubes (Spectrum) or by purification on a 2.5 x 100 cm Sephadex G10 column, with 10 % EtOH in H_2O as eluent and UV detection at 210 nm. Microwave-assisted reactions were performed on CEM Corporation Discover instrument, and mass spectra on Bruker Autoflex Maldi-Tof or Micro Q-Tof instruments. Flash chromatography was carried out on silica gel (Merck 230-400 mesh) and monitored by TLC silica gel 60 F_{254} plates.

Synthesis of heparin intermediates

*N-desulfated heparin: (**I-1**).* Heparin (8.01g) was dissolved in 60 mL of H_2O and converted in the acidic form with Amberlite IR 120 (H^+ form). An excess of pyridine was added to the aqueous solution then concentrated under reduced pressure. The resulting pyridinium salt was dissolved in 80 mL of a mixture of DMSO /H_2O (95:5) and stirred at rt overnight, to obtain an almost complete N-desulfation. Then, the solution was diluted with an equal volume of H_2O and dialyzed for 72 h. Subsequently, product was isolated by concentration under reduced pressure and freeze drying. The structure of the product, used as such in the next step, was confirmed by ^{13}C-NMR and SEC-TDA (15400 Da) analysis. (Fig.S5, *Supporting Information*)

*N-acetylated heparin: (**I-2**).* N-desulfated heparin **I-1** (2.5 g), dissolved in 57 mL of $NaHCO_3$ saturated solution was cooled to 4°C, then under vigorous stirring Ac_2O, (2.1 mL) was added and stirring was continued at 4°C for 30 min maintaining pH at about 8 by adding $NaHCO_3$. Finally, other 2.1 mL of Ac_2O were added and the mixture was maintained at 4°C for another 1.5 h, then, after dilution with an equal volume of H_2O was dialyzed for 72 h. The product was isolated by

concentration under reduced pressure and freeze dried. The structure of the final product, (2.1 g; yield=84%) used as such in the next step, was confirmed by ^{13}C-NMR and SEC-TDA (Mw= 15.400 Da) analysis (Fig.S5, *Supporting Information*)

*N-acetylated-gs-heparin-Aldehyde (**I-3**).* N-acetylated heparin **I-2** (1.15 g), dissolved in 33.5 mL of H_2O and cooled to 4°C, was added at an equal volume of a 0.2 M $NaIO_4$ solution and kept under magnetic stirring in the dark for 20 h. The excess of periodate was quenched by adding ethylene glycol (3 mL) and after 1 hr the reaction mixture was desalted by dialysis at 4°C in the dark for 16 hrs. The retentate was recovered, gently concentrated to half the initial volume and lyophilized to give the final intermediate I-3 (1.04 g, yield=90%) to be used as such in the next step.

*N-desulfated-gs heparin (**I-4**).* N-desulfated heparin **I-1** (1.2 g), in 35 mL of H_2O and cooled to 4°C, was added of an equal volume of aqueous 0.4 M $NaIO_4$ (33.5 mL). The pH value was adjusted to 3.5 with HCl (2% v/v, about 0.5 mL) and kept under stirring at rt in the dark for 6 h. Then, the oxidized heparin was precipitated by addition of 160 mL of EtOH (96% v/v) containing 3% (w/v) of CH_3COONa. The precipitate was collected by centrifugation (12.000 rpm) and washed several times with EtOH to remove excess of reagents and sodium acetate. To crude residue dissolved in 33 mL of H_2O, $NaBH_4$ (731 mg) was added in several portions, and the mixture was stirred for 3 hr at rt. Next, under stirring at 4 °C the pH value was adjusted to 4 with 5% (v/v) AcOH solution to quench the $NaBH_4$ in excess. After 10 min stirring, the mixture was neutralized with 0.1 N NaOH. After dialysis in membrane (cut-off 3500 Da at rt), concentration under reduced pressure and freeze drying the final product was obtained (1.05 g

yield=87%). Content of gs-residues was confirmed by ^{1}H, HSQC NMR spectra and molecular weight evaluated by SEC-TDA (Mw = 7500 Da).

*Reductive amination with alkyne amine of N-acetyl heparin reducing-end-units (**I-5**).* N acetylated heparin **I-2** (1.21 g, 80 µmol), solubilized in 15 mL of phosphate buffer (15 mM pH=5.5) under stirring at rt was added of propargylamine x HCl (266 mg, 2.9 mmol, 36 equiv.) and reaction mixture was heated up to 40°C and kept under stirring for 3 h. Then $NaBH_3CN$ (130 mg, 2.09 mmol) was added and the mixture stirred at 40°C for additional 24 h. A further 20 equiv. 130 mg $NaBH_3CN$ was added and the mixture heated for another day. The reaction mixture was diluted with H_2O and dialyzed against distilled H_2O at rt for 2 days. The retentate concentrated and freeze dried, from aqueous solution it was purified by alcoholic precipitation followed by SEC on G-10 Sephadex using H_2O/ethanol (9:1). After freeze-drying, 1.15 g of alkyne derivative was obtained with 95% (w/w) yield. The percentage of amino group attached to the reducing end was confirmed by ^{1}H-NMR and molecular weight evaluated by SEC-TDA (Mw = 15.300 Da) analysis.

*N-acetyl-gs-heparin reducing end-alkylamino-functionalized (**I-6**).* Alkylamino-N acetylated heparin- **I-5** (1.21 g), dissolved in 32 mL of H_2O and cooled to 4°C, was added of an equal volume of aqueous 0.2 M $NaIO_4$, and kept under stirring in the dark for 20 h. The excess of periodate was quenched by adding ethylene glycol (3.4 mL) and after 1 hr the reaction mixture was desalted by dialysis at 4°C for 16 hrs. The retentate was treated with $NaBH_4$ (740 mg), stirred for 3 hr at rt then, cooled at 4 °C adjusting the pH value to 4 with 5% (v/v) AcOH for quenching the $NaBH_4$ in excess. After 10 min stirring, Then the solution was neutralized with 0.1

N NaOH and dialysed at rt, concentrated and freeze dried. The crude gs-derivative was desalted on a Sephadex G-10 column (900 x 25mm) using H_2O /EtOH 9:1 (v/v) as a mobile phase at 2.1 mL/min. and monitoring the absorbance at 210 and 190 nm. After freeze-drying, 1.01 g of **I-6** was obtained with a 85% (w/w) yield. Content of gs-residues was confirmed by ^{1}H, HSQC NMR spectra and molecular weight evaluated by SEC-TDA (Mw = 14.700 Da) analysis.

Biotinylation of N-acetyl heparin glycol split units

General preparation (herein described for compound **A7**)**.** N-acetyl-gs-heparin intermediate **I-3** (0.95g, ca. 0.8 mmoli of aldehyde function) was dissolved in 15 mL phosphate buffer (15 mM, pH=5.5) under stirring at rt. Biotinylated linker 3, shown in Fig 3**,** (210 mg, 0.2 mmol, about 0.25 equiv. for each aldehyde function present on the polysaccharide chain) was solubilized in phosphate buffer (1 mL) and added to **I-3** solution that was heated up to 40°C and kept under stirring for 1 h, then treated with $NaBH_3CN$ (502 mg, 10 equiv.) the mixture was maintained at 40°C for overnight. After cooling to rt 10 equiv. of $NaBH_4$ (296 mg) were added and the reaction mixture was maintained at rt for other 3 h. After cooling at 4°C the pH value was adjusted to 4 with 5% (v/v) AcOH for quenching the $NaBH_4$ excess, and after 10 min stirring, neutralized with 0.1 N NaOH. After dilution with 10 mL of NaCl 2M, dialysis against distilled H_2O at rt for 1 day, the product was isolated by concentration and freeze drying to obtain the biotinylated-gs-heparin **A7**, which was purified by gel permeation chromatography (Shephadex G-10) to give the final product (about 810 mg, yield=84%). It was characterized by NMR (^{1}H, HSQC) and SEC-TDA analysis. Molecular weight and degree of biotinylation was evaluated by ^{1}H-NMR (Fig. S13 *Supporting Information*) as described in the characterization section and shown in the table

1. Other derivatives (**A1-6**) with different biotinylation contents (DS) were prepared by same procedure. The type of linkers, and molar ratio used to obtain each biotinylate compound are given in table 1. (Fig. S14 and S15 *Supporting Information*.)

Biotinylation of the glucosamine free amino group

*General procedure for N-desulfated-N-acyl-biotinylated gs-heparin (herein described for compound **B-3**).* N-desulfated-gs-heparin **I-4** (1.1g, ca 1.8 mmol) dissolved in 20 mL of 0.1N $NaHCO_3$ and cooled to 4°C was treated with Biotin-Peg_4-NHS **7** (320 mg 0.9 mmoli, about 0.5 equivalents for each glucosamine free amine function) in 1 mL of DMF and the mixture was stirred at rt for 2 h. Then further 0.2 equiv. of biotinylating agent 7 (ca 128 mg in 0.5 ml of DMF) was added and stirred for additional 12 h. The reaction medium was diluted with 10 mL of NaCl 2 M and dialyzed at rt for 1 day. The retentate was concentrated up to ca 15 mL and purified by alcoholic precipitation by addition of 30 mL of EtOH (96% v/v). The precipitate was collected by centrifugation (12.000 rpm) and washed several times with EtOH then redissolved in 15 mL of H_2O, and the alcoholic precipitation process repeated twice. The final biotin-gs-conjugated compound **I-7** was characterized by NMR (^{1}H, HSQC) and SEC-TDA analysis and used as such in the next step. Another biotinylated intermediate compound bearing the biotin-linker 6 was prepared by the same procedure.

*N-Acetylation of N-Biotinylated-gs-N-desulfated heparin (**B-3**)*. N-desulfated-gs-N-biotinylated heparin **I-7** (0.85 g, 1.4 mmoli), dissolved in 23 mL of saturated solution $NaHCO_3$

was cooled to 4°C then, under vigorously stirring, added Ac_2O, (1.1mL, 11 mmol) and stirred at 4°C for 30 min. During the reaction, pH was controlled and maintained at ca 8 by adding $NaHCO_3$. After a further addition of Ac_2O (1.1 mL) the mixture was maintained at 4°C for another 1.5 h, then, diluted with an equal volume of H_2O and dialyzed for 2 days. Concentration and freeze drying gave the crude N-biotinylated-gs-heparin derivative, which was purified by gel permeation chromatography (Sephadex G-10). The final product **B-3** (720 mg, yield=84%) was characterized by NMR (^{1}H, HSQC, Fig. S18 *Supporting Information*) and SEC-TDA (MW=9400 Da) analysis. The degree of biotinylation (DS= 6.7 for disaccharide unit) was evaluated by ^{1}H-NMR. Biotinylated compound (**B-1**), bearing the biotin spacer **6** was prepared by the same procedure described for **B-3**, using 1 equivalent of linker 7 instead of 0.5 with an overall yield of 87% showing a DS of 15.3 for disaccharide unit, while biotinylated compound (**B-2**), DS of 6.6.4 for disaccharide unit, was prepared using 0.5 equiv. of linker 7 instead of linker **6** with an overall yield of 89°%.

Reducing end unit conjugation by "click" chemistry approach (compounds **C1**, **C2** and **C3**)

*N-Acetylated-gs- heparin- reducing end triazole biotinylated (**C3**).* N-acetyl-gs-heparin-alkyne amine derivative **I-6** (1.01g, 0.067 mmol) in a H_2O/t-BuOH (12 mL, 70:30 v/v %) under magnetic stirring for 10 min was treated dropwise with biotinylated linker azide derivative **5** (54 mg, 0.13 mmol, about 2 equivalents for each alkyne function present on the polysaccharide chain) solubilized in a H_2O/t-BuOH (1 mL, v/v 1:1). Sodium ascorbate (300 µl, aqueous 0.2M, 1 equiv.) and $CuSO_4$ 5 H_2O (300 µl, aqueous 0.1M, 0.5 equiv.) were added, and the reaction mixture was stirred at rt for 2 h. Finally, other 2 equiv. of azide, 1 equiv. of sodium ascorbate and 0.5 equiv. of $CuSO_4$ 5 H_2O were added, and the mixture maintained at rt for further 4 h, then

dialyzed for 48 h. The retentate concentrated to 15-20 mL and freed from Cu using 3 mL of Chelex 100 resin, then neutralized with 0.1 N $NaHCO_3$. After dialysis, concentration under reduced pressure and freeze drying compound **C3** was obtained (ca 920 mg, yield=87%). Its structure was confirmed by NMR (^{1}H, HSQC, Fig. S19 *Supporting Information*) DS=1.9 % for disaccharidic units and SEC-TDA analysis (MW = 14200 Da).

Biotinylated compound **C2** was prepared by the same procedure with an overall yield of 86% and characterized by ^{1}H-HSQC showing a DS of 2.1% for disaccharide unit and by SEC-TDA analysis (MW= 9600 Da).

Biotinylated compound **C1**, bearing the biotinylated linker azide derivative **4** was prepared by the similar procedures described for **C3**, using 3 equivalents of biotinylating agent 5 instead of 2 with an overall yield of 84% and a DS of 3.4% for disaccharide unit.

*Anti-heparanase activity***.** In order to test the activity of the heparanase inhibitors, a homogenous assay based on the cleavage of the synthetic heparin pentasaccharide Fondaparinux (Arixtra; Aspen) has been used. The assay, which yields a disaccharide that is colorimetrically determinated (560 nm), was essentially performed as described by Hammond[21]. Serial dilutions of the compounds and of the reference Roneparstat were tested in triplicate with standard deviations (SDs) less than 10%. IC_{50} values for each compound, versus heparanase, were ultimately calculated by GraphPad software.

Cell lines and maintenance. HT1080 (fibrosarcoma), U87MG (glioblastoma) and U2OS (osteosarcoma) human cell lines and B16-F10 mouse melanoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured according to the ATCC recommendations. Briefly, HT1080, U87MG and mouse B16-F10 cells were grown in

Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific). Luciferase-labelled CAG human myeloma cells (kindly provided by Dr. R. Sanderson, University of Alabama at Birmingham) were cultured in RPMI 1640 medium, and U2OS cells were grown in McCoy's 5a medium (Thermo Fisher Scientific). All media were supplemented with 10% FCS (Thermo Fisher Scientific), 100 U/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific), and 2 mM L-Glutamine (Thermo Fisher Scientific). All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Proliferation Assay. HT1080, U87MG and U2OS exponentially growing cells were seeded into 96-well plates and then, 24h later, treated with test compounds at different concentrations or the solvent alone. Inhibition of cell proliferation was measured upon 3 days of treatment by means of a classical sulforhodamine B (SRB) assay performed in triplicate. Results were expressed as percentage of tumor cell proliferation as compared to vehicle-treated (control) cells.

Matrigel invasion assay. Cells were pretreated with test compounds at the indicated concentrations, in complete medium for 24h. Then, cells were detached, resuspended in serum-free medium and added (2.5×10^4 cells/filter) to the upper chamber of 24-well Transwell plates (Costar, Corning Inc., Corning, NY) previously coated with Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA). The same compound concentration used for cell pretreatment was added to both the upper and lower chambers. Cells were allowed to invade for 24 h at 37°C in a CO_2 incubator and then not-migrated cells were removed from the upper chamber with a cotton swab. The invading cells were fixed, stained with SRB, and then counted under a microscope, at 40X magnification. Activity of test compounds was compared to the reference compound.

Cell Adhesion Assay. U87MG (human glioblastoma astrocytoma) cells were treated for 18-20h with test compounds (B3, A7 and C3) in complete medium supplemented with 10% FCS. Then, cells were collected, washed in complete medium, and added to matrigel coated wells in triplicate, at previously identified optimal seeding density, and incubated at 37 °C in complete medium for times ranging from 15 minutes to 4h[25]. After incubation, the wells were washed three times with serum-free medium and the remaining firmly attached cells were stained with crystal violet 0.1 reagent. Optical density was measure at 500 nm by Victor 3 (Perkin-Elmer) plate analyzer. Each experiment was repeated three times with standard deviations (SDs) less than 10%.

CAG myeloma Assay. Luciferase-labelled CAG myeloma cells ($5x10^6$) were injected subcutaneously into NOD/SCID mice (n=5). 8-weeks old female mice were treated with biotinilated compounds C3, B3 and A7 (1000 µg/mouse, injected intraperitoneally twice a day, starting on day 9 of cell inoculation)[35]. Vehicle (PBS) alone was similarly injected as control (CTL).

Tumor growth was examined by IVIS imaging prior to the first injection of compounds and once a week thereafter. The experiment was terminated on day twenty-nine.

All the animals were handled as per protocols and procedures approved by the Technion Institutional Animal Care and Use Committee.

Bioluminescent imaging of the luciferase-expressing tumors was performed with a highly sensitive, cooled charge coupled device (CCD) camera mounted in a light-tight specimen box (IVIS; Xenogen Corp., Waltham, MA). Briefly, mice were injected intraperitoneally with D-luciferin substrate at 150 mg/kg, anesthetized and placed onto a stage inside the light-tight

camera box, with continuous exposure to isoflurane (EZAnesthesia, Palmer, PA). Light emitted from the bioluminescent cells was detected by the IVIS camera system with images quantified for tumor burden using a log-scale color range set at 5×10^4 to 1×10^7 and measurement of total photon counts per second (PPS) using Living Image software (Xenogen).

Experimental metastasis. B16-F10 mouse melanoma cells [29, 36, 37]:

B16-F10 mouse melanoma ($2x10^5$) cells were injected into the tail vein of C57BL/6 6-weeks old female mice without or together with the test compound (50 μg/mouse), essentially as described [29,35,36]. In some experiments, compounds were also injected (50 μg/mouse; intraperitoneally) to the mice 30 min prior to the tail vein injection of cells. Lungs were harvested 18 days thereafter, fixed in Bouin solution and metastases occurrence was determined by gross examination and counting of colonies using a stereomicroscope.

Real-Time Quantitative PCR (qPCR) Assay. Total RNA was extracted from HT1080 cells, after 24 h treatment with test compounds (10 μM), and then retrotranscribed using the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions. Real Time quantitative PCR analysis was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA) and the following PrimePCR SYBR Green Assays: qHsaCID0015228 (HPSE-1), qHsaCED0002206 (FGF-1), qHsaCID0015510 (FGF-2), qHsaCID0011597 (MMP-9), qHsaCED0043454 (VEGF-A) (Bio-Rad Laboratories Inc., Hercules, CA). The 7900HT

Sequence Detection System instrument and software (Applied Biosystems) were used to quantify the relative expression of the target genes by the DDCt method using total RNA to normalize gene expression.

FIGURES

Figure 1. Simplified formula of a representative chain of porcine mucosal heparin (a) and the corresponding glycol-split (gs) derivative Roneparstat (b), obtained by N-desulfation, N-acetylation and subsequent periodate oxidation/ borohydride reduction of nonsulfated glucuronic (G) and iduronic (I) acids.

Figure 2. Schematic representation of the pathway to obtain the linked spacer Biotin Roneparstat-like heparin derivatives: [A] by reductive amination of glycol-split residues, [B] by amide bond on N-desulfated glucosamine residues, and [C] through Click-grafting at the reducing end to give an alkenylamino linked biotin-spacer.

Figure 3. Structure of Biotin-spacers end functionalized.

Figure 4. Scheme of the synthetic pathway [A] exemplified for biotin-AA-NH_2 **3** spacer.

Figure 5. Scheme of the synthetic pathway [B] exemplified for Biotine-Peg_4-NHS **7** spacer.

Figure 6. Scheme of the synthetic pathway [C] exemplified for the case of biotin-N_3 **5** spacer.

Figure 7. Effect of compound **B3** and Roneparstat, tested at 10 μM, on the expression of selected genes**.** The expression levels of FGF-1, FGF-2, VEGF, MMP-9 and HSPE-1 mRNA in HT1080 cells, following 24 h of treatment, were measured by means of real-time qPCR analysis.

Results (mean ± SD of two measurements) are expressed as percent with respect to vehicle-treated cells.

Figure 8. Effect of heparanase-inhibiting compounds on lung colonization of B16 mouse melanoma cells (experimental metastasis). B16-F10 mouse melanoma cells ($1.5x10^5$/0.2 ml) were injected into the tail vein of C57BL mice (n = 5) together with the indicated compound (50 μg/mouse). Control (CTL) mice were injected with cells alone. Compounds were also injected (50 μg/mouse; intraperitoneal) to the respective group of mice 30 min prior to the tail vein injection of cells. On day 18 mice were sacrificed, the lungs fixed in Bouin solution and the number of melanoma colonies per lung (lung metastases) counted using a stereomicroscope. The results are expressed as number of cell colonies per lung ± SD. There was no significant difference between the inhibitory effect of compounds C3 and B3.

Figure 9. Effect of heparanase-inhibiting compounds on CAG myeloma tumor growth. Luciferase-labelled CAG myeloma cells ($5x10^6$) were injected subcutaneously into NOD/SCID mice (n=5). Mice were treated with compounds C3, B3, A7 and Roneparstat (RONE), administered intraperitoneally twice a day at 1000 μg/mouse, starting on day 9 of cell inoculation. Tumor growth was monitored by IVIS imaging prior to the first injection of compounds and once a week thereafter. Representative imaging results are presented in the figure (left panel). Graph bars in the right panel represent the average bioluminescence (light photons/sec/cm^2) ± SE, as measured by IVIS on day 29, prior to termination of the experiment. There was no significant difference between the inhibitory effect of compounds C3 and B3.

TABLES.

Table 1. Code numbers, reagents ratio, chemico-physical properties and heparanase inhibitory activity of Roneparstat-like derivatives.

Derivatization pathway and Referen€ce Compound	Biotin-spacer (Family Linkers)	Biotinylated Compounds		Molar ratio Linkers / disaccharide (equivalent)	Substitution degree (DS%)[a]	Number of biotin moities for heparin chain (NS)[b]	Glycol Split-RO (%)[c]		Molecular Weight[d] (Da)	Fondaparinux Hpse Assay (IC_{50} nM)[e]
							Residue gs-I (%)	Residue gs-G (%)		
Roneparstat	-	-		-	-	-	10	16	16200	2.08
Biotin-derivative on glycols-split residues [A]	Biotin-NH_2	**1**	A1	0.5	12.0	3.2	21	n.d.	16000	1.40
			A2	1.0	41.0	11.6	8	9	17000	3.22
			A3	0.75	31.0	8.1	7	5	15600	7.27
	Biotin-PEG-NH_2	**2**	A4	0.5	21.0	6.5	10	3	18700	2.42
			A5	1.0	28.0	9.9	7	5	21200	2.43
	Biotin-AA-NH_2	**3**	A6	0.5	6.6	1.7	16	14	15000	1.46
			A7	**0.25**	**3.0**	0.7	16	11	14100	2.76
Biotin-derivative on glucosamines residues [B]	Biotin-NHS	**6**	B1	1.0	15.3	2.3	12	15	9300	3.69
	Biotin-PEG-NHS	**7**	B2	0.5	6.4	0.9	8	8	8500	6.10
			B3	**0.5**	**6.7**	1.0	10	9	9400	6.92
Biotin-derivative at the reducing end unit [C]	Biotin-N_3	**4**	C1	3.0	3.4	0.5	7	16	9300	4.31
	Biotin-Gly-N_3	**5**	C2	2.0	2.1	0.4	17	16	9600	2.63
			C3	**2.0**	**1.9**	0.5	14	17	14200	4.11

[a] Degree of substitution (DS) was estimated from the ^{1}H-NMR spectra by comparing the peak integrated area of the proton H3$^-$ of biotin ring versus that of the N-acetyl protons of glucosamine and express as the percentage of the biotinylated disaccharide unit, with respect to the total number of disaccharide units.

[b]. Number of substituents (NS) indicates the number of biotin moieties bound for each heparin chain

[c] "Glycol-split (RO; %)" means the percentage of the modified uronic acid residues (RO) with respect to the total of the same present in the molecule. In particular, "(% gs-I)" and "(% gs-G)" means respectively the percentage of the iduronic acid and glucuronic acid residues (oxidated and reduced) with respect to the total of the disaccharides present in the polymer. The percent glycol split (gsI % and (gsG %) was calculated by HSQC experiment, from the area of the anomeric signal of the glycol split uronic acids at 4.94/106.9 ppm (gsI, I) and 4.71/106.5 ppm (gsG,) versus the area of the anomeric signal of the 2-O sulfated Iduronic acid at 5.2/102 ppm (I2S).

[d] Molecular weights were obtained from GPC analysis.

[e] Dose causing 50% Inhibition of heparanase enzymatic activity as determined from dose-response curves repeated at least twice in separate experiments]. **In bold** *are highlight the samples chosen for further biological tests.*

Table 2. Antiproliferative activity of derivatives **A7, B3**, **C3** and reference compound **Roneparstat** tested on HT1080, U87MG and U2OS tumor cells following 3 days of treatment

	Tumor cell proliferation (%)[a]								
Cell line	**HT1080**			**U87MG**			**U2OS**		
Concentration (µM)	100	33	11	100	33	11	100	33	11
Compounds									
Roneparstat	100	100	100	58	78	91	51	69	81
A7	75	99	100	73	75	84	69	79	85
B3	97	99	100	75	78	88	72	75	83
C3	95	100	100	76	86	91	67	76	81

[a] *Results are expressed as percentage of tumor cell proliferation compared to vehicle-treated (control) cells and are the mean of two independent experiments (SD always < 10%).*

Table 3. Inhibition of invasive potential of by derivatives **A7, B3**, **C3** and reference compound Roneparstat. Matrigel cell invasion assay performed on HT1080, U87MG and U2OS tumor cells upon 24 hours of treatment. Compounds were tested at the fixed concentration shown in brackets. Score symbols: "-"no inhibition; "+" < 50% inhibition; "++" 50-90% inhibition of cell invasion, with respect to invading cells in the absence of drugs.

	Cell lines					
Compounds	HT1080		U87MG		U2OS	
Roneparstat	(10 µM)	-	(10 µM)	-	(10 µM)	-
A7	(10 µM)	-	(10 µM)	-	(10 µM)	-
B3	(10 µM)	++	(1 µM)	+	(1 µM)	-
C3	(10 µM)	-	(10 µM)	-	(10 µM)	-

Table 4. Inhibition of glioblastoma cell adhesion by derivatives A7, B3, C3 and reference compound Roneparstat, performed on U87MG glioblastoma cells upon 24 hours of treatment. Compounds were tested at the fixed concentrations shown in brackets. Results expressed as percent cell adhesion with respect to vehicle-treated cells.

Compound Id	Cell adhesion *(% versus vehicle-treated cells)*	
	Concentration	
	(10 µM)	(0.1 µM)
Roneparstat	22.9	54.0
B3	27.2	62.9
A7	21.5	57.5
C3	12.8	83.2

ASSOCIATED CONTENT

Supporting Information. Characterization of biotinylated compounds, synthesis conditions and NMR spectra of biotinylated linkers This material is available free of charge via the Internet at http://pubs.acs.org."

The following files are available free of charge.

Supporting Information-Novel N-acetyl-Glycol-split heparin.pdf

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest. G.R. is employee of Takis srl. F.M.M. and G.G, are employees of Alfasigma SpA.

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ABBREVIATIONS

Biotin-spacer-NH_2, Biotinylated linkers amine end functionalized; Biotin-spacer-N_3, Biotinylated linkers azide end functionalized; Biotin-spacer-NHS, Biotinylated linkers activated ester end functionalized; gs, Glycol split; I, L-Iduronic acid; G, D-Glucuronic acid; RO, reduced oxidized; Boc-NH-PEG_4-CO_2H, 15-(tert-Butyloxycarbonyl)amino-4,7,10,13-tetra-oxa-pentanoic acid; tBuO-Peg_4-NH_2, 15-Amino-4,7,10,13-tetraoxa-pentadecanoic acid t-butyl ester; DS, Degree of substitution; NS, Number of substituents; MW-SPPS, solid phase synthesis -heating assisted. $(PEG)_n$, polyethylene glycol; DCC, Diciclohexyl carbodiimide; EDC, 1-ethyl-3-(3-dimethylamino)propyl) carbodimide; NHS, N-Hydroxysuccinimide; DIEA, *N*-Ethyldiisopropylamine; DMSO, Dimetyl sulfoxide; HCTU, O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tretramethyluronium; HT1080, human fibrosarcoma; U87MG, human glioblastoma astrocytoma; U2OS, human osteosarcoma cell lines;

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Fig.1

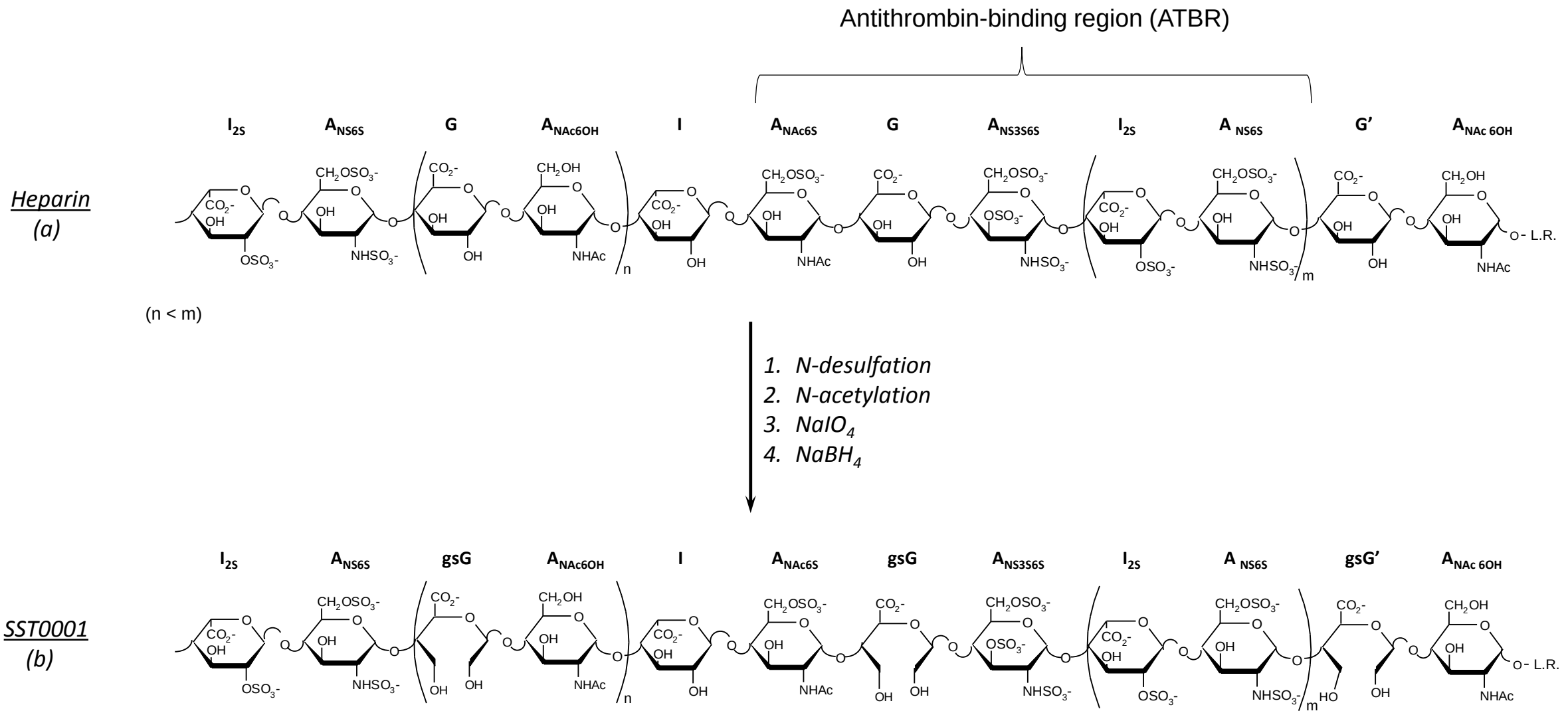

Antithrombin-binding region (ATBR)
I2S
ANS6S
G
ANAc6OH
I
ANAc6S
G
ANS3S6S
I2S
A NS6S
G'
ANAc 6OH
Heparin
(a)
(n < m)
1. N-desulfation
2. N-acetylation
3. NaIO4
4. NaBH4
I2S
ANS6S
gsG
ANAc6OH
I
ANAc6S
gsG
ANS3S6S
I2S
A NS6S
gsG'
ANAc 6OH
SST0001
(b)

Fig.2

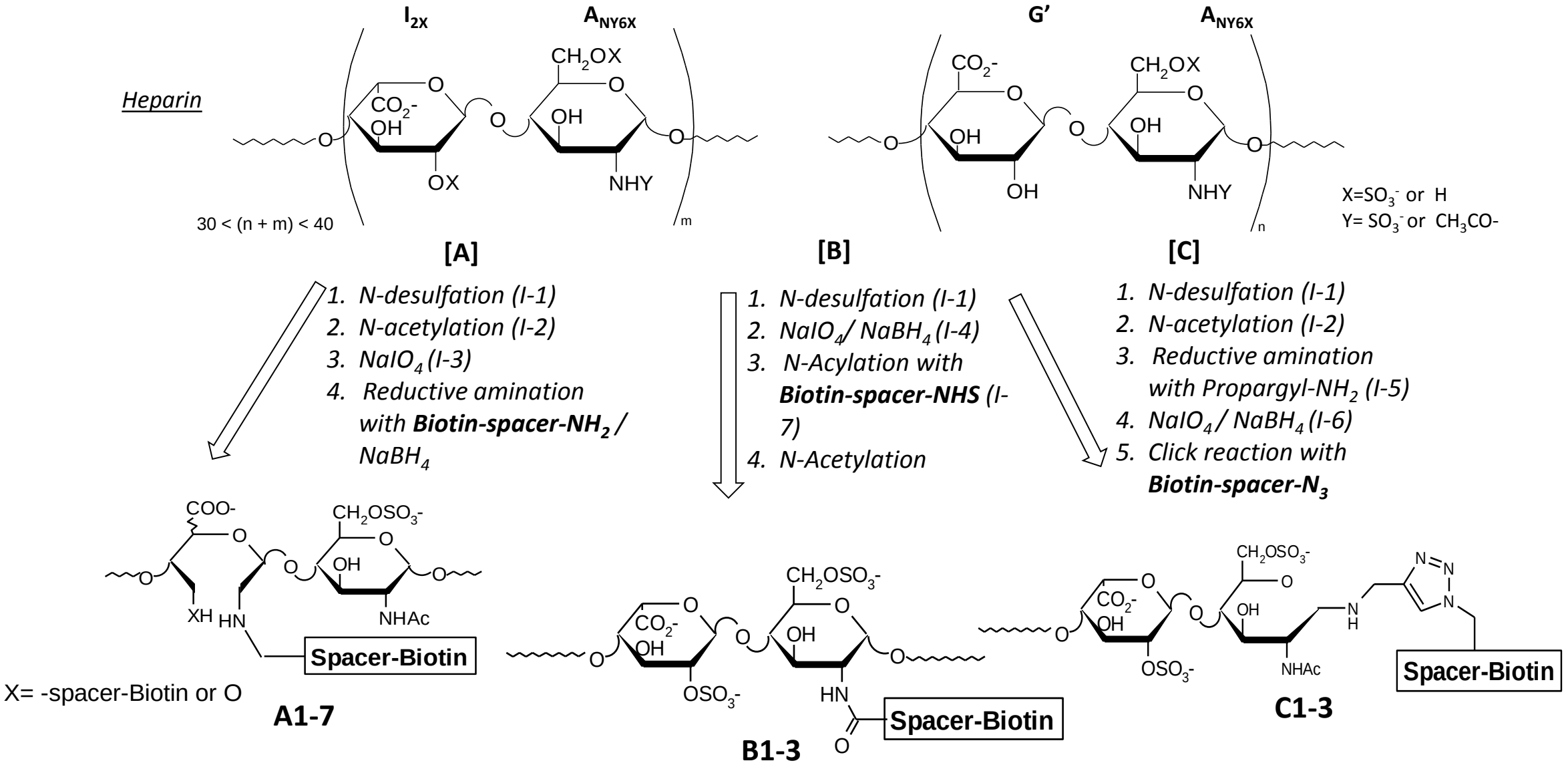
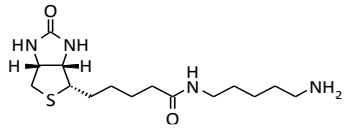
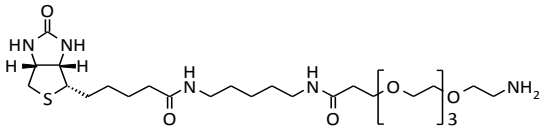

Heparin
I_{2X}
A_{NY6X}
G'
A_{NY6X}
30 < (n + m) < 40
X=SO_3^- or H
Y= SO_3^- or CH_3CO-
[A]
[B]
[C]
1. N-desulfation (I-1)
2. N-acetylation (I-2)
3. $NaIO_4$ (I-3)
4. Reductive amination with Biotin-spacer-NH_2 / $NaBH_4$
1. N-desulfation (I-1)
2. $NaIO_4$/ $NaBH_4$ (I-4)
3. N-Acylation with Biotin-spacer-NHS (I-7)
4. N-Acetylation
1. N-desulfation (I-1)
2. N-acetylation (I-2)
3. Reductive amination with Propargyl-NH_2 (I-5)
4. $NaIO_4$/ $NaBH_4$ (I-6)
5. Click reaction with Biotin-spacer-N_3
Spacer-Biotin
X= -spacer-Biotin or O
A1-7
B1-3
C1-3

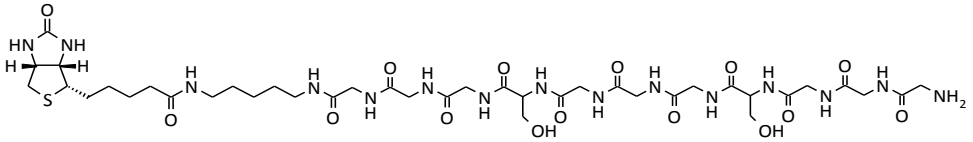
Fig.3



5-(Biotinamido)pentylamine **1**



2



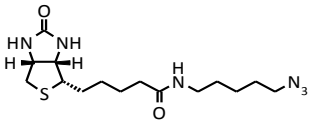
3

[Biotin-NH_2]

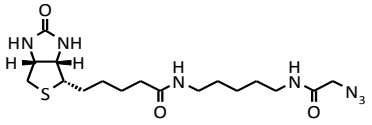
[Biotin-Peg_4-NH_2]

[Biotin-AA-NH_2]

Linkers Amine end functionalized
(Biotin- spacer-NH_2)



4

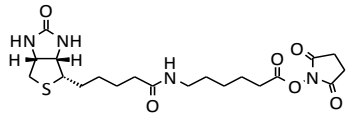


5

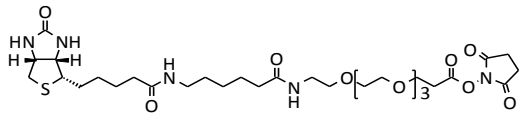
[Biotin-N_3]

[Biotin-Gly-N_3]

Linkers Azide end functionalized
(Biotin- spacer-N_3)



6



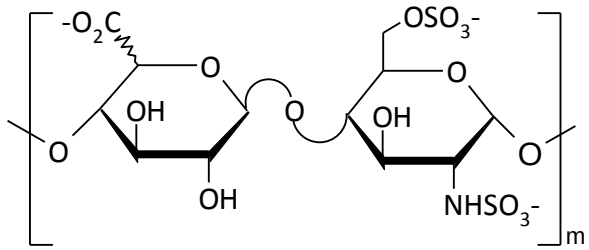
7

[Biotin-NHS]

[Biotin-Peg_4-NHS]

Linkers Carboxylate activated esters end functionalized
(Biotin-spacer-NHS)

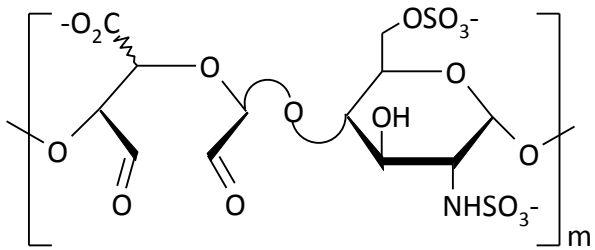
Fig.4



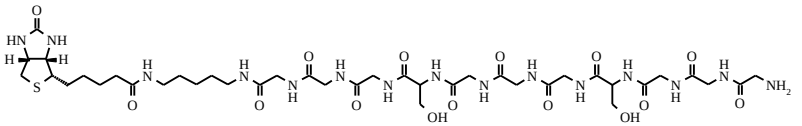
[Heparin]

1) N-Desulfation
2) N-Acetylation

$NaIO_4$

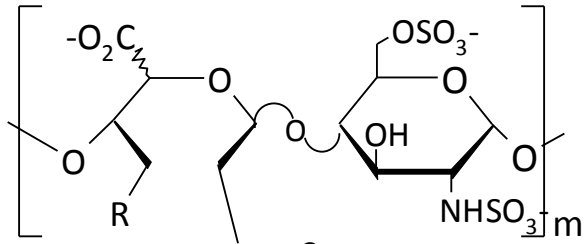


1) Phosphate buffer
$NaCNBH_3$

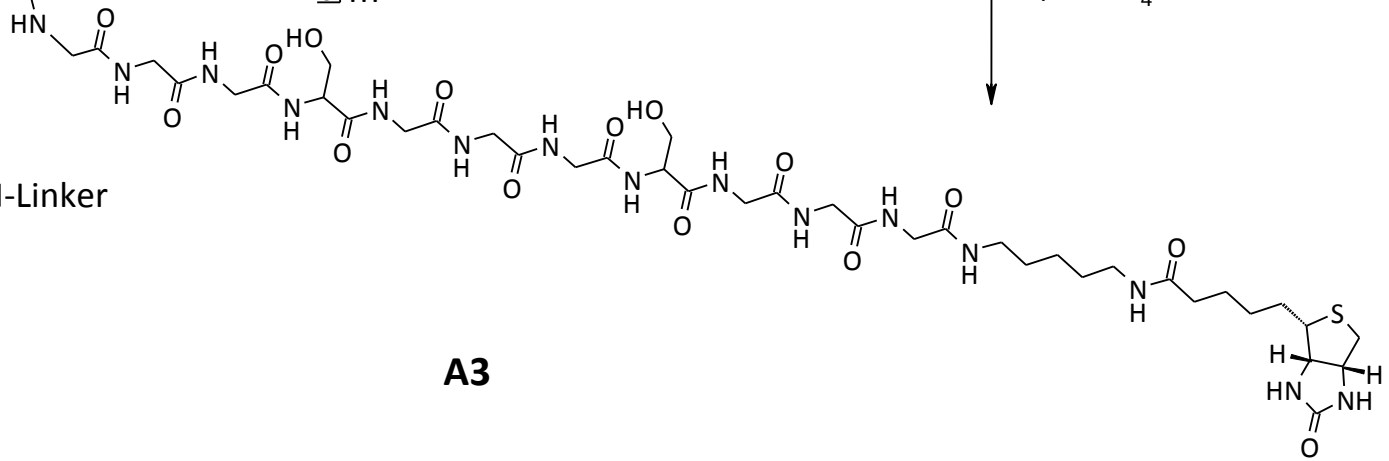


3

2) $NaBH_4$



R= OH or -HN-Linker



A3

Fig.5

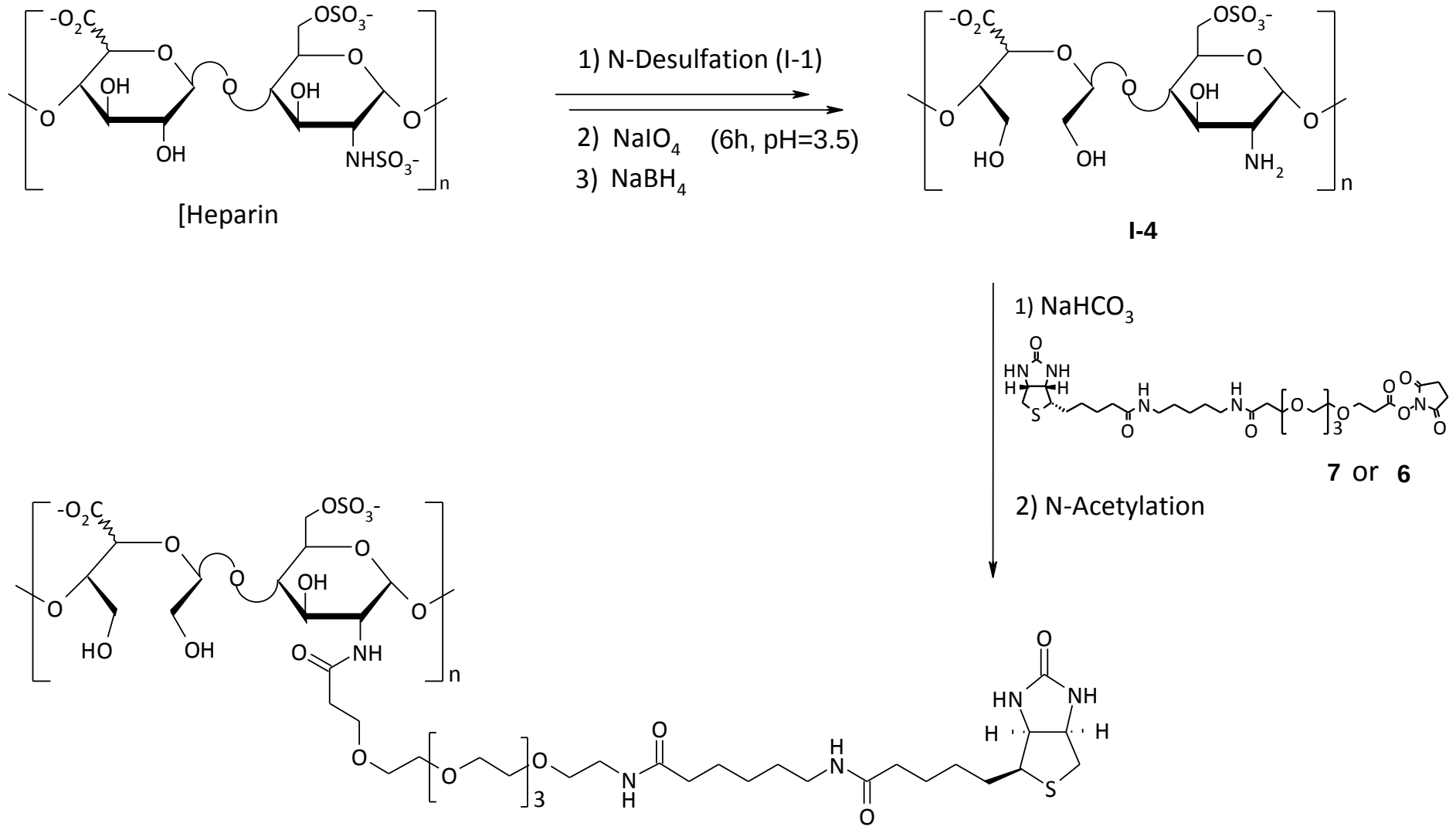

[Heparin
1) N-Desulfation (I-1)
2) NaIO4 (6h, pH=3.5)
3) NaBH4
I-4
1) NaHCO3
7 or 6
2) N-Acetylation
B2-3

Fig.6

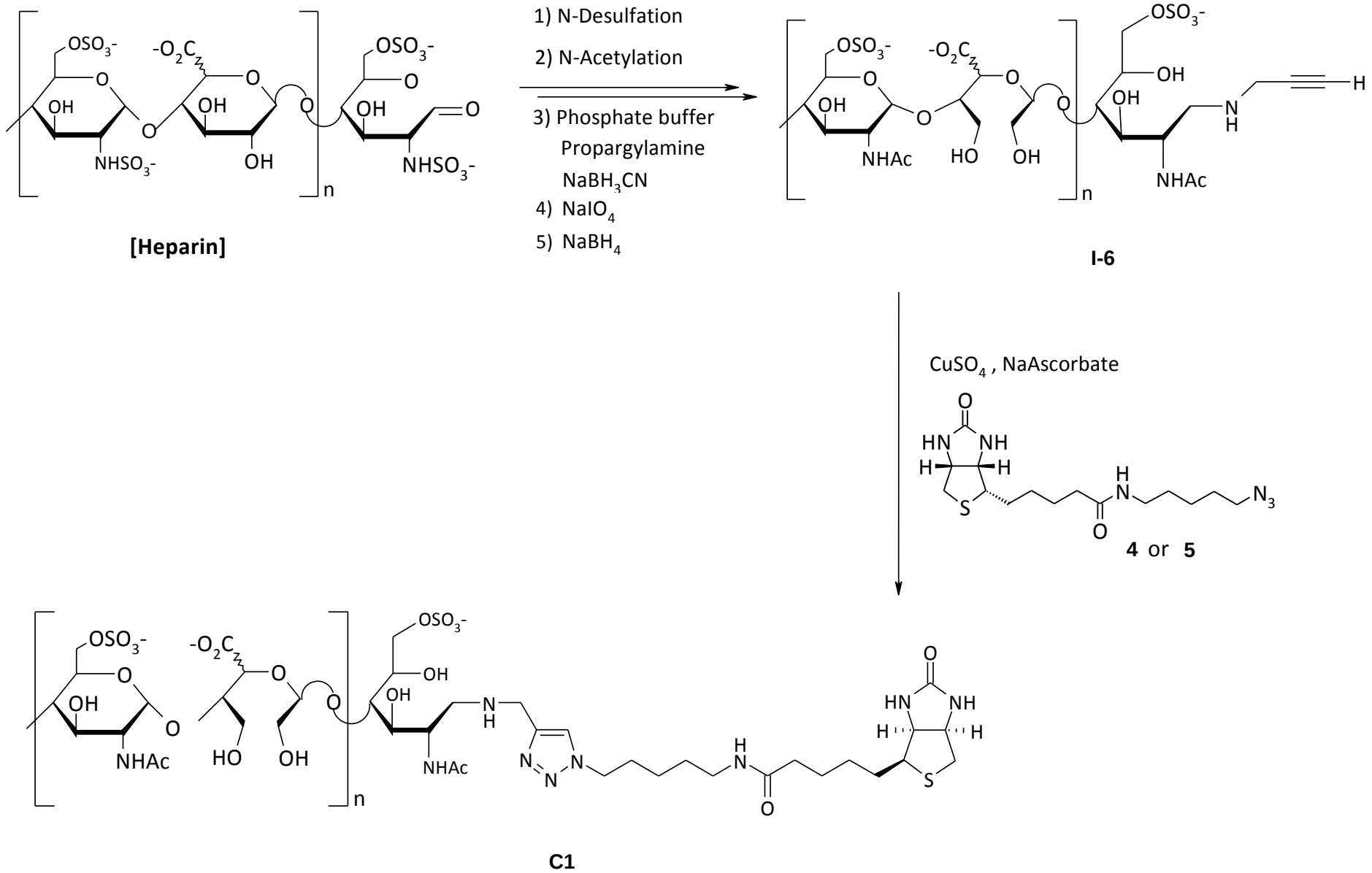

1) N-Desulfation
2) N-Acetylation
3) Phosphate buffer
Propargylamine
NaBH3CN
4) NaIO4
5) NaBH4
CuSO4 , NaAscorbate
[Heparin]
I-6
4 or 5
C1

Fig. 7

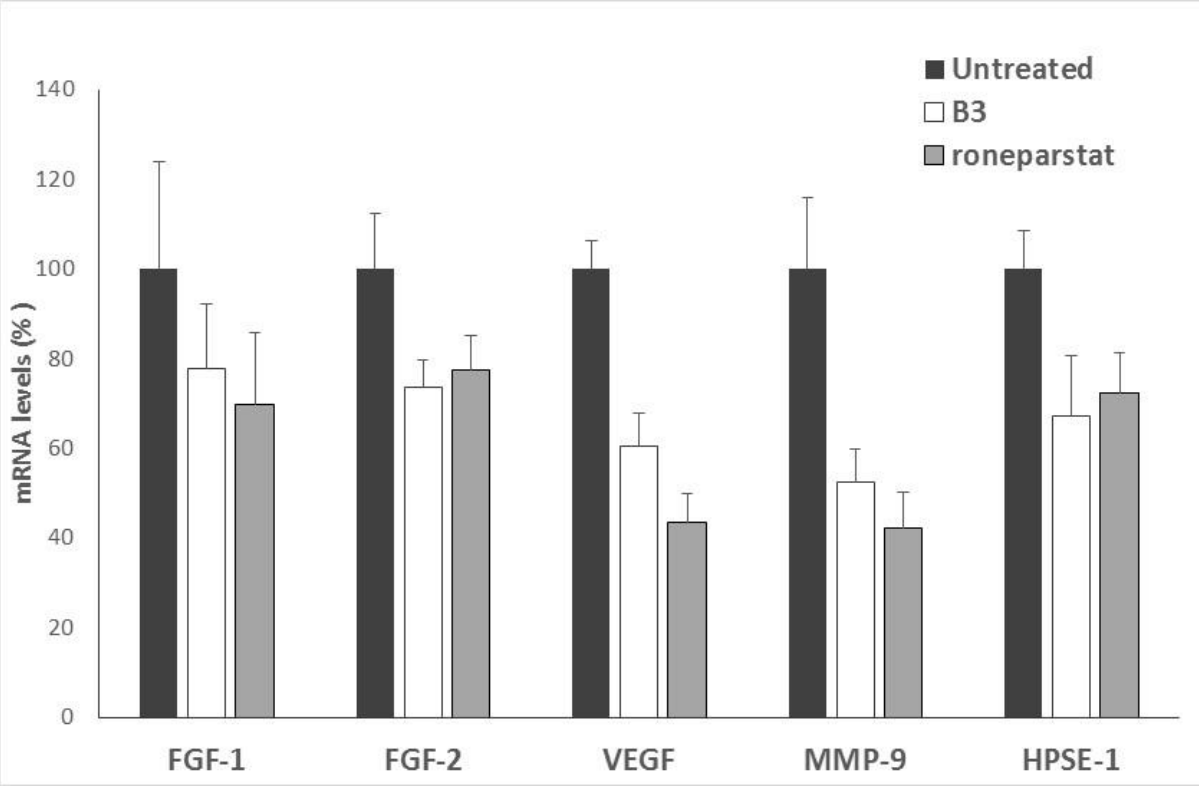

Untreated
B3
roneparstat
mRNA levels (%)
140
120
100
80
60
40
20
0
FGF-1
FGF-2
VEGF
MMP-9
HPSE-1

Fig.8

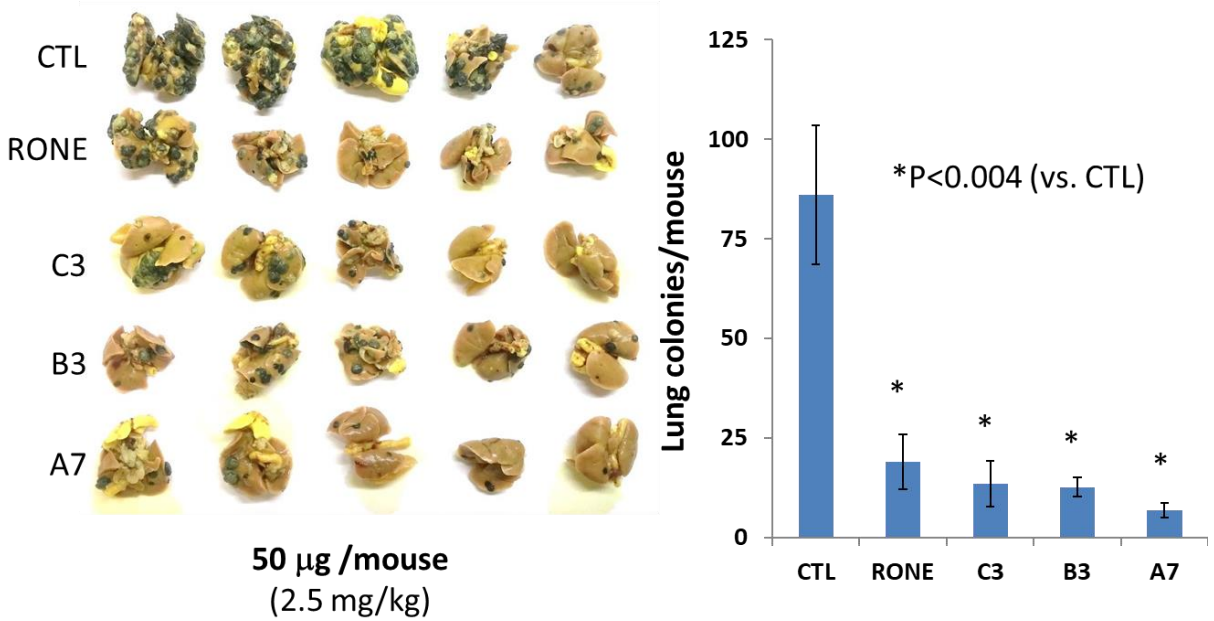
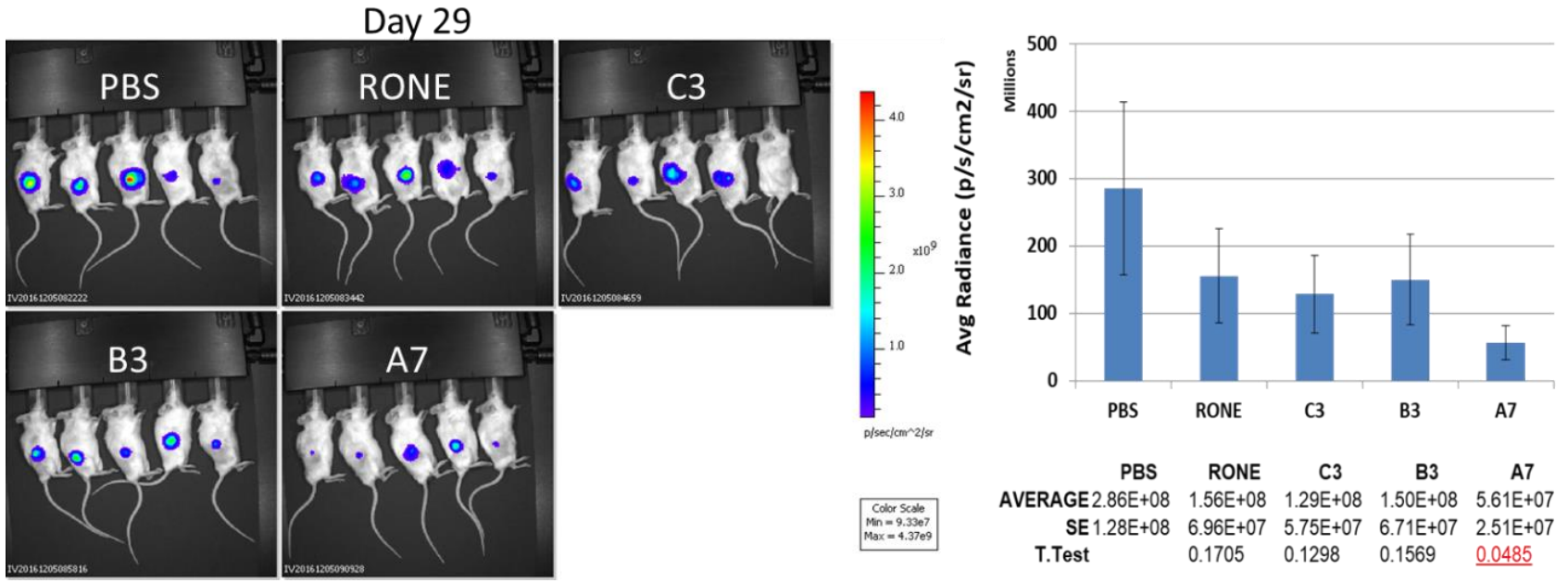

CTL
RONE
C3
B3
A7
50 μg /mouse
(2.5 mg/kg)
Lung colonies/mouse
125
100
75
50
25
0
*P<0.004 (vs. CTL)
*
*
*
*
CTL
RONE
C3
B3
A7

Fig.9



	PBS	RONE	C3	B3	A7
AVERAGE	2.86E+08	1.56E+08	1.29E+08	1.50E+08	5.61E+07
SE	1.28E+08	6.96E+07	5.75E+07	6.71E+07	2.51E+07
T.Test		0.1705	0.1298	0.1569	0.0485

Heparanase is regarded as a promising target for anticancer drugs and Ronepastat is one of one of its most promising inhibitors.

A new class of glycol-split heparins have been synthetized as biotinylated congeners of Roneparstat (N-desulfated-N-acetyl-gs-heparin through a linker of different functional groups.

Compounds were characterized by NMR and evaluation of molecular weight.

Derivatives tested “in vitro” as inhibitors of tumor cell adhesion and of expression of proangiogenic factors, and “in vivo” upon experimental tumor models, CAG myeloma, and murine B16F10 showed that the chemical modifications introduced do not hinder their biological activity.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: