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Synthesis of 6-amino-6-deoxyhyaluronan as an intermediate for conjugation with carboxylate-containing compounds: application to hyaluronan–camptothecin conjugates

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ABSTRACT

A novel methodology for making drug conjugates using hyaluronan as a carrier was developed. This strategy involves a completely regioselective two-step synthesis of 6-amino-6-deoxyhyaluronan, which is then easily functionalized with drugs through a suitable linker. The case of hyaluronan–camptothecin conjugates is described, making use of a simple succinate linker. The antitumor activity of new hyaluronan derivatives prepared is at present under evaluation.

1. Introduction

Hyaluronan (HA, Fig. 1) is a naturally occurring polysaccharide composed of a linear repeating disaccharide unit consisting of β-(1→4)-linked D-glucopyranuronic acid and β-(1→3)-linked 2-acetamido-2-deoxy-D-glucopyranose. It is a component of the mammalian extracellular matrix of the connective tissue, and participates in regulating the properties of pericellular matrices. HA plays a fundamental role in transducing signals in proliferating and migrating cells. HA–protein interactions have been shown to be involved in cell adhesion, growth, and migration processes, in inflammation and wound healing, and in cancer.[1–3]

Cellular HA receptors such as CD44, RHAMM (receptor for hyaluronan-mediated motility), and HARLEC (hyaluronan receptor of liver endothelial cells) play an important role in biological processes. Various tumors, for example, epithelial, ovarian, colon, stomach, and acute leukemia, overexpress the HA-binding receptors CD44[4] and RHAMM.[5] Consequently, these tumor cells show enhanced binding and internalization of HA.[6]

The high tumor specificity of these interactions and the high biocompatibility of HA prompted the design and synthesis of tumor-targeting bioconjugates of HA and cytotoxic agents.[7,8,4,9] Conjugation of low-molecular-weight HA to cytotoxic drugs such as paclitaxel,[10–12] doxorubicin,[13] mitomycin C,[14] and butyric acid[15] have been reported in the literature.

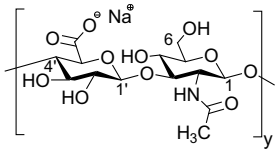

Figure 1. Structure of hyaluronan sodium salt (**1**) disaccharide repeating unit.

20-(*S*)-Camptothecin (CPT, Fig. 2) is a naturally occurring alkaloid isolated from *Camptotheca acuminata* with a significant antitumor activity against a variety of human solid tumors.[16] The CPT primary cellular target is type I DNA topoisomerase,[17] whose inhibition causes cell death by apoptosis.

At the beginning, the clinical use of CPT was hampered by its low water solubility, and initial trials made use of the sodium salt of the open lactone ring; however, severe toxicity was observed

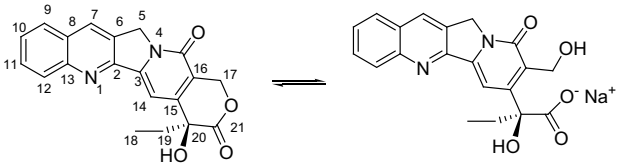

Figure. 2. Structures of camptothecin and its water-soluble sodium salt.

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and the trials were discontinued.[17] In order to overcome these drawbacks, water-soluble analogues of CPT such as topotecan (Hycamtin®) and irinotecan (Camptosar®) have been synthesized, which are now approved for clinical treatment of ovarian and colon cancers, respectively.[18–20]

The intact lactone ring is critical for CPT antitumor activity. At physiological pH, the equilibrium favors the less active and water-soluble carboxylate form. In human blood and tissues the equilibrium is shifted further toward the open lactone form, which strongly binds to serum albumin thus becoming inaccessible for cellular uptake.[21,22]

Esterification of 20-OH stabilizes the lactone ring in vivo and is a common prodrug strategy exploited to enhance CPT bioavailability and to reduce toxicity.[23]

Conjugation of CPT via an ester bond to water-soluble synthetic or natural polymers, such as poly(ethylene)glycol (PEG),[24,25] poly-*N*-(2-hydroxypropyl)methacrylamide (HMPA),[26] carboxymethyl dextran,[27] poly(L-glutamic acid),[28] and linear β-cyclodextrin-based polymers,[29] is a strategy used to produce effective drug delivery systems with improved cancer cell specificity and bioavailability.[30] Reported biological data support the rationale of conjugation of CPT to synthetic and natural polymers.

In this paper we describe a novel methodology for making drug conjugates using hyaluronan as a carrier. This strategy is based on the regioselective conversion of C-6 hydroxyl groups into amino groups that can serve as an anchor for conjugation through a suitable linker with a drug. The number of moles of amino group per mole of disaccharide repeating unit (i.e., degree of substitution or DS) introduced on hyaluronan backbone can be controlled by tuning reaction conditions (i.e., temperature and reaction time), thus allowing the production of derivatives with the desired content of conjugated drug.

In particular, CPT converted into the corresponding 20-*O*-hemisuccinate was conjugated on 6-amino-6-deoxyhyaluronan derivative by carbodiimide-mediated coupling.

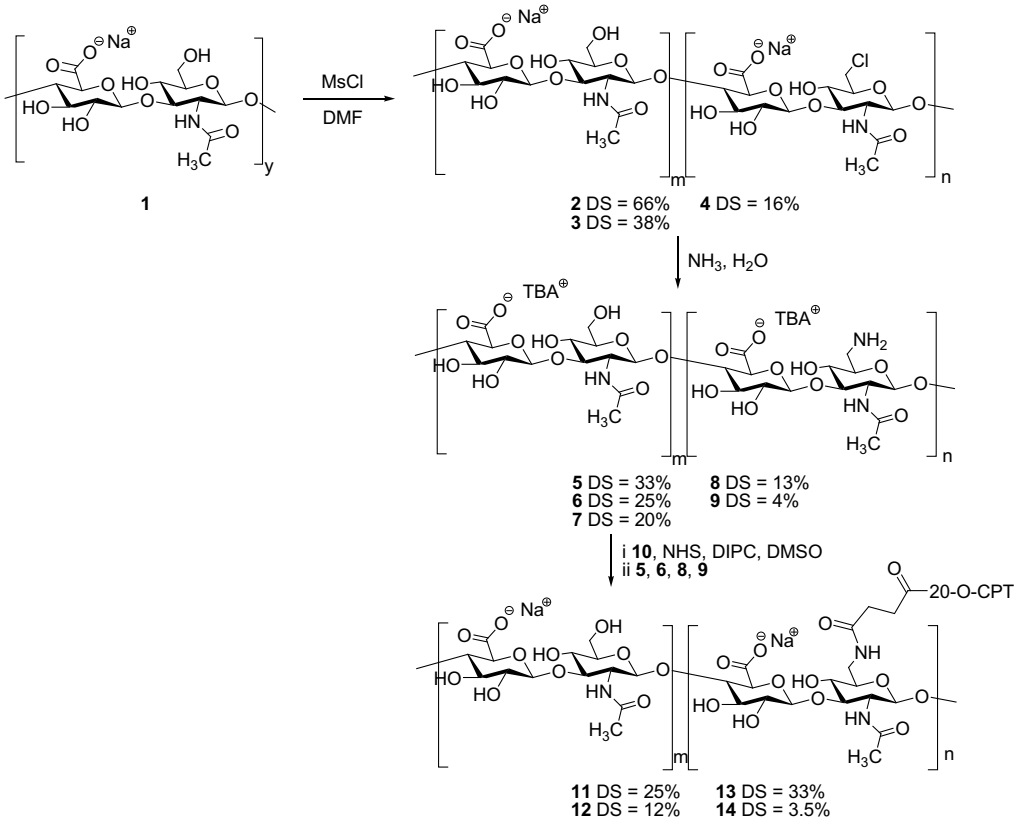
2. Results and discussion

Synthesis of primary amino derivatives of carbohydrates, and in particular of polysaccharides, has been achieved by substitution of the corresponding sulfonate ester or deoxyhalogeno derivative by azide, followed by reduction of the azido group to an amine.[31,32] The reaction of hyaluronan sodium salt (**1**) with methanesulfonyl chloride/*N*,*N*-dimethylformamide[33] resulted in the selective replacement of primary hydroxyl groups by a chloro group (compounds **2**–**4**, see Scheme 1).

This reagent has been successfully used for polysaccharides such as amylose,[34] pullulan,[35] laminaran,[31] cellulose,[36] and hyaluronan.[37]

Our initial reactions made use of the tetrabutylammonium salt of hyaluronan, which is soluble in *N*,*N*-dimethylformamide. However, this procedure was disappointing in terms of reproducibility of the degree of substitution (DS). This behavior could be attributed to the acidic environment of the reaction, leading to protonation and therefore gelification of hyaluronan, resulting in a non-homogeneous reaction mixture. Thus, we made use of hyaluronan sodium salt as a suspension in *N*,*N*-dimethylformamide, obtaining optimal reproducibility and control over the degree of substitution, which could be tuned by changing the reaction time. Quantification of chloro DS was based on the integration of ^{13}C NMR spectra, evaluating the peaks at 61 ppm (CH_2OH) and at 44.5 ppm (CH_2Cl).

The approximate linearity of chloro DS versus reaction time allowed the production of intermediates ranging from 16% to 66% DS mol/mol (with respect to the repeating disaccharide unit).



Scheme 1. Synthesis of conjugates starting from hyaluronan sodium salt (**1**).

Substitution by azide was then considered to obtain the amino derivative (Khan, R.; Vesnaver, R.; Konowicz, P. A.; Linda, P.; unpublished data). To this end, a simpler and one-step conversion to 6-amino-6-deoxyhyaluronan from the corresponding 6-chloro derivative was envisaged by heating the chloro compound in excess aqueous ammonium hydroxide, with the further advantage of using water as the solvent for the reaction. This procedure provided 6-amino-6-deoxyhyaluronans with a degree of substitution in the range 4–33% mol/mol as determined by ^{13}C NMR spectroscopy, with optimal control on the loading of amino groups, depending on starting chloride content and on reaction time. The structures of amino derivatives **5**–**9** were supported by their ^{13}C NMR, DEPT, and 2D heterocorrelated NMR spectra (Fig. 4), which showed in the ^{13}C NMR spectrum a decrease of the peak at 44.5 ppm due to CH_2–Cl and the appearance of a peak at 41 ppm due to CH_2–NH_2 (Fig. 3). A positive Kaiser test (ninhydrin test) confirmed the presence of primary amino groups.

The simplest linker suitable to anchor camptothecin to 6-amino-6-deoxyhyaluronan is succinic acid, whose use has already been reported for conjugates between paclitaxel and hyaluronan.[10,11,38] In that case, the 2′-*O*-hemisuccinate of paclitaxel could be activated as *N*-hydroxysuccinimidoyl ester, allowing mild coupling to hydrazide groups already introduced on hyaluronan.

We followed the same strategy making CPT-20-*O*-hemisuccinate (**10**),[39] by coupling CPT with succinic acid mono-*tert*-butyl ester, followed by treatment with trifluoroacetic acid. The synthesis of HA-CPT conjugates was achieved by classic activation of CPT-20-*O*-hemisuccinate (**10**) with *N*-hydroxysuccinimide/*N*,*N*-diisopropylcarbodiimide in dimethyl sulfoxide, followed by addition of 6-amino-6-deoxyhyaluronan, previously converted to its tetrabutylammonium (TBA) salt to achieve solubility in polar aprotic solvents.

The proton spectrum of conjugate **11** in D_2O (Fig. 4) shows a pattern of very broad signals that can be attributed to bound

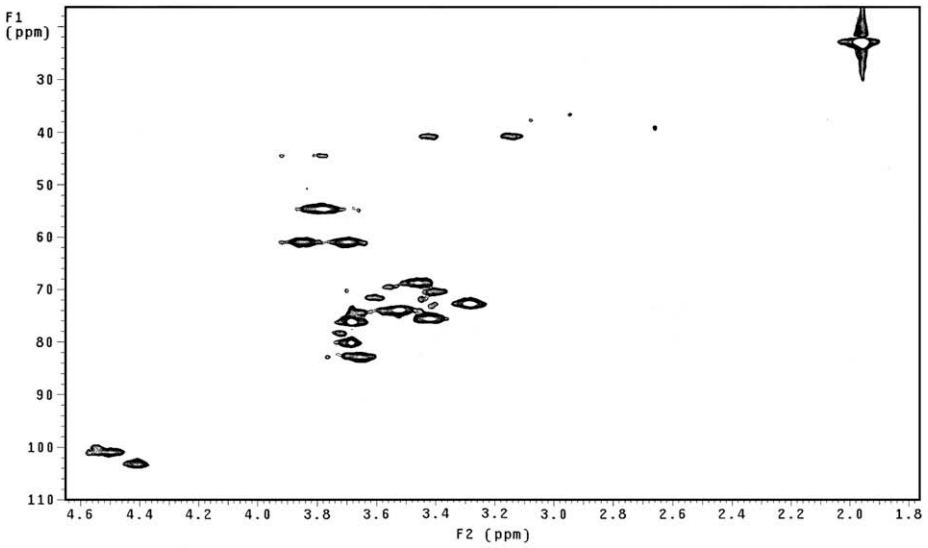


Figure 3. HSQC NMR spectrum of 6-amino-6-deoxyhyaluronan. Cross peaks between C-6 carbons and their diastereotopic protons are found at 41 ppm (^{13}C dimension) for $-CH_2NH_2$ and at 61 ppm for $-CH_2OH$.

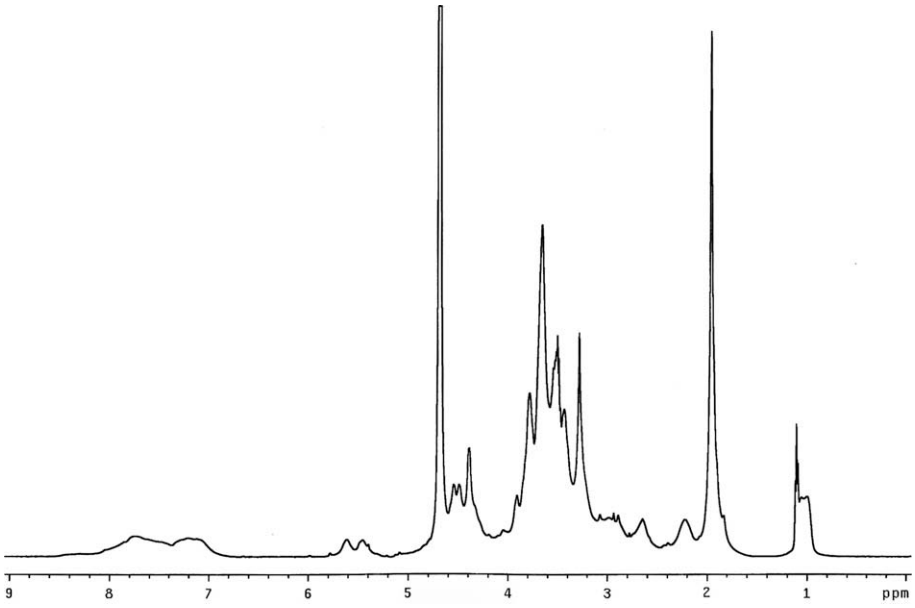


Figure 4. ^1H NMR spectrum of conjugate **11** in D_2O.

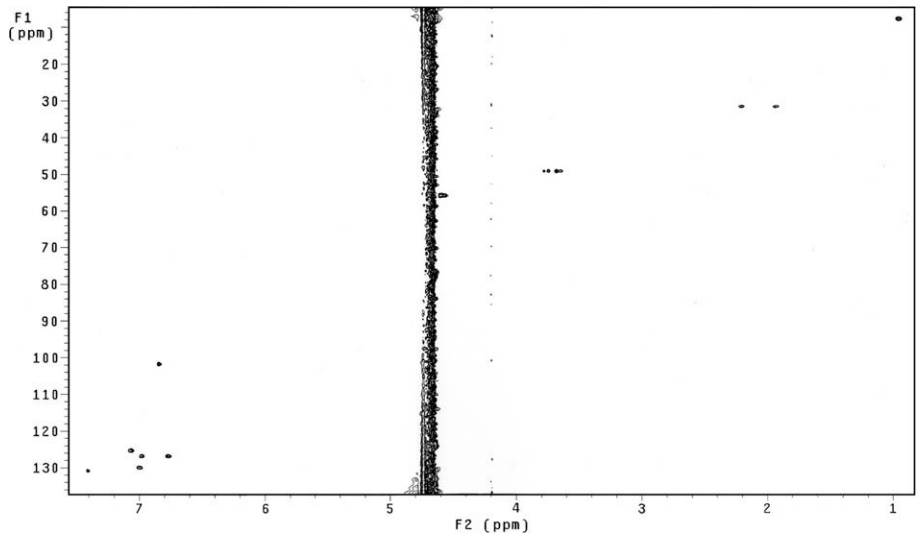


Figure 5. HSQC NMR spectrum of camptothecin in aq 0.1 N NaOH. The cross peaks of the exchangeable $ArCH_2N$ methylene group are visible at 3.62 and 3.68 ppm (^1H).

CPT-hemisuccinate. A DOSY (diffusion ordered spectroscopy) weighed proton NMR spectrum confirmed that these signals belong to a species bound to the hyaluronan chain.

The two doublets present between 5.5 and 5.8 ppm can be attributed to the lactone of bound CPT and integrate correctly with respect to other signals of bound CPT. Heterocorrelated NMR spectra supported this attribution and allowed the identification of both CPT-hemisuccinate signals and the newly formed amidic 6-CH_2 on the polymer.

Chemical shifts of bound CPT were also compared to reported CPT spectra in acidic and in basic D_2O,[40] showing high similarity to the spectrum in acidic D_2O, which suggests that CPT is present as its lactone form in the conjugate.

To complement the data of the paper reported above, spectra of CPT were recorded in aqueous base; the use of non-deuterated water avoided proton exchange of the $ArCH_2N$ methylene group, allowing complete assignment of peaks (Fig. 5).

Quantification of bound CPT in **11** was estimated to be around 25% mol/mol by ^1H NMR spectroscopy. The loading was confirmed by adding solid LiOH monohydrate to the NMR tube.

Complete hydrolysis at high pH was justified by the aforementioned study[16] of CPT in aqueous solution, which demonstrated that CPT is present solely in its lactone form at pH 8 or higher, and that no further transformations are observed even at pH 14. After a few minutes a new proton spectrum was taken, which showed complete hydrolysis of CPT from the conjugate; the sharp signals of the salt of the open-ring carboxylate form of CPT, freely soluble in water, could be precisely integrated against the signals of HA, confirming the DS to be 25% mol/mol. In this spectrum the signals of the succinate chain are present as two broad multiplets at two different chemical shifts for the two methylene groups, indicating that succinate is still bound to the polymer; this was confirmed by a DOSY weighed proton NMR spectrum. After a week at pH 13, NMR spectroscopy showed that hemisuccinate was still completely bound to the polymer.

In conjugate **11**, derived from 6-amino-6-deoxyhyaluronan **6** (amine DS 25% mol/mol), no traces of free primary amines were detected by NMR spectroscopy, indicating complete conversion of the amino groups.

These experiments clearly demonstrate that CPT-20-*O*-hemisuccinate was conjugated exclusively through amide bonds to 6-amino-6-deoxyhyaluronan. In an independent reaction, native HA in its TBA salt form was used, under the same conjugation conditions, and no bound CPT was detected after isolation of the polymer.

Finally, 1D and 2D NMR spectra of pure open ring CPT sodium salt in which the lactone is completely hydrolyzed were superimposed on the spectra of the hydrolyzed conjugate, indicating complete preservation of the structure of CPT during activation and conjugation reactions.

Reported hyaluronan–drug conjugates generally either exploit the carboxyl group of the glucuronic acid sugar or involve all the hydroxyl groups without control on the regioselectivity. Regarding the latter strategy, butyrate esters[15,41,42] showed a maximum efficacy for a DS of 19% mol/mol, for which the internalization mediated by CD44 was demonstrated, despite the rather significative substitution. It is known[43] that at least six sugars (three repeating dimers) are required for a monovalent binding of hyaluronan to CD44; this would mean that a modification of hyaluronan of up to 25% mol/mol could be allowed, leaving statistically portions of native chains consisting of three dimers between two substituents.

However, recent crystallographic structures of murine CD44–HA complexes[44] (Protein Data Bank: 2JCQ, 2JCR) show how the C-6 hydroxyl groups are basically not involved in recognition, justifying higher substitution levels on hyaluronan which should not significantly affect recognition.

The approach described in this work provides a tool to link molecules specifically on the C-6 of *N*-acetylglucosamine residues of hyaluronan, providing the best theoretical situation for efficient recognition.

Furthermore, the free water solubility of the conjugates, accompanied by a solubility, with respect to bound camptothecin, in the order of 10^{-3} M in physiological solution (0.15 M NaCl) makes these derivatives appealing for further development.

The antitumor activity of new hyaluronan derivatives prepared is at present under evaluation.

3. Experimental

3.1. Materials and methods

Hyaluronan sodium salt (20,000 g/mol) was purchased from Bioiberica (Spain). 20-(*S*)-Camptothecin was purchased from Sunray Pharmaceutical Company Ltd (China). NMR spectra were recorded on Varian Mercury 200 and on Varian Inova 500 NMR spectrometers, in D_2O solutions, unless otherwise noted.

Heteronuclear (^{1}H–^{13}C) 2D chemical shift correlation spectroscopy was performed using gHSQCAD (^{1}H–^{13}C) and gHMBCAD (^{1}H–^{13}C long range) pulse sequences.

The tetrabutylammonium (TBA) content in compounds **5–9** was measured by ^{1}H NMR spectroscopy, by integration of the TBA methyl peak at 0.9 ppm versus the HA methyl peak at 2.0 ppm. TBA content of 100–105% was measured for all derivatives.

The degree of chloro substitution (DS%, mole/mole) in compounds **2–4** was calculated from their ^{13}C NMR spectra, from the integral (I) of CH_2Cl signal and from the sum of CH_2Cl and CH_2OH integrals: $DS_{Cl} = 100 \times I_{CH_2Cl/(I_{CH_2OH}+I_{CH_2Cl})}$. A standard ^{13}C NMR sequence was used, assuming similar relaxation times for CH_2OH and CH_2Cl signals. Using a quantitative ^{13}C NMR sequence led to very small differences; however, these were within the usual error of this measurement.

Similarly, the degree of amine substitution (DS%, mole/mole) in compounds **5–9** was calculated from their ^{13}C NMR spectra, from the integral (I) of CH_2NH_2 signal and from the sum of CH_2NH_2, residual CH_2Cl, and CH_2OH integrals: $DS_{NH_2} = 100 \times I_{CH_2NH_2}/(I_{CH_2NH_2} + I_{CH_2OH} + I_{CH_2Cl})$.

The DS of CPT in HA–CPT conjugates **11–14** was calculated from their proton spectra, by integration of the six aromatic protons of CPT (6.6–8.7 ppm) and the three methyl protons of HA at 1.95 ppm.

Water content of the conjugates was measured by thermogravimetric analysis (TGA). The molecular weight (M_w) and molecular weight distribution of the hyaluronan derivatives were measured by HP–SEC (column: TSK PWxl from TosoBioscience, G6000 + G5000 + G3000 6, 10, 13 μm particle size) at 40 °C (mobile phase: NaCl 0.15 M + 0.01% NaN_3) coupled with a MALLS detector (WYATT DAWN EOS—WYATT, USA, λ = 690 nm, dn/dc = 0.167 mL/g), a UV detector (λ = 370 nm), and an interferometric refractive index detector. The analysis allows the measurement of M_w (weight average molecular weight), M_n (number average molecular weight), and P.I. (polydispersity index). Detector calibrations were performed using a standard BSA solution. Tangential flow filtrations were performed using a Centramate system from Pall, with 10,000 g/mol MWCO suspended screen membranes. The quantities in moles for hyaluronan and its derivatives in the examples are always referred to the repeating disaccharide unit.

3.2. 6-Chloro-6-deoxyhyaluronan, Na salt (2)

Hyaluronan sodium salt (50 g, 125 mmol) was suspended in 900 mL of dry DMF under nitrogen, with mechanical stirring at 20 °C. The suspension was then cooled to −10 °C, and 97 mL (1.25 mol) of methanesulfonyl chloride was added for 30 min. After an additional 30 min at −10 °C, the temperature was raised to 20 °C. After 1 h the temperature was gradually raised (during 1 h) to 60 °C, and stirring was continued for 18.5 h. The reaction mixture was then poured in portions into a mixture of ice and 1 M sodium carbonate (4 L) with vigorous mixing, maintaining the pH at 9.5 by addition of 1.5 M NaOH when required. The resulting brownish suspension was stirred at pH 9.5 at room temperature for about 48 h, whereupon a clear solution formed. This was filtered to remove traces of solids and then subjected to tangential flow filtration. The resulting solution was concentrated in a rotary evaporator to a final volume of about 1 L, and freeze-dried to afford 34.9 g (68%) of an off-white solid (DS 66% mol/mol). (NMR signals due to the Cl-substituted ring are marked by an underscript Cl.) ^{1}H NMR: δ 1.95 (br s, CH_3), 3.28 (m, H-2′), 3.42 (m, H-5), 3.45 (m, H-4), 3.52 (m, H-3′), 3.55 (m, H-4_{Cl}), 3.64 (m, H-5′), 3.65 (m, H-5_{Cl}), 3.67 (m, H-4′), 3.68 (m, H-3), 3.70 (m, H-6a), 3.79 (m, H-2), 3.79 (m, H-$6a_{Cl}$), 3.84 (m, H-6b), 3.92 (m, H-$6b_{Cl}$), 4.49 (m, H-1′), 4.55 (m, H-1), 4.60 (m, H-1_{Cl}); ^{13}C NMR: δ 22.6 (CH_3), 44.0 (CH_2Cl), 54.4 (C-2), 60.7 (CH_2OH), 68.6 (C-4), 69.3 (C-4_{Cl}), 72.6 (C-2′), 73.8 (C-3′), 74.1 (C-5_{Cl}), 75.5 (C-5), 76.3 (C-5′), 80.5 (C-4′), 82.3 (C-3), 101.0 (C-1), 103.4 (C-1′), 174.0, 174.1, 175.0; M_w 17,150, M_n 11,400, P.I. 1.5; water content 7.5% w/w (TGA).

3.3. 6-Chloro-6-deoxyhyaluronan, Na salt (3)

Following the procedure used for **2**, starting from 50 g (125 mmol) of hyaluronan sodium salt and maintaining the heating at 60 °C for 12 h, 33.5 g (66%) of an off-white solid was obtained (DS 38% mol/mol). ^{1}H and ^{13}C NMR spectra were similar, as for chemical shifts, to the spectra of **2**. M_w 18,650, M_n 8,900, P.I. 2.1; water content 8% w/w (TGA).

3.4. 6-Chloro-6-deoxyhyaluronan, Na salt (4)

Following the procedure used for **2**, starting from 50 g (125 mmol) of hyaluronan sodium salt and maintaining the heating at 60 °C for 7 h, 33.1 g (66%) of an off-white solid was obtained (DS 16% mol/mol). ^{1}H and ^{13}C NMR spectra were similar, as for chemical shifts, to the spectra of **2**. M_w 18,350, M_n 9670, P.I. 1.9; water content 9.2% w/w (TGA).

3.5. 6-Amino-6-chloro-6-deoxyhyaluronan, TBA salt (5)

Compound **2** (5.0 g, 12.5 mmol) was dissolved in 100 mL of concd NH_4OH, contained in a pressure-tight steel vessel. The solution was heated at 80 °C for 21 h, at the end of which time it was cooled, and excess ammonia was removed under vacuum. The solution was then neutralized with dilute HCl solution. After tangential flow filtration, the solution was sampled for NMR analysis on the sodium salt of **5**. The rest of the solution was treated with Amberlite IRA-120 (TBA form). Then it was filtered and freeze-dried to afford 5.43 g (70%) of **5** as an off-white solid (amino DS 33% mol/mol; chloro DS 31% mol/mol). ^{1}H NMR: δ 1.95 (br s, 3H, CH_3), 3.11 (m, 0.33H, CH_2NH$_2$), 3.25–3.70 (m, 7H), 3.42 (m, 0.33H, CH_2NH$_2$), 3.70 (m, H-6a), 3.77 (m, 1H, H-2), 3.79 (m, CH_2Cl), 3.85 (m, H-6b), 3.92 (m, CH_2Cl), 4.49 (m, H-1′), 4.55 (m, H-1), 4.60 (m, H-1); ^{13}C NMR: δ 22.6 (CH_3), 40.5 (CH_2NH_2), 44.0 (CH_2Cl), 54.4, 60.7 (C-6), 68.6, 69.3, 70.5, 72.0, 72.6, 73.8, 75.5, 76.3, 78.2, 80.1, 80.9, 82.3, 99.7, 101.0, 103.4, 174.0, 174.1, 175.0; M_w 17,200, M_n 9,600, P.I. 1.8; water content 6.9% w/w (TGA).

3.6. 6-Amino-6-chloro-6-deoxyhyaluronan, TBA salt (6)

Following the procedure used for **5**, starting from 5.0 g (12.5 mmol) of compound **2**, heating at 80 °C for 38 h, 5.9 g (76%) of an off-white solid was obtained. Amino DS 25% mol/mol; chloride DS 40% mol/mol. ^{1}H and ^{13}C NMR spectra were similar, as for chemical shifts, to the spectra of **5**. M_w 16,400, M_n 10,250, P.I. 1.6; water content 9.0% w/w (TGA).

3.7. 6-Amino-6-chloro-6-deoxyhyaluronan, TBA salt (7)

Following the procedure used for **5**, starting from 5.0 g (12.5 mmol) of compound **3**, heating at 80 °C for 22 h, 6.51 g (85%) of an off-white solid was obtained. Amino DS 20% mol/mol; chloro DS 18% mol/mol. ^{1}H and ^{13}C NMR spectra were similar, as for chemical shifts, to the spectra of **5**. M_w 14,500, M_n 9,700, P.I. 1.5; water content 7.9% w/w (TGA).

3.8. 6-Amino-6-chloro-6-deoxyhyaluronan, TBA salt (8)

Following the procedure used for **5**, starting from 5.0 g (12.5 mmol) of compound **4**, heating at 80 °C for 22 h, 6.43 g (83%) of an off-white solid was obtained. Amino DS 13% mol/mol; chloro DS <5% mol/mol. ^{1}H and ^{13}C NMR spectra were similar,

as for chemical shifts, to the spectra of **5**. M_w 14,450, M_n 10,350, P.I. 1.4; water content 6.9% w/w (TGA).

3.9. 6-Amino-6-chloro-6-deoxyhyaluronan, TBA salt (9)

Following the procedure used for **5**, starting from 5.0 g (12.5 mmol) of compound **4**, heating at 80 °C for 7 h, 5.95 g (77%) of an off-white solid was obtained. Amino DS 4% mol/mol; chloro DS 12% mol/mol. ^{1}H and ^{13}C NMR spectra were similar, as for chemical shifts, to the spectra of **5**. M_w 14,050, M_n 10,050, P.I. 1.4; water content 10.1% w/w (TGA).

3.10. Camptothecin-20-*O*-hemisuccinate[38] (10)

To a solution of 2.98 g (17.1 mmol) of succinic acid mono-*tert*-butyl ester and 1.40 g (11.5 mmol) of *p*-dimethylaminopyridine in 200 mL of CH_2Cl_2 were added, while stirring at room temperature, 2.68 mL (17.3 mmol) of diisopropylcarbodiimide and 3.00 g (8.62 mmol) of CPT. After stirring overnight, the resulting suspension was diluted with 80 mL of CH_2Cl_2 to obtain a solution that was washed with 0.1 N HCl solution and dried over anhyd Na_2SO_4. Then it was filtered and evaporated to dryness on a rotary evaporator. The residue was crystallized with 100 mL of MeOH, filtered, and washed with MeOH. After drying on the filter, the solid was treated with 50 mL of a 40% v/v solution of trifluoroacetic acid in CH_2Cl_2, and after 1 h standing at room temperature, the resulting greenish solution was evaporated to dryness on a rotary evaporator. The residue was crystallized with 100 mL of MeOH, filtered, and washed with MeOH and diethyl ether. After drying in vacuo, 3.67 g (95%) of a pale-yellow solid was obtained: mp 229 °C (dec.); ^{1}N NMR (DMSO-d_6): δ 0.92 (t, 3H, CH_3; J 7.3 Hz), 2.16 (q, 2H, CH_2CH$_3$; J 7.3 Hz), 2.49 (m, 2H, CH_2COOH), 2.77 (tq, 2H, CH_2COOR; J 17.8, 6.8 Hz), 5.28 (d, 1H, CH_2N; J 19.0 Hz), 5.31 (d, 1H, CH_2N; J 19.0 Hz), 5.49 (2 × d, 2H, CH_2O; J 17.8 Hz), 7.13 (s, 1H, C-14*H*), 7.72 (m, 1H, Ar), 7.87 (m, 1H, Ar), 8.13 (m, 1H, Ar), 8.18 (m, 1H, Ar), 8.69 (s, 1H, C-7*H*), 12.24 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6): δ 7.5 (CH_3), 28.4 (CH_2), 28.6 (CH_2), 30.4 (CH_2CH$_3$), 50.1 (CH_2N), 66.3 (CH_2O), 75.9 (*C*-Et), 95.1 (C-14), 118.9 (C-16), 127.7 (Ar), 127.9 (Ar), 128.5 (Ar), 129.0 (Ar), 129.7 (Ar), 130.4 (Ar), 131.5 (Ar), 145.3 (C-3), 145.9 (C-13), 147.8 (C-15), 152.3 (C-2), 156.5 (C-16a), 167.2 (C═O), 171.3 (C═O), 173.0 (C═O).

3.11. 20-*O*-Camptothecin 3-(6-carbamoyl-6-chloro-6-deoxyhyaluronan)propanoate, sodium salt (11)

To a solution of 1.03 g (2.30 mmol) of **10** and 318 mg (2.76 mmol) of *N*-hydroxysuccinimide in 30 mL of dimethyl sulfoxide was added, with stirring under nitrogen at room temperature, 356 μL (2.30 mmol) of diisopropylcarbodiimide. After 16 h, 1.50 g (2.30 mmol) of **6** was added and stirring was continued for 5 h. Satd NaCl solution (3.0 mL) was then added, and stirring was continued for 30 min. The mixture was poured into 120 mL of EtOH while stirring, and the resulting slurry was stirred for 10 min and then filtered, and washed with EtOH. The solid was suspended in 100 mL of DMF, slurried for 30 min, filtered and washed once with DMF and twice with MeOH. After drying on the filter, the solid was dissolved in 100 mL of water and dialyzed against purified water. Then the solution was filtered through a 0.22-μm pore size membrane and freeze-dried to give 1.01 g (86%) of a white solid. DS in CPT: 25% mol/mol; chloro DS 40% mol/mol. ^{1}H NMR: δ 1.1 (br m, 0.75 H, CH_3, CPT), 0.95 (br s, 3H, NHAc), 2.15 (br m, 0.5H, CH_2CH$_3$, CPT), 2.7 (br m, 0.5H, CH_2, hemisuccinate), 3.0 (br m, 0.5H, CH_2, hemisuccinate), 3.38 (br m, 1H, C2-*H*), 3.5–3.9 (m, HA), 3.7 (br m, 0.75H, CH_2OH, HA), 3.85 (br m, 0.75H, CH_2OH, HA), 3.82 (br m, 0.25H, C-6H_2NH, HA), 3.98 (br m, 0.25H, C-6H_2NH, HA), 4.5 (br m, 1H, HA), 4.6 (br d, 1H, HA), 5.52 (br d, 0.25H, CH_2O, CPT), 5.64 (br d, 0.25H, CH_2O, CPT), 6.9–8.6 (br m, 1.5H, Ar, CPT); M_w 18,540, M_n 11,600, P.I. 1.6; water content 9.0% w/w (TGA).

3.12. 20-*O*-Camptothecin 3-(6-carbamoyl-6-chloro-6-deoxyhyaluronan)propanoate, sodium salt (12)

Following the procedure used for **11**, starting from 1.03 g (2.30 mmol) of **10**, 400 mg (3.48 mmol) of *N*-hydroxysuccinimide in 30 mL of DMSO, 356 μL (2.30 mmol) of diisopropylcarbodiimide, and 1.50 g (2.30 mmol) of **8**, 0.99 g (95%) of a white solid was obtained. DS in CPT: 12% mol/mol; chloro DS <5% mol/mol. ^{1}H NMR: δ 1.1 (br m, 0.36 H, CH_3, CPT), 0.95 (br s, 3H, NHAc), 2.15 (br m, 0.24H, CH_2CH$_3$, CPT), 2.7 (br m, 0.24H, CH_2, hemisuccinate), 3.0 (br m, 0.24H, CH_2, hemisuccinate), 3.38 (br m, 1H, C2-*H*), 3.5–3.9 (m, HA), 3.7 (br m, 0.88H, CH_2OH, HA), 3.85 (br m, 0.88H, CH_2OH, HA), 3.82 (br m, 0.12H, C-6H_2NH, HA), 3.98 (br m, 0.12H, C-6H_2NH, HA), 4.5 (br m, 1H, HA), 4.6 (br d, 1H, HA), 5.52 (br d, 0.12H, CH_2O, CPT), 5.64 (br d, 0.12H, CH_2O, CPT), 6.9–8.6 (br m, 0.72H, Ar, CPT); M_w 16,550, M_n 11, 050, P.I. 1.5; water content 7.7% w/w (TGA).

3.13. 20-*O*-Camptothecin 3-(6-carbamoyl-6-chloro-6-deoxyhyaluronan)propanoate, sodium salt (13)

Following the procedure used for **11**, starting from 1.03 g (2.30 mmol) of **10**, 400 mg (3.48 mmol) of *N*-hydroxysuccinimide in 30 mL of dimethyl sulfoxide, 356 μL (2.30 mmol) of diisopropylcarbodiimide and 1.50 g (2.30 mmol) of **5**, 1.26 g (100%) of a white solid was obtained. DS in CPT: 33% mol/mol; chloro DS 31% mol/mol. ^{1}H NMR: δ 1.1 (br m, 0.99H, CH_3, CPT), 0.95 (br s, 3H, NHAc), 2.15 (br m, 0.66H, CH_2CH$_3$, CPT), 2.7 (br m, 0.66H, CH_2, hemisuccinate), 3.0 (br m, 0.66H, CH_2, hemisuccinate), 3.38 (br m, 1H, C2-*H*), 3.5 – 3.9 (m, HA), 3.7 (br m, 0.67H, CH_2OH, HA), 3.85 (br m, 0.67H, CH_2OH, HA), 3.82 (br m, 0.33H, C-6H_2NH, HA), 3.98 (br m, 0.33H, C-6H_2NH, HA), 4.5 (br m, 1H, HA), 4.6 (br d, 1H, HA), 5.52 (br d, 0.33H, CH_2O, CPT), 5.64 (br d, 0.33H, CH_2O, CPT), 6.9 – 8.6 (br m, 2.0H, Ar, CPT); M_w 14,750, M_n 9250, P.I. 1.6; water content 9.6% w/w (TGA).

3.14. 20-*O*-Camptothecin 3-(6-carbamoyl-6-chloro-6-deoxyhyaluronan)propanoate, sodium salt (14)

Following the procedure used for **11**, starting from 867 mg (1.94 mmol) of **10**, 334 mg (2.90 mmol) of *N*-hydroxysuccinimide in 30 mL of dimethyl sulfoxide, 300 μL (1.94 mmol) of diisopropylcarbodiimide, and 1.50 g (2.30 mmol) of **9**, 0.96 g (100%) of a white solid was obtained. DS in CPT: 3.5% mol/mol; chloride DS 12% mol/mol. ^{1}H NMR: δ 1.1 (br m, 0.11 H, CH_3, CPT), 0.95 (br s, 3 H, NHAc), 2.15 (br m, 0.07H, CH_2CH$_3$, CPT), 2.7 (br m, 0.07H, CH_2, hemisuccinate), 3.0 (br m, 0.07H, CH_2, hemisuccinate), 3.38 (br m, 1H, C2-*H*), 3.5–3.9 (m, HA), 3.7 (br m, 0.96H, CH_2OH, HA), 3.85 (br m, 0.96H, CH_2OH, HA), 3.82 (br m, 0.035H, C-6H_2NH, HA), 3.98 (br m, 0.035H, C-6H_2NH, HA), 4.5 (br m, 1H, HA), 4.6 (br d, 1H, HA), 5.52 (br d, 0.035H, CH_2O, CPT), 5.64 (br d, 0.035H, CH_2O, CPT), 6.9–8.6 (br m, 0.21H, Ar, CPT); M_w 20.050, M_n 15,400, P.I. 1.3; water content 10.1% w/w (TGA).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.09.027.

References

1. Turley, E. A.; Noble, P. W.; Bourguignon, L. Y. W. *J. Biol. Chem.* **2002**, *27*, 4589–4592.
2. Toole, B. P. *Glycobiology* **2002**, *12*, 37R–42R.

3. *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Ltd, 2004.
4. Day, A. J.; Prestwich, G. D. *J. Biol. Chem.* **2002**, *277*, 4585–4588.
5. Turley, E. A.; Belch, A. J.; Poppema, S.; Pilarski, L. M. *Blood* **1993**, *81*, 446–453.
6. Hua, Q.; Knudson, C. B.; Knudson, W. J. *Cell Sci.* **1993**, *106*, 365–375.
7. Huang, L.; Grammatikakis, N.; Yoneda, M.; Banerjee, S. D.; Toole, B. P. *J. Biol. Chem.* **2000**, *275*, 29829–29839.
8. Ponta, H.; Sherman, L.; Herrlich, P. A. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 33–45.
9. Hua, Q.; Knudson, C. B.; Knudson, W. J. *Cell Sci.* **1993**, 106, 365.
10. Luo, Y.; Prestwich, G. D. *Bioconjugate Chem.* **1999**, *10*, 755–763.
11. Luo, Y.; Ziebell, M. R.; Prestwich, G. D. *Biomacromolecules* **2000**, *1*, 208–218.
12. Rosato, A.; Banzato, A.; De Luca, G.; Renier, D.; Bettella, F.; Pagano, C.; Esposito, G.; Zanovello, P.; Bassi, P. *Urol. Oncol.* **2006**, *24*, 207–215.
13. Luo, Y.; Bernshaw, N. J.; Lu, Z.-R.; Kopecek, J.; Prestwich, G. D. *Pharm. Res.* **2002**, *19*, 396–402.
14. Akima, K.; Iwata, Y.; Matsuo, K.; Watar, N. U.S. Patent 5,733,891, 1998.
15. Coradini, D.; Pellizzaro, C.; Miglierini, G.; Daidone, M. G.; Perbellini, A. *Int. J. Cancer* **1999**, *81*, 411–416.
16. Giovanella, B.; Hinz, H.; Kozielski, A.; Stehlin, J.; Silber, R.; Potmesil, M. *Cancer Res.* **1991**, *51*, 3052–3055.
17. Thomas, C. J.; Rahier, N. J.; Hecht, S. M. *Bioorg. Med. Chem.* **2004**, *12*, 1585–1604 and references cited therein.
18. Saltz, L. B.; Cox, J. V.; Blanke, C.; Rosen, L. S.; Fehrenbacher, L.; Moore, M. J.; Maroun, J. A.; Ackland, S. P.; Locker, P. K.; Pirotta, N.; Elfring, G. L.; Miller, L. L. *New Engl. J. Med.* **2000**, *343*, 905–914.
19. Vanhoefer, U.; Harstrick, A.; Achterrath, W.; Cao, S.; Seeber, S.; Rustum, Y. M. J. *Clin. Oncol.* **2001**, *19*, 1501–1518.
20. Creemers, G. J.; Bolis, G.; Gore, M.; Scarfone, G.; Lacave, A. J.; Guastalla, J. P.; Despax, R.; Favalli, G.; Kreinberg, R.; Van Belle, S.; Hudson, I.; Verweij, J.; Ten Bokkel Huinink, W. W. *J. Clin. Oncol.* **1996**, *14*, 3056–3061.
21. Li, Q.-Y.; Zu, Y.-G.; Shi, R.-Z.; Yao, L.-P. *Curr. Med. Chem.* **2006**, *13*, 2021–2039.
22. Pizzolato, J. F.; Saltz, L. B. *Lancet* **2003**, *361*, 2235–2242.
23. Cao, Z.; Harris, N.; Kozielski, A.; Vardeman, D.; Stehlin, J. S.; Giovanella, B. *J. Med. Chem.* **1998**, *41*, 31–37 and references cited therein.
24. Conover, C. D.; Greenwald, R. B.; Pendri, A.; Shum, K. L. *Anti-Cancer Drug Des.* **1999**, *14*, 499–506.
25. Greenwald, R. B.; Pendri, A.; Conover, C. D.; Gilbert, C.; Yang, R.; Xia, J. *J. Med. Chem.* **1996**, *39*, 1938–1940.
26. Caiolfa, V. R.; Zamai, M.; Fiorino, A.; Frigerio, E.; Pellizzoni, C.; D'Argy, R.; Ghiglieri, A.; Castelli, M. G.; Farao, M.; Pesenti, E.; Gigli, M.; Angelucci, F.; Suarato, A. *J. Controlled Release* **2000**, *65*, 105–119.
27. Fujita, F.; Koike, M.; Fujita, M.; Sakamoto, Y.; Okuno, S.; Kawaguchi, T.; Yano, S.; Kiuchi, S.; Fujiwara, T.; Kudho, S.; Kakushima, M. *Clin. Cancer Res.* **2005**, *11*, 1650–1657.
28. Bhatt, R.; De Vries, P.; Tulinsky, J.; Bellamy, G.; Baker, B.; Singer, J. W.; Klein, P. *J. Med. Chem.* **2003**, *46*, 190–193.
29. Cheng, J.; Khin, K. T.; Jensen, G. S.; Liu, A.; Davis, M. E. *Bioconjugate Chem.* **2003**, *14*, 1007–1017.
30. Li, C.; Wallace, S. *Adv. Drug Delivery Rev.* **2008**, *60*, 886–898.
31. Khan, R.; Bosco, M.; Konowicz, P. A.; Stucchi, L.; Rizzo, R. *Carbohydr. Res.* **1996**, *292*, 39–46.
32. Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F. *Carbohydr. Res.* **2005**, *340*, 1403–1406.
33. Evans, M. E.; Long, L., Jr.; Parish, F. W. *J. Org. Chem.* **1968**, *33*, 1074–1076.
34. Cimecioglu, A. L.; Ball, D. H.; Kaplan, D. L.; Huang, S. H. *Macromolecules* **1994**, *27*, 2917–2922.
35. Mocanu, G.; Constantin, M.; Carpov, A. *Angew. Makromol. Chem.* **1996**, *241*, 1–10.
36. Ishi, T.; Ishizu, A.; Nakano, J. *Carbohydr. Res.* **1977**, *59*, 155–163.
37. Khan, R.; Vesnaver, R.; Stucchi, L.; Bosco, M. EP Patent 1,023,328, 1999; *Chem. Abstr.*, **1999**, 244688.
38. Leonelli, F.; La Bella, A.; Migneco, L. M.; Marini Bettolo, R. *Molecules* **2008**, *13*, 360–378.
39. Lu, H.; Lin, H.; Jiang, Y.; Zhou, X.; Wu, B.; Chen, J. *Lett. Drug Design Discovery* **2006**, *3*, 83–86.
40. Dey, J.; Warner, I. M. *J. Photochem. Photobiol. A: Chem.* **1996**, *101*, 21–27.
41. Coradini, D.; Pellizzaro, C.; Abolafio, G.; Bosco, M.; Scarlata, I.; Cantoni, S.; Stucchi, L.; Zorzet, S.; Turrin, C.; Sava, G.; Perbellini, A.; Daidone, M. G. *Invest. New Drugs* **2004**, *22*, 207–217.
42. Coradini, D.; Zorzet, S.; Rossin, R.; Scarlata, I.; Pellizzaro, C.; Turrin, C.; Bello, M.; Cantoni, S.; Speranza, A.; Sava, G.; Mazzi, U.; Perbellini, A. *Clin. Cancer Res.* **2004**, *10*, 4822–4830.
43. Lesley, J.; Hascall, V.; Tammi, M.; Hyman, R. *J. Biol. Chem.* **2000**, *275*, 26967–26975.
44. Banerji, S.; Wright, A. J.; Nobel, M.; Mahoney, D. J.; Campbell, I. D.; Day, A. J.; Jackson, D. G. *Nat. Struct. Mol. Biol.* **2007**, *14*, 234–239.