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New Conjugates of Hyaluronic Acid with γ-Cyclodextrin as Sorafenib Carrier in Cancer Cells

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In recent years, nanoparticles based on cyclodextrins have been widely investigated, mainly for drug delivery. In this work, we synthesized nanoparticles with a hyaluronic acid backbone (11 kDa and 45 kDa) functionalized with γ-cyclodextrins. We tested sorafenib in the presence of the new hyaluronan-cyclodextrin conjugates in A2780 (ovarian cancer), SK-HeP-1 (adenocarcinoma) and MDA-MB-453 (breast cancer) cell lines. We found that hyaluronan-cyclodextrin conjugates improve the antiproliferative activity of sorafenib. Remarkably, the system based on the 11 kDa hyaluronan conjugate was the most effective and, in the MDA-MB-453 cell line, significantly reduced the IC_{50} value of sorafenib cells by about 75%.

Introduction

In recent years, nanoparticles (NP) have proven to improve drug activity and increase the differential accumulation in tumor cells.[1] Cyclodextrins (CyD) are biocompatible molecules widely used to build a variety of NPs.[2] CyDs are cyclic oligosaccharides of α-1,4-linked d (+)-glucopyranose units.[3] They are widely employed in pharmaceutical formulations due to their ability to act as molecular containers. The three most common structures are α, β and γ, which contain six, seven and eight glucose units.[4] Furthermore, the ability to functionalize the hydroxyl groups of the CyD cavity allows for its incorporation into various systems. Polymeric NPs have been synthesized by conjugating CyDs with polymeric backbones such as polypeptides or polysaccharides.[5–9] CyD-based polymeric NPs can exploit the enhanced permeability retention (EPR) effect and active targeting by appropriate moieties recognized by specific receptors. Hyaluronic acid or hyaluronan (HA) has been widely used to build targeted systems.[10] HA is a linear glycosaminoglycan, composed of a repeating disaccharide of D-glucuronic acid and N-acetyl-D-glucosamine linked by β (1→3) and (1→4) linkages,[10] widely present as a component in the extracellular matrix (ECM).[11] It is broadly employed in targeted therapy against cancer because it can recognize different receptors. The principal receptor is CD44,[12] a transmembrane glycoprotein that plays a role in the initiation and progression of cancer. Tumor-derived cells over-express CD44 in a high-affinity state, which can promote the binding and internalization of HA.[13]

In the last years, HA has been conjugated with CyDs using a variety of strategies.[14–17] HACyD conjugates are not cytotoxic and have been studied as delivery systems of therapeutic agents such as phenol,[18] curcumin[19] or anticancer drugs.[14,19–22]

We recently synthesized polymers based on hyaluronic acid with βCyD.[23] We found that functionalizing the HA with CyDs can increase the solubility of doxorubicin and improve its antiproliferative activity. Expanding our research interests, we synthesized two new polymers (Figure 1) based on HA at

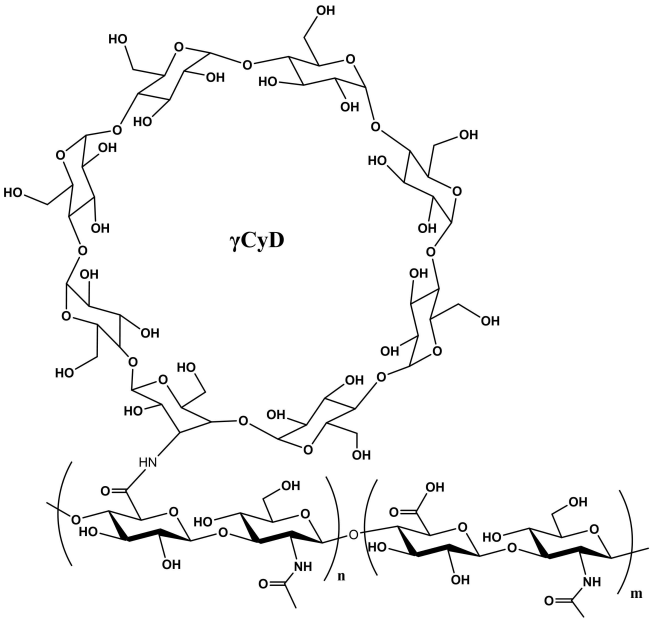

Figure 1. Hyaluronic acid γ-cyclodextrin conjugates HAHγCyD and HALγCyD.

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different molecular weights (11 kDa and 45 kDa) conjugated with γCyDs. Molecular weight (MW) of HA can be crucial for diverse physio-pathological functions and other biological activities.[24–26] The dimensions of the γCyD cavity may enhance the interaction with larger guests, increasing their bioavailability and solubility.[19] We tested these polymers as drug delivery systems of Sorafenib (SFN). SFN is an inhibitor of kinases approved for treating kidney, liver, and thyroid carcinoma.[27] SFN exhibits many side effects, such as hypertension and bilirubin elevation. In addition, SFN is poorly soluble in water. The SFN tosylate salt has improved water solubility compared to the free base form and has been used and prepared into tablets (Nexavar, Bayer HealthCare Pharmaceuticals-Onyx Pharmaceuticals).[28] SFN also has limited oral bioavailability, ranging from 38% to 49%, due to its limited solubility in aqueous solutions.[29]

Nevertheless, SFN remains one of the most used drugs against hepatocellular carcinoma.[28,30]

Various formulations of SFN-loaded nanoparticles have been evaluated to improve SFN efficacy.[31–33] The inclusion of SFN into the CyD cavity has been studied to enhance its solubility.[34,35] A study has analyzed different types of cyclodextrins, showing that SFN in γCyD achieved superior therapeutic efficacy and exhibited promising outcomes in the treatment by intratumoral injections compared to βCyD analogous.[35,36]

Here, we report the synthesis and characterization of HAγCyD conjugates as SFN carriers (Figure 1). We characterized the new HAγCyD conjugates by NMR and DLS and studied the HA conjugates/SFN systems in different cell lines: A2780 (ovarian cancer), SK-HeP-1 (adenocarcinoma) and MDA-MB-453 (breast cancer). We also studied the SFN solubility in the presence of HA conjugates in physiological-like conditions.

Results and Discussion

Synthesis and Characterization

HA was used at two low molecular weights, 11 kDa (HAL) and 45 kDa (HAH). HA conjugates with 3-deoxy-3-amino-γ-CyD ($CyD3NH_2$) were synthesized in water using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as the condensation agent. The conjugates were obtained with a good yield (about 50%) in all cases.

^{1}H NMR spectra (Figure 2) of HAHγCyD and HALγCyD are very similar, all signals are broad as typically found for similar systems.[19,20,23,37] The Hs-1 of CyD resonate at about 5 ppm. The peaks observed at 4.3–4.4 ppm are due to the Hs-1 of the glucuronic acid and glucosamine units of HA and the broad signals between 3 and 4 ppm are due to the HA and CyD protons. CH_3 of the N-acetyl group of HA resonates at 1.9 ppm as a singlet.

The polymer degree of substitution (DS) was determined by the ratio between the integral of the CH_3 signal at 1.9 ppm and CyD Hs-1 at 4.9 ppm. The integration of signals (Figure S1) showed that about 45% of the carboxylic groups of HAH were functionalized with CyD (about 52 cavities), and about 48% of carboxylic groups of HAL (Figure 2) were functionalized with CyD units (about 16 cavities). The DS was high and similar for both polymers.

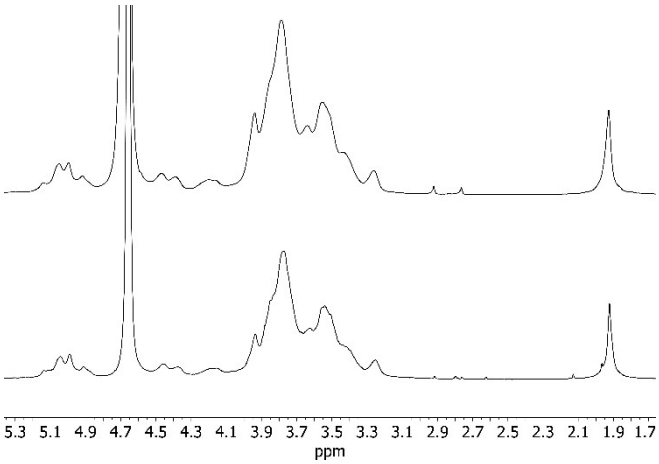

Figure 2. ^{1}H NMR spectra of HALγCyD (top) and HAHγCyD (down) (D_2O, 500 MHz)

The hydrodynamic diameter of HA conjugates was investigated by DLS at pH = 7.4 (Figure S2, S3). HA can self-assemble, forming nanoparticles.[38] The functionalization with CyD increased the hydrodynamic diameter compared to that of the native HA. All the systems showed were polydisperse. Moreover, HAHγCyD exhibited a larger Z average size compared to HALγCyD. This can be attributed not only to the higher molecular weight of the HA backbone but also to the greater number of CyD cavities in HAHγCyD (52 cavities) compared to HALγCyD (16 cavities).

Solubility Experiments

The water solubility of SFN in the presence of the HAHγCyD and HALγCyD polymers was determined at pH 7.4 by HPLC (Figure S2, S3). The graph reported in Figure 3 shows the concen-

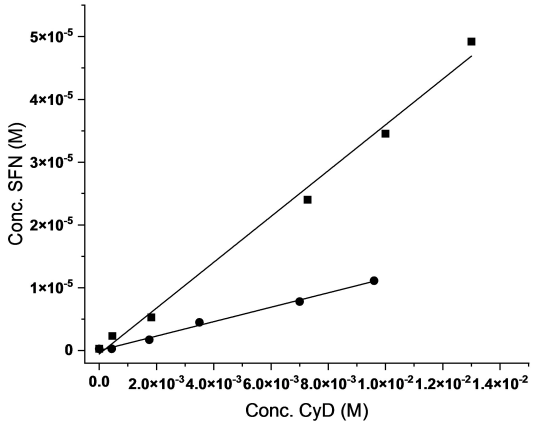

Figure 3. SFN solubility (phosphate buffer, pH 7.4) versus the amount of HAHγCyD (●) or HALγCyD (■) (reported as CyD cavity concentration).

tration of SFN versus the concentration of CyD cavities in the polymers for a clear comparison between the two conjugates.

The solubility experiment has proven a linear correlation between the SFN solubility and the concentration of the polymers (A_L-type graph). From the graph, the complexation efficiency (CE) can be calculated. The data indicate that the HALγCyD has CE 0.038 ± 0.005 and HAHγCyD has CE 0.012 ± 0.003. Given that the apparent stability constant value determined by $K = CE x S_0$ is inaccurate for compounds with $S_0 <$ 0.1 mg/ml,[39] such as sorafenib, we did not calculate the K values.

The HA conjugates have a similar functionalization degree (i.e., the number of CyD moiety for polymer unit) and HALγCyD has a three times higher CE value than HAHγCyD. A similar trend was reported for similar systems[40,41] suggesting that the higher molecular weight may reduce the accessibility of the cavities. Both the polymers improve the solubility of SFN; in the presence of HALγCyD at the highest concentration tested (5.2 mg/mL) SFN water solubility enhanced from 0.12 μg/ml to 23 μg/ml.

Antiproliferative Activity of SFN and HAγCyD Conjugates

The cell proliferation assays were performed for HAγCyD polymers/SFN systems on A2780, SK-Hep1, and MDA-MB-453 cells, with a molar ratio of 1/25 for HAHγCyD/SFN and 1/7 for HALγCyD/SFN (Table 1, Figure 4). The results indicate that the SFN complexes with HALγCyD exhibited significantly higher antiproliferative activity with the lowest IC_{50} values against all the cell lines. Notably, in MDA-MB-453 cells, the IC_{50} of HALγCyD/SFN becomes about four times lower than that of free SFN. In contrast, the complexation of SFN with HAHγCyD caused a slight increase of antiproliferative activity only in SK-Hep1 cells. We can hypothesize that HALγCyD can improve the SFN uptake more efficiently because of a better recognition of the receptor CD44 of HALγCyD/SFN system and/or a better affinity of HALγCyD for SFN than HAHγCyD, as suggested by CE values.

Table 1. Antiproliferative activity (IC_{50}, μM) of HAγCyDs polymers with SFN in A2780, SK-Hep-1 and MDA-MB-453 cell lines.

Cell line	SFN	HALγCyD/SFN	HAHγCyD/SFN
A2780	6.6 ± 1.0[a]	4.6 ± 1.0[b]	7.7 ± 2.1
SK-Hep1	11.7 ± 1.1	7.0 ± 2.8[b]	8.2 ± 2.6[c]
MDA-MB-453	14.9 ± 1.6	3.7 ± 0.7[d]	10.8 ± 2.4

[a] The values express the mean ± SD of IC_{50}; [b] $p < 0.02$; [c] $p < 0.05$; [d] $p < 0.001$, as compared to the SFN treatment.

Conclusions

We linked γ-cyclodextrin to linear hyaluronic acid backbones of 11 kDa and 45 kDa. This approach combines the solubilizing properties of cyclodextrins with the capability of hyaluronic acid to recognize CD44 receptors. The two conjugates demonstrated the capability to increase the sorafenib water solubility from 0.12 μg/ml up to 23 μg/ml (at the highest concentration of the lower molecular weight conjugate). The administration of sorafenib with cyclodextrin polymers enhanced the sorafenib antiproliferative activity in three cancer cell lines (A2780, SK-Hep-1 and MDA-MB-453). In the MDA-MB-453 cell line, the sorafenib IC_{50} value was reduced by about 75% in the presence of the lower molecular weight conjugate. The solubility and antiproliferative activity results suggest that the systems are promising sorafenib carriers.

Experimental Section

Materials

Commercially available reagents were used directly. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) and 3 A-amino-3 A -deoxy-2 A (S),3 A (R)-γ cyclodextrin ($CyD3NH_2$), were purchased from TCI. Hyaluronic acids sodium salt (8-15 kDa and 40–50 kDa) were purchased from Carbosynth. Dialysis was carried out with a tubing cellulose membrane (molecular weight cut-off of 3 kDa, Spectrum Chemical, VWR distributor, Milan, Italy).

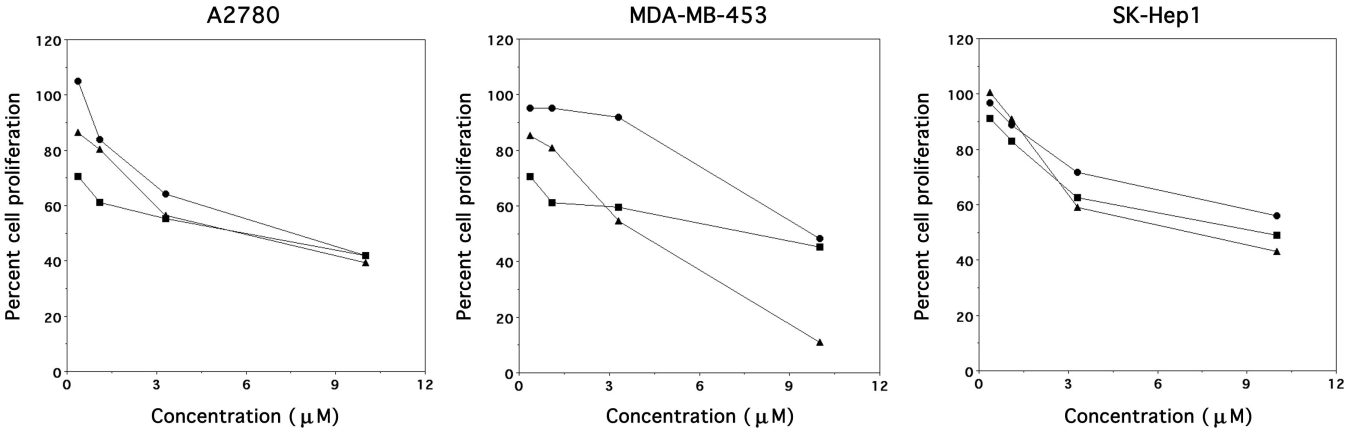

Figure 4. Mean concentration-response curves for the treatment of A2780, MDA-MB-453, and SK-Hep1 with SFN, (●), HALγCyD/SFN (▲), or HAHγCyD/SFN (·). The standard deviation, omitted for clarity of graphs, is reported in Table 1.

Thin Layer Chromatography (TLC) was carried out on silica gel plates (Merck 60-F254). Carbohydrate derivatives were detected on TLC by UV,the anisaldehyde and iodine test.

NMR Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 25 °C with a Varian UNITY PLUS-500 spectrometer at 499.9 and 125.7 MHz respectively. 2D NMR spectra (COSY, TOCSY, HSQC) were performed using 1 K data points, 256 increments. Proton spectra were referred to as the solvent signal.

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements were performed at 25 °C with Zetasizer Nano ZS (Malvern Instruments, UK) operating at 633 nm (He–Ne laser). The mean hydrodynamic diameter (d) of the NPs was calculated from intensity measurement after averaging the five measurements. The samples (1 mg/mL) were diluted in phosphate buffer (pH = 7.4) in ultrapure filtered water (0.2 μm filter).

Experiments of Solubility

SFN (3 μL, 0.17 M, DMSO solution) was added to 0.200 mL of seven solutions of the CyD polymers in phosphate buffer (100 mM, pH 7.4) at different concentrations as reported elsewhere.[42] The suspensions due to the SFN precipitation were sonicated for 10 min and incubated at 25 °C. After 18 h, suspensions were centrifuged at 10,800 rpm for 10 min at 25 °C. The SFN concentration in the supernatant was determined with analytical HPLC using a SHIMAZU LC-20A chromatography system equipped with an SPD-M20A photodiode detector. An Onyx Monolithic C_{18} column (4.6 mm i.d.×100 mm) was used. Eluents were A: MeOH/H_2O 50:50 and B: CH_3CN. The samples were eluted with a 2 ml/min flow rate under isocratic condition (40% B for 5 min, Rt 2.27). The detector λ selected was 265 nm. A linear calibration plot (Figure S3) was determined for free SFN dissolved in methanol.

The CE (complexation efficiency) was calculated from the straight-line slope obtained from SFN solubility data versus polymer concentration, CE = Slope/ (1 – Slope).[39]

Synthesis of HAHγCyD

DMTMM (46 mg, 0.17 mmol) and CyD3NH_2 (173 mg, 0.13 mmol) were added to HAH (50 mg, 1 μmol) in 10 mL water in three aliquots (every 30 min). The reaction mixture was stirred at 25 °C for 24 h. The final product was dialyzed against water (3 kDa molecular weight cut-off membrane).

Yield: 35%

^{1}H NMR: (500 MHz, D_2O) δ(ppm): 1.90 (s, CH_3 of N-Acetyl), 3.1–4.03 (m, H-3, −6, −5, −2, −4 of CyDs and HA), 4.17 (m, H-3-A of CyD), 4.37–4.47 (d, H-1 of glucuronic acid and glucosamine), 4.96–5.16 (m, H-1 of CyD).

Dimension (DLS, Z-Average), d: 531 ± 60 nm.

Synthesis of HALγCyD

The synthesis and purification were carried out as reported for HAHγCyD starting from HAL (50 mg, 5 μmol) DMTMM (63 mg, 0.23 mmol) and γCyD3NH_2 (177 mg, 0.14 mmol).

Yield: 53%

^{1}H NMR: (500 MHz, D_2O) δ(ppm): 1.92 (s, CH_3 of N-Acetyl), 3.21–4.03 (m, H-3, −6, −5, −2, −4 of CyDs and HA), 4.19 (m, H-3-A of CyD), 4.35–4.52 (d, H-1 of glucuronic acid and glucosamine), 4.83–5.16 (m, H-1 of CyD).

Dimension (DLS, Z-Average), d: 90 ± 5 nm.

Evaluation of Cell Proliferation Inhibition by the MTT Assay

Human cell lines A2780 (ovary, adenocarcinoma), MDA-MB-453 (breast, carcinoma), and SK-Hep-1 (liver, carcinoma) were plated at appropriate concentrations in 180 μl of opportune complete media (RPMI for A2780 cells and DMEM for MDA-MB-453 and SK-Hep-1 cells) into flat-bottomed 96-well microliter plates and centrifuged for 2 min at 1100 rpm. When adherent, after 7–8 h, cells were administered with 20 μl containing five 1:3 fold concentrations of SFN and the HACyD conjugate diluted in distilled water plus 1% DMSO. After 72 h, plates were processed as described elsewhere.[43] IC_{50} values were obtained by analyzing single concentration-response curves. Each experiment was repeated 3—6 times.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Cancer · Cyclodextrin · Hyaluronic acid · Drug delivery · Nanoparticles

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