Design, Synthesis, and Biological Evaluation of Camptothecin Loaded Biotinylated Cellulose Nanowhiskers as Anticancer Agents

Osamah N. Wennas1, Mohammed H. Mohammed2, Raid M. Al Abook3, and Dhulfiqar Ali Abed4

Abstract

Objective Using biotinylated cellulose nanowhiskers (CNWs), we designed and synthesized a Glutathione (GSH) sensitive- Camptothecin (CPT) prodrug for selective CPT delivery (compound 12).

Methods CPT-biotin (compound 9), was synthesized by direct conjugation of CPT to the biotin via GSH sensitive linkage to evaluate the role of CNWs in compound 12. The chemical structures of the synthesized prodrugs were confirmed by FT-IR, 1H NMR, 13C NMR, and ESI-MS, while the nanoparticles were characterized by DLS and TEM.

Results The in-vitro drug release assay demonstrated that only 18.6% of CPT was released from the nano conjugate under GSH stimulation at micromolar level (100 μM), while 83.1% accumulative release rate was achieved under GSH stimulation at millimolar level (10 mM). The in-vitro cytotoxicity assay (MTT assay) demonstrated that compound 9 showed higher inhibition ratios on biotin positive cells, MCF-7, and HepG2, and lower cytotoxicity on biotin negative, CHO. Compound 12 showed good activity against MCF-7, HepG2, and much lower cytotoxicity on CHO.

Conclusion This work demonstrates CPT-biotinylated cellulose nanowhiskers for selective chemotherapy and may have the potential to be used for cancer targeting.

Keywords Anticancer, camptothecin, cellulose nanowhiskers, biotin, glutathione.

Introduction

Cancer is a broad group of diseases that involves uncontrolled proliferation of abnormal cells that are capable of de-differentiation, invasion, and metastasis through the bloodstream or the lymphatic system. Among them, CNWs drew a lot of attention because of their numerous advantages, like being biocompatible and not triggering an immune response. CNWs have high hydrophilicity that impedes opsonin proteins’ adsorption. They are also suitable for different types of chemical linkages because the hydroxyl groups on the surface of CNWs can be easily modified to other chemical groups.

In the current study, reduction-sensitive nanoparticles were designed from biotin- decorated CNWs- CPT with disulfide linkage. The presence of disulfide between the CNWs and CPT was suggested to release the active drug in response to the elevated intracellular GSH. Biotin has been used as targeting moiety with many anticancers to prevent the non-specific normal cells attack and to increase the uptake by the target cells. The in vitro drug release was studied at different GSH concentrations, and the cytotoxic effects of the nano conjugate on MCF7, HepG2, and CHO cell lines were studied and compared with CPT prodrug without the nanocarrier.

Materials and Methods

Materials

Camptothecin, biotin, and 3,3’-dithiodipropionic acid were purchased from Beijing Yibai Biotechnology Co., Ltd. CNWs were purchased from the Process development center- The University of Maine U.S.A. Other chemicals were purchased...
from Sigma–Aldrich. All chemicals are of analytical grade, and they were used as received without further purification.

**Characterization of Compounds 5-9 and Compound 11**

Melting points, Fourier transform infrared spectroscopy: FTIR, NMR: $^1$H NMR and $^{13}$C NMR spectra, and Electrospray ionization mass spectrometry: ESI-MS were performed for compound characterization.

**Chemical Synthesis**

The target compounds were synthesized by multi-step reactions, as shown in schemes 1&2.

**Synthesis of Biotin Hydrazide (Compound 6)**

0.75 g (3.075 mmol) of biotin was suspended in 75 mL methanol, 0.5 g of the catalyst (Amberlite IR-120 resin) was added, and the suspension was stirred at room temperature for 24 h. After filtration and solvent's evaporation under reduced pressure, the title compound was obtained as a white powder, mp. 165-166°C, yield 88%. $^1$H NMR (500 MHz, DMSO-d6) 6.46 (1 H, s), 6.38 (1 H, s), 4.35 – 4.29 (1 H, m), 4.14 (1 H, m), 3.59 (3 H, s), 3.12 (1 H, dd, J 4.9, 3.2), 2.83 (1 H, dd, J 12.4, 5.1), 2.59 (1 H, d, J 12.5), 2.31 (2 H, t, J 7.5), 1.68 – 1.42 (4 H, m), 1.35 (2 H, tt, J 14.3, 6.6). $^{13}$C NMR (126 MHz, DMSO-d6) 173.78, 163.22, 61.52, 59.68, 55.81, 51.66, 40.33, 33.57, 28.48, 28.44, 24.95. ESI (MS) Calcd for C$_7$H$_8$N$_2$S (M+H)$^+$ 259.11, found 259.3.

**Synthesis of 3,3'-Dithiodipropionic Anhydride (Compound 7)**

3,3'-dithiodipropionic acid 2 g was dissolved in 5 mL of acetyl chloride in a round bottom flask and refluxed at 65°C for 4 h until the solution was clear. After most of the solvent is removed under reduced pressure at 50°C, iced ethyl ether was added to precipitate dithiodipropionic anhydride. After filtration and washing with petroleum ether, the title compound was obtained as a white powder, mp. 65-68°C, yield 38%.

**Synthesis of Camptothecin -3,3'-Dithiodipropionic Acid CPT-ss-COOH. (Compound 8)**

Compound 7 (1.923 g, 10 mmol) and compound 1 (0.348 g, 1 mmol) were dissolved in pyridine (30 mL), a solution of DMAP (0.61 g, 5 mmol) in 10 mL of pyridine was added dropwise at 10 °C under nitrogen atmosphere. The reaction mixture was heated to 70 °C, and the reaction proceeded for 48 h. Then, the reaction solution was precipitated by using an excess of methanol, washed with dilute HCl, and dried to give the title compound as a yellow powder mp. 228-230°C, yield 42%. $^1$H NMR (500 MHz, DMSO-d6) 8.66 (1 H, s), 8.15 (1 H, d, J 8.5), 8.10 (1 H, d, J 8.5), 7.89 – 7.82 (2 H, m), 7.70 (1 H, t, J 7.5), 7.18 (1 H, s), 5.51 (2 H, s), 5.26 (2 H, s), 3.09 – 2.89 (6 H, m), 2.59 (2 H, t, J 7.0), 2.18 (2 H, qdl, J 7.2, 3.8), 0.92 (3 H, t, J 7.4). $^{13}$C NMR (126 MHz, DMSO-d$_6$) 173.43, 170.84, 167.60, 157.00, 148.36, 146.36, 146.23, 145.71, 131.97, 130.87, 129.42, 128.95, 128.41, 128.15, 119.30, 95.71, 76.69, 66.76, 50.68, 34.37, 33.72, 33.62, 32.90, 30.69, 8.06. MS (ESI) Calcd for C$_{13}$H$_{14}$N$_2$O$_5$S (M+H)$^+$ 259.12, found 259.3.

**Synthesis of Camptothecin-SS-Biotin Hydrazide (Compound 9)**

To a stirred solution of compound 8 (540 mg, 1 mmol) in 10 mL of DMF, EDC (288 mg, 1.5 mmol) and NHS (172 mg, 1.5 mmol) were added. The solution was stirred at room temperature for 1 h to form NHS ester of compound 8. Compound 9 was obtained as a white powder, mp. 245-247°C, yield 88%. $^1$H NMR (500 MHz, DMSO-d6) 8.93 (1 H, s), 6.44 (1 H, s), 6.37 (1 H, s), 4.32 (1 H, dd), 4.15 (3 H, m), 3.10 (1 H, m), 2.83 (1 H, dd, J 12.4, 5.0), 2.59 (1 H, d, J 12.4), 2.02 (2 H, t, J 7.4), 1.67 – 1.41 (4 H, m), 1.31 (2 H, tt, J 14.3, 6.5). $^{13}$C NMR (126 MHz, DMSO-d6) 172.02, 163.21, 61.52, 59.68, 55.89, 33.71, 28.70, 28.49, 25.70. MS (ESI) Calcd for C$_{16}$H$_{19}$N$_2$O$_5$S (M+H)$^+$ 259.12, found 259.3.

**Scheme 1**

Synthesis schematics of compound 9. Reagents and conditions: (a) acetyl chloride, 65°C, 4 h, (38%); (b) DMAP, pyridine, 70°C, 48 h, (42%); (c) Amberlite IR 120 resin, methanol, rt, 24 h, 88%; (d) hydrazine, methanol, rt, 24 h, 90%; (e) EDC/NHS, DMSO, rt, 48h, 72%.
6 (258 mg, 1 mmol) was added to the reaction solution, and the mixture was stirred at room temperature for 48 h. The product was precipitated by the addition of water (100 mL). The product was purified by washing with dilute HCl, and then dried to give the title compound as a white powder, mp. 234-236, yield 72%. \(^1\)H NMR (500 MHz, DMSO-d6) 9.85 (1 H, d, J 2.1), 9.74 (1 H, d, J 2.1), 8.68 (1 H, s), 8.17 (1 H, d, J 8.5), 8.13 (1 H, d, J 8.2), 7.91 – 7.84 (1 H, m), 7.72 (1 H, t, J 7.6), 7.18 (1 H, s), 6.41 (1 H, s), 6.36 (1 H, s), 5.51 (2 H, s), 5.29 (2 H, s), 4.31 (1 H, m), 4.12 (1 H, m), 3.13 – 3.06 (1 H, m), 3.01-2.92 (6 H, m), 2.82 (1 H, dd, J 12.5, 5.1), 2.61 – 2.51 (3 H, m), 2.18 (2 H, qd, J 7.2, 3.8), 2.12 (2 H, t, J 7.4), 1.56 – 1.43 (4 H, m), 1.35 (2 H, tt, J 14.3, 6.5), 0.94 (3 H, t, J 7.4). \(^13\)C NMR (126 MHz, DMSO-d6) 171.35, 170.86, 169.41, 167.62, 156.97, 152.71, 148.32, 146.38, 145.71, 131.94, 130.86, 130.11, 129.40, 128.91, 128.36, 128.12, 119.31, 95.70, 76.67, 66.78, 61.50, 59.66, 55.87, 50.71, 33.93, 33.65, 33.42, 33.39, 32.82, 30.72, 28.55, 28.48, 25.51, 8.06. MS (ESI) Calcd for C\(_{37}\)H\(_{49}\)N\(_3\)O\(_5\)S (M+H)\(^+\) 575.21, found 575.19.

**Synthesis of Carboxylated CNWs (CNW-COOH, Compound 10)**

1.02 g of CNWs were suspended in 100 mL of distilled water and sonicated for 5 min. 29.5 mg, 0.188 mmol of TEMPO, and 324 mg, 3.15 mmol NaBr were added to the suspension. Then, 3.15 mmol of NaOCl was added slowly to the cellulose suspension. The pH of the mixture was kept at 10-11 by using 0.5 M NaOH while stirring the suspension. After about 45 min, the oxidation was terminated by adding 2 mL of methanol. 0.5 M HCl was used to adjust the pH 7. The oxidized CNWs were dialyzed against deionized water for 48 h. The concentration of the suspension was determined gravimetrically.

**Synthesis of Camptothecin -3,3'-Dithiodipropionic Hydrazide (Compound 11)**

Compound 8 (540 mg, 1 mmol) was dissolved in 15 mL of DMF at room temperature. HOBt (162 mg, 1.2 mmol) and EDC (230 mg, 1.2 mmol) were added. The mixture was stirred at room temperature; the progress of the reaction was monitored by TLC. After 2 h when all the acid was converted to activated ester intermediates. The activated intermediate was then slowly added (by inverse addition) to a solution of hydrazine (2 mmol) and cyclohexene (0.05 mL) in DMF (10 mL) while the temperature was kept at 0-10 °C. The reaction was complete upon the completion of the addition. The product was precipitated by the addition 100 mL of cold distilled water. After filtration, the compound was dried to give the title compound as a yellow powder, mp. 234-236, yield 72%.

**Conductometric Titration**

The carboxyl content of compound 10 was determined using conductometric titration, as shown in the titration curve (Figure 2).

**Determination of Biotin and CPT Content**

Elemental analysis was used to determine biotin content in compound 12 depending on the N%: LECO elemental analyzer was used. The amount of CPT was determined by UV-visible spectrophotometer at 372 nm using Carry 100 device.

**Particle Size and Zeta Potential**

Horiba SZ100 instrument was used to determine the particle size and Zeta potential.

**Transmission Electron Microscopes (TEM)**

After sonication in a water bath, very diluted suspensions of the nanoparticles (0.001 wt.%) were dropped on carbon film-covered copper grids. 2% uranyl acetate solution was applied.
used for staining the samples on the grid, and the drying was done at ambient conditions. Then, the samples were examined by TEM (PHILIPS Model: CM120).

**In Vitro Drug Release Study**

The CPT release experiments were carried out at 37°C, using two different media, acetate buffer (pH 5.8) with 10 mM GSH and phosphate buffer saline, pH 7.4 (FBS) with 100 μM GSH to simulate the different microenvironments in cancer cells and blood vessels, respectively.

**In Vitro Cytotoxicity Assay**

The in vitro cytotoxicity of compounds 9 and 12 was evaluated by MTT assay on biotin-positive cell lines, human breast cancer cell (MCF-7) and human hepatic carcinoma (HePG2), and biotin negative cell line, noncancerous Chinese hamster ovarian (CHO). MTT was performed to determine the cytotoxic effect of the samples at various concentrations. The results were given as the mean of three independent experiments and the IC_{50} values were then calculated.

**Results**

**Chemical Synthesis of (5-9) and 11**

Compound 5 was prepared by the esterification of biotin with methanol in the presence of high excess of methanol according to Fischer esterification using Amberlite IR120 resign as acid catalyst. FT-IR showed the disappearance of carboxylic acid carbonyl stretching band (1700 cm\(^{-1}\)) and the appearance of new band at 1743 cm\(^{-1}\) due to ester carbonyl stretching. \(^1\)H NMR of the compound 5 was characterized by the disappearance of COOH proton signal at 12.00 p.p.m. and the appearance of a new signal as an indication of biotin’s successful esterification, the signal was related to COOCH protons at 3.59 p.p.m. as singlet.

Compound 6 was obtained by the hydrazinolysis of biotin methyl ester in methanol using an excess of hydrazine hydrate. FT-IR showed the disappearance of ester carbonyl stretching band (1743 cm\(^{-1}\)) and the appearance of a new band at 1685 cm\(^{-1}\) due to hydrazide carbonyl stretching. \(^1\)H NMR of compound 6 was characterized by the disappearance of COOCH protons at 3.59 p.p.m. and the appearance of new signals to indicate the success of the reaction, the signals were related to CONHN\(_2\) protons at 9.06 p.p.m. and 4.36 p.p.m., respectively, both of them as a singlet.

Compound 7, the FT-IR showed the new bands at 1793 cm\(^{-1}\) and 1739 cm\(^{-1}\) due to asymmetric and symmetric stretching vibration of anhydride carbonyl. Compound 8 was obtained by the esterification of the hydroxyl of compound 1 with the anhydride (compound 7) using DMAP as an acylating catalyst. FT-IR showed a new band at 1720 cm\(^{-1}\) due to the carboxylic acid carbonyl stretching. \(^1\)H NMR of compound 8 was characterized by the disappearance of alcoholic proton signal at 6.53 p.p.m. and the appearance of new signals to indicate CPT esterification’s success; the signals related to aliphatic protons at 3.00-2.59 p.p.m.

Compound 9 was obtained by the conjugation of compound 8 and compound 6 (biotin hydrazide) using EDC as a coupling agent. FT-IR showed the disappearance of the carboxylic acid band at 1720 cm\(^{-1}\) due to amide formation. \(^1\)H NMR of compound 9 was characterized by the disappearance of CONHN\(_2\) signal at 4.15 p.p.m. and the appearance of new signals that were related to CONHNHCO protons at 9.85 p.p.m. and 9.74 p.p.m. both of them as a singlet. Compound 11 was obtained by the reaction of compound 8 with hydrazine using HOBt and EDC as coupling agents. FT-IR showed the disappearance of the carboxylic acid band at 1720 cm\(^{-1}\) due to azide formation. \(^1\)H NMR of compound 11 was characterized by the appearance of new signals to indicate the success of the reaction, the signals were related to CONHNH protons at 9.06 p.p.m. and 4.36 p.p.m., respectively, both of them as a singlet.

**Characterization of Nanoparticles**

**Conductometric Titration**

The determination of carboxyl content of compound 10 was done using a method derived for the titration of cellulose fibers by applying the following equation.

\[
[\text{COOH}] = \left( \frac{V \times C_{NaOH}}{m \text{CNWs}} \right) - [\text{OSO}_3H]
\]

Where V is the volume of NaOH (14.63 mL), C_{NaOH} is the NaOH concentration (0.01 mol/L), m CNWs is the weight of the sample (0.00015 kg), and [OSO\(_3\)H] was determined by elemental analysis and it was 175 mmole. By applying the equation, the [COOH] was calculated, and it was found to be 1150 mmole/kg.

**Table 1:** Characterization data of CNWs and CNW conjugates.

| Sample  | Particle size (nm) | PDI     | Zeta potential (mV) | Biotin % | CPT % |
|---------|-------------------|---------|---------------------|---------|-------|
| CNWs    | 68.1±4.19         | 0.383±0.124 | -34.33±1.59        | -       | -     |
| Compound 10 | 81.23±0.353     | 0.462±0.052  | -65.2±2.40         | -       | -     |
| Compound 12 | 87.26±3.496    | 0.369±0.035  | -45.16±1.20        | 8.67    | 5.6   |
Determination of Biotin and CPT Content

The biotin and CPT contents of compound 12 were shown in Table 1.

Particle Size and Zeta Potential

The studies via DLS and zeta potential analysis revealed that the nanoparticles (CNWs, compounds 10, and 12) have a hydrodynamic radius between 68 and 87 nm and an overall negative surface charge, as shown in Table 1.

Transmission Electron Microscopes

Figure 3 shows the TEM images of CNWs, and compound 12. No significant changes in the morphology or the degree of agglomeration were observed.

In Vitro Release Study

The disulfide linkage between the CNWs and CPT can be cleaved readily in the presence of a reducing agent such as GSH, as shown in scheme 3.

In Vitro Cytotoxicity Assay

The anticancer activity of compound 9, and compound 12 were examined in three cell lines with different expression levels of biotin receptor. MCF-7 and HepG2 cells overexpress biotin receptors on the surface, while biotin receptors were rarely expressed on CHO cells. Figure 5a, b, and c show the viability of the cells after 72 h incubation. The half-maximal inhibitory concentration was determined to show the effectiveness of the tested compounds on growth inhibition of the three cell lines (Figure 5d).
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Fig. 5

Cytotoxicity of free CPT, compound 9, and compound 12 against (a) MCF-7, (b) HepG2, and (c) CHO cells after incubation for 72 h (n = 3) (d) IC50s of free 6-MP, compound 9, and compound 12 in MCF-7, HepG2, and CHO cells.

Discussion

Chemical structure of the intermediates and the final compounds was confirmed using FT-IR, 1H NMR, 13C NMR, and ESI-MS. Characterization of nanoparticles was done using DLS and TEM. In Figure 4.

In vitro-release study results suggest that the release of CPT is accelerated in the cellular environment (especially tumors) with elevated GSH levels. In contrast, the release is minimum in the extracellular environment with a low GSH level.

Regarding the in vitro cytotoxicity, The IC50 of compound 9 is less than half or third of that of CPT in biotin positive cell lines, MCF-7 and HepG2, respectively, and about seven-folds higher than CPT for the biotin negative CHO cell line. For compound 12, it showed comparable activity to CPT against MCF-7 and higher activity against HepG2 with IC50 about half that of CPT, and much lower activity against CHO with IC50 is about eleven times higher than that of CPT. The relatively small molecule compound 9 has a cellular uptake by passive diffusion beside the cell endocytosis; conversely, compound 12 as a nanoparticle, mainly internalized only by cells endocytosis. These results suggest that the uptake and the cytotoxicity of compounds 9 and compound 12 were higher for cell lines with biotin overexpression than for cells with low biotin expression, and they can potentially reduce side effects of CPT by selectively killing biotin-positive tumor cells.

Conclusions

In the recent decade, there have been several CPT-macromolecule prodrugs to address CPT limitations. However, there is still an unmet need to develop different types of macromolecule prodrugs to reduce systemic toxicity and improve the therapeutic efficacy. In the present study, we synthesized two CPT prodrugs using a biotin receptor and a GSH sensitive disulfide linker for intracellular delivery of CPT. The chemical structures of the synthesized prodrugs were confirmed by FT-IR, 1H NMR, 13C NMR, and ESI-MS, while the nanoparticles were characterized by DLS and TEM. The release study showed that GSH could promote the drug release at the intracellular millimolar level compared with GSH at a micromolar level. MTT assay demonstrated that this system...
had a higher inhibition ratio on bovine positive cells, MCF-7, and HepG2, and lower cytotoxicity on negative bovine cells, CHO. Therefore, these bovine-guided, GSH dependent prod- 

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