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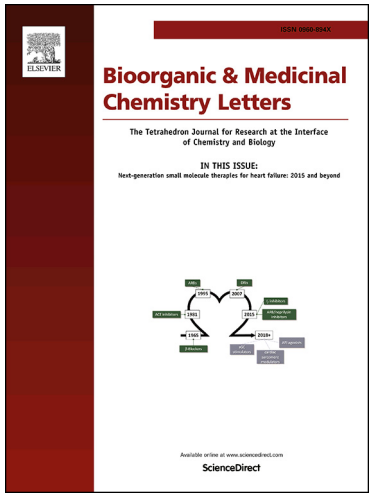

Design, characterization, and in vitro antiproliferative efficacy of gemcitabine conjugates based on carboxymethyl glucan

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PII: S0960-894X(18)30576-6
DOI: https://doi.org/10.1016/j.bmcl.2018.07.014
Reference: BMCL 25945

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 17 May 2018
Revised Date: 21 June 2018
Accepted Date: 7 July 2018

Please cite this article as: Ma, L., Chen, Y., Wang, X., Xiong, M., Sun, Y., Zhang, X., Zhao, Y., Design, characterization, and in vitro antiproliferative efficacy of gemcitabine conjugates based on carboxymethyl glucan, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.07.014

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Design, characterization, and in vitro antiproliferative efficacy of gemcitabine conjugates based on carboxymethyl glucan

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Gemcitabine
Carboxymethyl glucan
Amino acid linker
Conjugate
Antiproliferative activity

ABSTRACT

Gemcitabine (GEM) is widely used in clinical practice in the treatment of cancer and several other solid tumors. Nevertheless, the antitumor effect of GEM is partially prevented by some limitations including short half life, and lack of tumor localizing. Carboxymethyl glucan (CMG), a carboxymethylated derivative of β-(1-3)-glucan, shows biocompatibility and biodegradability as well as a potential anticarcinogenic effect. To enhance the antiproliferative activity of GEM, four water soluble conjugates of GEM bound to CMG via diverse amino acid linkers were designed and synthesized. ^{1}H NMR, FT IR, elementary analysis and RP-HPLC chromatography were employed to verify the correct achievement of the conjugates. In vitro release study indicated that conjugates presented slower release in physiological buffer (pH 7.4) than acidic buffer (pH 5.5) mimicking the acidic tumor microenvironment. Moreover, A549, HeLa and Caco-2 cancer cell lines were used to evaluate the in vitro cytotoxicity of conjugates and the results showed that binding GEM to CMG significantly enhanced antiproliferative activity of GEM on A549 cells. Therefore, these conjugates may be potentially useful as a delivery vehicle in cancer therapy and worthy of further study on structure-activity relationship and antiproliferative activity in vitro and in vivo, especially for lung tumor.

Gemcitabine (2', 2'-difluoro-2'-deoxycytidine, GEM), a nucleoside analogue, is widely used as antimetabolites in clinical practice to treat sevreral types of solid tumors including lung,[1] ovarian,[2] pancreatic cancer[3] and several others.[4] GEM has no efficacy outside the cell until undergoing phosphorylation in a stepwise manner to be final active triphosphate derivatives of GEM.[5] However, its chemotherapeutic efficacy is hampered by poor intracellular uptake depending on different nucleoside transporters,[6] rapid inactivation by cytidine deaminase (CDA), and indiscriminately targeting both cancer cells and normal cells, resulting in severe side effects.[7] Furthermore, the small molecular weight of GEM also contributes to a rapid renal clearance. To compensate for these drawbacks, a larger dose of GEM is administered to satisfy effectiveness of clinical treatment, further provoking adverse effects. To address these issues, conjugation of GEM with synthetic or natural polymers has been developed, for example, covalent attachment of GEM to synthetic polymers including polyethylene glycol (PEG),[8, 9] polyisoprene,[10] methoxy poly (ethylene glycol) - poly (lactic acid) (mPEG-PLA)[11] and poly (lactic-co-glycolic acid)[12] by modifying at its 4-amino group has been reported in literature, these conjugates can improve the cytotoxicity of GEM in vitro while reducing drug associated side effects, enhancing its stability and prolonging its circulation time in plasma. Poly-L-glutamic acid (PLGA)[13] and albumin,[14] nature carriers, introduced into 5'-hydroxyl and 4-amino group of GEM, respectively, have a similar efficacy with the above mentioned conjugates. Unfortunately, a mumber of drugs with PEGy-lated agents can activate the complement system, which may cause hypersensitivity reactions.[15]

Very few published reports are available regarding the use of GEM conjugated with polysaccharides. Compared with synthetic polymers, a polysaccharide has a variety of advantages, such as lower cost, higher safety, and adequate loading

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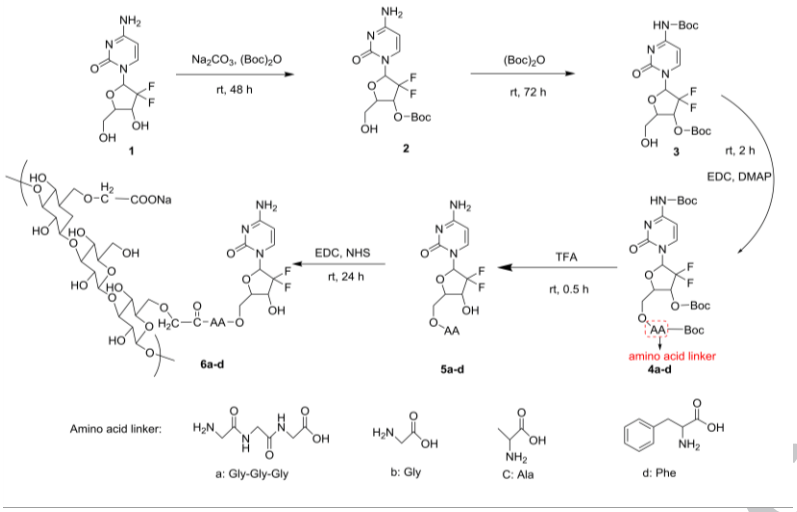

Scheme 1. Discovery route of conjugates **6a–d**.

capacity.[16] Among the polysaccharides being considered, carboxymethyl glucan (CMG), a carboxymethylated derivative of β-(1-3)-glucan, gained our attention because of its biocompatibility, biodegradability, excellent immunomodulation function,[17] potential anticarcinogenic and antimutagenic effects.[18] More importantly, no genotoxicity or inhibition on cells proliferation was reported in Chinese hamster epithelial cells (CHO-k1),[19] as well as no side effects associated with CMG administration were observed in prostate cancer patients.[20] A further study indicated that CMG can induce cell cycle arrest and apoptosis in MCF-7 cells,[21] increased production of tumor necrosis factor alpha (TNF-a) in mouse plasma and exhibited higher antitumor activity than native β-glucan in vitro on HL-60 tumor cells and in vivo in Sarcoma-180 solid tumor.[22] Besides, we were also inspired by a study showing that the combination of GEM with the polysaccharide SEP remarkably enhances antitumor activity and reverses apoptosis caused by GEM in both spleen and bone marrow.[23]

Based on the above studies, we hypothesized that conjugation of GEM with CMG could enhance the antiproliferative activity of GEM. Therefore, we designed and synthesized four GEM-CMG conjugates (**6a**, **6b**, **6c**, **6d**) with different amino acid linkers (Gly-Gly-Gly, Gly, Ala, Phe) (Scheme 1). These conjugates **6a-d** were determined using ^{1}H NMR, FT IR, elementary analysis and RP-HPLC. Subsequently, in vitro release study was performed in PBS at pH 7.4 and pH 5.5, mimicking the pH of physiological condition and tumor tissue microenvironment, respectively. Cytotoxicity study in comparison with native GEM was performmed on A549, HeLa and Caco-2 cancer cell lines. The key idea and working principle of GEM-CMG conjugates were schematically illustrated in Fig. 1.

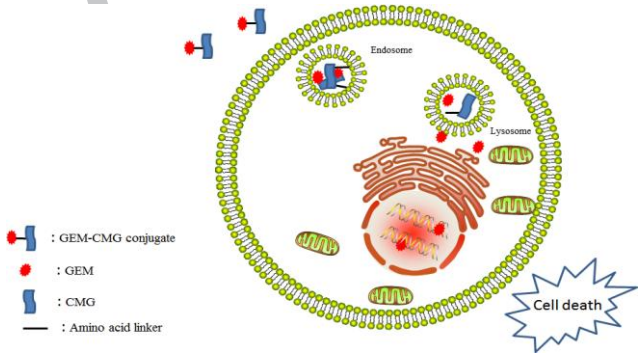

Fig. 1. Graphical illustration of GEM release from conjugates at the intracellular environment.

In view of the chemical structure of CMG and GEM, we attempted to bind GEM via its 4-amino or 5'- hydroxyl group to CMG by means of amino acid linker. Therefore, hydroxyl or amino groups that need not participate in the reaction should be selectively protected. According to previous study,[24] ditertbutyl pyrocarbonate ($(Boc)_2O$) was served as a selective protecting group in conjugate synthesis. Introducing amino acid linker into 4-amino group was regrettably unsuccessful due to the unideal product after the remove of Boc protecting group which might be caused by rearrangement.[25] Great efforts had been taken to link the amino acid linker into the 5'-hydroxyl group. The synthesis started with the protection of residual 3'-hydroxyl group and 4-amino group by stepwise in the presence of excess $(Boc)_2O$ and Na_2CO_3 in a solution of dioxane and water. Compound **2** was obtained in 81% isolated yield. Subsequent protecting of 4-amino group with $(Boc)_2O$ gave the fully armed compound besides exposed 5'-hydroxyl group. Following this, the amino acid linker **a-d** was introduced into 5'-hydroxyl group of GEM through an ester linkage. **4a-d** was prepared by adding N-Boc-L-amino acid to **3a-d** in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 4-dimethylaminopyridine (DMAP). All of Boc groups were removed by trifluoroacetic acid (TFA) in solution of dichloromethane (DCM) to give **5a-d**. The covalent attachment of **5a-d** to CMG was achieved by the formation of a amide bond between the primary amino group of amino acid and a carboxylic acid group of CMG in the presence of EDC and N – hydroxysuccinimide (NHS). Reaction mixture of conjugate was exhaustively dialyzed against distilled water for 2 days using dialysis bags (molecular weight cutoff, MWCO, 140,000) and precipitated by 75% ethanol. The precipitate was suffered from freeze drying to obtain the white conjugates, **6a-d**. The detailed description of the synthesis is reported in supporting information.

To quantify the GEM bound in **6a-d**, it was necessary to cleave GEM from **6a-d** by acid hydrolysis, 1 M hydrochloric acid was used to fracture the conjujates at 60 °C for 12 h to ensure complete hydrolysis and the hydrolysate was analyzed by RP-UV-HPLC. Conjugates **6a-d** with GEM contents varying from 2.4% to 6.63% was shown in Table. 1.

The structures of **6a-d** were verified by ^{1}H NMR spectra using D_2O solvent as shown in Fig. 2, the evident signals of native GEM were appeared at δ 7.80 ppm for H-6, δ 6.31 ppm for H-1' and δ 6.08 ppm for H-5, which were consistent with the reports,[26, 27] The cytidine protons of GEM in **6a-d** gave the signals for H-6 and H-5 at the range of δ 7.48-7.72 ppm and δ 6.00-6.17 ppm (red dotted line), respectively.[9, 28] Fig. 2a gives

Table 1

Drug loading of GEM in **6a-d** and percentage composition of different elements present in CMG and **6a-d**.

Compounds	C%	H%	N%	Drug loading of GEM (%)
6a	40.7961	6.4871	5.5748	6.63±0.68
6b	38.2329	6.2270	2.0700	3.33±0.34
6c	37.4122	6.1518	1.6536	2.40±0.22
6d	41.3529	6.7089	4.9967	4.70±0.35
CMG	29.4903	4.8976	/	/

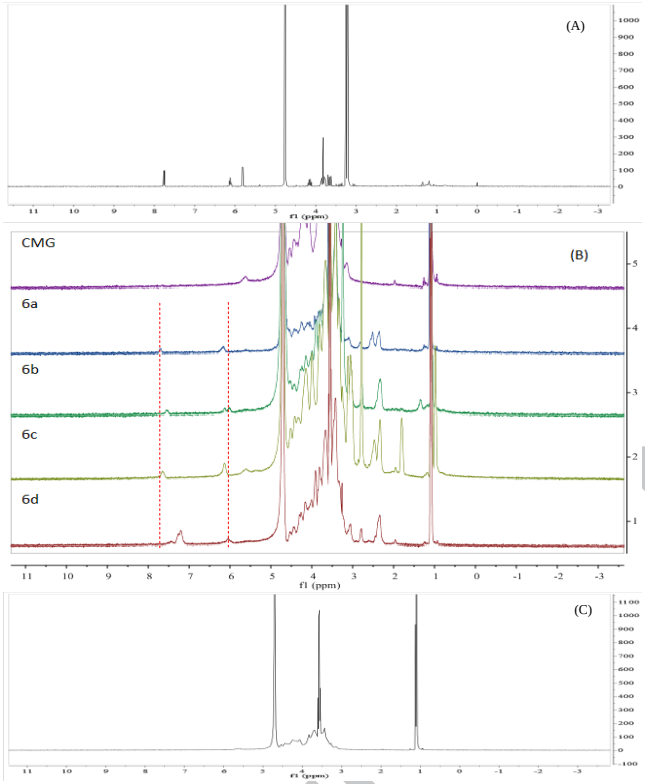

Fig. 2. ^{1}H NMR spectra of (A) GEM. (B) CMG, **6a**, **6b**, **6c** and **6d**. (C) Dialyzed and freeze-dried physical mixture of GEM and CMG (the molar ratio of GEM and CMG was 1:10).

the main broad signals at δ 3.46-4.54 ppm assigned to protons of β-D-glucose.[29] In order to eliminate the interference of unconjugated GEM in **6a-d** on structural identification, the physical mixture (the molar ratio of GEM and CMG was 1:10) was used as comparison to give the ^{1}H NMR spectrum portrayed in Fig. 2(C). Without doubt the physical mixture also undergone dialysis, alcohol-precipitation and freeze-drying which was the same as **6a-d**, and the result suggested that dialysis almost completely removed small molecular from crude conjugates because there were no other signals excepting CMG in Fig. 2(C). On the other hand, the appearance of characteristic signals at δ 7.48-7.72 ppm and δ 6.00-6.17 ppm owning to conjugated GEM in **6a-d**.

The characterization of **6a-d** was further performed by FT IR and elementary analysis. CMG exhibted the characteristic peaks of 3440 cm^{-1}, 2924 cm^{-1}, 1631 cm^{-1} and 1421 cm^{-1} as shown in Fig. 3(B). The absorption bands at 3440 cm^{-1} and 2924 cm^{-1} were ascribed to the stretching vibration of -OH group and -CH_2 stretching vibration, respectively. 1631 cm^{-1} and 1421 cm^{-1} were due to asymmetrical stretching vibration and symmetrical stretching vibration of carboxylate anion (-COO^-), the bands were similar to the observations reported for carboxymethylated polysaccharide.[30-32] Compared with CMG, the absorption bands at around 1615 cm^{-1} and 1500 cm^{-1} confirmed the formation of the amide linkage between CMG and amino acid modified GEM[33] (Fig. 3(B) **6a** and **6d**). Nevertheless, FT IR spectrum of **6b** and **6c** were not affected by the conjugation with GEM might due to the small amount of drug loading. In view of the increased intensity of the peaks for **6a** and **6d** at 1630 cm^{-1} along with 1050 cm^{-1} (-C-O- stretching vibration[34]) (Fig. 3(A)), it was deduced that there was an increase in the number of ester bonds and amide bonds, which reflects the amide and easter linkage had been formed in the conjugates from the side.[35] To validate this conclusion, elementary analysis was performed (Table. 1). Compared with **6b** and **6c**, the increase in the percentages of N and C clearly demonstrated the synthesis of conjugates, which was in accordance with the result of drug loading and FTIR.

Some research also had been performed on pH-responsive prodrug formulations to improve therapeutic efficacy, which was based on physiological pH conditions (pH 7.4) usually much higher than intracellular pH of tumor cells (pH 5.0-6.5).[36] Therefore, the release of GEM from **6a-d** and native GEM was carried out in PBS at pH 7.4 and pH 5.5. The release profiles of native GEM are portrayed in Fig. 4, 90% of native GEM was released at both conditions during initial 8 h and there was no significant pH dependency observed. Contrarily, **6a-d** showed much lower release rate which was plausibly ascribed to the presence of ester and amide linkage between GEM and CMG. During the 48 h period, the cumulative release at pH 5.5 was found to be 21.3%-52% while only 12.5%-18.4% at pH 7.4. However, the rate of GEM released from conjugates tended to be stationary in subsequent period of time. The most GEM was released from **6c** followed by **6a**, **6d** and **6b** in descending order. Amino acid-GEM compounds, for instance, 5'-gly-GEM could not be detected during the experiment probably due to the ester bond between amino acid and GEM cleaved easier in a lower pH environment than amide linkage between amino acid-GEM and CMG.

The antiproliferative activity of native GEM and **6a-d** was evaluated on lung tumor (A549), cervix epithelial adenocarcinoma (HeLa), colon adenocarcinoma (Caco-2) cell lines. All conjugates **6a-d** induced an increased cytotoxicity of GEM on A549 and HeLa cell lines compared with native GEM, especially on A549 cells. However, IC_{50} values of **6a-d** were higher than native GEM against Caco-2 cells. Furthermore, **6c**, which employed alanine as a linker, was the most cytotoxic when tested on A549 and HeLa cell lines. The better outcome of **6c** might be due to the fastest release rate of GEM from conjugate compared with **6a**, **6b** and **6d**. The antiproliferative activity of **6b** (glycin as the linker) was weaker than **6a** (tripeptide constructed of only glycine as the linker) on A549 and HeLa cell lines,

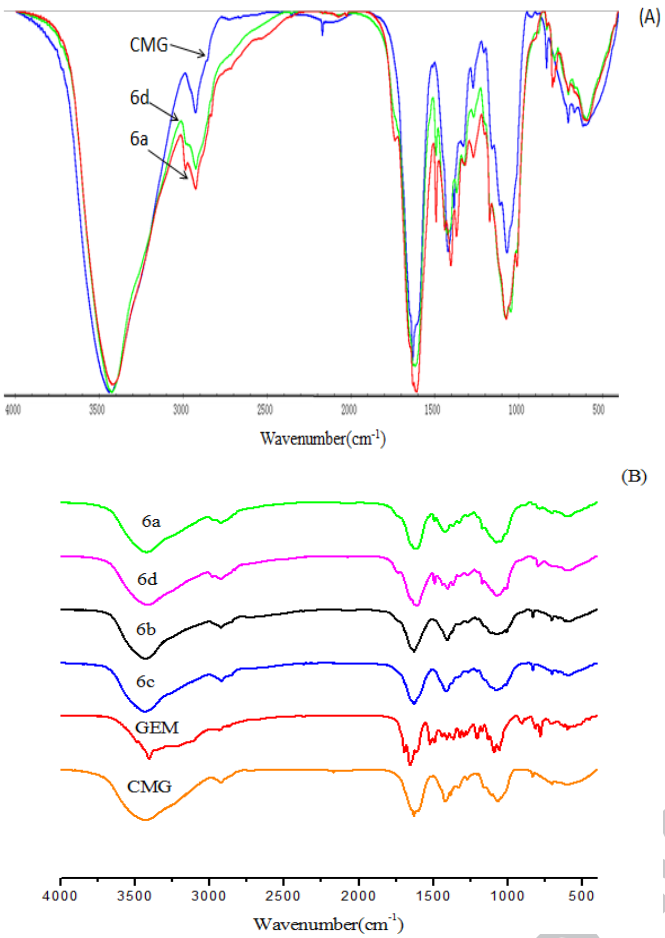

Fig. 3. FT IR of (A) overlapping spectra of CMG, **6d** and **6a**. (B) **6a**, **6d**, **6b**, **6c**, GEM and CMG in the region 4000 to 400 cm^{-1}.

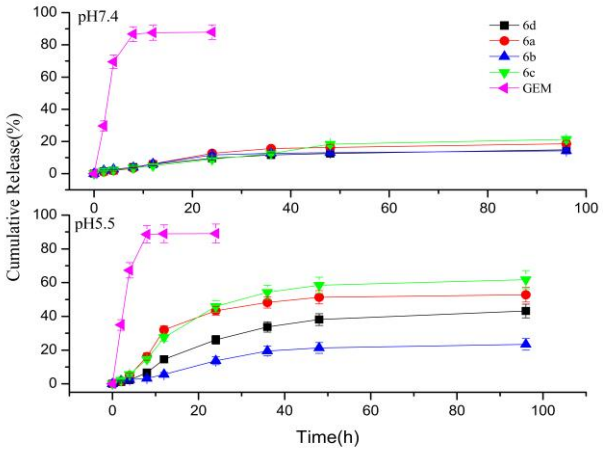

Fig. 4. Percentage of GEM release from **6a-d** in 0.1 M PBS at different pHs i.e., pH 5.5 and 7.4 at 37 °C. Experiment was terminated after 96 h and the error bar represents standard deviation (n = 3).

hinting that ~~proper~~ length and species of amino acid linker could have an effect on ~~significantly improve~~ the bioactivity of conjugate.

The results obtained here suggested the possibility of improving antiproliferative efficacy of GEM bound to CMG by means of amino acid linker. Although **6a-d** didn not exert an excellent therapeutic efficacy to all tested cell lines, fortunately, **6a-d** exhibited significant antiproliferative property on A549 cells. Among them, **6c** was the most active conjugate against A549 cell proliferation which also had the fastest release rate. **6a** was more cytotoxic than **6b** on A549 and HeLa cell lines, while the release rate of **6b** was slower than **6a**. Therefore, the amino acid linker is closely related to the release performance of GEM from conjugate, further having an effect on its therapeutic efficacy. More conjugates with amino acid linkers of different sequences, species and lengths should be synthesized to deeply explore the structure activity relationship. Moreover, antiproliferative activity on other lung tumour cell lines and in lung tumor animal modes should be investigated according to the performance of **6c** on A549 cells.

Table 2

IC_{50} values (μM) in A549, HeLa and Caco-2 cell lines (n=3, mean ± SD).

Compounds	A549	HeLa	Caco-2
6a	0.497±0.064	3.532±0.387	9.080±1.121
6b	0.646±0.071	5.194±0.591	12.437±1.622
6c	0.086±0.012	2.411±0.289	6.854±0.840
6d	0.336±0.043	12.553±1.390	10.712±1.241
GEM	0.802±0.096	>100	0.811±0.100

Acknowledgments

The research was supported by National Nature Science Foundation of China (Grant No. 81703389). In addition, we are glad to acknowledge the Analytical Center of Shenyang Pharmaceutical University for NMR measurements.

References and notes

1. Soni N, Soni N, Pandey H, Maheshwari R, Kesharwani P. Tekade RK. *Journal of colloid and interface science.* 2016;481: 107-116.
2. Hung SW, Marrache S, Cummins S, Bhutia YD, Mody H, Hooks SB, Dhar S. Govindarajan R. *Cancer letters.* 2015;359(2): 233-240.
3. Zhao X, Wang X, Sun W, Cheng K, Qin H, Han X, Lin Y, Wang Y, Lang J, Zhao R, Zheng X, Zhao Y, Shi J, Hao J, Miao QR, Nie G. Ren H. *Biomaterials.* 2018;158: 44-55.
4. Brown K, Weymouth-Wilson A. Linclau B. *Carbohydr Res.* 2015;406: 71-75.
5. Baraniak J, Pietkiewicz A, Kaczmarek R, Radzikowska E, Kulik K, Krolewska K, Cieslak M, Krakowiak A. Nawrot B. *Bioorganic & medicinal chemistry.* 2014;22(7): 2133-2140.
6. Mackey JRM, R. S. Selner. *Cancer Res.* 1998;58(19): 4349−4357.
7. Oluwasanmi A, Al-Shakarchi W, Manzur A, Aldebasi MH, Elsini RS, Albusair MK, Haxton KJ, Curtis ADM. Hoskins C. *Journal of controlled release : official journal of the Controlled Release Society.* 2017;266: 355-364.
8. Das M, Jain R, Agrawal AK, Thanki K. Jain S. *Bioconjugate chemistry.* 2014;25(3): 501-509.
9. Pasut G, Canal F, Dalla Via L, Arpicco S, Veronese FM. Schiavon O. *Journal of controlled release : official journal of the Controlled Release Society.* 2008;127(3): 239-248.
10. Maksimenko A, Mougin J, Mura S, Sliwinski E, Lepeltier E, Bourgaux C, Lepetre S, Zouhiri F, Desmaele D. Couvreur P. *Cancer letters.* 2013;334(2): 346-353.
11. Liang TJ, Zhou ZM, Cao YQ, Ma MZ, Wang XJ. Jing K. *International journal of pharmaceutics.* 2016;513(1-2): 564-571.
12. Khare V, Kour S, Alam N, Dubey RD, Saneja A, Koul M, Gupta AP, Singh D, Singh SK, Saxena AK. Gupta PN. *International journal of pharmaceutics.* 2014;470(1-2): 51-62.
13. Kiew LV, Cheong SK, Sidik K. Chung LY. *International journal of pharmaceutics.* 2010;391(1-2): 212-220.
14. Kushwah V, Agrawal AK, Dora CP, Mallinson D, Lamprou DA, Gupta RC. Jain S. *Pharmaceutical research.* 2017.
15. Szebeni J. *Toxicology.* 2005;216(2-3): 106-121.
16. Goodarzi N, Varshochian R, Kamalinia G, Atyabi F. Dinarvand R. *Carbohydr Polym.* 2013;92(2): 1280-1293.
17. Magnani M, Castro-Gomez RH, Aoki MN, Gregorio EP, Libos F, Jr., Morimoto HK, Reiche EM. Watanabe MA. *Natural product research.* 2012;26(10): 945-951.
18. Miadokova E, Svidova S, Vlckova V, Duhova V, Prazmariova E, Tothova K, Nadova S, Kogan G. Rauko P. *Biomedical Papers.* 2005;149(2): 493-496.
19. Magnani M, Calliari CM, de Macedo FC, Mori MP, de Syllos Cólus IM. Castro-Gomez RJH. *Carbohydrate Polymers.* 2009;78(4): 658-665.
20. Magnani M, Castro-Gomez RH, Aoki MN, GregÓRio EP, Libos F. Watanabe MAE. *Experimental and Therapeutic Medicine.* 2010;1(5): 859-862.
21. Zhang M, Cheung PCK, Chiu LCM, Wong EYL. Ooi VEC. *Carbohydrate Polymers.* 2006;66(4): 455-462.
22. Zhang M. *Carbohydrate Polymers.* 2004;57(3): 319-325.

Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.

23. Xie X, Zhou Y, Wang X, Guo J, Li J, Fan H, Dou J, Shen B. Zhou C. *Carbohydr Polym.* 2017;173: 360-371.
24. Zhi-wei Guo JMG. *J Org Chem.* 1999;64: 8319-8322.
25. Zhang D, Bender DM, Victor F, Peterson JA, Boyer RD, Stephenson GA, Azman A. McCarthy JR. *Tetrahedron Letters.* 2008;49(13): 2052-2055.
26. Peifer M, Berger R, Shurtleff VW, Conrad JC. MacMillan DW. *Journal of the American Chemical Society.* 2014;136(16): 5900-5903.
27. Gallo Z-wGaJM. *J Org Chem, .* 1999;64: 8319-8322.
28. Vale N, Ferreira A, Fernandes I, Alves C, Araujo MJ, Mateus N. Gomes P. *Bioorganic & medicinal chemistry letters.* 2017;27(13): 2898-2901.
29. Luan le Q. Uyen NH. *International journal of biological macromolecules.* 2014;69: 165-170.
30. Xing-Feng B AO YZH, Li R UAN. *Chem Pharm Bull* 2002;50(5): 623—629.
31. Tao Y, Zhang R, Yang W, Liu H, Yang H. Zhao Q. *Carbohydr Polym.* 2015;128: 179-187.
32. Wang X, Zhang Z. Zhao M. *International journal of biological macromolecules.* 2015;72: 526-530.
33. Manna PJ, Mitra T, Pramanik N, Kavitha V, Gnanamani A. Kundu PP. *International journal of biological macromolecules.* 2015;75: 437-446.
34. Jin Y, Zhang H, Yin Y. Nishinari K. *Carbohydr Res.* 2006;341(1): 90-99.
35. Yuen S-N, Choi S-M, Phillips DL. Ma C-Y. *Food Chemistry.* 2009;114(3): 1091-1098.
36. Pourjavadi A, Tehrani ZM. Moghanaki AA. *Pharmaceutical research.* 2016;33(2): 417-432.

Highlights

1. Gemcitabine derivatives were discovered by conjugating with carboxymethyl glucan via diverse amino acid linkers.
2. The release rate of gemcitabine from conjugates was increased under pH 5.5 compared with pH 7.4 in vitro release experiment.
3. Compounds **6c** exhibited excellent antiproliferative efficacy on A549 cells with IC_{50} values of 0.086 ± 0.012 μM.
4. Amino acid linker of different species and lengths could significantly improve the bioactivity of conjugate.

Graphical Abstract