Original Article

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Synthesis and *in vitro* Evaluation of Levothyroxine- Targeted Paclitaxel-Dextran Conjugate for Drug Delivery to Cancer Cells

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Abstract

Taxanes are a class of potent anticancer agents used in different cancers. Their low water solubility is the major challenge in formulation development that leads to the use of surfactants and organic solvents which causes severe hypersensitivity reactions. In this study, paclitaxel (PTX) was conjugated to a water-soluble and biocompatible polymer, dextran, to enhance solubility. Levothyroxine was also conjugated to dextran to provide targeted delivery. The cytotoxicity was studied on HepG2 and A375 cell lines before and after treating cell lines with levothyroxine. Results showed approximately 1250-fold water solubility enhancement by dextran conjugation. Drug conjugates presented higher cytotoxicity on A375 cell lines than its free drug counterparts at 10 and 50 nM concentrations after 24 hours. Pre-treatment with levothyroxine decreased the cytotoxicity on A375 as an integrin receptor rich cell line but did not show any significant effect on HepG2 cells which is low in expressing integrin receptor. In conclusion, preparation of levothyroxine targeted dextran conjugate might be an effective strategy for PTX delivery to different cell lines.

Keywords: Cytotoxicity, Conjugate, Dextran, L-thyroxine, Paclitaxel, Solubility.

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1. Introduction

Many studies have been conducted to find new drug delivery systems, because some common formulations in the world's pharmaceutical market have not been effective in delivering the drug to the site of the disease. Therefore, much effort have been done to design systems that are sensitive to the characteristics of the biological environment of the target tissues (1-3). One of these systems is prodrugs, through which the desired drug is conjugated to an inert carrier system and then released at the desired site with the help of enzymatic or chemical reactions (2, 4).

In recent years, macromolecular prodrugs have become popular, and many macromolecules with different molecular weights, such as antibodies, lipoproteins, proteins, polysaccharides, and synthetic macromolecules, have been used as drug carriers. Since the 1950s, several drugs have been covalently attached to various biodegradable and non-biodegradable polymers and the resulting prodrugs have been studied. Drugs are attached to polymers directly or via spacers to generate a wide range of prodrugs with different effects (3, 5-7). From the particle size point of view, these systems belong to nanocarriers. Therefore, all the advantages of nanoparticle systems could be added to

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their properties, including increased solubility and increased durability by enhanced permeability and retention effect (8).

One biocompatible polymer that can be applied in macromolecular-mediated prodrug delivery systems is dextran. This polymer is a hydrophilic, biocompatible natural polysaccharide that provides desirable delivery properties such as therapeutic targeting, increased drug residence time in the bloodstream, increased drug solubility, reduced side effects, and sustained release properties. Dextran is available in different molecular weights that present a wide range of solubility and can be conjugated to different molecules through hydroxyl functional groups (9-11).

Taxanes are broad-spectrum hydrophobic anticancer drugs widely used for clinical treatment of various types of cancers such as breast, prostate, colon, ovarian, and so on (12, 13). Their confirmed mechanism of action is stabilizing the microtubules and induction of abnormal spindles and microtubule dynamics failure which leads to blocking cell cycle progression (14). FDA-approved anticancer agents of taxanes are paclitaxel (PTX), docetaxel (DTX), and cabazitaxel (CTX). Reported side effects of taxanes are hypersensitivity reactions, edema, neurotoxicity, bone marrow suppression, diarrhea, vomiting, and nausea. Furthermore, because of their uptake by the normal tissues, other side effects such as hair loss have also been reported (14).

Due to the low water solubility of taxanes, surfactants (Cremophor EL® for PTX (15) and polysorbate 80 for DTX and CTX formulations) (16) are used to solubilize the drugs. Unfortunately, these surfactants cause severe life-threatening side effects such as edema, allergic reactions, and effusion (16). Therefore, many approaches have been attempted to diminish the use of toxic surfactants and provide targeted delivery of the taxanes to the tumor tissue (5, 6, 16-18). One of these approaches is preparation of prodrugs (8) that could improve the pharmacokinetics properties of taxanes and their solubility profile, as well (19).

In this study, a highly soluble conjugate of PTX was prepared. To eliminate the non-specific uptake of drug by the normal cells, thyroid hormone, levothyroxine (L-thyroxine) which has vital role in the growth and functions of cells, was covalently bound to the conjugate to induce carrier mediated uptake through integrin receptors.

Integrins as cell-surface anchor proteins regulate cell proliferation and migration as well as cell survival and apoptosis. The association of integrin receptors in tumor metastasis and angiogenesis and their overexpression has been approved in many studies. These receptors bind to the extracellular ligands and connect the extracellular environment and the intercellular signaling pathways. Several small molecules such as thyroid hormones have binding sites on cell surfaces for integrin to induce biological activities. L-thyroxine is transported into the cells through a carrier-mediated process. Integrin αVβ3 receptor overexpressed on vessels of tumors has a recognition site for L-thyroxine (20, 21).

These characteristics make L-thyroxine an attractive targeting molecule on integrin αVβ3 receptors (22). To examine the hypothesis, L-thyroxine-conjugated PTX-dextran prodrug was prepared and its effect on cell cytotoxicity was evaluated.

2. Materials and Methods

2.1. Materials

PTX was purchased from Sobhan Oncology Co. L-thyroxine was obtained from Iran Hormone Co. Dextran (molecular weight 70,000), carbonyldiimidazole (CDI), N-Hydroxy succinimide (NHS), Di-cyclohexyl carbodiimide (DCC), glutaric anhydride, pyridine, 1,6-hexamethylenediamine, sodium periodate (NaIO4) and (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide] (MTT) were purchased from Sigma Aldrich (St. Louis, USA). Anhydrous DMSO, methanol, ethanol, diethyl ether, and HPLC grade acetonitrile were obtained from Merck (Germany). All other chemicals were analytical grade and used without further modification.

2.2. Synthesis of conjugates

2.2.1. Synthesis of PTX- dextran conjugate

PTX glutarate hemiester was synthesized according to the method reported previously for CTX (5, 6). Briefly, 0.10 mmole PTX, 1.0 mmole glutaric anhydride and 0.5 mmole pyridine were

dissolved in 5 mL acetonitrile: chloroform (20:80) and stirred at room temperature (12 h). The reaction mixture was then washed with 5 mL of deionized water (three times) to eliminate pyridine and the excess amount of glutaric anhydride. Finally, the organic phase containing PTX-glutarate and unreacted PTX was chromatographed over silica gel for final purification.

Aminodextran was synthesized as previously reported by Mouaziz *et al*. with minor modifications (23). In this regard, 0.30 mmole dextran 70 KD was dissolved in 10 mL of distilled water, 0.10 mmole NaIO4 was added to dextran solution and stirred for 12 h. After that, the oxidized dextran solution was dialyzed (MWCO = 12000) for 24 h against distilled water. The obtained solution was cooled to about 5 °C and 0.3 mmol of 1,6-hexamethylenediamine was added to the reaction solution and stirred for 12 h until a homogeneous dark yellow solution was obtained. Then, 0.5 mmole sodium borohydride in 5 mL aqueous potassium hydroxide (1 mM) was added and stirred at room temperature for about 24 h. The final pale yellow solution was dialyzed (MWCO= 12000) for 24 h and the resulting aminodextran product was freeze-dried.

In the next step, aminodextran (1.0 mmol), 1.0 mmol CDI, 0.5 mL anhydrous triethylamine and 0.3 mmol PTX-glutarate were dissolved in 5 mL anhydrous DMSO and stirred at room temperature for 24 h. The obtained product was precipitated by adding a mixture of ethanol: diethyl ether (50:50) to the reaction medium followed by centrifugation. For the final purification, precipitate was washed with acetone three times to extract the unreacted drug hemiester.

2.2.2. Synthesis of PTX-dextran-L-thyroxine conjugate

A solution of 0.23 mmol L-thyroxine, 0.7 mmol NHS and DCC were prepared in 5 ml DMSO and stirred for 24 h to activate the carboxyl groups. The mixture was centrifuged at 10000 rpm for 15 minutes to precipitate dicyclohexylurea. Subsequently, 0.1 mmol PTX-dextran was added to the mixture and the reaction was continued at room temperature for 24 h. The obtained conjugate was purified by dialysis against water. The conjugation was confirmed by ^{1}H-NMR spectroscopy. ^{1}H-NMR spectrum (DMSO-d6) was recorded on a Bruker 400 MHz spectrometer (Avance III, Germany).

2.3. Determination of PTX content in the conjugate

HPLC method was applied to determine PTX content in the synthesized conjugate. A reversed-phase HPLC system consisting of WATERS C18 (150 mm×4.6 mm, 5 µm) column, SPD-10AVP with PDA detector LC-10AP pump (Shimadzu), and Class Nuchrom software was used. Mobile phase consisted of acetonitrile: water (30:70) and flow rate was set to 1 mL/min. Detection wavelength was set to 227 nm.

To determine drug content, basic hydrolysis was performed on sample. Briefly, defined amount of the conjugate was added to NaOH solution (0.1 N) and stirred for 3 h. Then methanol was added and vortexed for half an hour to precipitate the polymer. Defined volume of the resulting solution samples was injected to HPLC to determine the concentration of the drug using PTX standard curve. Standard curve of PTX was constructed in concentration range of 2.5, 5, 10, 20 and 50 µg/mL.

2.4. Determination of L-thyroxine content in conjugates

HPLC method was applied to determine L-thyroxine content by mentioned HPLC system after basic hydrolysis (mentioned in section 2.3). The mobile phase consisted of acetonitrile:water (50:50) and the flow rate was set to 1 mL/min at 227 nm. The standard curve of L-thyroxine was determined in concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 mg/ml.

2.5. Solubility studies

Solubility of conjugate was determined by adding an excess amount of synthesized prodrug to 1 mL of distilled water that was vortexed for 24 h at 25 °C. Then, the sample was filtered and the sediment was dried and the amount of conjugate was determined.

2.6. In vitro cytotoxicity assay

The cytotoxicity assay of the prepared prodrug was examined on HepG2 and A375 cells by MTT assay (21, 24). In brief, HepG2 hepatocellular carcinoma cell (C158, NCBI, Tehran, Iran) and A375 melanoma cells (C136, NCBI, Tehran, Iran) were cultured in DMEM media (1 g/l glucose, 2 mM glutamine), containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 µg/ml streptomycin. Cell cultures were incubated at 37 °C in a humidified 5% CO_2 before the experiment. A number of 5000 cells per well was seeded in a 96-well plate and allowed to adhere. After 24h, cells were exposed to PTX (in different concentrations (100, 50, 10, and 1 nM)) for 4 hours and prodrugs (with the equivalent concentrations of PTX) and incubated at 37 °C (5% CO_2 humidified atmosphere). MTT solution in DMSO, was then added to each well and cells were incubated for 48 h in the mentioned atmosphere. Cells incubated with culture medium were considered as the blank control group. Cell viability (%) was studied at 570 nm as the ratio of the absorbance of the treated cells (with different samples) to blank control cells.

2.7. In vitro comparative inhibition assay of L-thyroxine

To examine the possible effect of L-thyroxine on cell internalization via integrin receptors, competitive inhibition of the integrin receptors was evaluated.

HepG2 and A375 cells pre-treated with L-thyroxine at 10^{-3}mol/ml concentration were seeded for 30min at 37 °C as described in section 2.6. Then, 100nM of the conjugate was added to each well for 4 h. Cell viability (%) was then studied after 24 and 48 hours.

2.8. Statistical analysis

All data were reported as mean ± standard deviation (SD). Data comparison was evaluated by one-way ANOVA test and $p< 0.05$ was considered as a significant difference.

3. Results and discussion

3.1. Synthesis of conjugate

Successful synthesis of PTX-dextran-L-thyroxine conjugate was confirmed by ^{1}H-NMR data. Conjugation of PTX glutarate to the hydroxyl groups of dextran was studied by ^{1}H-NMR method. PTX- glutarate was synthesized successfully with high yield (73±3.1 %). ^{1}H-NMR data of the hemiester is presented in Figure 1, B that confirm the formation of related glutarate analog. Aromatic and glutarate related protons are shown and compared with intact PTX (Figure 1, A). An ester bond was formed between the hydroxyl groups of dextran and the carboxylic groups of glutarate linker

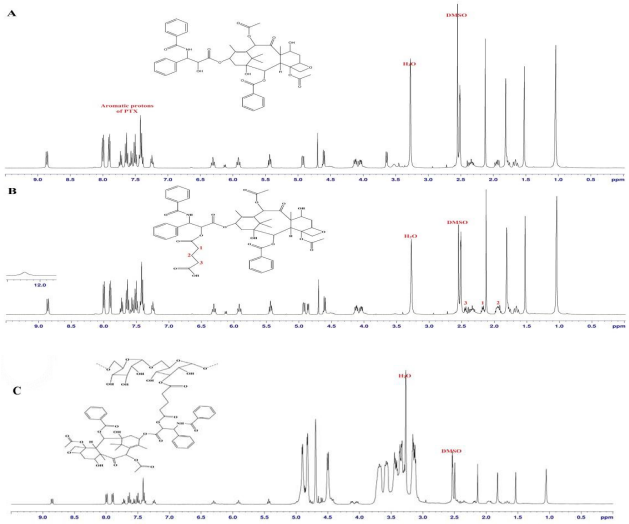

Figure 1. ^{1}H-NMR data of A) PTX, B) PTX-glutarate, C) PTX-dextran

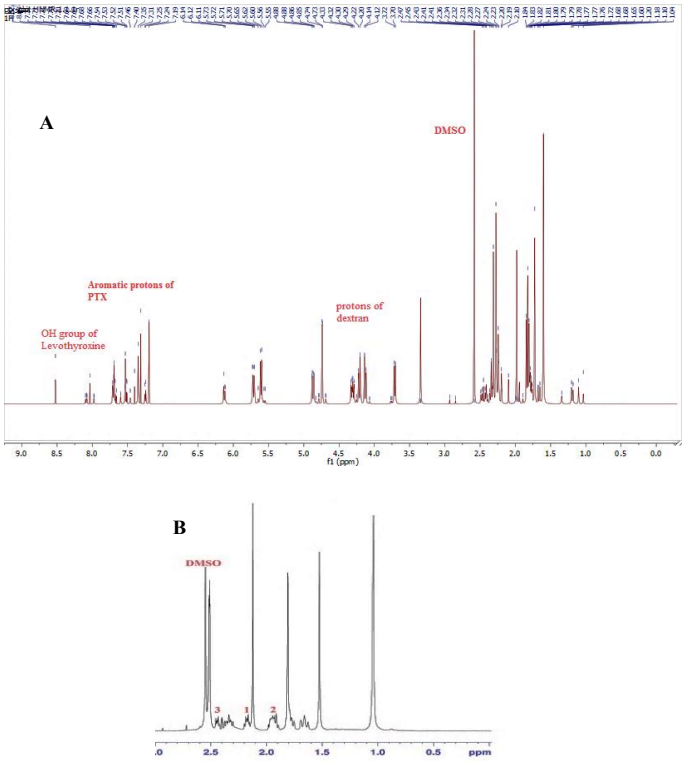

Figure 2. ^{1}H-NMR spectrum of the A) final conjugate, B) expanded spectrum ofof protons of glutarate linker.

(Figure 1, C). The results are in accordance with previous studies on other taxane drugs; cabazitaxel, docetaxel and PTX itself (5, 6, 25, 26).

An ester bond was formed between the hydroxyl groups of dextran and the carboxylic group of glutarate linker (peaks 1, 2 and 3, Figure 2, B). Aromatic protons of PTX and L-thyroxine and protons of dextran are presented at 7 to 8 ppm and 4 to 4.5, respectively. The peak at 8.5 ppm is related to the hydroxyl group of L- thyroxine.

3.2. PTX content in conjugate

PTX calibration curve was constructed in the range of 2.5 to 50 µg/mL. Precision (102.3±3.0 %), accuracy (98.5±0.8 %), LOD (0.77 µg/mL) and LOQ (2.34 µg/mL) were in acceptable ranges. Hydroxyl groups of dextran were activated by CDI and were conjugated to the carboxylic functional groups of PTX hemiester analog through ester bonds. By performing basic hydrolysis process, the formed ester bond was hydrolyzed and free drug was dissociated from dextran conjugate.

Content of PTX in conjugate was calculated to be 16.9±0.35%. This relatively high degree of drug content of conjugate is related to the presence of high number of hydroxyl functional groups in dextran polymer that provides a wide range of possible active sites for conjugation.

3.3. Determination of L-thyroxine content in conjugate

Validation results for determination of L-thyroxin showed an acceptable precision (98.4±0.5 %), accuracy (101.9±3.9 %), LOD (0.016 mg/ml) and LOQ (0.047 mg/ml). L-thyroxine content of the conjugate was 10.2±0.6 %. As described before, presence of hydroxyl groups in dextran polymer resulted in high degree of conjugation.

3.4. Solubility studies

A high value of 37.0±0.3 mg of conjugate was dissolved in 1 ml of deionized water. Based on the PTX content in each 100 mg of the conjugate, about 6 mg PTX is present in 37 mg of the

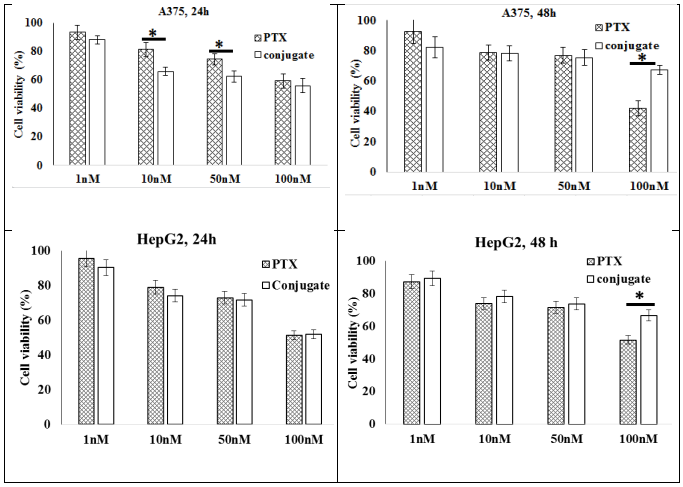

Figure 3. Results of cell viability of different concentration of conjugate after 24 and 48 h on A375 and HepG2 cell lines. *p<0.05, PTX compared to conjugate.

conjugate that was dissolved in water. In other words, the aqueous solubility of PTX (about 5 µg/mL) was increased to 1250 fold after conjugation. In our other studies on dextran conjugates of taxane drugs, different solubility values were reported. Solubility enhancement of cabazitaxel and docetaxel was more than 1000 fold that confirmed the effect of dextran conjugation on improving solubility of poorly soluble drugs (5, 6, 25).

3.5. In vitro cytotoxicity study

HepG2 is a human hepatoblastoma derived cell line, which expresses low integrin receptors in comparison to A375 which is an integrin receptor-rich cell line. *In vitro* cytotoxicity test showed (Figure 3) that on A375 cells, conjugate showed higher toxicity in 10 and 50 nM concentrations after 24 h. However, cytotoxicity was not significant (p> 0.05) in the concentration of 1 and 100 nM after 24 h and in all concentration after 48h. As an exception, only in 100 nM concentration after 48 h, conjugate presented less toxicity in comparison to its free PTX counterpart.

In HepG2 cell line, no significant difference was seen in all concentrations after 24 h (p> 0.05).

However, a significant difference between PTX and its conjugate was observed in 100 nM concentration after 48 h. Based on the results, cell toxicity of the conjugate was time and concentration dependent and higher toxicity on A375 cell line after 24 h in 10 and 50 nM concentrations was obtained. An explanation for the higher toxic effects of conjugate on A375 cell line is overexpression of integrin receptors on its cell membrane which leads to higher uptake of the conjugate, whereas drug uptake is not very efficient by HepG2 cells.

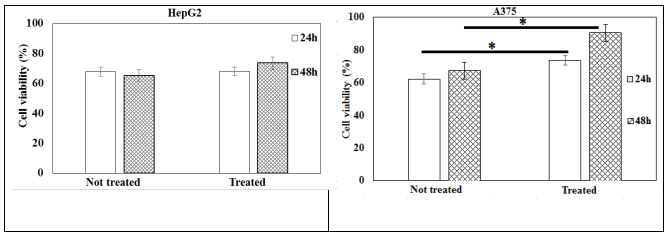

Figure 4. Results of cell viability of conjugate before and after treatment with L- thyroxine after 24 and 48 on HepG2 and A375 cell lines, , * p < 0.05.

3.6. In vitro competitive inhibition assay

To evaluate the mechanism of drug entrance and impact of integrin receptors on the enhanced uptake of the conjugate by A375 cell line competitive inhibition assay was performed. A375 cell line showed higher cell viability for 13 and 23% after 24 and 48 h, respectively when pretreated with L-thyroxine (Figure 4). In other words, L-thyroxine pre-treatment of A375 integrin overexpressing cells resulted in a substantial decrease in the entrance of the conjugate into cells because of saturation of the receptor mediated mechanism. The difference in cell death was not significant in HepG2 ($p > 0.05$). This was in accordance with previous studies that showed the effects of L-thyroxine pretreatment on cell viability (22). Meanwhile, more studies are needed in different concentrations of L-thyroxine and on various cell lines to prove the accuracy of these preliminary results.

4. Conclusion

PTX-L-thyroxine-Dextran conjugate was successfully synthesized via a simple chemical process. Conjugation to dextran improved the solubility of PTX which is a major solution to complications in pharmaceutical formulations of PTX. L-thyroxine conjugation to the conjugate increased cell line, cytotoxicity effect in a time and concentration dependent manner due to the higher uptake by integrin receptors on A375 cell line.

It seems that targeting and conjugating PTX to a water-soluble carrier would increase the solubility and cell uptake. Hence, targeted dextran derivative described in this study might serve as an efficient carrier for delivery of poorly soluble taxanes into the cells over expressing integrin receptors.

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Conflict of Interest

The authors declare no conflict of interest.

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