Preparation, characterization and \textit{in vitro} activity of a docetaxel–albumin conjugate

Jing Gao\(^1\), Shougang Jiang\(^1\), Xuewei Zhang, Yujie Fu, Zhiguo Liu\(^*\)

Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin 150040, People’s Republic of China
State Engineering Laboratory of Bio-Resource Eco-Utilization, Harbin 150040, People’s Republic of China

\textbf{ARTICLE INFO}

\textbf{Keywords:}
- Docetaxel
- Human serum albumin (HSA)
- 6-Maleimidocaproic acid
- Conjugate

\textbf{ABSTRACT}

Docetaxel is one of the most effective anticancer drugs. However, the current formulation of docetaxel contains Tween 80 and ethanol as the solvent, which can cause severe side effects. Consequently, the development of new type of formulation of docetaxel with high efficiency and low side effect is a very important issue. In this study, we explored the covalent linking of docetaxel and albumin via one organic linker. 6-Maleimidocaproic acid was applied to link the C2’ hydroxyl group of docetaxel with the cysteine-34 of albumin to obtain 1:1 docetaxel–albumin conjugate. The synthesized conjugate can control the release of docetaxel in the bovine serum. Furthermore, \textit{in vitro} cell cytotoxicity experiments indicated that the docetaxel–albumin conjugate have high activities for human prostate cancer cell line PC3 and human breast cancer cell line MCF-7. The present study provides a valuable strategy for further development of a new type of docetaxel–albumin prodrug.

\section{1. Introduction}

Docetaxel has been routinely used to treat breast cancer, ovarian and non-small cell lung cancers \cite{1}. It has been considered as one of the most effective chemotherapeutic agents over the past two decades \cite{2,3}. However, due to its low aqueous solubility, the current formulation of docetaxel in clinical use contains a large proportion of Tween 80 and ethanol (50:50, v/v), which can cause severe side effects. In order to avoid the adverse effects and increase the drug solubility, alternative formulations including liposomes, polymers and protein adjuvants have been investigated \cite{4–9}.

Among the variety of adjuvants, albumin has received considerable attention \cite{5}. Albumin is the most abundant plasma protein (35–50 g/L in human serum). It is an acidic, robust and very soluble protein. It is well known that a great number of therapeutic drugs can bind on albumin through reversible and covalent modes \cite{1,10,11}, which ultimately affects drug absorption, metabolism, distribution and excretion \cite{12}. Though albumin does not directly target an antigen or receptor on the surface of a tumor cell, it can accumulate in solid tumors by ‘enhanced permeability and retention’ (EPR effect) in relation to passive tumor targeting, which results from the enhanced vascular permeability of circulating macromolecules in tumor tissue and subsequent accumulation \cite{5,13}.

It has been revealed that most of the paclitaxel and docetaxel released from the formulations in a serum bound to the albumin \cite{14}. Nanoparticle albumin-bound paclitaxel (nab-P, Abraxane, Abraxis BioScience, USA) has shown greater activity and a better safety profile in comparison with standard solvent-based taxanes \cite{15,16}. Nab-P was approved by US Food and Drug Administration (FDA) in January 2005 and by the European Agency for the Evaluation of Medicinal Products (EMEA) in 2008 for the treatment of metastatic breast cancer \cite{14}. As for docetaxel, nanoparticle albumin-bound formulation was in a phase I/II trial to treat metastatic breast cancer and prostate cancer. In nanoparticle albumin-bound formulations, taxanes such as paclitaxel and docetaxel were physically adsorbed \cite{17–20}. There are some inherent disadvantages of nanoparticle albumin formulations such as instability and heterogeneity \cite{1}.

Conjugation of low molecular weight anticancer drugs to organic acid, muramyl dipeptide, antibodies, serum proteins or polymers through a cleavable linker has been an effective method for improving the therapeutic index of cytotoxic established agents \cite{21–25}. Covalent conjugation of docetaxel to albumin is an alternative approach to improve its water solubility and chemotherapeutic potency. Albumin has been exploited as an attractive protein carrier for drug delivery in recent years. The advantage of albumin as drug carrier is that it is non-toxic, non-immunogenic, biocompatible and biodegradable. Though it is not directly targeted towards an antigen or receptor on the surface of the tumor cell, albumin can accumulate in tumor tissues by ‘enhanced

\textsuperscript{*}Corresponding author.
\textsuperscript{+}E-mail address: zgooliu@nefu.edu.cn (Z. Liu).
\textsuperscript{1}These authors contributed equally to this work (co-first author).

https://doi.org/10.1016/j.bioorg.2018.10.032
Received 3 September 2018; Received in revised form 11 October 2018; Accepted 17 October 2018
Available online 17 October 2018
0045-2068/ © 2018 Elsevier Inc. All rights reserved.
permeability and retention’ (EPR effect) in relation to passive tumor targeting. Docetaxel covalently attached to human serum albumin can greatly improve its aqueous solubility, and avoid use the adjuvant vehicle such as polysorbate 80 and ethanol that can cause serious hypersensitivity reactions. Furthermore, covalent linking of docetaxel and albumin via a cleavable linker facilitate the control release of docetaxel. An additional advantage of albumin conjugate is that it shows improved pharmacokinetic behavior as well as escaping the cellular mechanisms underlying drug resistance.

Covalent cross-linking of taxanes and albumin has been attempted in recent years. For instance, the C2’ hydroxyl group of taxanes and lysyl amino groups of albumin can be utilized for the conjugation [26–28]. However, this strategy has some disadvantages such as the exact sites and the numbers of taxanes bound on albumin were difficult to control. Too many taxanes molecules bound on the albumin can induce the corresponding conjugates to be insoluble and even aggregative [26,28]. Consequently, quantitative conjugation of docetaxel and albumin is still an important research issue. Recently, Kratz et al. established an exact cross-linking approach to conjugate one doxorubicin to one HSA [5,13,22]. They found that the Cys34 position of albumin could be utilized to exactly cross-link doxorubicin [13,22]. Inspired by this innovative research, we explored a method to covalently conjugate docetaxel and albumin at the Cys34 position. Furthermore, the inhibitory activity of the docetaxel–albumin conjugate for the human breast cancer cell line and the human prostate cancer cell line has been investigated.

2. Materials and methods

2.1. Materials

Docetaxel was purchased from Sangon Biotech (Shanghai, China). 6-Maleimidohexanoic acid was provided by Jiaxing Bomei Biotech Lmt. Co. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), 4-(Dimethylamino) pyridine (DMAP) and human serum albumin (HSA) were purchased from Sigma-Aldrich (Shanghai, China). The human breast cancer MCF-7 cell line and the human prostate cancer PC-3 cell line were obtained from Harbin medical university (Harbin, China). Fetal bovine serum for cell culture was provided by ThermoFisher scientific (Beijing, China). All other reagents were analytically pure.

2.2. Synthesis of docetaxel-6-maleimidohexanoic acid-HSA conjugates

71.2 mg (0.09 mmol) docetaxel and 18.4 mg (0.09 mmol) 6-maleimidohexanoic acid were dissolved with 3 ml dichloromethane in a round bottomed flask. 3.4 mg (0.03 mmol) DMAP and 16.8 mg (0.09 mmol) EDAC were dissolved with 1 ml dichloromethane and then transferred into the flask. The mixed solution was stirred at room temperature for 3 h. 1 ml pure water was added to cease the reaction. The reacted solution was transferred into a 10 ml centrifuge tube. 1 ml saturated NaCl solution was used to wash the organic layer. After removing the water layer, the organic layer was evaporated under reduced pressure at 35 ºC. The obtained product was then dissolved by methanol and further purified by a preparative high performance liquid chromatography (HPLC) column (XBrige Prep C18 (10 * 250 mm, 5 μm), waters Co.). The obtained docetaxel-6-maleimidohexanoic acid crosslink was characterized by nuclear magnetic resonance (NMR) (Bruke Avance 300, Germany) and mass spectrometry (MS) (API3000, Applied Biosystems, CA, USA).

The docetaxel-6-maleimidohexanoic acid crosslink was then used to covalently conjugate with the HSA. 50 mg of HSA was dissolved with 2 ml pure water and 2 ml acetonitrile, and then 0.2 ml of equal molar docetaxel-6-maleimidohexanoic acid crosslink solution (dissolved by acetonitrile) was added. The mixed solution was irradiated with 254 nm ultraviolet light for 4 h to produce the final product. The obtained docetaxel-6-maleimidohexanoic acid-HSA was identified by Bruker Ultrafle Xtreme matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (25 kV, sinapinic acid substrate).

The release of docetaxel from docetaxel-6-maleimidohexanoic acid-HSA crosslink in bovine serum was investigated. 200 μl of 0.43 μM/ml docetaxel-6-maleimidohexanoic acid-HSA was added into a 5 ml of the bovine serum solution. The sample was maintained at 37 ºC in a water bath in the dark. 200 μl of the sample was removed at a scheduled time
and then added into 1 ml of bovine serum. The obtained solution was extracted with 2 ml ether for 20 min, and then rotary evaporated to dry. The obtained residue was dissolved by 200 μl acetonitrile. The concentration of docetaxel was determined by the HPLC method (mobile phase, acetonitrile/methanol 8.5:1.5).

2.3. In vitro cell cytotoxicity experiments

MCF-7 and PC-3 were incubated in the 10% fetal bovine serum and DMEM/high glucose culture medium at 37 °C in a 5% CO2 saturated humidity incubator. The passages of cells were digested with 0.25% trypsin. The cells (MCF-7, PC-3) in logarithmic growth phase were seeded into 96-well plates (100 μl/well). When the cells had grown to approximately 80% surface area of the well, the drugs were added at concentrations of 0.032, 0.16, 0.8, 4, 20, 50 and 100 μM. Each concentration was tested in five wells. After incubation for 72 h, 20 μl of methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to the wells. After further 4 h incubation in a 5% CO2 saturated humidity incubator, the 96-well plates were taken out and measured at absorption wavelength at 492 nm by a microplate reader.

3. Results and discussion

In this study, we demonstrate the covalent linking of docetaxel and HSA by 6-maleimidohexanoic acid. Fig. 1 shows the molecular structures of docetaxel (1), 6-maleimidohexanoic acid (2), HSA (3) and the final conjugate (4). 2′ Hydroxyl group of docetaxel and the Cys34 position of albumin were utilized as the conjugation sites as illustrated in Fig. 1. 6-Maleimidohexanoic acid was covalently conjugated with 2′ hydroxyl group of docetaxel by an esterification reaction, and then other side was linked with the HS-group of Cys34 of HSA by a Michael addition reaction.

3.1. Covalent conjugation of 6-maleimidohexanoic acid with docetaxel

6-Maleimidohexanoic acid has been applied for the conjugation reaction in this study. The reason is that its carboxylic acid moiety can be used for esterification reactions, whereas its unsaturated double bond inside the ring can be utilized for the Michael addition with the HS-group of Cys34 of HSA. Furthermore, 6-maleimidohexanoic acid has been successfully used for the preparation of a doxorubicin conjugate to bind albumin [5,13,22,29]. This doxorubicin derivative, named as DOXO-EMCH, is a carboxylic hydrazone in which doxorubicin is reacted at the C-13 keto position with 6-maleimidocaproic acid hydrazide (ε-maleimidocaproic acid hydrazide [EMCH]) and was chosen for further preclinical investigations [13,22]. It has been demonstrated that DOXO-EMCH bound rapidly and selectively to endogenous albumin, and indicated excellent antitumor activity [13,22]. The binding position of DOXO-EMCH on HSA has been identified to be the HS-group of Cys34. Based on these previous results, we investigate the use of 6-maleimidohexanoic acid to covalently conjugate docetaxel and HSA. To the best of our knowledge, this conjugation approach for docetaxel and HSA has not been thoroughly studied. Therefore, the conjugation of docetaxel and HSA in this study is believed to be a new type of docetaxel prodrg. The esterification reaction of docetaxel and 6-maleimidohexanoic acid was performed as described in the experimental section. As revealed by recent reports, the C2′ hydroxyl group of docetaxel is the very active position that was the first esterification hydroxyl group of docetaxel [28]. The obtained esterification product was purified by a preparative HPLC column, and identified by electrospray ionization mass spectrometry (ESI-MS) and NMR measurement. Fig. 2 shows the MS spectra of the conjugate of docetaxel and 6-maleimidohexanoic acid. The highest peak at m/z = 1023.7 in Fig. 2 is ascribed to the [M + Na]⁺ of the esterification product. This result confirmed that the 6-maleimidohexanoic acid has been attached to the docetaxel. The reaction site of 6-maleimidohexanoic acid on the docetaxel was further identified by NMR characterization as shown in Fig. 3. In the NMR
spectra of the conjugate, the presence of a peak at chemical shift $\delta$ 5.8 can be attributed to the hydrogen connected with 2′ carbon atom that has a chemical shift $\delta$ 5.4 in the pure docetaxel as shown in Fig. 3. The chemical shift of the hydrogen connected with C2′ carbon atom moved to low field can be attributed to the formation of an ester bond. Thus, the 6-maleimidohexanoic acid is believed to be linked at the 2′ hydroxyl group of docetaxel in the esterification reaction.

3.2. Docetaxel-linker-HSA conjugate

After 6-maleimidohexanoic acid was linked on the docetaxel, this intermediate was further used for conjugating with the HS-group of Cys34 of HSA via a Michael addition reaction. Before this cross-linking reaction, pure HSA was characterized by MS as shown in Fig. 4(a). The presence of the peak at $m/z$ of 66604.7 in Fig. 4(a) confirmed the existence and purity of HSA [30].

Fig. 4(b) shows the MS spectra of the final conjugate. The main peak at 67886.6 is 1281.9 mass units higher than that of the mass for pure HSA (66604.7). The calculated molecular mass of one docetaxel 6-maleimido-hexanoate is 1001. Based on these results, it can be inferred that one docetaxel 6-maleimido-hexanoate was conjugated on one HSA molecule. The additional mass (280.9) can be ascribed to the formation of matrix adducts of sinapinic acid (Mr 224.1 Da) with HSA and some unknown oxidation process. Sinapinic acid has usually been used as the matrix in MALDI-TOF-MS analysis. It has been confirmed that sinapinic acid can interact with HSA via covalent or electrostatic attachment [31,32]. In addition, according to the detection accuracy of MALDI-TOF-MS around 0.1%, the detection of a 60 kDa molecule will have 60 mass units uncertainty. The uncertainty may also derive from uncertainties in the isotopic abundance for each element in a complex biomolecules, such as proteins. Based on this MS result, it can be concluded that the conjugates were made up of 1:1 docetaxel and HSA. One merit in this study is that our approach realized the 1:1 conjugation of docetaxel and HSA by a relatively mild chemical reaction. To the best of our knowledge, the conjugate composed of 1:1 docetaxel and HSA has not been thoroughly studied in previous studies.
To verify the exact conjugation position of docetaxel on HSA, we have performed some control experiments to identify the exact reactive position on HSA in our preparation process. Iodoacetamide was used to block the reaction of Cys34 of HSA in a control experiment. The results indicated that the addition of iodoacetamide can significantly reduce the formation of docetaxel-HSA conjugate as determined by MALDI-TOF-MS analysis, which strongly supports the binding of one docetaxel 6-maleimido-hexanoate to the Cys34 on HSA [33].

It is critical for drug–protein conjugates to release their active components. In our study, the controlled release of docetaxel from the synthesized conjugate has also been investigated in the PBS buffer and bovine serum. Fig. 5 shows the release profile of docetaxel from the conjugate (docetaxel-6-maleimidohexanoic acid-HSA) in bovine serum.

The obtained results indicate that docetaxel was gradually released from the synthesized conjugates by a time-dependent process. After 14 h incubation, the concentration of docetaxel increased to a high level (release rate 65.7%). There was no further increase by further incubation. The release mechanism is believed to be a hydrolysis process in which the C2′ ester bond of conjugate is cleavable and hydrolyze into docetaxel and albumin-linker. It has been demonstrated that C2′ position of ester bond of the docetaxel–HSA conjugate can be hydrolysis in an aqueous solution [26,28]. The conjugate in this study undergoes more rapid ester bond hydrolysis in comparison with the release results of the docetaxel–albumin conjugate via a succinic linker [28]. The difference of hydrolytic lability between these two conjugates can be ascribed to the changes of the steric bulk at the C2′ ester bond. In this study, the use of a relatively long 6-maleimidohexanoic acid linker can reduce the steric bulk at C2′ position, which facilitates the hydrolysis process. As anticipated, increase in steric bulk at C2′ ester bond center by using short succinic linker slows its hydrolysis rate. In serum, the hydrolysis of the ester bond of the conjugate takes place by both chemical and enzymatic mechanisms (there are different type of esterase in serum). Furthermore, chemical degradation process of taxanes may occur in serum as revealed in the recent literature [34]. This is a possible reason that the measured concentration of the docetaxel was not further increased with increasing of the incubation time.

3.3. In vitro cell test of docetaxel-6-maleimido-hexanoate -HSA conjugate

Since docetaxel is one of the most effective anticancer drugs for the treatment of breast cancer and prostate cancer [35,36], the human breast cancer MCF-7 cell line and human prostate cancer PC-3 cell line were used for the In vitro test. An identical concentration of pure docetaxel was also investigated as the positive control. Fig. 6(a) shows the growth inhibition curve of MCF-7 cells subjected to different treatments.
for 72 h. The results indicate that the human breast cancer MCF-7 cells were inhibited by the obtained conjugate in a dose-dependent manner. Based on the cell test results, the IC50 (the dose which produces 50% inhibition of cell growth) of docetaxel and the final conjugate were calculated. The IC50 of pure docetaxel and docetaxel-6-maleimidohexanoate-HSA for MCF-7 cell line was 0.25 μM and 1.55 μM, respectively. Fig. 6(a) shows the growth inhibition curve of the PC-3 cells subjected to different treatments for 72 h. The IC50 of pure docetaxel and docetaxel-6-maleimido-hexanoate -HSA for the PC-3 cells was 11.8 μM and 0.96 μM, respectively.

All of the in vitro results indicate that the docetaxel-HSA conjugate in this study had high cytotoxicity, which can be ascribed to the controlled release of docetaxel from the conjugate. Furthermore, the cytotoxicity of the docetaxel-HSA conjugate varied with the different cell lines. For instance, the IC50 of docetaxel-HSA conjugate for the MCF-7 cell line is approximately 6 times of that of docetaxel whereas the IC50 for the PC-3 cell line is one twentieth of that of docetaxel. It has been noted that the docetaxel-HSA conjugate was nontoxic at a relatively low concentration (0.16 μM for MCF-7 and 0.4 μM for PC-3) in comparison to pure docetaxel, whereas it also indicates a gradual increase of toxicity with an increasing concentration. This phenomenon can be explained as follows. When the docetaxel-HSA conjugate was applied to cancer cell lines at relatively low concentrations (0.16 μM for MCF-7 and 0.4 μM for PC-3), there was only small amount of docetaxel hydrolysis from the conjugates whereas most of the HSA had been utilized as nutritional component, resulting in a very weak inhibition effect as indicated in Fig. 6. However, with an increase in the concentration of the docetaxel-HSA conjugate, there were more docetaxel released from the conjugates. Additionally, it may likely be ascribed to cell uptake of the nanoparticulate conjugate and/or better penetration of the conjugate into the cells which results in either case docetaxel being delivered to its site of action. Furthermore, the increased antitumor activity of the conjugates at high concentrations was also related to the enhanced intratumor delivery of docetaxel, after which the conjugate acts as an intracellular drug depot, slowly releasing the attached docetaxel into the cellular cytoplasm.

4. Conclusions

In this study, we successfully synthesized a docetaxel-6-maleimido-hexanoate -HSA conjugate. The MS and NMR results indicate that one side of the 6-maleimido caproic acid was conjugated with the 2’ hydroxyl group of docetaxel by an esterification reaction, while the other side was linked on the HS-group of Cys34 of HSA by a Michael addition. One merit of this study is that this approach realized the 1:1 conjugation of docetaxel and HSA by a relatively mild chemical reaction. In vitro cell results indicate that the docetaxel-linker-HSA conjugate had high cytotoxicity. Additional in vivo research on this conjugate is underway.

Acknowledgements

The authors gratefully acknowledge the financial support by National Key R&D Program of China (2016YFD0600805), the Fundamental Research Funds for the Central Universities (2572018CT01).

Conflict of interest

The authors declare that they have no conflict of interest.

References