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# *N*-acetylcysteine modified hyaluronic acid-paclitaxel conjugate for efficient oral chemotherapy through mucosal bioadhesion ability

Xin Jin[a,1], Sajid Asghar[b,1], Mei Zhang[a,1], Zhipeng Chen[c], Lin Huang[a], Qineng Ping[a], Yanyu Xiao[a,*]

[a] Department of Pharmaceutics, State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, China
[b] Faculty of Pharmaceutical Sciences, Government College University Faisalabad, Faisalabad, Pakistan
[c] Department of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210023, China

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ABSTRACT

*N*-acetylcysteine modified hyaluronic acid-paclitaxel (NAC-HA-PTX) conjugate was designed to improve the water solubility and oral bioavailability of PTX through mucosal bioadhesion ability. The average size of spherical NAC-HA-PTX micelles was 187 nm with a zeta potential of -25.38 mV. Mucin adhesion study showed that the amount of mucin adhered to NAC-HA-PTX micelles was 1.98-fold greater than that of hyaluronic acid-paclitaxel (HA-PTX) micelles. The fluorescence micrographs showed that the biodistribution sequence of coumarin 6-loaded micelles in the gastrointestinal tract was duodenum > jejunum > ileum, and NAC-modified micelles significantly exhibited better mucoadhesive properties than the corresponding unmodified ones. The pharmacokinetic study showed that the area under the curve ($AUC_{0\text{-}24h}$) of NAC-HA-PTX micelles was 2.32-fold and 2.56-fold higher compared to that of HA-PTX micelles and PTX solution (Taxol) after oral administration, respectively. NAC-HA-PTX micelles appear to be a promising drug delivery system to improve the bioavailability of insoluble drugs for efficient tumor therapy via oral administration.

## 1. Introduction

Cancer is seriously harmful to human society and is one of the most common causes of death in the world. In clinical application, the most usual route for administrating anticancer drug remains injection, which brings patients lots of inconvenience. Therefore, oral administration has become a preferable strategy for cancer treatments, as it is noninvasive, convenient and painless, removing risks of contamination and injection-related discomforts, and good patient compliance [1]. Nevertheless, oral delivery of most of the anticancer drug was limited owing to their low water-solubility in gastrointestinal fluids, poor permeability across intestinal mucosal barriers and high level of P-glycoprotein (P-gp) efflux [2].

In recent years, bioadhesive nanocarriers are widely used in oral delivery owing to their unique adhesion properties [3–6]. For oral delivery, the ability of nanocarriers to adhere to the mucosa is mainly achieved through electrostatic, hydrophobic, and van der Waals interactions, as well as through interpenetration or tangling of the polymer chains within the mucus mesh structure [7,8]. Among mucoadhesive polymers, thiolated polymers with reactive thiol groups immobilized on the polymeric structure have found increasing applications in oral delivery systems for their ability to enhance adhesion of micelles on intestinal mucosa, and thus prolong the residence time in the gastrointestinal (GI) tract and increase oral bioavailability [9–11]. The steady adhesion of the micelles to the mucus layer was ascribed to the S—S bonds formed by free thiol groups of thiolated micelles binding to

*Abbreviations:* HA, hyaluronic acid; PTX, paclitaxel; NAC, N-acetylcysteine; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, N,N-dimethylformamide; NAC-HA-PTX, NAC modified HA-PTX; CMC, critical micelle concentration; C6, coumarin 6; DS, the degree of substituent; PDI, polydispersity index; DLS, dynamic light scattering; TEM, transmission electron microscope; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; PAS, periodic acid/Schiff staining; KR, Krebs Ringer's buffer solution; DAPI, 4',6-diamidino-2-phenylindole; SD, Sprague-Dawley; Taxol, PTX solution; $C_{max}$, peak concentration; $T_{max}$, peak time; MCF-7, the human breast carcinoma; FBS, fetal bovine serum; DMEM/HG, Dulbecco's modified eagle's medium/high glucose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; GI, gastrointestinal; MW, molecular weight; MWCO, molecular weight cut-off; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline; AUC, the area under the curve; CL, eliminate rate; MRT, mean residence time; IC50, the half maximal inhibitory concentration

* Corresponding author at: Department of Pharmaceutics, State Key Laboratory of Natural Medicines, China Pharmaceutical University, No. 24 Tong Jia Xiang, Nanjing 210009, China.
*E-mail address:* cpuyanyuxiao@163.com (Y. Xiao).
[1] These authors contributed equally to this work.

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mucin [12–14]. Additionally, it has been demonstrated that polymers with free thiol groups can effectively improve drug permeability by suppressing the ATPase activity of P-gp expressed in the intestinal tract [15,16].

N-acetylcysteine (NAC) is the acetylated variant of the amino acid L-cysteine and is widely used for chronic obstructive pulmonary disease exacerbation and pulmonary fibrosis, besides, NAC is also commonly used as a specific antidote for acetaminophen overdose. Additionally, nanomaterials would exhibit marked bioadhesion and permeation enhancing effect with grafting of NAC. NAC conjugated biomaterial could tightly adhere to the mucus layer for a prolonged time via the formation of covalent S—S bonds between free thiol groups of NAC and the mucin glycoproteins, thus provide a steep drug concentration gradient at the absorption sites and exhibit an additional permeation enhancing effect [17]. A chitosan-NAC modified nanostructured lipid carrier developed for the ophthalmic drug delivery demonstrated signifcantly enhanced transcorneal penetration and delayed the clearance of the formulations in comparison with the uncoated ones [18].

Polymer-drug conjugate has aroused wide interests from more and more researchers over the last decades [19,20]. Polymer-drug conjugate, a hydrophilic block conjugated with a hydrophobic drug, can be self-assembled to form micelles with the inner core of hydrophobic segments and the outer shell of hydrophilic segments in an aqueous solution, which has been extensively used to improve the drug's solubility [21–23]. Among the polymers applied in polymer-drug conjugate, hyaluronic acid (HA) is widely conjugated with hydrophobic drugs due to its various advantages such as extensive sources, excellent biodegradability, nontoxicity and non-immunogenicity [24]. Besides, HA has abundant chemical groups such as —COOH and—OH, which are widely used in the modification reaction [25,26]. Furthermore, HA could effectively bind to certain receptors such as CD44 and RHAMM, overexpressed on the surface of many tumors [27–29]. Thus, polymer-drug conjugate based on HA exploiting CD44 receptor endocytosis pathway to enter glioma cells can increase the drug accumulation in the tumor cells [30].

Polymer-drug conjugates have recently been tried as an oral drug delivery system to enhance the bioavailability of insoluble drugs. For example, paclitaxel (PTX) conjugated trimethyl chitosan (TMC-PTX) has been developed for oral delivery of PTX, which increased $AUC_{0-\infty}$ value by 1.8-fold [31]. Indeed, HA-PTX conjugate has already been reported for enhancing antitumor activity of PTX via intravenous injection [32], and in the present study, NAC, with free thiol groups, was used as a mucoadhesive material, and then NAC modified HA-PTX (NAC-HA-PTX) conjugate was developed for increasing the oral bioavailability of PTX through the mucus bioadhesion and penetration ability (Fig. 1). In brief, the NAC-HA-PTX and HA-PTX conjugates were synthesizd and confirmed by $^1$H-NMR spectra. The degree of substitution (DS) of PTX and NAC, degree of thiolation, stability of thiol groups, the aqueous solubility of PTX and the critical micelle concentration (CMC) of NAC-HA-PTX conjugates were studied. The resulting NAC-HA-PTX and HA-PTX conjugates spontaneously self-assembled into core/shell micelles with hydrophobic PTX in the inner core and HA in the outer shell after being dispersed in distilled water. The micelles were characterized in terms of particle size, polydispersity index (PDI), zeta potential, morphology and stability. In addition, the permeability in small intestine of rats and oral bioavailability of NAC-HA-PTX and HA-PTX micelles were compared. Furthermore, Coumarin 6 (C6) loaded NAC-HA-PTX micelles were prepared to investigate the *in vitro* antitumor activity of NAC-HA-PTX micelles on MCF-7 cells.

## 2. Materials and methods

### 2.1. Materials

PTX (98% purity) was obtained from Jiangsu Taxus Biological Technology Co., Ltd. (Wuxi, China). HA sodium salt (MW = 10 kDa, determined by viscometry) was purchased from Freda Pharmaceutical Co. Ltd. (Shandong, China). N-acetylcysteine (NAC) was purchased from Aladdin (Shanghai, China). Dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai,China). All other chemicals and reagents were of analytical grade and used without further purification.

### 2.2. Animals

Male Sprague-Dawley (SD) rats (180–220 g body weight) were purchased from the Shanghai Silaike Laboratory Animal Co., Ltd. (Shanghai, China). All animal procedures were conducted in compliance with approved standards for laboratory animal care, and the study protocol was approved by the ethical committee of China Pharmaceutical University (Date 20170107, No. ACU-13(20170107)).

### 2.3. Synthesis of NAC-HA-PTX conjugate

#### 2.3.1. Synthesis of HA-PTX conjugate

HA-PTX conjugate was synthesized according to a previous research [33]. In brief, HA (100 mg, equivalent to 0.26 mmoL of —COOH group) was dissolved in anhydrous formamide (10 mL) at 50 °C to obtain polymer solution, and then DCC (0.31mmoL) and DMAP (0.052 mmoL) were added to the above HA solution. This reaction was continued for 1 h in an ice bath to activate the carboxyl groups of HA. At the same time, PTX (0.26 mmol) was dissolved in 10 mL of N,*N*-dimethylformamide (DMF), and then the activated HA solution was slowly added dropwise to PTX solution. The mixture was stirred at room temperature in nitrogen atmosphere for 24 h. After the reaction, the resultant mixture was dialyzed (MWCO 3500) against distilled water for 2 days. Finally, the solution was filtered and then lyophilized to obtain HA-PTX conjugate and stored at 4 °C for further use.

#### 2.3.2. Synthesis of NAC-HA-PTX conjugate

NAC-HA-PTX conjugate was also synthesized via esterification reaction in the same way according to a previous research [33]. Briefly, NAC (0.25 mmoL) dissolved in 10 mL of DMF was reacted with DCC (0.30 mmoL) and DMAP (0.05 mmoL) for 1 h in an ice bath to activate the carboxyl groups of NAC. After HA-PTX conjugate (100 mg, 0.25 mmoL of —COOH group, degree of substituent (DS) of PTX was 2.08%) was dissolved in 10 mL of formamide, the above mixture was dropped into the HA-PTX conjugate solution and stirred for 24 h at room temperature under $N_2$ protection. The resultant solution was dialyzed (MWCO 3500) against distilled water for 2 days. The solution was then filtered and lyophilized to obtain NAC-HA-PTX conjugate and stored at 4 °C for further use.

### 2.4. Characterizations of NAC-HA-PTX conjugate

#### 2.4.1. $^1$H-NMR spectra

The chemical structures of HA-PTX and NAC-HA-PTX conjugates were confirmed by $^1$H-NMR spectra on a Bruker Avance-300 spectrometer (AVACE, Kingswinford, UK) operated 300 MHz. HA, PTX, NAC, HA-PTX conjugate and NAC-HA-PTX conjugate (ca. 5 mg) powders were dissolved in 0.5 mL of $D_2O$ and the spectra were recorded in the 0–10 ppm interval at room temperature.

#### 2.4.2. The degree of substituent (DS) of PTX

The amount of PTX in HA-PTX conjugate was determined by Shimadzu LC-10AT HPLC system (Kyoto, Japan). The stationary phase and Kromasil $C_{18}$ column (150 mm × 4.6 mm, 5 μm) were maintained at 35 °C. The mobile phase was composed of methanol and water (70:30, v/v) with a flow rate of 1.0 mL/min. The effluent was detected at 227 nm. In addition, a calibration curve of PTX ($R^2$ = 0.999) was established to determine the PTX content in the conjugate. 0.4 mg of HA-PTX conjugate was dissolved in a 70:30 (v/v) mixture of