Toward the next generation of smart anti-tumor drugs: a highly water-soluble Nucleolin Aptamer-Paclitaxel conjugate with a Cathespin B-labile linker for tumor-specific targeting in ovarian cancer

Jun Lu

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Toward the Next Generation of Smart Anti-tumor Drugs:
A Highly Water-soluble Nucleolin Aptamer-Paclitaxel Conjugate
with a Cathepsin B-labile Linker
for Tumor-Specific Targeting in Ovarian Cancer

LU Jun

A thesis submitted in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

Principal Supervisor:
Prof. ZHANG Ge (Hong Kong Baptist University)

August 2017
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

I have read the University’s current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University’s Committee on the Use of Human & Animal Subjects in Teaching and Research (HASC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval, and acknowledged my obligations and the rights of the participants.

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ABSTRACT

Paclitaxel (PTX) is among the most commonly used first-line drugs for cancer chemotherapy. However, its poor water solubility and indiscriminate distribution in normal tissues remain clinical challenges. Here we designed and synthesized a highly water-soluble nucleolin aptamer-paclitaxel conjugate (NucA-PTX) that selectively delivers PTX to the tumor site. By connecting a tumor-targeting nucleolin aptamer (NucA) to the active hydroxyl group at 2’ position of PTX via a cathepsin B sensitive dipeptide bond, NucA-PTX could remain stable and inactive in the circulation. The tumor-recognition component NucA facilitates the uptake of the conjugated PTX specifically in tumor cells. Once inside cells, the dipeptide bond linker of NucA-PTX will be cleaved by cathepsin B and then the conjugated PTX will be released for action. The stability of NucA-PTX in human serum and the cathepsin-B dependent release of the conjugated PTX in tumor cells were verified by monitoring the fluorescence resonance energy transfer (FRET) of a dual fluorescence-labeled conjugate FAM-NucA-PTX-Rd. In addition, the PTX conjugation did not considerably affect the binding affinity between NucA and its target protein nucleolin, which was supported by both molecular dynamic simulation and isothermal titration calorimetry (ITC). The NucA modification was shown to facilitate the uptake of the conjugated PTX in ovarian cancer cells mainly by macropinocytosis in a nucleolin expression-dependent manner. Moreover, the NucA modification increased the in vitro cytotoxicity and mitosis inhibition of the conjugated PTX in ovarian cancer cell lines. The in vivo data collected from a human xenograft
model of ovarian cancer demonstrated that the NucA modification facilitated the selective accumulation of the conjugated PTX in ovarian tumor tissue, and subsequently resulting in notably improved antitumor activity and reduced toxicity.
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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADC</td>
<td>Antibody drug conjugate</td>
</tr>
<tr>
<td>ApDC</td>
<td>Aptamer drug conjugate</td>
</tr>
<tr>
<td>CCK8</td>
<td>Cell counting kit-8</td>
</tr>
<tr>
<td>CCNSC</td>
<td>Cancer Chemotherapy National Service Center</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DCU</td>
<td>1, 3-Dicyclohexylurea</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DME</td>
<td>Dimethoxyethane</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDCI</td>
<td>3-(Ethyliminomethylideneamino)-N,N-dimethylpropan-1-amine, hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEDQ</td>
<td>N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline</td>
</tr>
<tr>
<td>EIPA</td>
<td>Ethylisopropylamiloride</td>
</tr>
<tr>
<td>ESI</td>
<td>Electron spray ionization</td>
</tr>
<tr>
<td>FAM</td>
<td>Fluorescein amidate</td>
</tr>
<tr>
<td>FI</td>
<td>Fluorescence intensity</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>Fmoc-Cl</td>
<td>Fluorenlymethyloxycarbonyl chloride</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HCT-16</td>
<td>Human ileocecal carcinoma-16</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HAS</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrofluoric acid</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometer</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatograph-mass spectrometer</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl tert-butyl ether</td>
</tr>
<tr>
<td>NCVs</td>
<td>Nerve conduction velocities</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NucA</td>
<td>Nucleolin aptamer</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PABOH</td>
<td>4-Aminobenzyl alcohol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
</tr>
</tbody>
</table>
Phe-Lys  Phenylalanine-lysine
PNP  4-Nitrophenyl
PTX  Paclitaxel
Rh  Rhodamin B
SAR  Structure-activities relationship
SELEX  Systematic evolution of ligands by exponential enrichment
Sulfo-NHS  Sodium,1-hydroxy-2,5-dioxopyrrolidine-3-sulfonate,hydrate
TBSCl  tert-Butyl-chloro-dimethylsilane
TCEP  Tris(2-carboxyethyl)phosphine
TEAA  Triethylamine Acetate
THF  Tetrahydrofuran
TLC  Thin layer chromatography
TOFMS  Time-of-flight mass spectrometers
UV  Ultraviolet
Val-Cit  Valine-citrulline
WBC  White blood cell
Chapter 1 Introduction

1.1 Brief introduction of paclitaxel

Paclitaxel (Taxol®), a complex natural diterpene product originally isolated from the bark of *Taxus brevifolia*, has sparked a great interest and excitement of chemists, biologists, clinicians\(^1\)\(^{-6}\). The Cancer Chemotherapy National Service Center (CCNSC) in the United States executed a plant screening program to act as a public screening center for searching anticancer active compounds in 1955, and discovered the effective extracts of the bark of Pacific Yew, *Taxus brevifolia* showing significant antileukemic and cytotoxic activities in 1962\(^7\)\(^{,8}\). And then, Dr. Monroe Wall and Dr. M.C. Wani began to isolate the extract and obtained the pure compound named paclitaxel in the late 1960s\(^9\)\(^{,10}\). The structure of paclitaxel, which belongs to a novel kind of diterpenoid compounds, was established via X-ray Andrew McPhail at Duke University and published in 1971 (Figure 1)\(^{11}\). The structure of paclitaxel consists of baccatin III containing the unusual oxetane ring and a complex β-phenylisoserine ester esterified at the C-13 position, meanwhile, the structure was different from all previously known anticancer agents\(^12\).

![Paclitaxel chemical structure and 3D model](image)

**Figure 1** The chemical structure and 3D model of paclitaxel.

Because of the uniqueness of the structure, paclitaxel possessing a wide range of biological activities is used as an anticancer drug in clinics for treatment of several kinds
of metastatic tumors, including ovarian, breast, lung and pancreatic\textsuperscript{13-16}. In order to lead to the development of analogues with biological activities and optimization of the next generation agents, a surge of efforts had been made in the subsequent years to study the mechanism of action and establish the structure-activities relationship (SAR). Tubulin with a molecular weight of 55000 exists in two alternating protein monomers: tubulin $\alpha$ and tubulin $\beta$, which to some extent share structural similarities\textsuperscript{17}. Under the steady-state condition, the state of dynamic equilibrium between subunit tubulins and microtubules keeps the preferential growth and dissociation. The occurrences of synthesis of tubulins and assembly of microtubules are crucial to the prophase of mitosis and G2 phase in the cell cycle, however, the presence of paclitaxel shows its effect via the inference of dynamic equilibrium, promoting the irreversible tubulin polymerization into the microtubules and stabilizing the polymer\textsuperscript{18-20}. Comparing with other antimitotic agents inhibiting the process of assembly of tubulin into microtubules, such as vincristine, podophyllotoxin, as well as vinblastine, paclitaxel discovered as the first natural compound to promote the microtubules assembly has attracted significant interest and attention in the intensive research\textsuperscript{21-23}. This result concludes the interaction sites of paclitaxel binding to the tubulins are unique and different from the others. In the latter studies, the microtubule structure was improved to provide excellent starting sites in the construction via the refined tubulin structure, meanwhile, the interaction sites of paclitaxel were rarely modified and more efficient for binding\textsuperscript{24,25}.

The modification or imitation of natural products not only contributes to research and identification of structure features or active functional groups that could possess the
specific effects to interact or bind with receptors in the tissues, but also provides an opportunity for expand the amount and sources of natural products from natural metabolism, which was crucially important for the scarcely active natural products or endangered resources\textsuperscript{26,27}. The first total synthesis of paclitaxel was achieved by Nicolaou et al. in 1994, which provided a chemical pathway for obtaining the natural products or its derivatives\textsuperscript{3}. Paclitaxel consists of four rings (A, B, C and D ring) and a side chain at the position of C13. Medicinal chemists have conducted systematic and comprehensive structural modifications, mostly concentrated in the C1-hydroxyl group, C2-benzoyl group, C4-acetate, C7-hydroxyl group, C9-canbonyl group, C10-acetate, C2’-hydroxyl group, C3’-N-aryl group, and the D ring. Extensive studies have been carried out on the modification of paclitaxel and establishment of the SAR for paclitaxel over the last decade.

1.2 The structure-activity relationship of paclitaxel

In order to explore whether the hydroxyl group at the C1 position in the paclitaxel is a necessary group for stabling the microtubules and possessing cytotoxicity, Kingston et al. removed it and obtained some paclitaxel derivatives 1–3 (Figure 2)\textsuperscript{28}. According to IC\textsubscript{50} (\(\mu\text{M}\)), the cytotoxicity of compound 1, 2, 3 to HCT 116 human colon carcinoma was 0.0315, 0.046, 0.047, respectively, while that of paclitaxel was 0.0018 ± 0.0002. The datum from comparing derivatives with paclitaxel showed the hydroxyl group was no requirement for the bioactivities.
Figure 2 The structures of paclitaxel derivatives 1~3 with removing the hydroxyl group at the position C1.

Chen et al. removed the benzoyloxy group or changed the steric structure of paclitaxel at the C2 position and obtained two derivatives 4 and 5 (Figure 3), and then the experiments demonstrated the two compounds have no capacity of microtubule-binding and cytotoxic activities.

Figure 3 The structures of paclitaxel derivatives 4 and 5.

By comparing compounds microtubule assembly assay, the ED$_{50}$(6)/ED$_{50}$(PTX) and ED$_{50}$(7)/ED$_{50}$(PTX) was 1.2 and 1.1, respectively, while comparing the cytotoxicity to MCF7, the ED$_{50}$(6)/ED$_{50}$(PTX) and ED$_{50}$(7)/ED$_{50}$(PTX) was 1.3 and 12, respectively. Derivatives 6 and 7 replaced the benzene ring with aliphatic chain and synthesized by Gabetta et al. showed that they maintained certain capacity of microtubules-binding (Figure 4), though the cytotoxicity was significantly decreased.
Fang et al. replaced the benzoyl group with benzoyl amide group and produced the derivative 8 (Figure 4). Comparing with paclitaxel, compound 8, which showed antitumor activities towards three tumor cell lines, A-549, KB and A2780 as $IC_{50}/IC_{50($paclitaxel$)}$ 20.5, 16.8, 0.8, owned weaker capacity of inhibiting the proliferation of cancer cells$^{31}$.

The research of structure-activity relationship at the C2 position indicates the benzoyl group and stereo conformation are the essential ingredients to remain the antitumor activities.

According to the conclusion reported in the literatures, mainly modification at the C4 position includes deacetyl, deacetylation, etherification, esterification.

Neidigh et al. stripped the acetyl down and got a derivative 9 with a hydroxyl group at C4 position (Figure 5). Bioactivity tests, associated data such as maximum assembly rate of paclitaxel and compound 9 with the concentration of 40 μM is 0.98 and 0.015, respectively, showed this compound has lost the capacity of promoting microtubules-binding, and consequently could not inhibit the tumor cells$^{32}$.

Figure 4 The structures of paclitaxel derivatives 6-8.
Figure 5 The structures of paclitaxel derivatives 9 and 10.

Chordia et al. removed the acetylate and obtained the derivative 10 (Figure 5), which exhibited distinctly lower capacity of microtubules-binding and cytotoxicity\(^ {33}\), associated data such as maximum assembly rate of paclitaxel and compound 10 with the concentration of 40 μM is 0.98 and 0.13, respectively.

The research of structure-activity relationship at the C4 position in the paclitaxel suggests that acetylate is quite important for keeping bioactivity.

Gregory et al. replaced the hydroxyl group with a fluorine atom at the C7 and changed the stereo conformation, and then the produced derivative 11 still had the cytotoxicity on HCT-116 (ED\(_{50}(11)/ED_{50}(paclitaxel) = 3.04\)) while displayed the potential capacity of microtubules-binding (EC\(_{50}(11)/EC_{50}(paclitaxel) = 1.25\)) (Figure 6)\(^ {34}\).

Figure 6 The structures of paclitaxel derivatives 11-13.

Georg et al. removed the hydroxyl group and got the derivative 12 (Figure 6), whereas it had only a little decline in capacity of microtubules-binding in comparison
with paclitaxel (Microtubule assembly ED$_{50}$/ED$_{50}$(paclitaxel) = 1.4)$^{35}$. Since the position was unnecessary for keeping bioactivity, it was used as modified site connected with water-soluble sections. Deutsch et al. conjugated succinic acid to the position and constructed a paclitaxel sodium salt $^{13}$ (Figure 6), which greatly improved the poor water-solubility of paclitaxel$^{36}$.

Studies have shown that acetylate at the C10-hydroxyl group is not essential to remain the bioactivity of paclitaxel. Docetaxel as an obvious example possessed superior activity to paclitaxel.

Georg et al. removed the hydroxyl group at C10 and obtained a new derivative $^{14}$ (Figure 7). Compared with paclitaxel, compound 14 showed the similar anti-tumor activity against a broad spectrum of tumor cells, except HCT-16 cell.$^{37}$ Ojima et al. synthesized a series of acylated paclitaxel derivatives $^{15}$ and bioactivity research indicated these compounds exhibited significant cytotoxicity against tumor cells in comparison to paclitaxel and docetaxel (Cytotoxicity to ovarian carcinoma IC$_{50}$(15a)/IC$_{50}$(paclitaxel) = 0.019, IC$_{50}$(15b)/IC$_{50}$(paclitaxel) = 0.041, IC$_{50}$(15c)/IC$_{50}$(paclitaxel) = 0.047; Cytotoxicity to non-small-cell lung carcinoma IC$_{50}$(15a)/IC$_{50}$(docetaxel) = 0.32, IC$_{50}$(15e)/IC$_{50}$(docetaxel) = 0.9, IC$_{50}$(15f)/IC$_{50}$(docetaxel) = 0.6; Cytotoxicity to colon carcinoma IC$_{50}$(15g)/IC$_{50}$(paclitaxel) = 0.25, IC$_{50}$(15b)/IC$_{50}$(paclitaxel) = 0.167) (Figure 7)$^{38}$. 
Figure 7 The structures of paclitaxel derivatives 14 and 15.

A significant amount of efforts have been made into researching the position of C2'-hydroxyl group, which was a crucially active site both for chemical reactivity and biological activities. Meanwhile, many researchers have received abundant and meaningful achievements.

Joydeep Kant et al. modified the hydroxyl group by replacing with a fluorine atom, etherification and reduction and obtained some derivatives 16 (Figure 8). After cytotoxicity test in vitro, the result showed the derivatives lacked or decreased the cytotoxicity (Cytotoxicity to HCT116 IC$_{50}$(16a)/IC$_{50}$(paclitaxel) =216.5, IC$_{50}$(16b)/IC$_{50}$(paclitaxel) =74.25, IC$_{50}$(16c)/IC$_{50}$(paclitaxel) =118.75). The hydroxyl group also was silicon esterified and acetylated by Kingston’s group and produced derivative 17 and 18 (Figure 8), however compound 17 had little cytotoxicity and compound 18 decreased the capacity of tubulin assembly.
Research suggested that acylation of the hydroxyl group at C2’ could be hydrolyzed intracellular esterase, and then the released active paclitaxel exerted its antitumor activity\textsuperscript{41}. Taking this into consideration, this site could be esterified and introduced some hydrophilic groups to increase the water solubility of paclitaxel, which could be tested as a valuable paclitaxel prodrug.

Succinic anhydride and glutaric anhydride were the most frequently used as modified groups esterifying the hydroxyl group. Some derivatives 19 and 20 synthesized by Deutsch’s group as prodrugs had good water solubility and potential antitumor activities (Figure 9)\textsuperscript{36}.

In order to get a difunctional paclitaxel derivative, colchicine as an antitumor drug with inhibitory activity on microtubulin was linked to paclitaxel through an ester bond and formed a new derivative 21 (Figure 10). From the experimental data (Cytotoxicity to human mammary carcinoma cell line ED_{50(21)}/ED_{50(paclitaxel)} = 10), this compound exhibited higher cytotoxicity, whereas lost the capacity of tubulin assembly\textsuperscript{42}.

Figure 8 The structures of paclitaxel derivatives 16-18.

Figure 9 The structures of paclitaxel derivatives 19 and 20.
Natural unsaturated fatty acids have the advantages of its nonpoisonous, stable characteristics and harmless to the organism and belong to tumor-specific molecular used as a tumor-specific molecular served as tumor targeting drug carriers. In 2001, Charless S. Swindell et al. synthesized a new paclitaxel derivative 22 that was formed by docosahexaenoic acid linking to C2’-hydroxyl group via generating an ester band (Figure 10). Compared with paclitaxel, the compound 22 exhibited the increasement of antitumor activity, improvement of tumor-targeting and decline of cytotoxicity against normal cells. But by the same token, the compound 22 would exert its bioactivity after the intracellular esterase hydrolyzes the ester and release the paclitaxel.

Du’ group synthesized a series of water-soluble malic acid-docetaxel derivatives through esterifying C2’ hydroxyl group with malic acid. Compared with docetaxel, these derivatives exhibited evidently strong cytotoxicity and significantly inhibited the growth of solid tumors in vivo. Iwao Ojima et al. designed a series of paclitaxel derivatives in which the C2’ hydroxyl group was esterified with natural fatty acids, such as DHA, linolenic acid and linoleic acid, using as the prodrugs of paclitaxel. Biological data showed these derivatives had strong inhibitory activities against drug-resistant colon cancer cells and drug-sensitive ovarian cancer cells in vivo, meanwhile reduced the toxic...
Recently, Damain Plazuk et al. firstly introduced metal into paclitaxel molecule that bonded the ferrocenecarboxylic acid to C2’ hydroxyl group in the paclitaxel and docetaxel respectively, and produced four derivatives 23 and 24 (Figure 11). The bioactivity datum showed these derivatives possessed significant inhibitory activity against colon cancer cell, whereas its capacity of tubulin assembly was dramatic declined (Cytotoxicity to the colon adenocarcinoma SW620 cell line $\frac{IC_{50}(23b)}{IC_{50}(paclitaxel)} = 0.356$, $\frac{IC_{50}(24b)}{IC_{50}(paclitaxel)} = 0.066$, $\frac{IC_{50}(23a)}{IC_{50}(paclitaxel)} = 0.077$, $\frac{IC_{50}(24a)}{IC_{50}(paclitaxel)} = 0.045$)\(^{46}\).

**Figure 11 The structures of paclitaxel derivatives 23 and 24.**

Up to now, analogues of paclitaxel are continually extracted and separated or synthesized. Following this, comparing with the activities and refining the accurate relationships are incorporated into the results or conclusions, which are conductive to providing the summary of SAR (Figure 12)\(^{47}\).
1.3 Research and development of paclitaxel formulations

Paclitaxel has been approved by the FDA for the treatment of advanced ovarian in 1992 and breast cancer in 1994. But because of poor water solubility of paclitaxel (<0.03 mg/ mL\(^{48}\)), the mixture of cremophor EL and absolute ethanol was applied as a cosolvent to increase the paclitaxel solution in water. The cremophor EL must be preprocessed before the treatment. Otherwise it would cause a severe allergic reaction during the degradation in the body\(^{49}\). Meanwhile, paclitaxel was delivered in a non-specific manner, and the free paclitaxel would cause a serious dose-limiting toxicity, such as neutropenia, neurologic lesions\(^{50,51}\). What is more, these cosolvents would form small particles and encapsulated effective drugs in the blood circulation, which blocked the drug effects\(^{52}\).

Prodrug was a kind of modality that consisted of inactive derivatives by cross-linking with some compounds, and released the pharmacologically active drugs after hydrolysis or enzyomolysis in vivo\(^{53}\). Polymeric prodrugs could be self-assembled into nanometer-sized micelles. Due to the amphiphilic structural characteristics,
heparin-paclitaxel could be self-assembled into nanoparticles with a size of 140~180 nm. Compared to free drugs, the prodrugs exhibited a significantly increasing cytotoxicity against MCF-7 cell. In addition, the prodrug could maintain the integrity of the parent drugs and enhance the targeting selectivity to tumor cells\textsuperscript{54}. Transplanting the paclitaxel into the hydrophilic hyperbranched poly (ether-ester) would be self-assembled and form micelles with size of 50-120nm, whose drug loading is from 4.1% to 10.7%. This prodrug could improve the tolerance dose of paclitaxel in mouse model. Compared to 15mg/kg for traditional paclitaxel, the maximum tolerance dose for prodrug could overpass 45mg/kg, moreover, the solid tumor in tumor-bearing mice was completely disappeared after injecting the prodrug for 3~6 times\textsuperscript{55}.

Liposome was a kind of miniature vesicles formed by encapsulating drugs into lipoid bilayers. It could encapsulate hydrophilic drugs into its aqueous core or encapsulate lipophilic drugs into bilayer membrane. Due to the different preparing methods, the diameter of liposome with uniform sizes is from 50 nanometers to 1 micrometer\textsuperscript{56}. From 2006, the paclitaxel liposome for injection has been applied to clinical therapy, which was used in treatment of ovarian cancer, non-small cell lung cancer and other malignancies\textsuperscript{57}. It remained the growth inhibitory activity of free paclitaxel and reduced the side effects\textsuperscript{58}. Jain et al. prepared the polyelectrolyte stabilized multilayer liposomes using the skill of layer-by-layer assembly of the polyelectrolytes over liposomes and the formation with an average particles size of 226±17.61 nm had an entrapment rate of 71.91±3.16% and zeta potential of -39.9±3.79 mV. Compared to free drug, the liposomes exhibited higher uptake rate and lower toxicity in cell test.
Pharmacokinetics studies showed oral bioavailability of paclitaxel increase about 4.07 fold *in vivo* compared to that of traditional paclitaxel and the excellent antitumor efficacy in reduction in tumor growth as paclitaxel. Moreover, the developed formulation of paclitaxel loaded layersome revealed remarkably higher safety profile than Taxol. Mao et al reported a kind of paclitaxel liposome gel (PTX-lip-gel), whose efficacy and toxicity was examined by treating the pancreatic cancer in tumor-bearing mice model. Compared to paclitaxel liposome, the PTX-lip-gel exhibited a much more slowly release *in vitro*, which was proved to possess a favorable retention inside the tumor tissue. Compared with other groups at the same drug dose, PTX-lip-gel group revealed preferable balance between antitumor efficacy and side effects.

Abraxane was the first nanopartical-paclitaxel drug approved by FDA, which utilized human serum albumin (HSA) as a drug carrier. Nanopartical-pacliatxel lyophilized powder was made from HSA and paclitaxel by high pressure vibration technology, however, the process was not to conjugate paclitaxel with HAS by covalent bond. The particles of albumin bound paclitaxel were dissolved into a dissoluble albumin paclitaxel complex, and then the paclitaxel could associate and disassociate with injected or endogenic albumin or other biomolecules. The albumin bound paclitaxel utilized the natural albumin pathway and secreted protein acidic and rich in cysteine to assemble on tumor tissues with high concentration. At present, Abraxane was approved to treat metastatic breast cancer failed in curing by combined chemotherapy or to treat recurrent breast cancer in six months by receiving neoadjuvant chemotherapy. What is more, some explorations have been made in the new neoadjuvant chemotherapy applied
in treatment for locally advanced breast cancer in clinical trials, which obtained some encouraging results\textsuperscript{64,65}. Zhang’s group prepared a degradable poly (ethylene oxide) block polyphosphoester based paclitaxel drug conjugates (PEO-b-PPE-g-PTX) with multifunction, which has a loading capacity of paclitaxel up to 65wt\%\textsuperscript{66}. Compared to free paclitaxel, the water solubility of PEO-b-PPE-g-PTX has been improved more than 25000 times. In addition, the PEO-b-PPE-g-PTX exhibited its positive antitumor effects on a variety of cancer cell lines.

Compared to liposome and particle, polymeric micelle with smaller particle size is a kind of supramolecule, core-shell nanoparticle, whose size was kept down to 20–100 nanometers. Micelle facilitated some insoluble medicines dissolution through enhancing the penetration effect, increased the bioavailability and possessed the targeting property\textsuperscript{67,68}. Lian et al. prepared PTX-loaded chitosan/vitamin E succinate polymeric micelles (PTX-micelles) through self-assembling polymeric micelles, which were characterized by X-ray diffraction and dynamic light scattering\textsuperscript{69}. The PTX-micelles had a paclitaxel loading capacity as high as 21.37\% and controlled the particle size ranging from 77.6±11.4 nanometers, with an encapsulation efficiency of 65.0± 4.2\% and a zeta potential of +20 mV. Compared to Taxol, the cellular uptake experiments revealed an increased uptake efficiency of chitosan/vitamin E succinate micelles in MCF-7 cells, which were conducted by the confocal laser scanning microscopy. Because of the sustained-released properties of nanomicelles, the PTX-micelles showed it was more comparable and less cytotoxic against MCF-7 cells than Taxol. NK105 is a novel core-shell polymeric micellar nanoparticle system with a particle size of about 85
nanometers, which promoted the paclitaxel delivery to the solid tumors\textsuperscript{70}. The micelles consisted of hydrophilic PEG shell and modified polyaspartic acid as a lipophilic proportion, which did not require cremophor EL or ethanol as a solvent during the intravenous injection\textsuperscript{71}. Compared to paclitaxel, NK105 exhibited remarkable antitumor activities against LLC tumor cells. In addition, there was no significant difference between mice treated with NK105 and those treated with paclitaxel during the histopathological study.

Cyclodextrin with capacity of complexing was utilized to increase the solubility of insoluble drugs, dissolution rate and bioavailability, which encapsulated the lipophilic drugs into its hydrophobic core and formed a non-covalent complex. Mognetti et al. reported that paclitaxel was loaded on $\beta$-cyclodextrin based nanosponges, which was non-hemolytic and non-cytotoxic, forming a paclitaxel complex\textsuperscript{72}. The paclitaxel-loaded nanosponges with a particle size less than 500 nanometers formed a stable colloidal system, which released the paclitaxel in 2 hours \textit{in vitro} without initial burst effects.

At present, many researchers from the world have designed and prepared variable of novel derivatives and new formulations of paclitaxel, which have achieved the purpose of improving the water solubility of paclitaxel, reducing the side effects, enhancing the antitumor activity and realizing the targeting treatment. What is more, Abraxane and Lipusu have been applied into clinical trials and obtained the excellent clinical treatment results, which were a kind of encouragement and pressure. We hope a kind of novel paclitaxel formulation with more dissolvable solubility, less side effects, better antitumor activity and more precise targeting property will be applied into clinical trials to treat
various cancers.

At the beginning of 90’s, Turek and Ellington invented a screening and amplifying technology, which refers to systematic evolution of ligands by exponential enrichment (SELEX)\textsuperscript{73,74}. The oligonucleotide sequences obtained by this technology were known as aptamers, which were capable of binding to peptides, proteins, cells and even the whole organ with unique affinity and specificity. The selection of aptamers was generally carried out from a 10^{14}~10^{15} bp synthesized DNA molecular library. The middle of molecules in the library consisted of 25~30 nucleotides with random sequences and the ends with a fixed sequences are feasible for PCR combination and amplification. The process is firstly to fix the target on magnetic beads, affinity column or microplate and incubate with library\textsuperscript{75,76}. After fully combining, aptamers bound with the target were extracted from the unbound aptamers, which were separated from the complex by a denaturation. Then aptamers were through a polymerase chain reaction (PCR) to amplify and carried out the next round of selection, which needed to repeat about 8~15 times before obtained the aptamer with high affinity and excellent specificity\textsuperscript{77,78}.

Aptamers with a key feature of high specificity identified various target molecules through discriminating the different groups on the molecules, such as methyl, hydroxyl or enantiomer\textsuperscript{79}. In that case, the variation of target molecule might cause the aptamers wash away during diagnosing the small molecule. Aptamers also exhibited a strong affinity that its dissociation constant bound with target molecule could reach pmol/L, sometimes even more than that of antibody. For example, the aptamer has been applied to detecting the theophylline in the serum of asthmatic patients, which has no cross-reaction with
theobromine or caffeine with the similar structures, while the similar compounds often reacted with antibodies during detecting the theophylline. Unmodified oligonucleotides, particularly RNA, are easy to be degraded by the nuclease in the biological fluid. In order to improve the half-life period and bioavailability of nucleotides, their main chain skeletons were specially modified through chemical methods, which included modifying pyrimidine, such as 2-fluoro, 2-amine, replacing D-type ribose with L-type, modifying special sites. The nucleotide library with large amounts and complicated special structures could predict out a wide range of target molecules. So far, proved target molecules specifically binding with nucleotides including metal ions, organic dyes, drugs, amino acid, peptides. What is more, nucleotides also could be binding with target proteins, such as enzymes, growth factors, gene regulation factors, cell adhesion factors.

Compared to antibodies, nucleotides also have other properties. Firstly, they could be synthesized in vitro and do not rely on animals or cell cultures. Secondly, they had non-immunogenicity and no rejection reaction. Thirdly, they renatured after denaturation in several minutes and were reusable. Lastly, nucleotides needed a short selection period and were easy to be automatically generated.

1.4 The characteristics of AS1411

AS1411 with 26-base sequence (5'-GGTGGTGGGTTGTGGTGGTGGTGG) possessed a unique structure and bioactivities, due to including a large amount of guanines. From the early research, antisense effect did not lead to the special activities, instead, they related to G-quadruplex structure forming from a large number of repeated G sequences. In general, nucleic acids were easily hydrolyzed by exonuclease in the
serum because of the unstable phosphodiester bond. Thus the unmodified nucleic acids with a short half-life were usually considered too impossible to be applied in the clinical trials. However, due to the unique G-quadruplex structure, the nucleic acids with an appreciable amount of guanine showed the strong capability of resistance to nuclease degradation.

Nucleolin played an important role in cell proliferation and expressed in many kinds of tumors. Nucleolin was involved in various physiological activities of cells, such as regulation of ribosomal RNA transcription, assembly, transportation and DNA replication and recombination. As a cell surface receptor, nucleolin exhibited the properties of shuttling among nucleolus, nucleus and cytoplasm\textsuperscript{90,91}. Thus nucleolin could be used to mediate the transmembrane transportation of various small molecules including the monoclonal antibody and endostatin\textsuperscript{92}. When the cells were in the proliferation state, nucleolin migrated to cytoplasm and membrane. Nucleolin was over-expressed in the membrane due to the violent proliferation of tumor cells, thus it became an important target site for targeting drug delivery in cancer therapy.

AS1411 with unique G-quadruplex structure not only resisted to nuclease degradation, but also had specific affinity to nucleolin. In that case, the targeting of AS1411 to nucleolin is the most value as an antitumor drug. The study on mechanism of entering into cell revealed the G-quadruplex structure of AS1411 bound to the nucleolin on the membrane and mediated transport into cell\textsuperscript{86}, or cancer cells produced the phenomenon of endocytosis of AS1411 under the double actions of regulation of nucleolin and the stimulation of AS1411\textsuperscript{93}. Whatever the approach, the presence of
nucleolin is a necessary condition for AS1411 to enter into the cell.

From the 1990’s, the more aptamers with high affinity, specificity and unique structures were screened as the development of SELEX skills, which were applied in the areas of diagnosis, delivery system and treatments of diseases and made a significant progresses\textsuperscript{94-97}. After the discovery of AS1411, it has shown its excellent properties in the research of active targeting drug-delivery system. Thus AS1411 contributed to promote drug accumulation in cells via nucleolin-mediated endocytosis. From the fundamental researches to quickly enter clinical trials, AS1411 were at the front of research and development of nucleic acid drugs.

In order to meet the desirable requirements of cell killing activities of cytotoxic drugs, retaining properties of specifically targeted portion, maintaining the stability of conjugates and decreasing systemic toxicities, plenty of efforts have been invested in researching the variously appropriate linkers\textsuperscript{98-100}. Appropriate linkers are designed to attach the cytotoxic drugs to the monoclonal antibodies and to ensure the ADCs behave as prodrugs during the systemic circulation and finally release the free drugs at the targeted tumor cells (Figure 13).
An appropriate linker between the antibody and the cytotoxic drug provides a specific bridge, and thus facilitates the antibody to selectively deliver the cytotoxic drug to tumor cells and accurately releases the cytotoxic drug at tumor sites\textsuperscript{98,99,102}. Linkers are classified different categories in terms of the mechanism of drug release and their stability in circulation, including cleavable linkers and non-cleavable linkers\textsuperscript{103,104}. The peptide-based linkers are designed to keep ADCs intact in the systemic circulation, and allow to easily release the cytotoxic drugs upon cleavage by specific intracellular proteases, such as cathepsin B\textsuperscript{105}. Cathepsin B is a 30 KDa lysosomal cysteine protease existing in many cancer cells and can specifically hydrolyze the valine-citruline dipeptide linker\textsuperscript{106}. The protease-sensitive dipeptide accompanied with some kind of spacer as linker exhibited adequate stability in blood circulation and efficient release in tumor tissues\textsuperscript{107}. Due to the inconvenient pH conditions and serum protease inhibitors, these peptide linkers show greater systemic stability with rapid enzymatic release of the drug in...
the targeted cell, such as valine-citrulline (Val-Cit) dipeptide linker, phenylalanine-lysine (Phe-Lys) dipeptide linker. This linker has been utilized in many ADCs in the clinic, which displays an excellent balance between plasma stability and intracellular protease cleavage\textsuperscript{108}.

Ovarian cancer as a kind of malignant tumor seriously threatens to the lives and health of women and brings the mortality to the first place in gynecology oncology\textsuperscript{109,110}. Although the exact etiology is not fully understood as like other malignancies, the following factors are known to be closely related to ovarian cancer. Heredity family history was reported as approximately 10\% ovarian cancer patients are related to the malignancies in their relatives, especially first degree relatives suffered from breast cancer, non-polyposis colon cancer\textsuperscript{111}. The person with the BRCA1 or BRCA2 mutations has the 17\%\textendash{}44\% risk rate of suffering from ovarian cancer, compared with the general population with the danger of 1.8\%\textsuperscript{112,113}. Endocrine disorders, infertility, premature onset of menstruation or delayed menopause also have the high risk of suffering from ovarian cancer\textsuperscript{114,115}. Unreasonable dietary structure including excessive consumption of saturated fatty acids and low intake of vegetables could also increase the risk of ovarian cancer\textsuperscript{116}.

Surgical treatment is the traditionally key treatment for ovarian cancer, however it has some severe drawbacks, such as high risk with low success rate, large trauma and surgical complications.

Chemotherapy is still the basic therapeutic means of treatment for ovarian cancer\textsuperscript{117}. It has remarkable advantages of improving the cure rate in the term of sensitivity of cancer cells. Ovarian cancer as a kind of malignant tumor, which is relatively sensitive to chemotherapy, can be controlled or cured by many chemotherapeutic agents. Combined chemotherapy of cisplatin, doxorubicin and phosphoric amide acid has become the most widely used treatment for ovarian cancer\textsuperscript{118,119}. However, many patients are still resistant.
to the proposed scheme or have a relapse after treatment. In the late 1980s, the advent of paclitaxel advanced the treatment of ovarian cancer. Moreover, a large number of studies have revealed that patients, who were resistant to the cisplatin, still could be effectively treated by paclitaxel\textsuperscript{120}. In recent years, some studies have shown that some drugs, such as topotecan, ifosfamide, etoposide and adriamycin liposome had effective treatment for the relapsed and drug resistant patients, however the limited efficient rate of those drugs was from 10\% to 30\% and the remission period was not long\textsuperscript{121}.

Therefore, exploration of more effective and safe drugs for treatment of ovarian cancer has become an important task for chemists and clinical works.
Chapter 2 Design and Synthesis of Aptamer-PTX Conjugate

2.1 The design of aptamer-PTX conjugate

Paclitaxel (PTX) is one of the most commonly used first-line drugs for treating a variety of cancers in clinical chemotherapy which has markedly improved the survival time of cancer patients\textsuperscript{122,123}. However, its poor water solubility restricts its direct clinical applications\textsuperscript{124}. More importantly, PTX does not explicitly discriminate between cancer cells and normal cells which frequently lead to serious undesirable side effect\textsuperscript{125,126}. Attaching to tumor recognition elements has been proven beneficial for selective delivery of anti-cancer compounds to tumor cells, however few tumor-targeting elements were proved to be successful.

Aptamers are short single-stranded oligonucleotides which possess similar affinity and specificity to protein targets compared to antibodies, but have advantages in less immunogenicity and had been proved to be safe in clinical trial\textsuperscript{127,128}. Peptide bond has been reported to be relatively unreactive bond under physiological conditions, which will be ruptured in cancer cells. If we conjugate a nucleolin targeting aptamer with paclitaxel to form a nucleolin aptamer-paclitaxel conjugate to confer paclitaxel high water-solubility using the above peptide bond, the conjugate could facilitate paclitaxel selectively targeting cancer cells, which would enhance the antitumor activity and decrease the toxicity of paclitaxel\textsuperscript{129}. So we formulated the structure of an aptamer-paclitaxel conjugate and designed the synthetic route for the aptamer-paclitaxel conjugate (Figure 14).
conjugates were synthesized by the following route (Figure 14) and provided a red solid. The concrete fluorescence-labeled aptamer-paclitaxel was reacted with the hydroxyl group at the 7 position in paclitaxel by condensation, we also designed the aptamer-paclitaxel conjugate labeled by fluorescence. Rhodamine B

Figure 14 Synthetic route of aptamer-paclitaxel conjugates. Note for reagents and conditions: (i) Fmoc-Cl, Na₂CO₃, 1,4-dioxane, H₂O; (ii) NHS, DCC, THF; (iii) L-Cit, NaHCO₃, DME, THF, H₂O; (iv) PABOH, EEDQ, CH₂Cl₂/CH₃OH; (v) PNP chloroformate, pyridine, THF; (vi) PTX, DMAP, CH₂Cl₂; (vii) piperidine, DMF; (viii) succinic anhydride, DIPEA, THF; (ix) Sulfo-NHS, EDCI, aptamer, dd-H₂O, DMF, 0.5 M Na₂CO₃/NaHCO₃.

In order to investigate the tissue distribution of aptamer-paclitaxel conjugate in mice, we also designed the aptamer-paclitaxel conjugate labeled by fluorescence. Rhodamine B was reacted with the hydroxyl group at the 7 position in paclitaxel by condensation reaction and provided a red solid. The concrete fluorescence-labeled aptamer-paclitaxel conjugates were synthesized by the following route (Figure 15).
**Figure 15** Synthesis of fluorescence-labeled aptamer-paclitaxel conjugates. Note for reagents and conditions: (i) TBSCl, pyridine, CH₂Cl₂; (ii) Rhodamine B, EDCI, DMAP CH₂Cl₂; (iii) HF.Pyridine, THF, (iv) Compound 3, DMAP, CH₂Cl₂; (v) Piperidine, DMF; (vi) Succinic anhydride, DIPEA, THF; (vii) Sulfo-NHS, EDC, aptamer, dd-H₂O, DMF, 0.5 M Na₂CO₃/NaHCO₃.

In order to evaluate the serum stability of conjugate and enzyme sensitivity of dipeptide, we also need to design a double fluorescence-labeled aptamer-palitaxel conjugate according to the Fluorescence Resonance Energy Transfer (FRET) principle, which was linked with fluorescein amidate (FAM) at 5’ end of aptamer as a donor and bound with rhodamine B at 7 hydroxyl group in pacalitaxel as an acceptor. The double
fluorescence-labeled conjugate was synthesized by the following method (Figure 16).

Figure 16 Synthesis of double fluorescence-labeled aptamer-paclitaxel conjugate.

Note for reagents and conditions: (i) Sulfo-NHS, EDC, aptamer, dd-H$_2$O, DMF, 0.5 M Na$_2$CO$_3$/NaHCO$_3$.

2.2 Materials

Paclitaxel was purchased from Chengdu Biopurify Phytochemicals Ltd. Nucleolin aptamer (sequence: 5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3'ammc7-r/-3') and CRO (sequence 5'-TTTCCTCCTCCTCCTTCTCCTCCTCCTCC-/3ammc7-r/-3') were synthesized by GUANGZHOU RIBOBIO CO., LTD. Chemicals were obtained from Sigma-Aldrich. All the solvents used for extraction and isolation were of analytical grade. Silica gel (200–300 mesh) purchased from Qingdao Marine Chemical Group Co., P. R. China was used for separation and purification by column chromatography. Silica gel precoated aluminum cards with fluorescent indicator visualizable at 254 nm (Merck) were used for thin layer chromatography (TLC). The $^1$H NMR and $^{13}$C NMR spectra were performed on a Varian MERCURY plus-400 spectrometer using tetramethylsilane as an internal standard. Data were presented as follows: chemical shift, multiplicity (s =
singlet, br s = broad singlet, d = doublet, br d = broad doublet, t = triplet, m = multiplet),

\( J \) = coupling constant in hertz (Hz). LC-MS were performed on a LCMS-2020 Single
Quadrupole Liquid Chromatograph Mass Spectrometer. TOFMS were measured with a
Perkin–Elmer QSTAR mass spectrometer. HRMS was performed on a Micromass LCT
TM at the Instrumental Analysis Center of Hong Kong Baptist University. All air- and
moisture-sensitive manipulations were carried out with standard Schlenk techniques
under nitrogen. Dimethyl formamide (DMF), tetrahydrofuran (THF), petroleum ether
(PE), ethyl ether, methanol (MeOH) and dichloromethane (DCM) were dried according
to the published procedure.

2.3 Methods

2.3.1 Synthesis and characterization of the aptamer-PTX conjugates

**Synthesis and characterization of compound 1 (Fmoc-Val)**

![Chemical structure of Fmoc-Val](image)

To a solution of the **Val** (1.17g, 10 mmol) in 10% aqueous Na\(_2\)CO\(_3\) (20 mL) was
slowly added the solution of Fmoc-Cl (2.83 g, 11 mmol) in dioxane (40 mL) at 0 °C. The
mixture was stirred at the same temperature for 1 h and then allowed to warm to room
temperature. The solution was subsequently stirred overnight, poured into H\(_2\)O (100 mL)
and extracted with Et\(_2\)O (3 x 80 mL). The aqueous layer was cooled in an ice bath and
acidified with concentrated HCl, followed by extraction with EtOAc (3 x 50 mL). The
combined extracts were dried over MgSO\(_4\), filtered off and the solvent evaporated under
reduced pressure to afford the compound 1 as a white solid (yield 87%).

$^1$H NMR (400 MHz, DMSO-d6): $\delta$ 0.91 (s, 3H), 0.93 (s, 3H), 1.99-2.12 (m, 1H), 3.89 (q, J=8.4 Hz, 1H), 4.21-4.31 (m, 3H), 7.33 (t, J=7.2 Hz, 2H), 7.42 (t, J=7.2 Hz, 2H), 7.64 (d, J=8.8 Hz, 2H), 7.75 (d, J=7.2 Hz, 2H), 7.90 (d, J=7.6 Hz, 2H), 12.62 (br, 1H). MS (ESI): [M+Na]$^+$: calculated 362.2, found 362.1.

**Synthesis of compound 2 (Fmoc-Val-NHS)**

To a solution of compound 1 (2.71 g, 8 mmol) in THF (60 mL) was added HOSu (1.01 g, 8.8 mmol) and DCC (1.82 g, 8.8 mmol) at 0 °C. Then the mixture was allowed to warm to room temperature and stirred overnight. The solid DCU byproduct was filtered off and washed with cold THF. The solvent was removed under the reduced pressure, and the resulting glassy solid compound 2 was used without purification in the next step.

**Synthesis and characterization of compound 3 (Fmoc-Val-Cit)**

Compound 2 (8 mmol) in DME (30 mL) was added to a solution of Cit (1.54 g, 8.8 mmol) and NaHCO$_3$ (0.74 g, 8.8 mmol) in water (40 mL). THF (20 mL) was added to aid solubility, and the mixture was stirred at room temperature for 16 h. Aqueous citric acid (15%, 50 mL) was added, and the mixture was extracted with 10% 2-propanol/ethyl...
acetate (2 *100 mL). The solid product began to precipitate but remained in the organic layer. The suspension was washed with water (3*100 mL), and the solvent was evaporated under reduced pressure. The resulting white solid was dried in vacuo for 6 h and then treated with ether (100 mL). After brief sonication and trituration, the white solid compound 3 (yield 73%) was collected by filtration and drying.

$^1$H NMR (400 MHz, MeOD): $\delta$ 0.91-1.00 (m, 6H), 1.57-1.58 (m, 2H), 1.72-1.75 (m, 1H), 1.90-1.95 (m, 1H), 2.03-2.10 (m, 2H), 3.11 (t, $J$=6.8 Hz, 2H), 3.97-3.99 (m, 1H), 4.22-4.26 (m, 1H), 4.35-4.43 (m, 3H), 7.32 (t, $J$=7.2 Hz, 2H), 7.41 (t, $J$=7.2 Hz, 2H), 7.68 (t, $J$=8.0 Hz, 2H), 7.81 (d, $J$=7.6 Hz, 2H). MS (ESI): [M+H]$^+$: calculated 497.2, found 497.1.

**Synthesis and characterization of compound 4 (Fmoc-Val-Cit-PABOH)**

To a solution of compound 3 (1.72 g, 3.47 mmol) and PABOH (853.1 mg, 6.94 equiv) in 2:1 CH$_2$Cl$_2$/CH$_3$OH (60 mL) was added to EEDQ (1.71 g, 6.94 equiv). The mixture was stirred in the dark at room temperature for 36 hours. The solvents were removed under reduced pressure at 40 °C, and the white solid residue was triturated with ether (100 mL). The resulting suspension was sonicated for 5 min and then left to stand for 30 min. The compound 4 was collected by filtration, washed with ether, and dried in vacuo (yield 81%).
$^1$H NMR (400 MHz, DMSO-d6): $\delta$ 0.85-0.89 (m, 6H), 1.36-1.45 (m, 2H), 1.58-1.69 (m, 2H), 1.97-2.00 (m, 1H), 2.93-3.03.(m, 2H), 3.93 (t, $J$=7.2 Hz,1H), 4.21-4.31 (m, 3H), 4.40-4.44 (m, 3H), 5.10 (t, $J$=5.6 Hz, 1H), 5.41 (s, 2H), 5.98 (t, $J$=5.6 Hz, 1H), 7.23 (s, 2H), 7.32 (t, $J$=7.2 Hz, 2H), 7.40-7.46 (m, 3H), 7.54 (d, $J$=7.2 Hz, 2H), 7.74 (t, $J$=8.0 Hz, 2H), 7.89 (d, $J$=7.6 Hz, 2H), 8.11 (d, $J$=7.6 Hz, 1H), 9.91 (s, 1H). 13C NMR (100 MHz, DMSO-d6): $\delta$ 18.25, 19.21, 26.77, 29.52, 30.43, 48.06, 53.04, 60.07, 62.57, 65.66, 118.83, 120.07, 125.34, 126.90, 127.05, 127.62, 137.42, 137.49, 140.68, 143.75, 143.89, 156.09, 158.84, 170.34, 171.22 ppm. MS (ESI): [M+H]$^+$: calculated 602.3, found 602.2.

HRMS calcd. for [C$_{33}$H$_{39}$N$_5$O$_6$ + H]$^+$ 602.2973, found 602.2966.

**Synthesis and characterization of compound 5 (Fmoc-Val-Cit-PABC-PNP)**

A mixture of compound 4 (0.84 g, 1.4 mmol) and pyridine (224 μL, 2.8 mmol) in dry THF (60 mL) was added to the solution of PNP chloroformate (0.56 g, 2.8 mmol) at -40 °C under an argon atmosphere. The mixture was allowed to stir at room temperature and after 12, 24 hours were added respectively 1.5, 1.5 equiv of both 4-nitrophenyl chloroformate and pyridine. After 36 hours, the ethyl acetate was added. The organic layer was washed with 10% citric acid, water and brine, dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by column chromatograph to afford the compound 5 as a brown solid (yield 56%).
1H NMR (400 MHz, DMSO-d6): δ 0.85-0.90 (m, 6H), 1.23 (s, 1H), 1.34-1.47 (m, 2H), 1.56-1.62 (m, 1H), 1.58-1.71 (m, 2H), 1.97-2.02 (m, 1H), 2.89 (s, 1H), 2.92-3.06 (m, 2H), 3.94 (t, J=7.2 Hz, 1H), 4.21-4.31 (m, 3H), 4.40-4.46 (m, 1H), 5.24 (s, 2H), 5.42 (s, 2H), 5.98 (t, J=5.2 Hz, 1H), 7.32 (t, J=7.2 Hz, 2H), 7.40-7.45 (m, 5H), 7.55-7.59 (m, 2H), 7.65 (d, J=8.8 Hz, 2H), 7.75 (t, J=8.0 Hz, 2H), 7.89 (d, J=7.6 Hz, 2H), 8.11-8.16 (m, 1H), 8.30-8.34 (m, 2H), 10.15 (s,1H). 13C NMR (100 MHz, DMSO-d6): δ 18.26, 19.20, 26.80, 29.39, 30.44, 30.76, 35.77, 46.82, 53.12, 60.03, 65.65, 70.24, 115.77, 119.02, 120.08, 122.60, 125.34, 125.39, 126.18, 127.04, 127.61, 129.29, 129.48, 139.35, 140.68, 143.75, 143.88, 145.15, 151.94, 155.27, 156.09, 158.86, 170.72, 171.29 ppm. MS (ESI): [M+H]+: calculated 767.3, found 767.4 [M+Na]+: calculated 789.3, found 789.4. HRMS calcd. for [C40H42N6O10 + H]+ 767.3035, found 767.3020.

**Synthesis and characterization of compound 6 (Fmoc-Val-Cit-PABC-PTX)**

A mixture of compound 5 (460 mg, 0.6 mmol), paclitaxel (563 mg, 0.66 mmol) and DMAP (81 mg, 0.66 mmol) in dry dichloromethane (30 mL) was stirred at room temperature for overnight in the dark and then diluted with dichloromethane. The organic layer was washed with water and brine, dried by anhydrous sodium sulfate, evaporated under reduced pressure. The residue was purified by column chromatograph to afford the
compound 6 as faint yellow solid (yield 65%).

$^1$H NMR (400 MHz, DMSO-d6): $\delta$ 0.79 (t, J=4.0 Hz, 3H), 0.83-0.90 (m, 4H), 1.00-1.06 (m, 6H), 1.23 (s, 3H), 1.26-1.45 (m, 4H), 1.50 (s, 3H), 1.55-1.75 (m, 4H), 1.81 (s, 3H), 1.91-1.99 (m, 1H), 2.12 (s, 3H), 2.26 (s, 3H), 2.30-2.33 (m, 1H), 2.73 (s, 1H), 2.89 (s, 1H), 2.93-3.05 (m, 2H), 3.59 (d, J=6.4 Hz, 1H), 3.99-4.04 (m, 2H), 4.11-4.14 (m, 1H), 4.47 (br, 1H), 4.66 (s, 1H), 4.91-4.98 (m, 2H), 5.17 (s, 2H), 5.35 (d, J=8.8 Hz, 1H), 5.42 (s, 3H), 5.53 (t, J=8.8 Hz, 1H), 5.83 (s, 1H), 5.99 (t, J=6.0 Hz, 1H), 6.31 (s, 1H), 7.17-7.32 (m, 3H), 7.43-7.66 (m, 12H), 7.71-7.75 (m, 1H), 7.81-7.83 (m, 2H), 7.95-7.99 (m, 2H), 8.18-8.19 (m, 1H), 9.28 (d, J=8.4 Hz, 1H), 10.19 (s, 1H). 13C NMR (100 MHz, DMSO-d6): $\delta$ 9.77, 13.92, 18.26, 19.19, 20.67, 21.34, 22.53, 26.32, 26.75, 29.35, 30.42, 42.94, 46.08, 46.65, 53.14, 53.96, 57.39, 60.01, 65.66, 70.42, 71.18, 74.45, 74.71, 76.67, 77.15, 80.24, 119.02, 120.08, 125.34, 127.06, 127.37, 127.48, 127.63, 128.33, 128.68, 128.75, 129.32, 129.55, 129.89, 131.57, 133.48, 134.01, 136.93, 139.16, 139.26, 140.68, 143.72, 143.87, 153.79, 156.10, 158.92, 165.22, 166.37, 168.79, 168.98, 169.69, 170.72, 171.32, 202.38. MS (ESI): [M+H]$^+$: calculated 1481.5, found 1481.1. HRMS calcd. for [C81H88N6O21 + H]$^+$ 1481.6075, found 1481.6036.

**Synthesis and characterization of compound 7 (Val-Cit-PABC-PTX)**
Piperidine (0.88 ml, 0.89 mmol) was added to a solution of compound 6 (355 mg, 0.24 mmol) in DMF (5 ml) at room temperature. The solution was stirred at room temperature for 1 hour, and then solvent was evaporated. The residue was purified by column chromatograph to afford the compound 7 as faint yellow solid (yield 80%).

**Synthesis and characterization of compound 8 (Suc-Val-Cit-PABC-PTX)**

![Chemical structure](image)

To a solution of compound 7 (135.8 mg, 0.1 mmol) in THF (10 mL) was added succinic anhydride (24 mg, 0.2 mmol) and pyridine (20 μL, 0.25 mmol). The resulting solution was stirred at room temperature for 5 hours. The reaction mixture was concentrated in vacuo and diluted with ethyl acetate (50 mL) and then extracted with water, 0.1 N HCl (10 mL), brine and dried over. The combined organic layer was dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. The residue was purified by silica gel chromatography to afford compound 8 (yield 80%) as a white solid.

$^{1}$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 0.85–0.89 (m, 6H), 1.00 (s, 3H), 1.03 (s, 3H), 1.36-1.56 (m, 5H), 1.63-1.72 (m, 3H), 1.83 (s, 4H), 1.78-2.02 (m, 1H), 2.12 (s, 3H), 2.27 (s, 3H), 2.43-2.48 (m, 4H), 2.50 (s, 2H), 2.96-3.04 (m, 2H), 3.59 (d, J=6.8 Hz, 1H), 4.00-4.02 (m, 2H), 4.12-4.22 (m, 2H), 4.36-4.37 (m, 1H), 4.67 (s, 1H), 4.95 (t, J=7.2 Hz, 2H), 5.15 (s, 2H), 5.35-5.56 (m, 5H), 5.83 (t, J=8.8 Hz, 1H), 6.03 (s, 1H), 6.32 (s, 1H), 7.20 (s, 1H), 7.32 (d, J=8.4 Hz, 2H), 8.11 (d, J=6.8 Hz, 1H), 9.19 (d, J=8.4 Hz, 1H), 9.98
\( \delta 9.78, 13.87, 13.93, 14.06, 18.08, 19.17, 20.66, 21.35, 22.53, 26.32, 26.82, 28.78, 29.09, 29.26, 29.92, 30.42, 34.36, 36.52, 38.60, 42.94, 46.08, 48.59, 53.24, 53.91, 53.94, 57.40, 57.71, 57.75, 69.68, 70.39, 71.17, 74.46, 74.71, 75.27, 76.67, 77.14, 80.26, 83.62, 119.02, 121.18, 122.53, 127.34, 127.58, 128.33, 128.67, 128.75, 129.32, 129.52, 129.56, 129.91, 131.55, 133.48, 134.03, 136.94, 139.17, 139.27, 135.78, 158.94, 165.21, 166.37, 168.78, 168.97, 169.68, 170.72, 171.22, 171.50, 171.53, 173.63, 174.01, 190.50, 202.36 \) ppm. MS (ESI): \([M+H]^+\):

<table>
<thead>
<tr>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1359.2</td>
<td>1359.5</td>
</tr>
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</table>

HRMS calcd. for \([C70H82N6O22 + H]^+\) 1359.5555, found 1359.5555.

**Synthesis and characterization of conjugate 9a (Nucleolin aptamer-PTX conjugate)**

![Chemical structure](image_url)

**Synthesis of NucA-PTX conjugate:** Sulfo-NHS (2.6 mg, 12.0 μmol) dissolved in dd-H\(_2\)O (120 μL) was added to the solution of compound 8 (13.6 mg, 10.0 μmol) and EDC (2.5 mg, 13.0 μmol) in 200 μL N,N-Dimethylformamide (DMF). The mixture was stirred at 37°C for 2 hours. Activated compound 8 was then incubated with amino-modified nucleolin aptamer. Aptamer (0.34 mg, 0.04 μmol) was dissolved in 0.5 M Na\(_2\)CO\(_3\)/NaHCO\(_3\) buffer (60 μL, pH 8.4) in a 2 mL centrifuge tube, and 160 μL freshly prepared compound 8 N-Hydroxysulfosuccinimide ester reaction solution was added. After 2 hours reaction, an additional 160 μL of the active ester reaction solution was
added (320 μL, 10 μmol total). The reaction solution was mixed at 37°C overnight, then it was centrifuged and the residue was purified by RP-HPLC. The desired fraction was collected and lyophilized to obtain NucA-PTX conjugate (yield 52%). The product was characterized by ESI.

MS (ESI): calculated 9525.0, found 9526.0.

2.3.2 Synthesis and characterization of conjugate 9b (CRO aptamer-PTX conjugate)

CRO aptamer-PTX conjugate was provided with the same method as producing Nucleolin aptamer-PTX conjugate. The product was characterized by ESI.

MS (ESI): [M+H]^+: calculated 10057.0, found 10059.4.

2.3.3 Synthesis and characterization of fluorescence-labeled conjugate 16a (Nucleolin aptamer-PTX-Rh conjugate)

Synthesis and characterization of compound 10 (2’-TBS-PTX)

To a solution of imidazole (478 mg, 7.03 mmol) in 20 mL of DMF at room temperature was added tert-Butyldimethylsilyl chloride (TBSCl, 790 mg, 5.27 mmol). The solution was stirred at 50 °C for 30 minutes and then added PTX (3.0 g, 3.51 mmol). The solution was stirred at 80 °C for 4 hours and then diluted with EtOAc (200 mL) and washed with water (3 x 30 mL) and brine (3 x 10 mL). The organic solvent was dried over MgSO4 and concentrated, and the residue was by silica gel column chromatography to afford the desired compound 10 (yield 95%).
$^1$H NMR (400 MHz, MeOD-d4): δ 0.81 (s, 9H), 1.10 (s, 3H), 1.11 (s, 3H), 1.20 (t, J=7.2 Hz, 2H), 1.64 (s, 3H), 1.76 (s, 3H), 1.78-1.82 (m, 1H), 1.97 (s, 3H), 2.01 (t, J=9.2 Hz, 1H), 2.13 (s, 3H), 2.31-2.37 (m, 1H), 2.42-2.50 (m, 1H), 2.58 (s, 3H), 3.83 (d, J=7.2 Hz, 1H), 4.06 (q, J=7.2 Hz, 2H), 4.18 (s, 2H), 4.30 (q, J=6.8 Hz, 1H), 4.82 (d, J=5.2 Hz, 1H), 5.00 (d, J=7.6 Hz, 1H), 5.62 (d, J=7.2 Hz, 1H), 5.75 (d, J=5.2 Hz, 1H), 6.12 (t, J=8.8 Hz, 1H), 6.40 (s, 1H), 7.27 (t, J=7.6 Hz, 1H), 7.36-7.43 (m, 4H), 7.46-7.64 (m, 5H), 7.60-7.63 (m, 1H), 7.74-7.76 (m, 2H), 8.09-8.11 (m, 2H). (b) $^{13}$C NMR (100 MHz, MeOD-d4): δ 10.51, 14.51, 15.13, 19.19, 20.82, 20.91, 22.47, 23.72, 26.23, 26.93, 36.81, 37.61, 44.68, 47.92, 57.80, 59.31, 61.57, 72.38, 72.71, 76.29, 76.77, 77.07, 77.59, 79.06, 82.41, 85.96, 128.50, 128.85, 129.30, 129.77, 129.84, 131.25, 131.43, 132.88, 134.58, 135.01, 135.77, 139.32, 141.95, 167.69, 170.26, 171.31, 171.93, 173.17, 205.13. (c) MS (ESI): [M+H]$^+$: calculated 968.4, found 969.6. HRMS calcd. for [C53H65NO14Si+H]$^+$ 968.4247, found 968.4249.

**Synthesis and characterization of compound 11 (2'-TBS,7-Rh-PTX)**

A solution of compound 10 (2.1 g, 2.17 mmol) and 4-(N,N-dimethylamino)pyridine (DMAP, 530 mg, 4.34 mmol) in dry dichloromethane (50 mL) under nitrogen was treated with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 630 mg, 3.3 mmol) and Rhodamin B (1.58 g, 3.3 mmol) for 36 hours at 40 °C. The mixture was diluted with dichloromethane (150 mL) and extracted with H$_2$O (3 x 30 mL). The organic
solvent was dried over MgSO₄, filtered off and evaporated in vacuo. The resulting residue was purified by silica gel column chromatography to afford the desired compound 11 as red solid (yield 78%).

^1^H NMR (400 MHz, MeOD-d4): δ 0.85 (s, 9H), 0.99 (s, 3H), 1.10 (s, 3H), 1.27 (t, J=7.2 Hz, 6H), 1.33-1.39 (m, 9H), 1.66 (s, 3H), 1.87-1.90 (m, 2H), 2.02-2.08 (m, 5H), 2.27-2.34 (m, 1H), 2.58 (s, 3H), 2.88 (s, 2H), 3.01 (s, 2H), 4.06 (d, J=8.4 Hz, 1H), 4.15 (d, J=8.0 Hz, 1H), 4.80-4.84 (m, 2H), 5.52 (d, J=7.6 Hz, 1H), 5.56-5.60 (m, 1H), 5.77 (d, J=5.2 Hz, 1H), 6.10 (t, J=8.8 Hz, 1H), 6.15 (s, 1H), 6.98-7.04 (m, 3H), 7.14-7.18 (m, 3H), 7.29-7.34 (m, 1H), 7.35-7.37 (m, 1H), 7.64-7.68 (m, 1H), 7.75-7.89 (m, 5H), 8.10 (d, J=7.2 Hz, 2H), 8.26-8.29 (m, 1H). ^13^C NMR (100 MHz, MeOD-d4): δ 4.13, 4.32, 20.33, 22.03, 22.13, 23.64, 24.21, 28.36, 29.83, 29.83, 30.77, 31.24, 32.74, 32.93, 35.39, 35.89, 39.66, 39.79, 39.94, 41.03, 41.32, 42.26, 42.67, 43.51, 45.81, 53.79, 56.08, 56.21, 57.13, 66.01, 66.88, 75.72, 81.78, 83.69, 84.90, 85.46, 86.24, 86.32, 87.93, 89.96, 90.97, 93.90, 106.57, 106.60, 119.62, 124.08, 124.19, 124.56, 124.88, 136.61, 137.63, 138.43, 138.78, 138.93, 138.98, 140.27, 140.30, 140.36, 140.47, 140.78, 141.87, 142.01, 142.50, 142.75, 143.11, 143.49, 143.57, 143.83, 144.92, 148.46, 151.12, 166.16, 166.69, 168.29, 168.89, 169.05, 175.07, 176.77, 179.41, 179.45, 181.26, 182.33, 212.05 ppm. MS (ESI): [M+H]^+: calculated 1392.6, found 1393.7. HRMS calcd. for [C₈₁H₉₄N₃O₁₆Si]^+ 1392.6398, found 1392.6412.

Synthesis and characterization of compound 12 (7-Rh-PTX)
To a solution of compound 11 (1.8 g, 1.29 mmol) in 30 mL of THF at room temperature was added hydrogen fluoride-pyridine (0.35 mL, 3.87 mmol) and stirred for 4 hours. The solution was diluted with EtOAc (150 mL) and washed with saturated NaHCO$_3$ solution (2 x 20 mL) and brine (3 x 10 mL). The organic solvent was dried over MgSO$_4$ and concentrated, and the residue was by silica gel column chromatography to afford the desired compound 12 (yield 86%).

$^1$H NMR (400 MHz, MeOD-$d_4$): $\delta$ 0.88 (s, 3H), 0.99 (s, 3H), 1.11-1.24 (m, 17H), 1.67 (s, 3H), 1.84-1.94 (m, 6H), 2.04-2.10 (m, 1H), 2.21 (s, 3H), 3.24 (s, 1H), 3.50-3.57 (m, 4H), 3.58-3.67 (m, 4H), 3.92-4.02 (m, 3H), 4.61 (d, J=5.2 Hz, 1H), 4.65 (d, J=8.8 Hz, 1H), 5.38-5.46 (m, 2H), 5.51 (d, J=5.2 Hz, 1H), 5.99-6.03 (m, 2H), 6.85-6.92 (m, 3H), 7.02-7.05 (m, 3H), 7.15-7.19 (m, 1H), 7.26-7.46 (m, 10H), 7.53-7.56 (m, 1H), 7.64-7.74 (m, 4H), 7.95 (d, J=7.2 Hz, 2H), 8.13 (d, J=7.6 Hz, 1H).

$^{13}$C NMR (100 MHz, MeOD-$d_4$): $\delta$ 9.74, 11.40, 11.53, 13.09, 13.32, 19.25, 19.48, 20.57, 21.72, 25.34, 32.07, 35.07, 43.18, 45.48, 45.62, 46.61, 46.99, 47.20, 55.42, 56.26, 60.14, 70.76, 73.15, 73.45, 74.31, 74.96, 75.69, 80.32, 83.28, 95.99, 96.01, 113.49, 113.61, 113.96, 114.29, 127.10, 127.60, 128.19, 129.71, 129.76, 129.88, 130.21, 131.29, 131.43, 131.92, 132.15, 132.50, 132.90, 132.99, 133.28, 134.20, 138.60, 140.68, 155.59, 156.11, 157.71, 158.31, 158.49, 164.43, 166.16,
168.82, 168.89, 170.66, 173.02, 201.48 ppm. MS (ESI): [M+H]^+: calculated 1278.6, found 1278.7. HRMS calcd. for [C_{75}H_{80}N_{16}O_{16}]^+ 1278.5533, found 1278.5535.

Synthesis and characterization of compound 13 (Fmoc-Val-Cit-PABC-PTX-Rh)

A mixture of compound 5 (460 mg, 0.6 mmol), compound 12 (845 mg, 0.66 mmol) and DMAP (81 mg, 0.66 mmol) in dry dichloromethane (30 mL) was stirred at room temperature for overnight in the dark and then diluted with dichloromethane. The organic layer was washed with water and brine, dried by anhydrous sodium sulfate, evaporated under reduced pressure. The residue was purified by column chromatograph to afford the compound 13 as pink solid (yield 57%).

^1H NMR (400 MHz, MeOD-d4): δ 0.97-1.04 (m, 9H), 1.05-1.11 (m, 4H), 1.20 (t, J=8.8 Hz, 6H), 1.26 (t, J=6.8 Hz, 3H), 1.31 (t, J=6.8 Hz, 6H), 1.39 (s, 3H), 1.55-1.62 (m, 2H), 1.80 (s, 3H), 1.84-1.94 (m, 2H), 2.02-2.17 (m, 8H), 2.34 (s, 3H), 3.08-3.12 (m, 1H), 3.17-3.22 (m, 1H), 3.54-3.60 (m, 4H), 3.64-3.79 (m, 5H), 3.99 (d, J=6.8 Hz, 1H), 4.04 (d, J=8.4 Hz, 1H), 4.11 (q, J=7.2 Hz, 3H), 4.20 (t, J=6.4 Hz, 1H), 4.38 (d, J=8.0 Hz, 1H), 4.52-4.55 (m, 1H), 4.76 (d, J=8.8 Hz, 1H), 5.13 (d, J=4.4 Hz, 2H), 5.43 (d, J=6.0 Hz, 1H), 5.51-5.52 (m, 2H), 5.57-5.61 (m, 1H), 5.82 (d, J=6.0 Hz, 1H), 6.03 (t, J=8.4 Hz, 1H), 6.18 (s, 1H), 6.90-6.96 (m, 3H), 7.07-7.17 (m, 3H), 7.25-7.28 (m, 5H), 7.34-7.54 (m,
1H), 7.56-7.68 (m, 7H), 7.76-7.86 (m, 6H), 8.08 (d, J=7.2 Hz, 2H), 8.25 (d, J=7.8 Hz, 2H). 13C NMR (100 MHz, MeOD-d4): δ 9.83, 11.47, 11.56, 13.04, 13.60, 17.42, 18.45, 18.49, 19.31, 19.62, 20.18, 20.59, 21.75, 22.33, 24.78, 25.37, 26.51, 28.87, 28.99, 29.03, 29.21, 29.34, 30.43, 30.74, 31.67, 32.11, 32.92, 34.86, 34.88, 38.89, 43.14, 45.48, 45.60, 46.65, 53.54, 53.57, 53.83, 55.44, 61.13, 65.15, 66.65, 69.82, 71.66, 73.16, 74.29, 74.96, 75.68, 77.10, 77.32, 79.39, 80.31, 83.32, 95.95, 96.00, 113.52, 113.92, 114.22, 114.81, 119.08, 119.58, 119.70, 119.78, 124.80, 126.79, 126.82, 127.18, 127.42, 128.15, 128.29, 128.35, 128.68, 128.83, 129.00, 129.68, 129.73, 129.78, 129.88, 130.16, 130.69, 131.36, 131.50, 131.76, 132.12, 132.55, 132.94, 133.12, 133.32, 133.96, 133.98, 136.69, 138.63, 140.74, 141.18, 143.74, 143.88, 143.92, 154.34, 155.54, 155.99, 157.43, 157.47, 157.66, 158.20, 158.46, 160.91, 164.42, 166.17, 168.77, 168.84, 170.43, 170.87, 173.00, 174.22, 183.82, 201.61 ppm. [M+H]+: calculated 1906.8, found 1905.7.

Synthesis of compound 14 (Val-Cit-PABC-PTX-Rh)

Piperidine (0.88 ml, 0.89 mmol) was added to a solution of compound 13 (355 mg, 0.24 mmol) in DMF (5 ml) at room temperature. The solution was stirred at room temperature for 1 hour, and then solvent was evaporated. The resulting pink solid compound 14 was used without purification in the next step.
Synthesis of compound 15 (Suc-Val-Cit-PABC-PTX-Rh)

To a solution of compound 14 (obtained from the previous step) in THF (10 mL) was added succinic anhydride (24 mg, 0.2 mmol) and pyridine (20 μL, 0.25 mmol). The resulting solution was stirred at room temperature for 5 hours. The reaction mixture was concentrated in vacuo and diluted with ethyl acetate (50 mL) and then extracted with water, 0.1 N HCl (10 mL), brine and dried over. The combined organic layer was dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. The residue was purified by silica gel chromatography to afford compound 15 (yield 68%) as a pink solid.

$^1$H NMR (400 MHz, MeOD-$d_4$): $\delta$ 0.86-1.09 (m, 15H), 1.12-1.23 (m, 8H), 1.26-1.36 (m, 7H), 1.59-1.74 (m, 3H), 1.78 (s, 3H), 1.84-2.02 (m, 3H), 2.05 (s, 3H), 2.11-2.14 (m, 2H), 2.30-2.33 (m, 2H), 2.36 (s, 3H), 2.44-2.45 (m, 1H), 2.69-2.74 (m, 2H), 3.15-3.23 (m, 3H), 3.33 (s, 3H), 3.59-3.65 (m, 4H), 3.69-3.74 (m, 4H), 3.79 (d, J=6.4 Hz, 1H), 4.06 (d, J=6.4 Hz, 1H), 4.13-4.14 (m, 2H), 4.30-4.34 (m, 1H), 4.36-4.40 (m, 2H), 4.81 (d, J=9.2 Hz, 2H), 5.13 (s, 2H), 5.42 (d, J=6.0 Hz, 1H), 5.52-5.59 (m, 2H), 5.81 (d, J=6.0 Hz, 1H), 6.03 (t, J=8.4 Hz, 1H), 6.16 (s, 1H), 6.93-6.99 (m, 3H), 7.09-7.11 (m, 2H), 7.19-7.21 (m, 1H), 7.26-7.30 (m, 3H), 7.38-7.59 (m, 11H), 7.66-7.84 (m, 8H), 8.08 (d, J=7.2 Hz, 2H), 8.23 (d, J=4.4 Hz, 1H). $^{13}$C NMR (100 MHz, MeOD-$d_4$): $\delta$ 7.38, 9.24, 9.91, 11.63, 11.64,
11.75, 12.94, 13.78, 16.68, 18.30, 19.45, 20.64, 20.67, 21.88, 25.39, 25.87, 28.28, 29.24, 29.39, 31.86, 32.90, 34.87, 43.16, 45.53, 45.60, 45.62, 51.88, 55.46, 56.39, 60.28, 65.04, 67.88, 71.56, 75.13, 76.61, 77.30, 80.26, 88.77, 95.94, 96.01, 112.06, 113.45, 113.51, 114.16, 114.22, 118.10, 119.93, 127.17, 127.23, 127.29, 128.20, 128.39, 128.70, 128.74, 128.84, 129.77, 129.78, 129.92, 130.17, 130.50, 131.49, 133.92, 140.73, 147.45, 149.02, 154.36, 155.50, 155.52, 157.57, 157.64, 158.08, 161.37, 164.38, 166.14, 168.83, 168.89, 169.02, 170.47, 173.07, 173.78, 184.92, 185.32, 192.84, 214.61 ppm. MS (ESI): \([M+H]^+\): calculated 1784.8, found 1784.9. HRMS calcd. for \([C_{98}H_{111}N_8O_{24}]^+\) 1783.7706, found 1783.7713.

**Synthesis and characterization of fluorescence-labeled conjugate 16a (Nucleolin aptamer-PTX-Rh conjugate)**

![Nucleolin aptamer-PTX-Rh conjugate](image)

**Nucleolin aptamer-PTX-Rh conjugate** was provided with the same method as producing **Nucleolin aptamer-PTX conjugate**. The product was characterized by ESI. MS (ESI): \([M+H]^+\): calculated 10250.0, found 10249.7.

2.3.4 **Synthesis and characterization of fluorescence-labeled conjugate 16b (CRO-PTX-Rh conjugate)**

**CRO-PTX-Rh conjugate** was provided with the same method as producing **Nucleolin aptamer-PTX conjugate**. The product was characterized by ESI. MS (ESI): \([M+H]^+\): calculated 10481.0, found 10481.2.
2.3.5 Synthesis and characterization of fluorescence-labeled conjugate 17 (FAM-Nucleolin aptamer-PTX-Rh conjugate)

FAM-Nucleolin aptamer-PTX-Rh conjugate was provided with the same method as producing Nucleolin aptamer-PTX conjugate. The product was characterized by ESI. MS (ESI): [M+H]+: calculated 10786.0, found 10784.4.

2.4 Conclusion

To connect the cytotoxic PTX with the tumor-targeting NucA, the active 2’-hydroxyl group on PTX was the optimal site for substitution. However, the introduction of other functional groups on this site resulted in the loss of antitumor activity for the obtained PTX derivatives\textsuperscript{130}. Thus, a labile linker with a cathepsin B-sensitive dipeptide was utilized to facilitate the intracellular release of the conjugated PTX\textsuperscript{131,132}. In addition, spacers (PABC on PTX side and succinic acid on NucA side) were incorporated to the linker for reducing the steric hindrance for the protease.

So we designed the synthetic route for conjugates and successfully prepared the desired conjugates, which were characterized by high performance liquid chromatograph, mass spectrum or nuclear magnetic resonance. During the process of synthesis, we also optimized the reaction conditions, including the reaction temperature, solvents, material...
input, time and catalysts. We got a stable and mature synthetic route and the total yield was more than 6%.
Chapter 3 Determination of Physical Properties of Nucleolin Aptamer-Paclitaxel Conjugate

3.1 Materials

Paclitaxel was purchased from Chengdu Biopurify Phytochemicals Ltd. Nucleolin aptamer-paclitaxel was synthesized by the methods as mentioned before. DMSO, HEPES, NaCl, TCEP, glycerol (analytical grade) were obtained from Sigma-Aldrich. Pure water was produced by ECO series deionized water system.

3.2 Solubility test

The reported solubility of paclitaxel in water was less than < 0.004 mg/mL\(^{133}\), so we got 0.17mg paclitaxel and added to the 100 μL water and DMSO, respectively. The 1.96 mg nucleolin aptamer-paclitaxel conjugate was added to 100 μL water in the same method. All the solutions were vigorously shaken for 24 hours at the room temperature. In theory, all the concentrations of the solutions were 2 mM. However, the groups of paclitaxel in water and DMSO were still suspension or cloudy, and the group of conjugate was completely dissolved, showed as Figure 17.
The solution of nucleolin aptamer-conjugate was centrifugated by high speed centrifugator at 10000 rpm for 5 min at room temperature. The 10 μL upper layer was taken and diluted to 100 μL by water. We divided the 100 μL solution into 5 equal parts and determined each part by HPLC at 260 nm. The average value of five absorption peak areas was calculated and compared with the standard content (0.1 nmol NucA-PTX in 20μL water), showed as Table 1.

**Table 1 The peak areas of tested NucA-PTX and standard NucA-PTX.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested NucA-PTX</td>
<td>74752911</td>
<td>74496882</td>
<td>74895003</td>
<td>74920637</td>
<td>74658044</td>
<td>74744695</td>
</tr>
<tr>
<td>Standard NucA-PTX</td>
<td>1868825</td>
<td>1868337</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1868581</td>
</tr>
</tbody>
</table>

As been seen and calculated, the tested NucA-PTX was dissolved completely in water, which suggested PTX in the conjugate has improved the water solubility more than one thousand after conjugating with nucleolin aptamer. The condition of HPLC was following using a column of Xbridge RP-C18 (50 mm*4.6 mm 3.5 um) and an UV
detector set at 260 nm at 40 °C column temperature. The mobile phase consisted of A (100 mM TEAA pH 7.0) and B (acetonitrile) and the gradient was from 5% to 40% in 5 min, 40%-95% in 3 min and hold 95% for 2 min with the flow rate of 1.5 mL/min.

3.3 Affinity test

3.3.1 The design of molecular modeling of nucleolin aptamer

The three-dimensional model of NucA was generated by homology modeling based on a G-quadruplex structure (PDB ID: 2N3M). The structure of the C-terminal RGG-rich domain was calculated by I-TASSER \(^{134}\). Then the molecular dynamic for the coarse structure was implemented for energy minimization and optimization in amber force field \(^{135}\). The docking was performed to generate the initial complex of aptamer and nucleolin by using HADDOCK 2.2 \(^{136}\). Finally, the binding free energy was calculated with MM-PBSA \(^{137}\) algorithm to identify whether the paclitaxel had an influence on the interaction between aptamer and nucleolin.

3.3.2 The effect of the conjugated PTX on the interaction between NucA and nucleolin

Nucleolin binding and cell surface nucleolin-dependent uptake are essential for achieving the tumor-targeting property of NucA-PTX. Firstly, to investigate whether the conjugated PTX affects the interaction between NucA and its target protein nucleolin, their binding affinity before and after conjugation was studied by both molecular dynamic simulation. Using molecular dynamic simulation, it was found that the nucleolin of eukaryotic cell comprises several domains, including an acidic N-terminal domain, four RNA binding domains (RBD) and a C-terminal RGG-rich “tail”. In the simulated
model, it was found that the NucA-PTX conjugate interacted with RGG-rich domain of the C-terminal of the nucleolin, which was consistent with published paper\textsuperscript{138}. Structurally, the conjugated PTX segment laid at the end of the chain in the simulated model (Figure 18).

![Figure 18](image)

**Figure 18 The effect of the conjugated PTX on the interaction between NucA and nucleolin.** The prediction of the interaction models between NucA and nucleolin (left) and between NucA-PTX and nucleolin (right), respectively, by molecular dynamic simulation. C-terminal domain of nucleolin, NucA, RGG-rich domain and PTX molecule were indicated by red, green, yellow and blue, respectively.

3.3.3 The method of testing the binding affinities of NucA and NucA-PTX with nucleolin

The binding affinities of NucA and NucA-PTX with nucleolin were tested by MicroCal iTC 200 (isothermal titration calorimetry, Malvern). NucA and NucA-PTX at a concentration of 7.2 μM were injected into 720 nm nucleolin in 19 portions (each portion
2 μL) at 25 °C. All reagents were diluted with a HEPES Solution (20 mM HEPES, 600 mM NaCl, 0.3 mM TCEP, 25 % glycerol, pH 7.3). The injections were made over a period of 4 s with a 2-min interval between subsequent injections. The data were analyzed by Origin Software for ITC and dissociation constants (K_d) were calculated.

3.3.4 The effect of the conjugated PTX on the interaction between NucA and nucleolin

To investigate whether the conjugated PTX affects the interaction between NucA and its target protein nucleolin, their binding affinity before and after conjugation was studied by isothermal titration calorimetry (ITC). Quantitatively, no considerable difference was found in the binding affinities between NucA and NucA-PTX conjugate when docking to nucleolin. Moreover, the binding between NucA or NucA-PTX and Nucleolin was examined by titrating NucA or NucA-PTX into the nucleolin protein solution with a 10:1 ratio of concentration by isothermal titration calorimetry. The association constant (K_a) of the interactions by both titrations did not exhibit significant variation before and after the conjugation of PTX (Figure 19), with the K_a value 111 nM for NucA and 123 nM for NucA-PTX, respectively.
Figure 19 The interaction between NucA and nucleolin (left) and between NucA-PTX and nucleolin (right), respectively, detected by isothermal titration calorimetry (ITC).

3.4 Conclusion

Every molecule of synthesized NucA-PTX contains a nucleolin aptamer, which has better water solubility and higher molecular weight comparing to PTX. In theory, the NucA-PTX is similar to nucleolin aptamer in the terms of water solubility. After testing, the results verified the speculation that the water solubility of PTX in NucA-PTX has been improved than one thousand.

From the computer simulation results, the binding site between nucleolin aptamer and targeted protein nucleolin was far from the tail of nucleolin aptamer, to which the PTX derivative was conjugated. Additional, another evidence proved that there was little influence on the binding affinities before and after the conjugation between nucleolin
aptamer and targeted protein nucleolin through isothermal titration calorimetry.
Chapter 4 Biological Evaluation of Nucleolin Aptamer-Paclitaxel Conjugate in vitro

4.1 Materials

MTBE, rhodamine, FAM, methanol, acetonitrile, sodium acetate, DTT, EDTA were obtained from Sigma-Aldrich. Cathepsin B, transferrin, cholera toxin, and dextran were purchased from Chengdu WiseChoice Biotechnology Co., Ltd.. Human ovarian cancer cell line SKOV3 (ATCC HTB-77), OVCAR3 (ATCC HTB-161) and human normal liver cell line L02 were purchased from ATCC, USA. The cells were maintained in ATCC-formulated DMEM (for SKOV3 and OVCAR3) or RPMI-1640 (for L02) Medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and incubated at 37 °C with 5% CO₂ and 95% humidity.

4.2 The stability of nucleolin aptamer-paclitaxel conjugate in the serum and release of paclitaxel in vitro

4.2.1 Methods

The emission spectra of FAM-NucA, FAM-NucA-PTX-Rh and PTX-Rh were recorded by a fluorescence spectrophotometer ($\lambda_{ex} = 470$ nm) at a concentration of 2 μM. Then, FAM-NucA-PTX-Rh was incubated in human serum at a concentration of 2 μM at 37 °C. The fluorescence of FAM and Rhodamine of the sample at each time point (0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 12, 24, 30, 48 h) was recorded by a fluorescence spectrophotometer ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 520$ nm and 590 nm respectively). Aliquots of 100 μL NucA-PTX serum solution at the same concentration were also collected at each time point for concentration determination by HPLC. Then 250 μL methyl tert-butyl ether (MTBE) was added. After centrifugation, 200 μL supernate was collected and dried. Finally, the
residue was dissolved in 40 µL methanol for determining the concentration of NucA-PTX. The HPLC assay was conducted using a Phenomenex® C18 column (5 µm, 250×4.6 mm) kept at 35 °C and an UV detector set at 232 nm. Acetonitrile and water (53:47, V/V) were used as mobile phase at a flow-rate of 1.0 mL/min.

SKOV3 cells were seeded in a 24-wells plate at a density of $1\times10^5$ cells each well and incubated overnight. FAM-NucA-PTX-Rh at a concentration of 200 nM was added to the media of cells for incubation. A mixture of FAM-NucA and PTX-Rh of 1 : 1 ratio at 200 nM of each were also incubated with SKOV3 cells as a control. After 1 h, 3 h and 6 h incubation, cells was washed and run for fluorescence analysis by a FACScan cytometer (BD 831 Immunocytometry Systems).

### 4.2.2 Results

To further probe the intracellular release of PTX-Rh from FAM-NucA-PTX-Rh in ovarian cancer cells, SKOV3 cells were treated with FAM-NucA-PTX-Rh for 1 h, and the fluorescence of FAM and Rh of the washed cells were analyzed immediately or after continuous incubation for 3, 6 and 12 h by flow cytometry. In accordance with the results presented above (Figure 20), the fluorescence intensity ratio Rh/FAM kept decreasing upon time while the standardized Rh intensity was continuously increased, implying the successful intracellular release of PTX-Rh from NucA-PTX.
The intracellular release of PTX-Rh from FAM-NucA-PTX-Rh in SKOV3 cells monitored by flow cytometry. The relative median fluorescence intensity (MFI) of FAM and rhodamine in SKOV3 cells was examined after 1, 3 and 6 h incubation with FAM-NucA-PTX-Rh.

4.3 The sensitivity of the dipeptide linker in nucleolin aptamer-paclitaxel conjugate

4.3.1 Methods

To a 96-well plate was added 85.63 μL of buffer which contains 25 mmol/L sodium acetate, 5 mmol/L dithiothreitol (DTT) and 1 mmol/L Ethylenediaminetetraacetic acid (EDTA) (adjust to pH 5.0 with 1 N HCl, preincubated at 37 °C). 7.7 μL Cathepsin B buffer solution (0.5 unit/mL) then was added. Afterwards, 6.67 μL of a 30 μM FAM-NucA-PTX-Rh water solution was added to the enzyme solution. The final volume of this solution was 100 μL. The control group was 92.3 μL buffer and 7.7 μL Cathepsin B buffer solution. The whole system was incubated at 37 °C. The fluorescence changes of FAM and Rhodamine of the conjugate upon time were recorded by a fluorescence microplate reader ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 520$ nm and 590 nm respectively). Further, FAM-NucA-PTX-Rh was incubated at various Cathepsin B concentrations for 4 h and
fluorescence changes were recorded as the method described above. The concentration of NucA-PTX upon time in the presence of Cathepsin B was also detected by HPLC according to the method mentioned above.

4.3.2 Results

To verify the stability of NucA-PTX in circulation and the intracellular release of PTX, a dual fluorescence-labeled conjugate - FAM-NucA-PTX-Rh was designed according to the Fluorescence Resonance Energy Transfer (FRET) principle (Figure 21).

![Figure 21 Schematic diagram of tracking the release of paclitaxel from the conjugate via FRET.](image)

Fluorescein amidate (FAM) was linked to the 5’ end of NucA as the donor and Rhodamine B (Rh) was linked to the 7 hydroxyl group of PTX as the acceptor. When the dipeptide linker stayed intact, the fluorescence of both fluorophores in FAM-NucA-PTX-Rh remained stable, whereas when the linker was hydrolyzed in the presence of the specific protease - cathepsin B, FRET between FAM and Rhodamine B was interrupted, appeared as the decrease of Rh intensity and the increase of FAM.
intensity. With an excitation wavelength at 488 nm, compared to the individual fluorescence emission spectra of FAM-NucA and PTX-Rh, obvious FRET was noted in the fluorescence emission spectrum of FAM-NucA-PTX-Rh (Figure 22a). Whereas, in the presence of cathepsin B, an increased emission at 520 nm and a decreased emission at 590 nm upon time was observed in the spectra of FAM-NucA-PTX-Rh (Figure 22b).

**Figure 22** The fluorescence intensity of compounds. (a) Emission spectra of FAM-NucA-PTX-Rh, FAM-NucA and PTX-Rh (λ<sub>ex</sub> = 470 nm). All compounds were at a concentration of 2 μM. (b) Emission spectra of FAM-NucA-PTX-Rh (λ<sub>ex</sub> = 470 nm) in the presence of cathepsin B upon time. 0 h (black line), 6 h (red line) and 48 h (blue line). FAM-NucA-PTX-Rh was at a concentration of 2 μM and cathepsin B was added to a final concentration of 0.5 unit/mL.

Accordingly, the concentration of FAM-NucA-PTX-Rh could be reflected by the fluorescence intensity ratio at 590 nm and 520 nm (FI<sub>590/520</sub>), while the concentration of PTX-Rh could be presented by the fluorescence intensity of free PTX-Rh (FI<sub>590</sub>). When FAM-NucA-PTX-Rh was incubated in human serum at 37°C for 48 h, both FI<sub>590/520</sub> and FI<sub>590</sub> remained the same upon time (Figure 23), suggesting FAM-NucA-PTX-Rh existed as the intact form. In contrast, in the presence of cathepsin
B, FI $\lambda_{590}/\lambda_{520}$ gradually declined while FI $\lambda_{590}^0/\lambda_{590}$ increased, ultimately both remaining unchanged (Figure 23), implying the successful cleavage of the linker that connects FAM-NucA and PTX-Rh and the release of free PTX-Rh.

Figure 23 The change of fluorescence intensity on the present and absent of cathepsin B. The relative fluorescence intensity of FAM and rhodamine ($\lambda_{590}/\lambda_{520}$) indicating the concentration of FAM-NucA-PTX-Rh (left), and the fluorescence intensity of rhodamine ($\lambda_{590}$) indicating the concentration of the conjugated PTX-Rh (right) in the presence or absence of cathepsin B in human serum upon time.

4.4 The effect of NucA modification on the in vitro cellular uptake of the conjugated PTX

4.4.1 Methods

For time-dependent uptake experiment, SKOV3 cells were seeded in a flow tube at a density of $2\times10^5$ cells per tube and NucA-PTX-Rh or CRO-PTX-Rh with a final concentration of 500 nM were respectively added to the tubes. After 1, 2, 3, 4, 5 and 6 h of incubation, the cells were centrifuged (1000 rpm, 5 min) and the supernatants were removed. After re-suspended in 400 μL PBS, fluorescence of Rhodamine was determined.
with a FACScan cytometer (BD 831 Immunocytometry Systems) by counting 10,000 events. SKOV3 cells without any drug incubation were performed as a blank control to measure background signals which were subtracted from the final calculations. For concentration-dependent uptake assay, the final concentration of NucA-PTX-Rh and CRO-PTX-Rh was 125 nM, 250 nM, 500 nM, 1 μM and 2 μM respectively. After 3 h of incubation, the same procedures were conducted as the time-dependent uptake experiment. For aptamer blocking experiment, the SKOV3 cells were pe-incubated with 250 nM either nucleolin aptamer or CRO before adding NucA-PTX-Rh and nucleolin antibody. For nucleolin-dependent uptake assay, SKOV3, OVCAR3 and L02 cells were incubated with NucA-PTX-Rh or nucleolin antibody for 3 h and then centrifuged and re-suspended.

4.4.2 Results

The uptake of NucA-PTX-Rh in SKOV3 cells was examined by detecting the Rhodamine B fluorescence in SKOV3 cells after the treatment with NucA-PTX-Rh using flow cytometry. The uptake of NucA-PTX-Rh in SKOV3 cells was shown to increase upon time (Figure 24a) and concentration (Figure 24b), which was significantly higher than that of its counterpart CRO-PTX-Rh (Figure 24a, b). This suggested that NucA could facilitate the uptake of the conjugated PTX into SKOV3 cells in a both time and concentration dependent manner.
Figure 24 NucA modification facilitated the uptake of the conjugated PTX in SKOV3 cells, and the uptake of NucA-PTX-Rh was both time and concentration-dependent. (a) The time-dependent cellular uptake of the conjugated PTX-Rh examined by flow cytometry. (b) The concentration-dependent cellular uptake of the conjugated PTX-Rh examined by flow cytometry.

To examine whether the uptake of the conjugated PTX-Rh was nucleolin-dependent, the uptake of NucA-PTX-Rh in three cell lines: two human ovarian cancer cell lines – SKOV3 and OVCAR3, and one human healthy liver cell line – L02 were examined in this study. Different expression levels of nucleolin on the cell surface were observed for the three cell lines, with L02 the lowest and SKOV3 the highest (Figure 25). The uptake of NucA-PTX-Rh in the three cell lines was found increasing along with the nucleolin expression accordingly. The highest uptake of NucA-PTX-Rh was observed in SKOV3 cells with the top nucleolin expression, and the lowest uptake in L02 cells with the least nucleolin expression (Figure 25).
Figure 25 The uptake of NucA-PTX-Rh in different cell lines was correlated to the nucleolin expression on cell surface. The nucleolin expression (left) on the cell surface and the uptake of the conjugated PTX-Rh (right) in SKOV3, OVCAR3 and L02 evaluated by flow cytometry.

When the nucleolin on SKOV3 cells were partially blocked by pre-incubation with nucleolin aptamer, the uptake of NucA-PTX showed a significant decrease (Figure 26). While the pre-incubation with CRO aptamer resulted in a less blocking effect and consequently a smaller decrease of NucA-PTX-Rh uptake (Figure 26).
Figure 26 The block of surface nucleolin by NucA resulted from decreased NucA-PTX-Rh uptake. The cellular uptake of the conjugated PTX-Rh in SKOV3 pre-incubated with either NucA or CRO analyzed by flow cytometry.

4.5 The effect of the NucA modification on the cellular internalization of the conjugated PTX-Rh in NucA-PTX-Rh in SKOV3 cells in vitro

4.5.1 Methods

Confocal imaging for endocytosis pathways

SKOV3 cells (5×10^4/well) were seeded in glass bottom confocal dishes and incubated overnight. After washing, the cells were incubated with 250 nM of NucA-PTX-Rh or CRO-PTX-Rh and Alexa Fluor® 488-labeled endocytic markers (25 μg/ml dextran, 25 μg/ml transferrin and 5 μg/ml CTX-B) at 37 °C for 2 h. 2 μg/mL Hoechst 33342 then was added during the final 15 min of the incubation. After 2 h of incubation, the cells were washed and fixed with 4% paraformaldehyde for 10 min. The cells were visualized by confocal microscopy. To investigate the sub-cellular trafficking, the cells were incubated with 250 nM of NucA-PTX-Rh or CRO-PTX-Rh at 37 °C for 1
h. 75 nM LysoTracker Green DND-26 (Invitrogen) was added to the cells for another 1 h. 2 μg/mL Hoechst 33342 then was added during the final 15 min of the incubation. After 2 h of incubation, the cells were washed and visualized by confocal microscopy.

**Chemical inhibition of endocytosis pathways**

SKOV3 cells were seeded in flow tubes at a density of $2 \times 10^5$ cells per tube. Inhibitors of macropinocytosis (EIPA and cytochalasin D) with various concentrations were pre-incubated for 30 min prior to the addition of NucA-PTX-Rh at a concentration of 500 nM. After 2.5 h, the cells were centrifuged (1000 rpm, 5 min) and the supernatants were removed. After washed twice with PBS, the cells were re-suspended in 400 μL of PBS. Fluorescence was determined with a FACScan cytometer (BD 831 Immunocytometry Systems) by counting 10,000 events. SKOV3 cells with DMSO were performed as a blank control to measure background signals which were subtracted from the final calculations.

**4.5.2 Results**

To explore whether the conjugated PTX is taken up by SKOV3 cells through endocytosis resulted from the NucA modification, SKOV3 cells was incubated with NucA-PTX-Rh or CRO-PTX-Rh in the presence of Alexa Fluor 488–labeled endocytic markers and analyzed by confocal microscopy. A remarkable co-localization of NucA-PTX-Rh with Dextran (a marker for macropinocytosis) in SKOV3 cells was observed by confocal microscopy, and few instances of co-localization of NucA-PTX-Rh with transferrin (a marker for clathrin-mediated endocytosis) and choleratoxin (a marker for caveolae-mediated endocytosis) were detected (**Figure 27**), indicating that
NucA-PTX-Rh was mainly taken up by SKOV3 cells via macropinocytosis.

<table>
<thead>
<tr>
<th>Alexa488-labeled</th>
<th>Rhodamine</th>
<th>Hoechst 33342</th>
<th>Overlay</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choleratoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Dextran</td>
<td></td>
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Figure 27 The effect of NucA modification on the cellular internalization of the conjugated PTX-Rh in NucA-PTX-Rh in SKOV3 cells *in vitro*. The co-localization of the conjugated PTX-Rh (red) in NucA-PTX-Rh with Alexa Fluor 488-labeled endocytic markers (transferrin, choleratoxin and dextran; green) by confocal microscopy. The nucleus was counterstained with Hoechst 33342 (blue). The scale bar indicated was 10 μm.

In contrast, the distribution of CRO-PTX-Rh in SKOV3 cells was more dispersed compared to that of NucA-PTX-Rh, and no significant co-localization of CRO-PTX-Rh with any of the three markers was observed (Figure 28), indicating the non-specific uptake of CRO-PTX-Rh.
Figure 28 The co-localization of the conjugated PTX-Rh in CRO-PTX-Rh with Alexa Fluor 488-labeled endocytic markers (transferrin, cholera toxin and dextran) by confocal microscopy.

Inhibition of macropinocytosis pathway by EIPA and cytochalasin D of varied concentrations reduced the uptake of NucA-PTX-Rh, confirmed these findings from the confocal imaging (Figure 29).

Figure 29 The chemical inhibition of cellular uptake for the conjugated PTX-Rh in
NucA-PTX-Rh: the relative fluorescence of rhodamine was quantified after the treatment with inhibitors of macropinocytosis: EIPA (left) and cytochalasin D (right) by flow cytometry.

Additionally, confocal images showed that NucA-PTX-Rh was predominantly localized inside lysosomes, which could enable the break of labile linker and release of PTX in the conjugate (Figure 30). However only a small portion of CRO-PTX-Rh was observed localizing inside lysosomes, implying a slower release of PTX (Figure 30).

![Image](Image)

Figure 30 The effect of NucA modification on the cellular trafficking of the conjugated PTX-Rh in NucA-PTX-Rh in SKOV3 cells in vitro. The co-localization of the conjugated PTX-Rh with a lysosomal marker (LysoTracker Green DND-26) by confocal microscopy. The scale bar indicated was 10 μm.

4.6 The effect of NucA modification on the in vitro anti-tumor activity of the conjugated PTX

4.6.1 Methods

*In vitro* cell viability assay
The cell viability assay was conducted using a CCK8 kit. According to the provided protocol, SKOV3, OVCAR3 and L02 cells were seeded in 96-well plates with approximately 5000 cells in each well and incubate overnight for adherence. A stock solution of 500 nM of nucleolin aptamer, NucA-PTX and CRO-PTX were made up in medium and a serial two-fold dilution of the stock solution were performed to achieve a range of concentrations from 15.6 to 500 nM. The media of cells were removed and the solutions were added for 24 h and 48 h incubation. At the end of the incubation, 10 µL of CCK8 was added to each well and the fluorescence of the plate was read after 3 h incubation by a spectrometer at 450 nm.

**Cell cycle assay**

Cell cycle analysis was performed by following the instructions of the Propidium Iodide Flow Cytometry Kit (abcam). SKOV3 and OVCAR3 cells (5×10^5/well) were seeded in 6-well plates and incubated overnight. After washing, the cells were incubated with 200 nM of NucA-PTX or CRO-PTX at 37 °C for 48 h. The culture media was then removed, rinsed with PBS, trypsin was added to dissociate cells. The cells were collected and centrifugated at 1000 rpm for 5 min. Discarded the supernatant and washed cells with PBS. Then fixed the cells in 66 % ethanol on ice (gently resuspended the cells in 400 µL ice cold PBS, then slowly added 800 µL ice cold 100 % ethanol and mixed well). Stored at 4 °C overnight, then centrifugated at 1000 rpm for 5 min. Discarded the supernatant and washed cells with PBS. The cells were re-suspended in 200 µL 1X Propidium Iodide + RNase Staining Solution and incubated at 37 °C in the dark for 30 min. Finally, DNA content was measured by Flow Cytometry (Becton Dickinson), the percentage of cells in
each phase of the cell cycle was calculated using the ModFit software.

4.6.2 Results

To examine whether the NucA modification affects the anti-tumor activity of the conjugated PTX in ovarian cancer cells, the 24h cytotoxicity of NucA-PTX, CRO-PTX and NucA on two human ovarian cancer cell lines - SKOV3 and OVCAR3, and a normal liver cell line – L02, was examined by CCK8 assay. NucA-PTX exhibited a similar level of cytotoxicity as free PTX against two cancer cell lines (Figure 31). NucA exhibited no significant cytotoxicity in both cell lines. In contrast, NucA-PTX exhibited less cytotoxicity against L02 cells when compared to CRO-PTX (Figure 31).

**Figure 31 The effect of NucA modification on the cytotoxicity of the conjugated PTX in NucA-PTX in vitro.** The cell viabilities of SKOV3, OVCAR3 and L02 after 24h treatment of NucA, NucA-PTX or CRO-PTX evaluated by CCK8 assay. The concentration range of each compound was from 15.6 to 500 nM.

To confirm the effect of NucA-PTX on cell viability was led by PTX, cell cycle assay was performed to investigate whether the cancer cells were halted in G2/M phase after NucA-PTX treatment. The results showed that NucA modification increased the
percentage of SKOV3 and OVCAR cells in G2/M phase in the cell cycle to a greater extent after 48 h treatment with the conjugates (Figure 32).

Figure 32 The NucA modification affected the tumor cell population in the cell cycle caused by the conjugated PTX. The cell cycle distribution of SKOV3 and OVCAR3 cells after 48h treatment of NucA-PTX or CRO-PTX analyzed by flow cytometry.

The effects of NucA-PTX on the cell viability and cell cycle were consistent with its cellular uptake, which was related to nucleolin expression on cell surface of these cell lines (Figure 25).

4.7 Conclusion

By monitoring the FRET change of the dual fluorescence-labelled conjugate FAM-NucA-PTX-Rh, the dipeptide linker was shown to remain stable in human serum while release PTX both extracellularly in the presence of cathepsin B and intracellularly
inside the ovarian cancer cells. The intracellular localization of NucA-PTX-Rh in lysosomes, where cathepsin B was abundant and active at its optimal pH, implying the suitable environment for the cleavage of the linker in ovarian cancer cells.

The nucleolin aptamer for nucleolin-dependent cellular uptake and tumor cell targeting *In vitro* mechanism of the uptake and internalization of the conjugated PTX mediated by the NucA modification

The uptake of NucA-PTX-Rh was in a nucleolin-dependent manner, suggesting that the interaction between NucA and nucleolin played a crucial role in the entrance of the conjugated PTX into cancer cells. The co-localization assay with markers of three endocytosis pathways revealed that NucA-PTX-Rh was taken up by SKOV3 cells mainly by macropinocytosis, which is consistent with the findings previously reported\textsuperscript{139}. 
Chapter 5 Biological Evaluation of Nucleolin Aptamer-Paclitaxel Conjugate in vivo

5.1 Materials

Animal handing

All the animals were housed in Laboratory Animal House of the Institute for Advancing Translational Medicine in Bone & Joint Diseases with a temperature-controlled, 12 h light/dark cycle facility, and food and water were available ad libitum. The animals were acclimatized to the laboratory conditions for at least 7 days before being used in experiments. The animal study procedures were approved by the Animal Experimentation Ethics Committee of the Hong Kong Baptist University (Ref No. HASC/12-13/0032).

Mice modelling, drug administration and sampling

8 weeks old female BALB/c nude mice were inoculated subcutaneously with $2 \times 10^6$ SKOV3 cells in the left armpit. Tumors of around 10 mm diameter were observed two weeks after inoculation. For distribution assay, the tumor-bearing nude mice were randomly divided into two groups of 6 mice, and NucA-PTX-Rh or CRO-PTX-Rh at a single dose of 5 mg/kg were given to the mice of each group respectively by intravenous route (i.v.) via the tail vein. The mice were sacrificed 2 h after injection and the tumors and major organs (hearts, lungs, livers, spleens and kidneys) were collected for biophotonic imaging. For in vivo antitumor activity evaluation, the tumor-bearing nude mice were randomly divided into six groups of 6 mice. CRO, NucA, NucA-PTX, CRO-PTX or PTX at a dosage of 1.17 μmol/kg were given to mice of five groups by i.v. once a week for four weeks. Another group was given vehicle solution as the control.
group. At the end of the treatment, the mice were sacrificed, blood was collected for white blood cells count analysis and tumors were harvested for size and weight measurement.

5.2 The effect of NucA modification on the in vivo distribution of the conjugated PTX

5.2.1 Methods

Fluorescence imaging of the distribution of NucA-PTX-Rh and CRO-PTX-Rh in tissues at 2 h and 4 h after injection was performed using an IVIS® Lumina XR imaging system. Constant illumination settings, including exposure time (5 s), binning factor (4), f-stop (2) and field of view (15 cm for both width and length), were used during all image acquisition. Fluorescent and photographic images were acquired and overlaid. The pseudo-color image represented the spatial distribution of photon counts within the tissues. Background fluorescence recorded under a background filter (410-440 nm) was subtracted prior to the analysis.

Ex vivo localization of NucA-PTX-Rh in liver, kidney and tumor sections

Portions of the livers, kidneys and tumors were placed in 4% paraformaldehyde overnight after harvesting. Before embedding and sectioning, the tissue pieces were incubated in 30% sucrose for 6 h. The tissues were embedded in optimal cutting temperature medium (OCT), and cryosectioned. The sections were washed with PBS, permeabilized with 0.1 % Triton X-100 in PBS, stained with Alexa Fluor® 488 Phalloidin (Invitrogen, Eugene, OR) and mounted with ProLong Antifade Reagent with DAPI (Molecular Probes). Images were acquired with a confocal microscopy.
5.2.2 The results of NucA modification on the in vivo distribution of the conjugated PTX

The *in vivo* tumor-targeting property of the conjugated PTX-Rh within NucA-PTX-Rh after the NucA modification was evaluated by examining the tissue distribution of PTX-Rh in a xenografted mice model of human ovarian cancer. At 2 h and 4 h after intravenous injection of NucA-PTX-Rh or CRO-PTX-Rh, the Rh fluorescence intensities in ovarian tumor tissues collected from the NucA-PTX-Rh treated mice were shown to be significantly higher than those from the CRO-PTX-Rh treated mice, whereas the intensities in livers and kidneys from the NucA-PTX-Rh treated mice at 4h were remarkably lower when compared to those from the CRO-PTX-Rh treated mice (Figure 33). The fluorescence signal was almost undetectable in hearts, spleens and lungs in both groups (Figure 33).
Figure 33 The distribution of the conjugated PTX-Rh in tumor and major viscera (heart, liver, spleen, lung and kidney) at organ level 2h (upper) and 4h (bottom) after injection visualized by biophotonic imaging.

Furthermore, to confirm whether NucA could selectively deliver the conjugated PTX-Rh into tumor cells on which nucleolin was highly expressed, we examined the co-localization of PTX-Rh and the nucleolin$^+$ cells in cryosections from tissues of tumor and major organs (liver, lung, kidney and tumor) of the xenografted mice injected with NucA-PTX-Rh or CRO-PTX-Rh. We found numerous instances of nucleolin$^+$ cells and co-localization of PTX-Rh with nucleolin$^+$ cells in tumor tissue from the xenografted mice when NucA-PTX-Rh was administered, whereas there were few instances of such co-localization of PTX-Rh with nucleolin$^+$ cells in mice when CRO-PTX-Rh was administered. No obvious nucleolin$^+$ cells and co-localization of PTX-Rh with nucleolin$^+$
cells were detected in liver, lung and kidney form the mice in both NucA-PTX-Rh group and CRO-PTX-Rh group (Figure 34).

![Figure 34 The distribution of the conjugated PTX-Rh in tumor and major viscera (liver, lung and kidney) at tissue level examined.](image)

However, more instances of PTX-Rh were detectable in liver, lung and kidney in mice injected with CRO-PTX-Rh than those in mice injected with NucA-PTX-Rh (Figure 33), which was consistent with the above tissue distribution analysis (Figure 34).

5.3 The effect of NucA modification on the in vivo efficacy of the conjugated PTX

5.3.1 Methods

To determine tumor cell proliferation, the paraffin sections of the xenografted tumor were deparaffinized using xylene and rehydrated through graded series of alcohol concentrations. Endogenous peroxidase was quenched using 3% hydrogen peroxide for
10 min. Nonspecific binding of epitopes was blocked using 1:10 normal blocking serum. Slides were incubated at 4 °C overnight in a 1:100 dilution of mouse Ki-67 antibody (ab66155; Abcam). Sections were washed and incubated with biotinylated secondary antibody (sc-7207; Santa Cruz Biotechnology,) for 30 min followed by incubation with peroxidase substrate for 10 min. The sections were washed and incubated in deionized water for 5 min, counterstained with hematoxylin, and analyzed under a light microscope (Leica DMRB DAS; Leica, Heerbrugg, Switzerland).

5.3.2 Results

The *in vivo* anti-tumor effect of NucA-PTX was evaluated in a xenografted mice model of human ovarian cancer. Compared with PBS-treated group (control group) and CRO-treated group (aptamer control group), all the others treated groups exhibited significant decreases in tumor volumes, wherein the treatment with NucA-PTX showed a better inhibitory effect against tumor growth that PTX-treated group or nucleolin aptamer-treated group (Figure 35). Especially, the average tumor volume of the NucA-PTX treated group was significantly smaller than that treated with PTX ($P < 0.05$) and nucleolin aptamer ($P < 0.01$) on day 21 (Figure 35).
Figure 35 Macroscopic views of the xenografted tumor after 3 weeks different treatments from the groups indicated. Scale bar, 1 cm.

Then, Ki-67 antigen staining, a commonly used cell proliferation marker, and H&E staining were performed to further investigate the tumor suppression efficiency of NucA-PTX. For Ki-67 staining, we found numerous instances of Ki-67-positive cells in tumor tissue from PBS-treated and CRO-treated xenografted mice, indicating active cell proliferation. In contrast, the NucA-PTX treated group showed a lower Ki-67-positive cells than PTX and nucleolin aptamer treated groups (Figure 36), suggesting a higher tumor growth inhibition effect.
Figure 36 Analysis of tumor volume after 3 weeks different treatments indicated at a dosing frequency of once a week via intravenous injection. Data represent the mean ± SD (n=8). * P < 0.05.

For H&E staining, the nucleus was stained with hematoxylin in blue and the cytoplasm was stained by eosin in pink. The PBS-treated and CRO-treated group showed rhombic or polygonal cells with large spherical or spindle-shaped nuclei, which indicating the normal cells without apoptosis and necrosis (Figure 37).
Figure 37 H&E staining analysis of ovarian tumor sections from each group. Scale bars, 100 μm. Arrows indicate cell apoptosis and necrosis in the tumor sections. Scale bars, 100 μm. Note: PBS, the xenografted mice treated with PBS. PTX, the xenografted mice treated with paclitaxel. CRO, the xenografted mice treated with CRO aptamer. NucA, the xenografted mice treated with nucleolin aptamer. CRO-PTX, the xenografted mice treated with CRO-PTX. NucA-PTX, the xenografted mice treated with NucA-PTX.

5.4 The effect of NucA modification on the in vivo toxicity of the conjugated PTX

5.4.1 Methods

Histological examination

Isolated tissues and organs including the xenografted tumors, hearts, lungs, livers, kidneys and spleens were fixed in 10% neutral-buffered formalin followed by embedding in paraffin. 5-μm sections were made, mounted on glass slides, deparaffinized, and subjected to hematoxylin and eosin (H&E) staining using standard protocols. After mounting with coverslips, the specimens were viewed and analyzed under a light
microscope (Leica DMRB DAS; Leica, Heerbrugg, Switzerland).

**Neurophysiological assessment**

At the end of the experiment (4 days after the last administration) sensory/motor and sensory nerve conduction velocities (NCVs) were determined through stimulating respectively the caudal and the digital nerves respectively by using an electromyography apparatus (Myto2 ABN Neuro, Firenze, Italy). Caudal NCV was determined by placing a couple of recording needle electrodes at the base of the tail and a couple of stimulating needle electrodes 3.5 cm distally to the recording points. Similarly, the digital NCV was determined by placing the recording electrodes close to the ankle bone and the stimulating electrodes close to the fourth toe near the digital nerve. Both the caudal and digital NCVs were calculated by measuring the latency between the stimulus artefact and the onset of the first peak of the elicited action potential and the distance between the recording and the stimulating points. Ten responses were averaged for each recording. Prior to the initiation of dosing, the NCVs were evaluated for each animal and then mice were randomized into treatment groups in order to obtain homogeneous baseline caudal and digital NCV measures.

**5.4.2 Results**

Considering the toxicities of paclitaxel (e.g. hematological toxicity and neurotoxicity) reported in clinical application, we further evaluate the toxicity of NucA-PTX in the xenografted mouse model. Firstly, we measured the body weight of the treated mice every 3 days. PTX-treated group showed obvious weight loss (about 85%) throughout the experiment, indicating the potential of systematic toxicity. In contrast, no remarkably
body weight loss was observed in PBS-, CRO-, NucA- and NucA-PTX-treated groups, revealing a slightly low potential of systemic toxicity (Figure 38).

![Body weight changes after 4 weeks different treatments indicated in xenografted mice. Body weight was normalized to each body weight before treatment.](image)

Next, we investigated the myelosuppression of NucA-PTX through white blood cell (WBC) count test. No obvious change of WBC count was observed in xenografted mice after treatment with PBS and CRO, whereas PTX and CRO-PTX induced a significant decrease in the value of WBC count. The value of WBC count in the NucA-PTX-treated mice was also decreased compared with the value before administration but to a much lesser extent than that in PTX-treated group (Figure 39).
Figure 39 The white blood cell count determined by automated hematology analyzer before and after different treatments in xenografted mice.

Furthermore, we evaluated the potential neurotoxicity of NucA-PTX using an electromyography apparatus. PTX and CRO-PTX induced a marked and significant reduction in caudal and digital nerve conduction velocities (NCVs) when compared to the PBS- and CRO-treated groups, hinting the potential neurotoxicity of PTX. NucA-PTX also induced caudal and digital NCVs reduction compared to PBS control group, but the value of NCVs was higher than that in CRO-PTX-treated group (Figure 40).
Figure 40 Neurophysiological analysis of caudal NCV and digital NCV in the xenografted mice 4 days before the end of the different treatments indicated.

Finally, PTX-related toxicities to major organs were examined to evaluate the toxicity of NucA-PTX by pathological sections with H&E staining. Serious damage was observed in heart, liver, spleen and lung from the xenografted mice after PTX treatment. For heart injury, myofibrillar loss and atrophy of myocardial cells were examined in PTX-treated mice. For liver damage, hepatic cords loss, mild steatosis and dilatayion of blood sinus were observed. For lung injury, lung fibers were brokeed. For spleen, the atrophy of the white pulp was detected. However, the damage to these tissues was greatly lowered in NucA-PTX treated group when compared to those in PTX and CRO-PTX treated groups (Figure 41). There is no visible damage in kidneys from all of the treated groups (Figure 41).
Figure 41 Histological assessments of tissues using H&E staining in different treatment groups. Scale bars, 100μm. Arrows in “Heart images” indicate the myofibrillar loss and atrophy of myocardial cells. Arrows in “Liver images” indicate the hepatic cords loss, steatosis and dilatation of blood sinus. Arrows in “Spleen images” indicate the white pulp atrophy. Arrows in “Lung images” indicate the brokeed lung fibers. Scale bars, 100 μm. Note: PBS, the xenografted mice treated with PBS. PTX, the xenografted mice treated with paclitaxel. CRO, the xenografted mice treated with CRO aptamer. NucA, the xenografted mice treated with nucleolin aptamer. CRO-PTX, the xenografted mice treated with CRO-PTX. NucA-PTX, the xenografted mice treated with NucA-PTX. Data represent the mean ± SD, n=6. *P < 0.05.

5.5 Conclusion

In the human ovarian cancer xenografted mouse model, compared to the modification by
its CRO counterpart, the NucA modification facilitated the accumulation of the conjugated PTX in tumor tissue rather than major organs, subsequently resulting in improved anti-tumor efficacy-smaller tumor size and less active proliferation in tumor tissue. Emerging evidence showed that PTX induces severe leukopenia and organ damage\textsuperscript{141}. Whereas, NucA-PTX exhibited much lower myelosuppression and remarkably decreased organic damage, indicating that the NucA modification had a great potential of reducing systemic toxicities. The improved efficacy and reduced toxicity of NucA-PTX was most likely attributed to the stability in circulation and selective accumulation in tumor tissues with high nucleolin expression. There were previous studies utilizing NucA modified lipid/polymer nanoparticles for drug delivery\textsuperscript{142,143}, however concerns of those materials such as the inhomogeneity, instability and immunogenicity could not be ignored. By contrast, our synthesized NucA-PTX as one single molecule exhibited great potential in terms of stability and homogeneity with ignored immunogenicity.
Chapter 6 Conclusion

To connect the cytotoxic PTX with the tumor-targeting NucA, the active 2’-hydroxyl group on PTX was the optimal site for substitution. However, the introduction of other functional groups on this site resulted in the loss of antitumor activity for the obtained PTX derivatives\(^\text{130}\). Thus, a labile linker with a cathepsin B-sensitive dipeptide was utilized to facilitate the intracellular release of the conjugated PTX\(^\text{131,132}\). In addition, spacers (PABC on PTX side and succinic acid on NucA side) were incorporated to the linker for reducing the steric hindrance for the protease.

So we designed the synthetic route for conjugates and successfully prepared the desired conjugates, which were characterized by high performance liquid chromatograph, mass spectrum or nuclear magnetic resonance. During the process of synthesis, we also optimized the reaction conditions, including the reaction temperature, solvents, material input, time and catalysts. We got a stable and mature synthetic route and the total yield was more than 6%.

Every molecule of synthesized NucA-PTX contains a nucleolin aptamer, which has better water solubility and higher molecular weight comparing to PTX. In theory, the NucA-PTX is similar to nucleolin aptamer in the terms of water solubility. After testing, the results verified the speculation that the water solubility of PTX in NucA-PTX has been improved than one thousand.

From the computer simulation results, the binding site between nucleolin aptamer and targeted protein nucleolin was far from the tail of nucleolin aptamer, to which the PTX derivative was conjugated. Additional, another evidence proved that there was little influence on the binding affinities before and after the conjugation between nucleolin
aptamer and targeted protein nucleolin through isothermal titration calorimetry.

By monitoring the FRET change of the dual fluorescence-labelled conjugate FAM-NucA-PTX-Rh, the dipeptide linker was shown to remain stable in human serum while release PTX both extracellularly in the presence of cathepsin B and intracellularly inside the ovarian cancer cells. The intracellular localization of NucA-PTX-Rh in lysosomes, where cathepsin B was abundant and active at its optimal pH, implying the suitable environment for the cleavage of the linker in ovarian cancer cells.

In the human ovarian cancer xenografted mouse model, compared to the modification by its CRO counterpart, the NucA modification facilitated the accumulation of the conjugated PTX in tumor tissue rather than major organs, subsequently resulting in improved anti-tumor efficacy-smaller tumor size and less active proliferation in tumor tissue. Emerging evidence showed that PTX induces severe leukopenia and organ damage\textsuperscript{141}. Whereas, NucA-PTX exhibited much lower myelosuppression and remarkably decreased organic damage, indicating that the NucA modification had a great potential of reducing systemic toxicities. The improved efficacy and reduced toxicity of NucA-PTX was most likely attributed to the stability in circulation and selective accumulation in tumor tissues with high nucleolin expression. There were previous studies utilizing NucA modified lipid/polymer nanoparticles for drug delivery\textsuperscript{142,143}, however concerns of those materials such as the inhomogeneity, instability and immunogenicity could not be ignored. By contrast, our synthesized NucA-PTX as one single molecule exhibited great potential in terms of stability and homogeneity with ignored immunogenicity.
NucA-PTX exhibited comparable cytotoxicity in ovarian cancer cell lines with increased cell population in G2/M phase as PTX at the same molar concentration, suggesting the thorough release of the conjugated PTX from NucA-PTX for tubulin binding and inhibition of microtubule formation. In the meanwhile, the conjugated PTX did not significantly affect the nucleolin-binding affinity revealed in dynamic simulation modeling and ITC assay, which is consistent with the NucA facilitated uptake and tumor-targeting activity demonstrated above.

In summary, the highly water-soluble NucA-PTX with a protease-labile linker targeted ovarian tumor with high specificity, improved anti-tumor efficacy and reduced toxicity. As alternatives of ADCs, this work establishes an innovative technology platform for ApDC development. Moreover, by altering aptamers that target different cancer cells (e.g. Her2 positive breast cancer cells, triple negative breast cancer cells, non-small cell lung cancer cells, osteosarcoma cells etc.), the personalized paclitaxel derivatives could be developed for the patients with the according cancer types. Importantly, it provides an efficient approach to the next-generation of smart anti-tumor drug discovery toward precision medicine, i.e. personalized aptamer-drug conjugates. At present, we will do some more detailed and comprehensive research in NucA-PTX, including synthesis process, animal pharmacokinetics and pharmacology, in order to promote it enter into clinical trials.

Furthermore, the stable conjugation skills could be gradually applied in other active natural products, which possess following characters, such as high cytotoxicity, poor water solubility, easy modification site. After researching the structure-activity
relationship of natural products, we may ensure the biological activity groups or structures, choose the suitable linkers containing labile linkers and stable linkers, and derive the appropriate linking groups. Carrying different aptamers with targeting properties, conjugates could selectively bind to various targets, including proteins, small molecules, RNA, which could achieve the precision medicine. Through promoting the activities of medicine and reducing the dosage, this skill could increase the usage rate of natural products and improve the environment of precious plant and animal resources.
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Appendix

Figure S1 The mass spectrum of Fmoc-Val.

Figure S2 The H\textsuperscript{1}-NMR spectra of Fmoc-Val.

Figure S3 The mass spectrum of Fmoc-Val-Cit.
Figure S4 The $^1$H-NMR spectra of Fmoc-Val-Cit.

Figure S5 The mass spectrum of Fmoc-Val-Cit-PABOH.
Figure S6 The $^1$H-NMR spectra of Fmoc-Val-Cit-PABOH.

Figure S7 The $^{13}$C-NMR spectra of Fmoc-Val-Cit-PABOH.
Figure S8 The mass spectrum of Fmoc-Val-Cit-PABC-PNP.

Figure S9 The $^1$H-NMR spectra of Fmoc-Val-Cit-PABC-PNP.
Figure S10 The C$^{13}$-NMR spectra of Fmoc-Val-Cit-PABC-PNP.

Figure S11 The mass spectrum of Fmoc-Val-Cit-PABC-PTX.
Figure S12 The $^1$H-NMR spectra of Fmoc-Val-Cit-PABC-PTX.

Figure S13 The $^{13}$C-NMR spectra of Fmoc-Val-Cit-PABC-PTX.
Figure S14 The HH-COSY spectra of Fmoc-Val-Cit-PABC-PTX.

Figure S15 The mass spectrum of Val-Cit-PABC-PTX.
Figure S16 The $^1$H-NMR spectra of Val-Cit-PABC-PTX.

Figure S17 The mass spectrum of Suc-Val-Cit-PABC-PTX.
Figure S18 The $^1$H-NMR spectra of Suc-Val-Cit-PABC-PTX.

Figure S19 The $^{13}$C-NMR spectra of Suc-Val-Cit-PABC-PTX.
Figure S20 The HH-COSY spectra of Suc-Val-Cit-PABC-PTX.

Figure S21 The mass spectrum of Nucleolin-aptamer-PTX conjugate.
Figure S22 The HPLC spectra of Nucleolin-aptamer-PTX conjugate.
Figure S23 The $^1$H-NMR spectras of Nucleolin-aptamer-PTX conjugate (up) and Nucleolin (down).
Figure S24 The HH-COSY spectra of Nucleolin-aptamer-PTX conjugate.
Figure S25 The comparison of HH-COSY spectras of Nucleolin-aptamer-PTX conjugate, and Nucleolin.
Figure S26 The mass spectrum of CRO aptamer-PTX conjugate.

Figure S27 The HPLC spectra of CRO aptamer-PTX conjugate.
Figure S28 The mass spectrum of 2'-TBS-PTX.

Figure S29 The H$^1$-NMR spectra of 2'-TBS-PTX.
Figure S30 The C$^{13}$-NMR spectra of 2‘-TBS-PTX.

Figure S31 The mass spectrum of 2‘-TBS, 7Rh-PTX.
Figure S32 The H$^1$-NMR spectra of 2'-'TBS, 7Rh-PTX.

Figure S33 The C$^{13}$-NMR spectra of 2'-'TBS, 7Rh-PTX.
Figure S34 The mass spectrum of 7Rh-PTX.

Figure S35 The $^1$H-NMR spectra of 7Rh-PTX.
Figure S36 The C$^{13}$-NMR spectra of 7Rh-PTX.

Figure S37 The mass spectrum of Fmoc-Val-Cit-PTX-Rh.
Figure S38 The $^1$H-NMR spectra of Fmoc-Val-Cit-PTX-Rh.

Figure S39 The $^{13}$C-NMR spectra of Fmoc-Val-Cit-PTX-Rh.
Figure S40 The mass spectrum of Suc-Val-Cit-PTX-Rh.

Figure S41 The $^1$H-NMR spectra of Suc-Val-Cit-PTX-Rh.
Figure S42 The $^{13}$C-NMR spectra of Suc-Val-Cit-PTX-Rh.

Figure S43 The mass spectrum of Nuceolin aptamer-PTX-Rh conjugate.
Figure S44 The HPLC spectra of Nucolin aptamer-PTX-Rh conjugate.
Figure S45 The mass spectrum of CRO-PTX-Rh conjugate.

Figure S46 The HPLC spectra of CRO-PTX-Rh conjugate.
Figure S47 The mass spectrum of FAM-Nucleolin aptamer-PTX-Rh conjugate.

Figure S48 The HPLC spectra of FAM-Nucleolin aptamer-PTX-Rh conjugate.
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