Synthesis of New Branched 2-Nitroimidazole as a Hypoxia Sensitive Linker for Ligand-Targeted Drugs of Paclitaxel

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ABSTRACT: Because of the low selectivity and efficiency of normal antitumor agents, the strategy of ligand-targeted drugs was put forward. In this paper, we designed and synthesized a new bioreductive linker based on 2-nitroimidazole, which was used in three paclitaxel (PTX) prodrugs. The drug release mechanism via six-membered ring was demonstrated by chemical reduction and nitroreductase assay. Glucose and acetazolamide, which have been reported widely as ligands, were attached to compound 7 to afford Glu-PTX and AZO-PTX. The prodrugs were considerably stable in phosphate-buffered saline (pH 7.4) and plasma. What is more, PTX releasing could be triggered by nitroreductase rapidly. In in vitro cytotoxicity assay, the prodrugs exhibited moderate selectivity toward hypoxic tumor cells. We considered that the 2-nitroimidazole linker could accelerate the release of prodrugs under hypoxic condition. It was promising in the development of ligand-targeted drugs.

1. INTRODUCTION

PTX (paclitaxel) exhibits a high antitumor efficiency in clinical, especially for ovarian, breast, and lung cancer.1,2 However, PTX has many challenges in side effects, solubility, and drug resistance.3,4 In order to solve these problems and achieve precise treatment, the development of ligand-targeted drugs represents an attractive strategy.

Ligand-targeted drug links a targeting ligand and therapeutic agent through a spacer and cleavable linker. Among them, the cleavable linker acts as the most important role for drug release. An ideally designed cleavable linker could realize the drug stability during circulation in vivo and could be cleaved rapidly at the targeted site. Reductively cleavable linker, enzyme-cleavable linker, and acid-cleavable linker are often used in ligand-targeted drugs’ design.5,6

Hypoxia is one of the most notable features in solid tumors, as well as a compelling therapeutic target.7–9 The insufficient supply of blood from the disorganized vasculature results in the generation of low oxygen regions in the center of solid tumors. The presence of hypoxia in solid tumors brings about multiple contributions to chemo resistance and radio resistance and particularly induces the high expression of reductase.10,11 2-Nitroimidazole has been demonstrated to be selectively reduced via reductase under hypoxic condition. It has been widely used in the development of bioreductive prodrugs and imaging agents.12–15

In this paper, we synthesized a new branched linker on the basis of 2-nitroimidazole for the ligand-targeted drug system. PTX was used as a therapeutic agent. Usually, connecting functional fragment at 2’-OH of PTX via ester bond was a common strategy.16 However, some ester prodrugs were too stable in preclinical studies and ultimately could not sufficiently release the active drug. Therefore, we introduced pivalic acid at N - 1 position of 2-nitroimidazole and connected it to 2’-OH of PTX via ester bond. In our design, 2-nitroimidazole could be reduced to 2-aminoimidazole, then forming six-membered ring to release PTX. Furthermore, 2-nitroimidazole was branched at C-4 position to conjugate with the well-studied ligands, glucose and acetazolamide. In summary, we developed a new branched 2-nitroimidazole linker for ligand-targeted drugs of PTX, which could be stable in physiological conditions and rapidly release active drug under hypoxic condition.

2. RESULTS AND DISCUSSION

Compound 4 was synthesized facilely according to the following Scheme 1.

The chemical reduction of compound 4 by Pd/C in MeOH under H2 atmosphere was used to test whether the six-membered ring could be formed (Scheme 2). The reaction was confirmed to be totally converted to compound 4-b by H NMR (Supporting Information) and LC/MS (ESI: m/z: 8818).

Scheme 1. (a) LDA, Tetrahydrofuran (THF), −78 °C; (b) Cs2CO3, Dimethylformamide (DMF), 99%

Received: June 1, 2018
Accepted: July 24, 2018
Published: August 8, 2018

DOI: 10.1021/acsomega.8b01208
ACS Omega 2018, 3, 8813−8818
C₈H₁₃N₃O [M + H]⁺ calcd, 166.09; found, 165.99). This result indicated that the drug release via six-membered ring was reasonable.

PTX was connected to compound 5 to afford compound IMI-PTX (Scheme 3). We tested the stability of IMI-PTX in phosphate-buffered saline (PBS) (pH = 7.4), human plasma, and mouse plasma in vitro (Figure 1). After 24 h incubation in PBS, there was 65% IMI-PTX remained. The half time of IMI-PTX was 24 h in human plasma and 4 h in mouse plasma. The results were in accord with previous studies that the high level of esterase in mouse plasma could promote the ester bond hydrolysis. Overall, compound IMI-PTX was sufficiently stable for further evaluation.

Nitroreductase was used to test bioreductive efficiency. Compound IMI-PTX (10 μM) dissolved in pH 7.4 PBS and then incubated with NADPH (1 μM) and nitroreductase (50 μg/mL) at 37 °C; the drug release data were given by high-performance liquid chromatography (HPLC). As a result, 45% of IMI-PTX remained after 1 h incubation and 42% PTX was found to be released (Figure 2). It indicated that IMI-PTX could be metabolized by nitroreductase and release PTX rapidly via six-membered ring mechanism. The results encouraged us for further study of the 2-nitroimidazole linker.

On the basis of the above studies, we designed and synthesized the 2-nitroimidazole linker (compound 7), which was branched at the C-4 position of compound 5 (Supporting Information, SS). Compound 7 could be connected to PTX via ester bond and conjugated to ligands through click reaction.

Glucose and acetazolamide were well-studied ligands. Glucose was the basic energy molecule. The uptake of glucose would be increased in tumor cells especially in hypoxic tumor cells. Glucose was used as a targeting ligand in antitumor drugs such as glufosfamide. Acetazolamide was used as a CA IX inhibitor which was reported by Neri recently. For above reasons, glucose and acetazolamide were chosen as targeting ligands. At the same time, tetraglycol was connected between ligands and 2-nitroimidazole linker to improve the aqueous solubility. Finally, two PTX prodrugs, compound GLU-PTX and compound AZO-PTX, were synthesized (Scheme 4).

We evaluated the stability and the bioreductive efficiency of two compounds at the same conditions. In mouse plasma, the half time of AZO-PTX, GLU-PTX, and IMI-PTX was almost 4 h (Figure 3). The stability had no obvious change. In vitro nitroreductase assay, GLU-PTX released 95% PTX (Figure 4) and AZO-PTX released 63% PTX (Figure 5). The PTX releasing ratio was differently increased compared with IMI-PTX. We considered that the increase of aqueous solubility of two compounds may contribute to their bioreductive efficiency.

The compounds were tested for their in vitro inhibition of H460 and HT29 proliferation under hypoxic and normoxic conditions. H460 and HT29 cell lines have been proven that overexpress reductase, which could be conducive to increase the toxicity of the prodrugs under hypoxic conditions. The cells were incubated with the test compounds at various concentrations for 72 h under normoxic or hypoxic conditions. The in vitro cytotoxicity was assessed by MTT. The inhibition results are shown in Tables 1 and 2. Three compounds exhibited 1.1−2.2 selective ratios toward hypoxic tumor cells.

### 3. CONCLUSIONS

In conclusion, we synthesized a new bioreductive linker based on 2-nitroimidazole, which was used in three PTX prodrugs. The drug release mechanism via six-membered ring was demonstrated by chemical reduction and nitroreductase assay. The prodrugs were considerably stable in PBS (pH 7.4) and plasma. What is more, PTX releasing could be trigged by nitroreductase rapidly. In vitro cytotoxicity assay, the...
prodrugs exhibited moderate selectivity toward hypoxic tumor cells. We considered that the new 2-nitroimidazole linker could accelerate the release of prodrugs under hypoxic condition. The branched 2-nitroimidazole linker was promising in the development of ligand-targeted drugs.

Scheme 4. (a) (i) DMAP, EDCI, DCM, 51%; (ii) Cul, N,N-Diisopropylethylamine (DIPEA), n-BuOH, H2O, 40%

Figure 3. Stability of IMI-PTX, GLU-PTX, and AZO-PTX in mouse plasma.

Figure 4. In vitro nitroreductase assay of GLU-PTX.

Figure 5. In vitro nitroreductase assay of AZO-PTX.

Table 1. Inhibition of H460 Proliferation

| compound       | H460          |
|---------------|---------------|
|                | IC50 (μM)     | AIR/N2       |
| GLU-IMI-PTX    | 0.409 ± 0.031 | 0.183 ± 0.029 | 2.2 |
| AZO-IMI-PTX    | 0.163 ± 0.029 | 0.086 ± 0.009 | 1.9 |
| IMI-PTX        | 0.094 ± 0.012 | 0.043 ± 0.010 | 2.2 |
| PTX            | 0.007 ± 0.001 | 0.009 ± 0.002 | 0.8 |

*aData presented are the mean ± SD value of three independent determinations.

4. EXPERIMENTAL SECTION

4.1. Materials and Methods. 1H and 13C NMR spectra were given by a Bruker DRX-400 MHz spectrometer using TMS as an internal standard. The mass spectra (MS) were given by a Waters SDQ mass spectrometer, and high-resolution mass spectrometry (HRMS) was given by a Waters SYNAPT G2 ESI-TOF-MS analyzer. Melting points were taken on a SGW X-4 melting point apparatus. All of the
starting materials are commercially available and were used without further purification. PTX was purchased from Biocompounds Pharmaceutical Inc (Shanghai, China). The cell culture fluid and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific. Nitrroreductase from *Escherichia coli* and NADPH were purchased from Sigma-Aldrich. Mouse plasma was donated by an adult man. Human plasma was donated by a live subject.

### 4.2. Ethical Statement
We state that all experiments with live subjects were performed in compliance with the Laboratory Animal Management Regulations of the People’s Republic of China and the three R’s (replacement, reduction, and refinement) strategy of William Russell and Rex Burch, and the Animal Ethical and Welfare Committee of East China Normal University has approved the experiments.

### 4.3. Synthetic Procedure

#### 4.3.1. Ethyl 3-Iodo-2,2-dimethylpropanoate (3).
$n$-Butyllithium (44 mL, 105.6 mmol, 2.4 N in hexane) was added to the mixture of *N*,*N*-dissopropylamine (15.4 mL) in anhydrous THF (100 mL) at −30 °C dropwise, and the resultant solution was mixed for 16 h at 25 °C. Then, the reaction was cooled to −78 °C, and compound 1 (13.6 mL) was injected dropwise and maintained for another 1 h. Subsequently, diiodomethane (5.2 mL) was injected dropwise, and the resultant solution was mixed for 16 h at 25 °C. Then, the reaction was treated with aq NaHCl and then added EtOAc. The EtOAc phase was separated from aq. NaCl and water and treated with Na2SO4 (anhydrous). Concentrated in vacuum and afforded compound 2 as colorless oil (25 g, yield 82%).

#### 4.3.2. Ethyl 2,2-Dimethyl-3-(2-nitro-1H-imidazole-1-yl)propanoate (4).
Cs2CO3 (6.52 g) was added to the mixture of compound 3 (1.13 g) in DMF and kept for 15 min at 25 °C. Then, the reaction was treated with aq NH4Cl and then added DIPEA. The resultant solution was stirred for 1 h at 50 °C. The solution was concentrated in vacuum, and the residue was purified through a C-18 reverse column to give AZO-PTX as a white solid (30 mg, yield 71%, mp 194 °C).

#### 4.3.3. 2,2-Dimethyl-3-(2-nitro-1H-imidazol-1-yl)propanoic Acid (5).
The solution of compound 4 (2.4 g) in 1 M NaOH (20 mL) was stirred at 25 °C for 16 h. HCl (1 M) was added to adjust pH to 2 and then extracted with DCM. The DCM layer was treated with Na2SO4 (anhydrous) and filtered, and the filtration was concentrated in vacuum to give compound 5 as a yellow solid (1.78 g, yield 84%).

#### 4.3.4. IMI-PTX
Compound 5 (13 mg) and PTX (43 mg) were dissolved in 2 mL of DCM. DMAP (7 mg) and EDCI (12 mg) were added to the solution and then kept at 25 °C for 16 h. The solvent was concentrated under reduced pressure to provide crude product, and then the residue was purified through a silica gel column (DCM/MeOH = 3:1) to give IMI-PTX as a yellow solid (30 mg, yield 47%, mp 194–196 °C).

#### 4.3.5. AZO-PTX
Compound 6 (35 mg, Supporting Information), compound 27 (40 mg, Supporting Information), and Cul (14 mg) were added to a mixture of tert-butanol (3 mL) and water (1 mL). One drop of DIPEA was added to the mixture, subsequently, and the resultant mixture was stirred for 1 h at 50 °C. The solution was concentrated in vacuum, and the residue was purified through a C-18 reverse column to give AZO-PTX as a white solid (30 mg, yield 71%, mp 168 °C).

#### 4.3.6. GLU-PTX
Compound 7 (30 mg) and PTX (43 mg) were dissolved in 2 mL of DCM. DMAP (7 mg) and EDCI (12 mg) were added to the solution and then kept at 25 °C for 16 h. The solvent was concentrated under reduced pressure to provide crude product, and then the residue was purified through a silica gel column (DCM/MeOH = 3:1) to give GLU-PTX as a yellow solid (30 mg, yield 47%, mp 194–196 °C).

### 4.4. Determination of IC50

#### Table 2. Inhibition of HT29 Proliferation

| compound | HT29 IC50 (μM) | AIR | N2 | AIR/N2 |
|----------|----------------|-----|----|--------|
| GLU-PTX  | 0.076 ± 0.008  | 0.060 ± 0.005 | 1.3 |        |
| Azo-PTX | 0.040 ± 0.013  | 0.032 ± 0.009 | 1.3 |        |
| PTX      | 0.018 ± 0.003  | 0.017 ± 0.004 | 1.1 |        |
| 1H NMR   | 400 MHz, CDCl3 | δ 7.21 (s, 1H), 7.16 (s, 1H), 4.76 (s, 2H), 1.27 (s, 6H). MS (ESI): calcd for C8H12N3O4 [M + H]+, 214.1; found, 214.1. |
Biochemistry and Cell Biology (Shanghai, China). The cells in cell line HT29 were purchased from the Shanghai Institute of Human lung cancer cell line H460 and human colon cancer streptomycin, were used for H460 and HT29 cell culture. 

4.4. General HPLC Method. General HPLC method was used to test the purity of compounds and also used in stability studies. A mobile phase with the flow rate of 1.0 mL/min was gradient from 5% CH₃CN/H₂O to 95% CH₃CN/H₂O for 15 min, followed by 95% CH₃CN/H₂O for 5 min. The signal was detected at 254 nm. 

4.5. General Method for Stability Assay. The buffers were prepared as the following general method: PBS (pH 7.4, 10 mM) was composed of NaCl (137 mM), KCl (2.7 mM), Na₂HPO₄ (4.3 mM), and KH₂PO₄ (1.4 mM). The test compounds dissolved in different buffers and plasma (10 μM, 1% dimethyl sulfoxide) were incubated at 37 °C at different times, 100 μL of solution was transferred to a vial for analysis using HPLC.

4.6. General Method for Nitroreductase Assay. Test compound (10 μM), nitroreductase (50 μg/mL), and 1 mM NADPH was contained in the mixture and incubated at 37 °C for 12 h. The test compounds diluted in medium with diwater and quenched by 100 μL of cold methanol and then transferred to a vial for analysis using HPLC.

4.7. Cell Culture. RPMI 1640 medium and McCoy’s 5A medium, supplemented with 10% FBS and 1% penicillin/streptomycin, were used for H460 and HT29 cell culture. Human lung cancer cell line H460 and human colon cancer cell line HT29 were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in a standard cell-culture incubator (37 °C, 5% CO₂ and 95% humidity). Cells were seeded in 96-well plates for 12 h. The test compounds diluted in medium with different concentrations were added. Cells were incubated in normoxic (21% O₂, 5% CO₂, 37 °C) and hypoxic (94% N₂/5% CO₂/1% O₂) conditions for 72 h. Cell viability and proliferation were assessed by MTT (n = 3).

■ ASSOCIATED CONTENT

2 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01208.

Experimental procedures, characterization data, and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful for Shanghai Science and Technology Council (16DZ2280100).

■ ABBREVIATIONS

PTX, paclitaxel
PBS, phosphate-buffered saline
DMAP, 4-dimethylaminopyridine
DCM, dichloromethane
DMSO, dimethyl sulfoxide
DPEA, N,N-disopropylethylamine

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