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Title: Enhanced Killing of Cancer Cells with Nanopreparations Containing Cell-penetrating Moiety and MMP2-sensitive PEG-Paclitaxel Conjugate

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Enhanced Killing of Cancer Cells with Nanopreparations Containing Cell-penetrating Moiety and MMP2-sensitive PEG-Paclitaxel Conjugate

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ABSTRACT

With many attempts made to improve the therapeutic outcomes of the water-insoluble chemotherapeutics, there is still a need for the “smart” and robust nanocarriers for the enhanced tumor targeting. To increase the solubility, bioavailability and tumor targetability of the water-insoluble drug, paclitaxel (PTX), we designed a novel amphiphilic polyethylene glycol (PEG) 2000-paclitaxel conjugate (PEG2000-peptide-PTX), which self-assembled into a “core-shell” structure in an aqueous environment and responded to up-regulated extracellular matrix metalloprotease 2 (MMP2) resulting in tumor cell-specific drug release. Using this functional conjugate, an MMP2-sensitive nanopreparation composed of PEG2000-peptide-PTX, TATp-PEG1000-PE (cell-penetrating enhancer) and PEG1000-PE (building block), was constructed. Compared to conventional polymeric micelles, this nanopreparation ensured: (i) a high drug loading efficiency, (ii) a low risk of premature drug release/leakage, (iii) an **enhanced** tumor targeting, and (iv) **an enhanced tumor cell-selective drug internalization**. The functional paclitaxel conjugate and the suggested nanopreparation allowed superior cellular uptake and cytotoxicity over its non-sensitive counterpart. Intravenous injection of the nanopreparation in a non-small cell lung cancer xenograft mouse model resulted in tumor accumulation and significant tumor growth inhibition compared to free paclitaxel or conventional micelles. Moreover, decreased side toxicity was observed compared to free paclitaxel. Altogether, this novel nanopreparation is a promising drug delivery platform for cancer therapy.

Key words: matrix metalloprotease, nanomedicine, paclitaxel, stimulus-sensitive, TAT peptide.

\body

Drug-loaded nanocarriers such as liposomes, micelles, polymeric and inorganic nanoparticles, and drug conjugates have demonstrated various advantages over free therapeutic molecules. These nanopreparations can be further engineered with functional moieties to improve their performance in terms of circulation longevity, targetability, cellular penetration, and stimulus-sensitivity. The idea of a stimulus-sensitive drug delivery system is based on the abnormalities in the tumor microenvironment, such as acidic pH, altered redox potential and up-regulated proteins. These internal conditions as well as external stimuli such as hyperthermia, magnetic field, and ultrasound, can be used to change the behavior of nanocarriers, resulting in an enhanced tumor targeting and antitumor effects (1).

Matrix metalloproteases (MMPs), especially MMP2, are known to be involved and over-expressed in many stages of human tumors including breast cancer, colorectal cancer, lung cancer, liver cancer, prostate cancer, pancreatic cancer, and ovarian cancer (2, 3). Various MMP-sensitive substrates have been designed and showed stimulus-responsiveness when used in **drug delivery and imaging/diagnosis ? systems** (2-5). In our previous study, a synthetic octapeptide was used as the MMP2-sensitive linker in a PEGylated liposomal nanocarrier that could trigger PEG deshielding and the resultant enhanced cell internalization (6).

Although many targeted delivery strategies have shown the enhanced drug disposition in the tumor, low cellular bioavailability of chemotherapeutics due to insufficient cellular internalization could represent another barrier. To enhance the cellular uptake, intracellular delivery moieties [cell-penetrating proteins/peptides (CPPs) such as TATp] have been used to modify the nanocarriers/drugs since they are efficient in aiding translocation across the plasma membrane (7)+**article Rupa**.

Paclitaxel (PTX) is one of the most commonly used antineoplastic agents for the treatment of solid tumors (8). It inhibits tumor cells' proliferation by stabilization of microtubules and tubulin polymerization and arrests cells at the G2/M phase of the cell cycle, resulting in cell apoptosis (9). However, its clinical applications are complicated by its low water-solubility, off-target toxicity and acquired drug resistance. Among many attempts to deal with these issues, **the** "core-shell" polymeric micelles have shown successes in the solubilization and delivery of PTX (10). However, the low drug loading efficiency, risk of premature drug release, and insufficient targetability are major obstacles to clinical application.

In response to these challenges, we synthesized an amphiphilic drug-polymer conjugate/prodrug, PEG2000-peptide-PTX, which **contains** an MMP2-cleavable peptide between PEG and PTX. The synthesized drug-polymer conjugate self-assembles into micelles and forms mixed micelles with two amphiphilic polymers, PEG1000-PE (building block) and TATp-PEG1000-PE (cell-penetrating enhancer), in an aqueous environment, in which PTX is located in the hydrophobic "core" covered by a hydrophilic PEG "shell". To increase cell internalization, TATp conjugated to PEG1000-PE was used as a cell-penetrating enhancer in the nanopreparation as schematized in **Fig. 1A**. Upon administration, the assembled micellar nanopreparation accumulates in the tumor via the enhanced permeability and retention (EPR) effect (11) **use conditional?**. In **the** tumor's microenvironment, the peptide linker is cleaved by the up-regulated extracellular MMP2, allowing for the exposure of TATp moiety and subsequent cell internalization of the nanopreparation. Compared to conventional micelles, the proposed system ensures: (i) a high drug loading efficiency, (ii) a low risk of premature drug release/drug leakage, (iii) an enhanced tumor targeting, and (iv) **an enhanced tumor cell-selective drug internalization**.

Herein, we described the preparation and characterization of the PTX prodrug and the suggested MMP2-sensitive nanopreparation. The cell internalization, tumor tissue penetration and cytotoxicity of the nanopreparation were tested in **the** monolayer cancer cells **cultures** and **the** 3D cancer cell spheroids. Furthermore, after systemic administration, the tumor targeting, antitumor efficacy and side toxicity of the nanopreparation were **examined confirmed** in a non-small cell lung cancer (NSCLC) xenograft **mouse** model.

Results and Discussion

Amphiphilic molecules, such as phospholipids, have the potential to (self-) assemble into “core-shell” structures or other structures, such as bilayers, in an aqueous environment. This property has been used to design many different drug delivery systems including micelles, liposomes, emulsions, and other nanoparticles (12, 13). Since many drugs with high hydrophobicity result in poor solubility and bioavailability (10), the conjugation of a hydrophilic moiety (e.g. PEG) to a hydrophobic drug molecule (e.g. paclitaxel) should improve the drug’s solubility as well as impart amphiphilicity to the formed conjugates, so that the resultant amphiphilic molecules assemble into a “core-shell” structure. Although PEGylation has many advantages including stabilization of nanoparticles, protection of drug molecules, and prolongation of drug’s/carrier’s circulation time, various studies have shown that the stable PEG shell of nanocarriers are not always beneficial for drug delivery (6, 14). Ideally, the protective PEG shell should be removed before **specific** cellular internalization and the subsequent intracellular events. In our previous study, we showed that the octapeptide (GPLGIAGQ) demonstrated MMP2-sensitivity after conjugation with PEG and PE or incorporation into liposomal nanocarriers (6). Therefore, the same peptide sequence was used to link PEG2000 and PTX to impart the MMP2-sensitivity to the conjugate. We hypothesized that the MMP2-sensitive and amphiphilic PEG2000-peptide-PTX would serve not only as a tumor environment-sensitive water-soluble PTX prodrug, but also as an MMP2-responsive building block for **the design of a** PTX-containing tumor-targeted nanopreparation (**Fig. 1A**). With this in mind, we prepared a novel MMP2-sensitive nanopreparation composed of PEG2000-peptide-PTX, TATp-PEG1000-PE and PEG1000-PE and tested its *in vitro* and *in vivo* tumor targeting and antitumor efficacy.

Synthesis and Characterization of PEG2000-peptide-PTX and TATp-PEG1000-PE. To prepare the suggested MMP2-sensitive nanopreparation, two functional conjugates were synthesized. PEG2000-peptide-PTX represents an MMP2-sensitive amphiphilic PTX prodrug. This PTX conjugate was expected to have three functions by itself including solubilization of PTX, self-assembly into nanoparticles, and MMP2-sensitivity. TATp-PEG1000-PE was used as the moiety for intracellular delivery of PTX to tumor cells.

In our previous work, we successfully conjugated PEG3400 with the MMP2 cleavable peptide. The same method was used to link PEG2000 with the peptide. To link PEG2000-peptide with PTX, the coupling reagents (DCC/DMAP) were used to activate the carboxyl group of the peptide and accelerate the reaction (**Scheme 1A, SI**). In thin layer chromatography (TLC) (**Fig. S1A**), a new spot was visualized by both UV and Dragendorff’s reagent staining **to show together with** a significantly lowered retardation factor (R_f) value than that of PTX due to the increased hydrophilicity.

Fig. 1B shows the ^1H NMR spectra of PEG2000-peptide-PTX in both CDCl_3 (blue curve) and D_2O (red curve). The characteristic peaks of the PTX conjugate were clearly displayed when CDCl_3 was used as solvent. The PTX block is characterized with aromatic protons (7.35 to 7.55 ppm), N-H protons (7.0 ppm), the acetyl protons (2.17ppm and 2.35ppm), and methyl protons (1.6-1.7 ppm and 1.25 ppm) (15). The $-\text{CH}_2\text{CH}_2\text{O}-$ protons in the PEG block **is are** characterized by the peak at 3.65 ppm. The peaks of CH_3- , CH_2- and $\text{CH}-$ protons in the octapeptide can be found at 1.55 to 1.75, 1.25 and 0.8 ppm. However, most of **PTX** peaks **of PTX** disappeared when D_2O was used as solvent. The disappearance of PTX peaks in water could be due to the formation of “core-shell” structure in which the hydrophobic PTX is entrapped in its “core” and isolated by the hydrophilic PEG “shell”, whereas the conjugate would be fully dissolved in chloroform. The integration of the characteristic peaks showed that the molar ratio between PEG2000 ($-\text{CH}_2\text{CH}_2\text{O}-$ protons, ~ 3.65 ppm) and PTX (aromatic protons, 7.35 to 7.55 ppm) was about 1:1.

Although there are two hydroxyl groups available per PTX molecule, it has been reported that the 2’ hydroxyl group is more reactive than the sterically hindered 7’ hydroxyl (16). Moreover, with the 2-fold molar excess of PTX, PEG is more likely to be linked only to the 2’ position according to the 1:1 molar ratio, confirmed by ^1H -NMR. After reaction, the content of PTX per conjugate was about 24% (w/w) based on molecular weights.

For the synthesis of TATp-PEG1000-PE (**Scheme 1B, SI**), NHS-PEG1000-MAL was first linked to DOPE (PE) via an ester bond. Then, PE-PEG1000-MAL was conjugated with the cysteine of Cys-TATp. After conjugation, because of the increased hydrophilicity, TATp-PEG1000-PE remained near the starting point on the TLC plates and could be visualized by three staining methods (Dragendorff's reagent staining for PEG, Ninhydrin Spray reagent staining for peptides, and Molybdenum Blue Spray reagent staining for phospholipids) (**Fig. S1B**).

Characterization of the Micelle Formation. To understand the self-assembly process and physicochemical properties of PEG2000-peptide-PTX, the particle size, morphology, and critical micelle concentration (CMC) were **measured analyzed, you don't measure a morphology** (**Fig. 1C**). The CMC of PEG2000-peptide-PTX was about 3.2×10^{-5} M, which is in the range of the CMC of the micelles formed by PEG2000-PE (17), indicating the formation of a "core-shell" structure and in agreement with the $^1\text{H-NMR}$ data. The transmission electron microscopy (TEM) data showed that PEG2000-peptide-PTX formed non-spherical particles with a size of $61.3 \pm 15 \text{ nm}$. This suggests that the hydrophobic interaction/force among PTX molecules is not strong enough to "hold" them together tightly, resulting in a large and loose "core-shell" structure. In contrast, PEG1000-PE has a strong hydrophobic lipid moiety, which can "hold" extra molecules and facilitates the micelle formation (17), as confirmed by its spherical shape, small size ($11.9 \pm 2.0 \text{ nm}$) and low CMC (1×10^{-6} M). Mixing PEG1000-PE with PEG2000-peptide-PTX facilitated the micelle formation as evidenced by a decreased CMC (3.9×10^{-6} M), near-spherical and "compact" shape, and small size ($22.5 \pm 2.7 \text{ nm}$). **Lin, could you try to measure respective LogP to explain?** The measured CMC of PEG2000-peptide-PTX/PEG1000-PE micelles was similar to the calculated CMC (1.9×10^{-6} M) using the equation: $1/\text{CMC} = X_1/\text{CMC}_1 + X_2/\text{CMC}_2$ (18), suggesting the formation of a mixed micelle. Here, the formed mixed micelles had a high drug loading (15% w/w) and stable structure, which is superior to the conventional PTX polymeric micelles with low drug loading [in most cases, less than 5% w/w (17)] and higher risk of premature drug release/leakage.

Cleavage of PEG2000-peptide-PTX by MMP2. The cleavability of PEG2000-peptide-PTX was determined by enzymatic digestion followed by TLC analysis as in our previous study (6). After incubation with $5 \text{ ng}/\mu\text{L}$ of active MMP2, the spot of the PTX conjugate completely disappeared while two new spots were seen in the TLC plate (**Fig. 1D**). This indicated that the peptide linker was cleaved by MMP2 resulting in two **digested** fragments (IAGQ-PTX and PEG-GPLG) since this peptide sequence could be specifically cleaved by MMP2 at the site between glycine and isoleucine, which is consistent with our previous data (6). Furthermore, incubation of PEG2000-peptide-PTX/PEG1000-PE mixed micelles with MMP2 showed similar results to PEG2000-peptide-PTX alone (**Fig. 1D**), indicating sufficient accessibility of MMP2 to the peptide even in this "compact" micellar structure. In contrast, incubation of the PTX conjugate with the mouse plasma was not able to cleave the linker and release PTX (**Fig. S1C**). The MMP2-induced cleavage was also supported by the *in vitro* and *in vivo* data.

MMP2-triggered Tumor Cell-specific Cytotoxicity of PEG2000-peptide-PTX. PEG2000-peptide-PTX and its uncleavable counterpart were tested in A549 tumor cells and H9C2 normal cardiomyocytes (**Fig. 2A**). The decreased cytotoxicity of PTX was observed in both cell lines after PEGylation. In tumor cells, PEG2000-peptide-PTX and free PTX showed comparable strong toxicity at high concentrations (29.5 and 2.95 mg/mL) as evidenced by around 20% cell viability, while PEG2000-peptide-PTX was much safer in normal cells with about 70% cell viability compared to less than 40% cell viability of PTX at the same doses. Higher cytotoxicity of PTX in A549 cells than H9C2 cells is understandable since tumor cells have higher proliferation rates than normal cells, resulting in the different response to the same treatment (19). **I think higher sensitivity was better**

The cytotoxicity of both PTX and its conjugate was dose-dependent. This indicates that the released PTX moiety from its prodrug is still cytotoxic after MMP2-induced cleavage, which is in agreement with the previous reports that esterification at either C-2' or C-7 did not significantly influence PTX's cytotoxicity (20). However, the cytotoxicity of PEG2000-peptide-PTX was lower than that of free PTX,

since PEGylation inhibits the cellular uptake of the conjugate (**Fig. 2C**), and only released PTX can be efficiently taken up by cells. The drug release process (cleavage) delayed PTX's action and lowered its activity. This can be compensated by a higher dose (**Fig. 2A**), appropriate nanocarriers (**Fig. 2D**), or a longer treatment time (**Fig. 2F**). In contrast, the uncleavable PTX conjugate did not show significant cytotoxicity with all doses in both cell lines (**Fig. 2A**).

These data are consistent with the corresponding MMP2 levels in the cell media (**Fig. S2**). In SDS-PAGE gel, both cells secreted proteins with a molecular weight close to 66.5 KDa, which is just close to that of the active MMP2 (EMD Biosciences). Their gelatinase activity was observed with the as bright bands on a zymogram gel. However, compared with each other, the MMP2 level in A549 cells was much higher than that in H9C2 cells. Higher MMP2 in cancer cell media could efficiently cleave the peptide linker allowing for the PTX release from its nontoxic prodrug and the resultant cytotoxicity. The cytotoxicity of PEG2000-peptide-PTX at the high doses in normal cells is probably due to the basal MMP2 which is required to maintain their normal physiological activity (21). We also cannot rule out an induction/activation of MMP2 in normal cells by the toxic chemotherapeutics like PTX (22) and doxorubicin (23). However, compared to pathological MMP2 in tumors, its influence on the PTX conjugate is limited, which is supported by the MMP2 level (**Fig. S2**) and cytotoxicity data (**Fig. 2A**). These data suggested that PEG2000-peptide-PTX has the imparts MMP2-triggered tumor cell-specific cytotoxicity.

The ability of PEG2000-peptide-PTX to induce cell apoptosis was analyzed by fluorescence-activated cell sorting (FACS) using Annexin V / Propidium Iodide (PI) double staining (**Fig. 4D**). The percentage of viable cells of PTX (69.7%) and its conjugate (75.4%) treated groups were much lower compared to untreated cells (above 90%). The similar percentage of early apoptotic cells (Annexin positive only) were detected in PTX (2.8%) and PEG2000-peptide-PTX (2.0%) groups. As expected, the percentage of late apoptotic cells (both Annexin and PI positive) in PEG2000-peptide-PTX was lower than that of PTX (4.7% versus 10.4%), but it is still much higher than 2.3% of untreated cells. Both treatments significantly increased the number of the necrotic/dead cells (PI positive only) with 17.1% for PTX and 17.9% for its conjugate compared to only 4.6% in untreated cells. The data suggested that both PTX and its conjugate could induce significant tumor cell apoptosis and death, while compared to PTX, its conjugate had lower apoptosis-inducing ability because of the delayed drug release upon MMP2-mediated cleavage.

Cellular Uptake and Cytotoxicity of the MMP2-sensitive PTX-containing Nanopreparation. The MMP2-sensitive nanopreparation composed of PEG2000-peptide-PTX, PEG1000-PE and TATp-PEG1000-PE (**Fig. 1A**) was prepared. Upon micelle formation, the drug loading decreased from 24% w/w of PEG2000-peptide-PTX to about 15% of the total nanopreparation, which is still much higher than most reported PTX micellar formulations (17).

The cellular uptake of NBD-PE labeled nanopreparations by A549 cells in monolayer was evaluated by FACS and confocal microscopy (**Fig. 2C**). PEG2000-peptide-PTX with/without MMP2 treatment and its nanopreparation without MMP2 treatment had no difference in the cellular uptake compared to PEG1000-PE micelles. PEGylation inhibits the interaction between the nanoparticles and cell membrane, resulting in the low cell internalization. In contrast, pre-treatment of the MMP2-sensitive nanopreparation (TATp-PEG1000-PE/PEG2000-peptide-PTX) with MMP2 removed PEG2000 and exposed the TATp moiety, resulting in an increased in cellular uptake similar to that obtained with of TATp-PEG1000-PE micelles. As a result, the MMP2-sensitive nanopreparation treatment caused higher cytotoxicity (44% cell viability) compared to its non-sensitive counterpart (83%), PTX conjugate (91%) and uncleavable conjugate (97%) at the dose of 29.5ng/mL in A549 cells monolayers (**Fig. 2D**). Therefore, the combined use of PEGylation, MMP2-sensitivity, and TATp improved the anticancer drug's targetability and therapeutic effect.

However, the two-dimensional cell culture cannot fully represent *in vivo* tumors since they are different in terms of cellular heterogeneity, nutrient and oxygen gradients, cell-cell interactions, matrix

deposition and gene expression profiles, resulting in different drug responses and poor *in vitro-in vivo* correlation(24, 25). Recently, the idea of 'multicellular resistance' (MCR) with a set of more complicated mechanisms was spotlighted (25). In this theory, the resistance to drug penetration caused by tumor's heterogeneity **should be is** considered one of the critical parameters, **and thus cannot be** reproduced in monolayer cell cultures (25). However, it can be well-reproduced in 3D cultures of cancer cells (spheroids) (24).

In this study, the **3D**-A549 multicellular spheroids were established to better mimic the real tumor conditions *in vitro*. **Fig. 2E** shows the penetration and cell internalization of the rhodamine-PE-labeled nanopreparations in A549 spheroids. The TATp modification significantly increased the penetration of the nanopreparations **in on** spheroids (**b, c, and d**) compared to the nanopreparations without TATp. **As expected**, The presence of the long-chain PEG in the nanopreparation lowered their cell association, while MMP2 pre-treatment increased spheroid fluorescence, i.e. penetration (**c, e and g vs. d, f and h**). Stronger red fluorescence around cell nuclei was observed upon pre-treatment of TATp-PEG1000-PE/PEG2000-peptide-PTX with MMP2 (**j vs. i**) **at higher magnification** in “zoom-in” images, indicating that the MMP2 deshielded PEG2000 and triggered the TATp-mediated cell internalization.

After 3 treatments of PTX formulations at 29.5ng/mL in 6 days, all PTX formulations except TATp-PEG1000-PE/PEG2000-peptide-PTX showed similar cytotoxicity with about 2-fold increase of the lactate dehydrogenase (LDH) release compared to untreated spheroids, while the empty nanocarriers (TATp-PEG1000-PE) showed no cytotoxicity. **The observed cytotoxicity of the non-sensitive nanopreparations was probably a result of the non-specific cell internalization and accumulative effect of the treatments, which would not be reproducible reproduced in the *in vivo* dynamic conditions.** It was also notable that free PTX didn't cause the highest cytotoxicity probably due to its limited penetration in **3D** spheroids (26). In contrast, the TATp-modified MMP2-sensitive nanopreparation showed much higher cytotoxicity with a more than 4-fold LDH release.

***In Vivo* Tumor Targeting and Antitumor Efficacy.** The tumor targetability and antitumor efficacy of the MMP2-sensitive nanopreparation were evaluated in a NSCLC xenograft mouse model. The biodistribution of rhodamine-labeled nanopreparations were analyzed by flow cytometry after cell dissociation at 2h after systemic administration (**Fig. 3A**). No significant fluorescence in heart, spleen, lung and kidney cells were observed after injection of fluorescent nanopreparations, indicating negligible accumulation of the nanopreparations in these organs. This is probably the result of the PEG's stealth effect and the nano size of the particles. Although a slight liver accumulation was observed, in agreement with the previous report (5), the up-regulated MMP2 in the tumor's microenvironment led to significant tumor homing and tumor cell internalization of the MMP2-sensitive nanopreparation. The strong red fluorescence around blue cell nuclei was observed with the injection of the MMP2-sensitive nanopreparation in confocal micrographs of the tumor sections, indicating that the efficient cell internalization occurred after tumor accumulation (**Fig. 3B**). These data suggested that small particle size and hydrophilic PEG “corona” could minimize the distribution and cell internalization of nanoparticles in non-target tissues but maximize their tumor accumulation via EPR effect. The tumor cell-selective internalization of the nanopreparations was further enhanced by incorporation of MMP2-sensitivity and cell-penetrating enhancer into the nanoparticles.

To test the therapeutic activity of the MMP2-sensitive nanopreparation, tumor-bearing mice were injected with PTX formulations twice a week for 4 weeks at the dose of 5mg/Kg PTX. The tumor growth of the MMP2-sensitive nanopreparation group (**e**) was significantly inhibited compared to HBS (**a**), the non-sensitive nanopreparation (**b**), PEG2000-PE/PTX micelles (**c**), and free PTX (**d**) (**Fig. 3C**). It was also notable that the best inhibition against tumor growth by the MMP2-responsive treatment was well correlated with the highest apoptosis induction in tumors (green fluorescent dots in the TUNEL assay) (**Fig. 3D**). However, no significant changes were observed after treatment of mice with the MMP2-sensitive nanopreparation in terms of mouse body weights, activities of alanine transaminase (ALT) and aspartate transaminase (AST), and white blood cell counts (**Fig. 4 A-C**). The free PTX group

showed significantly low white blood cell counts (around 30% of HBS group) after treatments since its poor *in vivo* pharmacokinetic profile, a decrease consistent with (ref). The hematoxylin and eosin (H&E) staining also showed that there were no histological signs of toxicity in major organs between the MMP2-sensitive nanopreparation and control (HBS) groups. However, the necrotic area was significantly shown in the MMP2-sensitive nanopreparation-treated tumor compared to HBS group (Fig. 4D), which confirmed the high antitumor activity observed in the *in vivo* efficacy studies (Fig. 3C and D). The high tumor targeting led to effective *in vivo* antitumor activity and low side toxicity of the MMP2-sensitive nanopreparation, which is correlated with the results from the *in vitro* penetration, cellular uptake and cytotoxicity studies. The high therapeutic index of the MMP2-sensitive nanopreparation is resulted from the longevity of PEGylation (27), the MMP2-sensitivity (5), the efficient TATp-mediated cell internalization (7), and the enhanced penetration/diffusion (28, 29).

Conclusion

This work demonstrates a new strategy for tumor targeting and anticancer therapy with a novel drug delivery platform, which can work as a tumor-targeted nanocarrier as well as a nanomedicine of its own. The tumor cell-specific drug delivery and cytotoxicity are controlled by a functional paclitaxel conjugate, which has the ability to self-assemble and the MMP2-sensitivity. This MMP2-sensitive micellar nanopreparation with high paclitaxel loading, low risk of drug leakage, and enhanced tumor targeting and cellular penetration is superior to free paclitaxel and the conventional polymeric micelles, as the promising results shown in both *in vitro* and *in vivo*. This MMP2-sensitive paclitaxel-containing nanopreparation may be a good candidate for cancer therapy involving paclitaxel.

Materials and Methods

Materials, Synthesis and Characterization of PEG2000-peptide-PTX and TATp-PEG1000-PE, Apoptosis Analysis, and Establishment of A549 Tumor Cell Spheroids. The description is presented in detail in *SI Materials and Methods*.

Preparation of the MMP2-responsive PTX-containing Nanopreparation. To prepare the MMP2-responsive PTX-containing MMP2-sensitive nanopreparation (TATp-PEG1000-PE/PEG2000-peptide-PTX) shown in Fig. 1, PEG2000-peptide-PTX (50 mol%), PEG1000-PE (40 mol%), and TATp-PEG1000-PE (10 mol%) were dissolved in chloroform and dried on a freeze-dryer overnight, followed by hydration with HBS at room temperature. The non-sensitive nanopreparation (TATp-PEG1000-PE/PEG2000-peptide-PTX uncleavable), nanopreparations without TATp modification (PEG1000-PE/PEG2000-peptide-PTX and PEG1000-PE/PEG2000-peptide-PTX uncleavable), and the empty micelle (TATp-PEG1000-PE) were prepared using the same method. The particle size and morphology of these nanopreparations were analyzed by transmission electron microscopy (TEM) (model XR-41B) (Advanced Microscopy Techniques, Danvers, MA) using negative staining with 1% phosphotungstic acid (PTA).

***In Vitro* Cellular Uptake of PEG2000-peptide-PTX and Its Nanopreparations.** To study the cellular uptake in cell monolayers, the A549 cells were seeded at 1.6×10^5 cells/well in 24-well plates and treated with NBD-PE-labeled formulations in serum-free media. After 2 h incubation, the cells were analyzed by FACS and confocal microscopy. To study the cellular uptake by spheroids, the 6-day old spheroids were incubated with rhodamine-PE-labeled formulations in serum-free medium for 2h. The spheroids were then imaged by confocal microscopy. The detailed information on these procedures can be found in *SI Materials and Methods*.

***In Vitro* Cytotoxicity of PEG2000-peptide-PTX and Its Nanopreparations.** To study cytotoxicity of the PTX conjugate, cancer cells (A549 cells) and normal cells (H9C2 cells) were seeded at 2×10^3 cells/well in 96-well plates and incubated with PTX or PTX conjugates, respectively. To study the cytotoxicity of the PTX-containing nanopreparations, various PTX formulations were incubated with

A549 cells at 2×10^3 cells/well in 96-well plates. After 72h incubation, the cell viability was determined. To study the response of spheroids to PTX conjugates and its nanopreparations, various formulations were incubated with the 6-day A549 spheroids at the dose of 29.5ng/mL PTX every other day for 6 days. The cytotoxicity was determined on day 12. The detail information can be found in *SI Materials and Methods*.

Biodistribution and Intratumor Localization Study. The establishment of the NSCLC xenograft mouse model was described in *SI Materials and Methods*. HBS, the rhodamine-labeled MMP2-sensitive nanopreparation, and its non-sensitive counterpart were intravenously injected in tumor-bearing mice with a tumor size of about 400mm^3 at the dose of 5mg/Kg PTX via tail vein, respectively. At 2 h post-injection, mice were anesthetized and sacrificed. The tumor and major organs (heart, liver, spleen, lung, and kidney) were collected. The fresh tissues were minced into small pieces and incubated in 400 U/ml of collagenase D solution for 30 min at 37°C to dissociate cells (30). The single-cell suspension was analyzed immediately by FACS. The rest part of the tissue was stored at -80°C and sectioned with a microtome, followed by Hoechst 33342 staining. The tumor sections were analyzed by confocal microscopy detected by a confocal microscope at $630\times$. Each group will consisted of 5 mice.

Antitumor Efficacy and Side Toxicity Study. Tumor-bearing mice were randomized to 5 groups (7 mice per group), when the tumor size was around 200mm^3 . HBS, PTX in Cremophor EL/ethanol (1:1, v/v), PEG2000-PE/PTX, TATp-PEG1000-PE/PEG2000-peptide-PTX, and the non-sensitive nanopreparation were injected intravenously in mice at the dose of 5mg/Kg PTX twice a week for 4 weeks. The tumor volume and body weight were monitored every 3 days until the end of the experiment. At the end of the experiment, whole blood was collected from the retro-orbital plexus. White blood cells were isolated using HISTOPAQUE® 1083 as a density gradient medium and counted using a haematocytometer by microscopy. The activities of alanine transaminase (ALT) and aspartate transaminase (AST) were measured by the ALT and AST assay kits. The tumor and major organs were collected and sectioned. Tumor sections were stained by Hoechst 33342, and the tumor cell apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using FragEL™ DNA Fragmentation Detection Kit. The pictures were taken by confocal microscopy at $400\times$. Additionally, Hematoxylin and Eosin (H&E) Staining of organs (at $200\times$) and tumor sections (at $200\times$ and $400\times$) was conducted for histological study of toxicity.

Statistical Analysis. Data were presented as the mean \pm standard deviation (SD). The difference between any two groups was determined by ANOVA. $P < 0.05$ was considered statistically significant.

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Figure Legends

Fig. 1. Drug delivery strategy and characterization of the MMP2-sensitive nanopreparation. (A) Drug delivery strategy. (B) ^1H NMR of PEG(2000)-peptide-PTX. Both CDCl_3 (blue) and D_2O (red) were used to determine the chemical and nano- structure of the PTX conjugate. (C) Transmission electron microscopy (TEM) and critical micelle concentration (CMC). For CMC measurement, pyrene was used as the fluorescent probe. The CMC value was obtained as the crossover point of two tangents of the curves. The particle size and morphology of the nanopreparations were analyzed by TEM using negative staining with 1% PTA. (D) Enzymatic cleavage. To determine the digestion of PEG2000-peptide-PTX (left) and its nanopreparation (right), the samples were treated with $5\text{ng}/\mu\text{L}$ of MMP2 and run in chloroform/methanol (8:2, v/v) and visualized by Dragendorff's reagent. PTX, paclitaxel; TATp, trans-activating transcriptional activator peptide; PEG, poly(ethylene glycol); PE, phosphatidylethanolamines; MMP2, matrix metalloproteinase-2.

Fig. 2. *In vitro* evaluation of paclitaxel conjugate and its nanopreparation. (A) Cytotoxicity of PEG2000-peptide-PTX in A549 and H9C2 cells. The cytotoxicity of monolayer cells was determined by Cell Titer-Blue[®] Cell Viability Assay after 72 h treatments. (B) Apoptosis analysis. The apoptosis of A549 cells was determined by FACS using Annexin V / Propidium Iodide double staining after 72h treatments. (C) Cellular uptake in monolayer A549 cells. The cells were treated with NBD-PE-labeled formulations (green) for 2 h before measurements. For FACS (left), cells were trypsinized and washed by PBS before FACS analysis. For confocal microscopy (right), cells were fixed and stained with Hoechst 33342, and observed at $630\times$. Scale bar (D) Cytotoxicity of PTX nanopreparations in A549 cells monolayers. Cells were treated with moderate to low doses of PTX formulations for 72 h before Cell Titer-Blue[®] Cell Viability Assay. (E) Penetration of PTX nanopreparations in A549 spheroids. The spheroids were treated with rhodamine-PE-labeled formulations for 2h before confocal microscopy at $100\times$ (a-h). The sections from c and d were stained by Hoechst 33342, and observed at $400\times$. (F) Cytotoxicity of PTX nanopreparations in A549 spheroids. The 6-day spheroids were treated with PTX formulations at the dose of $29.5\text{ng}/\text{mL}$ every other day for 6 days and the cytotoxicity was estimated by the LDH release.

Fig. 3. *In vivo* tumor targeting and antitumor efficacy. (A) Biodistribution analyzed by FACS. HBS, the rhodamine-labeled nanopreparation, and its non-sensitive counterpart were i.v. injected in tumor-bearing mice at the dose of $5\text{mg}/\text{Kg}$ PTX, respectively. At 2 h post-injection, tumor and major organs (liver, kidney, spleen, heart, and lung) were collected. The cells were dissociated from fresh tissue and analyzed immediately by FACS. (B) Intratumor localization. The tumor tissue sections were stained by Hoechst 33342 and detected by confocal microscopy at $400\times$. (C) Tumor size reduction (% of the starting tumor volume). Tumor size was measured every 3 day and calculated as $V=1w^2/2$. (D) Tumor cell apoptosis. Tumor sections were stained by Hoechst 33342, and the cell apoptosis was analyzed by TUNEL assay using FragEL[™] DNA Fragmentation Detection Kit and recorded by confocal microscopy at $200\times$.

Fig. 4. *In vivo* side toxicity assessment. (A) Mouse body weight (% of starting body weight). (B) White blood cell counts. At the end of the experiment, white blood cells were isolated by centrifugation using HISTOPAQUE[®]-1083 and counted by microscopy. (C) Activity of alanine transaminase (ALT) and aspartate transaminase (AST). The serum was separated from blood and the activity of transaminase was measured by the ALT and AST assay kits. (D) H&E staining ($200\times$ and $400\times$). No histological difference was observed between the MMP2-sensitive nanopreparation treated group and negative control in the organs, while the necrotic area was significant after the MMP2-sensitive nanopreparation treatment in the tumor tissue.