Targeting Bone Tumor and Subcellular Endoplasmic Reticulum via Near Infrared II Fluorescent Polymer for Photodynamic-Immunotherapy to Break the Step-Reduction Delivery Dilemma

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Specific localization of photosensitizers (PSs) to a certain organelle could result in targeted attack to cause greater trauma to cancer cells, eventually maximizing photodynamic therapy (PDT). However, currently, efficient and precise transportation of PSs via drug delivery to tumor cells and subcellular organelles is still challenging, due to a so-called step-reduction delivery dilemma (SRDD) which also threatens anticancer drug delivery to exert their efficacy. Herein, a cascade targeting near infrared II (NIR II) fluorescent nanoparticles (NPERS/BO-PDT) is designed that can target bone tumor first and then target the subcellular organelle of endoplasmic reticulum (ER). It is found that NPERS/BO-PDT achieves the targeted accumulation of the bone tumor and then ER. NPERS/BO-PDT generates reactive oxygen species (ROS) in the subcellular organelles of ER under near infrared light irradiation. The continuous ER stress by ROS promotes the release of more damage-associated molecular patterns, induces immunogenic cell death, stimulates the adaptive immune response, and further synergistically inhibits tumor growth, achieving the so-called photodynamic-immunotherapy. Overall, this study exemplifies a safe and efficient nano-drug delivery system for a bone and ER cascade targeting via delivery of PSs to break the SRDD and highlights potential clinical translation.

1. Introduction

Multiple physiologic physical barriers of solid tumors exist including diseased organs, tissues, cells, and even subcellular organelles which results in the delivery of any drugs to the final targeted following a step-reduction manner and greatly threatens anticancer drugs to exert their efficacy. This phenomenon greatly hinders the limited progress and clinical translation of nanomedicine. To some extent, it could be actually considered as a step-reduction delivery dilemma (SRDD). It seems imperative to propose new strategies to break this SRDD to improve current cancer therapy.

Photodynamic therapy (PDT) is a promising anti-tumor therapy with high effectiveness and low incidence of adverse effects. Recent studies unveil PDT can efficiently kill tumor cells through photodynamic-immunotherapy. However, there is still SRDD which hampered the efficient delivery of photosensitizers (PSs) to exert PDT. Therefore,
it is of great significance to deliver PSs to cancer cells and even organelles before they are irradiated.

Osteosarcoma (OS) is a malignant tumor that originates from bone and destroys healthy bone tissues.\(^6\) The complex anatomical characteristics and abnormal cancer cell proliferation make the limited vascular systems more unevenly distributed in OS, resulting in the fact that few anticancer drugs can reach the bone tumor tissues.\(^7\) Therefore, high-doses of anticancer agents were used to achieve desirable therapeutic outcomes, which in turn inevitably results in adverse cytotoxic effects and significantly narrows the treatment options for OS.\(^8\) Fortunately, the high concentration of minerals in bone tissues is unique for targeted drug delivery.\(^9\) Bisphosphonates are such a class of bone targeting agents that can chelate with Ca\(^{2+}\) of hydroxyapatite and adhere to the bone tissues.\(^10\) Endoplasmic reticulum (ER) is a cellular organelle for protein synthesis which determines the function, fate, and survival of cells.\(^11\) Moreover, ER stress determines a variety of tumor-promoting properties, making targeting subcellular organelle ER promising.\(^12\) Recent advances in biomaterials and biosafety materials provide new possibilities to overcome these limitations.\(^13\) Therefore, ideally, PSs could be delivered to the tumor cells and then translocated to ER for photodynamic-immunotherapy.

Herein, we designed a polymer containing an aggregation-induced emission (AIE) molecular unit in the main chain, which can produce reactive oxygen species (ROS) and emit near infrared II (NIR II) fluorescence (designated as P\(^{\text{DPT}}\)) for photodynamic-immunotherapy. Subsequently, an ER-targeting ligand (N-Tosylethlenediamine) and a bone targeting ligand (alendronic acid) were introduced into P\(^{\text{DPT}}\) respectively to prepare an ER-targeting polymer (designated as P\(^{\text{ER-PDT}}\)) and a bone targeting polymer (designated as P\(^{\text{BO-PDT}}\)). Therefore, P\(^{\text{ER-PDT}}\) and P\(^{\text{BO-PDT}}\) were mixed and self-assembled into NIR II fluorescent cascade targeting nanoparticle (NPER/BO-PDT) (Scheme 1A). NPER/BO-PDT with cascade targeting bone tumor and ER performance could break the SRDD. To address this, NPER/BO-PDT were injected into mice bearing orthotopic OS and they reached the tumor site through blood circulation. The targeted accumulation of NPER/BO-PDT in bone tumor tissues is achieved by alendronic acid mediated chelation with the Ca\(^{2+}\) of hydroxyapatite at the bone tumor site, and then the NPER/BO-PDT were effectively endocytosed by tumor cells. Finally, NPER/BO-PDT were selectively accumulated on the subcellular organelles of ER. Under 808 nm light irradiation, on the one hand, NPER/BO-PDT can generate ROS in ER for killing tumor cells; on the other hand, the ROS generated in situ destroys the calcium homeostasis, triggers stronger ER stress, increases damage-associated molecular patterns (DAMPs) released by dying tumor cells, and amplifies the immunogenic cell death (ICD) effect. Moreover, under the stimulation of DAMPs, immature dendritic cells (DCs) become mature, thereby enhancing their antigen presentation ability and stimulating the effective adaptive immune response. Meanwhile, NPER/BO-PDT can promote the M1 polarization of tumor-associated macrophages (TAMs) and reduce the percentage of regulatory T cells (Tregs) in the tumor microenvironment, and further synergistically activate T cells and regulate the secretion of inflammatory factors, and ultimately achieve efficient photodynamic-immunotherapy (Scheme 1B). We further established a patient-derived OS animal model (PDX\(^{\text{OS}}\)), and demonstrated NPER/BO-PDT mediated PDT significantly inhibited the tumor growth on a PDX\(^{\text{OS}}\) model (Scheme 1C). Overall, we demonstrated here NPER/BO-PDT target bone and ER for ROS generation, resulting in induction of persistent ER stress, amplifying the ICD effect, which breaks the SRDD and finally highlights the use of cascade targeting strategy for more effective photodynamic-immunotherapy.

2. Results and Discussion

2.1. Preparation and Characterization of NPER-PDT and NPER/BO-PDT

P\(^{\text{DPT}}\) was synthesized via a condensation polymerization as previously described (Scheme S1 and Figure S1, Supporting Information).\(^4\) Next, an ER targeting ligand, that is, the N-Tosylethlenediamine was introduced into P\(^{\text{DPT}}\) to prepare an ER-targeting polymer (P\(^{\text{ER-PDT}}\)) (Scheme S2 and Figure S2, Supporting Information). Similarly, alendronic acid was introduced into P\(^{\text{DPT}}\) to prepare a bone targeting polymer (P\(^{\text{BO-PDT}}\)) (Scheme S3, Figures S3 and S4, Supporting Information). Subsequently, P\(^{\text{DPT}}\) and P\(^{\text{BO-PDT}}\) were used to prepare NPER/BO-PDT, respectively. Moreover, P\(^{\text{ER-PDT}}\) and P\(^{\text{BO-PDT}}\) were mixed to prepare cascade targeting nanoparticles NPER/BO-PDT.

First, we found NPER-PDT and NPER/BO-PDT exhibited uniform spherical shapes with a homogenous diameter around 100 nm (Figure 1A,B). The average hydrodynamic diameter of NPER-PDT and NPER/BO-PDT were further confirmed by dynamic light scattering to be 109 and 112 nm with polydispersity indexes (PDI) at 0.11 and 0.14, respectively (Figure 1C,D). To further verify the photostability of NPER/BO-PDT, we then irradiate NPER/BO-PDT under 808 nm laser for 10 min with Indocyanine Green (ICG) as a control. Results showed that the color of NPER/BO-PDT was basically unchanged, while ICG has an obvious photobleaching effect, indicating NPER/BO-PDT had a better light stability (Figure S5, Supporting Information). Moreover, the diameter of NPER/BO-PDT did not change after 10 days in PBS, further proving the good stability of NPER/BO-PDT (Figure S5B, Supporting Information).

Next, we study the absorbance and light emitting properties of nanoparticles. The results showed that NPER/BO-PDT had a strong absorption in the range of 300–850 nm with a maximum absorption peak at 688 nm. Moreover, under 808 nm light irradiation, NPER/BO-PDT demonstrated strong fluorescence emission in the second NIR window (NIR II, 950–1700 nm) with a major peak at 1001 nm (Figure 1E). In addition, NPER/BO-PDT was supposed to be ROS sensitive as there are numerous thioketal bonds and AIE molecular units in the polymer main chain. Under NIR light irradiation, NPER/BO-PDT can generate ROS, thereby breaking down the thioketal bonds, resulting in the dissociation of nanoparticles. To verify this process, on the one hand, H\(_2\)O\(_2\) was used to dissociate the NPER/BO-PDT with Nile red, and the changes in the absorption peak of Nile red were recorded for the nanoparticle dissociation kinetics.\(^16\) The results showed that the dissociation half-life of NPER/BO-PDT is \(\approx 2.71\) h (Figure 1F). On the other hand, since robust ROS generation was a prerequisite for PDT, we continued to study the ROS generation ability of NPER/BO-PDT under NIR light irradiation (808 nm, 1.0 W cm\(^{-2}\)). DBPF (1,3-Diphenylisobenzofuran) is selected as a ROS indicator because it would be oxidized and degraded by ROS, making the absorbance of DBPF at 415 nm ideal for monitoring the level of ROS generation. Results showed that under NIR light...
Scheme 1. Schematic illustration of NIR II fluorescent NPER/BO-PDT with cascade targeting performance for breaking SRDD to amplify photodynamic-immunotherapy. A) The ER targeting N-Tosylethylenediamine and bone targeting alendronic acid were respectively introduced into polymer PDT (ROS generation) to prepare a new ER-targeting polymer (PER-PDT) and a bone targeting polymer (PBO-PDT), respectively. Finally, PER-PDT and PBO-PDT were mixed and co-assemble to NPER/BO-PDT. B) Inhibition of tumor growth via NPER/BO-PDT on a K7M2 cancer model of OS. NPER/BO-PDT was injected into mice bearing orthotopic K7M2 OS, and they were accumulated in bone tumor tissues via bone targeting. Subsequently, NPER/BO-PDT within the tumor cells could be further delivered to the ER lumen. Upon near infrared (NIR) light irradiation, NPER/BO-PDT could generate ROS in ER, which then killed tumor cells directly and triggered the imbalance of Ca\(^{2+}\) homeostasis. The persistent imbalance of intracellular Ca\(^{2+}\) homeostasis could induce the continuous ROS-based ER stress, which then increased DAMPs releasing by dying tumor cells, and amplified the ICD effect and activated the adaptive immunity. C) Inhibition of tumor growth via NPER/BO-PDT on a second cancer model of PDX OS.

Irradiation for 180 s, the absorbance of NPER/BO-PDT and DPBF aqueous mixture solution significantly reduced with a half-life of 110 s, and only 35.6% of DPBF is not oxidized (Figure 1G), indicating that NPER/BO-PDT can quickly generate ROS (the singlet oxygen, \(^{1}\)O\(_2\)) under NIR light irradiation. Finally, to demonstrate the bone targeting ability, on the one hand, PBO-PDT with phosphate was characterized by XPS with obvious peaks at 132.9 eV (Figure 1H), indicating PBO-PDT was successfully conjugated with alendronic acid. On the other hand, the binding ability of NPER/BO-PDT to hydroxyapatite was studied and we found that
Figure 1. Characterization of NPER/BO-PDT. A, B) The transmission electron microscope images of NPER-PDT and NPER/BO-PDT. Scale bar: 200 nm. C) The diameter and D) PDI of NPER-PDT and NPER/BO-PDT by dynamic light scattering. E) Absorption spectra and fluorescence emission spectra of NPER/BO-PDT. F) NPER/BO-PDT dissociation kinetics monitored by Nile red assay. G) Decomposition rates of DPBF by ROS, generated from NPER/BO-PDT under NIR light irradiation (808 nm, 1.0 W cm⁻²). H) Characterization of BO-PDT by XPS. I) The normalized hydroxyapatite-binding capacity of NPER/BO-PDT. Data are shown as mean ± SD. **p < 0.01.

NPER/BO-PDT can bind to hydroxyapatite faster, and the binding force of NPER/BO-PDT to hydroxyapatite is about three times that of NPER-PDT (Figure 1I). The above results together indicated that NPER/BO-PDT had bone targeting ability.

2.2. ER-Targeting and Cytotoxicity of NPER/BO-PDT

The uptake of NPER/BO-PDT by tumor cells and further selective accumulation in the ER play a critical role in its anti-tumor activity. On the one hand, the ER-targeting property of NPER/BO-PDT was examined in MNNG/HOS cells by labeling NPER/BO-PDT with Cy5.5 dye (NPER/BO-PDT @Cy5.5). As shown in Figure 2A, the co-location rate exhibited a time-dependent cellular uptake, which gradually enhanced as the incubation time increased. Upon incubation for 5 h, the red fluorescence signal from NPER/BO-PDT @Cy5.5 and the green signal from ER tracker matched well (the Pearson correlation coefficient is 0.61), making yellow fluorescent spots within ER, which demonstrated the specific ER targeting ability of NPER/BO-PDT. Subsequently, the red and green fluorescence intensity at lines m, n, and k in Figure 2A was further analyzed. The line-scan profiles also denoted the co-localization of NPER/BO-PDT within ER compartments (Figure 2B). These results verified that NPER/BO-PDT possessed selectivity for ER in cells. On the other hand, NPER/BO-PDT @Cy5.5 was used to treat the cells for intracellular uptake study and we found the fluorescence intensity in MNNG/HOS cells was gradually intensified with increasing incubation time from 1 h to 7 h by flow cytometry (FCM).

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A

1h

3h

5h

DAPI / ER-tracker / NPER/BO-PDT@cy5.5 / Merge

B

- NPER/BO-PDT - ER

n

k

Distance (µm)

C

Counts

Florescence intensity

1h

4h

7h

Control

Florescence intensity

D

1h

4h

7h

DAPI / Actin / NPER/BO-PDT / Merge

E

Relative cell viability

Con. (µg/mL)

NPER/BO-PDT

NPER/BO-PDT + L

F

G1

G4

G7

Calcein AM / PI / Merge

G

Counts

Florescence intensity

PBS

NPER/BO-PDT

NPER/BO-PDT + L

G4

G5

G7

DAPI / DCF / Merge

H

G1 = PBS

G4 = NPER/BO-PDT

G5 = NPER/BO-PDT + L

G7 = NPER/BO-PDT + L
Specifically, the fluorescence intensity at 7 h was nearly ten fold higher than that at 1 h (Figure 2C). Fluorescence with high spatial and temporal characteristics can help one understand the physiological function of nanoparticles. Here, we could observe NPER/BO-PDT via NIR II imaging for cellular endocytosis. Results showed similar trends that the red fluorescence was gradually intensified with increasing incubation time of NPER/BO-PDT in K7M2 cells (Figure 2D). 3D multi-cellular tumor spheroids can better mimic the in vivo cell microenvironment, and narrow the gap between in vivo and in vitro experiments. Subsequently, we further verified the uptake of NPER/BO-PDT@Cy5.5 with 3D multi-cellular tumor spheroids. We found the red fluorescence intensity in the same depth portion of 3D tumor spheres was also gradually intensified from 1 h to 7 h (Figure S6A, Supporting Information), hence confirming the effective uptake of NPER/BO-PDT by 3D multi-cellular tumor spheroids. In addition, NPER/BO-PDT may enter tumor cells through endocytosis. To prove this process, we treated 3D multi-cellular tumor spheroids with an endocytosis inhibitor genistein. Results unveiled that genistein can inhibit the endocytosis of NPER/BO-PDT (Figure S6B, Supporting Information), indicating NPER/BO-PDT can indeed be effectively endocytosed by tumor cells.

After tumor cells take up NPER/BO-PDT, it will generate ROS under NIR light irradiation, and then kills tumor cells. First, the anti-tumor activity of NPER/BO-PDT was evaluated in vitro. As shown in Figure 2E, NPER/BO-PDT + L had a 62% inhibition rate at 20 μg mL–1 in K7M2 cells, while NP + L only had a 30% inhibition rate at the same concentration. Similar results were found in other OS cells (Figure S7A,B, Supporting Information). Moreover, the apoptosis rate of cells treated with NPER/BO-PDT revealed that NPER/BO-PDT + L induced significantly higher level of apoptosis (47.6%) as compared to NP + L (26.9%) in K7M2 cells (Figure S8A,B, Supporting Information). Taken together, these results indicated that NPER/BO-PDT with ER-targeting ability had stronger anti-tumor activity and pro-apoptotic effect than NP + L. Second, live and dead assay was applied to investigate the cell killing effect of NPER/BO-PDT in 2D and 3D tumor spheroids. The results revealed that the tumor cells and spheroids treated with PBS or NPER/BO-PDT displayed mainly green fluorescence (live cells), while those treated with NPER/BO-PDT + L showed great red fluorescence (dead cells) (Figure S9, Supporting Information and Figure 2F). Third, we continued to evaluate the ROS generation ability of NPER/BO-PDT via DCFH-DA probes by FCM and confocal laser scanning microscopy (CLSM). We found that on the one hand, the fluorescence intensity of cells treated with NPER/BO-PDT + L was nearly ten fold higher than that of cells treated with NPER/BO-PDT without laser irradiation by FCM (Figure 2G and Figure S10A, Supporting Information). On the other hand, cells treated with NP + L or NPER/BO-PDT + L all exhibited strong green fluorescence by CLSM, further proving the efficient ROS generation (Figure 2H). As the half-life of ROS in cells is very short (<40 ns) and the intracellular diffusion distance is limited (<20 nm), the ROS generated in situ in subcellular organelles prompts us to design cascade targeting nanoparticles for enhancing the anti-tumor efficacy.

2.3. NPER/BO-PDT induces the ER Stress and ICD

ER is a complex dynamic organelle with an important intracellular Ca2+ store. Generally, intracellular ROS levels are always maintained in a state of dynamic equilibrium. Once there is too much intracellular ROS generated, the excessive ROS will impair the ER function, which subsequently results in the imbalance of intracellular Ca2+ homeostasis. To confirm whether the NPER/BO-PDT would induce more dramatic imbalance of Ca2+ homeostasis under continuous NIR light irradiation, the intracellular Ca2+ fluorescence intensity was detected by CLSM and FCM. A Ca2+ probe Fluo-3 AM was employed which could be cleaved in cancer cells by enzymes to form Fluo-3. Once the Fluo-3 meets with Ca2+, there is green fluorescence. We showed that cells treated with NPER/BO-PDT + L exhibited stronger green fluorescence by CLSM, which was 1.9 times higher than that of NP + L (Figure S10B,C, Supporting Information). The further quantification analysis via FCM revealed the fluorescence intensity of cells treated with NPER/BO-PDT + L was 1.6 times higher than that of NP + L (Figure 3A). Taken together, these results proved NPER/BO-PDT can dramatically impair the stability of Ca2+ homeostasis than NP + L under NIR light irradiation.

The persistent imbalance of intracellular Ca2+ homeostasis would impair the proper synthesis and folding of proteins, which consequently induces the accumulation of unfolded or misfolded proteins in the ER lumen, accompanied by ER stress. Moreover, the degree of ER stress determines whether to return homeostasis or to activate the cell death program. At the initiation of ER stress, misfolded proteins inflicted by the excessive ROS would bind to the ER chaperone binding immunoglobulin protein (BiP), which then leaves the ER stress sensors protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme 1a (IRE1α) free to be activated. Additionally, these activated ER stress responsive proteins would further upregulate the unfolded protein response (UPR), which facilitates the clearance of unfolded or misfolded proteins and the maintenance of ER homeostasis. However, once the status of ER stress continues for a long time, the expression of proapoptotic C/EBP homologous protein (CHOP), which is a key mediator of the ER stress-mediated apoptosis pathway, would be upregulated by the excess ROS through PERK/ERF2/ATF6/CHOP pathway, and thereby promoting cell apoptosis.[12,21] To briefly prove the above mentioned ER stress
Figure 3. NPER/BO-PDT induced the imbalance of Ca^{2+} in the ER lumen and stimulated persistent ROS-based ER stress, resulting in a stronger ICD effect and more DCs maturation under NIR light irradiation in vitro. A) The cytosolic Ca^{2+} level in cells after various treatments by FCM. B) CLSM images of CHOP-stained cells after various treatments. Scale bar: 20 μm. C) Western blot results of the ER stress proteins after various treatments. D) CLSM images of the exposure of CRT in K7M2 cells after various treatments. Scale bar: 20 μm. E) Quantitative study of the exposure of CRT by FCM. F) CLSM images of the release of HMGB1 in K7M2 cells after various treatments. Scale bar: 20 μm. G) The relative ATP release of cells after various treatments. H) Quantitative study of the maturation of BMDCs co-cultured with K7M2 cells with various pretreatments by FCM. I) Schematic illustration of ER-targeting of NPER/BO-PDT for PDT, resulting in the ICD effect and further maturation of BMDCs in vitro. Data are shown as mean ± SD. Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.
process in MNNG/HOS cells under various treatments, we carried out a relative protein expression analysis via CLSM and Western blot. CLSM imaging revealed that the red fluorescence signal from CHOP and the blue signal from cell nucleus stained with DAPI showed pink fluorescence in the merged images in cells treated with NPER/BO-PDT + L, demonstrating the significant upregulated expression of CHOP (Figure 3B). Furthermore, the Western blot results revealed that the expression of CHOP, p-IRE-1α, p-eIF2α, ATF6, and XBP-1s were significantly upregulated in cells treated with NPER/BO-PDT + L, as compared to those treated with NPD + L, especially for CHOP (Figure 3C). Taken together, the aforementioned results fully validated that NPer/BO-PDT was capable of inducing a considerable ROS generation in ER, which then contributed to the redox balance disorder and the imbalance of Ca2+ homeostasis, and stimulated continuous ROS-based ER stress and cell death through activation of the PERK/eIF2α/ATF6/CHOP pathway.

Recently, more and more studies have demonstrated that PDT can induce ICD effect through the ROS-based ER stress effect.19,22) Tumor cells that undergo ICD would generate a series of DAMPs, such as surface-exposed calreticulin (CRT) from the ER lumen, passively released high mobility group Box 1 (HMGB1), and secreted adenosine triphosphate (ATP) from the cytoplasm, which are also the hallmarks of ICD.24,25) Specifically, the surface-exposed CRT is a proapoptopic “eat me” signal, and the secreted ATP can serve as a “find-me” signal that jointly elicits phagocytosis of the dying tumor cells by the DCs.26) HMGB1 can promote DCs maturation and antigen presentation.27) Taken together, all these DAMPs coopted in triggering enhanced immune responses. Hence, to verify whether the ROS-based ER stress triggered by NPer/BO-PDT can induce effective ICD, promote the release of DAMPs, and induce the DCs to mature under NIR light irradiation, we conducted a series of experiments. First, the surface-exposed CRT was detected by CLSM and FCM. On the one hand, the result of CLSM study showed that K7M2 cells treated with NPer/BO-PDT + L exhibited stronger green fluorescence than those treated with NPD + L, indicating higher exposure of CRT (Figure 3D). The quantification of the fluorescence pixel intensity in each cell further revealed that the green fluorescence in cells treated with NPer/BO-PDT + L was ~1.4 times higher than that of NPD + L (Figure S11A, Supporting Information). On the other hand, the quantitative FCM result further suggested that the fluorescence intensity of CRT in cells treated with NPer/BO-PDT + L was 1.5 times higher than that of NPD + L (Figure 3E). The aforementioned results prove that NPer/BO-PDT triggered higher CRT expression under NIR light irradiation. Second, we continued to investigate the translocation of HMGB1 from nucleus to extracellular matrix. As shown in Figure 3F, the HMGB1 (red) was primarily merged with the nucleus (blue) of K7M2 cells treated with PBS. However, compared with NPD + L, more HMGB1 was released from the nucleus in the cells treated with NPer/BO-PDT + L (Figure S11B, Supporting Information). Taken together, the above findings indicated that NPer/BO-PDT promotes more translocation of HMGB1 under NIR light irradiation. Third, ATP secretion in the cell culture medium was evaluated by ATP assay. As shown in Figure 3G, ATP secretion in the supernatant of NPer/BO-PDT + L treated cells was almost 2.2 times that of NPD + L, indicating that cell treated with NPer/BO-PDT promoted the higher level of ATP secretion under NIR light irradiation. Finally, to confirm whether the ICD effect induced by DAMPs can promote the maturation of bone marrow-derived dendritic cells (BMDCs), we continued to evaluate the DCs maturation in vitro through co-incubation with K7M2 cells following various treatments. The results showed that the maturation ratio of co-cultured DCs treated with NPer/BO-PDT + L (25.3%) was 2.2 times higher than that of the PBS treatment group (11.6%), while the ratio in NPD + L treatment group was 17.0%, which substantially demonstrated the stronger DCs mature effect of NPer/BO-PDT + L (Figure 3H and Figure S12, Supporting Information).

In summary, the persistent ROS-based ER stress triggered by NPer/BO-PDT under NIR light irradiation can effectively induce the ICD effect of tumor cells and further promote DCs maturation (Figure 3I).

2.4. Biodistribution and Tumor Suppression of NPer/BO-PDT in vivo

Biosafety is a prerequisite for nanomedicine to achieve anti-tumor ability in vivo. Hence, we conducted a safety assessment of NPer/BO-PDT first. Healthy KM mice were used and intravenously injected with a single dose of NPer/BO-PDT. The body weight of mice in each group was recorded every 2 days from the day of injection. Fourteen days after the administration, the blood and main organs of mice were taken for physiological and biochemical examination. On the one hand, the results of blood count and biochemical parameters showed that mice treated with NPer/BO-PDT were relatively normal as compared to the PBS group (Figures S13 and S14, Supporting Information). Compared with the PBS group, there was no significant difference in the average body weight of the mice in the NPer/BO-PDT treatment group (Figure S15A, Supporting Information). On the other hand, no obvious pathological abnormality was found in the hematoxylin and eosin (H&E) staining images of main organs in the NPer/BO-PDT treatment group (Figure S15B, Supporting Information). Altogether, the aforementioned results proved the excellent biosafety of NPer/BO-PDT, which is important for in vivo applications.

Subsequently, we explored the in vivo biodistribution of NPer/BO-PDT on an orthotopic K7M2 OS model based on BALB/c mice (Figure 4A).27) The in vivo imaging showed that the fluorescence intensity of tumor site continued to increase from 0.5 h to 24 h after an intravenous injection by an in vivo Imaging System (IVIS, PerkinElmer). Moreover, the fluorescence intensity of tumor site in NPer/BO-PDT treatment group was higher than that of the NPer-PDT treatment group at 24 and 48 h after an intravenous injection (Figure 4B, left panel). The above results indicated that NPer/BO-PDT had excellent stability and could achieve rapid accumulation and retention in the bone tumor tissues compared to NPer-PDT. Then, the main organs and tumors of mice at 48 h after injection were further collected and analyzed quantitatively ex vivo. We found that the ex vivo accumulation of NPer/BO-PDT in tumor tissues is greater than that of NPPer-PDT, and the fluorescence intensity of NPer/BO-PDT in the tumor tissues is ~1.6 times that of NPer-PDT (Figure 4B (right panel) and 4C), further confirming the superior bone targeting ability of NPer/BO-PDT.

To further evaluate the in vivo anti-tumor effect of NPer/BO-PDT, we constructed an orthotopic K7M2 OS model based on BALB/c mice. As shown in Figure 4A, NPer/BO-PDT was intravenously in-
NPER/BO-PDT had higher therapeutic efficacy in vivo under NIR light irradiation, compared to NP-PDT (Figure 4G, lower). In summary, compared with NP-PDT, the infiltration of CD8+ T cells was higher than other different treatments (Figure 5A). More importantly, the CRT exposure, HMGB1 release, and CD8+ T cell infiltration, and the effectively induces a adaptive immune response via FCM. We found that in tumor tissues the results showed that the percentages of CD4+ T cells (54.4%) and CD8+ T cells (20.5%) in mice treated with NPER/BO-PDT + L are higher than any other treatments, which are 2.6 times and 2.5 times that of PBS (Figure 5G,H and Figure S21, Supporting Information), respectively. Moreover, the percentage of CD8+ T cells by FCM was also consistent with the previous observations with IF staining. Additionally, in the spleens, a more remarkable increase in CD4+ T cells and CD8+ T cells was observed in mice treated with NPER/BO-PDT + L, which showed 1.4 times and 1.8 times that of the PBS (Figures S22A,B and S23, Supporting Information), respectively. The above results fully illustrated that NPER/BO-PDT could dramatically accelerate DCs mature in tumors and TDLNs under NIR light irradiation. Second, to verify whether the mature DCs could evoke the adaptive immune response by activating T lymphocytes, we then evaluated and quantified the infiltration of CD4+CD8+ T cells in tumor tissues and spleens via FCM. We found that in tumor tissues the results showed that the percentages of CD4+ T cells (54.4%) and CD8+ T cells (20.5%) in mice treated with NPER/BO-PDT + L are higher than any other treatments, which are 2.6 times and 2.5 times that of PBS (Figure 5G,H and Figure S21, Supporting Information), respectively. Moreover, the percentage of CD8+ T cells by FCM was also consistent with the previous observations with IF staining. Additionally, in the spleens, a more remarkable increase in CD4+ T cells and CD8+ T cells was observed in mice treated with NPER/BO-PDT + L, which showed 1.4 times and 1.8 times that of the PBS (Figures S22A,B and S23, Supporting Information), respectively. The above results fully illustrated that NPER/BO-PDT + L could promote greater antitumor immune response. However, there still exist various immunosuppressive mechanisms protecting tumor cells from being eliminated by the immune system. Hence, we further evaluated the M1 polarization of TAMs and the infiltration of the immunosuppressive Tregs in tumor tissues. On the one hand, the results showed that the percentage of M1 polarized macrophages (CD80+CD86+) in mice treated with NPER/BO-PDT + L was obviously greater than other treatments (Figures S22C and S24, Supporting Information), whereas the changing trend of M2 polarized macrophages (CD80-CD86+) was just the opposite (Figure S22D, Supporting Information). The further ratio of M1/M2 in mice treated with NPER/BO-PDT + L was 1.5 times of NPPDT + L and 2.0 times of NP-PDT + L (Figure S22E, Supporting Information), respectively, implying NPER/BO-PDT + L can promote the M1 polarization of TAMs. On the other hand, as shown in Figure 5D,E, a

Figure 4. Biodistribution and antitumor effect of NPER/BO-PDT on an orthotopic K7M2 tumor model. A) Establishment of an orthotopic K7M2 tumor model for in vivo therapeutic and imaging studies. B) In vivo and ex vivo biodistribution of NPER/BO-PDT in K7M2 tumor bearing mice (left panel) and major organs (right panel) via fluorescence bio-imaging. T, H, L, L, S, L, K represent tumor, heart, liver, spleen, lung, and kidney, respectively. C) Semiquantitative biodistribution of NPER/BO-PDT in the dissected tumors and major organs by fluorescence quantification at 48 h after intravenous injection. D) Tumor growth curves in mice with various treatments. E) The body-weight variation and F) survival curve analysis of mice with various treatments. Scale bar: 50 μm. Data are shown as mean ± SD. *p < 0.05, **p < 0.01.
Figure 5. Cascade targeting NP<sup>ER/BO</sup>-PDT enhanced the antitumor immunity in vivo. A) Immunofluorescence staining CD8<sup>+</sup> positive T cells in mice treated with NP<sup>ER/BO</sup>-PDT. Scale bar: 10 μm. B) The representative FMC analysis images of DCs and C) the percentages of mature DCs (CD80<sup>+</sup>CD86<sup>+</sup>) populations within tumor tissues. D) The representative FCM analysis images of Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>) and E) the percentages of Tregs populations with tumor tissues. F) The percentages of mature DCs (CD80<sup>+</sup>CD86<sup>+</sup>) populations within TDLNs. The percentages of G) CD8<sup>+</sup> T cells populations and H) CD4<sup>+</sup> T cells populations with tumor tissues. Data are shown as mean ± SD. *p < 0.05, **p < 0.01.
Figure 6. Antitumor effect of NP<sub>ER/BO</sub>-PDT on a PDX<sup>OS</sup> tumor model. A) Schematic illustration of establishment of PDX<sup>OS</sup> model for in vivo therapeutic study. B–E) Tumor growth profiles of mice with various treatments. F) Tumor weights and G) images after the mice were sacrificed at day 21, and the tumors were isolated. H) Representative photographs of H&E staining images from mice with various treatments. Scale bar: 100 μm. Data are shown as mean ± SD. **p < 0.01, ***p < 0.001.
significant down-regulation of Tregs was shown in the mice treated with NPER/BO-PDT + L. The percentage of Tregs (CD4+ FoxP3+) in mice treated with NPER-PDT + L (23.7%) is 1.5 times that of NPER/BO-PDT + L (15.4%) (Figure S19B, Supporting Information). Taken together, these results clearly demonstrated that the bone and ER cascade targeting NPER/BO-PDT + L effectively induced adaptive immunity in K7M2 tumor-bearing mice via robust ICD reprogrammed immunosuppressive microenvironment, achieved the synergistic enhancement of PDT and immunotherapy, and provided a new strategy for OS. To further investigate the in vivo antitumor efficacy of NPER/BO-PDT, we further constructed a PDXOS model, which was derived from patients who had not received any chemotherapy or other regimes for OS. When the tumor size reached \( \approx 100 \text{ mm}^3 \), mice bearing PDXOS tumor were randomly divided into five groups. Then, as shown in Figure 6A, NPER/BO-PDT was intravenously injected, and the tumor volume and body weight in each group were monitored. The results showed that the tumors in mice of NPER/BO-PDT treatment group grew rapidly (Figure S25A–E, Supporting Information), and the average relative tumor volume at day 21 in NPER/BO-PDT treatment group was 9.8, while it was 0.13 in NPER/BO-PDT + L treatment group (Figure 6B,C). Compared with that of the NPER-PDT + L treatment group, the tumor growth in NPER-PDT + L treatment group was significantly inhibited, and the relative tumor volume at day 21 in NPER-PDT + L treatment group was seven times that of NPER-PDT + L treatment group (Figure 6D,E). However, there was no significant difference in the average body weight of the mice in different treatment groups (Figure S25F, Supporting Information). More importantly, tumors in the mice of NPER/BO-PDT + L treatment group shrunk drastically, and the tumor weight at day 21 was only 5% of the PBS treatment group (Figure 6F,G). Further H&E staining images of the tumor slides showed a larger number of apoptotic cells in the NPER/BO-PDT + L treatment group (Figure 6H). Altogether, under NIR light irradiation, NPER/BO-PDT also induced a significant therapeutic effect on a PDXOS model.

3. Conclusions

PDT is a promising cancer treatment modality via photodynamic-immunotherapy. However, the SRDD is one of the main limiting factors that restrict the anticancer activity. In this study, a cascade targeting NIR II fluorescent nano-drug delivery system was successfully designed with AIE effects and breaking the SRDD for strong anti-tumor immunity, which exhibited efficient accumulation and retention at bone tumor site and ER lumen, and excellent therapeutic effect on OS tumor-bearing mice. We found that NPER/BO-PDT could fundamentally improve the effect of photodynamic-immunotherapy under NIR light irradiation through more efficient accumulation of PSs in tumor tissues and even the subcellular organelles. Specially, potent ICD induced by NPER/BO-PDT via persistent and intense ROS-based ER stress in situ of ER could promote immune recognition, boost strong system immune response, and re-modulate the immunosuppressive microenvironment. Overall, our finding proposed a promising strategy to enhance photodynamic-immunotherapy with potential clinical application and transformation prospects.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

cascade targeting, endoplasmic reticulum stress, photodynamic-immunotherapy, step-reduction delivery dilemma

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