Multimodal Image-Guided Surgical and Photodynamic Interventions in Head-and-Neck Cancer: From Primary Tumor to Metastatic Drainage

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Abstract

Purpose: The low survival rate of head-and-neck cancer (HNC) patients is attributable to late disease diagnosis and high recurrence rate. Current HNC staging has inadequate accuracy and low sensitivity for effective diagnosis and treatment management. The multimodel porphyrin lipoprotein-mimicking nanoplatform (PLP), intrinsically capable of positron emission tomography (PET), fluorescence imaging, and photodynamic therapy (PDT), shows great potential to enhance the accuracy of HNC staging and potentially HNC management.

Experimental Design: Using a clinically relevant VX-2 buccal carcinoma rabbit model that is able to consistently develop metastasis to regional lymph nodes after tumor induction, we investigated the abilities of PLP for HNC diagnosis and management.

Results: PLPs facilitated accurate detection of primary tumor and metastatic nodes (their PET image signal to surrounding muscle ratios were 10.0 and 7.3, respectively), and provided visualization of the lymphatic drainage from tumor to regional lymph nodes by both pre-operative PET and intra-operative fluorescence imaging, allowing the identification of unknown primaries and recurrent tumors. PLP-PDT significantly enhanced cell apoptosis in mouse tumors (73.2 % of PLP-PDT group versus 7.1 % of PLP alone group), and demonstrated complete eradication of primary tumors and obstruction of tumor metastasis in HNC rabbit model without toxicity in normal tissues or damage to adjacent critical structures.

Conclusion: PLPs provide a multimodal imaging and therapy platform that could enhance HNC diagnosis by integrating PET/CT and fluorescence imaging, and improve HNC therapeutic efficacy and specificity by tailoring treatment via fluorescence-guided surgery and PDT.

Translational Relevance
This translational study in a clinically relevant large animal HNC model, provides a firm basis for future applications of PLPs in HNC management: 1) non-invasive PET imaging of unknown primary and recurrence/remnant disease based on the ability of $^{64}$Cu-PLPs to selectively accumulate in primary tumors and metastatic nodes with sensitive detection of lymphatic drainage; 2) real-time intraoperative fluorescence guidance to augment current surgical procedures, including a trans-oral approach for oral and oropharynx primary disease and trans-cervical approach for neck dissection and metastatic lymph node dissection; 3) PLP-PDT intervention either for surgically inaccessible tumors or those which are adjacent to critical anatomical structures that may be sensitive to damage during surgery.

Introduction

Head and neck cancer (HNC) annually accounts for more than 550,000 new cancer cases (1) and approximately 350,000 cancer deaths, worldwide.(2) HNCs are a heterogeneous group of tumors that arise in the head and neck area and are notorious for their high morbidity, aggressive behavior and requirement for multidisciplinary care. HNCs have large variations in aetiologies, anatomical origins, prognoses and tumor stages.(3-6) HNC patients with oral cavity cancer exhibit an average 50%–55% 5-year survival rate. Prognosis is dependent on the stage of the tumor at the initial presentation and its accuracy is critical for appropriate treatment management.(7, 8) The proximity of HNC to several adjacent critical structures, such as major vessels, cranial nerves, sensory organs and the brain, also increases the importance of accurate assessment of local and regional disease to optimize effective tumor removal and disease-specific treatment.

Surgical resection and radiation therapy, often combined with chemotherapy, are the mainstays of HNC treatment and have increased the need for imaging modalities to guide precise
treatment since any tumor that remains undetected outside of the treatment field could adversely affect the patients’ prognosis and survival. The most common imaging modalities in HNC are computed tomography (CT), magnetic resonance imaging (MRI), single-photon-emission computed tomography (SPECT) and positron emission tomography (PET) using $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG). However, they are often limited by inadequate sensitivity, specificity and spatial resolution for detection of small, early stage lesions and distant metastases. Additionally, successfully utilizing these modalities to directly guide treatment remains a challenge. For example, determining the tumor free margin during surgery is still done by visual inspection and palpation. Theranostics, which integrates imaging with therapeutic functionalities into the same multimodal agent, is uniquely positioned in cancer management applications, where imaging modalities can not only noninvasively detect and functionally characterize disease, but also provide quantitative assessments of distribution and delivery of therapeutics. Theranostics thus holds great promise to traverse the gap between diagnosis and treatment to permit image-guided disease stratification and treatment.

In addition to the aggressive and recurrent nature of HNC, current HNC treatments involve a very high risk of functional and cosmetic debilitation to the head and neck area, which may cause collateral healthy tissue damage and long-term side effects. Therefore, a significant clinical interest is to explore alternative treatment modalities that have fewer and smaller risks but retain a high level of efficacy in order to improve treatment outcomes and overall quality of life. Photodynamic therapy (PDT), which generates cytotoxic singlet oxygen through interactions between optical light and a photosensitizer in the presence of oxygen, has emerged as a viable tool for localized treatment of malignant tissues. Due to the extremely short life time and subsequent short diffusion distance (10-300 nm) of singlet oxygen, PDT damage is restricted to
photosensitizer accumulation, enabling local tumor ablation without damaging underlining connective tissues unlike other ablation techniques. (18, 19)

Recently, we developed a novel biomimetic, porphyrin lipoprotein-mimicking nanoparticle (PLP), which integrates multiple functionalities, including PET, near-infrared (NIR) fluorescence imaging and PDT into an ultra-small (~20nm) nanoscaffold. (20) Intrinsic copper-64 labeling allows for pre-operative PET imaging of PLP delivery as well as sensitive and accurate detection of various primary and metastatic tumor types in mouse models. Its smaller-size and prompt intracellular uptake compared to previously reported porphysome nanoparticles (21, 22) result in more efficient nanostructure accumulation and dissociation in tumors. This fast accumulation and dissociation releases fluorescence and photodynamic reactivity, which are highly-silenced in intact PLP, providing an attractive activation mechanism for low-background NIR fluorescence imaging and tumor-selective PDT. (20)

Here, we propose novel strategies for effective HNC management using PLP-based imaging and intervention in a large animal model (orthotopic VX2 rabbit HNC tumor model) (Figure 1): 1) PET imaging for pre-operative detection of primary tumor and metastatic disease including lymph node mapping; 2) selective fluorescence activation in tumors to enable intra-operative visualization of tumor tissue and metastatic lymph node drainage for surgical guidance; 3) for the first time, proof of complete ablation of primary tumors and effective prevention of tumor metastasis by image-guided PDT. Importantly, with both PLP administration and PLP induced-PDT, minimal toxicity to healthy tissues was observed. Therefore, the intrinsic multimodal and biomimetic nature of PLP confers high potential as a cancer theranostic agent for clinical translation and targeted cancer therapy.

**Materials and Methods**
1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids Inc. (AL, USA). Porphyrin-lipid (pyropheophorbide-lipid) was prepared using previously reported protocols.(22) Cholesteryl oleate (CO) was obtained from Sigma-Aldrich Co. (MO, USA). The ApoA-1 mimetic peptide (R4F), Ac-FAEKFKEAVKDYFAKFWD, was purchased from GL Biochem Ltd. (Shanghai, China). Cell culture media was obtained from the ATCC (American Type Culture Collection, Manassas, VA). Fetal bovine serum (FBS) and trypsin-ethylenediaminetetraacetic acid (EDTA) solution were all purchased from Gibco-Invitrogen Co. (CA, USA). ⁶⁴CuCl₂ was obtained from Washington University (MO, USA).

**PLP preparation and ⁶⁴Cu labeling**

A lipid film was prepared by evaporation of lipid mixtures in chloroform under nitrogen. The lipid mixture for PLP consists of 0.9 μmol porphyrin-lipid, 2.1 μmol DMPC and 0.3 μmol cholesterol oleate. The completely dried lipid films were hydrated with 1.0 mL PBS buffer (150 mM, pH 7.5) and sonicated (Bioruptor®) at low frequency (30s on/ 30s off) for 30 cycles at 40 °C. R4F peptide (2.3 mg, 5 mg/mL) was titrated into the rehydrated solution. After overnight shaking at 4 °C, the solution was filtered with 0.1 μm membrane (Millex®, Sigma-Aldrich) to gain PLP. PLP was labeled with ⁶⁴Cu using the previously reported method (20). Briefly, PLP was 1:1 diluted with 0.1M NH₄OAC (pH 5.5), and then mixed with ⁶⁴CuCl₂ solution and incubated at 37 °C for 60 min. The mixture was purified with the centrifugal units (30K, Amicon® Ultra) and the radiochemical purity and yield were assessed.

**PDT study on mouse xenograft models**

All animal experiments were performed in compliance with University Health Network guidelines. The PDT efficacy of PLP was investigated on KB xenograft mice. Four groups were included in the treatment study: blank control group, PDT laser alone, PLP injection alone, and
PLP plus PDT laser treatment (n=3 for each group). When the tumor reached 4.0 to 5.0 mm in diameter, PLP was intravenously injected into mice for the PLP group and PDT group at a dose of 5 mg/kg of porphyrin content. At 24 h post-injection, mice were anesthetized and imaged by the in vivo Maestro imager to evaluate tumor accumulation and porphyrin fluorescence activation. Tumors were subsequently irradiated with a 671 nm laser (DPSS LaserGlow Technologies, Toronto, Canada) with a light dose of 75 J/cm², laser intensity of 100 mW/cm², and irradiation area of 9 mm diameter. Temperature changes of tumors were monitored using an infrared thermal camera (Mikroshot, LUMASENSE Technologies). Tumors from each treatment group were harvested at 24 h post-treatment, sliced and subjected to H&E staining and TUNEL staining analysis. Cells showing DAB staining positive and with morphology of cytoplasmic condensation, loss of cell–cell contact and shape of shrinkage were counted as TUNEL positive cells.

**VX-2 buccal carcinoma rabbit model**

The VX-2 buccal squamous cell carcinoma model was developed using the method described elsewhere.(23, 24) Briefly, the tumor was harvested under sterile conditions from the freshly euthanized rabbit, placed in Hanks Balanced Salt Solution (HBSS, Sigma), washed twice with sterile HBSS, cut into small pieces, and stored at -80°C until used. To obtain a single tumor cell suspension, the tumor pieces were thawed, minced and pressed through a 70 μm cell strainer. 300 μL of a high-density single cell suspension (~ 5 × 10⁶/mL) was injected into the buccinators muscle (Buccal area) of an anaesthetized New Zealand white rabbit (2.8-3.3 kg).

**Pharmacokinetic study on HNSCC rabbits**

About 2 weeks after tumor induction when the tumor size reached 1.5~2.0 cm, rabbits were intravenously injected with ⁶⁴Cu-PLP through a catheter in the marginal ear vein (0.33
mg/kg for porphyrin, ∼5 mCi). Arterial blood was collected at 5 min, and 0.5, 1, 4, 8, 21, 30 h post-injection (n=4). The radioactivity of the plasma was determined as a function of concentration on a gamma-counter (Wizard 1480: PerkinElmer Inc., MA, USA). The clearance half-life was determined by log-linear regression.

**PET/CT imaging of HNC rabbits**

At 24 h post-injection of $^{64}$Cu-PLP (0.33 mg/kg for porphyrin, ∼5 mCi), rabbits were anesthetized and subjected to PET imaging on a microPET system (Focus 220: Siemens, Munich, Germany), and CT imaging on a microCT system (Locus Ultra: GE Healthcare, U.K.) following a 5 mL injections of Omnipaque 350 (GE Healthcare, Mississauga ON). PET/CT Images were registered and merged using Amira (FEI Visualization Sciences Group, Bordeaux, France). Volumes of interest were drawn on the merged CT images with Inveon Research Workplace (Siemens, Munich, Germany), and the standard uptake values (SUV) of $^{64}$Cu-PLP were quantified from the registered images.

**Biodistribution and ex vivo fluorescence imaging of PLP on HNC rabbits**

After PET/CT imaging of the organs of rabbits including tumor, lymph node, salivary gland, lung, heart, liver, muscle, spleen, and kidneys, the organs were excised, weighed, and radiolactivity was measured on a gamma-counter. Organ uptake was calculated as percentage of injected dose per percentage of total animal mass of the sample (SUV) for each rabbit. Ex vivo fluorescence imaging was performed with the Maestro (Caliper Life Sciences, MA, U.S.A.) with a yellow filter setting (excitation:575-605 nm; emission:≥ 645 nm detection, 200 ms exposure time).

**Rabbit tissue pathology and microscopic imaging**
Frozen tissue sections were fixed and treated with DAPI, H&E and Pan-Cytokeratin staining. High-resolution images of the stained sections were acquired on a scanning laser confocal microscope (TISSUEscope 4000, Huron Technologies).

**Intraoperative Fluorescence Imaging**

Real-time fluorescence-guided surgery on VX-2 rabbits was performed with an in-house fluorescence imaging endoscopy system (650 ± 20 nm excitation, 700 ± 25 nm emission) at 24 h after intravenous injection of 4 mg/kg of PLP. Guided with fluorescence, tumor and suspicious lymph nodes were dissected until only non-fluorescent nodules were left on the surgical bed of the animals.

**PDT on HNC rabbits**

Four groups of VX-2 rabbits were included in the treatment study: blank control (n=3); PDT laser alone (n=3); PLP injection alone (n=3); PLP plus PDT laser treatment (n=4). When the tumor size reached ~ 300 mm³, PLP was intravenously injected into rabbits for the PLP group and PLP-PDT group (4 mg/kg of porphyrin dose). For PDT treatment, rabbits were anesthetized and subjected to a two-step PDT procedure at 24 h post-injection. The first step was a straight laser irradiation (671 nm) on the exterior surface of the tumor with a light dose of 125 J/cm², laser power of 200 mW and irradiation area of 15 mm in diameter. Temperature changes of tumors during laser irradiation were monitored using the infrared thermal camera. The second treatment step involved the insertion of a fiber-optic cable (9 mm diffuse laser fiber) into the tumor to irradiate from the interior of the tumor with a light dose of 120 J/cm² and laser power of 100 mW. After the treatment, rabbits were put under a standard protocol of care and the tumor growth was continuously monitored with microCT scanning. Terminal surgeries were performed on rabbits when the tumor size reached 5000 mm³. All four PLP-PDT rabbits were found tumor-
free at about 30 days after treatment. They were euthanized at day 34-36 post-PDT for further evaluation of treatment efficacy.

To evaluate the toxicity of the treatment, comprehensive biochemistry and haematology blood test of all treated rabbits were performed at 24 h post-injection, right before PDT, 1 week post- and 3 weeks-post- PDT treatment. After terminal surgery, tissues from tumor region and other major organs were harvested at 24 h post-treatment, subjected to H&E and Pan-cytokeratin staining, and imaged with Aperio ImageScope to determine the remnant of malignancy. Two experienced pathologists evaluated all histopathology slides for malignancy identification and tumor eradication confirmation.

**Statistical analysis**

The Student’s t-test (two tailed) was used to determine significant differences in TUNEL and toxicity study. P-values less than 0.05 were considered significant.

**Results**

**PLP preparation and its multifunctional nature**

PLP nanoparticles were prepared by assembly of porphyrin-phospholipids and DMPC-phospholipids (3:7 mol/mol) on a cholesteryl oleate core in aqueous solution, followed by size-constraint with an 18-amino acid ApoA-1 mimetic peptide R4F to obtain an ultra-small spherical structure with a 20 nm average diameter (**Figure 1**). The porphyrin fluorescence of the PLP was effectively silenced (> 95 % quenching efficiency) due to intermolecular fluorescence quenching caused by high density packing of the porphyrin molecules. Photodynamic activity was also suppressed in the intact PLPs.(20) Both fluorescence and photodynamic reactivity can be promptly restored by disruption of the nanostructure. The intrinsic metal chelating capability of
the porphyrin allowed for direct labeling with the radionuclide copper-64 through a robust procedure to generate $^{64}$Cu-PLPs with a labeling yield greater than 98%.

**PLP-PET enabled detection of primary tumor and sentinel lymph nodes in HNC rabbit model**

We investigated the feasibility of PLPs for HNC detection and treatment using a VX-2 buccal squamous cell carcinoma rabbit model which is a particularly inimitable model for developing lymphatic metastases within approximately 2 weeks after tumor induction. The blood clearance profile of $^{64}$Cu-PLP was fitted to a two-compartment model, showing a favorable slow half-life of $27.7 \text{ h}$ (Figure 2a). PET imaging was performed on VX2 rabbits at 24 h post intravenous injection of $^{64}$Cu-PLP (0.34 mg/kg of porphyrin, ~5 mCi) to match its biological half-life and radionuclide half-life ($^{64}$Cu $t\frac{1}{2} = 12.7$ h). As shown in the PET/CT co-registered image (Figure 2b, Figure S1), the tumor and sentinel lymph node (SLN) were clearly distinguishable with high contrast. Moreover, the 360° view of the image clearly displays the drainage from tumor to lymph node (Video S1), which is unprecedented for intravenous administration of organic nanoparticles. Consistent with the rendered image, tumor and SLN showed significantly higher standard uptake values (SUV) quantified from PET volume-of-interest (VOI) measurements compared to that of surrounding muscle, which was $3.58 \pm 0.53$, $2.57 \pm 0.53$ and $0.35 \pm 0.02$ respectively (n=5, P< 0.05, Figure 2c).

The distribution of $^{64}$Cu-PLPs in major organs was further evaluated by gamma-counting, which revealed similar distribution patterns in healthy tissues of PLP in tumor-bearing and healthy rabbits (Figure 2d). The relatively high SUV of the liver ($9.34 \pm 0.92$ SUV and $10.54 \pm 1.68$ SUV for tumor-bearing and healthy rabbits, respectively) was likely due to hepatobiliary clearance of $^{64}$Cu-PLPs. However, this high uptake would not affect HNC detection considering
the relatively remote location of the liver from the head and neck region. The average uptake of tumor and SLN from gamma-counting was $3.14 \pm 0.26$ SUV and $2.21 \pm 0.26$ SUV respectively (Figure 2d, n=5), which is consistent with their corresponding SUVs from PET image VOI quantification (Figure 2c). The SLN of tumor-bearing rabbits exhibited significantly higher uptake than that of healthy rabbits ($0.87 \pm 0.13$ SUV, n=3, P<0.01) and is likely due to the elevated lymphatic flow and the presence of metastatic lesions that were identified by H&E analysis and Pan-Cytokeratin (PanCK) staining (Figure S2). Therefore, $^{64}$Cu-PLPs were capable of delineating malignant SLNs from healthy ones.

Ex vivo fluorescence imaging of the resected tissues further confirmed the significantly higher accumulation and fluorescence activation of PLPs in the tumor and draining SLN of tumor-bearing rabbits (Figure 2e). Negligible fluorescence signal was observed in the salivary glands despite the relatively high accumulation of $^{64}$Cu-PLPs (Figure 2e). This is likely due to PLP non-specifically accumulating in salivary glands like other PET imaging agents (e.g. $^{18}$F-FDG), but remaining intact and non-fluorescent. These results indicate that by utilizing both PET and fluorescence imaging, PLP was able to provide complementary information for accurate detection of metastatic lymph nodes and potentially could be employed for image-guided resection of lymph nodes with low background fluorescence of the salivary glands.

Fluorescence-guided resection of primary tumor and metastatic disease

By taking advantage of the selective fluorescence activation of PLPs in the tumor and metastatic lymph node(s), we evaluated the capacity of PLPs for fluorescence intraoperative guidance of surgical resection of primary tumors and SLN(s) in tumor-bearing rabbits. As shown in Figure 3a, the tumor (with skin intact) was sufficiently fluorescent for visualization compared to surrounding tissue under an in vivo fluorescence imaging system. Upon raising the skin flap
during surgical exploration, the tumor was exposed and was clearly delineated by the porphyrin fluorescence (Figure 3b). Guided by the fluorescence, all suspicious malignancies around the cheek were surgically removed. The surgical bed exhibited negligible fluorescence signal, suggesting complete tumor resection (Figure 3c). The resected tissues were confirmed to be malignant by histological analysis (Figure 3d). The porphyrin fluorescence in the tissue histology slides corresponded well with cancer cell morphology and positive PanCK staining, indicating that PLP fluorescence highlighted the primary tumor with considerable specificity and accuracy at the cellular level (Figure 3d). Likewise, PLP fluorescence also delineated the draining SLN in vivo (Figure 3e). Notably, the lymphatic network from the primary tumor to SLN, and to regional lymph nodes was exquisitely mapped by the fluorescence signal (Figure 3f). Following the orientation of the lymphatic network (zoomed-in images, positions 1-5 in Figure 3f), the secondary positive lymph node and lymphatic spread pattern was identified. Histology confirmed metastasis in the lymph node and strong porphyrin fluorescence was observed in the PanCK-positive area, indicating uptake of PLP in the metastatic region (Figure 3g). Altogether, PLP fluorescence not only clearly delineates the primary tumor and malignant lymph node(s), but also the regional lymphatic network, which may potentially aid in nodal staging of HNC patients and reveal malignant lymph nodes prior to resection and pathological analysis.

**PLP-enabled PDT induced apoptosis**

By knowing that the fluorescence can be promptly restored upon tumor accumulation, we next investigated if the photodynamic reactivity of PLPs can be activated efficiently for PDT. PDT effectiveness was first evaluated in a KB-xenograft mouse model with four groups including blank control, PLP control, laser control and PLP-PDT group (n=3). At 24 h after
intravenous injection of PLP, high-contrast porphyrin fluorescence was observed in tumor regions, indicating selective tumor accumulation and porphyrin fluorescence activation (Figure 4a). The fluorescent tumors subsequently received localized PDT laser treatment (671 nm, 100 mW/cm², 75 J/cm²) that did not cause significant temperature increase in the tumor or the surrounding area, demonstrating that there were no photothermal effects of the treatment (Figure 4b). The PDT efficacy was examined by H&E histological analysis at 24 h post-PDT, which showed that only the PLP-PDT group experienced cellular damage in the tumor, while the control groups did not exhibit similar changes (Figure 4c). The PLP-PDT induced cell death was further confirmed by a TUNEL assay, which demonstrated that PLP-PDT significantly enhanced cell apoptosis in the tumor (73.2 % positive) compared to the control groups (1.5 % positive for blank control, 6.9 % positive for laser control and 7.1 % positive for PLP control) (Figure 4d). In addition, no obvious cellular damage or morphology changes were observed in the healthy organs of the PLP-PDT treated groups in comparison to the blank controls (Figure S3), indicating that PLP-enabled PDT does not cause toxicity to healthy tissues.

The long-term therapeutic effect of PLP-PDT was assessed on HNC rabbits. Tumor-bearing rabbits with average tumor sizes of 300 mm³ were categorized into four groups, including blank control (n=3), laser only control (n=3), PLP only control (n=3) and PLP-PDT group (n=4). As shown in Figure 5a, a two-step laser irradiation strategy was used for the PDT at 24 h post-PLP injection in order to irradiate the entire tumor volume. The absence of significant temperature increase during the laser treatment confirmed no thermal effect of the treatment, precluding the concern that thermal effect may cause unintended side effects on neighbouring healthy tissues (Figure S4). PLP-PDT caused scarring around the tumor beginning from 24 h post-PDT, until 26 days post-treatment. Ultimately, all PLP-PDT rabbits had no
palpable tumor at day 34 post-treatment (Figure 5b). Post-treatment tumor volumes were quantitatively determined by the volumetric measurement of 3D microCT images. The PLP-PDT group showed a slight tumor size increase within the first week post-treatment, which was likely attributable to the expected inflammatory response and edema caused by PDT (Figure 5c). However, the tumor size gradually declined from 6 days post-PDT until no tumor was detected on day 34 post-PDT. In contrast, the control groups that received either laser irradiation or PLP administration alone showed accelerating tumor growth, similar to the blank control, indicating that neither of them induced any therapeutic effects (Figure 5d, Figure S5). The control groups reached the end point (tumor volume > 5000 mm$^3$) at day 6 for blank control, day 8 for laser control, and day 9 for PLP control (Figure 5d), respectively. PLP-PDT enabled complete tumor ablation was further confirmed by pathological analysis, which demonstrated that the tissues resected from the original tumor area at terminal surgery did not exhibit pathological cell morphology, in addition to its negative PanCK staining (Figure 5e). Notably, although they did not receive direct laser irradiation, the lymph nodes of the PLP-PDT group showed a gradual decrease in size from 14 days post-PDT (Figure S6). All lymph nodes from the PLP-PDT group were found metastasis-free at 34 days post-PDT evidenced by pathology and PanCK staining analysis (Figure 5f). These results strongly suggest that for HNC subtypes that are surgically inaccessible or adjacent to critical anatomical structures, such as the oropharynx, nasopharynx, hypopharynx and for recurrence cases, PLP-PDT may serve as an alternative approach to radiation treatment and chemotherapy to increase therapeutic efficacy and decrease long-term toxicity. PLP-PDT appears to be exceedingly effective, highly localized, and allows for the preservation of healthy tissue function.

**PLP is a safe multi-functional nanoplatform**
The toxicity of PLP-PDT to rabbits was assessed by blood tests periodically (Figure 6a). The hepatic function of rabbits after treatment were normal with no significant changes, except for alkaline phosphatase (ALP), which showed a moderate decrease within the normal range (from 68.1 ± 8.66 to 43.5 ± 9.67 U/L) at 1 week after treatment and returned to the baseline level over time (normal range 12-98 U/L). Red blood cell level remained stable after treatment, indicating that there was no interference with the physiological regulation of endogenous porphyrin (heme). White blood cell counts also remained unaffected, suggesting that no immunogenic effects were caused by PLP. Post-mortem histological analysis on PLP-PDT rabbits did not show abnormal cellular morphology in the heart, lung, liver, spleen, adrenal or muscle (Figure 6b). These results suggest that PLP-enabled PDT treatment is a safe therapeutic approach.

Discussion

HNC management is often limited by inappropriate tumor detection. In this study, we have shown that PLP nanoparticles could enhance HNC diagnosis and improve therapeutic intervention and management. $^{64}$Cu-PLP enabled PET/CT imaging of primary tumors and lymphatic drainage from tumor to metastatic lymph node at 24 h post intravenous injection in a clinically relevant large animal HNC model. Although some non-specific uptake of $^{64}$Cu-PLP in the salivary gland was detected, selective fluorescence activation of PLP resulted in high fluorescence signals in the tumor and the subsequent lymphatic drainage network, while the salivary gland and healthy lymph node(s) displayed background-level fluorescence. Therefore, fluorescence imaging following PET imaging could have tremendous potential to enable tumor localization and determine the invasion status of the draining lymph nodes.

Lymph node metastasis is a significant indicator for low survival and poor prognosis and
is predictive of a higher risk for distant metastases,(25, 26) which are responsible for most HNC deaths.(27) Thus, accurate detection of lymph nodes metastasis close to the primary tumor is fundamental for appropriate treatment, especially for individuals diagnosed with oral cavity or oropharyngeal squamous cell carcinoma.(28) However, clinically, it is challenging to evaluate lymph node metastasis status reliably, which results in inappropriate treatment to many of HNC patients.(29) For example, elective neck dissection is currently recommended for all HNC patients with an occult metastatic rate greater than 20%–30%,(30) which actually might not be necessary for 60-70% of patients (31, 32) and is associated with high risks and morbidity.(33-35) The standard technetium-99-based SLN biopsy procedure currently suffers from several limitations including lack of real-time intra-operative visual information.(36-38) PLPs offer a promising method for accurately detecting malignant SLNs with pre-operative PET/CT imaging and complementary fluorescence imaging for non-invasive diagnosis, pre-operative stratification, and more accurate cancer staging. Additionally, PLP would provide insight of metastatic lymphatic pathways for the identification of unknown primaries and recurrent tumors with greater sensitivity to significantly improve cancer patients’ surgical outcome. Furthermore, reliable staging of SLNs with PLP would dramatically decrease unnecessary dissection of the neck.

HNC surgical management is often limited by inappropriate intraoperative tumor delineation and inability to visualize occult nodal metastasis, which leads to increased tumor recurrence, and decreased survival. PLP-enabled real-time intraoperative fluorescence imaging provides a useful tool for image-guided surgical resection of primary tumor and malignant lymph node(s) to achieve complete disease tissue resections.
PLPs are also capable of enhancing targeted delivery of PDT in tumor-bearing rabbits. PDTs may be particularly clinically advantageous for patients with tumors that are surgically inaccessible or adjoining to critical anatomical structures. Moreover, the absence of temperature changes during PDT is excellent for treating tumors close to tissues that are sensitive to heat. Interestingly, all of tumor-bearing rabbits at one month after PLP-PDT displayed pathologically non-malignant cervical lymph nodes, though lymph nodes did not receive directly laser irradiation. PLP administration combined with PDT did not result in detectable functional or histological side effects on rabbits. Therefore, the intrinsic multimodal and biomimetic nature of PLPs confer high potential for clinical translation as a cancer theranostic agent. In addition, the core-shell structure of PLP (hydrophobic core enveloped in hydrophilic shell) provides an amiable environment for stable loading of various cargos, such as chemotherapeutics and siRNAs, thus offering potential for additional chemo- or/and gene therapy. The exact mechanism of PLP uptake into tumors in vivo is still not well understood. One possible explanation for the high tumor selectivity of PLP is a combination of two factors. First, the ultra-small size of PLP (<30nm) might provide an advantage for efficient penetration through the permeable tumor vasculature into the tumor interstitial space, resulting the local enrichment of porphyrins within the tumor.(39) Secondly, porphyrin molecules have their own tumor affinity that could further drive their selective tumor uptake as reported previously.(40)

Conclusion

With the combination of PET imaging, real-time intraoperative NIR fluorescence guidance, and selective PDT intervention, PLPs hold great potential for cancer management. Direct labelling of PLPs with copper-64 enabled accurate, pre-operative PET/CT imaging of primary tumors, SLNs, and lymphatic drainage following intravenous administration. The
selectively activated fluorescence of PLPs facilitated the accurate delineation of tumors and metastatic lymph nodes. The feasibility of PLPs for intraoperative fluorescence-guided tumor and lymph node resection and tumor-selective PDT were validated in a large HNC animal model. Thus, PLPs provide a multimodal imaging and therapeutic platform that could enhance HNC diagnosis by integrating PET/CT and fluorescence imaging, and improve HNC therapeutic efficacy and specificity by tailoring treatment via fluorescence-guided surgical along with selective PDT.

Disclosure of Potential Conflicts of Interest

All authors disclosed no potential conflicts of interest.

Authors’ Contribution


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References


Figure 1. Schematic presentation of PLP illustrating its core-shell spherical structure (shown in the center) with TEM image showing its size around 20 nm. Surrounding images demonstrate its intrinsic multimodalities of positron emission tomography, fluorescence imaging and photodynamic therapy in a large rabbit HNC model.

Figure 2. PLP-enabled non-invasive detection of primary tumor and lymphatic drainage in rabbit HNC model; a) Pharmacokinetic profile of PLP in HNC rabbits (n=4); b) Representative PET/CT 3D image of HNC rabbit at 24 h after intravenous injection of $^{64}\text{Cu}$-PLP (red arrow: tumor, white arrow: regional lymph node); c) Distribution of $^{64}\text{Cu}$-PLP in muscle, tumor and lymph node quantified by PET volumetric analysis. The uptake was presented as standard uptake values (SUV). Tumor and lymph node uptake of PLP were significantly higher than the muscle uptake (n=4, P<0.05); d) Distribution of $^{64}\text{Cu}$-PLP in major organs in HNC rabbits (n=5) and healthy rabbits (n=3) measured by $\gamma$-counting; e) Ex vivo fluorescence of resected tumor, regional lymph node and other major organs of HNC rabbits after PET/CT imaging.

Figure 3. PLP-enabled fluorescence-guided resection of tumor and metastatic lymph nodes. In vivo fluorescence imaging of HNC tumor in rabbits at 24 h after intravenous injection of PLP: a) before incision with the skin intact; b) during surgery upon skin flap removal; c) post-surgery with the surgical bed non-fluorescent confirming the completion of the procedure; d) Representative H&E, Pancytokeratin staining and fluorescence microscopy of tissue slices of the resected tumor; e) Intra-operative fluorescence imaging of the sentinel lymph node upon skin flap removal; f) Lymphatic network mapped by PLP fluorescence. A series of zoom-in images (position 1-5) were acquired following the lymphatic flow from the sentinel lymph node to the regional lymph node; g) Representative H&E, pancytokeratin staining and fluorescence microscopy of tissue slices of the resected suspicious lymph nodes detected by PLP.

Figure 4. PLP-enabled PDT in a mouse xenograft model. a) Fluorescence activation of PLP in KB xenograft model at 24 h after intravenous injection of PLP; b) Averaged tumor temperature during laser irradiation for laser control and PLP-PDT groups (n=3 for each group); c) H&E and TUNEL staining of tumor sections from blank control, laser control, PLP control and PLP-PDT groups at 24 h post-treatment; d) Percentage of TUNEL positive cells out of total cells in the tumor region of all groups. Significantly higher cell apoptosis in the tumor was observed in the PLP-PDT group compared to the controls (n=3, P<0.05).

Figure 5. PLP-enabled PDT in HNC rabbits. a) Illustration of the 2-step PDT laser irradiation treatment strategy at 24 h after intravenous injection of PLP; Representative photographs (b) and axial CT images (c) of rabbits before and after PLP-PDT; d) Average tumor growth curve determined by volumetric CT measurements; Representative H&E and Pancytokeratin staining of tissues resected from the original tumor region (e) and lymph node resected (f) at day 34 after PLP-PDT. All tissues showed malignancy-free.

Figure 6. Evaluation of the toxicity of PLP-PDT. a) Blood assay of rabbits before PLP administration and 1 week and 3 week after PLP-PDT treatment (n=4); b) Representative H&E
staining sections of the main organs including heart, lung, liver, spleen, adrenal and muscle from PLP-PDT rabbits, indicating no side effects on healthy tissues after tumor ablation.
Fig. 2
Fig. 4

Panel a: Image of a mouse with fluorescence and thermal imaging.

Panel b: Graph showing temperature change over time with different treatments:
- PLP + Laser
- Laser Only

Panel c: Micrographs showing histological sections:
- H&E
- TUNEL
  - Blank
  - Laser Only
  - PLP
  - PLP + Laser

Panel d: Bar graph showing TUNEL positive cell percentage for:
- Blank
- Laser
- PLP
- PLP + Laser

** indicates statistical significance.
Fig. 5

PDT treatment strategy

<table>
<thead>
<tr>
<th>1st treatment</th>
<th>2nd treatment</th>
</tr>
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<tbody>
<tr>
<td><img src="image1" alt="Diagram" /></td>
<td><img src="image2" alt="Diagram" /></td>
</tr>
<tr>
<td>Tumor</td>
<td>Tumor</td>
</tr>
<tr>
<td>Straight laser</td>
<td>Diffusion laser</td>
</tr>
<tr>
<td>Laser</td>
<td>Laser</td>
</tr>
<tr>
<td>Irradiation</td>
<td>Irradiation</td>
</tr>
</tbody>
</table>

b) Day 1, pre-PDT | Day 5 | Day 21 | Day 34
Tumor

c) Day 1 | Day 5 | Day 21 | Day 34
CT scans

d) Tumor volume (mm³)
- Blank
- Laser
- PLP
- PLP+ Laser

e) Tumor
H&E
PanCK

f) Lymph node
H&E
PanCK

1 mm
500 μm
500 μm
Multimodal Image-Guided Surgical and Photodynamic Interventions in Head-and-Neck Cancer: From Primary Tumor to Metastatic Drainage

Nidal Muhanna, Liyang Cui, Harley Chan, et al.

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