ABSTRACT

Approximately 50% of all cancer patients undergo radiotherapy (RT) as part of their treatment regimen. However, the development of genetic mutations can severely impair cell death arising from radiation-induced DNA damage, leading to cancer recurrence and poor disease prognosis after treatment. Photodynamic therapy (PDT) offers an alternative approach to induce localized cancer cell death by damaging the cell and organelle membranes instead of relying on DNA damage. Yet, its clinical application is typically limited to surface-level lesions due to the poor tissue-penetration properties of visible light photons, which are required as an activation source.

Herein, we report the usage of calcium tungstate nanoparticles (CWO NPs) as energy transducers for potentiating PDT using X-ray photons from RT as the activation source. CWO NPs undergo "radioluminescence" wherein they can absorb incident high energy X-ray photons and emit lower energy UV-A and blue photons. Therefore, by intratumorally administering NPs, visible light photons can be generated *in situ* during RT. Since X-ray photons can penetrate tissue more efficiently than visible light photons, this strategy addresses the limitations of both RT and PDT.

Firstly, we demonstrate the compatibility of CWO NPs as energy transducers for activating two different photosensitizers: bilirubin (BR) and protoporphyrin IX (PPIX). In the case of bilirubin, we conjugated it with poly(ethylene glycol) (PEG) to form amphiphilic chains that self-assembled to encapsulate CWO NPs. For PPIX, CWO NPs were formulated by encapsulating them with poly(ethylene glycol-b-D,L-lactic acid) block copolymer (PEG-PLA/CWO NPs), while systemically delivering PPIX through its hydrophilic prodrug 5-aminolevulinic acid (ALA). In both scenarios, mechanistic studies revealed that X-ray irradiated CWO NPs generated sufficient blue light photons *in situ* to activate photosensitizers. This yielded significant improvement in cell-killing effects compared to RT alone, as demonstrated by clonogenic assays conducted in radio-resistant 4T1 and HN31 cell lines. The inherent non-toxicity of both formulations was also demonstrated through MTT assays. *In vivo* efficacy studies using intratumorally administered NPs demonstrated a significant improvement in tumor growth control and mouse survival compared to conventional RT treatments.

These studies highlight the potential of RT-PDT in achieving enhanced local tumor control. However, a notable limitation of this approach is its inability to effectively treat

metastatic lesions. To address this challenge, recent research has explored the combination of RT-PDT with immune checkpoint inhibition, particularly targeting indoleamine-2,3-dioxygenase (IDO) to induce systemic abscopal responses. To investigate this idea, we conducted efficacy studies in mice upon simultaneous treatment with Epacadostat, a small molecule IDO inhibitor. Although some improvement in tumor control and survival was observed across two separate studies, these results did not reach statistical significance. Consequently, further optimization of treatment schedules and immune checkpoint inhibitor delivery is necessary to obtain a more conclusive understanding of the compatibility of these treatment modalities.

Next, computed tomography (CT) imaging studies revealed that the current formulation of PEG-PLA/CWO NPs exhibits limited spreading in collagen-dense tumors like 4T1 when administered intratumorally. To overcome this, a modified formulation was developed by surface-functionalization with collagenase (Col-PEG-PLA/CWO NPs) to degrade collagen within tumors. The results suggest an approximately 2.4× improvement in intratumoral spreading volume relative to non-functionalized NPs. In the context of RT-PDT, this could imply significantly improved illumination of the tumor volume.

Lastly, one limitation of the current platform design is the requirement of intratumoral administration to deliver NPs. When administered systemically, less than 1% of NPs passively accumulate in the tumor. To address this, NPs were loaded into chimeric antigen receptor-functionalized neutrophils (CAR-neutrophils) differentiated from human pluripotent stem cells. Specifically, the receptors were modified with chlorotoxin peptide which is capable of selectively targeting glioblastomas. The results presented in this study demonstrate the optimal conditions for uptake of NPs by CAR-neutrophils. Furthermore, purification steps to separate NP-loaded CAR-neutrophils from unloaded NPs are described.

In summary, these studies describe the development and biological evaluation of two distinct NP platforms for RT-PDT. However, a few key limitations currently hinder the clinical translation of these technologies, including the inability to treat metastases, poor intratumoral spreading, and the need for intratumoral injections. Preliminary solutions have been identified for each of these challenges, providing a foundation for future investigations.