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RESEARCH ARTICLE

In Vivo Photoactivation Without “Light”: Use of Cherenkov Radiation to Overcome the Penetration Limit of Light

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Abstract

Purpose: The poor tissue penetration of visible light has been a major barrier for optical imaging, photoactivatable conversions, and photodynamic therapy for *in vivo* targets with depths beyond 10 mm. In this report, as a proof-of-concept, we demonstrated that a positron emission tomography (PET) radiotracer, 2-deoxy-2-[¹⁸F]fluoro-D-glucose (¹⁸FDG), could be used as an alternative light source for photoactivation.

Procedures: We utilized ¹⁸FDG, which is a metabolic activity-based PET probe, as a source of light to photoactivate caged luciferin in a breast cancer animal model expressing luciferase.

Results: Bioluminescence produced from luciferin allowed for the real-time monitoring of Cherenkov radiation-promoted uncaging of the substrate.

Conclusion: The proposed method may provide a very important option for *in vivo* photoactivation, in particular for activation of photosensitizers for photodynamic therapy and eventually for combining radioisotope therapy and photodynamic therapy.

Key words: Cherenkov radiation, Bioluminescence, Photoactivation, Caged luciferin

Introduction

Photoactivatable conversions such as photosynthesis in plants, photoswitchable chemical reactions, photoactivatable probes for imaging, and photosensitization in photodynamic therapy exist throughout nature and are exploited in many laboratory techniques. However, the poor tissue penetrating ability of light undoubtedly limits its broad application *in vivo* [1–3]. To address light penetration issues, long wavelength light sources are often used, but this can come as a tradeoff with excitation losses due to poor overlap

with absorption spectra. Ideally, incident light for these applications would exhibit both high-depth penetration and a high spectral overlap with photoactive molecule’s absorbance spectrum. An emerging way to address depth is to “move” the light source from outside of the tissue to the depth where it is co-localized with photoactive agents. Importantly, placement of an excitation “light source” *in vivo* can be accomplished noninvasively with Cherenkov radiation.

Recently, there has been growing interest in the use of photons from Cherenkov radiation for optical imaging [4–12] and for excitation of quantum dots and fluorophores *in vivo* [6, 8]. Charged particles such as β^+ and β^- which are generated from radioactive isotope decay can result in visible light with a broad energy range (ca. 6.1 to 1.23 eV, 200–1,000 nm). As a charged particle travels, it can polarize the molecules of its medium into a high-energy (excited)

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state. When the polarized molecules relax back to the ground state, they emit light in the form of radiation luminescence. The spectrum of radiation luminescence consists of continuous wavelengths throughout the ultraviolet and visible spectrum, with the intensity distribution inversely proportional to the square of the wavelength [4, 5]. The use of this light for *in vivo* optical imaging has, to date, been limited to the lower energy and intensity portion of the continuous spectrum. To the best of our knowledge, harnessing the higher intensity portion of the spectrum (200–400 nm) has not yet been demonstrated.

Photoactivatable conversions, such as photosynthesis in plants, photoswitchable chemical reactions, photoactivatable probes for imaging, and photosensitization in photodynamic therapy are widely observed in nature and have been applied in many chemical biology applications [13]. We hypothesized that a radioactive isotope that produces charged particles and thus Cherenkov radiation could be used as an “internal light source” to photoactivate caged compounds *in vivo*. The radiation luminescence in the higher energy and intensity portion of the spectrum could be considered as an alternative for external ultraviolet light, which is necessary for photoactivation (uncaging; Fig. 1a). Recognizing that many medicinal applications of caged drugs have been impeded by the poor penetrating ability of the light source [13–16], we feel that this method could provide a critical step forward for *in vivo* diagnostics and therapy. Since radioactive isotopes can be tuned and localized to tissues within the body, the proposed photoactivation reaction should have no depth limitation. Obviously, the distribution of an administered radioisotope within tissue will be an issue, but one that is likely controllable

through chemical modifications. We anticipate that the use of Cherenkov radiation could have potential clinical value for therapies and diagnostics associated with many forms of photoactivatable conversions.

In this proof-of-concept study, we utilized 2-deoxy-2-[^{18}F] fluoro-D-glucose (^{18}F FDG), which is a metabolic activity-based positron emission tomography (PET) probe [17–19], as a source of light to photoactivate caged luciferin in a breast cancer animal model expressing luciferase. Bioluminescence produced from luciferin allowed for the real-time monitoring of Cherenkov radiation-promoted uncaging (Fig. 1a).

In this report, we used luciferin 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNP-luciferin) to demonstrate the *in vitro* and *in vivo* feasibilities of photoactivation with ^{18}F FDG. The 1-(2-nitrophenyl)ethyl functional group has been used widely as a caging group for various biological molecules [13–15]. To uncage/photoactivate 1-(2-nitrophenyl)ethyl-bearing molecules, the covalent bond between the active molecule and (2-nitrophenyl)ethyl group has to be cleaved by light (Fig. 1b). Normally, irradiation with 365 nm (ultraviolet) light is used to photocleave the bond and thus release the active form of a compound [13–15]. In this report, we demonstrated that the ester bond could be cleaved by ^{18}F FDG.

Materials and Methods

^{18}F FDG-Promoted Uncaging in Solution

DMNP-luciferin (25.0 μg , 50.0 nmol, Invitrogen, Molecular Probes) was suspended in distilled water (1.0 mL) and treated

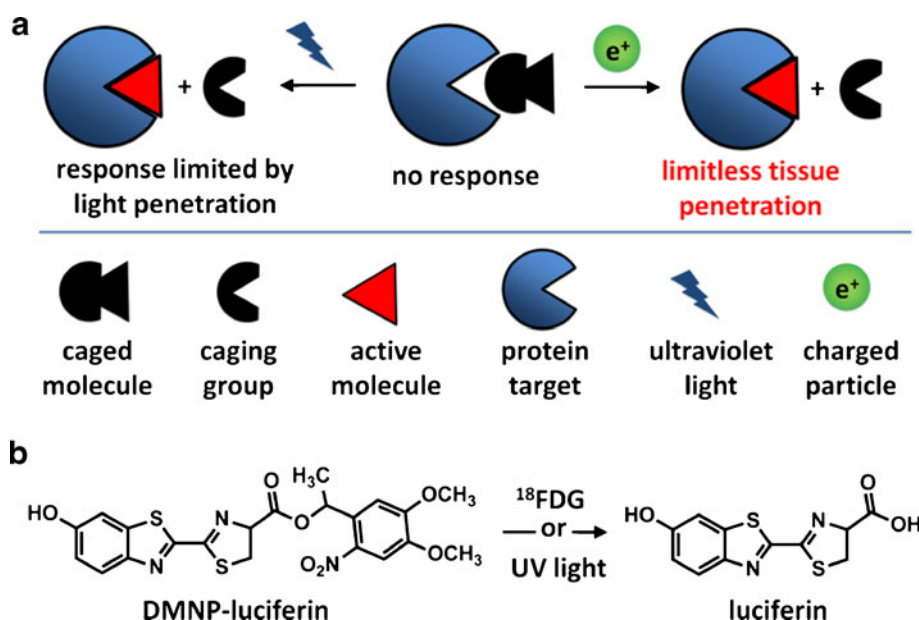


Fig. 1. A new method for uncaging photoactivatable compounds. **a** The illustration of the activation processes of caged active molecules. UV light can be used for uncaging but exhibits limited tissue penetration for *in vivo* applications. Charged particles from radioactive decay such as β^+ or β^- produce radiation luminescence capable of affecting the transformation. In principal, this approach has limitless tissue-penetrating capability. **b** The release of luciferin by uncaging reaction from DMNP-luciferin with UV 365 nm irradiation or with radiation luminescence generated from ^{18}F FDG.

with ^{18}F FDG (500 μCi , IBA Molecular) in water (0.1 mL) in the dark. The resulting suspension was incubated in the dark at room temperature for 12 h. LC-MS analysis of the sample was accomplished using a C18 reversed-phase column on a HP 1100 LC/MSD LC-MS spectrometer.

For light-promoted uncaging, a distilled water solution of DMNP-luciferin (25.0 μg , 50.0 nmol) was irradiated with UV 365 nm light (UVGL-58 lamp, 6 W). The solution in a 1.5-mL Eppendorf tube was placed directly under the lamp and irradiated for 5 min. The delivered UV dose was $\sim 1.8 \text{ kJ/m}^2$. After the UV treatment, LC-MS was recorded.

Cell Imaging

Luciferase-expressing breast adenocarcinoma cells (MDA-MB-231-luc-D3H1, Caliper) were seeded in a 96-well black clear-bottom plate (2,000 cells/well). Cells were incubated with a 100- μL solution from each of the three treatment groups ($n=4$ wells/group) as follows: (1) a solution (1.0 mL) of ^{18}F FDG (500 μCi) and DMNP-luciferin (25.0 μg ; for this cell study, before adding to cell media, the solution was kept for 24 h to deconvolute the signal from the radiation luminescence of ^{18}F FDG itself); (2) a solution (1.0 mL) of UV 365 nm irradiated DMNP-luciferin (25.0 μg); (3) a solution (1.0 mL) of DMNP-luciferin (25.0 μg) alone. Wells with the above-listed solutions without cells served as controls. The plate was incubated at 37°C for 5 min and then imaged using IVIS Spectrum (Caliper, Hopkinton MA). The imaging parameters were the following: block excitation; open emission filter; Bin=8, FOV=C, $f=1$, and exposure time=120 s. The quantification was based on photon radiance (photon per second per square centimeter per steradian).

Animal Imaging

Animal Preparation Nude mice (nu/nu, Massachusetts General Hospital Radiation Oncology breeding facilities) were injected with 2×10^6 breast adenocarcinoma cells (MDA-MB-231-luc-D3H1, Caliper) in the mammary fat pad. Tumors were allowed to grow for 3 weeks.

Imaging Procedure An IVIS Spectrum imaging system was used to record the images (exposure time, 120 s; bin=8, $f=1$, FOV=D). Tumor-bearing mice were divided into five groups. In the first group ($n=5$), each animal received an i.p. injection of luciferin (0.5 mg/mouse, Caliper, Hopkinton MA). Imaging was performed before injection and at 2, 10, 20, 30, 40, and 60 min post-injection. In the second group ($n=6$), each animal received an i.p. injection of DMNP-luciferin (0.5 mg/mouse). Images were acquired before injection and at 10, 30, 60, 90, and 120 min post-injection. The third group of mice ($n=3$) was injected i.p. with DMNP-luciferin and the whole bodies were irradiated with 365 nm UV lamp (UVGL-58, 6 W) for 3 min (UV dose was $\sim 10.8 \text{ kJ/m}^2$) before each image acquisition [20] of the five imaging time points. Images were acquired before injection and at 10, 30, 60, 90, and 120 min after injection. The fourth group of mice ($n=5$) was injected with an aqueous solution of ^{18}F FDG (400 μCi , 100 μL , i.p.). Images were acquired before injection and 10, 20, 30, 40, 50, 70, 100, 130, and 160 min post-injection (in Fig. 4b, imaging was started at 40 min after ^{18}F FDG injection). The fifth group of mice ($n=6$) was first injected with an aqueous solution of ^{18}F FDG (400 μCi , IBA Molecular, 100 μL , i.p.). Images were acquired before injection and at 10, 20, 30, 40 min post-injection. Next, the same animals were injected

with DMNP-luciferin (0.5 mg/mouse, i.p.). Images were acquired at 50, 70, 100, 130, and 160 min post- ^{18}F FDG injection (i.e., 10, 30, 60, 90, and 120 min post-DMNP-luciferin injection).

Image Analysis A region of interest (ROI) was selected to encircle the tumor in each mouse. The analysis was performed using ROIs of the same size. The quantification was based on photon radiance (photon per second per square centimeter per steradian).

Results

Photoactivation of Caged Luciferin with ^{18}F FDG in Solution

In this report, we first tested whether DMNP-luciferin [21] could be converted to its active form, luciferin, by using ^{18}F FDG (as a source of Cherenkov radiation luminescence) instead of 365 nm UV light (Fig. 1b). Indeed, incubation of an aqueous ^{18}F FDG solution (400 μCi in 1.0 mL) with DMNP-luciferin in the dark promoted the ester bond cleavage as revealed by LC-MS analysis (Fig. 2a, b), with a yield of about 25%. Positive control study where 365 nm UV irradiation was used to promote the cleavage to release luciferin yielded similar results (SI Fig. 1), with a yield of about 20%.

Photoactivation of Caged Luciferin with ^{18}F FDG in Cells

To verify that the product of DMNP-luciferin uncaged by radiation luminescence could serve as an active substrate for luciferase, we used luciferase-expressing breast adenocarcinoma cells (MDA-MB-231-luc-D3H1). First, we confirmed that DMNP-luciferin alone and the products of DMNP-luciferin uncaging by UV 365 nm light and radiation luminescence (via ^{18}F FDG) had no significant signal without the cells (Fig. 3a, three lower rows). To image the bioluminescence of the uncaged luciferin with cells, we treated DMNP-luciferin with 0.5 mCi ^{18}F FDG for 24 h to ensure the complete decay of ^{18}F before adding to the cell culture media. This was necessary to avoid the direct luminescence signal from ^{18}F FDG itself (the half-life of FDG is 109.8 min). Then, the solution was applied to the cells (Fig. 3a, top row). Cells treated with DMNP-luciferin irradiated with UV 365 nm light (Fig. 3a, second row from the top) and cells treated with DMNP-luciferin alone (Fig. 3a, third row from the top) were used as the positive and negative controls, respectively. We found that the signal from the cells treated with the product uncaged by ^{18}F FDG incubation was significantly higher than that from DMNP-luciferin-treated cells (Fig. 3b) and was similar to the treatment with the product of UV 365 nm irradiation, suggesting that radiation luminescence-promoted uncaging released an active form of luciferin from caged DMNP-luciferin. Signal from the control wells (Fig. 3a, three lower rows) was negligible ($<10\%$ of the positive control).

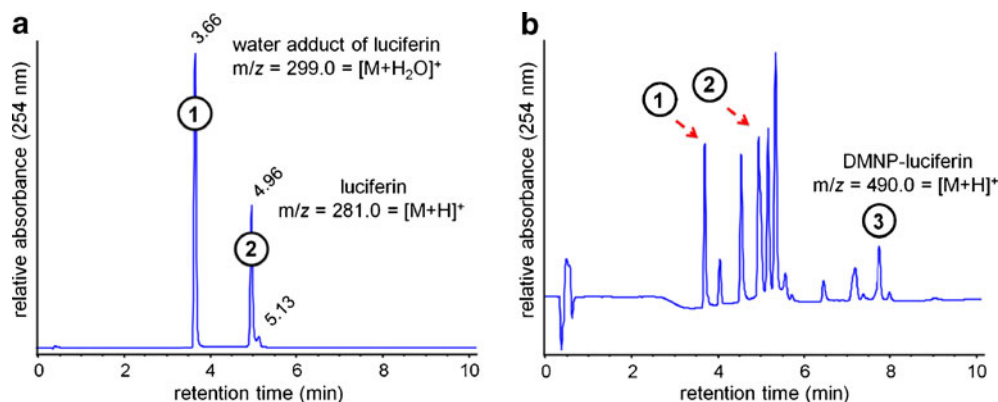


Fig. 2. LCMS characterization of luciferin and the products of ^{18}F (radiation luminescence) promoted uncaging of DMNP-luciferin. **a** LCMS data from analysis of D-luciferin standard (Caliper). Chromatogram has two major peaks, corresponding to a luciferin water addition product (retention time, 3.7 min) and luciferin (retention time, 5.0 min). The parent ion observed as major species. **b** Chromatogram of the products of ^{18}F FDG-promoted uncaging reaction of DMNP-luciferin. Peak 1 corresponds to luciferin hydrate; peak 2—the released luciferin; peak 3—DMNP-luciferin.

Photoactivation of Caged Luciferin with ^{18}F FDG Radiation Luminescence *In Vivo*

In this study, we hypothesized that ^{18}F FDG could be used *in vivo* for uncaging/photoactivation of a substrate in a model system. To test this hypothesis, we used mice bearing luciferase-expressing breast tumors. The experimental group of animals was first injected with ^{18}F FDG (0.4 mCi, i.p.) followed by DMNP-luciferin at 40 min after ^{18}F FDG administration (Fig. 4a). This time point was selected to allow for ^{18}F FDG accumulation, which normally reaches peak

levels in tumors at approximately 1 h post-injection [17]. For this group, the total signal in tumor site was the sum of radiation luminescence of ^{18}F FDG and bioluminescence of luciferin released by uncaging (Fig. 4a). Four control groups were used to support the findings from this study. The first control group included animals injected with luciferin as a positive control, which showed the expected BLI signal (SI Fig. 2). The second control group was injected with only ^{18}F FDG to measure the signal from the direct radiation luminescence of ^{18}F FDG (Fig. 4b). The third control group was injected with DMNP-luciferin and then subjected to *ex*

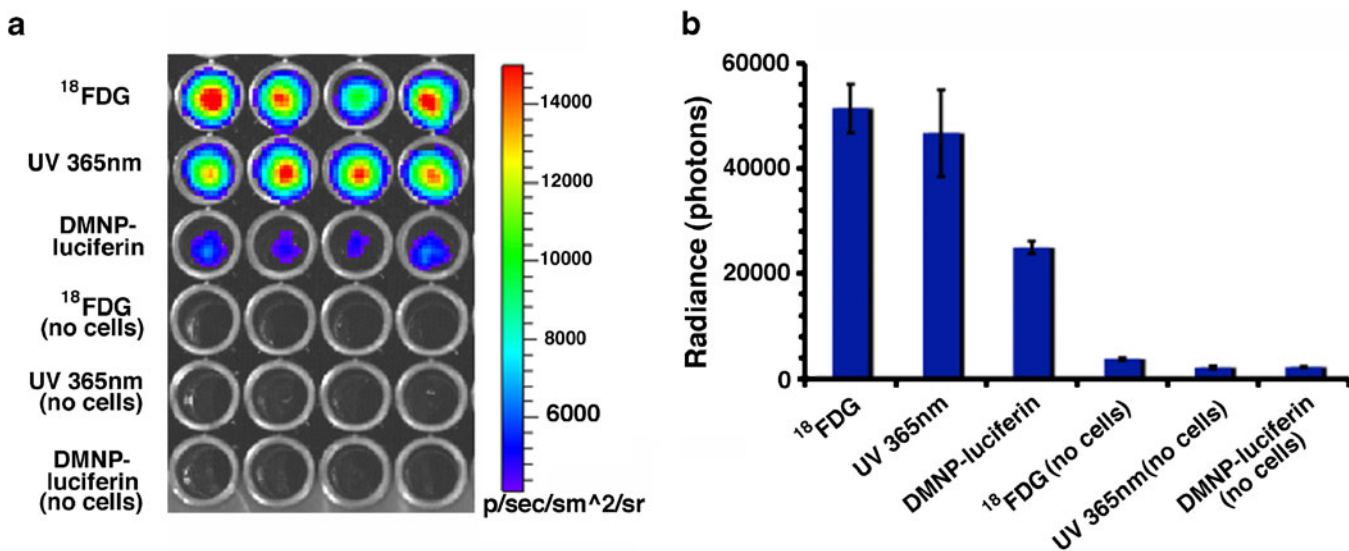


Fig. 3. Bioluminescence imaging of MDA-MB-231-luc-D3H1 cells (Caliper). **a** Rows 1–3 cells were treated with the products of ^{18}F FDG-promoted (radiation luminescence) uncaging of DMNP-luciferin, UV 365 nm irradiated DMNP-luciferin, and DMNP-luciferin (25.0 μg) alone. Rows 4–6 blank controls of the three solutions described above without cells. **b** Quantitative analysis of the image in (a). Radiation-luminescence-promoted uncaging and UV-light-promoted uncaging showed significantly higher bioluminescence signal than that of DMNP-luciferin alone.

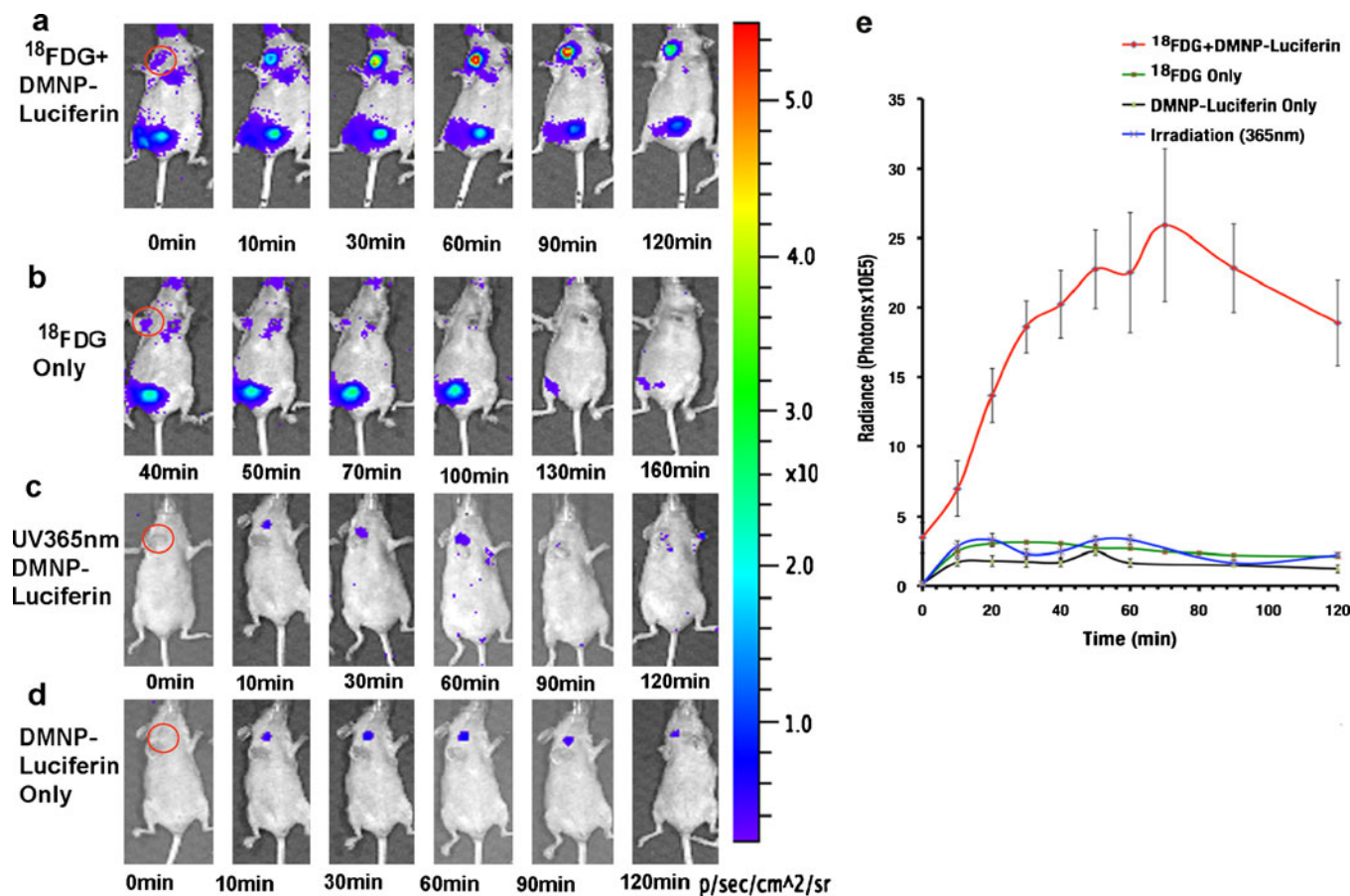


Fig. 4. Luminescence imaging of tumor-bearing mice subjected to different treatments regimens. The tumor in each group was marked with *red circle* in the first image. **a** Mice were first treated with ^{18}F FDG and then with DMNP-luciferin 40 min later. Images are from 0 min (pre-injection), 10, 30, 60, 90, and 120 min post-injection of DMNP-luciferin. **b** Mice were injected with ^{18}F FDG only. For comparison purposes, images shown here were obtained 40 min after ^{18}F FDG injection (for the whole-time ^{18}F FDG course, see SI Fig. 3). **c** Mice were treated with DMNP-luciferin and irradiated with UV 365 nm light for 3 min before each image acquisition. **d** Mice were i.p. injected with DMNP-luciferin only. **e** Quantitative analysis of the images in (a–d). The signal from the group treated with both ^{18}F FDG and DMNP-luciferin (*red line*) was significantly higher than that of DMNP-luciferin alone group (*black line*), UV irradiated group (*blue line*), or ^{18}F FDG only group (*green line*).

in vivo irradiation using 365 nm UV light (Fig. 4c). The final control group was treated with only DMNP-luciferin to test the inherent background signal (Fig. 4d).

Our results indicate that the bioluminescence signal from the tumor of ^{18}F FDG-injected animals significantly increased after DMNP-luciferin administration (Fig. 4a). The signal from tumors in the ^{18}F FDG-only control group increased gradually until peaking around 40 min. This is consistent with PET imaging results reported by Fueger (SI Fig. 3, full-time course is shown) [17]. The signal in the experimental group (DMNP-luciferin+ ^{18}F FDG) was 12.5-fold higher of that in the ^{18}F FDG-only group and reached its peak at 70 min post-DMNP-luciferin injection (Fig. 4e). Our data suggest that the radiation luminescence signal from ^{18}F FDG contributed to only about 8% of the total signal observed in the experimental group. The contribution of background to the signal as determined by the DMNP-luciferin-only control group (Fig. 4d) was approximately 5% of the highest signal observed in the experimental group (Fig. 4e). Taken together, our data suggested that ^{18}F FDG,

which had accumulated in the tumor, had a remarkable capacity to uncage DMNP-luciferin and thus release the active substrate for luciferase.

As expected, we found that irradiation with 365 nm light could indeed activate caged luciferin (Fig. 4c), similar to previously reported findings [20]. However, the signal in this group was only slightly higher than that of DMNP-luciferin-only group (Fig. 4d) and significantly lower than the signal from the experimental group, indicative of the low penetrating ability of UV light for *in vivo* uncaging.

Discussion

Although radiation luminescence from charged particles has been used successfully for *in vivo* optical imaging [4, 5], to the best of our knowledge, its application beyond imaging has not yet been explored. In this proof-of-concept report, we demonstrated that the phenomenon of radiation lumines-

cence could be used for photoactivatable chemical conversion. This suggests that a radioactive isotope could serve as an alternative “light source,” which could be used to overcome the tissue penetrating inadequacy of conventional light sources. We believe that our approach will open new directions for various light-promoted conversions, particularly for *in vivo* applications.

It has been shown recently that the 400–500-nm portion of the continuous spectrum of radiation luminescence could be used to excite quantum dots *in vitro* and *in vivo* [5–8]. However, the exact excitation mechanism of this phenomenon is still not clear. Cherenkov resonance energy transfer [6] to quantum dots and the excitation of quantum dots by radiation luminescence are likely the major contributors. In our experiments, the observed uncaging effect could probably be ascribed to: (1) Cherenkov (i.e., radiation luminescence), (2) direct interaction/polarization by positrons, (3) scintillation light from positron interaction with the media and container. However, it is not possible at this point to experimentally verify this assumption.

The application of the technology that we have described is certainly not limited to photo-uncaging of molecules *in vivo* with ^{18}F FDG. Previously, it was demonstrated that in addition to radionuclides that generate β^+ particles (such as ^{18}F and ^{64}Cu), β^- emitters such as ^{131}I and ^{90}Y could be used for optical imaging [5]. In this report, we demonstrated that radiation luminescence generated by ^{18}F FDG was capable of activating caged luciferin *in vivo*; thus, a natural extension of this work is the use of other radionuclides, which emit either β^+ or β^- particles, for the same reaction.

Similarly, this approach could be used for other photo-conversions including photodynamic therapy, which we are currently exploring. We expect this could increase the efficacy of such treatments. Most photosensitizers of photodynamic therapy have to be activated using light in the near-infrared range [1–3]. However, porphyrin compounds, the drugs that are most commonly used in photodynamic therapy, exhibit stronger absorption and excitation in the range of 400–600 nm [22–24]. Radiation luminescence could be used to excite molecules for photodynamic therapy in this higher energy portion of the spectrum.

We anticipate a possible synergy between photodynamic therapy and administration of therapeutic radioisotopes given that excitation of photosensitizers could occur by local radiation luminescence. This has not been explored despite the fact that photodynamic and radioisotope therapies are widely used in combination for surface tumors. To date, the synchronized combination of the two has not been practiced widely. We feel that concurrent treatment with a therapeutic radioisotope with unlimited depth penetration and photodynamic therapy using a photosensitizer could be synergistic and could produce clinical benefits not only for surface tumors but also for deep tumors. By extension, proton therapy, which both uses and produces charged particles (such as positrons [25]), could likely be harnessed

to excite a photosensitizer in photodynamic therapy, thus enhancing the overall therapeutic efficacy.

Conclusion

In summary, in this proof-of-principle study, we have demonstrated that Cherenkov radiation may have chemical and therapeutic applications beyond radiation luminescence optical imaging. We accomplished this using a straightforward DMNP-luciferin uncaging experiment, which showed that Cherenkov radiation from ^{18}F FDG could serve as an internal “light source” for photoactivation. We are currently exploring the application of this finding to new imaging and therapeutic strategies. However, challenges associated with optimization of radiation dose for maximum effect should be considered, especially when the radiation risk may limit the utility of this approach.

Conflict of Interest Statement. A.M. declares the payment for lectures outside the submitted work.

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