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A dinuclear ruthenium(II) complex excited by near-infrared light through two-photon absorption induces phototoxicity deep within hypoxic regions of melanoma cancer spheroids

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ABSTRACT: The dinuclear photo-oxidizing Ru_{II} complex $[\{Ru(TAP_2)\}_2(tpphz)]^{4+}$ (TAP = 1,4,5,8- tetraazaphenanthrene, tpphz = tetrapyrido[3,2-a:2′,3′-c:3′′,2′′- h:2′′′,3′′′-j]phenazine), $\mathbf{1}^{4+}$ is readily taken up by live cells localizing in mitochondria and nuclei. In this study, the two-photon absorption cross-section of $\mathbf{1}^{4+}$ is quantified and its use as a two-photon absorbing phototherapeutic is reported. It was confirmed that the complex is readily photo-excited using near infrared, NIR, light through two-photon absorption, TPA. In 2-D cell cultures, irradiation with NIR light at low power results in precisely focused photo-toxicity effects in which human melanoma cells were killed after 5 minutes of light exposure. Similar experiments were then carried out in human cancer spheroidsthat provide a realistic tumor model for the development of therapeutics and phototherapeutics. Using the characteristic emission of the complex as a probe, its uptake into 280 µm spheroids was investigated and confirmed that the spheroid takes up the complex. Notably TPA excitation results in more intense luminescence being observed throughout the depth of the spheroids, although emission intensity still drops off toward the necrotic core. As $\mathbf{1}^{4+}$ can directly photo-oxidize DNA without the mediation of singlet oxygen or other reactive oxygen species, photo-toxicity within the deeper, hypoxic layers of the spheroids was also investigated. To quantify the penetration of these phototoxic effects, $\mathbf{1}^{4+}$ was photo-excited through TPA at a power of 60 mW, which was progressively focused in 10 µm steps throughout the entire z-axis of individual spheroids. These experiments revealed that, in irradiated spheroids treated with $\mathbf{1}^{4+}$, acute and rapid photo-induced cell death was observed throughout their depth, including the hypoxic region.

Introduction

The potential of photodynamic therapy, PDT, as a possible treatment regime was first identified over a hundred years ago, [1-3] but it only became clinically available from the 1980s.[3-8] This light driven modality requires a photosensitizer, PS, which is essentially a prodrug. [9-12] In fact, photo-excitation of the PS results in the generation of reactive molecular species and it is these species that are responsible for the therapeutic action of PDT; they are created by two common pathways,[13-15] both of which begin with the PS excited into a triple state.

In Type I reactions, the photo-excited PS participates in redox processes leading to generation of reactive oxygen species (ROS) that trigger cell death. [16] On the other hand, Type-II reactions involve the direct energy transfer from the triplet state of the PS to dioxygen thus forming highly reactive singlet oxygen ($_1O_2$), that is capable of damaging virtually all biomolecules[17-19]

PDT is still an emerging treatment technique. Consequently, although PS leads are in development, only a few systems are currently licensed. [10,20] Nevertheless, PDT offers particular potential for the treatment of skin cancers.[21-23] However, whilst some FDA-approved PS molecules have been tested in preclinical melanoma models demonstrating tumor regression and prolonged survival rates,[24,25] remission and reoccurrence of melanoma have also been reported.[26-28] Full clinical trials on the treatment of choroidal melanoma and metastatic skin melanoma using a clinically established sensitizer Verteporfin, and also Chorin e_6, have been reported and photo-excitation at fluences of 100- to 120 J/cm_2 produced promising therapeutic responses.[29,30] However, one of the complications with classical PDT regimes arises from hypoxia. Due to rapid growth many tumors possess hypoxic regions, particularly at, and close to, their core.[31] Hence, incomplete treatment of tumors by PDT leading to relapse often involves these hypoxic regions where phototoxic effects are diminished. [32]

A second potential drawback to current PDT modalities used in treating solid cancers is poor selectivity toward cancer cells. One approach to improve tumor tissue targeting over normal stroma is to activate a PS through two-photon absorption, TPA. As this requires the absorption of two photons simultaneously, activation through TPA is

proportional to the square of the light source intensity and therefore only occurs at, or very close to, the focal point of the laser.[33] TPA also has an added advantage of providing a method to excite the PS in the near-infrared, NIR, therapeutic window where biological materials are most transparent, thereby providing PDT up to depths of 8 mm.[34] However, this requires a PS with an appreciable two photon absorption cross section, TPACS (σ) and unfortunately, current commercial PS molecules have low TPACS; for example for photofrin, σ = 3 BM at 800 nm, [35] which results in low therapeutic effects under two photon irradiation conditions.[36,37]

An ideal PS should be amphipathic in nature, so that it can readily transverse the cellular membrane but display good water-solubility, exhibit a minimum toxicity in the dark, and be photostable. [38,39] Additionally, a good TPACS would enhance treatment depths within tumors. [34,40] It is becoming apparent that these demanding criteria can be met by certain transition metal complexes; [41]. The fact that complexes of d_6-metal centers can display large optical nonlinearity is well established. [42-44]. At the same time, this class of complexes have been investigated as photosensitizers for PDT, [45-48] with Ru^{II} complexes attracting particular attention. [49-53]. In recent work the potential of d_6 complexes for TPA-PDT has also been delineated. [41,54-59]

In separate research, it has been found that new cancer treatments often fail clinical trials even after successful preclinical studies. [60,61] This is attributed to the fact that pre-clinical models usually involve either 2D cell cultures or animal models. 2D monolayer models are not capable of mimicking the complexity and heterogeneity of clinical tumors, [62,63] while *in vivo* tumors models involving non-human species can display discrepancies due to physiological differences between species. In response to these difficulties, humanized 3D models[64] are being developed; in particular, multicellular spheroids possess several features of *in vivo* tumors such as cell-cell interaction, hypoxia, drug penetration, response and resistance, and production/deposition of extracellular matrix. [65-69]

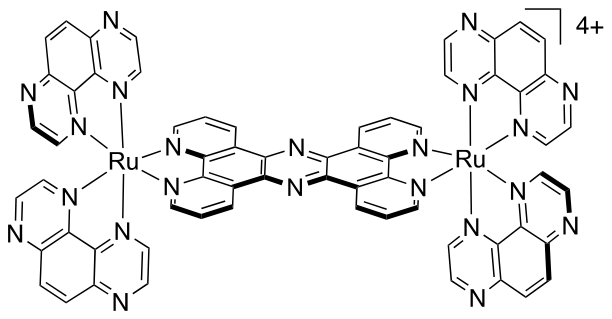

Figure 1. Structure of complex $\mathbf{1}^{4+}$.

As part of a program to identify novel therapeutics[70-73] and phototherapeutics[74,75] based on d_6-metal complexes, we recently reported on a novel dinuclear Ru^{II} complex $[\{Ru(TAP)_2\}_2(tpphz)]^{4+}$, $\mathbf{1}^{4+}$, Figure 1, (TAP = tetraazaphenanthrene, tpphz = tetrapyridophenazine) that binds to duplex and quadruplex DNA with high affinities.[76] This complex is water-soluble, stable in serum free media, and is internalized into live cells, where it predominately localizes in the nucleus and mitochondria of melanoma cells.

Earlier studies have established that complexes containing electron deficient $Ru^{II}(TAP)_2$ units possess strongly oxidizing $Ru^{II}\rightarrow TAP$ ^{3}MLCT excited states capable of *directly* photo-oxidizing guanine sites within DNA.[77-82] In agreement with these previous reports, detailed photophysical studies on $\mathbf{1}^{4+}$ revealed that both quadruplex and duplex DNA quench its excited state and this results in the generation of photo-oxidized guanine radical cation sites within the DNA. Furthermore, within 2-D melanoma cultures, we found that whilst $\mathbf{1}^{4+}$ is not intrinsically cytotoxic, it is activated by light; for example, irradiation with low fluences of LED light at 405 nm rapidly induced apoptosis in human C8161 melanoma cells, effectively resulting in zero cell-viability. [76]

Complex $\mathbf{1}^{4+}$ was developed as a photo-redox active analogue of the metal complex $[\{Ru(phen)_2\}_2(tpphz)]^{4+}$ (phen = phenanthroline) which has been successfully used as a live cell TPA optical imaging probe.[83] Given that $\mathbf{1}^{4+}$ is capable of directly damaging biomolecules without the mediation of $^1O_2$ or other ROS, that it localizes in both nuclei and mitochondria, which are specific targets for PDT,[9,48,84,85] and the fact that it is related to a system with an appreciable TPACS, we reasoned that this complex could be developed as a PS to initiate PDT effects at tissue depths where hypoxic occurs and normal PDT regimes are ineffective.

Herein, we report that $\mathbf{1}^{4+}$ functions well as a PS in TPA conditions and as a consequence the complex facilitates high spatial resolution phototoxicity within an engineered spheroid model of the highly invasive and spontaneously metastatic, recalcitrant human skin cancer, C8161 melanoma.[28] Furthermore, as the complex is luminescent, and its emission properties are compatible with optical microscopy, we can directly track the penetration of this theranostic deep into this tumor model.

Virtually no studies on metal complex photosensitizers and spheroids have been reported. The Chao group recently reported on Ru^{II}-complexes for TPA-PDT in HeLa-based spheroids but their work involved PSs that function through a classic oxygen-dependent Type II mechanism, whereas the photo-damaging mechanism of $\mathbf{1}^{4+}$ is independent of ROS and $^1O_2$. As the employed spheroid model contain healthy, hypoxic, and necrotic layers these studies have afforded insights into the effectiveness of the new PS in a realistic tumor environment containing both quiescent and proliferative areas. Consequently, the use of this preclinical human tumor model has provided detailed information on PS penetration, light penetration, responsiveness dosage and resistance area that could only previously be obtained by animal studies in immune compromised animals or clinical trials.

Results and Discussion

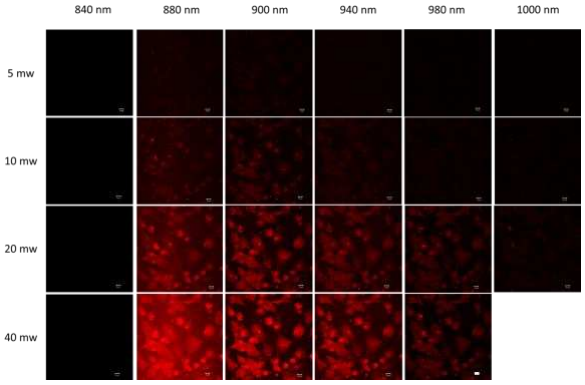

Figure 2. Emission from $\mathbf{1}^{4+}$ internalized within human melanoma cells following a TPA laser excitation over a wavelength range of 840 -1000 nm and power range of 5 – 40 mW (Scale Bar =10μm).

We first investigated the TPACS of $\mathbf{1}^{4+}$ through an adaption of a previously reported method[86] (see Materials and Methods for details). This led to an estimate maximum TPACS of σ = 90 GM, which is up to double that of commercial sensitizers that have been investigated for TPA-PDT.[87 88]

We then went on to investigate emission within the melanoma cells using TPA conditions, which revealed optimal emission outputs from the internalized complex on excitation at 850 - 900 nm (Figure 2), confirming that it could be photo-excited in these conditions using NIR light, well within the therapeutic window.

The TPA-PDT potential of $\mathbf{1}^{4+}$ was next investigated in a monolayer of human C8161 melanoma cells incubated with 100 μM of $\mathbf{1}^{4+}$ for 24 hour to ensure complete internalization. A discrete 250x250 pixel region of interest (ROI) was marked in a 512x512 pixel frame and irradiated with 900 nm laser light (10 and 20 mW). The ratio of live and dead cells were determined before and after the irradiation (every 5 minutes) using Syto-9 (cell indicator) and PI as live and dead cell markers respectively - see SI and Figure 3. This experiment revealed that at 10mW, 30min exposure time was required before any phototoxicity was observed in irradiated cells within the ROI (see SI), while at 20mW the entire ROI showed complete melanoma death after 5 minutes of exposure – Figure 3. The photoxic-effect was observed in melanoma cells only in the presence of Ru-Ru TAP, and not in response to two-photon-laser irradiation alone under identical conditions (See SI).

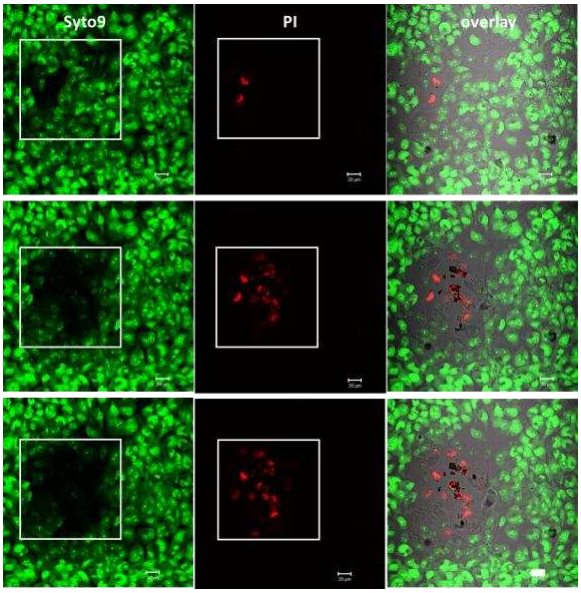

Figure 3. Two-photon photo-toxicity of $\mathbf{1}^{4+}$ in human melanoma cells treated with 1^{4+} (100 μM) after irradiation within the marked white square at 900nm. Live/dead cells imaged with Left column: Syto-9 (2 μM), Middle column: Propidium iodide (500 nM), and Right column: combined image. Recorded at 0 mins (top row), 5 mins (middle row), and 10 mins (bottom row) (scale bar = 20 μm).

Apoptosis and necrosis are cell death defined responses to a stimulus. In response to Ru(II) plus light activation cell death was studied as apoptosis more specifically using Annexin V and propidium iodide (PI). Furthermore, morphology changes of cells (by H and E) and cytoskeleton filament (F-actin) structures were also studied. Both apoptosis and necrosis was noticed within the cell population after PDT treatment. Cell size was decreased, actin filaments were damaged, with shrinkage in nuclear size and cell blebbing, suggesting necrosis as the prominent cause of death (See SI).

We have previously established that C8161 human melanoma spheroids cultured for 10 days (at an initial seeding density of 12,000 cells) have an outer proliferative area of approximately 100 μm in depth over a second layer of hypoxic cells that is also approximately 100 μm thick. The remaining central volume of the spheroid forms a necrotic core.[89] These features provide an opportunity to study the PDT effect of $\mathbf{1}^{4+}$ in a live multicellular 3-D tumor model that has both proliferative and hypoxic regions and thus assess the therapeutic effectiveness of this PS in low oxygen conditions. In these experiments we first explored photo-excitation of $\mathbf{1}^{4+}$ at various depths within tumor spheroids using conventional one-photon excitation at 458 nm and two-photon excitation (λex = 900nm). Although luminescence was observed throughout the 280 x 280 μm spheroid, indicating that the complex penetrates deep into the spheroids, emission intensity using either excitation regime noticeably decreases toward their center.

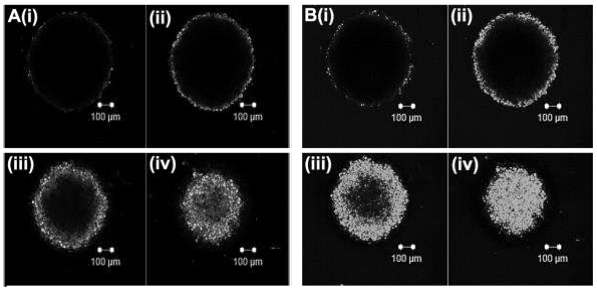

Figure 4 Comparing the luminescence of $\mathbf{1}^{4+}$ in a 280 280 µm C8161 human melanoma spheroids following: (A) one-photon excitation (458 nm) and (B) two-photon excitation (900 nm) at a power of 10 mW. Intensities measured at z-stack depths of: (i) 0 µm, (ii) 60 µm, (iii) 180 µm, and (iv) 240 µm. (scale bar = 100 µm).

This decrease in signal is probably caused by a combination of two effects:1) there is a concentration gradient for the complex that diminishes with spheroid depth and/or 2) light penetration into the center of the spheroid is too low to photo-excite $\mathbf{1}^{4+}$. Nevertheless, a comparison of emission produced by 1PA and TPA at analogous laser powers reveals that, as expected, NIR excitation results in higher intensity emission at depths (Figure 4). These results reveal that photoexcitation of the complex to a depth of ~240 ± 20 µm in the z-axis can be readily accomplished.

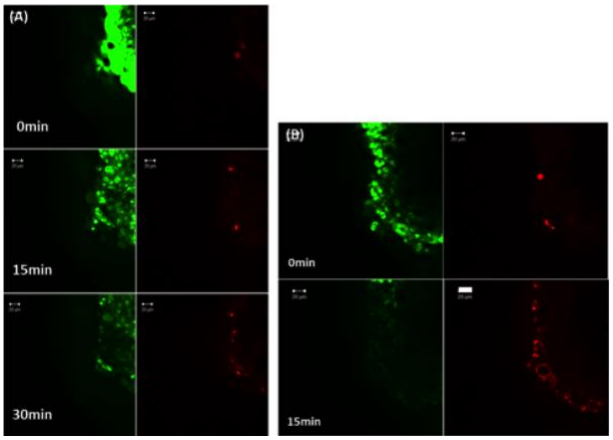

Figure 5. Two-photon PDT in a human melanoma spheroid outer proliferative area region with a 900 nm laser and powers of: (A) 40 and (B) 60 mW after treatment with 100 µM of $\mathbf{1}^{4+}$ for 24 hours. Left: live cells imaged with Syto-9. Right: dead cells imaged with PI. In spheroids untreated with $\mathbf{1}^{4+}$, illumination at either power produced no increase in cell death (scale bar = 20µm)

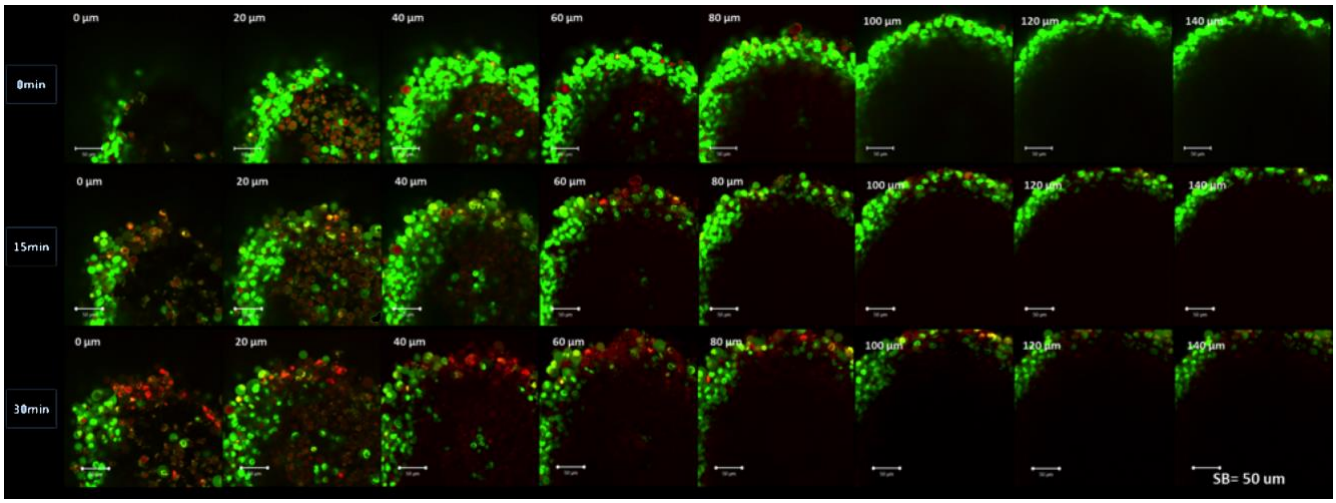

Figure 6. Figure 6. TPA-PDT on human melanoma spheroids cultured for 10 days. Spheroids were then allowed to settle on a 35mm plate overnight and incubated with $\mathbf{1}^{4+}$ (100uM), propidium iodide (500nM) and Syto-9 (2µM) in SFM for 24 hours. Spheroids were irradiated with a 900nm laser (power = 60mW) using a continuous z-stack scan (10µM apart) for 30 min. This was followed by a live and dead cell scan through the whole spheroid. Live/dead scan at specific depths before treatment revealing hypoxic/necrotic central region (before treatment, top). Analogous live/dead scans 15 minutes (middle) 30 minutes (bottom) after irradiance. (scale bar = 50 µm).

Consequently, the therapeutically effective dose of $\mathbf{1}^{4+}$ in melanoma spheroids using a two-photon excitation regime was evaluated. In particular, the effect of laser power on exposure to $\mathbf{1}^{4+}$ at a concentration of 100 µM was quantified. Initially excitation through laser irradiation at 900 nm was focused on the spheroid outer proliferative layer at fluxes of 20, 40 and 60 mW respectively. Live and dead cells were quantified before irradiation and then at every 15 minutes using Syto-9 and PI respectively – Figure 5.

These experiments showed that although no cell death occurred at 20 mW flux, at 40mW the proliferative region of the spheroid was eradicated after 30 to 45 min of exposure, Figure 5a, and irradiation at 60 mW produced the same effect after only 15 minutes of light exposure – Figure 5b. Negative control experiments confirmed that spheroids untreated with $\mathbf{1}^{4+}$ showed no increase in cell death on laser irradiation confirming that the photo-sensitization of the spheroids requires $\mathbf{1}^{4+}$. The differences in the irradiance dose for monolayer cultures compared to melanoma spheroids illustrates how the use of 3D spheroids provides an improved preclinical model for lead development.

Next we investigated the depth of the phototherapeutic effect of $\mathbf{1}^{4+}$ within the spheroid. Depending on cancer stage, human melanoma tissue thickness can vary, consequently PDT treatment will be optimized when a PS penetrates and activates at specific depths. To investigate this possibility, a

point on the surface of the spheroid was chosen and then laser irradiation at a power of 40 mW or 60 mW was focused at z-axis depths that progressively increased in 10 µm steps (Figure 6 and SI). Again, the ratio of live and dead cells in this z-stack was determined before irradiation and every 15 minutes thereafter using Syto-9 and PI. At 40 mW laser power, minimal evidence of phototoxicity at depth was observed after 30 to 45 min, but at 60 mW the continuous z-stack scan revealed cell death within 15 to 30 minutes. Significantly, although the phototoxic effect was at its greatest between 0 to 80 µm, cell death was observed throughout the entire z-axis depth of the spheroid - Figure 6C, showing that cell killing occurred in the hypoxic regions of the spheroid. A similar analysis of light-induced cell death across the entire 300x300 µm x-y plane at 60 mW revealed that phototoxic effects can be seen through the entire xy plan, even at depth.

Conclusion

Lamp light sources commonly used for conventional PDT are in an energy range of 60 to 200 J/cm$_2$ (commonly with flux rates of 150 mW/cm$_2$). [90] Although a high flux rate does reduce treatment time, it often causes hyperthermia and a reduced photodynamic effect due to oxygen depletion. [91-94] Furthermore, although lamp sources are suitable for treatment of large skin area they cannot be focused on small specific lesions. In contrast, as laser excitation volumes of a few femtoliters can be attained by two-photon excitation, high spatial selectivities can be obtained providing the potential to selectively treat melanoma without deleterious effects on surrounding tissue. Furthermore, excitation of a PS through TPA at 900 nm avoids a known mechanism of PDT resistance in melanoma; the abundance of melanin in such pigmented tumors means that light that should excite the PS is instead absorbed by melanin.[26,95,96] This "filter" effect means that transmittance through melanoma only occurs at wavelength about 700 nm.[27,97]

In summary, given that NIR photo-excitation of **1**$^{4+}$ through TPA allows phototoxic effects to be delivered with high precision into the depths of a therapeutically relevant and realistic tumor model to induce cell death even in hypoxic conditions, this complex is a highly promising lead for focused PDT regimes. By exploiting structural "ground rules" used to optimize TPACS, [33,57] new derivatives of this lead complex with enhanced two-photon excitation properties are currently being targeted. Ongoing work exploring the use of this PS in more sophisticated preclinical models will form the basis of our future reports.

Material and Method

Complex **1**$^{4+}$ was synthesized through a reported method[76]

Estimate of two-photon absorption cross-section

The value of two-photon absorption cross-section was calculated according to the following modified equation presented by Rebane and co-workers[86]

$$\sigma_s = (c_r/c_s)*()I_s/I_r)*(Fl\ \lambda_s/Fl\ \lambda_r)*(\sum I_r/\sum Fl_s)*(\phi_r\phi_s)*\ \sigma_r \quad (1)$$

where σ is the two-photon absorption cross-section, I is two photon intensity, Fl (one photon emission intensities at l) $\sum$Fl is the integrated one photon intensity, c is the molar concentration (r,s are reference and sample), and φ is the differential emission quantum yield in the spectral range: 608-620 nm. The differential emission quantum yield of Rhodamine B, was taken as 0.5. The value σ_r for Rhodamine B in MeOH was taken as 180 GM at 850 nm and 13 GM at 900 nm. [86] The differential quantum yield φ was obtained on a Jobin-Yvon Fluoromax 4 fluorimeter under one photon excitation. However, in order to achieve good spectral agreement for the reference under one- and two photon excitation, a lower solution concentration required for single photon measurements. The emission maxima and QY of Rhodamine B in MeOH is known to vary with sample concentration Therefore, the two-photon absorption cross section were calculated using Rhodamine B QY which corresponds to the solution used i) for two-photon measurements (QY = 0.4[5]) and ii) for single photon measurements (QY= 0.65[5]). The difference between the two calculations are approximately 50%. Thus a range of ~ 7 (@ 900 nm) to 90 (@850 nm) GM was estimated. These errors are due to several factors including the small values and uncertainties of rhodamine B two-photon cross section at 900 nm (which is 13 GM).

Human melanoma cell culture

The C8161 human melanoma cell line was isolated from an abdominal wall metastasis from a recurrent malignant melanoma menopausal woman (and a gift from Professor F. Meyskens UC Irvine (USA) via Dr. M. Edwards (University Glasgow, UK)). C8161 melanoma cells were grown in melanoma culture medium consisted of EMEM media (Sigma-Aldrich) supplemented with FCS (10%v/v), L-glutamine (2uM), Pencillin (100U/mL), streptomycin (100ug/mL) and Amphotericin (0.625ug/mL).

Intracellular localization in human melanoma cell line

C8161 melanoma cells were grown in 6-well plates for 24 hours at 37˚C. The cells were washed with SFM and incubated with 100 µM RuRuTAP for 1, 6, 12 and 24 hours in dark at 37˚C. For mitochondria, nucleus and lysosome co-localisation, the cells were further incubated with Mitotracker (200 nM), Lysotracker (100 nM) and DAPI (300 nM, for 15min) for an hour. Cells were washed with SFM and fixed with formaldehyde (3.7%, 15 min). Cell imaging was performed after cells were washed with PBS (thrice, 5min).

Photo-cytotoxicity using 405±20nm lamp in human melanoma cell line

C8161 melanoma cells were grown in 24 well plates (1x10$_5$ cells/well) for 24 hours at 37˚C. Cells were then incubated with increasing concentrations of **1**$^{4+}$ (0, 10, 50, 10, 200 µM) for another 24 hours in the dark at 37˚C. The compound was then removed and cells were washed with SFM and replenished with SFM. The plates were then irradiated (6.01 J (1 hour), 12.04 J (2 hours) and 18.03 J (3 hours), using a ThorLabs LED (M405LP1) with an emission of 405 nm (±20nm) and power output of 1500 mA. The LED was fixed on a metal stand (20cm from base). After the irradiance, the SFM was replaced with serum containing medium and cells were incubated for 18 hours. Alamar blue (Resazurin Na salt, 100 µM for 4 hours in SFM) was used to measure the cell viability.

Apoptosis assay (Annexin V and PI staining)

Apoptosis and/or necrosis cell death are two defined pathways. To distinguish the cellular death pathway after the stimuli (**1**$_{4+}$ at 100µM for 24 hours with and without 1-hour 405nm light irradiance) was studied using Annexin V Fluor-488 and PI (Invitrogen, V13241). Briefly, C8161 melanoma cells were seeded on 6 well plate for 24 hours, then treated with or without **1**$_{4+}$ (100µM for 24 hours in SFM), the plates were then irradiated or kept in dark for 1 hour using lamp (405nm). After which the plates were incubated with Annexin V and PI (10ug/mL and 500nM respectively) for 30 min and washed with annexin binding buffer (thrice, 5min). Cells were then fixed (formaldehyde 3.7%, 15 minute) and washed with PBS (x3, 5min). Counterstained with DAPI and imaged under a Zeiss LSM510 confocal microscope using an Achroplan water dipping objective (40X, NA 0.75, WD 2.1).

Two-photon absorption cross-section imaging

C8161 melanoma cells were incubated with **1**$_{4+}$ at 100 µM concentration (in SFM) for 24 hours. Cells were then washed with PBS (x3) and fixed with 3.7% formaldehyde (15 min). Luminescence images of **1**$_{4+}$ within were taken between 800 to 1000 nm by confocal microscopy (attached to Ti-sapphire Chameleon FD900 laser) and an Achroplan objective 40X/0.75 W, Scan speed of 6 r). The average optical power of each wavelength studied was altered to four different powers (5, 10, 20, and 40 mW). The resultant emission images were collected at λem = 630-700nm from varied excitation wavelengths and four different power settings. To attain ideal cross-section value the emission profile was used to calculate corrected fluorescence emission intensity using ImageJ.

Uptake and distribution 1$_{4+}$ in 3D melanoma spheroids

Melanoma spheroids were formed using C8161 human melanoma cell lines using a liquid overly method as described previously.[89] Briefly, an initial cell seeding density of 12K were cultured in a 96-well plate coated with agarose gel (1.5% w/v) for 10 days (at 37˚C and 5% CO_2).

To measure **1**$_{4+}$ distribution through the melanoma spheroids (MCTS), spheroids after 10 days culture were removed and transferred to 35mm glass bottomed dishes (3-4 MCTS in each dish) and allowed to incubate at 37°C (5% (v/v) CO2) overnight to settle. **1**$_{4+}$ at 100 µM concentration was incubated for a period of 24 hours. Before analysis, MCTS were washed with SFM (x3) and kept immersed in SFM during data collected upon two-photon excitation (λex = 900nm, λem = 630-700nm). A Zeiss LSM510 META upright confocal microscope, connected to a two-photon class 4 tuneable Ti-sapphire Chameleon laser (FD900, Coherent) and an Achroplan water dipping 40X objective lens (WD 2.1 mm, NA 0.75) was used to image the **1**$_{4+}$ emission through the full depth of the spheroid. Optical slices were taken at 10 µm apart in each sample to create a 3D (Z-stack) construct image. The depth of penetration was between first and last fluorescence optical slice. Frame size (512 x 512) scan direction (single), scan speed (6), laser power (20mW), and detector gain (874) was kept constant for all repeats.

Two-photon photo-cytotoxicity in melanoma cells

Human melanoma C8161 cells were seeding on a 35 mm dish plate (5x10$_5$ cells/well). After incubation for 24 hours the cells were treated with or without **1**$_{4+}$ at 100 µM concentration (in SFM) for 24 hours. The cells were washed with serum free culture medium (x3) and replenished with live and dead medium (propidium iodide (PI at 500 nM) and Syto-9 (2 µM) in SFM) for 15 minutes and through the length of time of experiment. The monolayer of cells was imaged for live cells (Syto-9, λex = 488nm (Ar-ion), λem = 500-550nm) and dead cells (PI, λex = 543 nm, λem = 565-615 nm). Live and dead image were taken from the same area (512x512 pixel) after every 5 minutes of irradiation at 900nm at 10 and 20 mW (scan speed= 6) on a marked region of interest (250x250 pixel). Irradiation was carried using a Ti:sapphire laser (Cameleon, Coherent) connected to a Zeiss confocal microscope (LSM510) using an Achroplan water dipping objective (40X, NA 0.75, WD 2.1).

Two-photon photo-cytotoxicity in melanoma spheroids

Photo-cytotoxicity in 3D melanoma spheroids was also imaged using the same method discussed above. However, the laser power for irradiation was increased to 20, 40 and 60mW. After attaining the ideal cytotoxic response at 60mW at a single optical slice, spheroids were irradiated with a 60mW 900nm 2-photon laser using a continuous z-stack (10 µM optical slice apart) scan (scan speed = 6), irradiance dose though the whole thickness of spheroid was followed by live and dead cell scan through the whole spheroid.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

Intracellular localization and uptake in melanoma cells; apoptosis and necrosis caused by PDT on melanoma; Two-Photon photo-toxicity without **1**$_{4+}$; Imaging showing TPA-PDT of whole thickness of melanoma spheroid

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