Photo-Chemotherapy of Infrared Active BODIPY-Appended Iron(III) Catecholates for in vivo Tumor Growth Inhibition

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Methods for Cellular Experiments.

**MTT Assay:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the photocytotoxicity of the complexes. In this assay, the mitochondrial dehydrogenases of viable cells cleaved the tetrazolium rings of MTT forming dark purple insoluble formazan crystals that were soluble in DMSO and was quantified from spectral measurements. Approximately, $5.8 \times 10^4$ BT474luc cells were plated separately in two different 96 wells culture plate and incubated with various concentrations of 2 from 3.15 to 100 μM in 1% DMSO/Dulbecco’s modified Eagle’s medium (DMEM) for 4 h in the dark. After 4 h of incubation, the media containing compounds was removed and replaced with DPBS buffer for one set of the cells which were exposed to red light ($\lambda = 600$-720 nm, light dose = 30 J cm$^{-2}$), using Waldmann PDT 1200 L, whereas the other set was kept in the dark for same time period using standard protocols. After exposure to light, DPBS was removed and replaced with fresh medium and incubation was continued for a further period of 16 h in dark for the plate thus making the total incubation time of $\sim 21$ h. After the incubation period, 5 mg/mL of MTT (20 μL) was added to each well and incubated for an additional 3 h. The media was removed entirely from the wells and then DMSO (200 μL) was added and spectral measurement was taken at 570 nm using TECAN microplate reader. Cytotoxicity of the complexes was measured as the percentage ratio of the absorbance of the treated cells to the untreated controls. The IC$_{50}$ values were determined by nonlinear regression analysis (Graph Pad Prism 6). Data were obtained by using three independent sets of experiments done in triplicate for each concentration.
**Cellular Localization:** By using confocal microscopy the intracellular localization of the fluorescent complexes 1 (10 μM) in 1% DMSO/DMEM was investigated using confocal microscope (Zeiss LSM 880 with Airyscan) with an oil immersion lens having a magnification of 63X. About 5 x 10^4 BT474luc cells were plated on glass cover slips in 12 well tissue culture plates and incubated at 37 °C and 5% CO₂ atmosphere for 24 h. BT474luc cells were then treated with the complexes for 4 h in dark and fixed. Cells were later incubated with DAPI (1 mg/mL) for 5 min to stain the nucleus. The cover slips were subjected to confocal microscopy after being mounted on slides. Similar procedure was used for sub-cellular localization without any prior fixation. Live cells were stained with Mitotracker® Deep Red (MTR, 100 nM) and incubated for 20 min at room temperature and then observed under the microscope. Multiple images were recorded, and experiments were done in duplicates to confirm the results.

**DCFDA Study:** Cellular reactive oxygen species (ROS) was detected by 7'-dichlorofluorescein diacetate (DCFDA) assay.² Cellular ROS oxidizes cell permeable DCFDA generating a fluorescent DCF having emission maxima at 528 nm. The percentage population of cells generating ROS was determined by flow cytometry analysis. BT474luc cells were incubated with the complex 2 at its IC₅₀ value (6 μM) for 4 h and then irradiated with red light (λ = 600-720 nm, light dose = 30 J cm⁻²) using Waldmann PDT 1200 L. The cells were harvested by trypsinization and a single cell suspension was prepared. The cells were subsequently treated with 1.0 μM DCFDA (solution prepared with DMSO) in dark for 20 min at room temperature. The distribution of DCFDA stained BT474luc cells was obtained by flow cytometry in the FL-1 channel.
**Animal Studies**

All animal experiments with mouse models were done in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Mice were maintained in pathogen-free facility with a 12-hour light dark cycle and *ad libitum* access to standard diet and water. Athymic *nu/nu* male mice (4-6 weeks old) were used for xenografting studies. For subcutaneous tumours, $10^6$ luciferase expressing BT474 cells were washed and harvested in DMEM and subsequently injected subcutaneously over the flank on each side in a volume of 0.1 ml. After all tumours were about 5 mm in diameter, the mice were divided into two groups; a test group which was treated with complex 2 at 5 mg/kg body weight, and a control group. The mice were protected from light. For each mouse, the right tumour was photo-irradiated with red light (685 nm ± 10%, 20 mW/cm$^2$, BTL 4110 premium with 50mW) 4 hour after injection of complex 2 and the left tumor was protected from any light exposure. The tumors were monitored on a weekly basis by tumor diameter measurements and *in vivo* imaging for 15 days. Tumour volume was calculated as $\pi LW^2/6$ (L is length and W is width of tumour).

**Bioluminescent Imaging**

Mice were injected intra-peritoneally with 1.5 mg of D-luciferin (15 mg/ml) in PBS and anaesthetized in an isofluorane chamber which is saturated with oxygen. Imaging was done within 5 min after injection with a Xenogen IVIS system coupled to LivingImage acquisition and analysis software. For BLI plots, photon flux was obtained by using a region of interest encompassing each tumour. The obtained value was normalized to the value obtained at Day 3 after injection of Complex 2.
Figure S1. Cell viability plots as obtained from the MTT assay in BT474luc cells treated with complex 2, for an initial 4 h incubation period in dark. Cells were exposed to red light (40 min, $\lambda = 600$-720 nm, light dose = 30 J cm$^{-2}$) (black symbols in the dark; red symbols in red light).

Figure S2. Scatter plot of merged green and red pixel intensities of complex 1 and mitotracker deep red (MTR) and their confocal merged image.
Figure S3. Survival curve of mice over a period of fifteen days showing a decline in survival of mice to ~40% by the fifteenth day.

References:

1. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer–drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107-1112.