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Chemistry & Drug Discovery

Supporting Information

Near-Infrared Intraoperative Chemiluminescence Imaging

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MATERIALS AND METHODS

Commercially available compounds were used without further purification unless otherwise stated. Tris(2,2'-bipyridyl)dichloro ruthenium(II) hexahydrate ($[Ru(bpy)_3]Cl_2 \cdot 6H_2O$) and ammonium cerium(IV) nitrate $(NH_3)_2Ce(NO_3)_6$ were purchased from Sigma Aldrich (St. Louis, MO, USA). Water (>18.2 $M\Omega cm^{-1}$ at 25 °C) was obtained from an Alpha-Q Ultrapure water system from Millipore (Bedford, MA, USA). High performance liquid chromatography (HPLC) purification and analysis was performed on a Shimadzu UFLC HPLC system equipped with a DGU-20A degasser, a SPD-M20A UV detector, a RF-20Axs fluorescence detector, a LC-20AB pump system, and a CBM-20A communication BUS module. HPLC solvents (Buffer A: 0.1% TFA in water, Buffer B: 0.1% TFA in MeCN) were filtered before use. HPLC analysis was performed on a reversed phase Phenomenex Gemini column (C18, 5 µm, 4.6 mm, 250 mm). Analysis were performed with this method: flowrate: 1 mL/min; gradient: 0-15 min 5-95% B; 15-18 min 95% B; 18-20 min 100%-5% B).

Mouse models

All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of MSKCC and followed National Institutes of Health guidelines for animal welfare. Female nude (outbred) mice at age 5 - 6 weeks and NU/J male mice at age 6 – 8 were purchased from Taconic Laboratories (Hudson, NY, USA) and Jackson Laboratories (Farmington, CT, USA) respectively.

Blood and Serum Stability

10 µL of a solution of $[Ru(bpy)_3]Cl_2$ (approx. 1M) was added to 250 µL of fetal bovine serum (FBS, Gemini Bio-products) or whole blood and incubated at 37 °C for increasing lengths of time (0, 30, 60, 120, 180 and 240 minutes). $[Ru(bpy)_3]^{2+}$ was then extracted by first adding 500 µL of MeCN, followed by centrifugation to pellet blood cells and proteins (5 minutes at 5000 rpm). The supernatant was collected diluted with 750 µL and prepared for HPLC injection by filtering. The blood and serum stability was quantified by HPLC.

Nebulizer Components

1 Harmon Face Values 3oz mini sprayer (Bed, Bath and Beyond, New York, NY, USA); 1 Hitech HS-82MG Mirco Servo Motor, 3.4kg/cm output torque @ 6V (Hitech RCD USA Inc., Poway, CA, USA); 1 Energizer 9V alkaline battery (Energizer Holdings Inc., St. Louis, USA); 2 28 cm plastic cable ties (General Electric Inc., Fairfield, CT, USA); 1 role of duct tape (3M Inc., St. Paul, MN, USA); 1 copper speaker cable 1m (RCA Inc., New York, NY, USA); 1 pencil Papermate Classic HB (Sanford L.P., Oak Brook, IL, USA); 1 littleBits w1 wire (littleBits, New York, NY, USA); 1 littleBits p1 power (littleBits, New York, NY, USA); 1 littleBits i2 toggle switch (littleBits, New York, NY, USA); 1 littleBits 011 servo (littleBits, New York, NY, USA); 3 Wood parts: 12.5x2.5x1.8 cm (A), 11x2.5x1.8cm (B), 12.7x10.7x1.8cm (C); 3 wood cutting screws (4x25 mm); 1 paper clip (Staples, New York, NY, USA); 2 20 cm plastic covered wire twist ties (Staples, New York, NY, USA); 1 10cm of 1/16" stainless steel rod (Metals Depot Int. Inc., Winchester, KY, USA).

Nebulizer Construction

Wood part A was attached upright in the center of part C using two screws. Wood part B was attached to the middle of part A using one screw so the B can still be moved a bit. Two holes were drilled trough the lower tip of the spray bottle trigger. The stainless steel rod was pushed through to form two loops one on either side of the trigger. The spray bottle was attached to wood part B using the two plastic cable ties. The littleBits 011 servo motor was cut off, and the cables of the littleBits servo control unit reconnected to the HS-82MG servo motor. The servo motor was attached to the top of wood part A using duct tape. The pencil was attached to the servo motor lever using the paper clip. The outermost parts of the pencil were connected tightly to the steel rod loops using plastic covered twist wires. The littleBits servo motor control units magnetic cable connector was cut off, reattached to the speaker cable and taped to wood part C. A littleBits w1 cable was cut in half and one part attached to the loose end from the speaker cable. The (magnetic) littleBits parts i2 and p1 were connected to the available w1. Figure S1 shows a photograph of the nebulizer.

ICI Detection limit determination

A droplet of 100 µL of a solution containing 260 µg, 52 µg, 26 µg , 5.2 µg, 2.6 µg, 520 ng, 260 ng, 52 ng, 26 ng, 5.2 ng or 2.6 ng $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$ was mixed with 100 µL of a solution of $(NH_3)_2Ce(NO_3)_6$ in water (25mM) on a microscopic slide. The IVIS bioluminescence reader was set up by initializing the Live Image 4.2 software. After signing in to the user profile the "Initialize" button was

clicked in the acquisition control panel. In the “Imaging Mode” “Luminescent” and “Photograph” were checked, and “Fluorescent” was unchecked. The “Exposure Time” setting for “Luminescent” was changed to 20 seconds. Remaining settings for “Luminescent” were set to “Binning”: Medium; “F/stop”: 1; and “Emission Filter”: Open. Settings for “Photograph” were set to “Exposure Time”: Auto; “Binning”: Medium; and “F/stop”: 8. “Subject Height” was adjusted according to imaging target. In the “Field of View” drop down menu stage position was changed to “B”. The experiment was then set up by placing a microscopic slide on a sheet of black construction paper on the floor of the imaging chamber. The nebulizer was prepared by detaching the plastic spray bottle from the wooden support and filling it with a solution of Et_3N (1:3 in water/ethanol) before reattachment. The nebulizer was placed inside the bioluminescence reader such that the spray flow was pointed towards the area of interest on the imaging subject, and also such that the nebulizer was not obstructing the camera’s field of view. Small black pieces of construction paper were placed over any potential hot spots (e.g. white marks on microscopic slides). At least 40 cm of the nebulizer remote chord were placed inside the IVIS chamber such that it did not interfere with the imaging subject, nebulizer, or the magnetic door latch. After closing the instrument door, image sequences were acquired. Specifically, after the camera shutter opened, three bursts of the solution of Et_3N were sprayed by switching the nebulizer on/off three times (0.24 ± 0.04 mL per spray burst). All images were quantified using Live Image 4.3 software. ROIs were drawn over the droplets and the average flux was reported. For background determination a water droplet was mixed with Ce-solution and sprayed with Et_3N-solution.

***In vivo* ICI after intravenous injection**

In vivo ICI was performed in female nude mice. Mice received $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$ (20 µg, 27 nmol, in 100 µL, in sterile PBS) via tail vein injection after sterilizing the tail with an alcohol pad. For all intravenous injections, mice were gently warmed with a heat lamp and placed on a restrainer. The tails were sterilized with alcohol pads, and injection took place via the lateral tail vein. Mice were sacrificed by CO_2 asphyxiation at 10 min p.i. The mouse abdomen were opened by a longitudinal cut and the organ of interest exposed superficially cut. The IVIS bioluminescence reader was set up by initializing the Live Image 4.2 software. After signing in to the user profile the “Initialize” button was clicked in the acquisition control panel. In the “Imaging Mode” “Luminescent” and “Photograph” were checked, and “Fluorescent” was unchecked. “Exposure Time” setting for “Luminescent” was changed to 20 seconds. Remaining settings for “Luminescent” were set to “Binning”: Medium; “F/stop”: 1; and “Emission Filter”: Open. Settings for “Photograph” were set to “Exposure Time”: Auto; “Binning”: Medium; and “F/stop”: 8. “Subject Height” was adjusted according to imaging target. In the “Field of

View" drop down menu stage position was changed to "B". The experiment was then set up by placing the mouse carcass on a sheet of black construction paper on the floor of the imaging chamber. The nebulizer was prepared by detaching the plastic spray bottle from the wooden support and filling it with a 25mM solution of $(NH_3)_2Ce(NO_3)_6$ in water before reattachment. The nebulizer was placed inside the bioluminescence reader such that the spray flow was pointed towards the area of interest on the imaging subject, and also such that the nebulizer was not obstructing the camera's field of view. Small black pieces of construction paper were placed over any potential hot spots (e.g. injection sites). At least 40 cm of the nebulizer remote chord were placed inside the IVIS chamber such that it did not interfere with the imaging subject, nebulizer, or the magnetic door latch. After closing the instrument door image sequences were acquired. After the camera shutter opened, three bursts of a solution of $(NH_3)_2Ce(NO_3)_6$ in water were sprayed by remotely switching the nebulizer on/off three times (0.24 ± 0.04 mL per spray burst). Quantification was performed the same way as for the determination of the detection limit using Live Image 4.3 software. Control animals were injected with PBS and treated the same way.

***In vivo* biodistribution**

Biodistribution studies were performed in female nude mice (n = 5). Mice administered with $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$ (20 µg, in 100 µL, in sterile PBS) via tail vein injection. Mice were sacrificed by CO_2 asphyxiation at 10 min p.i. and major organs were collected, sliced in half and sprayed with a solution of $(NH_3)_2Ce(NO_3)_6$ in water (25mM), using the same protocol and settings as described in the in vivo ICI section. All images were quantified using Live Image 4.3 software. ROIs were drawn over regions of interest and the average flux was reported. For background determination PBS injected control animals were used.

ICP-MS quantification

Female athymic mice (n = 3) at 10 weeks of age (Taconic, Hudson, NY, USA) were injected with $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$ (20 µg, in 100 µL, in sterile PBS) through tail veins. One mouse was injected with 100 µl PBS. Mice were sacrificed by CO_2 asphyxiation at 10 min p.i. and perfused with 20 ml PBS. Kidneys, liver, lungs, spleen, brain, heart, and femur muscle were collected from each animal, weighed, and preserved at -20 °C before ICP-MS analysis. Ruthenium (Ru) analysis and quantification was performed using inductively coupled plasma-mass spectrometry (ICP-MS). Tissues were weighed and acid digested before ICP-MS analysis. For each gram of tissue, 2 mL of nitric acid

and 1 mL of hydrogen peroxide was added. Samples were left at room temperature for 2 days and vortexed to aid tissue breakdown before the addition of hydrogen peroxide. After dilution in Milli-Q water, samples were centrifuged and then analyzed using an Agilent Technologies 8800 ICP-MS. Indium was used as an internal standard, added via t-piece before the nebulizer. Calibration standards were prepared from a 1000 µg/mL Ru certified reference material (Inorganic Ventures, Christiansburg, VA, USA). Blanks were inserted during the run, and all reagents used were ultra-trace grade.

Subdermal injection

For subcutaneous injections, NU/J male mice were anesthetized with 2% isoflurane (Baxter Healthcare, Deerfield, IL, USA) (2 l/min medical air), food pads were sterilized with alcohol pads and injected with 6 µg (8 nmol) $[Ru(bpy)_3]Cl_2 \cdot 6H2O$ (dissolved in 10 µL sterile PBS) left and 10 µL sterile PBS right. For comparisons between imaging modalities, the contralateral (right leg) was injected with indocyanine green (ICG) (13 nmol in 10 µL PBS) for parallel ICI and ICG imaging. 15 min p.i. mice were sacrificed by CO_2 asphyxiation.

***In vivo* lymph node imaging**

For *in vivo* imaging, skin was removed on both the control (right) and $[Ru(bpy)_3]Cl_2$ (left) side. The exposed area was subsequently sprayed with a solution of $(NH_3)_2Ce(NO_3)_6$ in water (25mM), protocol and settings as described in the *in vivo* ICI section. All images were quantified using Live Image 4.3 software. ROIs were drawn over regions of interest and the average flux was reported. For background determination the contralateral side was imaged.

***In vivo* lymph node quantification**

Following *in vivo* imaging, the left and right popliteal lymph nodes were excised and cut in half. They were then sprayed and imaged as described in the *in vivo* lymph node imaging section.

Statistical analysis

Unpaired t-tests were done to determine statistical relevance. $P < 0.05$ was considered statistically significant. Data is presented as mean ±SD.

SUPPLEMENTARY FIGURES

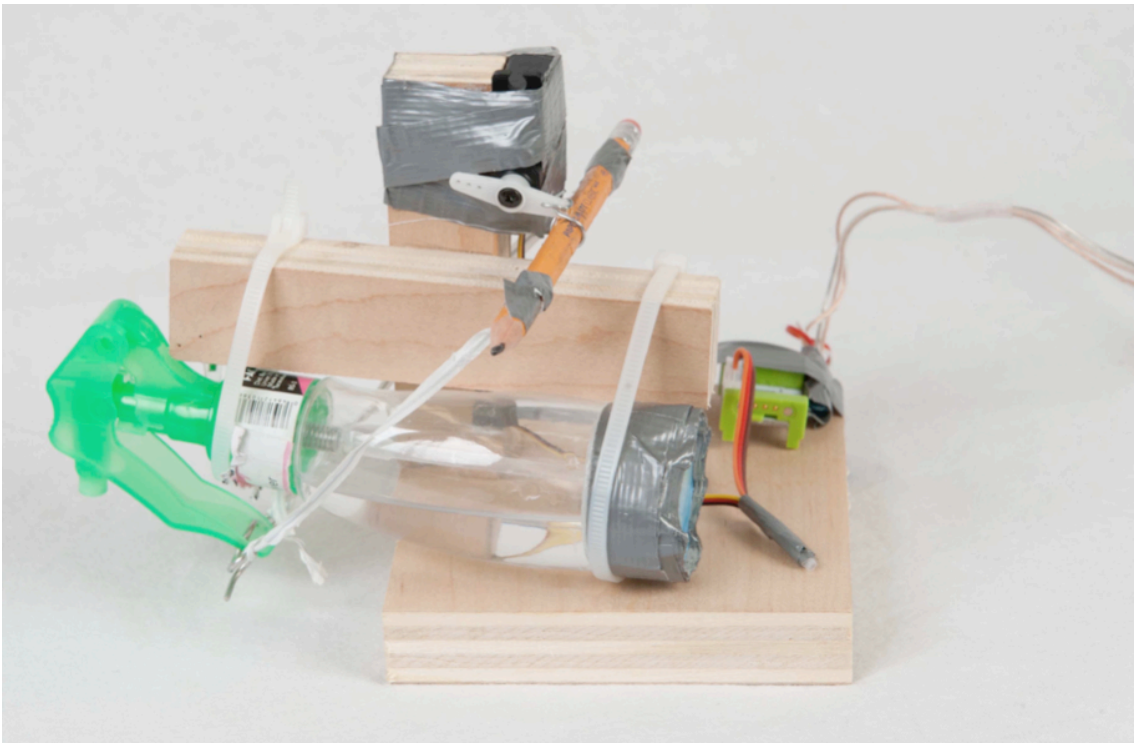

Fig. S1 The Nebulizer.

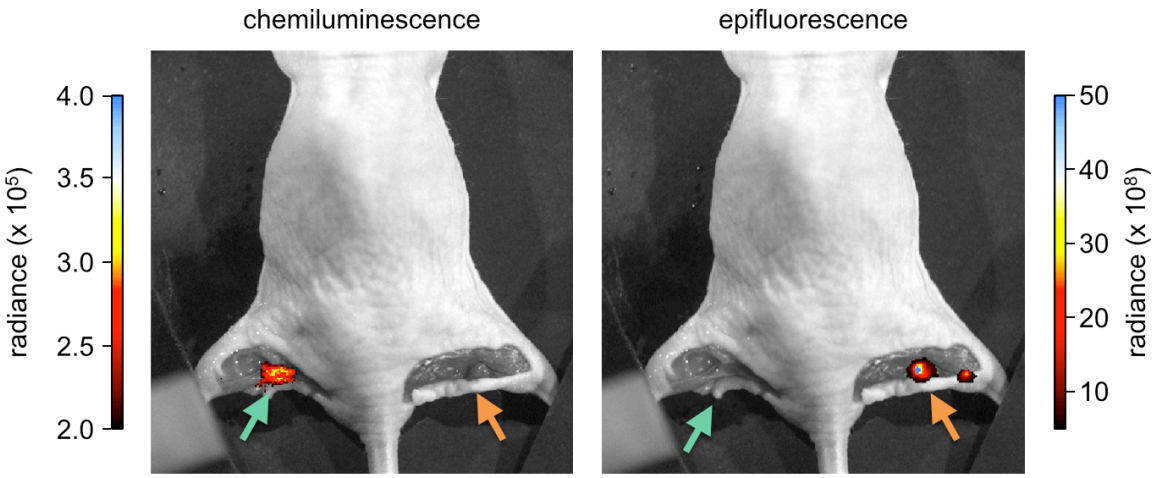

Fig S2. Parallel imaging of ICI and ICG. White light / chemiluminescence composite image (left) and white light / epifluorescence image (right) of a mouse injected with $[Ru(bpy)_3]^{2+}$ into the left hind limb (8 nmol in 10 µL PBS) and ICG into the right hind limb (13 nmol in 10 µL PBS) 15 min after injection. Turquoise arrow: popliteal lymph node lighting up with ICI imaging; Orange arrow: popliteal lymph node lighting up with epifluorescence imaging.

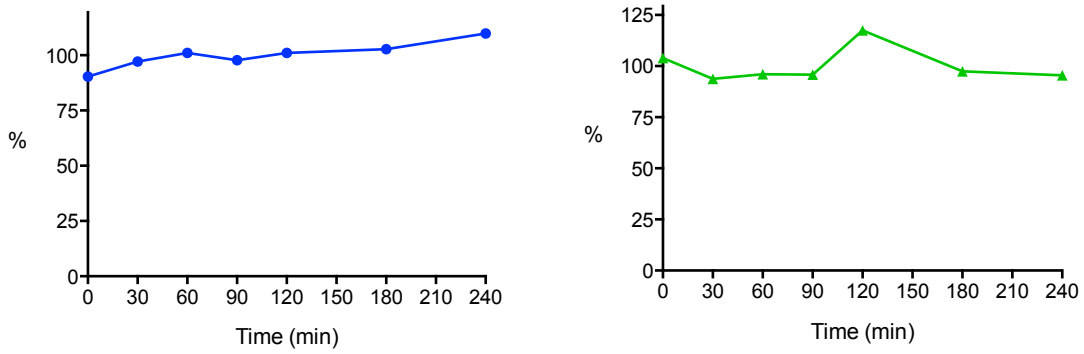

Fig. S3 Stability of $[Ru(bpy)_3]Cl_2 \cdot 6H_2O$ in FBS (left) in whole blood (right).