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Supporting Information

Metalloimmunotherapy with Rhodium and Ruthenium Complexes: Targeting Tumor-Associated Macrophages

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Experimental

Cell Lines and Cell Culture

MDA-MB-231(Female), and MCF-10A (Female), were provided by the American Type Culture Collection and were maintained in DMEM. Bone marrow macrophages (BMMs) for 3D experiments were extracted from femurs and tibiae of 10- to 12-week-old FVB mice. The cells were spun out by placing the bones, cut on each end, in a 0.5 ml tube with a 18G hole at the bottom, into a 1.5 ml tube into a microcentrifuge and pulsing at ~4,500g for 15 sec. Cells were resuspended in BMM growth media (MEMα containing 20% FBS and 30% L929 conditioned media as the source of MCSF). Cells were plated on four 100mm Petri dishes with 12 ml media per dish and left to grow undisturbed for 4-5 days in a 37 °C and 5% CO_2 incubator. All media for 2D experiments were supplemented with 5% penicillin/streptomycin. MDA-MB-231 media was supplemented with 10% FBS. MCF-10A media was supplemented with 5% HS, 20 ng/mL EGF, 0.5 mg/mL Hydrocortisone, 100 ng/mL cholera toxin, and 10μg/ml insulin. Cells were grown at 37 °C and 5% CO_2 atmosphere. M0 macrophages were differentiated from BALB/c femoral bone marrow cells and cultured in RPMI with 10% FBS, 1% penicillin/streptomycin and 30% L-929 conditioned medium (as a source of M-CSF). Differentiation into M1 and M2 was achieved by culture for 5-7 days with 50 ng/mL IFN-γ (M1, Peprotech) and 10 ng/mL LPS (M1, Sigma-Aldrich) or 20 ng/mL IL-4 (Peprotech).

Materials and General Methods

Commercial reagents were purchased from the stated sources: MTT reagent (Sigma Aldrich), alamar blue, doxorubicin (ThermoFisher). Flow cytometry antibodies and dyes include BV605-conjugated anti-CD45 (BD Biosciences), APC-R700-conjugated anti-F4/80 (BD Biosciences), Alexafluor488-conjugated anti-iNOS (eBioscience), PE-Cy7-conjugated anti-Arginase 1 (eBioscience), anti-calreticulin (Invitrogen), Alexafluor594-conjugated donkey anti-rabbit (for anti-calreticulin detection), and Ghost Dye Violet 510 viability dye (Tonbo Biosciences). DMEM was obtained from Gibco.

MTT absorbance assays were performed by adding 10 µL MTT reagent (5 mg/mL) to the wells of a 96-well plate containing 100 µL of media. Plates were incubated at 37 °C and 5% CO_2 atmosphere for 2 h. Plates were aspirated and to each well 100 µL DMSO were added. Plates were shaken for 20 min. Absorbance at 570 nm was read in each well using a Tecan M200 spark plate reader. Average absorbance values for blank wells were subtracted from absorbance values for each experimental and control well value to eliminate the background. Viability data were obtained by averaging blank-normalized absorbance values for control cells and expressing average absorbance for the treated samples as percent control.

General Toxicity Screen

M0 macrophages, M1 macrophages, and M2 macrophages were seeded at 5,000 cells per well in 96-well plates in 50 µL of their respective growth media. Cells were incubated overnight (~18 h). Experimental media stocks for each compound **1-22** were prepared in the respective medias at 20 µM and 2% DMSO. On two separate plates, to

quadruplicate wells, 50 µL of each experimental treatment was added, resulting in cells exposed to 10 µM and 1% DMSO. Octuplicate wells on each plate were also treated with vehicle (media containing 1% DMSO). Plates were then incubated for 1 h. After incubation plates were removed from the incubator and either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min. Plates were then incubated for 72 h before assessing for viability by MTT assay.

MDA-MB-231 breast cancer and MCF-10A breast epithelial cells were seeded at 7,000 cells/well in 96-well plates in 100 µL of their respective cell growth media. Cells were incubated overnight (~18h). Experimental media was prepared for each compound **1-30** at 10 µM and 1% DMSO. The media was aspirated from each well and quadruplicate wells were treated with 100 µL of treatment media. Octuplicate wells on each plate were treated with vehicle (media containing 1% DMSO). Plates were then incubated for 1 h. After incubation plates were removed from the incubator and either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min. Plates were then incubated for 72 h before assessing for viability by MTT assay.

EC_{50} Determination

M0 macrophages, M1 macrophages, and M2 macrophages were seeded at 5,000 cells per well in 96-well plates in 50 µL of their respective growth media. Cells were incubated overnight (~18 h). Experimental media stocks for each compound were prepared in the respective medias at concentrations ranging from 50 µM- 200 nM and with 2% DMSO. On two separate plates, to quadruplicate wells, 50 µL of each experimental treatment was added, resulting in cells exposed to treatments between 25

μM-100 nM in 1% DMSO. Octuplicate wells on each plate were also treated with vehicle (media containing 1% DMSO). Plates were then incubated for 1 h. After incubation plates were removed from the incubator, their media was aspirated, and 100 μL of vehicle was added to each well. Plates were then either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min. Plates were then incubated for 72 h before assessing for viability by MTT assay. EC_{50} values were determined using the Igor Pro graphing software.

MDA-MB-231 breast cancer and MCF-10A breast epithelial cells were seeded at 7,000 cells/well in 96-well plates in 100 μL of their respective cell growth media. Cells were incubated overnight (~18h) Experimental media stocks for each compound were prepared in the respective medias at concentrations ranging from 25 μM-100 nM in 1% DMSO. On two separate plates, to quadruplicate wells, 100 μL of each experimental treatment was added. Octuplicate wells on each plate were also treated with vehicle (media containing 1% DMSO). Plates were then incubated for 1 h. After incubation plates were removed from the incubator, their media was aspirated, and 100 μL of vehicle was added to each well. Plates were then either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min. Plates were then incubated for 72 h before assessing for viability by MTT assay. EC_{50} values were determined using the Igor Pro graphing software.

3D Cell Viability Assays

Cells were plated in a black-walled, clear-bottomed 96-well plate. Cultrex was added to each well (20 μl) while on ice. The plate was spun at 300 g for 3 min at 4 °C to evenly coat each well followed by incubation at 37 °C for 15 min to solidify the Cultrex prior to plating the mixture of MDA-MB-231 and BMM (2,000 cells each/well) in 50 μl of

media. After setting (45 min at 37 °C) cultures were overlayed with 150 µl of media with 2% Cultrex according to our published protocols.[1, 2] After 48h, media was aspirated and replaced with media containing 25 µM **3** or **22** or no metal complex either with or without 1.25 µM doxorubicin. Each condition was treated in quadruplicate wells. Plates were then incubated for 24 h. After incubation media was aspirated and replaced 180 µL vehicle. Plates were then either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min. Spheroids were then incubated for 72 h. To each well 20 µL alamar blue was added, resulting in 10% alamar blue in vehicle and plates were returned to the incubator for 24 h. Plates were read on a BioTek Synergy 2 plate reader at an excitation/emission of 560/590 nm. Average fluorescence values for blank wells were subtracted from fluorescence values for each experimental and control well value to eliminate the background. Viability data were obtained by averaging blank-normalized fluorescence values for control cells and expressing average fluorescence for the treated samples as percent control

Fluorescence Assisted Cell Sorting

Acid-washed coverslips were added to 35mm dishes, coated with 45 µl cultrex, and placed in a 37 °C incubator for 15 min to polymerize. A mixture of 50,000 MDA-MB-231 and 50,000 BMM cells in 60 µl of media were added to the top of each coverslip, allowed to settle for 45 min in a 37 °C incubator, then 3 ml of media with 2% cultrex as overlay was added per dish. After 72 h, the cells were removed from the incubator and the media was aspirated and replaced with media containing 25 µM **3** or **22** or no metal complex either with or without 1.25 µM doxorubicin. 24 h later, media was changed,

dishes were either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min, and incubated for either 4 h or 72 h at 37°C prior to collection. To obtain samples for cell sorting, cultures were washed in PBS and 3 ml of cold PBS with 5 mM EDTA (pH 7.4) was added to each dish. Samples along with the PBS/EDTA, were collected in. a 15 ml tube using transfer pipets (8 coverslips per condition). Tubes were incubated on a rocker in ice for 10 min, spun down at ~100g for 5 min at 4 °C, then PBS/EDTA was removed. This wash was repeated 3 times, followed by 2 washes with regular PBS. Viability dye and extracellular marker staining was conducted in Flow Buffer (1xPBS with 2% FBS). Cells were stained with Ghost Viability Dye (1:1000) for 15 minutes on ice followed by 2 washes with Flow Buffer. Fc Block (1:200) was applied for 15 minutes on ice, followed by incubation with unconjugated antibody to calreticulin (1:200) for 15 minutes on ice and two washes in Flow Buffer. Conjugated antibodies to CD45, F4/80, and anti-rabbit IgG (for calreticulin) were applied (1:200) for 15 minutes on ice followed by two washes in Flow Buffer. Cells were then fixed in Intracellular (IC) Fixation Buffer (eBioscience) for 10 minutes on ice, followed by two washes in 1x Permeabilization Buffer (eBioscience). Intracellular iNOS and Arginase 1 were detected by staining in 1x Permeabilization Buffer followed by two washes in 1x Permeabilization Buffer and one wash in Flow Buffer. Cells were resuspended in Flow Buffer and analyzed on an LSR II (BD Biosciences) with downstream analysis in FCS Express software (De Novo).

Confocal Microscopy Imaging

BMMs were stained using Cell Tracker Orange (1:1000 in SFM for 1 h at 37 °C) for easy identification during imaging. Acid-washed coverslips were added to 35mm

dishes, coated with 45 µl cultrex and placed in a 37 °C incubator for 15 min to polymerize. A mixture of 10,000 MDA-MB-231 and 10,000 BMM cells in 60 µl of media were added to the top of each coverslip, allowed to settle for 45 min in a 37 °C incubator, then 3 ml of media with 2% cultrex as overlay added per dish. After 72 h, the cells were removed from the incubator and the media was aspirated and replaced with media containing 25 µM **3** or **22** or no metal complex either with or without 1.25 µM doxorubicin. 24 h later, media was changed, dishes were either wrapped in foil or irradiated with blue light (460/70 nm) for 20 min, and incubated for 4 h before imaging.

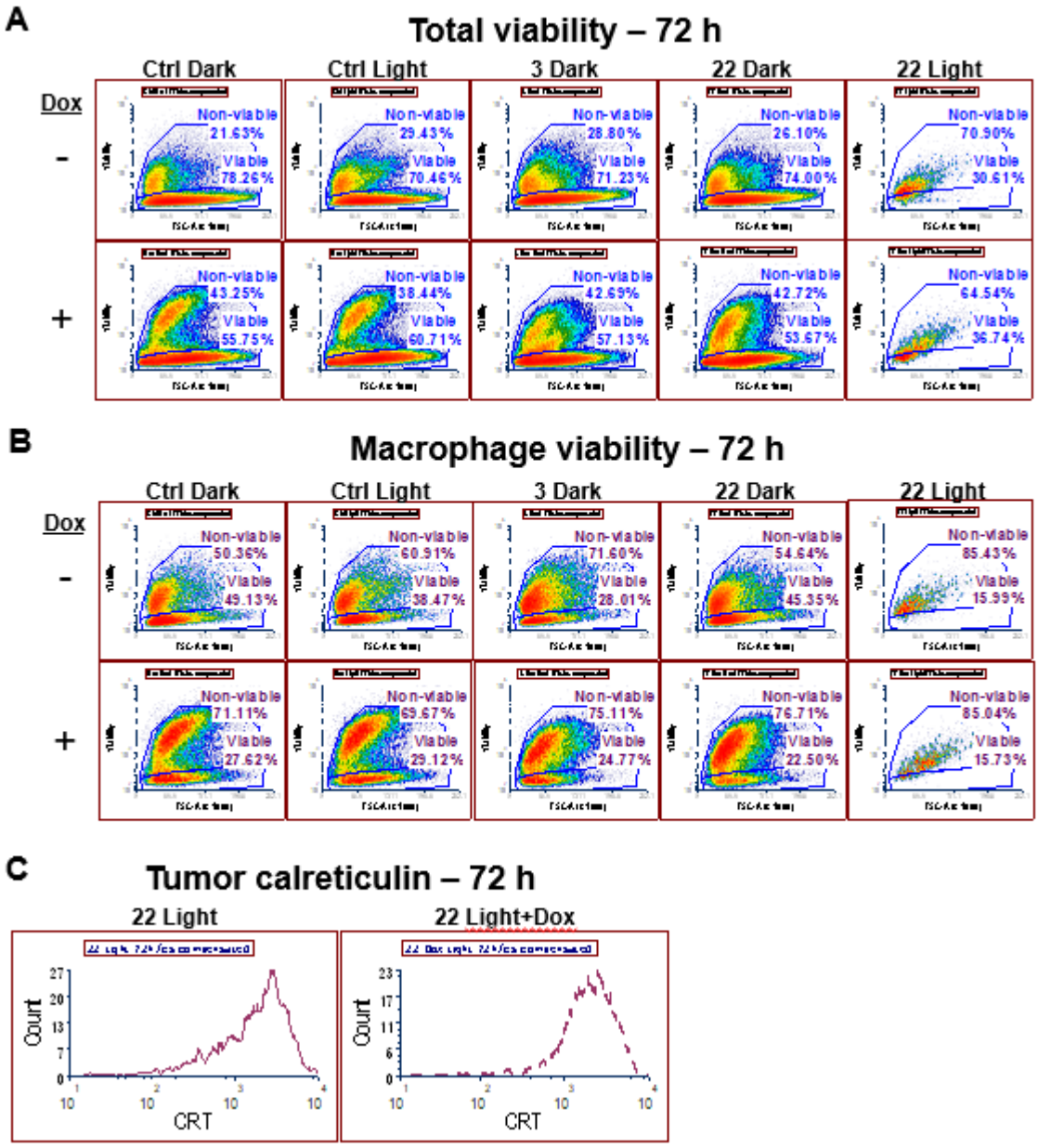
For calreticulin immunocytochemistry, coverslips were washed with PBS, fixed in a 2% formaldehyde + 0.1% glutaraldehyde in PBS solution for 30 min, washed, and then the aldehyde groups were quenched with a 10 mM solution of sodium borohydride (2x 5 min). Cultures were then washed, permeabilized with 0.2% Triton-X, and blocked with 0.2% BSA for 1 h. Incubation with rabbit anti-calreticulin antibody (1:50) in PBS + 0.2% Triton-X was performed overnight. The following day, cultures were washed and incubated with AlexaFluor 488 secondary antibody (1:1000) in PBS + 0.2% Triton-X + 5% Normal Donkey Serum for 1 h. They were then washed and imaged using a Zeiss LSM 780 confocal microscope. 3D reconstruction of calreticulin staining was performed using Volocity Software (Perkin Elmer, Waltham, MA). Surface staining of calreticulin in each optical slice through entire Z-stack was quantified using Image J.

For Calcein AM staining (live/dead), cells were seeded and treated as above. Prior to imaging on Zeiss LSM 780 confocal microscope, coverslips were stained with Calcein AM at 2 µM for 30 min. Imaging was performed using a 40x dipping lens. Live cells (green; Calcein A/M) were captured using excitation at 488nm and emission at 507nm.

3D reconstruction of Calcein AM-stained spheroids and the quantification of the sum of channel intensities were performed using Volocity Software (Perkin Elmer, Waltham, MA).

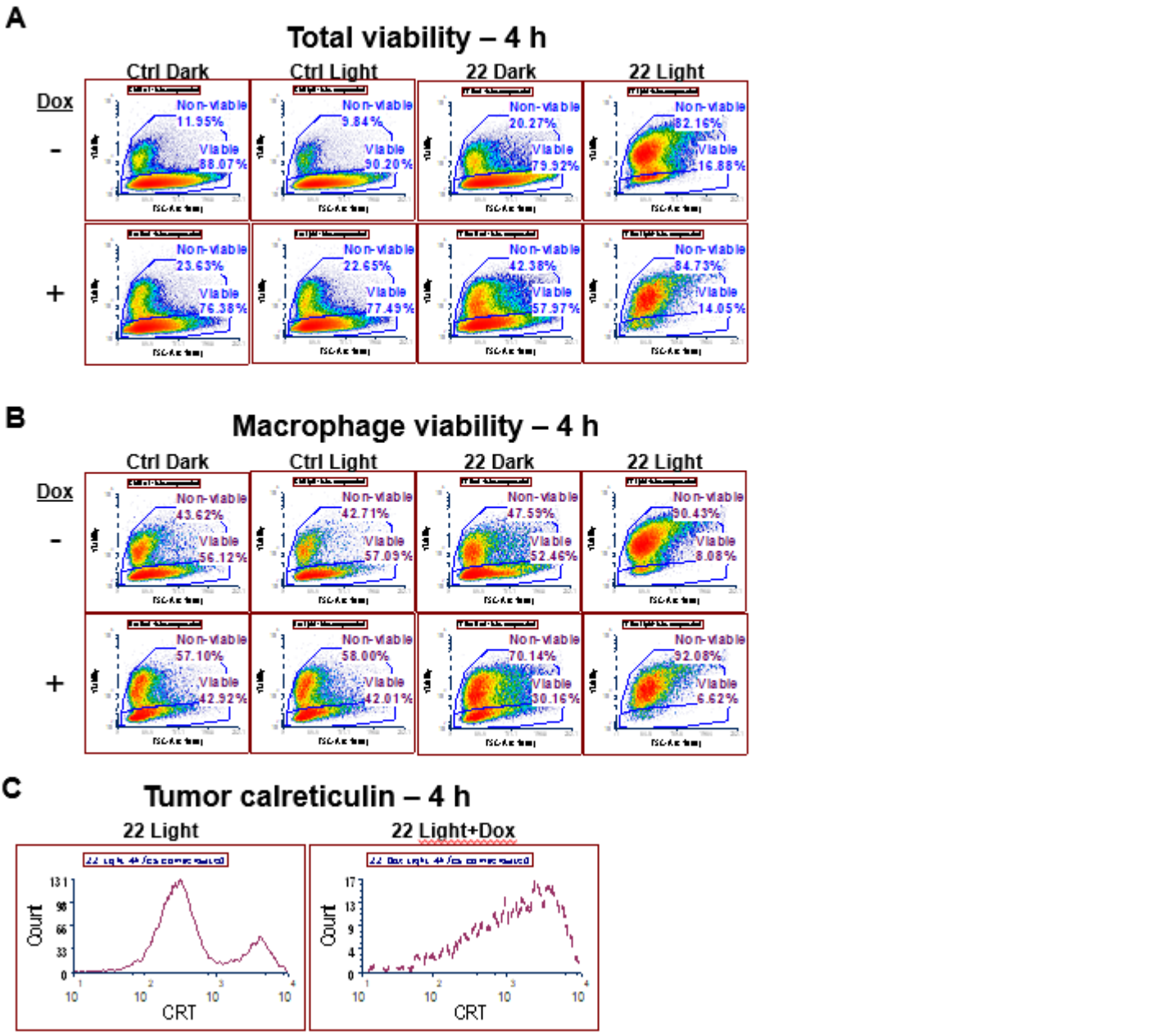
1. Bram van der Eerden, A.v.W., *Meeting report of the 2016 bone marrow adiposity meeting*. 2017. p. 304-313.
2. Alaaeddine, R.A.E., Perihan A.; AlZaim, Ibrahim; Abou-Kheir, Wassim; Belal, Ahmed S.F.; El-Yazbi, Ahmed F., *The Emerging Role of COX-2, 15-LOX and PPARγ in Metabolic Diseases and Cancer: An Introduction to Novel Multi-target Directed Ligands.* Curr. Med. Chem., 2021. **28**(11): p. 2260-2300.

Figure S1: Supporting Information Flow Data-72 h



Flow cytometric viability plots of (**A**) total 3D spheroid dissociate and (**B**) F4/80+ macrophages after 72 h treatment as described in Figure 4. **C**: Individual histograms of tumor calreticulin exposure from samples treated with 22 and light, or 22, doxorubicin, and light from Figure 4D, where levels of detectable cells were lowest among treatment groups.

Figure S2: Supporting Information Data-4 h



Flow cytometric analysis of dissociated 3D spheroids after 4 h treatment as described in Figure 4. Viability plots of (**A**) total 3D spheroid dissociate and (**B**) F4/80+ macrophages **C**: Individual histograms of tumor calreticulin exposure from samples treated with 22 and light, or 22, doxorubicin, and light from Figure 4H.

Table S1: Phototherapeutic Indices

Entry	Compound	M0	M1	M2	MDA-MB-231	MCF-10A
1	**1**	>1.1	>1.0	>1.3	**<1**	N/A
2	**2**	>1.5	>2.0	>2	N/A	N/A
3	**3**	>1.3	<1	1.2	1.1	N/A
4	**5**	1.1	1.2	2.2	1.0	1.4
5	**7**	1.5	2.2	4.3	1.2	<1
6	**8**	3	<1	2.0	1.6	1.3
7	**14**	<1	2.8	2.1	<1	2.5
8	**19**	N/A	N/A	N/A	N/A	N/A
9	**20**	N/A	N/A	N/A	N/A	N/A
10	**21**	>18	>12	>36	N/A	N/A
11	**22**	>25	>100	>45	7.4	>3.6

Phototherapeutic indices are a measure of the difference in toxic potency of a compound in the light vs. in the dark. PI's are expressed as the ratio of EC50 in the dark to EC50 in the light. Values were calculated for each compound in each cell type