Supporting Information

Tunable Cytotoxicity of Rhodamine 6G via Anion Variations

Paul K. S. Magut,[†] Susmita Das,[†] Vivian E. Fernand,[‡] Jack Losso,[§] Karen McDonough,[¶] Brittni M. Naylor,[†] Sita Aggarwal,[□] and Isiah M. Warner[†*]

[†] Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA.

[‡]Department of Chemistry and Physics, LeTourneau University, Longview, TX 75602

[§]Department of Food Science, Louisiana State University Baton Rouge, LA 70803.

[¶]Veterinary Science Department, Louisiana State University, Baton Rouge, LA 70803.

[□]William Hansel Cancer Prevention, Pennington Biomedical Research Center, Baton Rouge, LA 70808.

Email: iwarner@lsu.edu

Reagents and Experimental Procedures

Reagents. Human serum and glutaraldehyde were purchased from Sigma Aldrich (St. Louis, MO) and used as received. Sodium tetraphenyl borate (99.5%), sodium trifluoromethanesulfonate (98%), sodium L-ascorbate (98%), rhodamine 6G (95%), phosphate buffered saline, methylene chloride, anhydrous acetonitrile, ethanol (spectroscopic grade),crystal violet, digitonin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and 0.2 μm nylon membrane filters were purchased from Sigma Aldrich (Milwaukee, WI). Lithium bis (perfluoroethylsulfonyl) imide was donated by Dr. Gary Baker (Oak Ridge National Laboratory, Oak Ridge, TN). Cell viability MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and Mitochondrial ToxGlo^{TM} Assay kits were purchased from Promega Corporation (Madison, WI). Cell death ELISA assay kit was purchased from Roche Applied Sciences (Indianapolis, IN). Triply deionized water (18.2 MΩ·cm) from an Elga model PURELAB ultra water filtration system (Lowell, MA) was used for all preparations of GUMBOS. A BRANSON 3510RDTH model bath ultrasonicator (335 W, 40 kHz frequency) (Branson Ultrasonics Corporation, Danbury, CT) was used at room temperature for preparation of nanoGUMBOS.

Synthesis and Characterization of Rhodamine 6G-based GUMBOS. The rhodamine 6G-based organic salts (GUMBOS) were prepared using ion exchange procedures similar to those previously reported in the literature with slight modification.[1,2] The synthesis of rhodamine tetraphenyl borate ([R6G][TPB]) is herein described as a representative procedure for the hydrophobic GUMBOS. Thirty (30) mg (0.063 mmol) of [R6G][Cl] and 23.6 mg (0.068 mmol) of sodium tetraphenyl borate ([Na][TPB]) salt were dissolved in a mixture of methylene chloride (DCM) and water (2:1, v/v) and allowed to stir for 24 h at room temperature (Scheme 1). Afterwards, the DCM bottom layer was washed several times with water to remove the sodium chloride by-product, and the product was dried by removal of solvent *in vacuo*. Further freeze-

drying to remove traces of water afforded 46.1 mg (96.4% yield, Table S1) of [R6G][TPB]. A different procedure was employed for synthesis of the hydrophilic GUMBOS, [R6G][Asc] and [R6G][OTf] since these products are relatively more soluble in water [2] (Scheme 1). The starting materials, [R6G][Cl] and sodium ascorbate ([Na][Asc]) were stirred in anhydrous acetonitrile for 72 h. The NaCl byproduct was removed by filtration and [R6G][Asc] or [R6G][OTf] GUMBOS were obtained by removal of acetonitrile under vacuum. The GUMBOS obtained were characterized by use of ^{1}H NMR (Bruker Avance 400, $CDCl_3$) (Fig. S 10a, 11, 12a and 13) and elemental analysis (Table S5). For [R6G][BETI] and [R6G][OTf], ^{19}F NMR (Bruker DPX 250, $CDCl_3$) was used to confirm anion exchange (Fig. S 10b and 12b). Melting points of the GUMBOS were determined using a MEL-TEMP capillary melting point apparatus (Stanford Research Systems, Sunnyvale, CA)

Determination of Hydrophobicity. 1-Octanol/ water partition coefficient was used to gauge relative hydrophobicities of [R6G] - based GUMBOS. High performance liquid chromatography (HPLC) coupled with a UV-vis detector was used.[3] Separation and quantification of the GUMBOS in 1-octanol was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of an SCL-10A system controller, two LC-10AD pumps, a DGU-14A degasser, a SIL-10AD autosampler and an SPD-10AV UV-vis detector (λ = 530 nm). Separation of the analytes was performed at room temperature using a Phenomenex Luna C18 column, 100 Å pore size, 4 µm particle size, 250 × 4.6 mm i.d. column containing a guard column (Phenomenex, Torrance, CA, USA). The [R6G]-based GUMBOS were eluted isocratically at a flow rate of 0.6 mL/ min using an acetonitrile/ water mobile phase (45/55, v/ v) containing phosphoric acid buffer (pH= 3.0). An injection volume of 20 µL was employed for this study. The concentration of GUMBOS in water phase was determined using mass balance. In a typical study, a known amount of [R6G][TPB] was dissolved in a 1-octanol and water

mixture (equal volumes) and shaken for 24 h. The upper 1-octanol phase was then analyzed by use of HPLC with UV detection and quantified using an external calibration method. The equation $K_{(o/w)} = [GUMBOS]_{o, e} / [GUMBOS]_{w, e}$ was used to calculate the partition coefficient, where 'K', 'o', 'w,' and 'e' represents partition coefficient, octanol, water, and equilibrium state, respectively. It is worth noting that the 1-octanol used in the partition experiments was pre-saturated with water overnight before use in order to correct for mutual solubility of the two solvents.[3]

Quantum Yields and Lifetime Measurements. Quantum yields (QY) of the GUMBOS were measured relative to [R6G][Cl], QY= 0.90 ± 0.02.[4] The GUMBOS and solution of [R6G][Cl] in deionized water were optically matched at the excitation wavelength (530 nm) and the QY calculated with corrections for the absorbance of all dispersions and solutions. The optical density was set at around 0.1 at 530 nm, and the integrated intensities of the emission spectra, corrected for differences in index of refraction and concentration.[5] Fluorescence Lifetime measurements were performed at Horiba Scientific (Edison, NJ) using a time domain mode. A picoseconds pulsed laser source of 495 nm was used and emission was collected at 550 nm with a TBX detector. The Time Correlated Single Photon Counting (TCSPC) mode was used for data acquisition with a resolution of 50 ps per channel.

Photostability Measurements. Photostability fluorescence measurements were collected as follows: the excitation/ emission slit widths were maintained at maximum value (14 nm/ 14 nm) for maximum light exposure of the samples. Appropriate neutral density filters were used in order to avoid saturation of the detector. Data were collected over a period of 5000 seconds. All measurements were performed at room temperature by use of a Spex Fluorolog-3 spectrofluorimeter (model FL3-22TAU3; Jobin Yvon, Edison, NJ) equipped with a 450-W xenon lamp and R928P PMT emission detector.

Stability Studies. The stability of the nanoGUMBOS in phosphate buffered saline (PBS) at a pH 7.4 and ionic strength, I = 0.15 M, and in blood serum (10 % Serum in PBS) was monitored by use of absorption and fluorescence measurements. In a typical experiment, nanoGUMBOS were freeze-dried and lyophilized to remove water and then re-suspended in 5 mL PBS. Absorbance and fluorescence emission measurements of the re-suspended nanoGUMBOS were acquired at various time intervals. A similar procedure was used for a solution containing 10% blood serum in PBS.

Cell Culture. Normal human breast fibroblast (Hs578Bst, ATCC no. HTB-125), human breast carcinoma (Hs578T, ATCC HTB-126), hormone-independent human breast adenocarcinoma (MDA-MB-231, ATCC no. HTB-26) and hormone- dependent human breast adenocarcinoma (MCF7, ATCC no. HTB-22) cell lines were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). All cell lines were grown to 90% confluence according to ATCC's instructions before use in further experiments.

Cell Viability Assay. Cytotoxicities of [R6G]- based GUMBOS and nanoGUMBOS for each cell line was determined by use of MTT Assay kit(Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Briefly, in a 96-well plate, 5,000 cells in 0.1 mL culture medium were seeded to each well. After 24 h, the culture medium was removed and discarded followed by addition of 0.1 mL culture medium containing 0-100 μM test compounds. Each concentration was performed in triplicates. Use of cell media and maintaining a sterile environment during the synthesis of nanoGUMBOS allowed their introduction directly into mammalian cells *in vitro* without the need to wash or re-suspend. The cells were then incubated for 48 h at 37 ºC, in 5% CO_2 atmosphere. At the end of the incubation period, the cells were treated with 15 μL MTT and incubated for 4 h. After four hours, 100 μL stop solution was added per well and incubated overnight. Afterwards, the plate was shaken for 20 seconds to

homogenize and cell viability determined using a micro plate spectrophotometer (Benchmark plus Bio-Rad Laboratories, Hercules, CA, USA). Absorbance was read at 570 nm with a reference wavelength of 650 nm. Cell viability as a percentage was determined by computing the ratio between absorbance of the treated cells and the absorbance of untreated (control) cells taken as 100%. Data was expressed as mean ± SD

Apoptosis. Apoptotic cell death was determined using a cell death ELISA assay kit (Roche Applied Sciences, Indianapolis, IN) as per the manufacturer's instruction. In a typical experiment, 10^6 cells/ well were incubated with 0-70 μM of the test compound for 48 h to induce apoptosis. The cells were then lysed to produce nucleosomes and apoptotic cell death detected using a micro plate spectrophotometer (Benchmark Plus, Bio-Rad Laboratories, Hercules, CA, USA). Absorbance was read at 405 nm with a reference wavelength of 490 nm.

Clonogenic Assay. Clonogenic assay was performed according to a procedure described in literature with slight modifications.[6] Briefly, breast cancer cell line MDA-MB-231 was cultured in triplicate in 6-well plates at a density of 50 cells/ well and allowed to attach for two hours. Cells were observed under a microscope to confirm attachment and then incubated with the test compound for 48h. Control wells contained cells with only cell culture medium. After 48 h, the medium was changed and the cells were allowed to form colonies over a period of one week. The colonies were then fixed with glutaraldehyde (6 % v/ v), stained with crystal violet (0.5 % w/ v) and counted using a stereomicroscope.

Microscopy. Five thousand Hs578Bst cells, Hs578T cells and MDA-MB-231 cells were plated on glass bottom culture dishes (35 mm petri dish, 10mm Micro well; Ashland, MA, USA) for cell adherence a day before use. After 24 h, cells were incubated with the test compound at a final concentration of 50 μM at 37 °C and 5% CO_2 in a humidified cell culture incubator for 48 h. Cell images were obtained under a light microscope (Nikon Diaphot 300; Hoffman

Modulation Contrast) equipped with camera Nikon D70s at a magnification of 20X. Similarly, for confocal microscopy, cells were incubated with test compounds at a final concentration of 50 nM for 30 min. Cell images were acquired under an oil immersion objective (x40) with a confocal laser microscope (Leica TCS SP5 AOBS confocal microscope) equipped with an argon-krypton laser.

Cellular Uptake. Cellular uptake was evaluated using flow cytometry following a protocol reported in literature,[7] with slight modifications. Briefly, 3 x 10^4 cells per well were seeded in a 24- well plate and incubated for 24 h. The medium was replaced with medium containing [R6G][BETI] or [R6G][TPB] at 0-50 μM. In another set of experiments to investigate the effect of extracellular pH on uptake of nanoGUMBOS by normal breast cell line, Hs578Bst, media was adjusted to pH 6.5 and 7.4. After 4 h, cells were washed three times with PBS and harvested by trypsinization. They were then washed again with centrifugation and resuspended in FACS buffer (0.3% BSA in PBS). Mean Fluorescence intensities were determined by flow cytometry (Beckton & Dickinson, Mountain View, CA, USA).

Determination of Mitochondrial Responses to [R6G][BETI] and [R6G][TPB]. To determine whether these compounds inhibited oxidative phosphorylation, a Mitochondrial ToGlo™ Assay kit developed by Promega Corporation (Madison, WI) was used. This experiment was performed following the manufacturer's protocol with slight modification. The experiment comprised two parts. In the first part, membrane integrity (MI) was assessed while in the second part ATP was measured. Briefly, 10,000 cells/ well were grown using standard media in a white 96- well plate (costar®) and allowed to adhere. These cells were then washed in serum-free, galactose-containing medium in order to restrict ATP production to oxidative phosphorylation. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control mitochondrial toxin and digitonin was used as a positive control toxicity compound. All test compounds and controls

were prepared in serum-free galactose-containing medium. After washing, cells were incubated with the test compounds and controls for 2 h. At the end of 2h, 20 µL of a 5x diluted fluorogenic peptide substrate (bis- AAF-R110) was introduced to each well and mixed by use of orbital shaking for 1 min at 600 rpm. The sample was then incubated for 30 min at 37 °C followed by measurement of fluorescence using excitation at 485 nm and measuring emission at 530 nm. This test was considered valid when the signal from the cytotoxicity control was at least twofold higher than that of the untreated media control. To determine the amount of ATP, the sample plate was equilibrated to room temperature for 5-10 min and 100 µL of prepared ATP detection reagent were added to each well followed by 5 min mixing on an orbital shaker at 600 rpm. Data was expressed as percentage of vehicle control.

Results

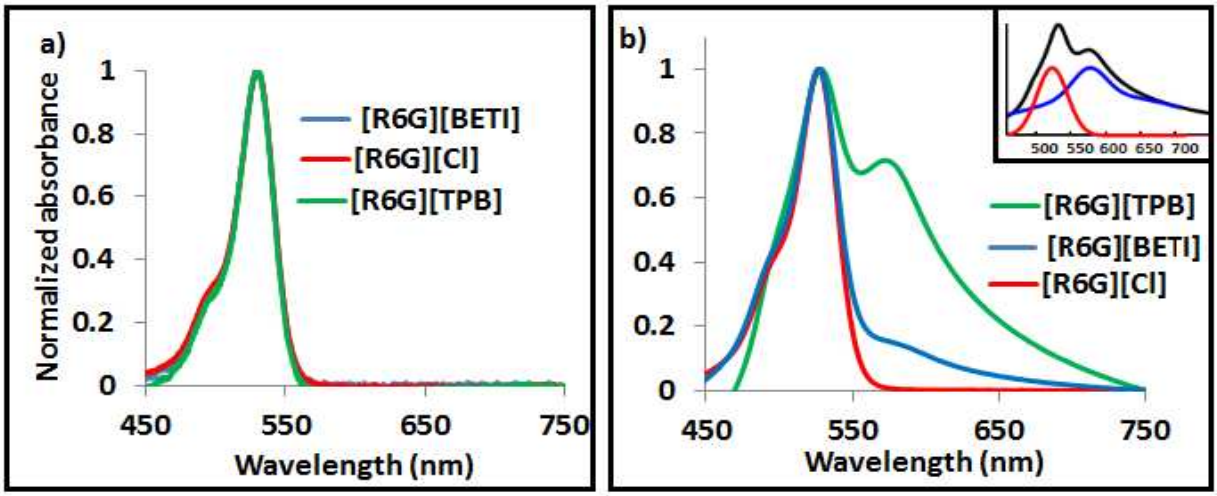


Fig. S1 Absorption spectra of [R6G]-based **a**) GUMBOS in ethanol **b**) nanoGUMBOS in PBS. Inset; deconvoluted absorption Spectrum of [R6G][TPB] and [R6G][BETI] nanoGUMBOS (black line), representing randomly oriented aggregates absorbing at λ= 525 nm (red line) and J-aggregates absorbing at λ= 582 nm (blue line)

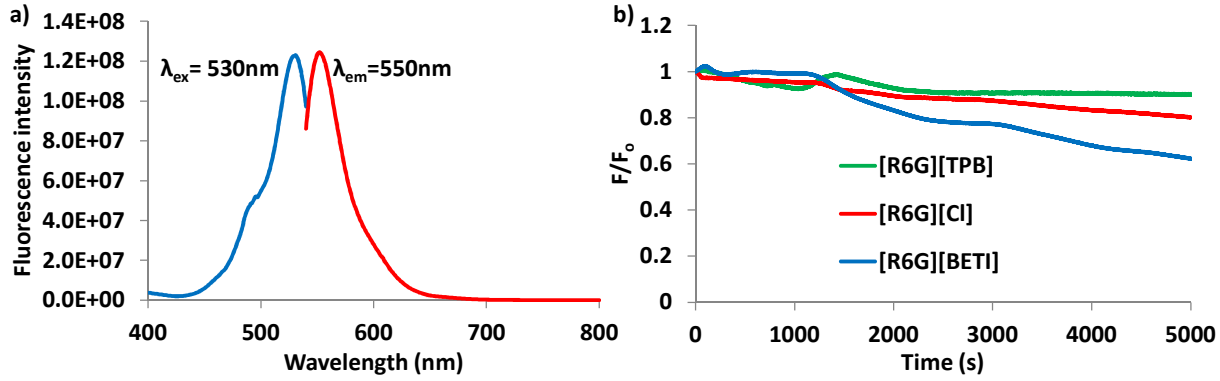


Fig. S2 Fluorescence studies in PBS displaying **a**) Fluorescence excitation and emission spectra of 4 µM [R6G][TPB] nanoGUMBOS and **b**) Photostability of 0.1 µM [R6G] - based compounds. $\mathbf{F_o}$ and **F** are fluorescence intensities at t= 0 and at different times respectively. Maximum slit widths of 14 nm were maintained on both excitation and emission.

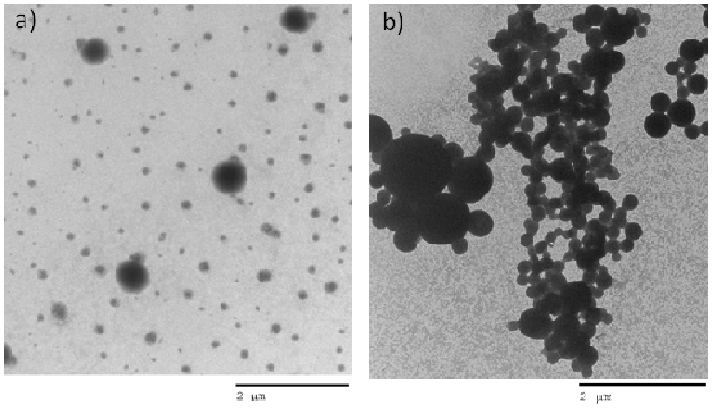


Fig. S3. TEM images of a suspension of **a)** [R6G] [BETI] and **b)** [R6G] [TPB] GUMBOS. This displays micro- and nano-sized particles. Bars represent 2 µm

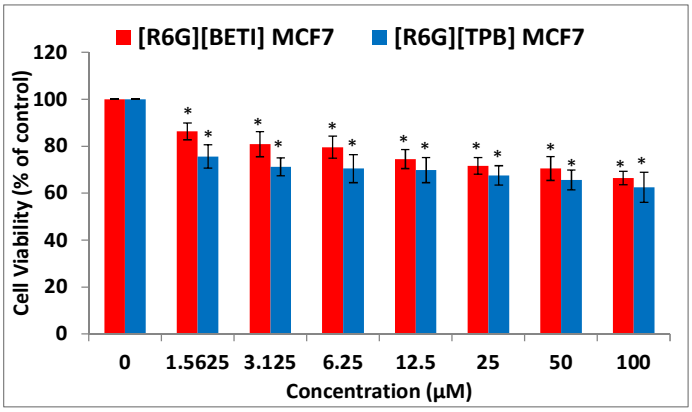


Fig. S4. Effect of [R6G][BETI] (red) and [R6G][TPB] (blue) on MCF7 breast cancer cell line. The inhibitory activity was measured after 48 h using MTT assay. *Statistically different from corresponding control, $P < 0.0001$.

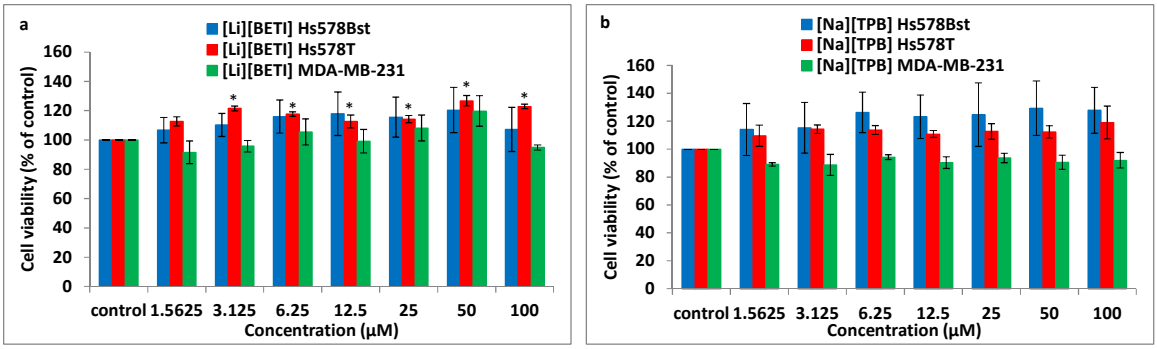


Fig. S5. Cell viability assay of Hs578Bst, Hs578T and MDA-MB-231 cell lines upon treatment with 0–100 μM of; **a)** [Li][BETI] and **b)** [Na][TPB] controls. *Statistically different from control, $P < 0.0001$ for [Li][BETI] Hs578T. Statistical analysis shows no difference from control for all the others ($P > 0.05$)

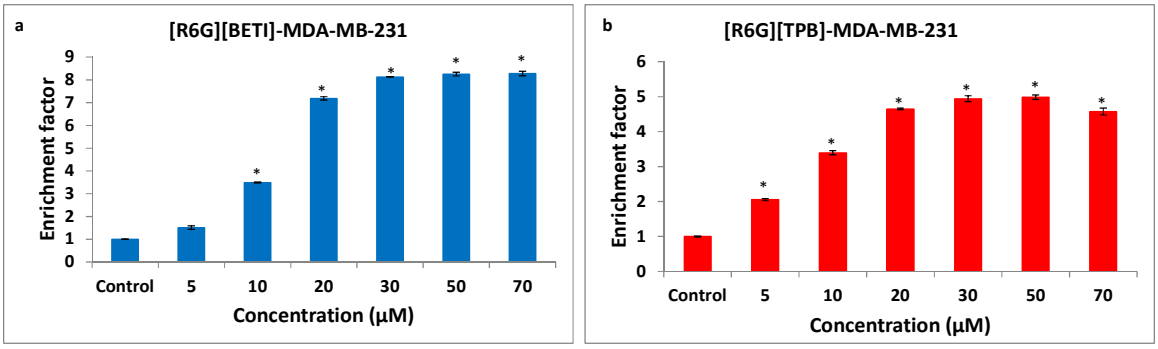


Fig. S6. Effect of **a)** [R6G][BETI] and **b)** [R6G][TPB] on cell death of MDA-MB-231 breast cancer cells. MCF7 and Hs578T cancer cell lines displayed similar trends. The enrichment factor is the ratio of the absorbance of the sample (dying/dead cells) and the absorbance of the control (viable cells). *Statistically different from control, $P < 0.0001$.

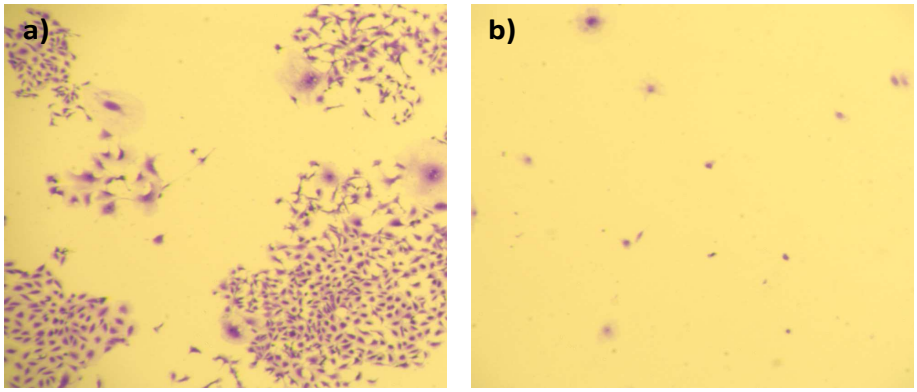


Fig. S7. Clonogenic assay images of MDA-MB-231 breast cancer cells showing **a**) Colonies formed from control wells and **b**) zero colonies formed in wells treated with 12.5 μM [R6G][BETI]. Similar results were obtained for [R6G][TPB].

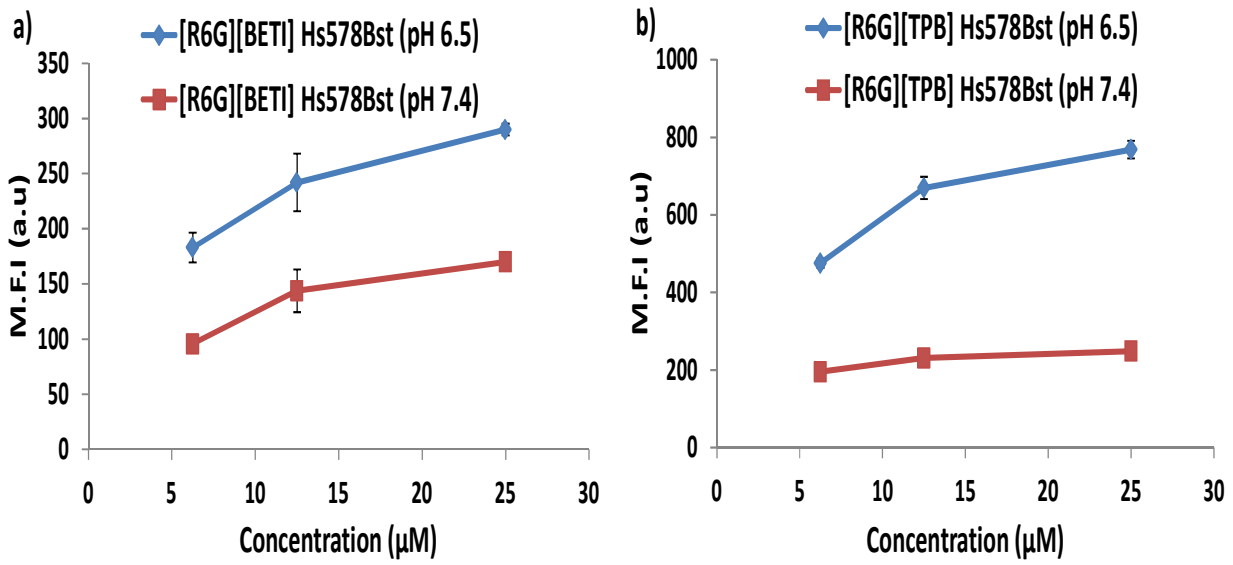


Fig. S8. Mean Fluorescence intensity (M.F.I) of normal breast cell line, Hs578Bst treated with **a)** [R6G][BETI] and **b)** [R6G][TPB] at a pH of 6.5 (blue) and 7.4 (red).

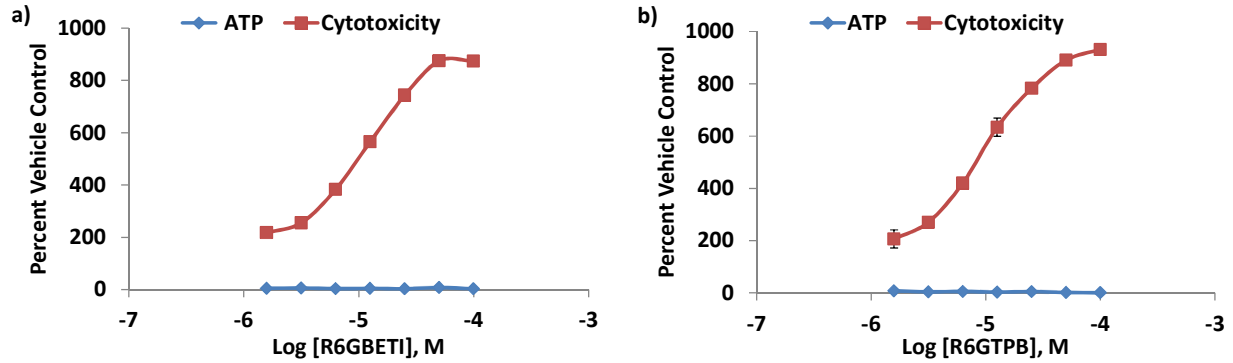


Fig. S9. Profiles of mitochondrial toxicity of **a)** [R6G][BETI] and **b)** [R6G][TPB] using a Mitochondrial ToxGlo^{TM} Assay. MDA-MB-231 cells were plated at 10,000 cells/ well and treated in serial dilutions of compounds resuspended in glucose free (galactose supplemented) DMEM media for 2 h. A reduction in ATP with discordant changes in membrane integrity (cytotoxicity) indicates that the two compounds are mitochondrial toxins.

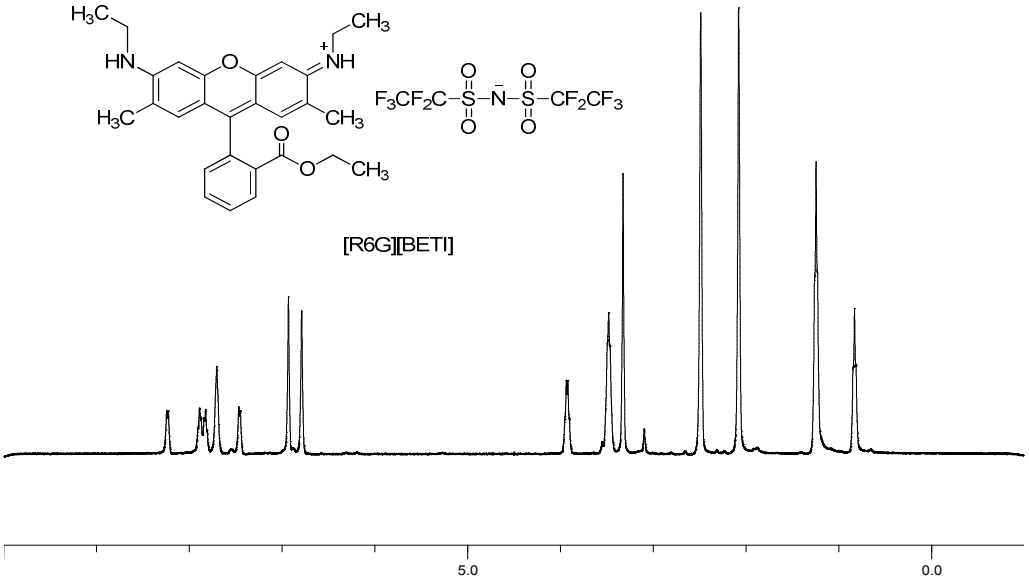


Fig. S10a. ^{1}H NMR (d_6 DMSO, 400MHz) of [R6G][BETI]

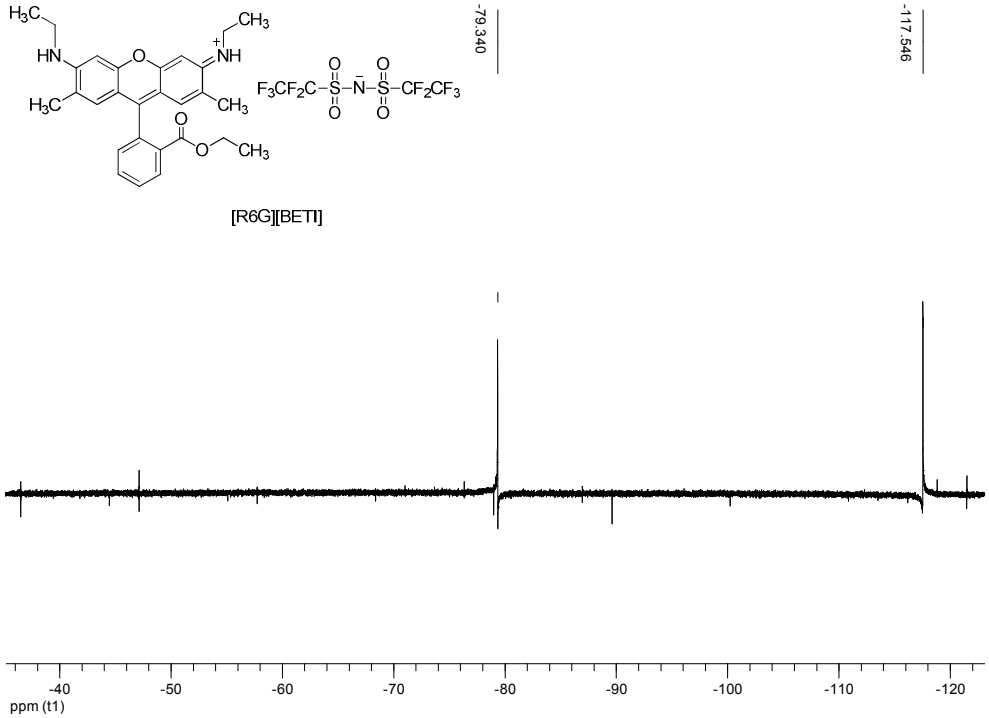


Fig. S10b. ^{19}F NMR (d_6 DMSO, 400MHz) of [R6G][BETI]

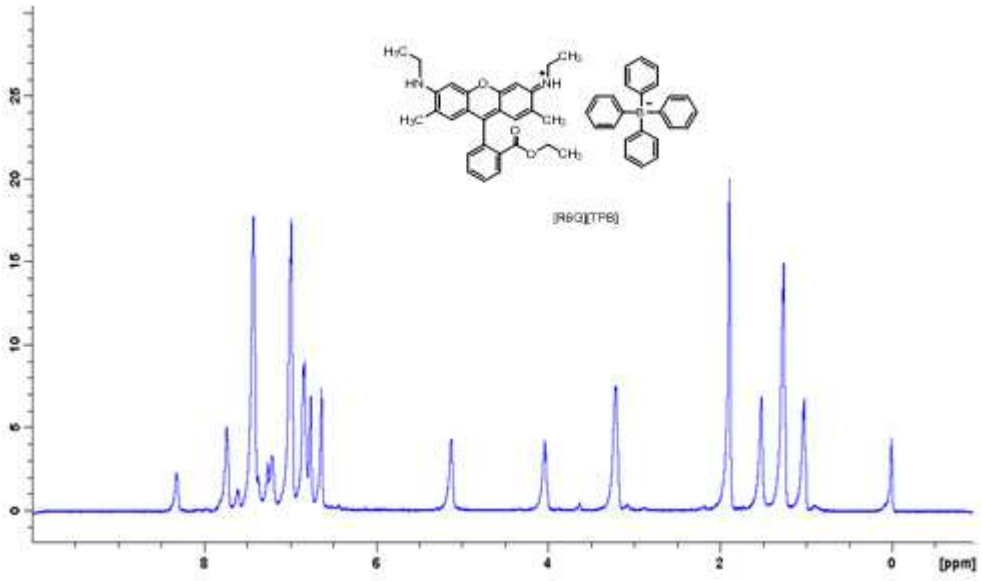


Fig. S11. ^{1}H NMR (d_6 DMSO, 400MHz) of [R6G][TPB]

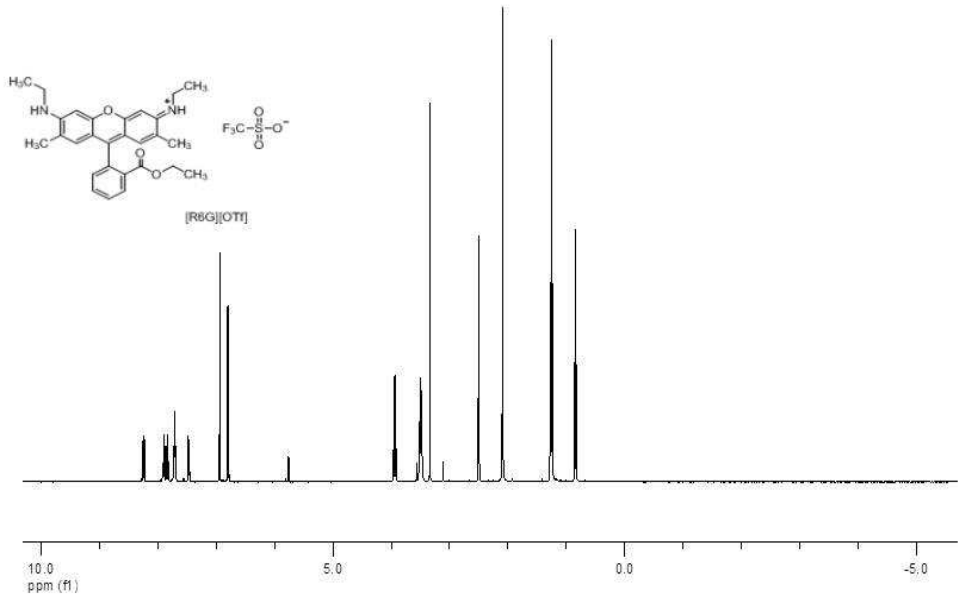


Fig. S12a. ^{1}H NMR (d_6 DMSO, 400MHz) of [R6G][OTf]

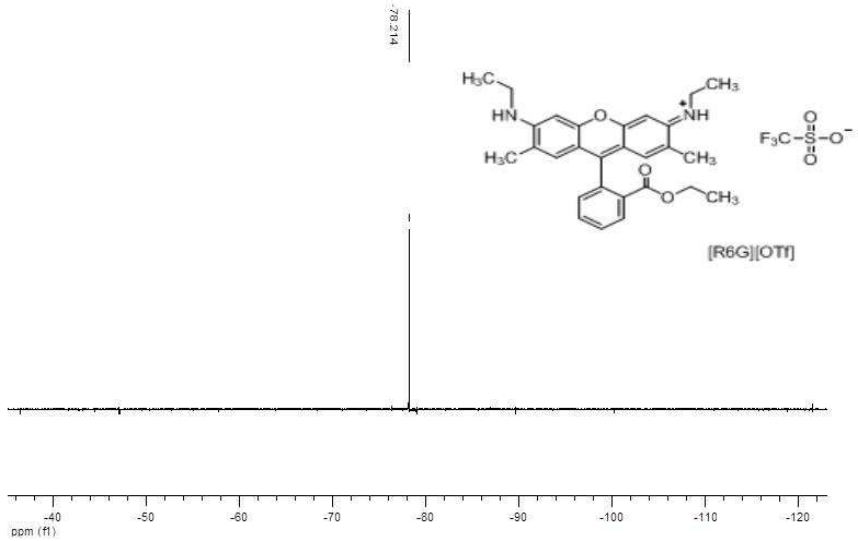


Fig. S12b. ^{19}F NMR (d_6 DMSO, 400MHz) of [R6G][OTf]

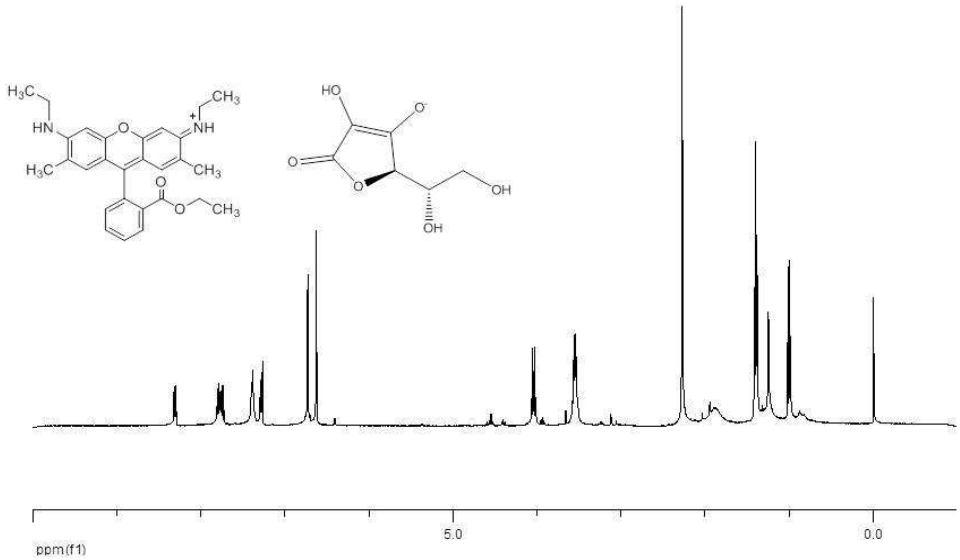


Fig. S13. ^{1}H NMR (d_6 DMSO, 400MHz) of [R6G][Asc]

Table S1: Yields, melting points, logarithm of 1-octanol/water partition coefficients of rhodamine 6G-based GUMBOS and size of corresponding particles.

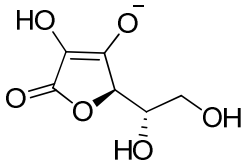
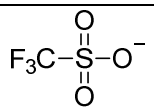
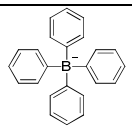
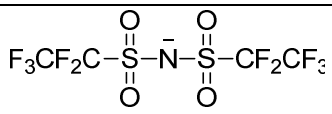
GUMBOS	Yields (%)	Anion structure	MW of Anion (g/mol)	mp (°C)	Partition Coefficients (Log $K_{o/w}$)	Particle Size (nm)
[R6G][Asc]	89		176	138	-0.5	N/A
[R6G][OTf]	94		149	239	0.1	N/A
[R6G][TPB]	96		319	83	0.3	92 ± 17
[R6G][BETI]	96		381	64	1.0	101 ±21

Table S2: Dissociation constants of Rhodamine-based GUMBOS in buffer solutions

Compound	pH	Solubility (g/L)	Solubility ($molL^{-1}$)	Dissociation constants Ks (mol^2L^{-2})
[R6G][BETI]	6.5	9.56×10^{-4}	1.16×10^{-6}	$(1.35 \pm 0.70) \times 10^{-12}$
	7.4	2.23×10^{-3}	2.71×10^{-6}	$(7.36 \pm 0.06) \times 10^{-12}$
[R6G][TPB]	6.5	2.82×10^{-3}	3.71×10^{-6}	$(1.37 \pm 0.05) \times 10^{-11}$
	7.4	2.97×10^{-3}	3.89×10^{-6}	$(1.52 \pm 0.040) \times 10^{-11}$
[R6G][OTf]	6.5	3.28×10^{-2}	5.54×10^{-5}	$(3.07 \pm 0.20) \times 10^{-9}$
	7.4	3.61×10^{-2}	6.10×10^{-5}	$(3.73 \pm 0.40) \times 10^{-9}$
[R6G][Asc]	6.5	0.50	8.05×10^{-4}	$(6.49 \pm 0.08) \times 10^{-7}$
	7.4	0.43	6.95×10^{-4}	$(4.83 \pm 0.20) \times 10^{-7}$
[R6G][Cl]	6.5	1.37	2.85×10^{-3}	$(8.11 \pm 0.60) \times 10^{-6}$
	7.4	1.68	3.50×10^{-3}	$(1.23 \pm 0.07) \times 10^{-5}$

Table S3: Quantum yields and lifetimes of the GUMBOS

GUMBOS	Quantum Yields	Lifetimes (ns)
[R6GAsc]	0.87	3.92
[R6G][Cl]	0.90	3.94
[R6G][OTf]	1.01	3.92
[R6G][TPB]	0.97	3.91
[R6G][BETI]	1.01	3.91

Table S4: IC_{50} (μM) of R6G-based compounds towards breast cell lines

Compounds	**Hs578Bst IC_{50} (μM)**	**Hs578T IC_{50} (μM)**	**MDA-MB-231 IC_{50} (μM)**	**MCF7 IC_{50} (μM)**
[R6GAsc]	10.2	21.0	5.0	13.3
[R6G][Cl]	34.6	19.2	5.1	30.7
[R6G][OTf]	40.6	18.6	10.2	14.2
[R6G][TPB]	-	25.6	12.2	>100
R6G][BETI]	-	23.8	11.4	>100

Table S5. Elemental analysis of the rhodamine 6G-based GUMBOS

GUMBOS	**C**		**H**		**N**		**S**	
	Theory (%)	Found (%)	Theory (%)	Found (%)	Theory (%)	Found (%)	Theory (%)	Found (%)
[R6GAsc]	66.04	65.96	6.19	5.98	4.73	4.69	5.41	5.30
[R6G][OTf]	58.78	58.60	5.27	5.20	4.68	4.63	-	-
[R6G][BETI]	46.67	47.02	3.91	3.79	5.10	5.14	7.78	7.57
[R6G][TPB]	81.88	81.71	6.74	6.75	3.67	3.69	-	-

References

(1) Bwambok, D. K.; El-Zahab, B.; Challa, S. K.; Li, M.; Chandler, L.; Baker, G. A.; Warner, I. M. *Acs Nano* **2009**, *3*, 3854.

(2) Fraga-Dubreuil, J.; Famelart, M. H.; Bazureau, J. P. *Org. Process Res. Dev.* **2002**, *6*, 374.

(3) Lee, S. H.; Lee, S. B. *J. Chem. Technol. Biotechnol.* **2009**, *84*, 202.

(4) Magde, D.; Wong, R.; Seybold, P. G. *Photochem. Photobiol.* **2002**, *75*, 327.

(5) Williams, A. T. R.; Winfield, S. A.; Miller, J. N. *Analyst* **1983**, *108*, 1067.

(6) Franken, N. A. P.; Rodermond, H. M.; Stap, J.; Haveman, J.; van Bree, C. *Nature Protocols* **2006**, *1*, 2315.

(7) Vader, P.; van der Aa, L. J.; Engbersen, J. F. J.; Storm, G.; Schiffelers, R. M. *J. Controlled Release* **2010**, *148*, 106.