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Supporting information:

Aromatic long chain cations of ionic liquids permeabilise the inner mitochondrial membrane and induce mitochondrial dysfunction at cytotoxic concentrations

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1. Supporting Tables

Table S1 Viability of HeLa cells relative to DMSO-treated control cells after treatment with AmILs at their MTS IC_{50} concentrations for 4 hours, as measured by the MTS assay.

Compound	**HeLa IC_{50} (mM**	**Cell viability (% of control)**
$[C_4quin][Br]$	2.24 ± 0.06	93 ± 2.7
$[C_4py][Br]$	13.31 ± 1.18	99 ± 1.6
$[C_4mim][Br]$	13.88 ± 1.84	96 ± 2.8
$[C_4TMA][Br]$	11.43 ± 1.23	96 ± 2.8
$[C_{10}quin][Br]$	0.0268 ± 0.0003	93 ± 0.98
$[C_{10}py][Br]$	0.0888 ± 0.0003	97 ± 0.47
$[C_{10}mim][Br]$	0.0848 ± 0.0006	97 ± 1.8
$[C_{10}TMA][Br]$	0.197 ± 0.0073	95 ± 1.2

2. Supporting Figures

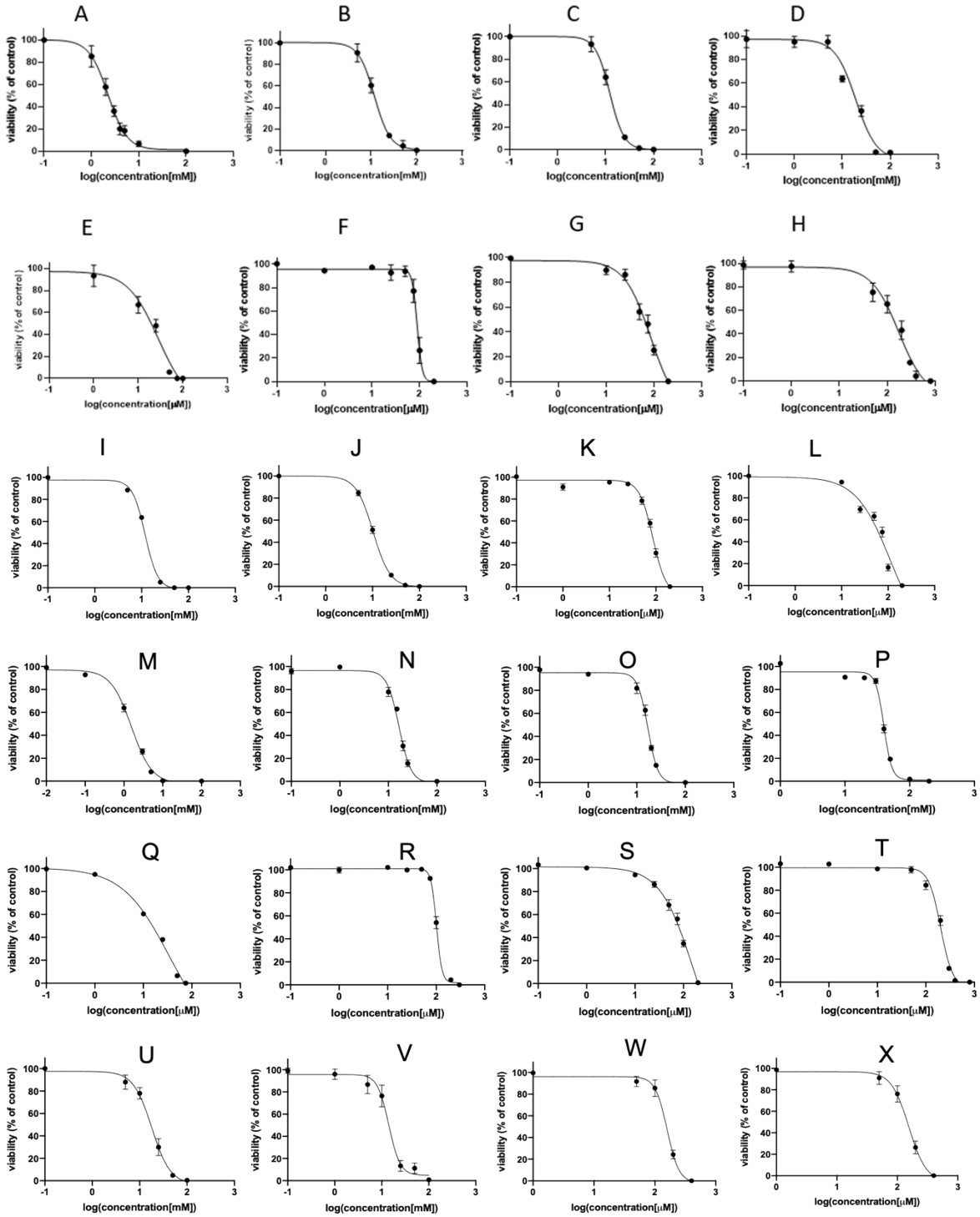


Figure S1 Dose-response curves showing the effect of AmILs on the cell viability after 48 h treatment. In HeLa cells, A) [C_4Quin][Br], B) [C_4Py][Br], C) [C_4Mim][Br], D) [C_4TMA][Br], E) [C_{10}Quin][Br], F) [C_{10}Py][Br], G) [C_{10}Mim][Br], H) [C_{10}TMA][Br], I) [C_4Mim][BF_4], J)

[C_4Mim][CF_3SO_3], K) [C_{10}Mim][BF_4], L) [C_{10}Mim][CF_3SO_3]. In Beas-2b cells, M) [C_4Quin][Br], N) [C_4Py][Br], O) [C_4Mim][Br], P) [C_4TMA][Br], Q) [C_{10}Quin][Br], R) [C_{10}Py][Br], S) [C_{10}Mim][Br], T) [C_{10}TMA][Br], U) [C_4Mim][BF_4], V) [C_4Mim][CF_3SO_3], W) [C_{10}Mim][BF_4], X) [C_{10}Mim][CF_3SO_3]. Data represents the mean ± SEM of 3 independent experiments.

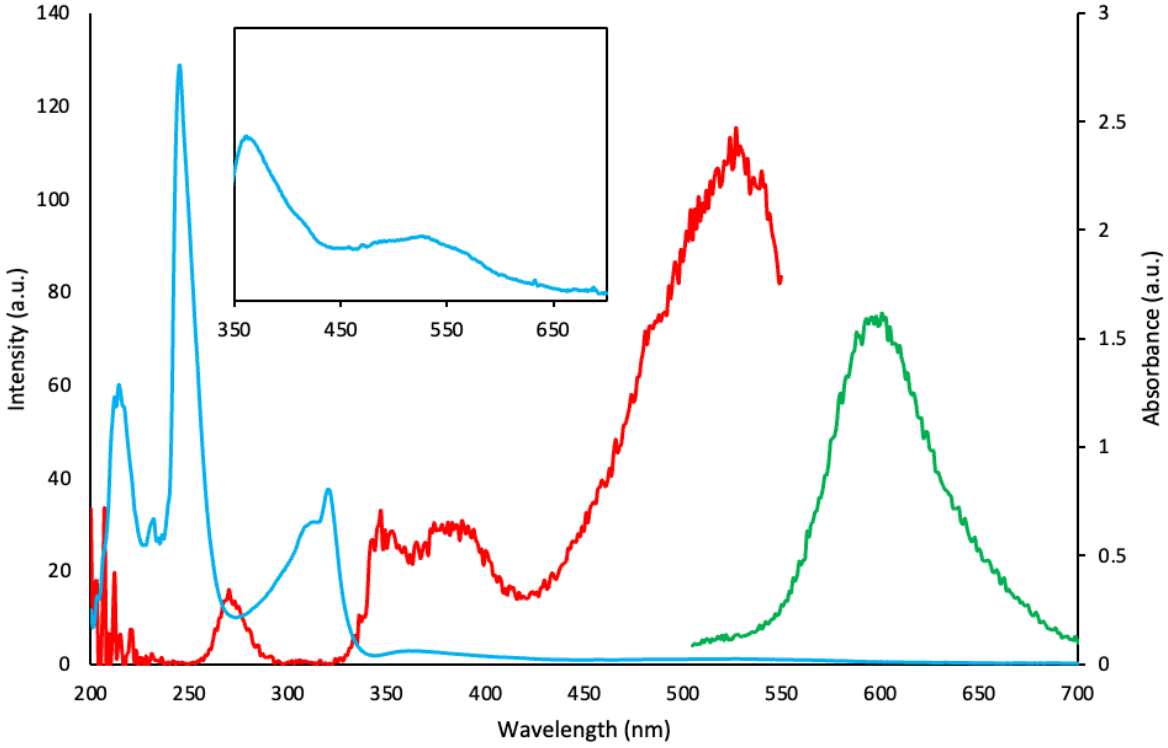


Figure S2 Optical absorbance (blue), excitation (red) and emission from 488 nm (green) spectra for [C_4MeQuin] [I] in acetonitrile. Inset shows absorbance between 350 – 700 nm for clarity.

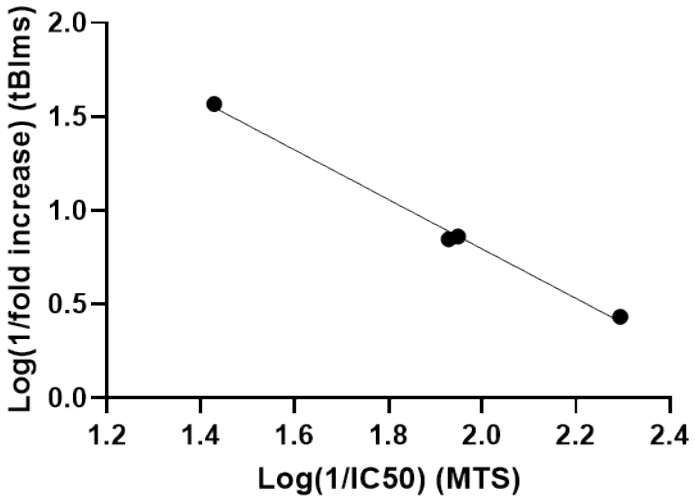


Figure S3 Correlation plot of tBLMs conductance Log(1/fold increase) against MTS Log(1/IC_{50}) for C_{10}-AmILs.

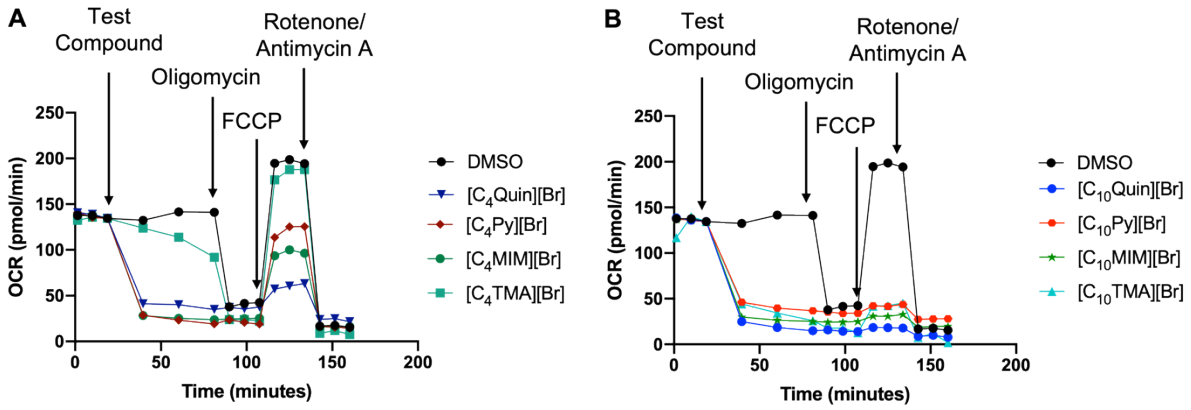


Figure S4 (A) The effect of C_4-ILs on oxygen consumption rate (OCR) in HeLa cells. (B) The effect of C_{10}-ILs on oxygen consumption rate (OCR) in HeLa cells. The sequential addition of the ILs at IC_{50} concentrations, oligomycin a mitochondrial ATP-synthase inhibitor (1 μM), the protonophore FCCP (2 μM) and the electron transport chain complex inhibitors rotenone/antimycin A (1 μM). Data represents the average of 2 wells from the same experiment.

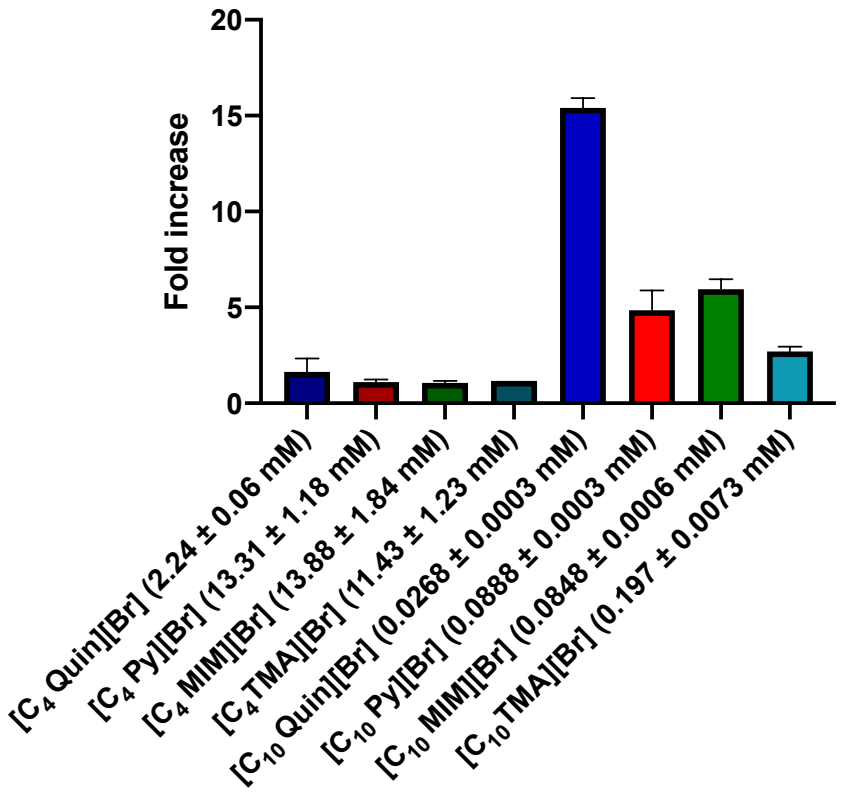


Figure S5 Effects of AmILs (at MTS IC_{50} concentrations) on the conductance of DOPC lipid bilayers tethered to gold electrodes, as measured by electrical impedance spectroscopy. Data normalised to membrane conductance prior to AmIL treatment at pH = 7. Data represents the mean 2 independent experiments.

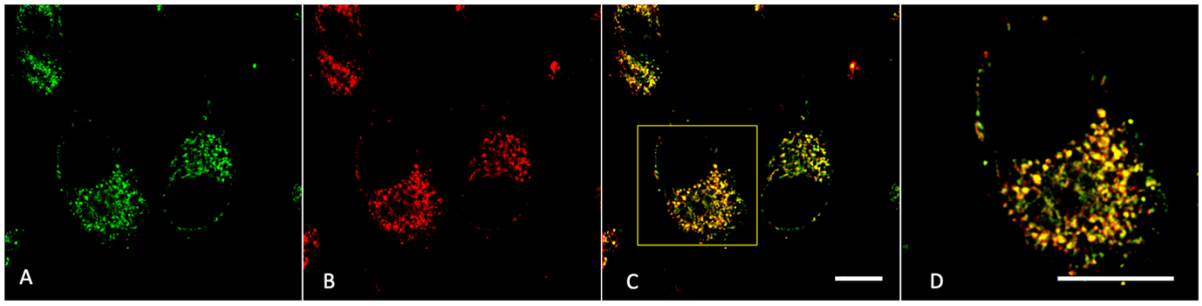


Figure S6 Confocal fluorescence microscopy of [C_4MeQuin][I] in HeLa cells without the Hoechst stain. (A) Green channel [C_4MeQuin][I] (500nM, λ_{ex}= 488 nm). (B) Red channel Mitotracker Deep Red (200 nM, λ_{ex}= 644 nm). (C) Merged channels. (D) Magnified view of region in (C). Scale Bar (10 μm).

3. Experimental procedures - Chemistry

General Chemistry

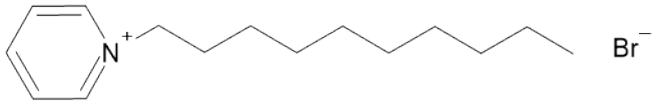
[C_4Py][Br], [C_4MIM][Br], [C_4TMA][Br], [C_{10}TMA][Br], all chemical reagents and solvents were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) or Chem-Supply (Port Adelaide, SA, Australia). The purity of all test compounds was confirmed to be ≥ 95% by absolute quantitative ^{1}H nuclear magnetic resonance (qNMR) spectroscopy. ^{1}H and ^{13}C NMR spectra were acquired using an Agilent 500 MHz spectrometer (500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C) in deuterated chloroform ($CDCl_3$) unless otherwise specified. Melting point determination was preformed using Stuart automatic melting point. High- resolution mass spectroscopy (HRMS) was performed on an Agilent 6510 Accurate-Mass Q-TOF Mass Spectrometer equipped with an ESI source.

General Procedure for synthesis of Ionic liquids.

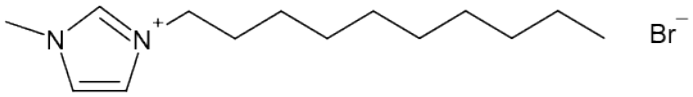
The alkyl bromide (5 mmol) and appropriate heterocycle (5 mmol) were heated at 140 °C for 24 hours. The reaction mixture was cooled to room temperature and the resulting crude products were purified by one of the following methods:

Method 1 – Crude solids were triturated with hexane. The solids were then dissolved in a minimum volume of chloroform, and diethyl ether was added dropwise until precipitation. The resultant solid was then collected using vacuum filtration and used without further purification.

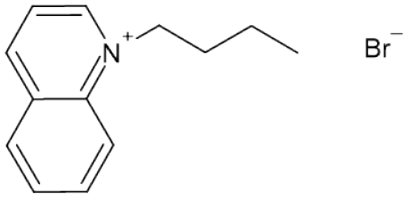
Method 2 – Crude oils were washed with hexane which was subsequently removed by decantation. The washed oil was then dissolved in a minimum volume of chloroform, and diethyl ether was added dropwise until the oil reformed. The ether layer was removed via decantation and the resultant oil was dried under reduced pressure.



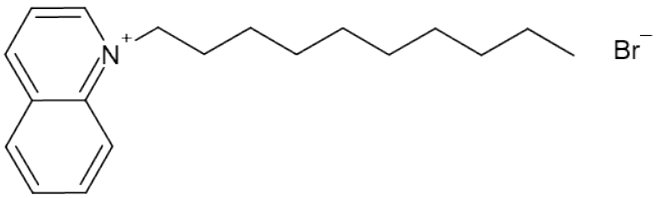
1-Decylpyridinium bromide ([C_{10}Py][Br]) was obtained as a yellow wax. Product formed by method 1. Yield = 90%. ^{1}H NMR ($CDCl_3$): δ 9.47 (d, *J* = 6 Hz, 2H), 8.51 (t, *J* = 8 Hz, 1H), 8.13 (t, *J* = 6.5 Hz, 2H), 5.03 (t, *J* = 7.5 Hz, 2H), 2.03 (pent, *J* = 8 Hz, 2H), 1.22-1.42 (m, 14H), 0.88 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 145.31, 144.99, 129.71, 128.56, 62.21, 31.90, 31.80, 29.44, 29.34, 29.21, 29.05, 26.07, 22.63, 14.09. HRMS (ESI) *m/z* $[M]^+$ calculated for $C_{15}H_{26}N$ 220.2054; found 220.2061. Purity by Q-NMR = 96.3 %. The ^{1}H and ^{13}C NMR spectrum were in agreement with previously reported data.[1]



1-Decyl-3-methylimidazolium bromide ([C_{10}Mim][Br]) obtained as a colourless liquid. Product formed by method 1. Yield = 96 %. ^{1}H NMR ($CDCl_3$): δ 10.46 (s, 1H), 7.45 (t, *J* = 1.5 Hz, 1H), 7.32 (t, *J* = 2 Hz, 1H), 4.30 (t, *J* = 7.5 Hz, 2H), 4.12 (s, 3H), 1.86-1.94 (m, 2H), 1.18-1.37 (m, 14H), 0.86 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 137.36, 123.68, 121.51, 60.45, 36.75, 31.79, 30.29, 29.41, 29.33, 29.20, 28.96, 26.23, 22.61, 14.07. HRMS (ESI) *m/z* $[M+H]^+$ calculated for $C_{14}H_{27}N$ 223.2163; found 223.2177. Purity by Q-NMR = 99.3 %. The ^{1}H NMR spectrum were in agreement with previously reported data.[2]



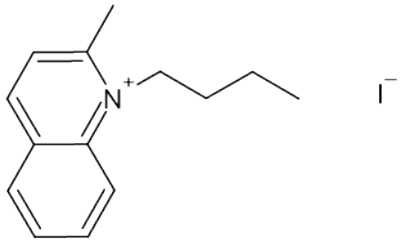
1-Butylquinolinium bromide ([C_4Quin][Br]) obtained as a brown solid. Product formed by method 2. Yield = 93 %. Mp = 159.3 °C. ^{1}H NMR ($CDCl_3$): δ 10.63 (d, *J* = 5.5 Hz, 1H), 9.00 (d, *J* = 8 Hz, 1H), 8.30-8.35 (m, 2H), 8.21 (t, *J* = 7.5 Hz, 2H), 7.97 (t, *J* = 8 Hz, 1H), 5.42 (t, *J* = 7.5 Hz, 2H), 2.10 (pentet, *J* = 8 Hz 2H), 1.57 (sext, *J* = 8 Hz, 2H), 1.01 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 150.48, 147.25, 137.67, 136.06, 131.18, 130.20, 129.71, 122.61, 118.40, 58.01, 32.26, 19.81, 13.63. HRMS (ESI) *m/z* $[M]^+$ calculated for $C_{13}H_{16}BrN$ 186.1272; found 186.1280. Purity by Q-NMR = 95.1 %. The ^{1}H and ^{13}C NMR spectrum were in agreement with previously reported data.[3]



1-Decylquinolinium bromide ([C_{10}Quin][Br]) obtained as a brown solid. Product formed by method 2. Yield = 81 %. Mp = 53.4 °C. ^{1}H NMR ($CDCl_3$): δ 8.93 (dd, *J* =1.5, 4 Hz, 1H), 8.17 (d, *J* = 8.5 Hz, 1H), 8.12 (d, *J* = 8 Hz, 1H), 7.83 (d, *J* = 8 Hz, 1H), 7.73 (t, *J* = 8 Hz, 1H), 7.56 (t, *J* = 8 Hz, 1H), 7.41 (dd, *J* = 4.5, 8.5 Hz, 1H), 3.41 (t, *J* = 7 Hz, 2H), 1.85 (pent, *J* = 8 Hz, 2H), 1.20-1.34 (m, 14H), 0.88 (t, *J* = 7 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 150.59, 147.16, 137. 65, 135.94, 131.17, 130.15, 129.72, 122.72, 118.30, 58.25, 31.79, 30.41, 29.43, 29.33, 29.20, 29.15, 26.50, 22.62, 14.09. HRMS (ESI) *m/z* $[M]^+$ calculated for $C_{19}H_{28}N$ 270.2211; found 270.2217. Purity by Q-NMR = 97.9 %. The ^{1}H NMR spectrum were in agreement with previously reported data.[4]

Procedure for the synthesis of [C_4MeQuin][I]

Quinaldine (1 mmol) was heated with 1-iodobutane (5 mmol) at 140 °C for 24 hours in a sealed pressure tube. The crude mixture was cooled to room temperature and the resulting solid was triturated with hexane and then diethyl ether to afford pure product.



1-butyl-2-methylquinolinium iodide ([C_4MeQuin][I]) obtained as a black solid. Product formed by method 2. Yield = 48 %. Mp = 156.9 °C. ^{1}H NMR (DMSO): δ 9.10 (d, *J* = 11 Hz, 1H), 8.58 (d, *J* = 10 Hz, 1H), 8.25-8.21 (m, 1H), 8.12 (d, *J* = 11 Hz, 1H), 7.99 (t, *J* = 9 Hz, 1H), 4.92 (t, *J* = 10 Hz, 2H), 3.12 (s, 3H), 1.89 (pentet, *J* = 10 Hz 2H), 1.59 (sext, *J* = 9 Hz, 2H), 0.99 (t, 9 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 161.06, 146.14, 138.75, 135.77, 131.09, 129.52, 128.73, 126.05, 119.42, 51.72, 30.47, 22.96, 19.77, 14.00. HRMS (ESI) *m/z* $[M]^+$ calculated for $C_{14}H_{18}N$ 200.1428; found 200.1432. Purity by Q-NMR = 98.2 %.

Procedure for anion exchange

[C_{10}Mim][[BF_4] and [C_{10}Mim][$CH_3SO_3^-$] were prepared by a metathesis reaction between [C_{10}Mim][Br] and saturated solutions of sodium tetrafluoroborate ($NaBF_4$) and sodium trifluoromethanesulfonate (CF_3NaO_3S) respectively. The precipitate was collected with vacuum filtrations and washed then dried under vacuum.

4. Experimental procedures - Biology

General cell culture and cell-based assays

HeLa cervical cancer cells and BEAS-2B lung epithelial cells were provided by **Professor Michael Murray** (University of Sydney) and Distinguished Professor Brian Oliver (University of Technology Sydney), and were originally obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies, Victoria, Australia). Confluent cells (80 – 90%) were harvested using Trypsin/EDTA after washing with PBS. Cells were treated with various concentrations of the test compounds in media (for C_4AmILs) or in media with 0.1% DMSO (for C_{10}AmILs); matched control cells were treated media only or media with 0.1% DMSO.

MTS cell viability assay: The ability of ILs to decrease the cell viability in HeLa cervical cancer cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) *in vitro* cytotoxicity assay. Cells (HeLa and Beas-2b) were seeded in 96-well plates (5 x 10^3 cells per well) and allowed to adhere overnight. The cells were then treated with various concentrations of ILs for 48 h. Control cells were treated with DMEM supplemented with 10% FBS and 0.1% DMSO. The cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer's recommendation. The IC_{50} dose response curves were defined as the test compound concentrations which prevented cell growth of more than 50% (relative to the vehicle control) and were constructed using non- linear regression analysis with Prism 8.4.2 (GraphPad Software, CA, USA).

Mitochondrial function: Mitochondrial function was measured by determining the OCR of cells with a Seahorse XF24 extracellular flux analyser (Seahorse Bioscience, MA, USA) according to the manufacturer's protocol. HeLa cells were seeded in a 24-well XF cell culture

microplates (2.5×10^4 cells per well) and allowed to adhere overnight (37 °C, 5% CO_2). After 24 h, the culture media was replaced with buffered XF Base Medium supplemented with 2 mM L-glutamine, 10 mM glucose and 2 mM sodium pyruvate at pH=7.4. The cells were incubated at 37 °C without CO_2 for an hour, and then, the OCR was measured utilizing an XF Cell Mito Stress Test Kit (Seahorse Bioscience, MA, USA). Test compounds (final concentration at MTS IC_{50}), oligomycin (final concentration 1 μM), carbonylcyanide m-chlorophenylhydrazone (FCCP-final concentration 2 μM) and rotenone/antimycin A (final concentration 0.5 μM) were added to the sensor cartridge, and the OCR was measured using a modified cycling program.

Fluorescence microscopy: HeLa cells were seeded on a 24-well glass bottom plate (1 x 10^4 cells/well) and allowed to adhere overnight. Cells were treated with [C_4MeQuin][I] (500 nM) and stained with Hoechst (5 μM) and mitotracker deep red (100 nm) and then incubated (37 ^oC, 20 min). The cells were washed twice with media, and then incubated with 4% paraformaldehyde/PBS to fix the cells (37 ^oC, 20 min). The cells were washed twice with PBS prior to examination of the cells on a Nikon A1 laser scanning confocal microscope with a 60X Plan Apo (NA 1.4) objective. Hoechst 33342 stained nuclei were detected using a 405 nm laser and a 400-450 nm bandpass filter. [C_4MeQuin][I] labelled structures were detected with a 458 nm laser and a 500-550 nm bandpass filter. Mitotracker Deep Red labelled Mitochondria were detected with a 640 nm laser and a 662-737 nm bandpass filter.

JC-1 assay: HeLa cells were seeded in triplicate in black 96-well plates (1 x 10^4 cells/well) and allowed to adhere overnight. The cells were treated with test compounds at their individual cell viability IC_{50} concentrations for 1 h, control cells received DMEM with 10% FBS and 0.1% DMSO. Cells were incubated with the JC-1 dye in media with 10% FBS (37^oC, 20 min) and the JC-1 red: green ratio was measured on the teacan plate reader. (JC-1 Mitochondrial Membrane Potential Assay Kit; Cayman Chemical, Ann Arbor, MI).

ATP assay: ATP formation was assessed in HeLa cells which were seeded in triplicate in black

96-well plates (1 x 10^4 cells/well) and allowed to adhere overnight. Cells were treated with ILs at their respective cell viability IC_{50} concentration at various time points (0-4 hr) in DMSO (final concentration of 0.1 %), control cells were treated with 0.1% DMSO alone. CellTiter-Glo solution was added to cells (RT, 30 min) and the luminescence was determined (CellTiter-Glo® luminescent cell viability assay, Promega; Annandale, NSW, Australia).

ROS assay: HeLa cells were seeded in triplicate in a 96-well black plate (2 x 10^4 cells/well) and allowed to adhere overnight. The following day cells were washed with 200 µL prewarmed PBS, then cells were incubated with 100 µL of DCFDA solution for 20 mins at 37 ^oC. Next, cells were treated with ILs at their individual cell viability IC_{50} values for 6 h and H_2O_2 (200 µM, positive control) and ROS was measured on the teacan plate reader.

Statistical analysis: For in vitro assays, the data obtained was analysed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. $P < 0.05$ were accepted as being statistically significant. All statistical analyses were performed using GraphPad Prism version 8.4.2 (GraphPad Software, CA, USA). The results are expressed as the means ± SEM unless otherwise specified.

5. Experimental procedures – Tethered bilayer lipid membranes

Tethered bilayer lipid membranes: Lipid bilayers were anchored across a gold electrode according to "T10" architecture.[5,6] This consists of 10% benzyl-disulfide (tetra-ethyleneglycol) n=2 C20-phytanyl "tethering" molecules interspersed with 90% benzyl-disulfide-tetra-ethyleneglycol-OH "spacer" molecules. Spacer and tether molcules are all coordinated onto a 2.1 mm2 gold tethering electrode. To these first layer chemistries were added a second layer of mobile phase lipid molecules of 3 mM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids Inc., Alabaster, USA) which was left to incubate with the tethering molecules for exactly 2 min before a rapid exchange of 3 x 400 μL 100 mM NaCl tris buffer induced the formation of a completed tBLM.

Electrical impedance spectroscopy: Swept frequency electrical impedance spectroscopy ranging from 0.1 Hz to 2000 Hz was applied at 25 mV peak-to-peak, using a Tethapod™ electrical impedance spectrometer (SDx Tethered Membranes Pty Ltd). Impedance and phase profiles were fitted to an equivalent circuit consisting of a constant phase element, representing the tethering gold electrode and reservoir region, in series with a resistor, to represent the impedance of the surrounding electrolyte solution, and a resistor/capacitor representing the lipid bilayer. Data fitting utilized a proprietary adaptation of a Levenberg–Marquardt fitting routine incorporated into the TethaQuickTM software (SDx Tethered Membranes Pty Ltd).

6. References

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