Electronic Supplementary Information

Ratiometric Raman imaging reveals the new anti-cancer potential of lipid targeting drugs

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Fluorescence staining for viability

Cell viability was assessed using Molecular Probes™ LIVE/DEAD® Viability/Cytotoxicity Kit *for mammalian cells* (Invitrogen). A staining solution of calcein AM (2 µM) and ethidium homodimer-1 (4 µM) in medium was prepared. Media was removed from the dish and replaced with 1 ml staining solution. After *ca.* 30 min incubation at room temperature, staining solution was removed and sample was imaged using a Nikon Eclipse LV100 microscope with a Photometrics CoolSNAP HQ camera, Nikon Lu Plan 20×/NA 0.40 objective, and a Semrock BrightLine® FITC-3540B-NTE filter cube for live cells or a Semrock BrightLine® TXRED-4040B-NTE filter cube for dead cells. Images were acquired using MetaMorph software with a 0.1 s (Figure S1) or 0.05 s (Figure S3) acquisition time. Brightfield images were takes using a 0.01 s acquisition time (Figure S1). Images were processed in ImageJ to merge channels and manually adjust brightness and contrast.

Live cell viability pre and post Raman analysis

PC3 human prostate cancer were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and PNT2 human normal immortalized prostate epithelium cells were cultured in Rosewell Park memorial Institute medium (RPMI) in both cases supplemented with 1% penicillin/streptomycin (10,000 units/ml), 1% fungizone, and 10% heat-inactivated foetal bovine serum (FBS). Cells were incubated at 37 °C and 5% CO2 in a humidified incubator.

Cells were seeded (*ca.* 2.5×10^5 cells) in 35 mm glass bottomed imaging dishes (Ibidi). After overnight incubation at 37 °C and 5% CO_2 in a humidified incubator, cell medium was removed and replaced with phenol red free DMEM supplemented with 1% penicillin/streptomycin (10,000 units/ml), 1% funizone, and 1% heat-inactivated FBS. After further overnight incubation at 37 °C and 5% CO_2 in a humidified incubator, 5 µl of DMSO (control, 0.25% v/v), 5 µl of 40 mM Orlistat in DMSO (final concentration 100 µM) or 5 µl of 200 µM CAY10566 in DMSO (final concentration 500 nM) was added. After 24 hr incubation at 37 °C and 5% CO_2 in a humidified incubator, Raman measurements were carried out.

Raman spectra were acquired on a Renishaw inVia Raman microscope equipped with a 532 nm Nd:YAG laser, 1800 l/mm grating and a Nikon NIR Apo 60×/1.0W DIC water immersion objective.

For Raman mapping measurements, the imaging dish was removed from the incubator following

treatment with DMSO, Orlistat or CAY10566. A 60× water immersion objective was used to focus on the adherent cells growing on the glass bottomed surface of the imaging dish. Maps of two areas were acquired for each condition using a step size of 1 µm in x and y, 0.2 s acquisition time and 50% laser power. Two maps were acquired per area, firstly using a center of 3000 cm^{-1} and immediately subsequently using a center of 1400 cm^{-1}.

Cell viability was assessed for cells not undergoing Raman analysis (before Raman) and after Raman analysis of cells (after Raman) (Figure S1).

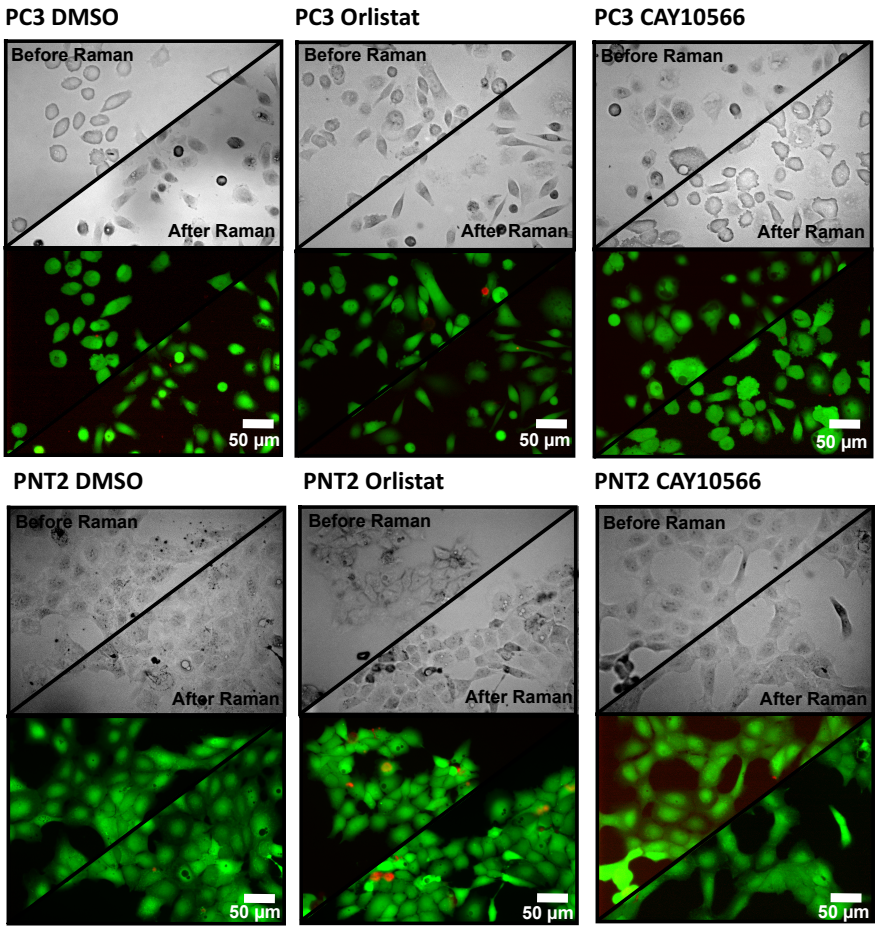

Figure S1 Cell viability pre and post Raman analysis Cells were stained using Molecular Probes™ LIVE/DEAD® viability/cytotoxicity fluorescence assay and analyzed using a fluorescence microscope with FITC filter (green, live) and TexasRed filter (red, dead). Both PC3 prostate cancer cells and PNT2 prostate epithelial cells were treated with DMSO (vector, 0.25% v/v), orlistat in DMSO or CAY10566 in DMSO before viability assessment. Viability was assessed on live cells not undergoing Raman measurement (before Raman) and on live cells after undergoing Raman measurement (after Raman). Brightfield and merged live/dead fluorescence viability images are shown.

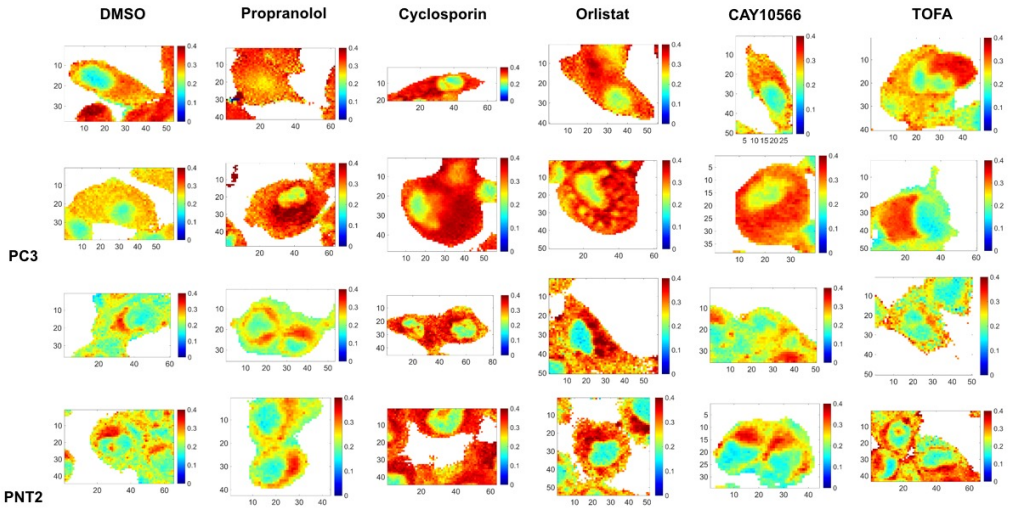

Figure S2 Ratiometric Raman imaging of intracellular lipid distribution Additional examples of fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2851 cm^{-1} were created as a reflection of lipid/protein ratio. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

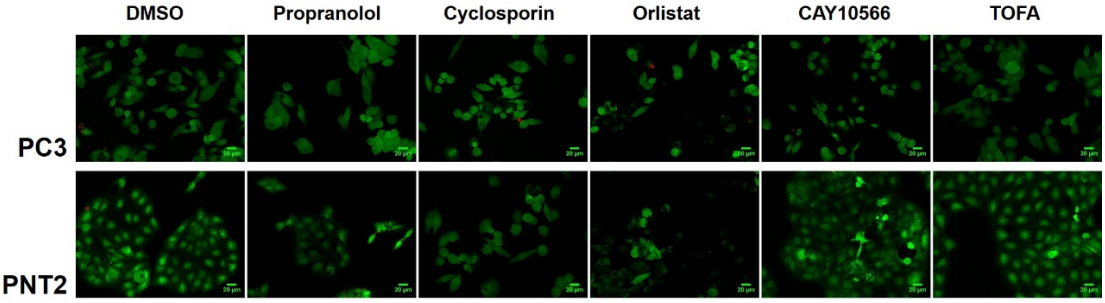

Figure S3 Cell viability assessment PC3 and PNT2 cells were stained using Molecular Probes™ LIVE/DEAD® viability/cytotoxicity fluorescence assay and analysed using a fluorescence microscope with FITC filter (green, live) and TexasRed filter (red, dead). Both PC3 prostate cancer cells and PNT2 prostate epithelial cells were treated with DMSO (vector), or one of a number of lipid altering drugs before viability assessment. Scale bar = 20 µm.

Fluorescence staining for phospholipidosis and steatosis

For fluorescence staining for intracellular accumulation of phospholipids and neutral lipids, a HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit (Invitrogen) was used. After overnight incubation following cell seeding, medium was removed and replaced with medium containing both the drug of interest and LipidTOX™ Red phospholipidosis detection reagent, which had been filtered through a 0.2 µm filter. Following 48 hr incubation at 37 °C and 5% CO_2 in a humidified incubator, medium was removed before adding Hoechst 33342 in 4% paraformaldehyde for *ca.* 30 mins. The

solution was then removed and cells were washed with PBS (×4) before adding LipidTOX™ Green in PBS for *ca.* 30 mins. Cells were imaged immediately using a Leica Microsystems SP8 confocal microscope equip with a 63×/NA 1.20 HC PL water immersion objective. Hoechst 33342 was imaged using a 405 nm diode laser for excitation and photomultiplier tube (PMT) detector detecting emission wavelengths from 410 nm to 500 nm. LipidTOX™ Green was imaged using a 488 nm argon laser for excitation and hybrid detector (HyD) for detection of emission wavelengths from 500-543 nm. LipidTOX™ Red was imaged using a 561 nm diode-pumped solid-state laser for excitation and hybrid detector (HyD) for detection of emission wavelengths from 580-755 nm.

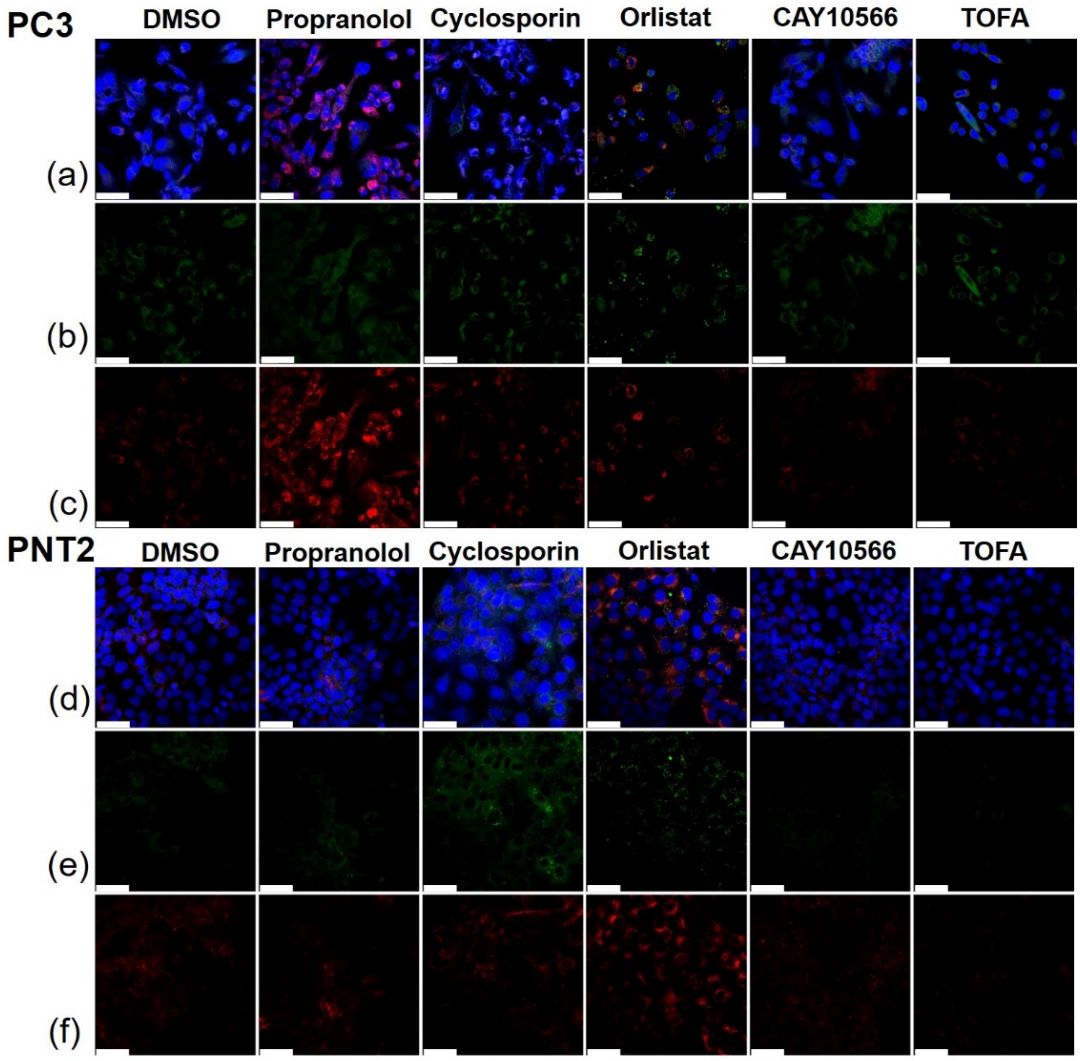

Figure S4 Fluorescent detection of phospholipidosis and steatosis Representative fluorescent images of PC3 (a) and PNT2 (b) cells treated for 48 hrs with DMSO (control) and a number of lipid altering drugs and stained with Hoechst 33342 (blue) for nuclei, LipidTOX™ Green (green) for accumulation of neutral lipids (steatosis) and LipidTOX™ Red (red) for accumulation of phospholipids (phospholipidosis). Images of all three channels (top), LipidTOX™ Green only (middle) and LipidTOX™ Red only (bottom) are shown. Scale bar = 50 µm.

Table S1 details the mean and standard deviation values for the intensity ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2935 cm^{-1} and 2851 cm^{-1} for spectra extracted from cell regions in a total of three different Raman mapped cells per condition. The calculated effect size for each condition is also given. This data corresponds to that given in Figure 4 in the main text.

2851 cm^{-1}/ (2933 cm^{-1} +2851 cm^{-1})	DMSO		Propranolol		Cyclosporin		Orlistat		CAY10566		TOFA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PC3	0.27	0.04	0.31	0.04	0.32	0.04	0.30	0.04	0.27	0.04	0.24	0.04
Effect Size			0.88		1.13		0.87		−0.02		−0.84	
PNT2	0.22	0.04	0.22	0.03	0.31	0.05	0.28	0.06	0.23	0.04	0.26	0.05
Effect Size			−0.06		1.92		1.12		0.12		0.69	

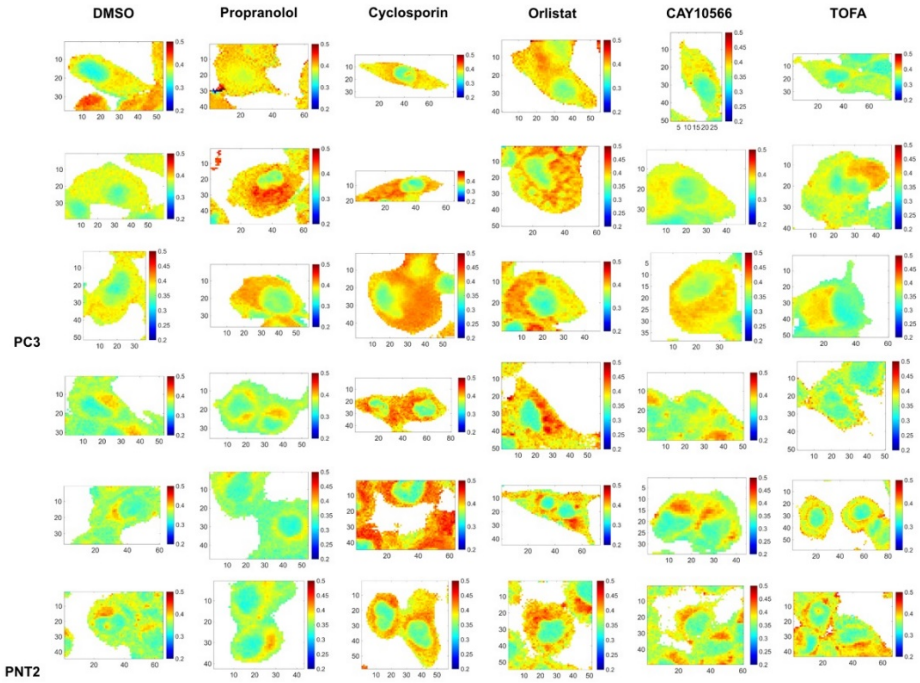

Figure S5 Ratiometric Raman imaging of intracellular lipid distribution Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2881 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2881 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

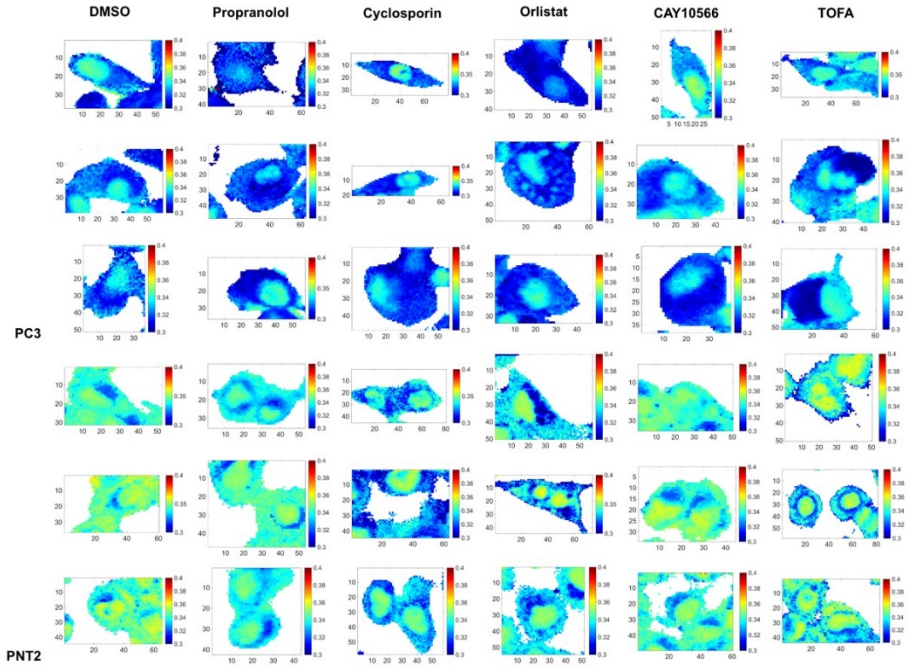

Figure S6 Ratiometric Raman imaging of intracellular lipid distribution Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2974 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2974 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

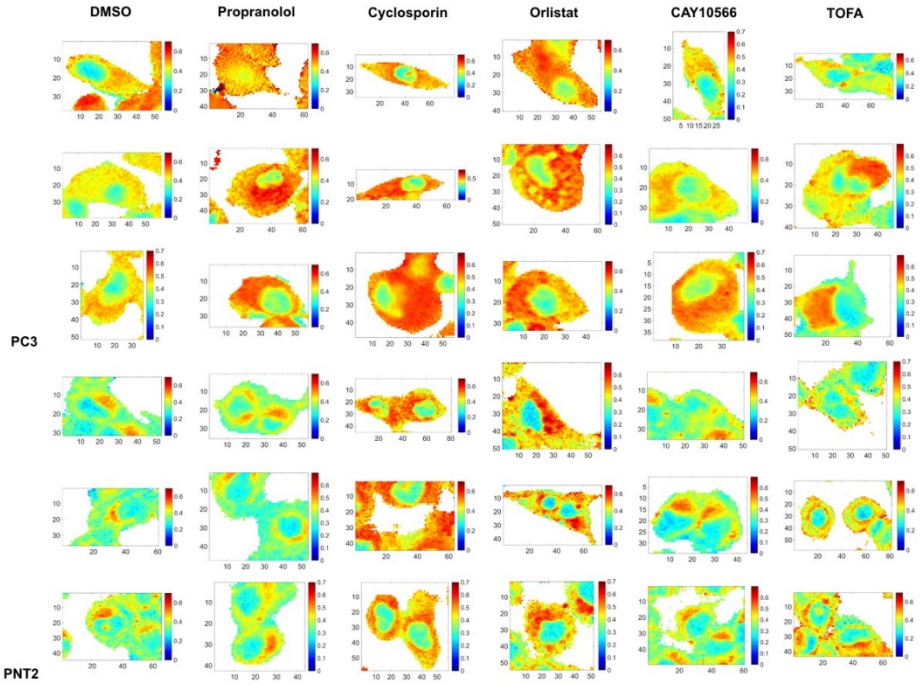

Figure S7 Ratiometric Raman imaging of intracellular lipid distribution Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2974 cm^{-1} and 2851 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

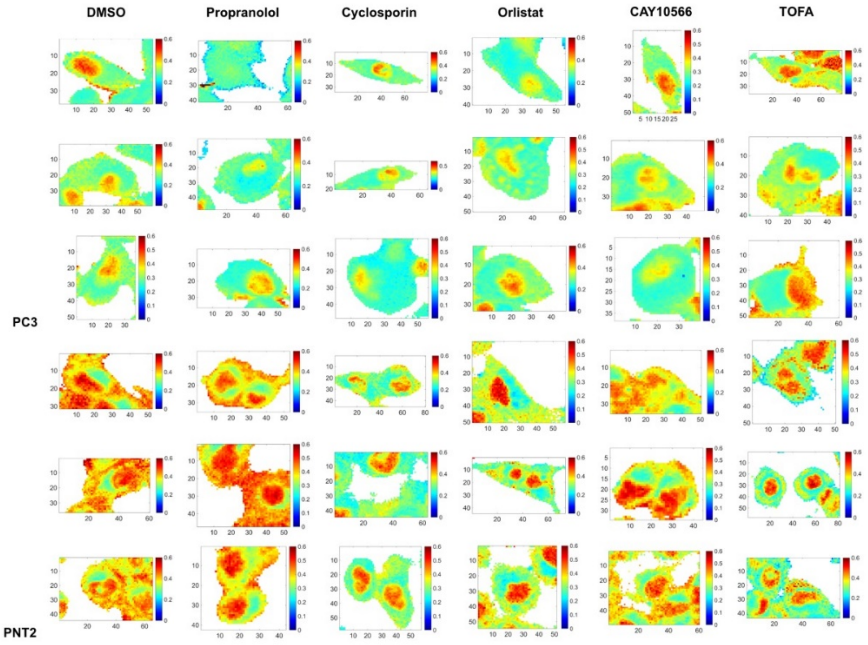

Figure S8 Ratiometric Raman imaging of intracellular lipid distribution Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 3013 cm^{-1} and the sum of the peak intensities at 2851 cm^{-1} and 3013 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

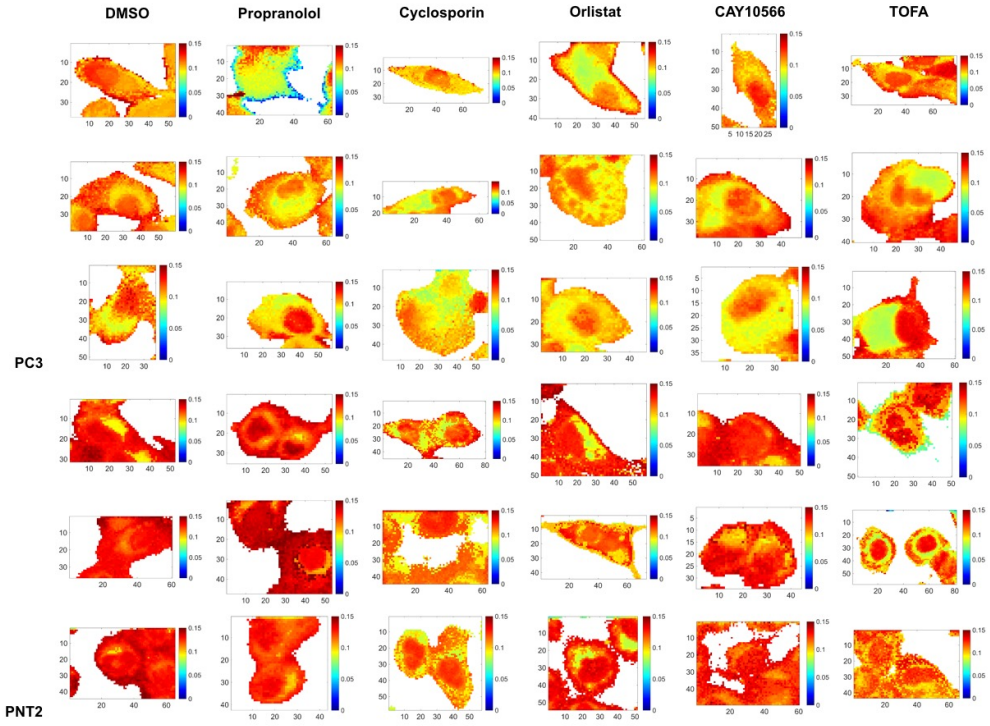

Figure S9 Ratiometric Raman imaging of intracellular lipid distribution Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 3064 cm^{-1} and the sum of the peak intensities at 2851 cm^{-1} and 3064 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

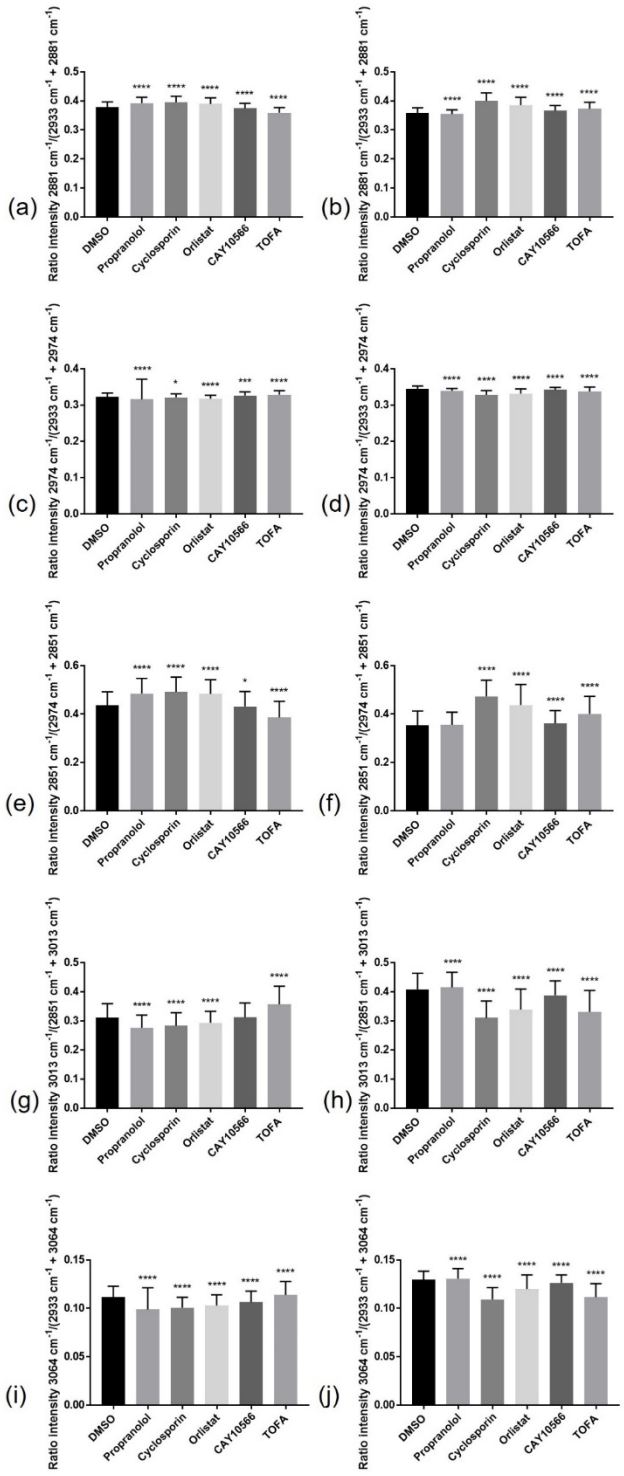

Figure S10 Quantitative Raman assessment of global lipid response The intensity ratios of a selection of Raman peak intensities for spectra extracted from cell regions in a total of three different cells per condition, were compared, where the mean and standard deviation for control PC3 cells (DMSO) and drug treated PC3 cells, and control PNT2 cells (DMSO) and drug treated PNT2 cells are shown. Ratio 2 = intensity 2881 cm^{-1}/(intensity 2881 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (a) and PNT2 (b); ratio 3 = intensity 2974 cm^{-1}/(intensity 2974 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (c) and PNT2 (d); ratio 4 = intensity 2851 cm^{-1}/(intensity 2851 cm^{-1} + intensity 2974 cm^{-1}) for PC3 (e) and PNT2 (f); ratio 5 = intensity 3013 cm^{-1}/(intensity 3013 cm^{-1} + intensity 2851 cm^{-1}) for PC3 (g) and PNT2 (h); ratio 6 = intensity 3064 cm^{-1}/(intensity 3064 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (i) and PNT2 (j). One-way ANOVA tests were performed between control (DMSO) and each drug treatment for both cell lines.
* p≤0.05; ** p≤0.01; *** p ≤0.001; **** ≤0.0001.

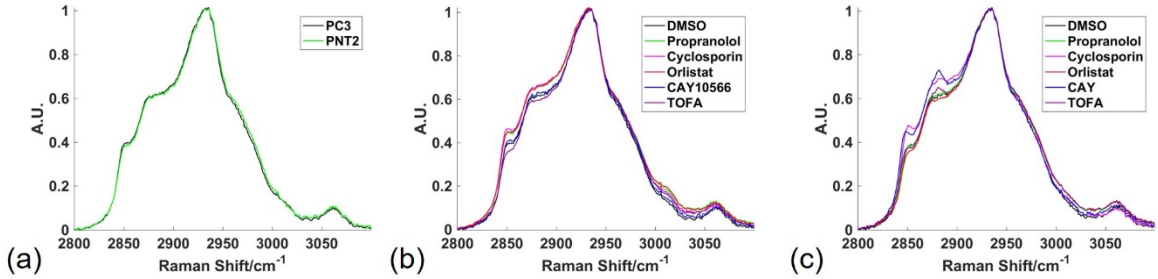

Figure S11 High wavenumber spectral response to lipid altering drugs biological replicate 2 Average Raman spectra from the high wavenumber spectral region from all spectra extracted from cell regions of three different Raman maps were compared between PC3 and PNT2 control (DMSO) cells (a); PC3 cells treated with DMSO (control), or a lipid altering drug (b); and PNT2 cells treated with DMSO (control), or a lipid altering drug (c) for a second biological replicate. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}.

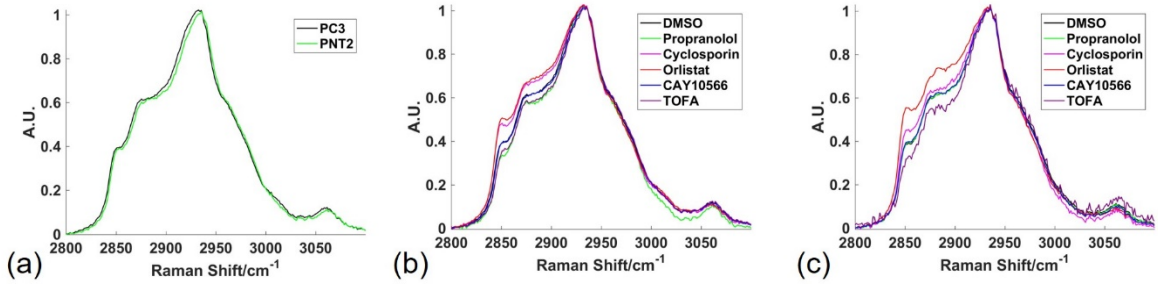

Figure S12 High wavenumber spectral response to lipid altering drugs biological replicate 3 Average Raman spectra from the high wavenumber spectral region from all spectra extracted from cell regions of three different Raman maps were compared between PC3 and PNT2 control (DMSO) cells (a); PC3 cells treated with DMSO (control), or a lipid altering drug (b); and PNT2 cells treated with DMSO (control), or a lipid altering drug (c) for a third biological replicate. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}.

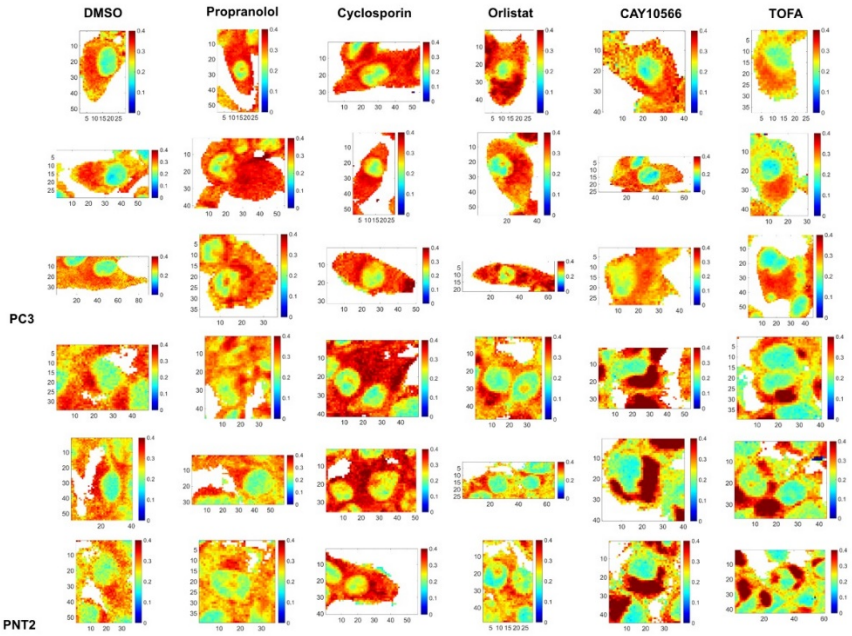

Figure S13 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 2 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a second biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2851 cm^{-1} were created as a reflection of lipid/protein ratio. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

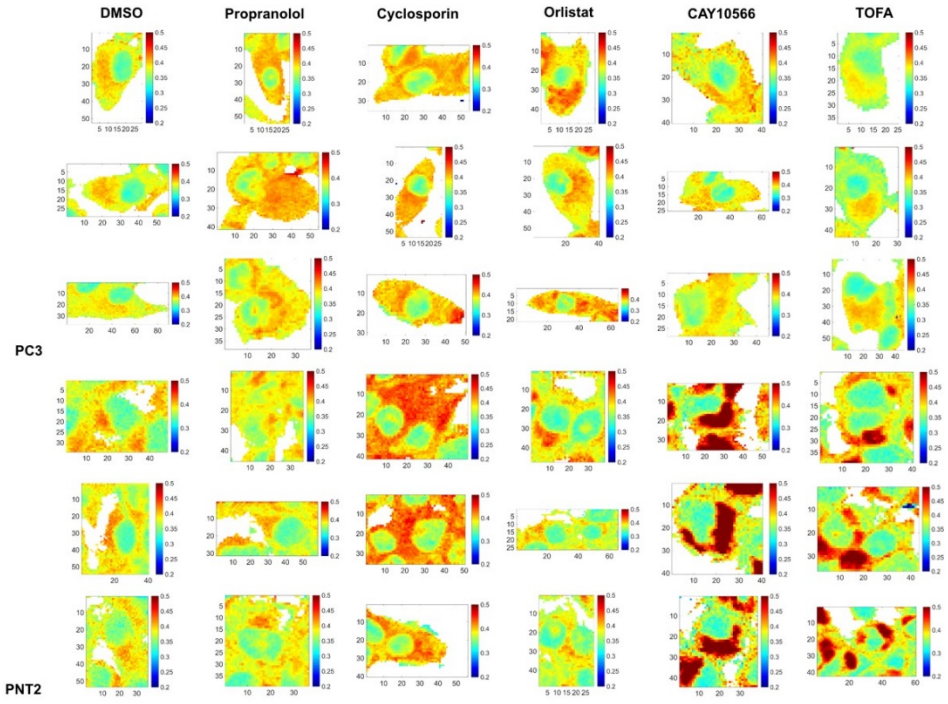

Figure S14 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 2 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a second biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2881 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2881 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

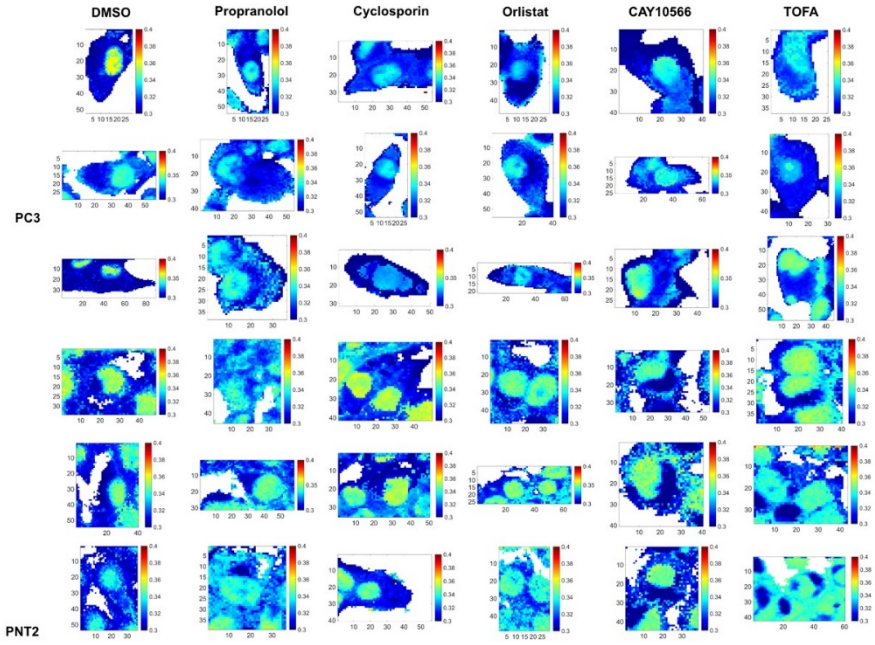

Figure S15 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 2 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a second biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2974 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2974 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

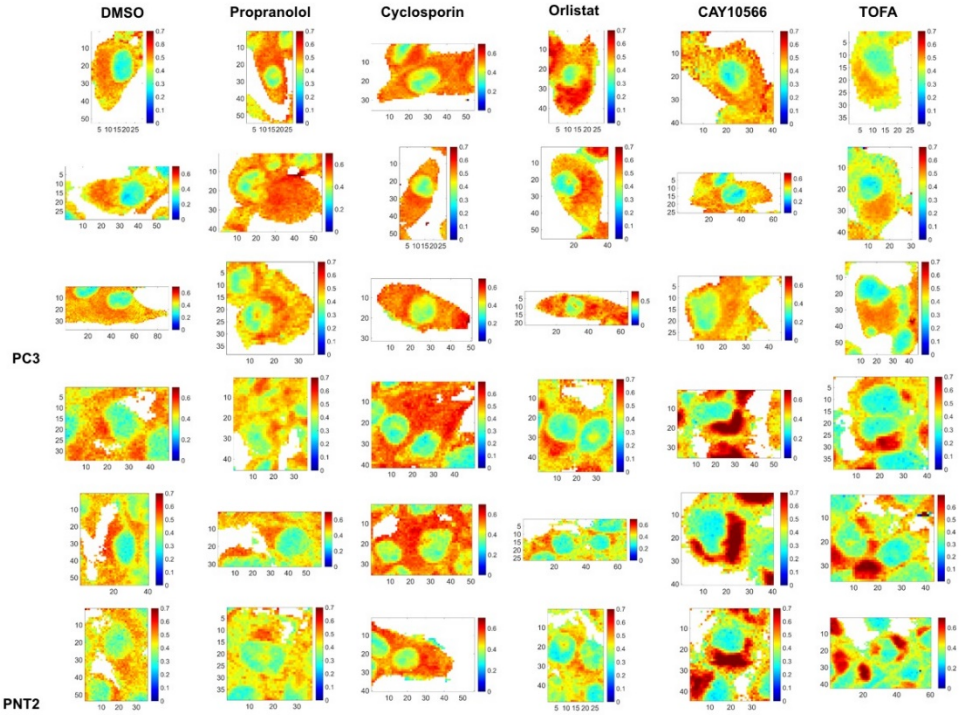

Figure S16 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 2 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a second biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2974 cm^{-1} and 2851 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

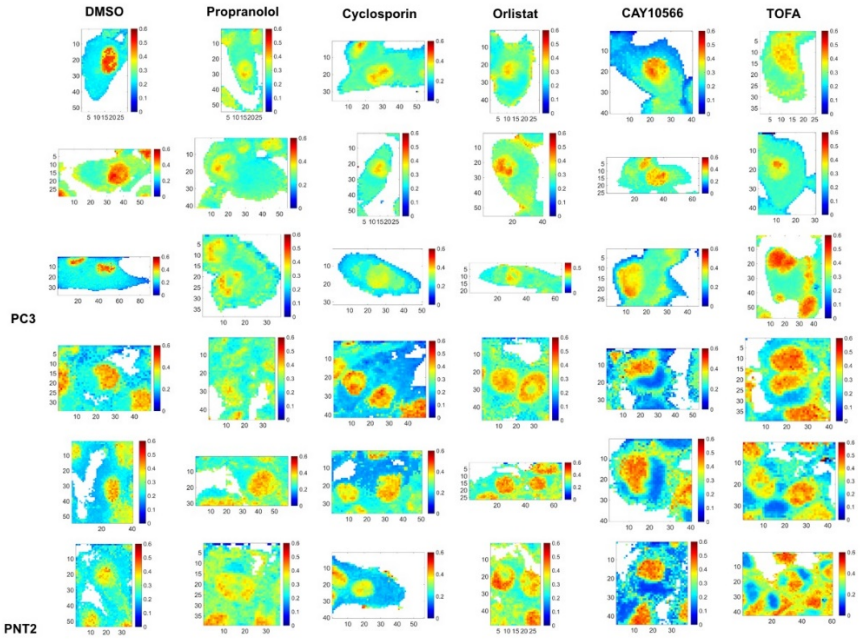

Figure S17 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 2 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a second biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 3013 cm^{-1} and the sum of the peak intensities at 2851 cm^{-1} and 3013 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

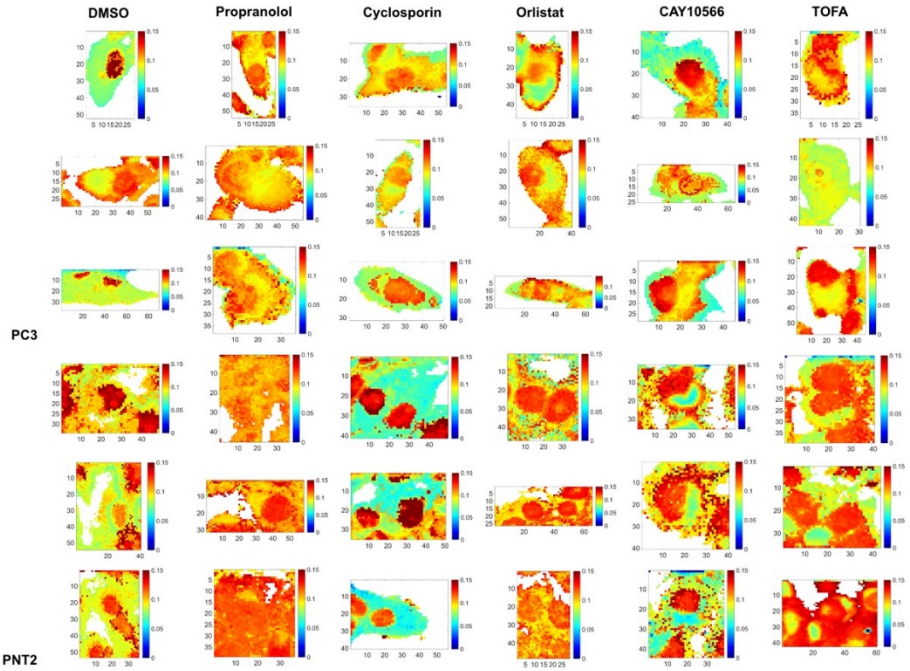

Figure S18 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 2 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a second biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 3064 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 3064 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

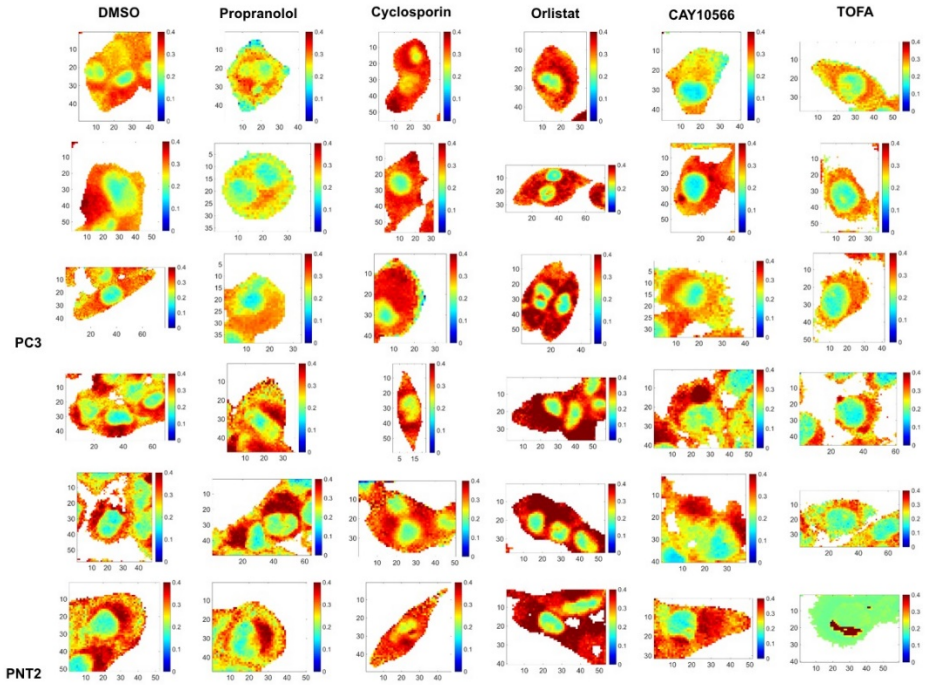

Figure S19 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 3 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a third biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2851 cm^{-1} were created as a reflection of lipid/protein ratio. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

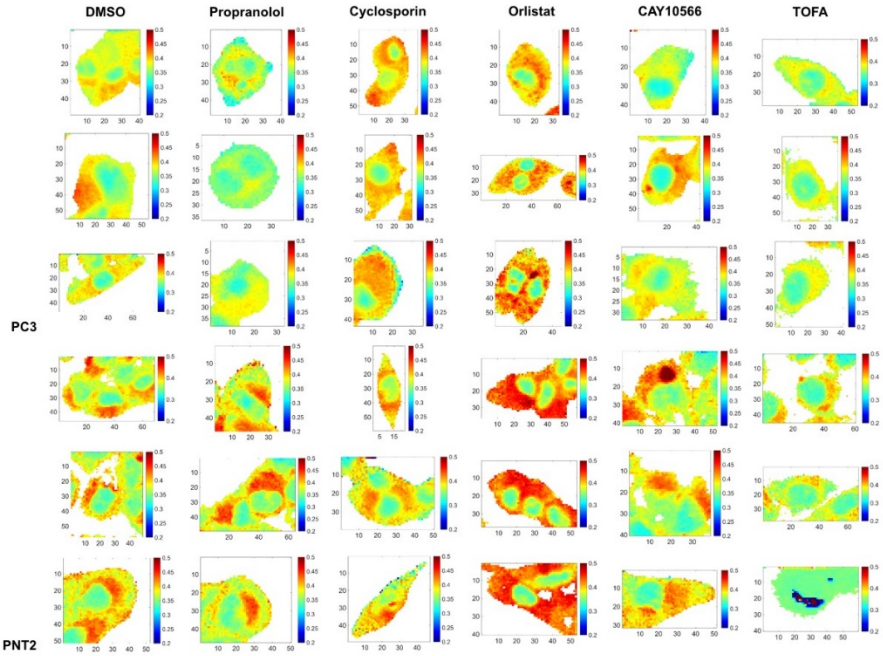

Figure S20 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 3 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a third biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2881 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2881 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

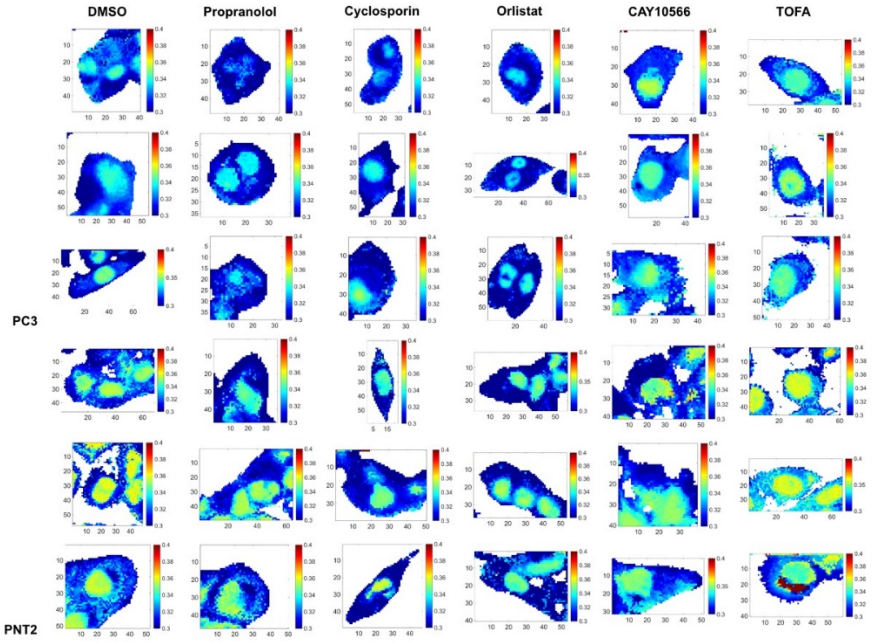

Figure S21 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 3 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a third biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2974 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 2974 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

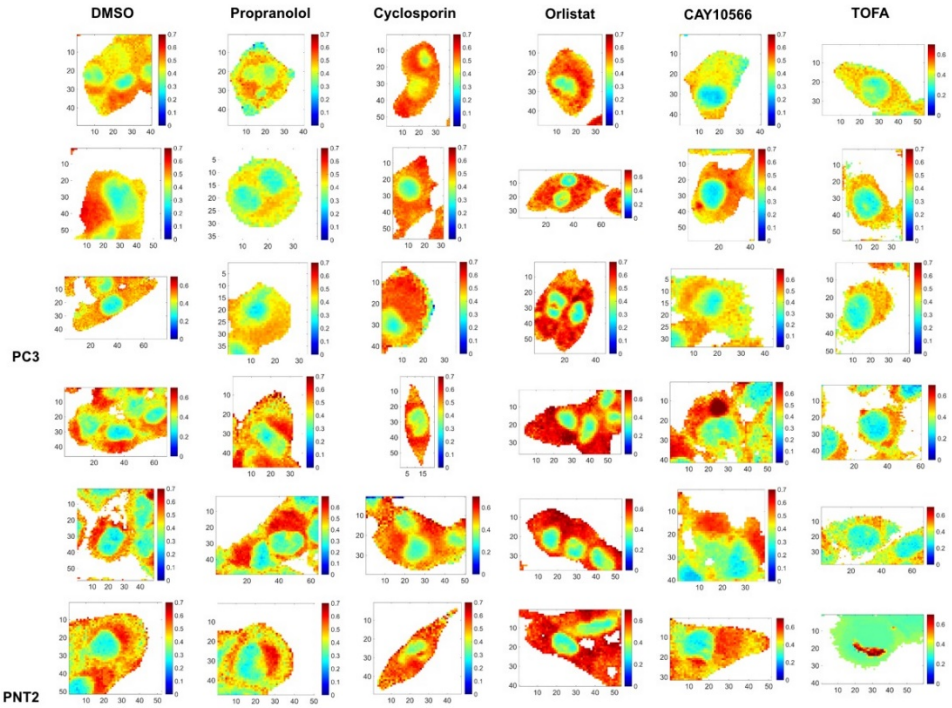

Figure S22 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 3 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a third biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 2851 cm^{-1} and the sum of the peak intensities at 2974 cm^{-1} and 2851 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

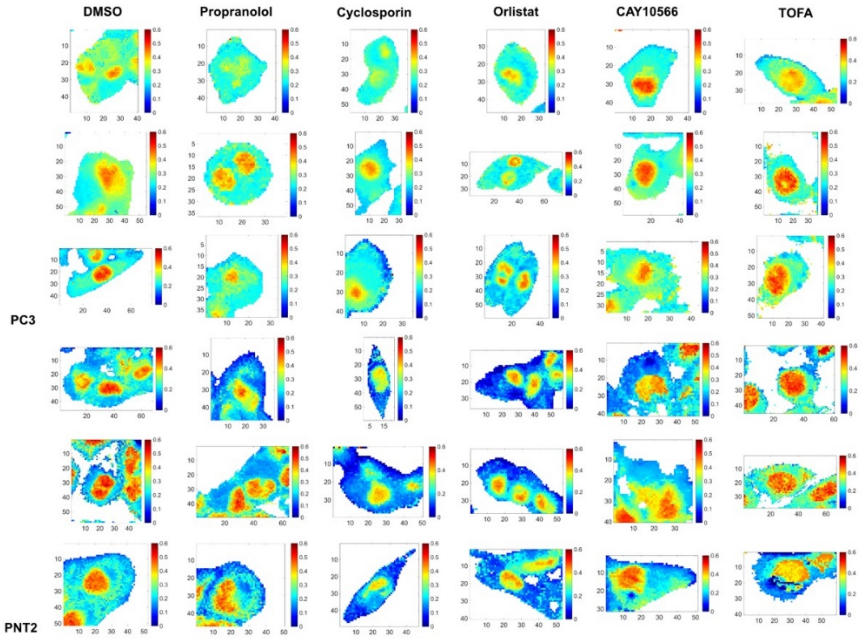

Figure S23 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 3 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a third biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 3013 cm^{-1} and the sum of the peak intensities at 2851 cm^{-1} and 3013 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

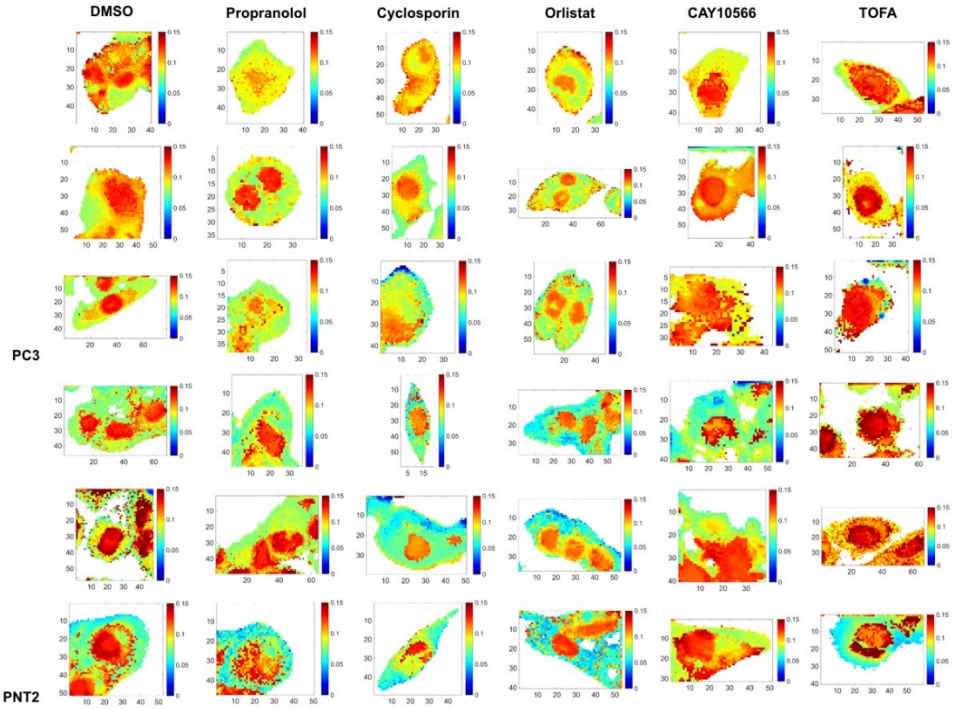

Figure S24 Ratiometric Raman imaging of intracellular lipid distribution biological replicate 3 Fixed cell regions mapped using a Raman microscope for two prostate cancer cell lines, PC3 and PNT2, treated with DMSO (control), or a lipid altering drug, for a third biological replicate. After processing in Wire 4.1 and MATLAB®, false color images of the ratio of peak intensity at 3064 cm^{-1} and the sum of the peak intensities at 2933 cm^{-1} and 3064 cm^{-1} were created. Cells were mapped using 532 nm, 0.5 s acquisition, 15 mW laser power, 1 µm step size in x and y and a spectral centre of 3000 cm^{-1}. Spatial coordinates on images are in µm.

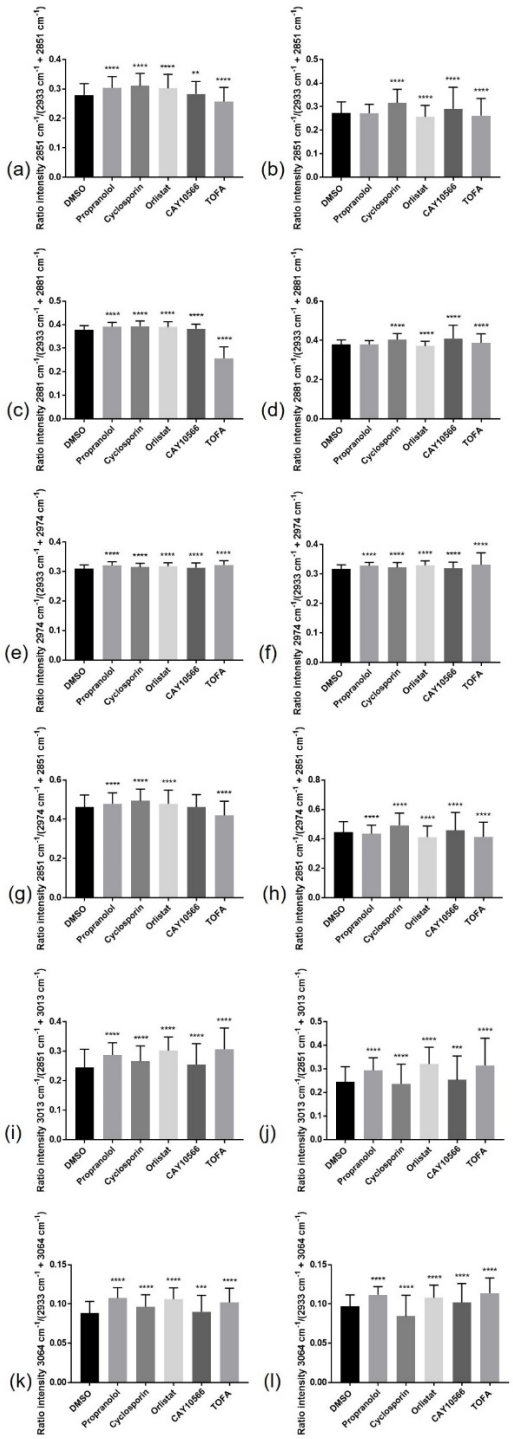

Figure S25 Quantitative Raman assessment of global lipid response biological replicate 2 The intensity ratios of a selection of Raman peak intensities for spectra extracted from cell regions in a total of three different cells per condition, were compared, where the mean and standard deviation for control PC3 cells (DMSO) and drug treated PC3 cells, and control PNT2 cells (DMSO) and drug treated PNT2 cells are shown for a second biological replicate. Ratio 1 = intensity 2851 cm^{-1}/(intensity 2851 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (a) and PNT2 (b); ratio 2 = intensity 2881 cm^{-1}/(intensity 2881 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (c) and PNT2 (d); ratio 3 = intensity 2974 cm^{-1}/(intensity 2974 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (e) and PNT2 (f); ratio 4 = intensity 2851 cm^{-1}/(intensity 2851 cm^{-1} + intensity 2974 cm^{-1}) for PC3 (g) and PNT2 (h); ratio 5 = intensity 3013 cm^{-1}/(intensity 3013 cm^{-1} + intensity 2851 cm^{-1}) for PC3 (i) and PNT2 (j); ratio 6 = intensity 3064 cm^{-1}/(intensity 3064 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (k) and PNT2 (l). One-way ANOVA tests were performed between control (DMSO) and each drug treatment for both cell lines. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** ≤ 0.0001.

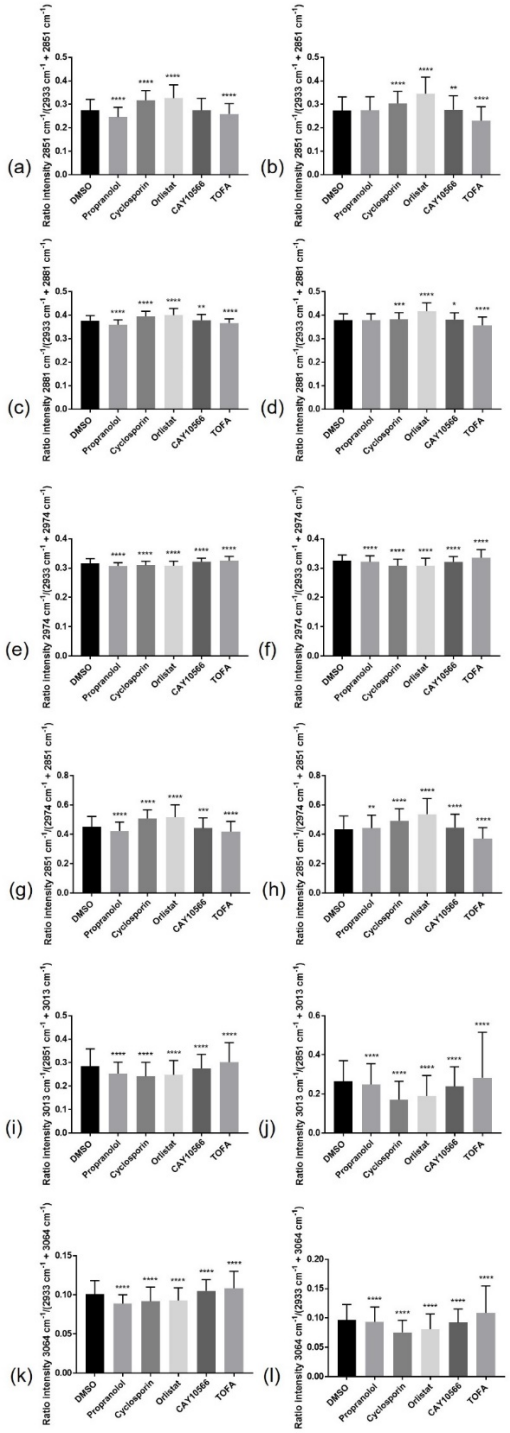

Figure 26 Quantitative Raman assessment of global lipid response biological replicate 3 The intensity ratios of a selection of Raman peak intensities for spectra extracted from cell regions in a total of three different cells per condition, were compared, where the mean and standard deviation for control PC3 cells (DMSO) and drug treated PC3 cells, and control PNT2 cells (DMSO) and drug treated PNT2 cells are shown for a third biological replicate. Ratio 1 = intensity 2851 cm^{-1}/(intensity 2851 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (a) and PNT2 (b); ratio 2 = intensity 2881 cm^{-1}/(intensity 2881 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (c) and PNT2 (d); ratio 3 = intensity 2974 cm^{-1}/(intensity 2974 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (e) and PNT2 (f); ratio 4 = intensity 2851 cm^{-1}/(intensity 2851 cm^{-1} + intensity 2974 cm^{-1}) for PC3 (g) and PNT2 (h); ratio 5 = intensity 3013 cm^{-1}/(intensity 3013 cm^{-1} + intensity 2851 cm^{-1}) for PC3 (i) and PNT2 (j); ratio 6 = intensity 3064 cm^{-1}/(intensity 3064 cm^{-1} + intensity 2933 cm^{-1}) for PC3 (k) and PNT2 (l). One-way ANOVA tests were performed between control (DMSO) and each drug treatment for both cell lines. * p≤0.05; ** p≤0.01; *** p ≤0.001; **** ≤0.0001.

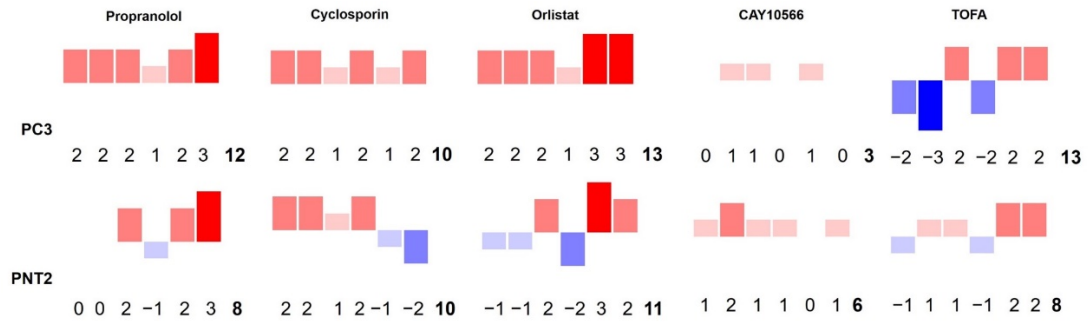

Figure S27 A Raman based phenotypic 'barcode' biological replicate 2 Barcodes were created for PC3 and PNT2 cells treated with a number of lipid altering drugs in comparison to a control cell population for a second biological replicate. Three Raman cell maps per condition per cell line were measured and for each point corresponding to a cell region, a total of six different intensity ratios were calculated. For each ratio, an effect size was calculated and a whole number value between −3 and 3 was assigned reflective of the size of this. The assigned numbers in the order corresponding to ratio 1, ratio 2, ratio 3, ratio 4, ratio 5, ratio 6 created the first six digits of the barcode. The final digit was the sum of the absolute values of all previous numbers. This was also represented pictorially where the size, direction and color of bars corresponded to the assigned number for each ratio. Ratio 1 = 2851 cm^{-1}/(2851 cm^{-1} + 2933 cm^{-1}); ratio 2 = 2881 cm^{-1}/(2881 cm^{-1} + 2933 cm^{-1}); ratio 3 = 2974 cm^{-1}/(2974 cm^{-1} + 2933 cm^{-1}); ratio 4 = 2851 cm^{-1}/(2851 cm^{-1} + 2974 cm^{-1}); ratio 5 = 3013 cm^{-1}/(3013 cm^{-1} + 2851 cm^{-1}); ratio 6 = 3064 cm^{-1}/(3064 cm^{-1} + 2933 cm^{-1}).

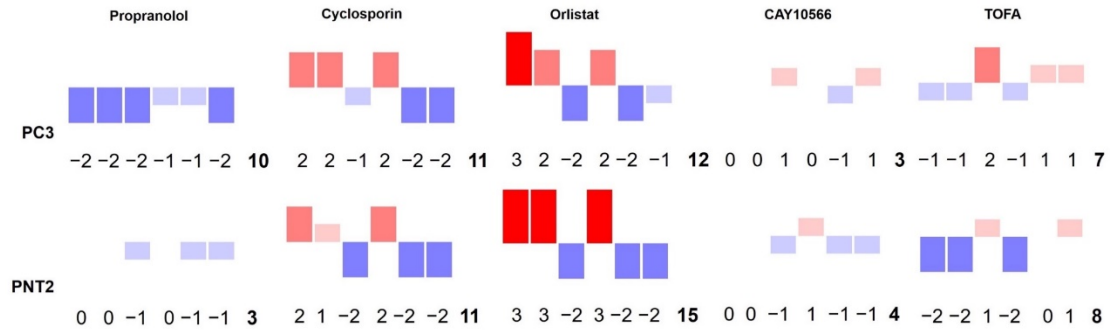

Figure S28 A Raman based phenotypic 'barcode' biological replicate 3 Barcodes were created for PC3 and PNT2 cells treated with a number of lipid altering drugs in comparison to a control cell population for a third biological replicate. Three Raman cell maps per condition per cell line were measured and for each point corresponding to a cell region, a total of six different intensity ratios were calculated. For each ratio, an effect size was calculated and a whole number value between −3 and 3 was assigned reflective of the size of this. The assigned numbers in the order corresponding to ratio 1, ratio 2, ratio 3, ratio 4, ratio 5, ratio 6 created the first six digits of the barcode. The final digit was the sum of the absolute values of all previous numbers. This was also represented pictorially where the size, direction and color of bars corresponded to the assigned number for each ratio. Ratio 1 = 2851 cm^{-1}/(2851 cm^{-1} + 2933 cm^{-1}); ratio 2 = 2881 cm^{-1}/(2881 cm^{-1} + 2933 cm^{-1}); ratio 3 = 2974 cm^{-1}/(2974 cm^{-1} + 2933 cm^{-1}); ratio 4 = 2851 cm^{-1}/(2851 cm^{-1} + 2974 cm^{-1}); ratio 5 = 3013 cm^{-1}/(3013 cm^{-1} + 2851 cm^{-1}); ratio 6 = 3064 cm^{-1}/(3064 cm^{-1} + 2933 cm^{-1}).

Comparing biological replicates

In a final step, a quick way to visualise and compare between these ratiometric based barcodes for multiple biological replicates i.e. a complete replicate experiment where an additional three cells were mapped per condition, was investigated. The barcodes, consisting of seven variables, could be compared using the unsupervised multivariate analysis technique principal component analysis (PCA). While it was possible to perform PCA on the full spectrum from each condition, by selecting key peaks from visual inspection of the spectra themselves, the data sets were simplified to account for the most pronounced and meaningful spectral variations, which resulted in PCA plots less confounded by numerous data points with nominal variation between conditions. The strategy to establish the statistical significance, in terms of ratiometric Raman response, of a drug on a particular cell line has already been demonstrated, using one-way ANOVA tests, and more meaningfully, effect size. To combine these into a multivariate barcode based on a selection of ratios gave a stamp for Raman phenotypic response, where the final number in the barcode again gave a strong indication of the magnitude of the effect of any particular drug. These descriptors already give a strong means of comparing between treatments. However, particularly when incorporating further biological replicates, an easy visualisation of the difference in response between drug treatments, and between different cell lines to the same drug treatment, is necessary. PCA was performed using MATLAB® firstly using the barcodes generated from three biological replicates treated with the five drug compounds, for both PC3 and PNT2 cell lines (Figure S29). For PC3 cells, it was clear that propranolol, cyclosporin and orlistat treated cells all gave a similar phenotypic response, with points clustering together in the plot. In contrast, both CAY10566 and TOFA clustered separately, suggesting different and distinguishable responses. In comparison, for PNT2 cells the plot suggests propranolol and CAY10566 to have similar phenotypic responses, with one propranolol outlier, and all other drug treatments to have a similar response. Analysis of the principal component loadings (Figure S30) can also be used to indicate what part of the barcode i.e. what ratio, was most prominent in causing the separation between treatments. It was also highly important to consider the response of different cell lines to one drug as this could be an indicator of selectivity. For propranolol treatment a clear difference in response was observed when considering all biological replicates, as already indicated from previous data (Figure S29(c)). While most other drug treatments showed no clear separation between the two cell lines (Figure S31), in

agreement with many of the previous observations, CAY10566 treatment clearly separated the two cell lines based on principal component 1 (Figure S29(d)). This suggested, that while the effect of CAY10566 was very minimal in both cell lines, the small effect it was having could be selective between cell lines.

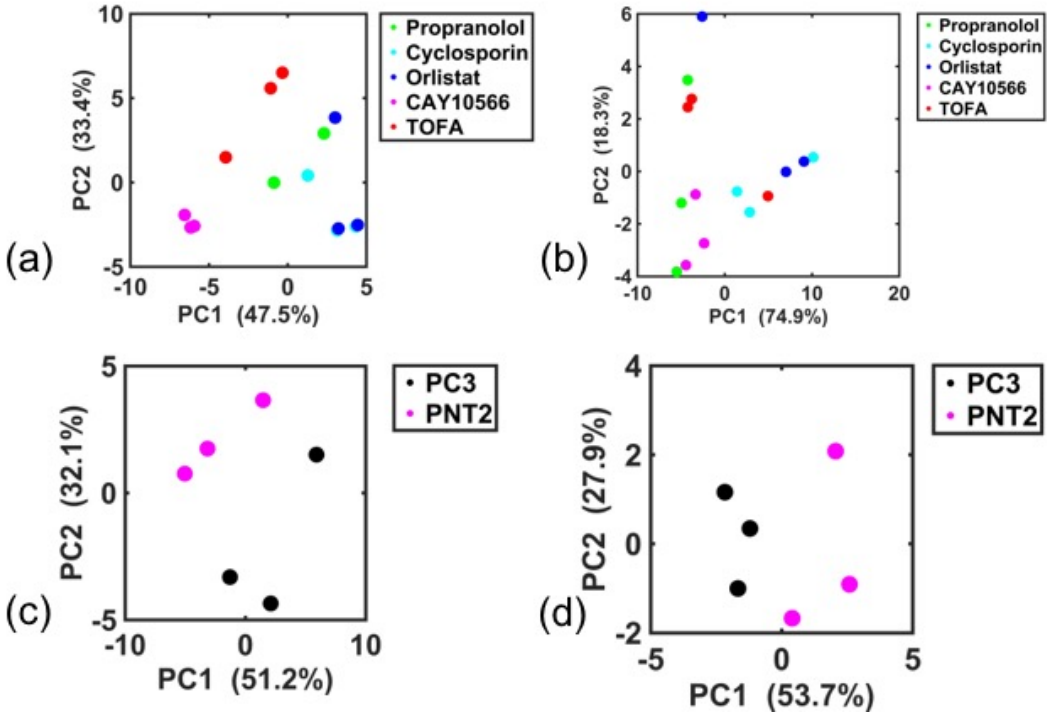

Figure S29 Principal component analysis of phenotypic barcodes Barcodes were generated based on effect size for difference in intensity ratios after Raman mapping of three cells per biological replicate per condition and comparing between drug treated and control (DMSO) cells. A total of three barcodes for each condition were generated representing three full biological replicates. These multivariate barcodes were compared using principal component analysis for PC3 cells treated with five lipid altering drugs (a), PNT2 cells treated with five lipid altering drugs (b), PC3 and PNT2 cells both treated with propranolol (c), and PC3 and PNT2 cells both treated with CAY10566 (d).

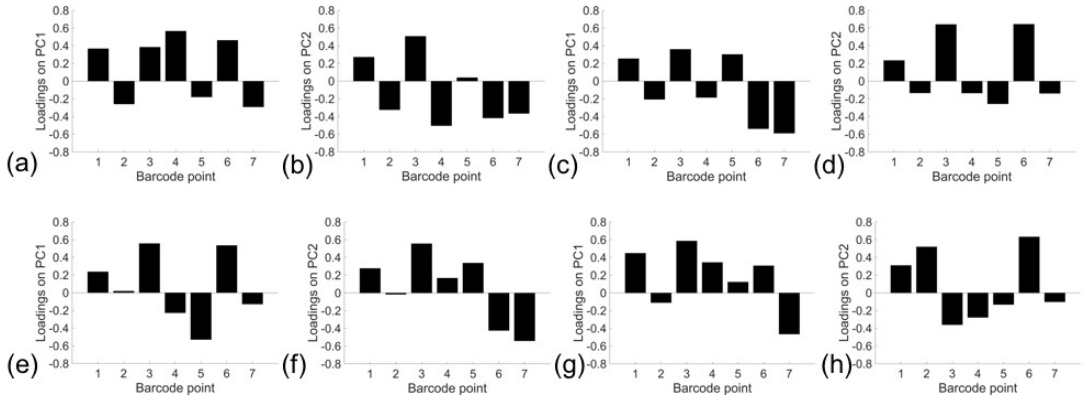

Figure S30 Principal component analysis of phenotypic barcodes loadings Barcodes were generated based on effect size for difference in intensity ratios after Raman mapping of three cells per biological replicate per condition and comparing between drug treated and control (DMSO) cells. A total of three barcodes for each condition were generated representing three full biological replicates. These multivariate barcodes were compared using principal component analysis for PC3 cells treated with five lipid altering drugs giving principal component 1 loadings (a) and principal component 2 loadings (b); PNT2 cells treated with five lipid altering drugs (b) giving principal component 1 loadings (c) and principal component 2 loadings (d); PC3 and PNT2 cells both treated with propranolol giving principal component 1 loadings (e) and principal component 2 loadings (f), and PC3 and PNT2 cells both treated with CAY10566 giving principal component 1 loadings (g) and principal component 2 loadings (h).

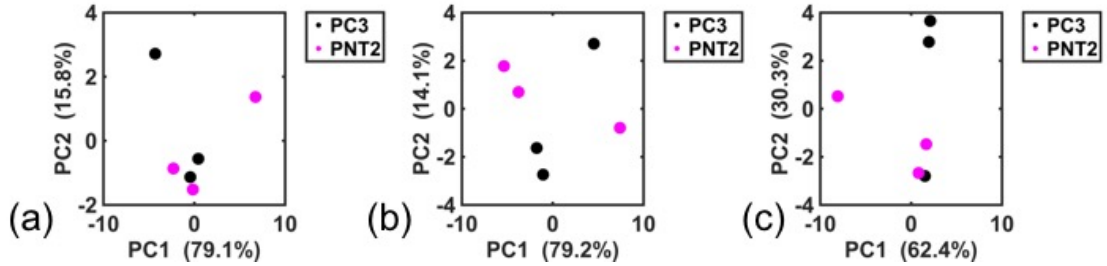

Figure S31 Principal component analysis of phenotypic barcodes Barcodes were generated based on effect size for difference in intensity ratios after Raman mapping of three cells per biological replicate per condition and comparing between drug treated and control (DMSO) cells. A total of three barcodes for each condition were generated representing three full biological replicates. These multivariate barcodes were compared using principal component analysis for PC3 and PNT2 cells both treated with cyclosporine (a), orlistat (b) and TOFA (c).

Table S2 Useful interpretation of effect size sourced 22/11/2017 from https://www.leeds.ac.uk/educol/documents/00002182.htm.

Effect Size	Percentage of control group that would fall below the average of the experimental group
0.0	50%
0.1	54%
0.2	58%
0.4	66%
0.6	73%
0.8	79%
1.0	84%
1.2	88%
1.4	92%
1.6	95%
1.8	96%
2.0	98%
2.5	99%
3.0	99.9%