Utilizing stimulated Raman scattering microscopy to study intracellular distribution of label-free ponatinib in live cells

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Materials and Methods

DFT Calculations

For each molecule shown in Table S1, 20 sample conformers were generated using the distance-based algorithm implemented in RDKit and a basic energy minimisation was performed using the MMFF94 forcefield. The geometry of individual structures was then further optimized at a DFT level according to Becke's three-parameter hybrid method with LYP correlation (B3LYP) using the 6-31G(d) basis set in the gaussian09 program package. Due to the large size of the molecules, Pulay's DIIS method was applied during SCF optimisation.[1] Force constants, and the resulting Raman vibrational frequencies were computed with the same DFT method and basis set. To correct for the an-harmonic nature of bonded atoms, a scaling factor of 0.96 was used to re-scale the high range frequency values.[2] The 10 structures with the lowest single point free energy were used for the data reported in Table S1.

Cell Lines and Drug Treatments

Human CML cell lines were cultured in RPMI-1640 Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% L-glutamine and maintained in a humidified atmosphere at 37 °C and 5% CO_2. All cell lines were obtained from Dr Helgason's group at the University of Glasgow. Ponatinib (Synkinase) and chloroquine (Sigma Aldrich) stock solutions were prepared in DMSO and diluted in media before addition to the cells at the indicated concentrations.

In Vitro Drug Sensitivity Assay

Cells were seeded onto 96 well plates and incubated for 48 h at 37 °C before addition of increasing concentrations (0.1 to 5000 nM) of ponatinib. After 48 h AlamarBlue cell viability reagent (ThermoFisher Scientific) was added and fluorescence measured at 590 nm using a PerkinElmer EnVisionTM 2101 Multilabel reader after 3 h. Control plate readings (taken before addition of ponatinib) were subtracted from drug treated plate readings. These absorption values from six replicate wells were normalised as a percentage of vehicle (DMSO) treated values and GI_{50} values generated using GraphPad Prism. Results presented from 3 independent experiments.

Western Blotting

Cell lysates were prepared in 100 µL lysis buffer (10 mL RIPA buffer (50 mM TRIS pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, prepared in H_2O), 1 tablet PhosSTOP (Roche), 1 cOmpleteTM ULTRA tablet (Roche)) for 10 min on ice. Cleared lysates were resolved by SDS-PAGE and immunoblotted with the following primary antibodies: phospho-CRKL (Tyr207, 1:1000), α-tubulin (1:2000) (both Cell Signalling Technologies), CYP3A4 (1:500, Abcam), ATG7 (1:1000, Sigma Aldrich).

Spontaneous Raman Spectroscopy

Spontaneous Raman spectra of solid ponatinib were acquired using a confocal Raman spectrometer (inVia™ confocal Raman microscope, Renishaw). A 297 mW 785 nm diode laser, or a 200 mW 532 nm laser excitation source was used to excite the sample through a 20× or 50× objective. The recorded spectral range for grating 1200 g mm^{-1} was 100–3200 cm^{-1}, while the total data acquisition time was 10 s for spectra. All spectra were background subtracted using the background correction algorithm available on the WiRE 4.4 software.

Stimulated Raman Scattering

Images were acquired using a custom-built multi-modal microscope setup previously described.[3] Briefly: A picoEmerald (APE, Berlin, Germany) laser provided both a tunable pump laser (720-990 nm, 7 ps, 80 MHz repetition rate) and a spatially and temporally overlapped Stokes laser (1064 nm, 5-6 ps, 80 MHz repetition rate). The output beams were inserted into the scanning unit of an Olympus

[1] P. Pulay, *J. Comp. Chem.*, **1982**, *3*, 556-560 10.1002/jcc.540030413.
[2] M. L. Laury, M. J. Carlson, A. K. Wilson, *J. Comp. Chem.*, **2012**, *33*, 2380–2387 10.1002/jcc.23073.
[3] W. J. Tipping, M. Lee, A. Serrels, V. G. Brunton, A. N. Hulme, *Chem. Sci.*, **2017**, *8*, 5606-5615 10.1039/c7sc01837a.
[4] K. Aljakouch, T. Lechtonen, H. K. Yosef, M. K. Hammoud *et al*., *Angew. Chem*., **2018**, *57*, 7250-7254.

FV1000MPE microscope equipped with an Olympus XLPL25XWMP N.A. 1.05 objective lens using a short-pass 690 nm dichroic mirror (Olympus). Backscattered emission signals from two-photon fluorescence were separated from scattered excitation light using a short-pass 690 nm dichroic mirror and IR cut filter (Olympus). A series of filters and dichroic mirrors were then used to deconvolve the different emission signals onto one of 4 available photo-multiplier tubes (PMT). Lysotracker® Green two-photon fluorescence signals were filtered using FF552-Di02, FF483/639-Di01 and FF510/84 (Semrock).

For SRS measurements, the Stokes beam was modulated with a 20 MHz EoM built into the picoEmerald laser. Forward scattered light was collected using a 20× Olympus XLUMPLFLN N.A. 1.00 objective lens and Stokes light was removed by filtering with an ET890/220m filter (Chroma). A telescope focused the light onto an APE silicon photodiode connected to an APE lock in amplifier with the time constant set to 20 µs except for fast acquisitions where it was set to 2 µs. The lock in amplifier signal was fed into an Olympus FV10-Analog unit. Laser powers after the objective were measured up to 40-70 mW for the pump laser and up to 70 mW for the Stokes laser. All images were recorded at 512 × 512 or 1024 × 1024 pixels with a pixel dwell time between 2 and 20 µs using FluoView FV10-ASW scanning software (Olympus). Where SRS signal quantification was calculated, all samples were imaged under identical conditions on the same day. ImageJ was used to add false colour assignments, overlays and scale bars to images. Consistent brightness settings were used throughout.

Liquid Chromatography Mass Spectrometry

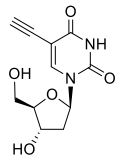
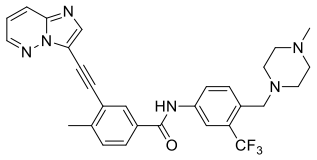
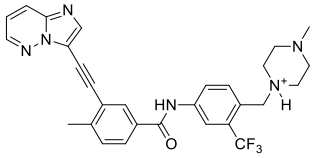
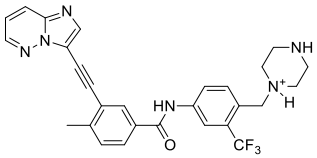
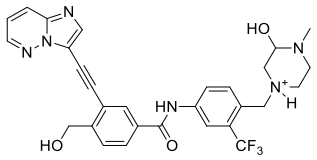
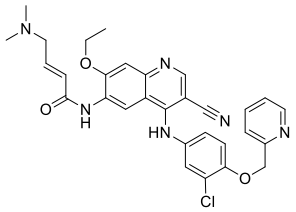
Cells were incubated with ponatinib (5 µM, 1 h) then washed with serum free RPMI-1640 by centrifugation (301 g, 3 min). The aspirated cell pellet was extracted using MS extraction buffer (1 mL; 50% MeOH, 30% MeCN, 20% H_2O). The insoluble material was pelleted (15 000 g, 4 °C, 5 min) and the supernatant collected for analysis. Extracts were vacuum centrifuged to remove solvents before being adjusted to 0.1 % formic acid in a total volume of 50 µL. 10 µL were injected onto a Dionex BioRS using a 100 mm x 2.1 mm C18 Accucore 150 for separation over a 10 min gradient from 5% to 95% acetonitrile (with 0.1 % formic acid throughout). Eluting molecules were ionized with 3.4 kV and analysed on a Thermo Q Exactive with a scan range of 150-600 m/z and resolution 35k. Ion chromatograms were extracted using RawTraces (gitlab.com/jimiwills/rawtraces) and analyzed with associated Perl scripts.

Immunofluorescence

Cells were adhered to Cell-Tak coated fluorodishes prior to fixing (4% paraformaldehyde in PBS) and permeabilising (0.1% Triton X-100 in PBS, (PBST)). Samples were blocked with 1% BSA in PBS and incubated with primary antibody overnight (LAMP 1, 1:200, Cell Signaling Technology; TFEB, 1:100, Invitrogen) in 1% BSA in PBS. Samples were washed thrice with PBS, incubated with secondary antibody (AlexaFluorTM 488 goat anti-rabbit, 1:400, ThermoFisher), 1 h, RT) in 1% BSA in PBS and labelled with DRAQ7 (1:60, Abcam).

Fluorescence Activated Cell Sorting

Samples were stained with Lysosensor™ Green DND-189 (1 µM, 1 h, ThermoFisher Scientific), washed with serum free RPMI-1640 by centrifugation (301 g, 3 min), resuspended in PBS +10% FBS and kept on ice prior to immediate processing. Samples were run on a BD LSRFortessa X-20 (SORP) (Becton Dickinson), processed using BD FACSDiva software (Becton Dickinson) and data analysed using FlowJo software (TreeStar).

Compound	Wavenumber[a] (cm^{-1})	I_{Ram} ($Å^4$ amu^{-1})	cRIE
EdU 	2132 ± 0.74 (2120)[b]	693 ± 57	1.0
Ponatinib 	2217 ± 0.5 (2221)	14649 ±1581	21.1 ± 2.3
Ponatinib protonated 	2217 ± 0.9	12135 ± 1059	17.5 ± 1.5
Ponatinib *N*-desmethyl 	2218 ± 0.5	15011 ± 1194	21.7 ± 1.7
Ponatinib dihydroxylated 	2218 ± 0.9	11613 ± 1136	16.8 ± 1.6
Neratinib[c] 	2228 ± 0.1 2238[d] (2208)[d]	819 ± 16	1.2 ± 0.1

[a] DFT calculated values ± S.D. with experimentally determined values in brackets. [b] Value reported by Tipping *et al.*[3]. [c] C≡N rather than C≡C. [d] Values reported by Aliakouch *et al.*[4]

Table S1. DFT calculated wavenumbers, intensities (I_{Ram}) and calculated relative intensity to EdU (cRIE) values for ponatinib and its metabolites. Standard deviation for 10 lowest energy structures shown.

Cell line	GI_{50} ± SEM (nM)
KCL22	2.4 ± 0.1
KCL22$^{Pon\text{-}Res}$	593 ± 88
KCL22$^{Pon\text{-}Res}$ CRISPR-Ctrl	339 ± 20
KCL22$^{Pon\text{-}Res}$ CRISPR-*ATG7*	702 ± 55

Table S2. GI_{50} values expressed as a mean of n = 3 ± standard error of the mean (SEM) for a panel of CML cell lines. GI_{50} for each cell line was determined using AlamarBlue assay, where cells were treated with a range of ponatinib concentrations for 48 h.

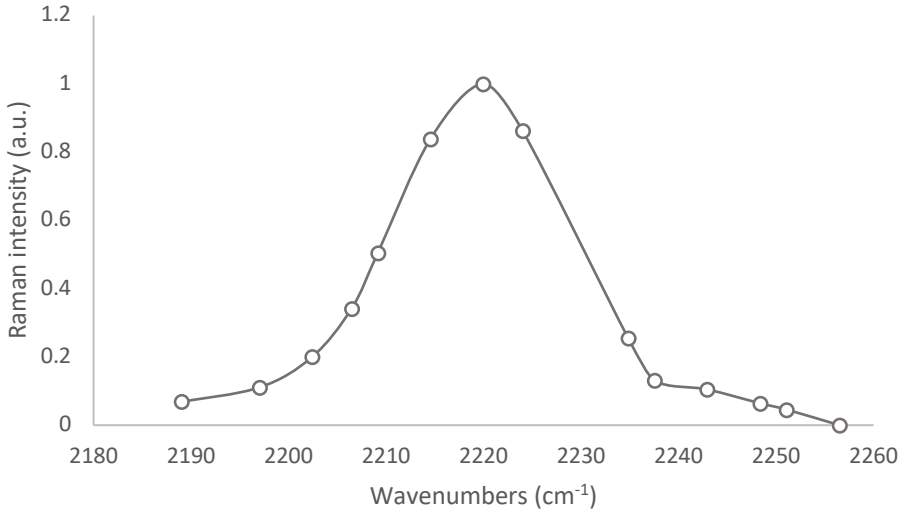


Figure S1. SRS sweep of puncta in KCL22$^{Pon\text{-}Res}$ cells treated with ponatinib (500 nM, 24 h).

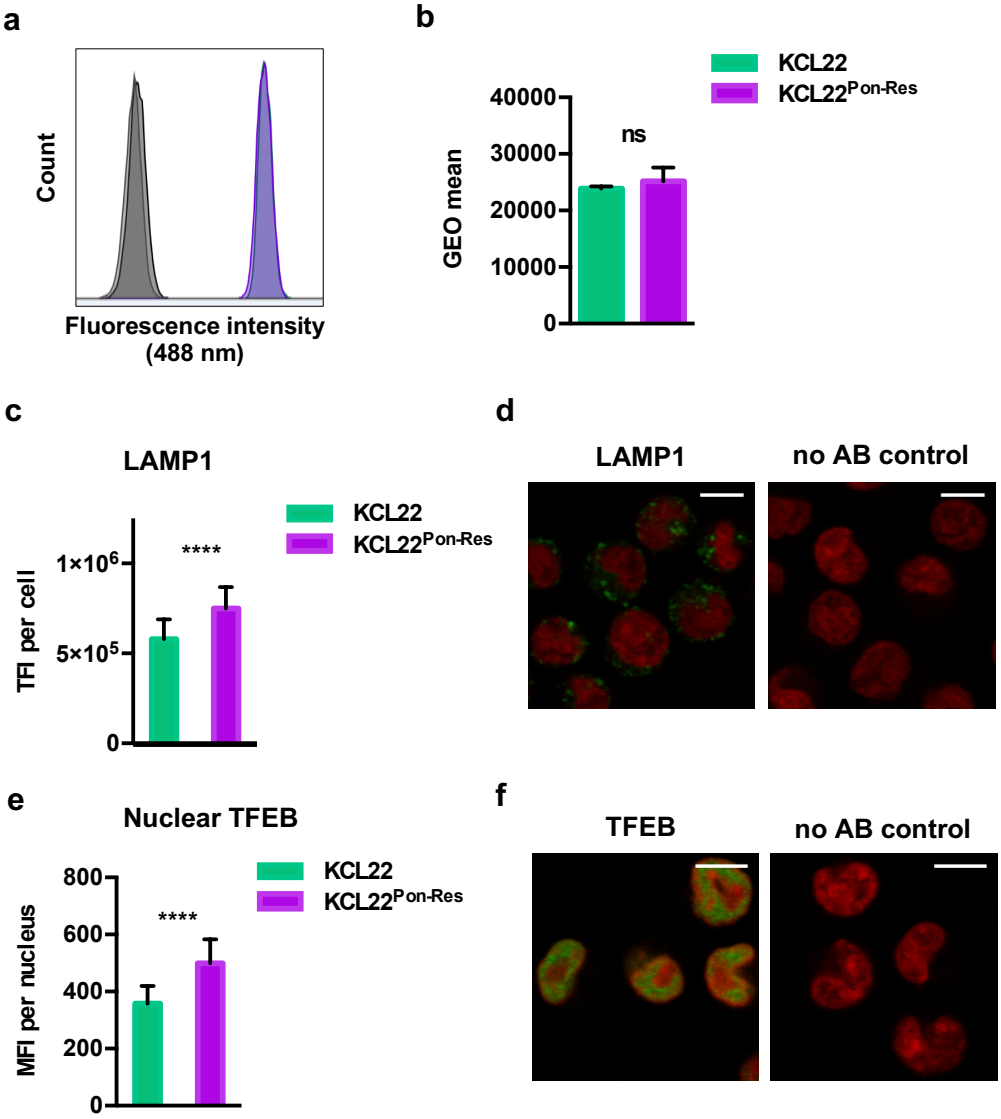


Figure S2. (**a**) Representative FACS histogram of KCL22 (green) and KCL22$^{Pon\text{-}Res}$ (purple) cells labelled with Lysosensor™ Green DND-189 (1 µM, 1 h). Grey and Black lines represent unlabelled KCL22 and KCL22$^{Pon\text{-}Res}$ control cells respectively. (**b**) Geometric mean values derived from n=3 biological repeats of FACS data using FlowJo software. One-way ANOVA (Tukey's multiple comparisons test) was used to compare the geometric means. (**c**) Immunofluorescence of LAMP1 quantified in KCL22 (green) and KCL22$^{Pon\text{-}Res}$ (purple) cells. Nuclei were stained with DRAQ7. Total fluorescence intensity in individual cells from six fields of view was quantified from 3 biological repeats. Welch's t-test was used to compare LAMP1 levels, P<0.001. (**d**) Representative images used for quantification in (**c**). DRAQ7 = red; LAMP1 = green. (**e**) Immunofluorescence of TFEB quantified in KCL22 (green) and KCL22$^{Pon\text{-}Res}$ (purple) cells. Mean fluorescence intensity in individual nuclei of n=6 fields of view was quantified from 3 biological repeats. The Mann-Whitney test was used to compare TFEB levels, P<0.001. (**f**) Representative images used for quantification in (**e**). DRAQ7 = red; TFEB = green. Scale bars: 10 µm.

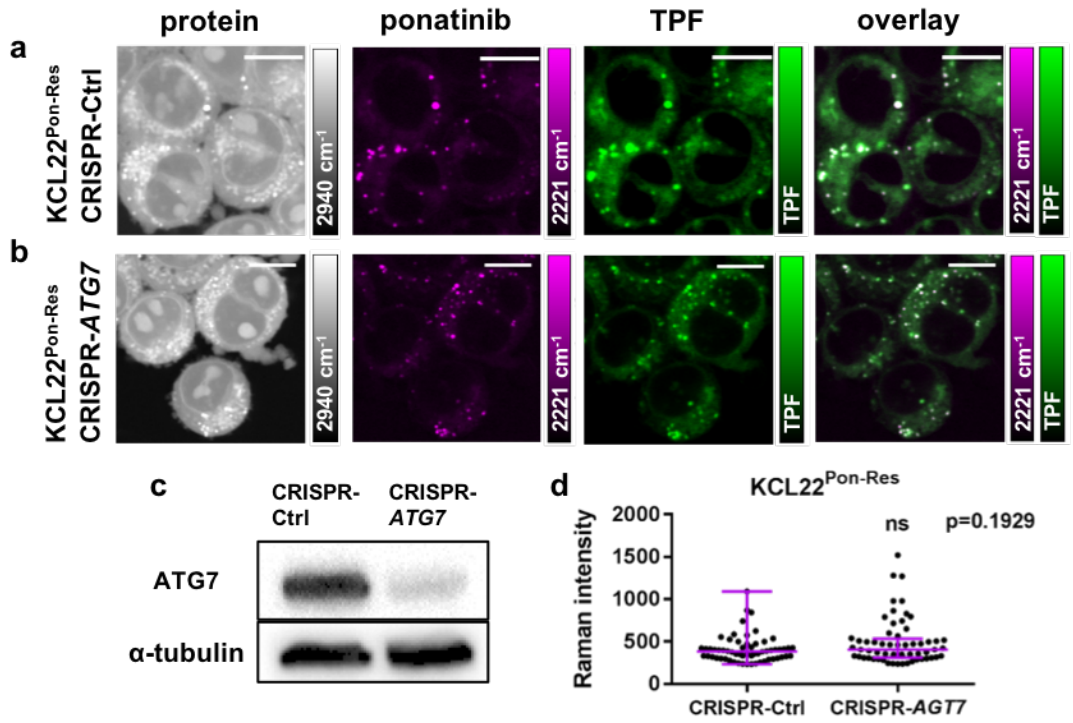


Figure S3. Multimodal imaging and quantitative assessment of ponatinib uptake in (**a**) KCL22$^{Pon\text{-}Res}$ CRISPR-Ctrl and (**b**) KCL22$^{Pon\text{-}Res}$ CRISPR-*ATG7* cell lines that were treated with ponatinib (5 μM, 1 h). SRS images acquired at (from left to right) 2940 cm^{-1} (CH_3, proteins); 2221 cm^{-1} (C≡C, ponatinib); TPF image acquired at 861 nm (Lysotracker® Green); overlay of ponatinib and TPF. Scale bars: 10 μm. (**c**) ATG7 protein expression in KCL22$^{Pon\text{-}Res}$ CRISPR-*ATG7* cell lines. α-tubulin was used as a loading control. (**d**) Mean ponatinib Raman intensity at 2221 cm^{-1} inside the vesicles of each individual cell quantified from for KCL22$^{Pon\text{-}Res}$ CRISPR-Ctrl and KCL22$^{Pon\text{-}Res}$ CRISPR-*ATG7* cells treated with 5 μM ponatinib for 1 h, n=20 cells, 3 biological repeats.

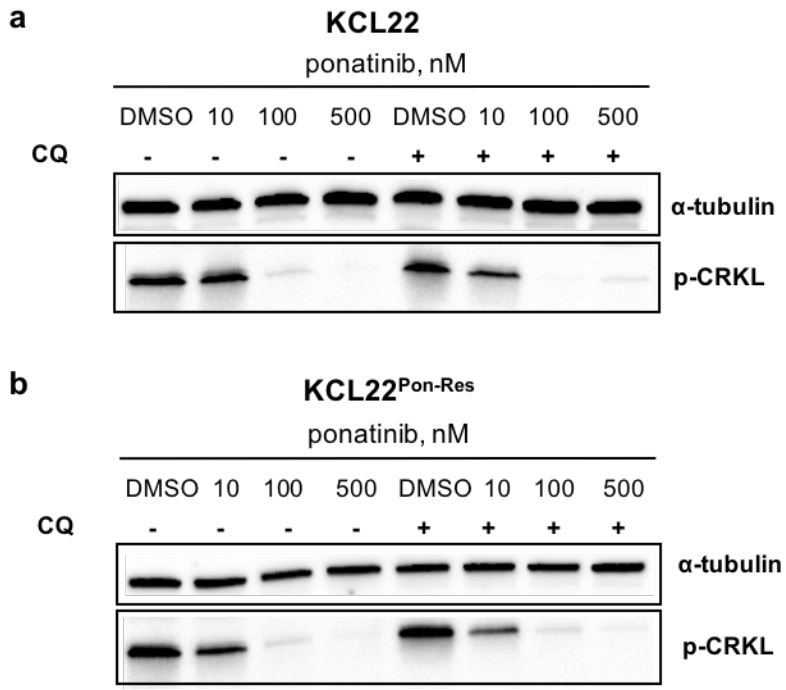


Figure S4. Effect of ponatinib on the phosphorylation of CRKL in KCL22 and KCL22$^{Pon—Res}$ cells. (**a**) KCL22 and (**b**) KCL22$^{Pon-Res}$ cells were treated with (left to right) either DMSO (0.0003%, v/v), ponatinib (10 nM, 100 nM, 500 nM, 1 h) or a combination of chloroquine (CQ; 20 μM, 2 h) pre-treatment and ponatinib (10 nM, 100 nM or 500 nM, 1 h). Western blot analysis was carried out with an antibody specific to phosphorylated CRKL (p-CRKLTyr207). α-tubulin was used as a loading control.