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Quantitating drug-target engagement in single cells *in vitro* and *in vivo*

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Abstract

Quantitation of drug target engagement in single cells has proven difficult, often leaving unanswered questions in the drug development process. Here we show that intracellular target engagement of unlabeled new therapeutics can be quantitated using polarized microscopy combined with competitive binding of matched fluorescent companion imaging probes. We quantitate the dynamics of target engagement of covalent BTK inhibitors as well as reversible PARP inhibitors in populations of single cells as two examples using a single companion imaging probe for each target. We then determine average *in vivo* tumor concentrations and show marked population heterogeneity following systemic delivery, revealing single cells with low target occupancy at high average target engagement *in vivo*.

Introduction

To achieve the desired biological response, drugs must first reach the intended organ/tissue, enter the cell (for intracellular proteins) and engage the target¹. The duration, completeness and cellular heterogeneity of drug-target engagement dictates success. Most pharmacokinetic studies rely on bulk sampling of plasma or tissue providing modest information on drug activity at the cellular target. Furthermore, novel mechanistic targets are not easily invalidated in failed treatments if target engagement was not confirmed, which, for example, was the case in 43% of Phase II failures in a recent study². Given the complexity of *in vivo* drug action³ and recent clinical failures of drugs that are not properly characterized⁴, methods to determine cellular drug binding could, in theory, reduce the considerable clinical failure rates and associated high costs.

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J.M.D., C.V. and R.W. designed the experiments. E.K. and L.G.M. synthesized CIPs. E.K. performed in-gel experiments. J.M.D., K.Y. and R.J.G. performed cell experiments. J.M.D. performed *in vivo* experiments. J.M.D. and M.C. analyzed data. J.M.D. and C.V. performed imaging experiments. J.M.D. and R.W. wrote the paper, all authors reviewed and approved the final manuscript.

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Direct chemical modification of drugs provides small labels such as biotin or fluorophores enabling tissue distribution and target engagement measurements by pull down assays or imaging^{5–8}. However, the addition of a label changes the physiochemical properties of a small molecule, and thus results may not be directly relevant to the parent drug candidate. Conversely, labeling target proteins with genetic fluorescent labels, such as GFP, may alter protein activity or trafficking⁹. Among several creative label free approaches to measure target engagement^{10–12} PET imaging is currently the most commonly used at multiple stages in drug development¹³. Radiolabelled drug measures tissue accumulation¹⁴ while lack of accumulation following drug administration indicates parent drug target occupancy¹⁰. However, this approach does not consider non-specific accumulation¹⁵, lacks single cell spatial resolution, and some radio-labels, such as carbon-11, have a limiting half-life¹⁶. Alternatively, the cellular thermal shift assay (CETSA) measures bound protein thermal stabilization to determine target engagement and can be extended to *in vivo* measurements¹⁷. Yet, CETSA obtains cell population averages, results are difficult to quantitate and *in vivo* measurements have only been demonstrated with covalent drugs. Enzymatic drug inhibition can be measured using activity based probes¹⁸ or molecules that become fluorescent upon enzyme cleavage¹⁹. While these approaches provide valuable insight into target inhibition, they require reactive or cleavable probes, are limited to certain protein classes and lack spatial resolution. Therefore, measuring engagement of clinical drug with target at the cellular level and *in vivo* with reversible inhibitors has remained elusive.

Here we establish a new approach to quantitate target occupancy of unlabeled drugs at cellular resolution using competitive binding with fluorescently labeled companion imaging probes (CIP) and fluorescence polarization microscopy. Our approach takes advantage of the target specificity of a CIP and the subcellular spatial resolution of microscopy. Importantly, this technique measures unlabeled drug engagement, and, although not a direct measurement of drug concentration in the cell, we determine engagement of drug to the target, which, ultimately, is the therapeutic objective. Here, we quantitate intracellular target engagement of unlabeled covalent and reversible drugs in live cells in culture and *in vivo*.

Results

Measuring binding with companion imaging probes

We hypothesized that an unmodified molecularly targeted drug will compete for target binding with a matched fluorescent CIP. Target engagement of the CIP, detectable by fluorescence polarization microscopy, is thus indicative of unlabeled drug binding. Detection is enabled by the large mass differences between the small fluorescent molecule in free and protein-bound states (Supplementary Results, Supplementary Fig. 1a). With polarized light, only CIP molecules with absorption dipole moments aligned along the plane of excitation become excited through photoselection. Subsequent Brownian rotation of the excited molecules during the fluorescence lifetime is inversely related to the remaining polarization anisotropy of the emission photons. When bound to the target protein, CIP rotation slows and the excited molecules retain orientation, producing polarization anisotropy. Yet, in the presence of unlabeled drug the CIP cannot bind as completely and the ensemble average polarization, representing the fraction of target bound CIP, becomes more isotropic,

indicating unlabeled drug target engagement (Fig. 1a). To obtain spatial information we image cells with polarized two-photon excitation light and collect emission in channels parallel and perpendicular to the excitation polarization (Supplementary Fig. 1b).

As proof of principle, we focused on two different types of small molecule drugs: covalent, irreversible inhibitors and reversible inhibitors. As an example of the former we chose ibrutinib (1), a Bruton's tyrosine kinase (BTK) inhibitor⁷ and, as an example of the latter, olaparib (3), a poly(ADP ribose) polymerase (PARP) inhibitor²⁰ (Fig. 1b and c). To obtain fluorescent CIP of each drug (ibBFL (2) and olBFL (4)) we labeled them with BODIPY-FL using standard chemistries^{21–23} (Fig. 1d and e).

Because anisotropy is an ensemble average of all excited fluorescent molecules it is dependent on the concentration of CIP in each measurement, which may not be uniform in complex *in vivo* settings. This phenomena is demonstrated with olBFL target engagement in HT1080 fibrosarcoma cell nuclei (Fig. 1f–h). At higher CIP concentrations, more unbound olBFL accumulates and the intensity increases, which decreases the anisotropy. Thus, non-specific accumulation prevents measurement of total target engagement with intensity or anisotropy alone. Therefore, we derived a value, the difference in measured and unbound (non-specific) anisotropy multiplied by the fluorescence intensity, $\Delta r \cdot \text{int}$ (Supplementary Text), which represents the concentration of CIP-bound target protein, or uninhibited target. We indeed found that $\Delta r \cdot \text{int}$ is, unlike anisotropy or intensity, independent of CIP concentration under target saturating conditions, with single cell values that correlate with primary target expression across three different cell lines (Fig. 1i). Although, because olaparib binds to PARP1–3 in the nucleus²⁴, the correlation is not unity. To assess the measurement sensitivity we determined the coefficient of variation (COV) for measurement noise, non-specific heterogeneity and target engagement heterogeneity of olBFL (Supplementary Fig. 2). We found a low COV for measurement noise (2%) and non-specific heterogeneity (2.8%) but a high COV for target engagement heterogeneity (12%), indicating that measured heterogeneity largely arises from engagement heterogeneity across a population of cells.

Covalent inhibitors

Toledo cells, a B-cell lymphoma model expressing BTK, show high cytoplasmic ibBFL anisotropy. However, as expected, incubating Toledo cells with native ibrutinib for 20 minutes prior to ibBFL loading (Supplementary Fig. 3a) reduced the cellular CIP anisotropy in a concentration dependent manner (Fig. 2a). To measure this change we quantitated cytoplasmic $\Delta r \cdot \text{int}$ as a function of ibrutinib concentration (Fig. 2b) and found an intracellular ibrutinib K_i (50% engagement) of 2 nM, which was validated by traditional measurements (Supplementary Fig. 3c). We also extended our approach to another covalent BTK inhibitor, AVL292²⁵, and quantitated binding constants using ibBFL as the CIP (Supplementary Fig. 3d and 4b). However, with covalent inhibitors, target engagement depends on both the concentration and duration of exposure to the target, producing K_i values that are reliant on drug incubation time (Supplementary Fig. 4a and Supplementary Table 1). Therefore, dynamic cellular properties that cannot be simulated *in vitro*, such as membrane penetration and compartmentalization inside the cell, will affect binding kinetics

of these non-equilibrium drugs. After determining binding rate constants at each inhibitor concentration we found the intracellular second order binding constant, which also considers covalent activity, was higher for ibrutinib than AVL292 ($k_2/K_i = 4.5 \times 10^5$ and 1.2×10^5 $\text{s}^{-1}\text{M}^{-1}$, respectively) (Supplementary Fig. 4c–e).

To extend these measurements into the *in vivo* setting of complex tumor environments we grew HT1080 tumors transfected with BTK-mCherry in nude mice. As expected, target engagement is dose dependent in these cells (Supplementary Fig. 5 and 6). To measure drug target engagement *in vivo* and accurately control exposure time, we administered ibrutinib intravenously, removed the tumor and incubated tissue in ibBFL (Supplementary Fig. 7a). CIP anisotropy was dependent on the ibrutinib dose delivered and allowed quantitation of ibrutinib target engagement within the tumor (Fig. 2c and d). Several factors contribute to the distribution of unoccupied target cellular levels; measurement noise (represented by BTK free tumor data (Fig. 2d)), expression levels in single cells²⁶, and the distribution of ibrutinib within the tumor - the latter two dictate the level of engaged target and thus drug efficacy. To analyze total drug exposure *in vivo* we compared average measurements to *in vitro* data (Supplementary Fig. 7b). Cytoplasmic measurements reveal cells with unoccupied target levels that may allow survival at otherwise effective concentrations (Supplementary Fig. 7c). For example, at one hour time points, average measurements indicate 0.5 mg/kg intravenous ibrutinib is similar to ~ 3 nM constant exposure in cell culture engaging 83% of total target in the tumor, yet 7% of the cells *in vivo* have unoccupied target levels that fall within the untreated tumor cell population.

Reversible inhibitors

We next investigated reversible small molecule inhibitor target engagement; a class of drugs that has proven particularly difficult to measure in cells and *in vivo* because of loss of equilibrium during sample processing and analysis. When olBFL was added to HT1080 cells expressing H2B-mApple, anisotropy was only high in the nucleus, where the majority of target PARP protein resides²⁴ (Fig. 3a). The presence 1000 nM olaparib reduced nuclear anisotropy values to cytoplasmic levels, indicating complete target occupancy by the unlabeled drug and specificity of the CIP for olaparib target. Importantly, the spatial resolution of our approach enables analysis on the nuclei only, which prevents errors that can occur in bulk measurements of accumulation arising from olaparib independent CIP accumulation in the cytoplasm of cells. Interestingly, punctate foci of olBFL binding in the nucleoli, where PARP has a higher abundance, are present in intensity-weighted anisotropy images. We found similar anisotropy values in foci and whole nucleus measurements, yet increased intensity and $\Delta r \cdot \text{int}$ demonstrating the higher target concentrations (Supplementary Fig. 8a–c). Here, however, we analyze total nuclear signal to measure all PARP inhibitor target engagement in the nucleus. Quantitating cell nuclei $\Delta r \cdot \text{int}$ showed a target engagement dose dependence (Fig. 3b), with greater heterogeneity at lower concentrations arising from target expression levels. We determined the influence on drug resistance in PARP inhibitor target engagement (Supplementary Fig. 9). The activity of MDR1 produced an 8.4 and 3.5 fold decrease in olaparib and talazoparib target engagement, respectively. While cells that were grown to resistance to olaparib displayed similar olaparib target engagement to control cells, yet had decreased target expression levels.

We also used Schild analysis²⁷, due to the competition mechanism of olBFL (Supplementary Fig. 8d–f), to determine apparent intracellular k_D values for olaparib in HT1080 (1.97 ± 0.24 nM), HCC1937 (breast cancer, 1.93 ± 0.21 nM), and MHHES1 (Ewing's sarcoma, 1.92 ± 0.27 nM) cell lines, producing no significant difference across cell lines with different target expression levels (Fig. 1i, 3c and d and Supplementary Fig. 10). However, we measured the apparent intracellular k_D of two other PARP inhibitors in HT1080 cells, using olBFL, and found talazoparib has a higher affinity ($k_D = 1.38 \pm 0.18$ nM, $p < 0.05$) while veliparib has a lower affinity ($k_D = 3.68 \pm 0.72$ nM, $p < 0.05$) than olaparib (Fig. 3d), which correlates to previous non-cellular measurements^{20,28,29} (Supplementary Fig. 10f). The similarity between intracellular and previous, *in vitro*, values demonstrate that NADH pocket targeting PARP inhibitors are quite specific inside cells.

We next determined PARP inhibitor intracellular activity in tumor models *in vivo* using HT1080 H2B-mApple xenograft tumors and intravital microscopy³⁰ (Fig. 4a). Administering drug and CIP locally to control concentration we found similar olaparib target engagement *in vivo* as *in vitro* (Supplementary Fig. 11b). However, the cellular distribution of occupied target was much higher *in vivo* (average coefficient of variation at each concentration (except 1 μ M) 28 ± 14 % higher) (Supplementary Fig. 11c), potentially arising from increased transcriptional heterogeneity that can occur *in vivo*³¹. Importantly, we only analyzed cells with saturating olBFL (based on intensity) to ensure measurements were similar to *in vitro* conditions. Perhaps more relevant to clinical pharmacokinetic strategies, heterogeneity was more pronounced following systemic delivery. Single cell analysis demonstrates *in vivo* olaparib target engagement is widely distributed around the *in vitro* average (using average, controlled *in vivo* data (Supplementary Fig. 11c) to determine drug concentration) with unoccupied target heterogeneity that extends beyond *in vitro* measurements (Fig. 4b). For example, at 200 nM olaparib, average values indicate that target is nearly saturated in both *in vitro* and systemic *in vivo* measurements, with little *in vitro* heterogeneity. Yet, a substantial fraction of tumor cells in animals treated systemically have unoccupied target levels outside the observed *in vitro* range, indicating *in vitro* assays may not accurately represent *in vivo* conditions.

We then assessed target engagement dependency on dose and time after delivery (Fig. 4c). At lower doses and longer circulation times the average target occupancy and the percentage of cells with complete target engagement were lower while cellular distribution was higher (Fig. 4c and d and Supplementary Fig. 11c). Kinetic analysis of average values reveals an olaparib engagement half life of 16.5 hours (Supplementary Fig. 11d) following 2 mg/kg intravenous delivery. While, following the same dose, comparison of average values to controlled measurements indicates the overall olaparib concentration in the tumor is ~550 nM at 1 hour, ~80 nM at 8 hours, and ~20 nM at 16 hours (Supplementary Fig. 11e). However, single cell analysis shows that a subpopulation of cells have substantial unoccupied target at these high concentrations. For example, 2 hours after intravenous 2 mg/kg administration, the measurement average indicates all target is engaged yet cellular analysis demonstrates that 25% of the cells have incomplete target occupancy. These results indicate that bulk measurements, such as plasma concentration, may not accurately represent drug binding in each targeted cell.

To be most effective our approach needs to be sensitive and generalizable to other drugs. Using theoretical modeling we found a limit of PARP detection for olBFL in the nucleus of 1.3 nM (Supplementary Fig. 8i and j, and Supplementary Text). This limit is influenced by the affinity of olBFL and nuclear solubility of unbound olBFL (Supplementary Fig. 8h). Additionally, to demonstrate the general applicability of our approach we performed intracellular measurements of vinblastine (5), which is a clinically used tubulin binder, using a vinblastine CIP vinBFL (6) (Supplementary Fig. 12)³². Single cell uninhibited heterogeneity is more pronounced at higher vinblastine concentration than that seen with olaparib and ibrutinib. Tubulin concentration is likely more dependent on cell size and cycle than are PARP and BTK, which may explain the increased heterogeneity observed.

Discussion

Currently, we can only infer target engagement through downstream, pharmacodynamic measurements or estimate it from measurements of bulk concentration in tissue. Both approaches are problematic, yet drug discovery science has depended on them. Therefore, we are unable to analyze heterogeneous target engagement that may arise from cell cycle status³³, efflux pump expression³⁴, variable protein expression³⁵, the tumor microenvironment³⁶, or spatially heterogeneous exposure through vasculature³⁷. Our approach demonstrated here measures specific target engagement of unlabeled drug with high spatial resolution. The importance of measuring specific binding is well known in pharmacology research, and emphasized by the high level of non-specific interaction for the CIPs used here (demonstrated by cytoplasmic olBFL intensity).

Our approach could be extended to other compounds and target classes using fluorophore attachment to amine modified solvent exposed sites. However, binding affinity and competition with unlabeled inhibitors would first need to be determined for any new CIP. Furthermore, fluorescent labels must be chosen to ensure CIPs locate intracellularly with the binding target³⁸. CIP intracellular location may be more limiting for nuclear targets where a fluorescent label could prevent nuclear permeability in live cells. To ensure accuracy in the measurements such that values can be compared across cell lines the $r_{\min}$ value, or non-specific interaction, for each cell type and CIP combination needs to be determined. This can be accomplished through analysis of target-free regions of a cell during CIP saturation or through measurements under unlabeled drug target saturation. In essence, non-specific heterogeneity needs to be determined before a given CIP can be confidently employed.

With this approach the target engagement, or lack thereof, can be determined. Some inhibitors, however, have been shown to bind to multiple cellular targets with similar, or slightly decreased, affinities. With these pan-target inhibitors the relative abundance and affinity of each target will influence the target engagement of the clinical drug, and thus the measured engagement of the CIP. The sensitivity of our approach also depends on CIP affinity and target abundance. Importantly, off target (targets that the CIP but not the clinical drug engages) may also influence measurements (Supplementary Text).

Since measurements are made on live cells, complimentary methods could be combined with our approach to develop a broader understanding of cellular pharmacology. For

example, single cell methods such as mass cytometry³⁹, RNAseq⁴⁰ or multi-target fluorescent immunohistochemistry (through cycle imaging)⁴¹ would provide insight into how target engagement is effected by or affects RNA and protein expression, signaling or location at the single cell level. However, measuring target engagement with our approach is limited by the need for a valid CIP and, currently, the use of window chambers for *in vivo* experiments (primarily to stabilize tissue). Although, when using covalent drugs, tissue can be analyzed *ex vivo*. Existing, alternative methods to determine target engagement do not provide single cell resolution or work effectively using reversible inhibitors. Single cell resolution not only provides information on the heterogeneity of target engagement, but allows measurements to be specific for cells of interest omitting stromal or immune cells from being included in target engagement data. However, other approaches, such as CETSA¹⁷, provide specific protein measurements through antibody detection. Otherwise, as the only method to measure single cell target occupancy, this approach should be a valuable tool to better understand small molecule inhibitor target engagement.

Online Methods

Cell Culture

HT1080 cells (ATCC) stably expressing BTK-mCherry²¹ were cultured in DMEM supplemented with 10% FBS and 1% Pen/Strep (Invitrogen). Virus generated from pMSCVpuro-BTK-mCherry retroviral vector was a generous gift from Dr. Hidde Ploegh (Massachusetts Institute of Technology, Cambridge, MA, USA). Viral supernatant was added directly to HT1080 cells for 48 hours, and BTK-mCherry-expressing cells were then selected with DMEM media containing 2 µg/mL puromycin for 96 hours. HT1080 MDR cells were constructed using components of the MDR1 expression plasmid (Addgene, plasmid no. 10957: pHaMDRwt) and a lentiviral vector pLVX (Clontech)⁴². HT1080 cells stably expressing H2B-mApple (addgene) were cultured in DMEM supplemented with 10% FBS and 1% Pen/Strep under geneticin (100 µg/ml, Invitrogen) selection. Toledo (B lymphocytes, ATCC), MHES1 (Ewing's sarcoma, CLS Cell Line Services), UWB1.289 (cervical cancer, ATCC) and HCC1937 cells (breast cancer, kind gift of Prof. Timothy Mitchison, Harvard Medical School) were cultured in RPMI (Invitrogen) with 10% FBS and 1% Pen/Strep. Cells were imaged in phenol red free DMEM (Invitrogen) with 10% FBS and 1% Pen/Strep. All cell lines tested negative for mycoplasma before use and at the end of experiments.

Companion imaging probes

ibBFL²¹, olBFL²² and vinBFL³⁷ were synthesized as previously described. A detailed analysis is in the Supplementary Note.

In vitro cell experiments

Non-adherent cell experiments—Toledo cells (ATCC) ($\sim 5 \times 10^4$) were centrifuged in 1 ml volumes (300 g for 3 minutes), resuspended in the desired concentration of BTK inhibitor and incubated in a cell incubator (37 °C and 5% CO₂) in 12 well plates. Following incubation, cells were centrifuged and washed once to removed unbound drug. Cells were resuspended in 1 ml of RPMI containing 150 nM ibBFL and returned to the incubator for 3

hours. The 3 hour incubation time was used to ensure complete binding of ibBFL (Supplementary Fig. 3b). Cells were then centrifuged and resuspended in 50 μ l of phenol-red free media containing 150 nM ibBFL. Cells (10 μ l) were then transferred to a microscope slide, covered with a no. 1 cover glass and imaged in the presence of ibBFL. As a control (no BTK expression) we delivered spCAS9 and the guide RNA CTTACCGGAATCTGTCTTTC using a lentiviral approach (GenScript) to Toledo cells. However, knockout of BTK proved to be lethal. We thus used HT1080 H2BmApple cells (known to have very low BTK levels) and compared them to HT1080 BTKmCherry cells (high BTK levels) to better characterize BTK inhibitor engagement.

HT1080 BTKmCherry cell experiments—Cells were grown to ~75% confluency on 12 mm glass coverslips in 12 well plates. **1)** To determine the binding rate of ibBFL the CIP was added at 250 nM in phenol red free media and the cells were returned to the incubator. Cells were removed at 30 minute intervals and imaged in the presence of incubation media with ibBFL. **2)** For intensity of bound drug experiments ibrutinib was added at 20 nM in media for various times. The drug was then washed off and ibBFL was added at 250 nM for 3 hours to ensure complete binding of ibBFL (Supplementary Fig. 3b). The cells were then washed in phenol red free media for 18 hours and imaged. **3)** To determine the binding affinities of BTK inhibitors in HT1080 BTK-mCherry cells, drug was added at the desired concentration and the cells were returned to the incubator for the desired time. Media was then removed, the cells were washed once with drug free media, and phenol red free media containing 250 nM ibBFL was added to each well. The cells were then returned to the incubator for 3 hours before imaging in the presence of incubation media with ibBFL, to ensure complete binding the CIP (Supplementary Fig. 3b).

PARP inhibitor experiments—HT1080, HCC1937 or MHHES1 cells were grown to ~75% confluency on 12 mm cover glass in 12 well plates. PARP inhibitor and olBFL, at the desired concentrations, were brought up in phenol red free media from 10 mM DMSO stocks and added to the cells. The cells were then incubated for 20 minutes and transferred to the microscope for imaging in incubation media with olBFL.

Resistance—HT1080 MDR cells were grown to ~75% confluency on 12 mm cover glass in 12 well plates. Olaparib was added at the desired concentration for 30 minutes in the presence or absence of 1 μ M tariquidar (SelleckChem). The cells were then washed and 1000 nM olBFL was added in the presence of 1 μ M tariquidar for 15 minutes. Cells were immediately transferred to the microscope for imaging. UWB1.289 cells were created resistant to olaparib by increasing olaparib in the growth media from 20 nM to 1 μ M over the course of a month while maintaining a sub-confluent population. Viability response to olaparib was determined through the presto blue assay following manufacturers directions (Life Technologies).

Western blot

Cells were grown to confluence, washed twice with ice-cold PBS and then lysed in RIPA with protease inhibitor. Lysates were passed through a 23g syringe, incubated five minutes on ice, sonicated for one minute and centrifuged at $14,000 \times g$ for 15 min at 4°C to remove

cellular debris. Total protein was measured using the BCA assay (Pierce) and equal protein was loaded on a 4–12% NuPAGE Bis-Tris gel (Life Technologies). The blot was blocked in SuperBlock T20 (TBS) (Pierce) for one hour, followed by brief washing in TBS containing 0.1% Tween-20 (TBST). Blots were incubated overnight at 4°C in PARP1 (9532, Cell Signaling Technology) primary antibody diluted 1:1000 in 10% SuperBlock/TBST. Blots were washed three times, 5 min each, followed by a one hour incubation in HRP-conjugated secondary antibody 1:2000 in 10% SuperBlock/TBST. Blots were again washed three times, 5 min each in TBST followed by detection using SuperSignal West Pico chemiluminescent substrate (Pierce). Blots were stripped with restore western blot stripping buffer (Thermo) and staining was repeated with GAPDH (AF5718, R and D Systems) primary antibody.

Animal Experiments

All animal experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. Animals (Female, 20 week old nude mice (Cox-7, Massachusetts General Hospital)) were anesthetized with 2% isoflurane in oxygen at a flow rate of 2 l/min for both surgical and imaging procedures. All surgical procedures were performed under sterile conditions. The body temperature of mice was maintained at 37 °C during surgical and imaging procedures. Between one and three animals were used per condition. If multiple animals were used, the average cell values of each measurement were within 10% to eliminate artifacts in heterogeneity. Animals were excluded if no tumors were visible via fluorescent protein expression. No randomization was performed and the experimenter was not blinded.

For ibrutinib experiments, nude mice were injected subcutaneously with 10^6 HT1080 BTK-mCherry or HT1080 H2B-mApple cells in 50 μ l of PBS on both sides of the flank. Once the tumors reached at least 100 mm³ (14–21 days), ibrutinib was injected i.v. through the tail vein (30 gauge needle). Ibrutinib (10 mM in DMSO) was diluted first in DMSO to 10 μ l volume, then diluted in 10 μ l of 1:1 DMAC:Solutol and further diluted in PBS to reach the desired concentration in 100 μ l volume. One hour after injection, the animals were sacrificed and perfused with 10 ml of PBS through the left ventricle to flush residual blood from the tumor. The tumors were removed and incubated in 200 nM ibBFL in DMEM media supplemented with 10% FBS and 1% pen/strep for 3 hours in a cell incubator prior to imaging. Lectin-fluorescein (Vector Labs) was injected i.v. 30 minutes prior tumor removal to image the vasculature.

For olaparib experiments, dorsal window chambers were implanted on nude mice and HT1080 H2B-mApple cells were injected into the skin fascia, 5×10^5 cells in 50 μ l of PBS. Tumors were allowed to grow for at least 10 days. To determine binding, the glass cover slip was removed from the window and olaparib and olBFL were added topically at the desired concentrations in sterile PBS. Images were taken after 20 minutes to allow for diffusion and binding equilibrium. For systemic measurements olaparib (10 mM in DMSO) was diluted first in DMSO to 10 μ l volume, then diluted in 10 μ l of 1:1 DMAC:Solutol and further diluted in PBS to reach the desired concentration in 100 μ l volume. The drug was then delivered i.v. through the tail vein (30 gauge needle). Twenty minutes prior to imaging the cover glass of the chamber was removed and 500 nM olBFL in PBS was added topically.

Data analysis

Images were analyzed in ImageJ, Matlab, and Prism. The detector noise was first removed and the anisotropy, total fluorescence, and $\Delta r \cdot \text{int}$ were then calculated at each pixel. Regions of interest were used to define cells and subcellular compartments and the average anisotropy and intensity of each region was then determined. Data were transferred to Prism where all curve fitting was performed. De-noised images were created in Matlab and anisotropy images were generated using a custom look up table. Single cell dose response data were binned into groups based on anisotropy or $\Delta r \cdot \text{int}$ value, the number of cells in each bin was then used to assign a color based on a jet look up table.

To analyze PARP inhibitor cell data, nuclei were segmented using the H2B-mApple fluorescence channel. The average intensity and anisotropy in each nucleus was then calculated. For *in vitro* experiments the minimum anisotropy ($r_{\min}$) was determined by segmenting multiple cell cytoplasm and finding the average value for each cell type used. For *in vivo* experiments, the intensity and anisotropy of each cell cytoplasm were measured by assigning a region adjacent to the cell nucleus. The cytoplasmic anisotropy was used to determine $r_{\min}$ for each cell, which normalizes the measurement for any scattering artifacts. Additionally, the cytoplasmic intensity was used to ensure olBFL had saturated the cells. Cells in which olBFL intensities were below the 1.2 times the average intensity of saturation, derived from Fig. 1f, were omitted from analysis. If cytoplasmic values of anisotropy were below 0.22 or the intensity was too low, the cell nucleus was not considered for analysis.

To analyze BTK inhibitor data in Toledo cells, cells were segmented to remove cell debris and dead or dying cells. The average intensity and anisotropy in each cell cytoplasm was then calculated. To analyze HT1080 BTK-mCherry cells *in vitro* and *ex vivo*, cells were segmented using the empty nucleus and mCherry channel. The average BODIPY FL channel fluorescence and anisotropy and mCherry intensity were then found for each region. The minimum anisotropy ($r_{\min}$) was determined by segmenting the cytoplasm of HT1080 H2B-mApple cells loaded with ibBFL, and average value of 0.23 was found. For *ex vivo* measurements the intensity was used to confirm cell saturation.

Ex vivo tumor images were created from 2 micron section z-stacks using a 20 $\times$ water objective in both confocal (fluorescein and mCherry) and two-photon (SHG) modes. The images were processed using Amira software (FEI).

Statistical analysis

Student's t test and F tests were performed when necessary in Excel and Prism.

Imaging

Images were taken on an Olympus BX61-WI upright microscope with two-photon excitation adapted to make polarization measurements as previously described²³. Briefly, a Glan-Thompson polarizer and half wave plate were inserted in the excitation laser line (MaiTai DeepSee Ti:sapphire pulsed laser (Spectra Physics) with a pulse width of 110 fs and a repetition rate of 80 MHz) to polarize the excitation light. Emission was collected through a

690 nm shortpass filter. Light was split with a 570 nm dichroic mirror and filtered through emission bandpass filters (495–540 nm) and (575–630 nm). Green emission (BODIPY FL) was split into orthogonal polarizations with a polarizing beam splitter in a custom filter cube and detected with photomultiplier tubes (PMT). The PMT gains were adjusted such that 2 μ M fluorescein in water produced an anisotropy of 0.004 at room temperature. The alignment and measured intensity of the system was tested before each experiment using fluorescent calibration slides and adjusted if necessary. Images were acquired at 910 nm excitation through a 25 $\times$ 1.05 NA water immersion objective (XLPlan N, Olympus). Laser power was constant throughout experiments. Confocal images were taken with 2 $\times$ 0.14 NA (XFluor, Olympus) and 20 $\times$ 1.00 NA water immersion (XLUMPlan FL N, Olympus) objectives. Second harmonic generation images were taken in two-photon with 880 nm excitation and emission collected between 420 – 460 nm with a 20 $\times$ 1.00 NA water immersion objective.

The intensities of each image were determined through summation of the intensity of each polarization channel at each pixel. Fluorescence anisotropy at each pixel was calculated from the equation: $r = (I_{||} - I_{\perp}) / (I_{||} + 2I_{\perp})$, where r is anisotropy, $I_{||}$ is the intensity in the parallel channel and $I_{\perp}$ is the intensity in the perpendicular channel. A custom look up table was generated for each CIP to assign color to anisotropy value. Images were cropped to remove polarization artifacts at the edges. For visualization, the anisotropy color image was weighted by the intensity image.

ibBFL binding experiments

Purified BTK (Promega) was diluted in PBS to a concentration of 1 μ M. ibBFL was diluted from a 10 mM stock in DMSO to 1 μ M in phenol red free media. Solutions were then created from the two stocks to have a concentration of 500 nM ibBFL and varying concentrations of BTK (0 – 600 nM) with volume made up with PBS. 5 μ l of solution was then trapped between two spaced pieces of cover glass and imaged. Therefore, ibBFL concentration was constant across the binding curve.

BTK in-gel fluorescence

Toledo cells (3×10^6 / mL, 1 mL) were incubated with different concentrations of Ibrutinib and AVL292 in growth media (ranging from 25 to 0.025 μ M). After 20 minute incubations, cells were washed once with growth media and incubated with 150 nM ibBFL solution in growth media at 37°C 5% CO₂ incubator for 3 hr with gentle mixing at every hour. After incubation, cells were washed with ice-cold PBS 1 $\times$ and then lysed with 100 μ L of radioimmunoprecipitation buffer (RIPA, Cell Signaling Technology) containing HALT protease inhibitor cocktail (Pierce) on ice for 1 hr with gentle vortexing every 20 minutes. After incubation, each tube was centrifuged at 10,000g for 10 min at 4°C and cell lysate in supernatant was obtained. Protein concentration of cell lysates was quantified with BCA protein assay (Pierce) and were brought to the same concentration by dilution with 1 $\times$ RIPA buffer (Cell Signaling). Resulting cell lysates were mixed with NuPAGE LDS sample buffer (Life Technology) and heated at 85°C for 5 minutes. 20 μ L of sample per lane was loaded onto a 12-well NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen). Gels were run in NuPAGE MES SDS running buffer (Life Technology) at 200 V for 35 minutes in the XCell SureLock

Mini-Electrophoresis system (Invitrogen). The gels were removed from the cassette and fluorescent intensity of each band was measured using a Typhoon 9410 fluorescent scanner (GE Healthcare, Pittsburgh, PA, USA) using 488 nm excitation and 520 nm emission filter. Fluorescent intensity was quantified using ImageJ.

Schild Analysis

Sigmoidal curve fits (Prism) were used to find the IC₅₀ of the olBFL binding curve for each concentration of PARP inhibitor. These values were then used to create dose ratios (IC₅₀ at a given PARP inhibitor concentration divided by the IC₅₀ when no PARP inhibitor is present). The log of the dose response minus one was plotted against the log of the PARP inhibitor concentration. A linear line with a slope equal to one was then fit to the data and the y intercept (y = 0) was used to determine the apparent intracellular k_D.

Derivation of Δr_{int}

Anisotropy is defined by the Perrin equation⁴³:

$$\frac{r_o}{r} = 1 + \frac{\tau}{\tau_\theta} \quad (1)$$

where r is the anisotropy, r_o is the fundamental anisotropy (no rotation) of the molecule - dictated by the angle between the absorption and emission dipole moments, τ is the fluorescence lifetime and τ_θ is the rotational lifetime. Assuming fluorescence lifetime is constant²¹, when a fluorescent drug binds to the much larger protein target the rotational lifetime increases and the anisotropy becomes closer to the fundamental anisotropy.

Anisotropy is an ensemble measurement representing the average of all molecules within the measured sample or volume. This can be represented by:

$$r = \sum_{n=1}^i \frac{r_n N_n}{N_{tot}} \quad (2)$$

where r_n is the anisotropy of a given state, N_n is the number of molecules in that state and N_{tot} is the total number of fluorescent molecules measured. For the system of a fluorescent drug binding to the protein target there can exist two molecular states, bound and unbound. Here, we assume that off target effects are minimal and thus do not represent a potential third state. This assumption is validated by the lack of non-specific heterogeneity of a CIP, discussed below.

The two state system can be represented by:

$$r = \frac{r_{bound}[RD_{fluo}] + r_{free}[D_{fluo}]_{free}}{[D_{fluo}]_{tot}} \quad (3)$$

where r_{bound} is the anisotropy value of bound CIP, $[RD_{fluo}]$ is the concentration of bound CIP, r_{free} is the anisotropy value of unbound CIP, $[D_{fluo}]_{free}$ is the concentration of unbound CIP, and $[D_{fluo}]_{tot}$ is the total concentration of CIP.

The total CIP in the measurement is the sum of the two states, bound and unbound:

$$[D_{fluo}]_{tot} = [D_{fluo}]_{free} + [RD_{fluo}] \quad (4)$$

Equations (3) and (4) can be rearranged to:

$$[RD_{fluo}] = \frac{r - r_{free}}{r_{bound} - r_{free}} [D_{fluo}]_{tot} \quad (5)$$

Assuming minimal photobleaching and a linear relationship across the measurement range, the total fluorescence drug concentration can be related to the measured intensity through the constant γ :

$$Int = \gamma [D_{fluo}]_{tot} \quad (6)$$

Substituting equation (6) into equation (5) we get:

$$[RD_{fluo}] = \frac{r - r_{free}}{r_{bound} - r_{free}} \frac{Int}{\gamma} \quad (7)$$

Finally, grouping constants into a single term, C , equation (7) can be rearranged into:

$$C = \frac{1}{\gamma(r_{bound} - r_{free})} \quad (8)$$

$$[RD_{fluo}] = C(r - r_{free})Int \quad (9)$$

or

$$[RD_{fluo}] = C \cdot \Delta r \cdot Int \quad (10)$$

Here, the difference between the measured anisotropy and the unbound anisotropy, a constant, multiplied by the intensity has a linear relationship to the concentration of bound fluorescent drug through the constant C .

Under target saturation conditions in the absence of any other inhibitors, Δr_{int} represents the total amount of target. This relationship is demonstrated in Figure 1f – h. The nuclear intensity of HT1080 cells loaded with olBFL increases with increasing fluorescent drug concentration. However, the increasing intensity arises from unbound fluorescent drug in the nucleus, which produces a lower measured anisotropy signal. Yet, Δr_{int} is independent of the olBFL concentration. Therefore, the total target concentration is Δr_{int} multiplied by the constant C .

Limit of detection

Because our approach relies on the stoichiometric engagement of CIP to target there will be a limit of detection that depends on the CIP affinity and target expression levels. For covalent inhibitors the limit is theoretically one target copy number, as the affinity goes to infinity over time. For reversible inhibitors, the detection limits are higher. Specifically, the detectable expression level depends on the amount of CIP added. Two opposing factors dictate the detection limit: the measurable difference in anisotropy and saturation of the target with the CIP. Higher CIP concentrations will more likely saturate the target, yet provide more unbound CIP thus reducing the measured anisotropy.

For olBFL we found a theoretical limit of detection in the nucleus of 1.3 nM of protein target when 50 nM olBFL is applied to cells (Supplementary Fig. 8i,j). At this olBFL concentration and 1.3 nM of target, the Δr is above measurement noise yet the difference in Δr_{int} and saturated Δr_{int} is below measurement noise. This limit of detection is determined by the affinity of olBFL for the target and the solubility of unbound CIP in the nucleus. However, if olBFL had a decreased affinity the limit of detection would be higher. To make target engagement measurements the expression levels of the target need to be higher than the limit of detection. Therefore, low affinity CIP may not be capable of detecting low abundance target and drug engagement.

We validated anisotropy dependence on binding of ibBFL with pure BTK protein in solution (Supplementary Fig. 1c). At constant ibBFL (500 nM) increasing concentration of BTK increased the imaged anisotropy. The anisotropy value plateaued at 500 nM when ibBFL was saturated by the target protein. Binding of olBFL was previously demonstrated²¹.

Multiple targets

Drugs that bind to multiple targets with high affinity will produce target engagement measurements that reflect the average engagement across all target. It is not possible to distinguish engagement to one protein species from another. That is, however, unless the size of the targets are significantly ($> \sim 2$ orders of magnitude) different. The anisotropy of CIP engaged to different sized targets may be different, as anisotropy is imparted by the rotational speed of the target. Therefore, different targets rotating at different rates would introduce a third anisotropy state into the measurement. However, because most proteins are within a couple orders of magnitude the anisotropy differences between CIP bound to different proteins is negligible. If the drug engages other macromolecular targets, such as mRNA, as well as protein the differences could influence the measurements.

Off target effects

Since anisotropy is an ensemble average of all states in the system it is important to establish that only two states are present for equation 3 to be valid. The target engaged CIP is one state, while non-specific, or unbound, is the second state. Off target binding or a non-selective CIP would represent a third state. However, these additional states can be assumed to constitute a single unbound state if the heterogeneity of the unbound measurements is low. A single unbound state integrates all the non-specific interactions of the CIP inside a cell. If the measured anisotropy is similar amongst a population of cells than these non-specific interactions can be removed. The resulting target engagement values represent only what the unlabeled drug engages. Any heterogeneity in the non-specific anisotropy above the error of measurement would prevent the reliable removal of the unbound fraction of CIP from target engagement values and thus produce inaccurate results. Therefore, the CIP non-specific anisotropy ($r_{\min}$) heterogeneity needs to be determined for each CIP-cell combination. Here we found similar $r_{\min}$ values that were independent for cell type for each CIP used (0.24 and 0.23 for olBFL and ibBFL, respectively).

Generality of approach to different experimental systems

Once off target effects have been determined and ruled out as potential contributors to measurement noise, the use of $\Delta r \cdot \text{int}$ provides a means to compare measurements between experimental systems (e.g. different microscopy setups). However, because $\Delta r \cdot \text{int}$ incorporates the intensity of the measured signal, only relative differences between conditions can be compared across systems. For example, $\Delta r \cdot \text{int}$ provides an approach to determine the apparent intracellular k_D of a drug through measurements at different CIP and drug concentrations using Schild analysis (Fig. 3 and Supplementary Fig. 10). Anisotropy is an absolute measurement and should be directly comparable between systems. However, anisotropy cannot be used to determine k_D because of the competitive binding mechanism (Supplementary Fig. 8e).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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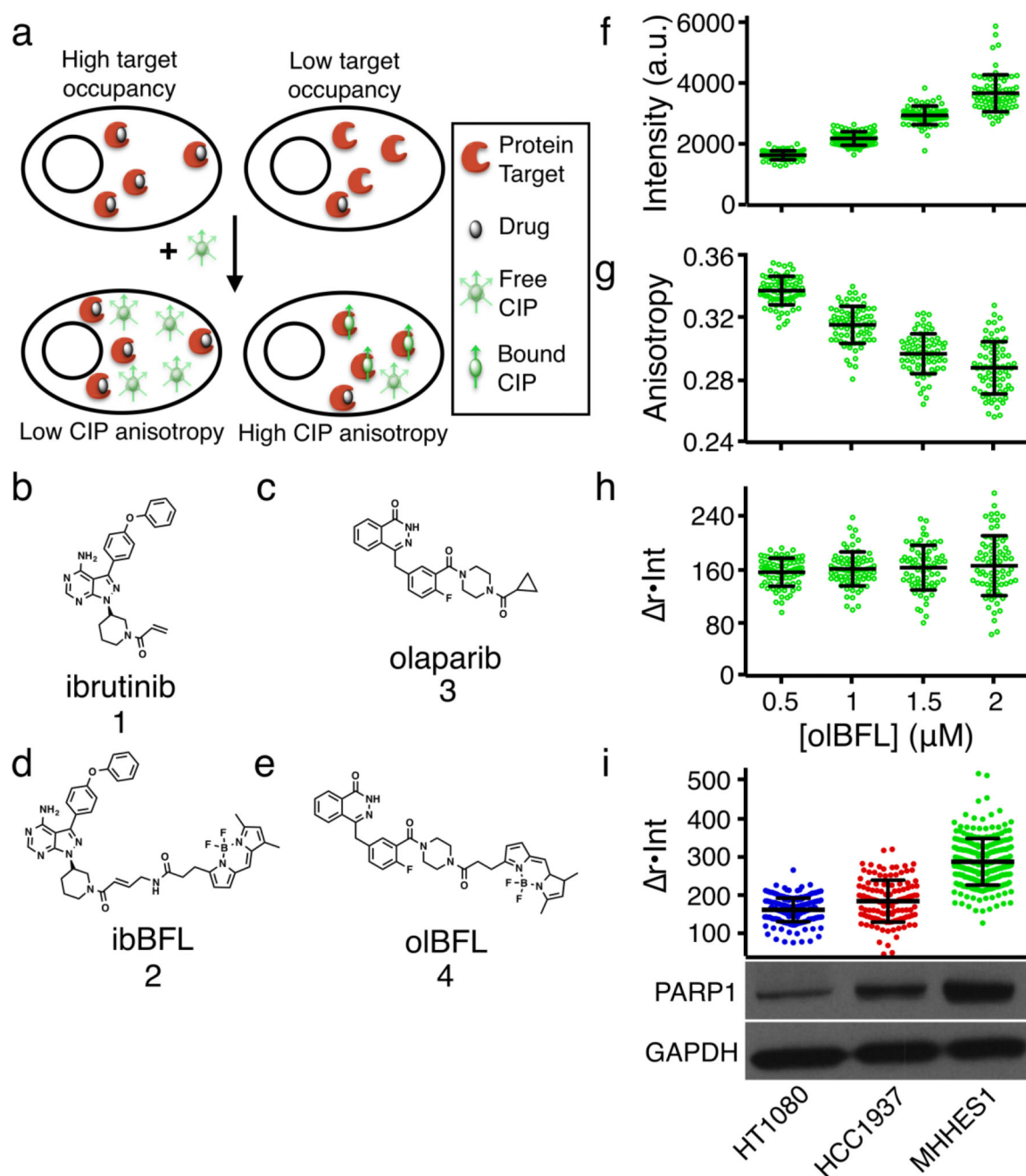


Figure 1. Measuring single cell drug binding through anisotropy competition imaging

(a) CIP measures unoccupied target through CIP rotational speed slowing when bound to the target, which increases the anisotropy and represents the degree of clinical drug target engagement. Chemical structures of ib Brutinib **(b)** and olaparib **(d)** and corresponding CIP linked to BODIPY FL **(c and e)**. Single cell HT1080 nuclei olBFL intensity **(f)**, anisotropy **(g)** and $\Delta r \cdot \text{int}$ **(h)** incubated with different concentrations of olBFL. Data are individual nuclei (green open circle) with mean $\pm$ s.d. (black), $n > 68$ per olBFL concentration, one technical replicate. **(i)** Single cell nuclear $\Delta r \cdot \text{int}$ measurements of HT1080 (blue), HCC1937 (red) and MHHES1 (green). Data are individual nuclei with mean $\pm$ s.d. (black), $n > 123$ per

cell line, one technical replicate. Western blot (bottom) of HT1080, HCC1937 and MHHES1 cells for PARP1 (the most abundant PARP in the nucleus) and GAPDH, full gels in Supplementary Fig. 1. PARP1 expression normalized to GAPDH is 1, 1.7 and 2.9 for HT1080, HCC1937 and MHHES1, respectively.

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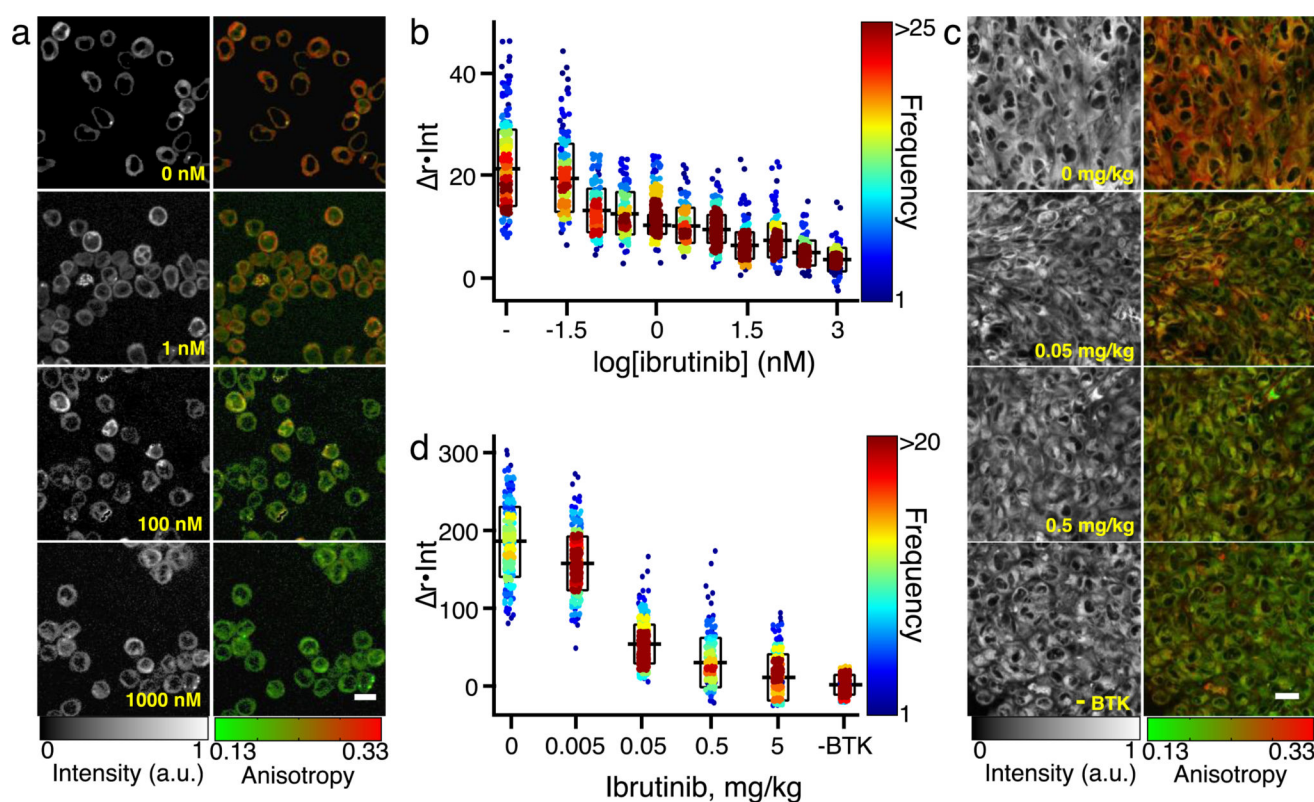


Figure 2. Ibrutinib target engagement

(a) Representative CIP fluorescence intensity (left) and anisotropy (right) images of Toledo cells loaded with varying concentrations of ibrutinib (yellow text) followed by ibBFL (150 nM). Scale bar: 12 μ m. **(b)** Single cell $\Delta r \cdot \text{int}$ measurements of Toledo cells incubated with varying concentrations of ibrutinib for 20 minutes followed by ibBFL (150 nM). Shown are mean (black line) $\pm$ s.d. (black box), $n > 133$ cells per ibrutinib concentration, one technical replicate. Corresponding anisotropy measurements are shown in Supplementary Figure 3e. **(c)** Representative CIP fluorescence intensity (left) and anisotropy (right) images of HT1080 BTK-mCherry and BTK-free HT1080 H2B-mApple (bottom) tumors following systemic ibrutinib delivery (yellow text) and *ex vivo* loading of ibBFL (200 nM). Scale bar: 20 μ m. **(d)** Single cell $\Delta r \cdot \text{int}$ measurements of cell cytoplasm following systemic ibrutinib delivery and *ex vivo* ibBFL loading. Shown are mean (black line) $\pm$ s.d. (black box), $n > 200$ cells per ibrutinib concentration, one technical replicate.

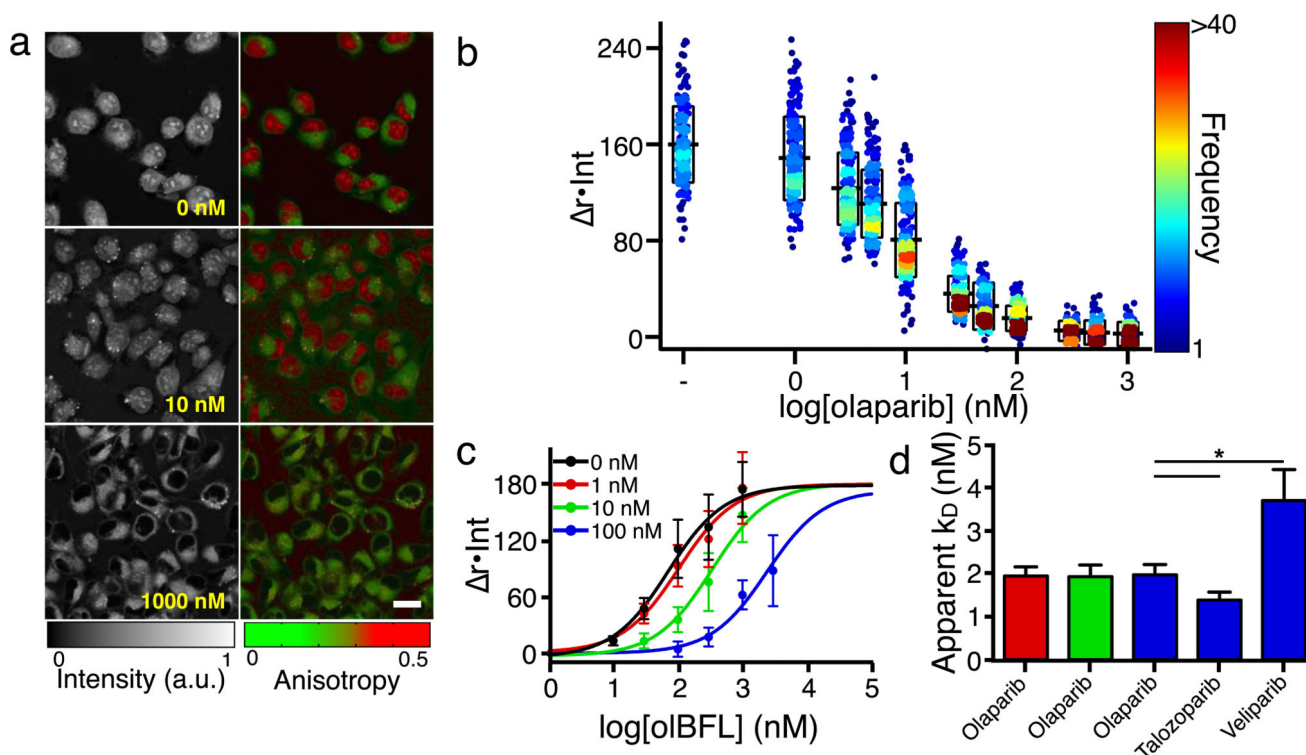


Figure 3. Intracellular PARP inhibitor target engagement

(a) Representative CIP fluorescence (left) and anisotropy (right) images of intracellular olaparib activity *in vitro* in HT1080 H2B-mApple cells incubated with olaparib (yellow text) and olBFL (500 nM). Scale bar: 20 μ m. **(b)** Single cell nuclei $\Delta r \cdot \text{Int}$ in HT1080 H2B-mApple cells *in vitro* incubated with olaparib and olBFL (500 nM). Shown are mean (black line) $\pm$ s.d. (black box), $n > 187$ cells per olaparib concentration, one technical replicate. Corresponding *in vitro* anisotropy measurements are shown in Supplementary Figure 11a. **(c)** Representative Schild curves of olBFL $\Delta r \cdot \text{Int}$ in HT1080 cell nuclei in the presence of different olaparib concentrations. Data are total cell mean $\pm$ s.d., $n = 3$, with sigmoidal fit. **(d)** Apparent intracellular K_D of PARP inhibitors in HCC1937 (red), MHHES1 (green) and HT1080 (blue) cells (* $p < 0.05$, Student's t-test). Data are mean $\pm$ s.d., $n = 3$.

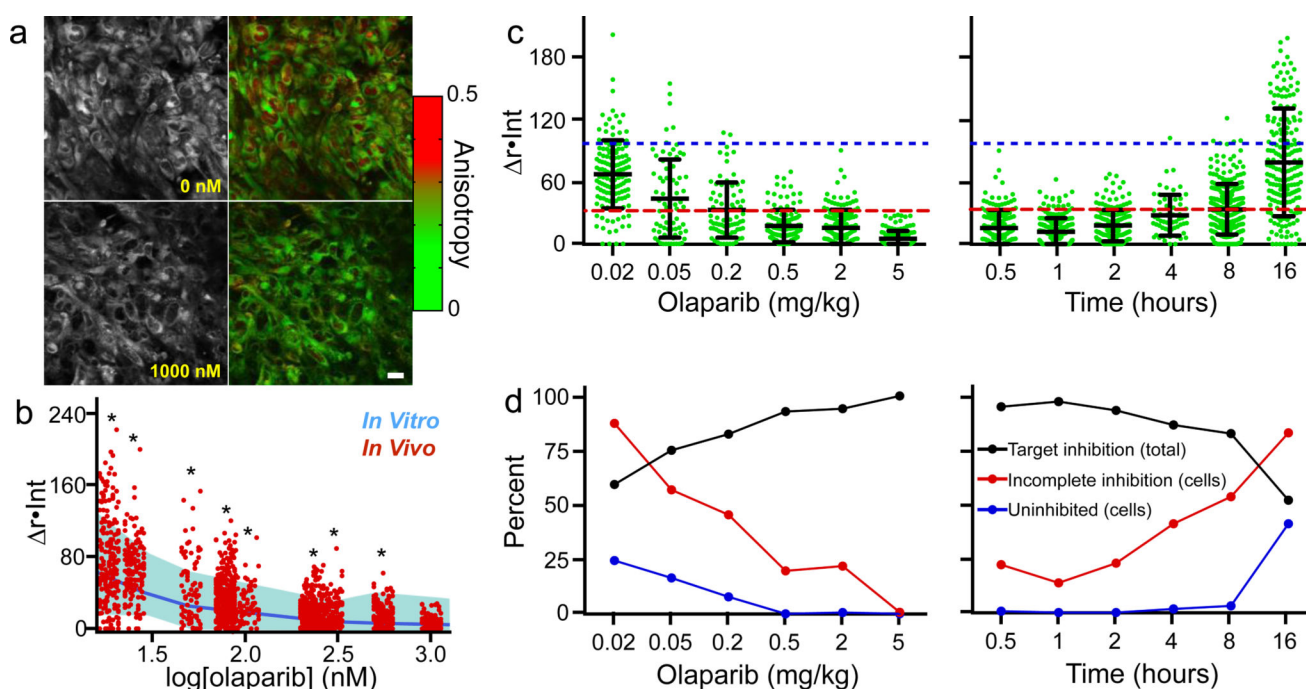


Figure 4. *In vivo* olaparib target engagement

(a) Representative CIP fluorescence (left) and anisotropy (right) images of intracellular olaparib activity *in vivo* in HT1080 H2B-mApple cells incubated with olaparib (yellow text) and oBFL (500 nM). **(b)** *In vivo* single cell nuclei $\Delta r \cdot \text{int}$ (red) following systemic olaparib delivery (concentration determined from calibration (Supplementary Figure 11c)) with average (blue line) and range of (minimum to maximum, light blue) *in vitro* cell nuclei $\Delta r \cdot \text{int}$ measurements, $n > 77$ for each measurement, one technical replicate. * indicates significant ($p < 0.001$) difference in variance (F test) between *in vivo* and *in vitro* measurements, $n = 1$ measurement. **(c)** Single cell nuclei $\Delta r \cdot \text{int}$ values of *in vivo* HT1080 H2B-mApple tumors 30 minutes after different olaparib concentration systemic (i.v.) delivery (left) and at different times following systemic (i.v.) delivery of 2 mg/kg olaparib (right), $n > 65$ for each condition, one technical replicate. Red dashed line, 3 standard deviations above the average $\Delta r \cdot \text{int}$ at 1 μM olaparib (Supplementary Figure 11b) (99% confidence) - the threshold above which intracellular target is not completely saturated by olaparib. Blue dashed line, minimum cell nucleus $\Delta r \cdot \text{int}$ in absence of olaparib. **(d)** Percent of total target engagement (black), cell nuclei with incomplete engagement (red) and cell nuclei with unoccupied target levels within the range of untreated cell population.