Single cell imaging of Bruton’s Tyrosine Kinase using an irreversible inhibitor

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A number of Bruton’s tyrosine kinase (BTK) inhibitors are currently in development, yet it has been difficult to visualize BTK expression and pharmacological inhibition in vivo in real time. We synthesized a fluorescent, irreversible BTK binder based on the drug Ibrutinib and characterized its behavior in cells and in vivo. We show a 200 nM affinity of the imaging agent, high selectivity, and irreversible binding to its target following initial washout, resulting in surprisingly high target-to-background ratios. In vivo, the imaging agent rapidly distributed to BTK expressing tumor cells, but also to BTK-positive tumor-associated host cells.

**Results**

We sought to design a bright, fluorescent derivative of an irreversible BTK inhibitor that would preserve the selectivity of the parent drug. As such a model agent, Ibrutinib fits tightly into the ATP binding pocket of BTK, forms an irreversible bond with Cys481, and has a suitable modification point for fluorochrome attachment...
A BODIPY-FL modified Ibrutinib (Ibrutinib-BFL) was designed and synthesized de novo in seven steps (Fig. 1b). Briefly, iodination of commercially available pyrazolopyrimidine compound with N-Iodosuccinimide, followed by Suzuki coupling of the product with 4-phenyloxybenzene boronic acid, resulted in compound 2. Mitsunobu reaction of compound 2 with N-Boc-3-hydroxypiperidine resulted in compound 3. After deprotection of the Boc protecting group in acidic conditions, the crude product was coupled with the linker (compound 5) to introduce a Michael acceptor for the irreversible binding affinity. Coupling of the crude Boc-deprotected compound 6 with BODIPY-FL-NHS finalized the synthetic steps to produce Ibrutinib-BFL (7) at an overall yield of \( \approx 11\% \).

To confirm the effect of BFL modification on the inhibition efficacy of the drug, half-maximal inhibitory concentration (IC\(_{50}\)) of Ibrutinib and Ibrutinib-BFL were determined against purified BTK enzyme. Ibrutinib-BFL had an IC\(_{50}\) of \( \approx 200 \) nM, which is less potent than the parent drug (\( \approx 2 \) nM IC\(_{50}\); data not shown). Although it may be possible to further optimize the affinity of Ibrutinib-BFL by testing various linkers, we found the current generation probe to be quite acceptable for imaging, as shown in subsequent experiments.

We next determined whether Ibrutinib-BFL would bind to purified BTK in vitro, endogenous BTK in live cells, and ultimately in vivo. Purified BTK was incubated with varying concentrations of the imaging probe for one hour at room temperature, denatured at 70°C for 10 minutes and then processed for SDS-PAGE gel analysis. There was a clear dose-response increase of the fluorescent signal around 80 kDa (BTK molecular weight is 76 kDa), as well as at the bottom of the gel (unbound fraction of Ibrutinib-BFL) (Fig. 2a). Additionally, binding could be blocked by pre-incubation with the parent compound and silver staining of the gel showed equal loading of BTK protein (Supplementary Fig. S1). These results clearly confirmed the covalent binding property of Ibrutinib-BFL toward purified BTK.
Figure 2 | Characterization. a. Target binding. Denaturing gel electrophoresis of decreasing concentrations of Ibrutinib-BFL incubated with 0.1 µg purified BTK for one hour, imaged with 488 nm excitation/520 nm emission. Note the dose dependent binding of Ibrutinib-BFL. Size marker on the far left. b. Denaturing gel electrophoresis of cell lysates following incubation of decreasing concentrations of Ibrutinib-BFL with Toledo (BTK+, left half of gel) or Jurkat (BTK-, right half of gel) cells at 37°C for two hours. Note the superb specificity of the probe.

We next performed a similar experiment in lymphoma cells. We first determined BTK expression in several lymphoma cell lines (Daudi Burkitt’s Lymphoma line, and DB, Toledo, and RC-K8 Diffuse Large B-Cell Lymphoma (DLBCL) lines) and one T-cell leukemia line (Jurkat) by Western blot (Supplementary Fig. S1). As expected, T cells did not express BTK. We found high BTK expression in Daudi and Toledo cell lines, and henceforth used Toledo as model BTK-positive cells and Jurkat as negative control cells. Toledo and Jurkat cells were incubated with different doses of Ibrutinib-BFL, and cell lysates were processed for SDS-PAGE and analyzed by fluorescent gel scanning.

The imaging probe showed remarkable specificity, with binding observed only at a single band (Fig. 2b). The specificity was further confirmed by the absence of a band in BTK-negative Jurkat cells, even at the highest concentration of probe (Fig. 2b), as well as by silver staining of the gel (Supplementary Fig. S1).

We next performed live cell imaging experiments using an imaging flow cytometry system. To prepare Toledo and Jurkat cells, we incubated them with 100 nM Ibrutinib-BFL for two hours, followed by washing. Figure 3 and Supplementary Fig. S2 summarize some of the results confirming target binding, specificity via blocking, and the ability to perform live cell imaging. To quantify co-localization between the imaging probe and BTK at the subcellular level, we created a stable transgenic cell line expressing a BTK-mCherry fusion protein in HT1080 human fibrosarcoma cells. In vitro cell experiments showed excellent co-localization and blocking ($r^2 = 0.9851$; Fig. 4).

We next performed in vivo experiments using three-color (blue: vasculature, green: Ibrutinib-BFL, red: BTK-mCherry-HT1080 cells) time-lapse intravital imaging. The intravascular half-life of Ibrutinib-BFL was ~10 minutes (Supplementary Fig. S3). Within an hour after systemic administration, there was extensive leakage of the compound into the tumor interstitium. At later time points, cellular uptake became apparent, presumably due to interstitial washout and/or intracellular accumulation. The ability to image in multiple channels allowed us to ask whether Ibrutinib specifically localized in tumor cells. We show that greater than 99% of all BTK-mCherry-HT1080 cells had achieved therapeutic drug concentrations within one hour. This effective intracellular dose persisted for prolonged periods of time and the compound was still detectable inside cancer cells 24 hours after administration (Fig. 5). Interestingly, there was also accumulation of Ibrutinib-BFL in non-tumor cells even at late time points. Given the exquisite specificity of the drug (see Fig. 2), we hypothesized that these non-target cells also contain BTK. We thus performed correlative immunohistochemistry using anti-BTK antibody. Our data indicates that Ibrutinib-BTK also accumulates in tumor-associated macrophages and lymphocytes (Fig. 6).

Discussion

Inhibition of BTK is emerging as a promising target for B-cell malignancies, other cancers with BTK over-expression, and certain autoimmune diseases where BTK is involved. Ibrutinib, an irreversible inhibitor, is approved for treatment of mantle cell lymphoma and CLL, and is currently undergoing late-stage efficacy studies in patients with various B-cell malignancies. Based on its covalent target binding, we hypothesized that the molecule could serve as a companion imaging agent. Here we show that this is indeed the case. Ibrutinib-BFL co-localized with BTK in BTK-positive malignant cells and had low background accumulation in non-BTK cells, including those expressing structurally related interleukin-2-inducible T-cell kinase (ITK), which is expressed in T cells and Jurkat cells (see Supplementary Fig. S1). The companion imaging drug, Ibrutinib-BFL, also showed a predictable dose response curve, could be competitively inhibited, allowed drug concentrations to be quantitated in vivo, and enabled mapping of drug distributions at the single cell level. As such, we believe that Ibrutinib-BFL could have several applications, including use as a companion diagnostic for flow cytometry in haematologic malignancies, as an imaging agent to localize and map BTK-positive tumors, as a method to track subcellular localization of endogenous BTK, and as a tool to measure pharmacokinetics and pharmacodynamics in experimental settings during development of novel BTK-pathway inhibitors.

BTK is a cytoplasmic tyrosine kinase belonging to the Tec family. It is expressed in the B-cell lineage, plays a pivotal role in signaling and development, and is highly active in several haematological malignancies. Some previous BTK imaging has been done with fluorescent protein tags (BTK-GFP and BTK-mCherry) to understand its activation and nucleocytoplasmic shuttling, and its roles in myeloid cell chemotaxis and infection. Alternative research methods have primarily involved fluorescently labeled antibodies for immunohistochemistry and flow cytometry applications. The
The approach developed here, utilizing a small molecule affinity ligand, is compatible with live cells, can be used in vivo, and has potential clinical applicability. Not only does Ibrutinib-BFL specifically bind to BTK, but also it remains bound until protein turnover due to the virtually nonexistent off-rate of covalent inhibitors. This feature will allow for long-term study of endogenous BTK in live cells, providing a window into drug pharmacodynamics, as well as innate heterogeneity in responses to drugs targeting the BCR signaling pathway. Beyond utilizing Ibrutinib-BFL in pharmacologic studies of next generation inhibitors, there are future diagnostic opportunities in which BTK-expressing lymphomas could be imaged in the clinic. While the current work focused on single cell imaging in vivo, we also anticipate whole body imaging applications. For example, the fluorine in BODIPY-FL could be exchanged for 18F for positron emission tomography (PET) imaging, or entirely replaced via bioorthogonal ligands or direct 18F attachment. Alternatively, longer-lived isotopes such as Zirconium-89 could also be utilized in order to take full advantage of the probe’s irreversible binding kinetics. Such molecules may be useful in clinical imaging-based tests for whole body distribution and inhibition of BTK. Other areas of interest are to use these molecules for imaging BTK in macrophages during infection, or to use them as a readout during gene therapy for the immunodeficiency disorder X-linked agammaglobulinemia, which results from loss of functional BTK. Irrespective of the contemplated use, we believe that the developed agent should be useful in a number of different applications. As covalent inhibitors have gained interest, we anticipate covalent imaging agents to follow, and Ibrutinib-BFL can provide a roadmap for such development.

### Methods

**Synthesis and Characterization of Probe.** All reagents were obtained from commercial sources and used without further purification. Dry THF, MeOH, DCM, and DMF were obtained from Sigma-Aldrich (St. Louis, MO). 1H and 13C NMR spectra were recorded at 23°C on a Bruker 400 MHz spectrometer. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant (J, Hz), and integration. LC-ESI-MS analysis and HPLC-purifications were performed on a Waters (Milford, MA) LC-MS system. For LC-ESI-MS analyses, a Waters XTerra C18 5 µm column was used. For preparative runs, an Atlantis® Prep T3 OBDTM 5 µm column was used [eluents 0.1% TFA (v/v) in water (solution A) and MeCN (solution B); gradient: 0–1.5 min, 5–100% B; 1.5–2.0 min, 100% B for analysis and 0–0.75 min, 5% B; 0.75–9.0 min, 5–100% B; 9.0–10.0 min, 100% B for prep.].

**3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1).** A solution of 4-amino-1H-pyrazolo[3,4-d]pyrimidine (780 mg, 5.77 mmol) and N-ido-succinimide (2.02 g, 8.98 mmol) in DMF (6 mL) was stirred at 80°C overnight. Resulting brown solution was filtered and sticky solid was washed with water and cold ethanol. Resulting light yellow solid was dried in vacuo to give compound 1 (1.50 g, 99.6% yield). Crude product was used for the next reaction without further characterization.
3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (2). A solution of compound 1 (200 mg, 0.77 mmol), tetrakis-(triphenylphosphine)palladium (124 mg, 0.11 mmol), potassium phosphate tribasic (488 mg, 2.3 mmol), and 4-phenoxyphenylboronic acid (492 mg, 2.3 mmol) in 1,4-dioxane (2.5 mL) in a microwave vial was heated to 180 °C for 10 minutes under microwave irradiation. Resulting reaction mixture was diluted with water and organic material was extracted with EA three times. Combined organic material was dried over Na2SO4 and concentrated in vacuo. Resulting yellow solution was dissolved with DCM and resulting turbid solution was filtered to give compound 2 as a white solid (138 mg, 59.4% yield). 1H NMR (400 MHz, DMSO) δ 13.55 (s, 1H), 8.24 (s, 1H), 7.67 (d, J = 5.8 Hz, 2H), 7.44 (t, J = 7.8 Hz, 2H), 7.17 (m, 5H); 13C NMR (101 MHz, DMSO) δ 158.0, 157.0, 156.3, 156.0, 155.7, 143.9, 130.1, 130.0, 128.4, 123.7, 119.0, 118.9, 96.9; LRMS (ESI) m/z calcd for C17H13N5O [M+H]+ 304.12, found 304.14.

(R)-tert-butyl 3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carboxylate (3). A solution of compound 2 (57 mg, 0.19 mmol), (S)-3-hydroxy-N-Boc-piperidine (80 mg, 0.40 mmol), DIAD (150 μL, 0.764 mmol), and polymer-TPP (0.5 g, 1.6 mmol) in THF (4 mL) was stirred at ambient temperature overnight. After reaction completion, polymer-TPP was removed by filtration, filtrate was concentrated in vacuo and purified with silica gel column chromatography (EA : Hex = 0 : 100 to EA only) to give compound 3 (45 mg, 48.7% yield) as a clear oil. 1H NMR (400 MHz, CDCl3) δ 8.37 (s, 1H), 7.65 (d, J = 7.5 Hz, 2H), 7.38 (m, 2H), 7.19 – 7.12 (m, 3H), 7.08 (d, J = 8.6 Hz, 2H), 5.53 (s, 2H), 4.84 (dq, J = 10.4, 5.1, 4.3 Hz, 1H), 4.35 – 4.23 (m, 1H), 4.16 – 4.06 (m, 1H), 3.46 (t, J = 11.5 Hz, 1H), 2.88 (td, J = 12.3, 2.8 Hz, 1H), 2.34 – 2.14 (m, 2H), 1.96 – 1.85 (m, 1H).

Figure 4 | Imaging of adherent BTK-mCherry cells to determine co-localization with Ibrutinib-BFL. a. Imaging co-localization between 500 nM Ibrutinib-BFL (green) and HT1080 cells stably transfected with BTK-mCherry (red), following a 2-hour incubation with Ibrutinib-BFL and then a 24-hour incubation in probe-free media (top). Center: competitive inhibition with 1 μM Ibrutinib prior to Ibrutinib-BFL addition. Bottom: Ibrutinib-BFL incubated with non-BTK expressing parent HT1080 cells. b. Note the exquisite co-localization. Scale bar: 50 μm.

Figure 5 | In vivo tumor imaging. Serial imaging before, and at 2, 5 and 24 hours after intravenous administration of Ibrutinib-BFL to a representative mouse harboring a BTK-positive HT1080 tumor (red; first column). Note extensive drug accumulation in all cells, persisting even at the 24-hour time point. * Indicates accumulation in non-tumor cells (see Fig. 6). Scale bar: 50 μm.

Figure 6 | Histology. To corroborate intravital serial imaging, tumors were examined histologically. Anti-BTK staining showed BTK signal in HT-1080-BTK-mCherry cells as expected, but also in tumor-associated macrophages (white). These regions of drug accumulation correspond to those seen by intravital imaging (* in Fig. 5). Scale bar: 10 μm.
1.78 – 1.65 (m, 1H), 1.45 (s, 9H). 13C NMR (101 MHz, CDCl3) δ 74.8, 170.9, 155.7, 154.6, 154.3, 136.0, 130.9, 129.7, 124.0, 119.5, 119.1, 98.6, 79.8, 52.9, 41.2, 44.0, 30.2, 28.4, 24.5. LRMS (ESI) m/z calculated for C11H19NO4 [M+H]+ 270.20, found 270.20.

(E)-4-(tert-butoxycarbonyl)amino)but-2-enolic acid (5)

A solution of compound 4 (110 mg, 0.21 mmol) in 2 mL of TFA and DCM mixture (1:3) was stirred for 1.5 hours at 37°C. 1H NMR (400 MHz, CDCl3) δ 7.20 – 7.02 (m, 2H), 6.65 (t, J = 9.8 Hz, 1H), 5.75 (br s, 2H), 4.94 – 4.74 (m, 4H), 4.74 – 4.60 (m, 1H), 4.52 (d, J = 13.0 Hz, 0.5H), 4.10 – 3.97 (m, 0.5H), 3.86 (d, J = 13.2 Hz, 0.5H), 3.66 (dd, J = 13.2, 10.4 Hz, 0.5H), 3.33 (t, J = 12.0 Hz, 0.5H), 3.26 – 2.93 (m, 2H), 2.83 – 2.65 (m, 5H), 2.50 – 2.15 (m, 2H), 2.06 – 1.70 (m, 1H), 1.80 – 1.58 (m, 1H), 1.46 (s, 9H); LRMS (ESI) m/z calculated for C11H19NO4 [M+H]+ 256.20, found 257.20.

(E)-tert-butyl 4-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-4-oxobut-2-en-1-yl)carbamate (6)

A solution of compound 5 (100 mg, 0.21 mmol) in 2 mL of TFA and DCM mixture (1:3) was stirred at ambient temperature for 30 minutes. After 30 minutes stirring, reaction mixture was concentrated in vacuo. After azeotropic distillation with DCM and ACN three times, crude product was concentrated in vacuo. Reaction mixture was purified with silica gel column chromatography (EA:Hex = 0:1 to 10:1) to give compound 6 (5 mg, 0.009 mmol) in 2 mL of TFA and DCM mixture (1:3). 1H NMR (400 MHz, CDCl3) δ 7.07 – 6.90 (m, 2H), 4.81 (s, 1H), 4.76 (q, J = 7.2 Hz, 2H), 3.89 (s, 2H), 1.42 (s, 9H), 1.26 (t, J = 7.1 Hz, 3H). 13C NMR (101 MHz, CDCl3) δ 166.2, 155.7, 147.7, 149.1, 124.7, 79.9, 60.5, 41.8, 28.4, 14.3. LRMS (ESI) m/z calculated for C27H30N6O3 [M+H]+ 570.20, found 570.20.

 Imaging of non-adherent lymphoma cells by flow cytometry

Jurkat and Takeda cells were single- or triple-stained with the following, followed by washing: Ibrutinib-BFL (2 hours in growth media, 37°C, Hoechst 33342, nuclear dye (Ibrutinib) or APC-conjugated anti-human-CD45 antibody (Clone HI30, BioLegend, San Diego, CA, USA)(both 30 minutes in PBS containing 2% BSA, 4°C). Stained cells were transferred to Clear-view Snap-Cap microtubes (Sigma-Aldrich) for Amnis ImageStream® Mark II Imaging flow cytometry (Amnis Corporation, Seattle, WA, USA). Single-stained samples were used to create a compensation table, then 30,000 images from each triple-stained sample were collected using excitation lasers 405-nm, 488-nm, 592-nm, and bright-field excitation, and 430–500 nm (Ch2), 480–560 nm (Ch2), 640–745-nm (Ch11) and 430–480 (Ch1) emission filters. Representative images were manually selected from this data set.

 Imaging of adherent cells by microscopy

HT1080-BTK-mCherry cells were seeded into a 96-well plate at 20,000 cells per well and allowed to grow to confluence overnight. Cells were incubated in growth media containing 1 μM Ibrutinib in final 0.1% DMSO, or control 0.1% DMSO, at 37°C for 1.5 hours. Without washout, a 50-μL 100-fold serial dilution of Ibrutinib-BFL was added for final 0.1% concentration of 500 nM. Control wells contained equivalent DMSO without Ibrutinib-BFL. Cells were incubated for one hour at 37°C and then washed once with media for five minutes. The media was then replaced and cells were incubated at 37°C overnight. The live cells were subsequently visualized on the DeltaVision imaging system (Applied Precision, A GE Healthcare Company). Images were processed with Fiji software, an open-source version of ImageJ.

In vivo tumor imaging

Nu/nu mice were implanted with 2 x 10⁶ HT1080-BTK-mCherry cells into a dorsal skinfold window chamber (APT Trading Company, Ventura, CA, USA) according to established protocols and with guidelines from the Institutional Subcommittee on Research Animal Care. Tumors were allowed to grow and vascularize for two weeks. 75 nmol Ibrutinib-BFL in 150 μL solution containing DMAC and solute was injected via tail vein as reported previously. Mice were anesthetized with 2% isoflurane in 2 L/min oxygen. Time-intensity curves were calculated by measuring the fluorescence intensity at 2, 5, and 24 hours post-injection. Images were processed with Fiji software.

Histology

HT1080-BTK-mCherry tumors were harvested from nu/nu mice and embedded in an O.C.T. compound (Sakura Finetek, Torrance, CA, USA). Serial 6 μm-thick frozen sections were prepared for histological analysis. Fluorescence immunohistochemistry staining was performed using Mac-3 (clone: M83-84, BD Biosciences, San Jose, CA, USA) and BTK (clone: D8S, Cell Signaling Technology), followed by Alexa Fluor 647 goat anti-rabbit IgG (both Invitrogen) secondary antibodies, respectively. Images were captured using a BX63 fluorescence microscope (Olympus America) equipped with a Neo sCMOS camera (Andor Technology, Belfast, UK) and processed with Fiji software.

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