Supplemental data

Supplementary Table 1. List of primary antibodies used for WB and IF

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Antigen	Source	Company	Catalog number
BTK	Rabbit	Cell Signaling Technology	8547
pBTK (Y223)	Rabbit	Cell Signaling Technology	5082
AKT	Rabbit	Cell Signaling Technology	4685
pAKT (S473)	Rabbit	Cell Signaling Technology	9271
ERK1/2	Rabbit	Cell Signaling Technology	4695
pERK1/2	Rabbit	Cell Signaling Technology	9101
MEK1/2	Rabbit	Cell Signaling Technology	8727
pMEK1/2	Rabbit	Cell Signaling Technology	9154
SYK	Rabbit	Cell Signaling Technology	2712
pSYK	Rabbit	Cell Signaling Technology	2710
CRBN	mouse	R&D Systems	MAB9574-100
GAPDH	Rabbit	Cell Signaling Technology	2118

Supplementary table 1

Supplementary materials and methods

Cell culture

DOHH2, MOLM13, and MINO cells were purchased from DSMZ (Braunschweig, Germany) and grown in Hyclone RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% HyClone fetal bovine serum (FBS).

In vitro target binding assay

The binding assay for WT-BTK was performed using the Lantha Screen^{TM} Eu kinase binding assay kit (Thermo Fisher) per the manufacturer's protocol with some modifications. The assay was performed on white 384-well microplates (Corning, 6008280) in a buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35) by mixing the final concentrations of 5 nM BTK (Thermo Fisher, PV3363), 30 nM kinase Tracer 236 (Thermo Fisher, PV5592), and 2 nM Eu-anti-His-tag antibody (Thermo Fisher, PV5596) with compounds used. All compounds were prepared in duplicate to calculate half-maximal inhibitory concentration (IC_{50}) values. The mixture was incubated at room temperature (RT) and time-resolved FRET (TR-FRET) signals were collected at 615 nm (donor) and 665 nm (acceptor) using the Bio-Tek Synergy H1 microplate reader. IC_{50} values of the compounds were calculated against those of BTK using GraphPad Prism 5.

The homogeneous TR-FRET (HTRF) assay was performed to measure CRBN binding activity using a CRBN binding assay kit (Cisbio, 64BDCRBNPEG). The assay was performed by combining GST-tagged CRBN protein, Eu-anti-GST antibody, thalidomide-red, and serially diluted compounds in a Cisbio's PROTAC binding buffer using white 384-well microplates (Corning, 6008280). Following incubation at RT, the plate was read using a Bio-Tek Synergy H1 microplate reader. The HTRF signals were collected at 620 nm and 665 nm and calculated using the following equation: signal 665 nm/signal 620 nm × 10,000. GraphPad Prism 5 was used to plot dose-response curves and calculate IC_{50} values for each compound.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

Total lysates from TMD-8 were separated on SDS–PAGE gel. Proteins were transferred using Trans-Blot Turbo Transfer Kit. For conventional western blotting, membranes were blocked with 5% skim milk powder in Tris (tris(hydroxymethyl)aminomethane)-buffered saline and Tween 20 (TBST) (25 mM Tris pH 7.5, 150 mM NaCl, 0.2% Tween-20) for 45 min at RT, incubated overnight at 4°C with a primary antibody (anti-BTK, Cell Signaling Technologies, cat. #8547, 1:1,000 dilution in a blocking buffer), and washed thrice with TBST before adding anti-rabbit immunoglobulin (Ig) G (Cell Signaling Technologies, cat. #7074, 1:5,000 dilution in a blocking buffer) for 45 min at RT. The mixture was then washed thrice in TBST and developed by electrochemiluminescence (Super Signal Chemiluminescent Substrate, Thermo Fisher Scientific).

Western blotting was performed using LI-COR Odyssey® Infrared Imaging System. The experiments were performed per the manufacturer's instructions. Briefly, proteins were separated as described above alongside Odyssey pre-stained molecular weight markers (Licor, fluorescence in a 700-nm channel).

Immunofluorescence

The TMD-8 cells were fixed using 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), and permeabilized with 0.25% Triton X-100. The cells were then washed with PBS and blocked with 1% bovine serum albumin/PBS with Tween 20. Coverslips were incubated overnight at 4°C with 1:250 anti-BTK (Cell Signaling Technologies, cat. #8547) and 1:50 anti-CRBN (R&D Systems, cat. MAB9574-100). The following day, the coverslips were washed with PBS and incubated with 200 µL of 1:500 a secondary anti-rabbit (goat anti-rabbit IgG (H + L) Highly Cross-Adsorbed Secondary antibody, Alexa Fluor Plus 488, cat. A32731, Invitrogen) or anti-mouse antibody (goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 647 cat: A21235, Invitrogen) for 45 min in the dark. A fluorophore-conjugated secondary antibody was added to check positive signals using Zeiss LSM900 (Carl Zeiss).

C-C motif chemokine ligand (CCL) 3 & CCL4 enzyme-linked immunosorbent assay (ELISA)

TMD-8 cells were seeded at 2×10^6 cells/well on a 12-well plate (Corning, CLS3513) in a 1 mL of cell culture medium and were treated with 0.01 µM PROTAC, ibrutinib, acalabrutinib, ARQ-531, Binder, or MT-802 for 24 h followed by stimulation with 10 µg/mL anti-IgM (Southern Biotech, 2022-01) for 8 h before harvesting. Supernatant from the TMD-8 cells was then collected to measure CCL3 and CCL4 levels using a human CCL3/MIP-1α Quantikine ELISA kit (R&D Systems, DMA00) and a human CCL4/MIP-1β Quantikine ELISA kit (R&D Systems, DMB00) per the manufacturer's instructions. Absorbance was detected using a Synergy H1 microplate reader (Bio-Tek) and Gen5 (version 3.08).

Stable cell line generation

pLVX-BTK WT or pLVX-BTK C481S plasmids were co-transfected with a packaging plasmid and VSV-G envelope plasmid into 293T cells. Lentiviral supernatant was collected at 48 h and added to the TMD-8 cells with polybrene 72 h after transfection (8 µg/mL). Puromycin (3 µg/mL) selection was performed for 2 weeks to produce TMD-8 BTK WT and TMD8 BTK C481S cell lines.

Immunohistochemistry

Spleen tissues and TMD-8 xenografted tumors were extracted from mice, fixed in 4% paraformaldehyde, and paraffinized. Paraffin blocks were sectioned, deparaffinized with xylene, rehydrated, and treated with citrate for 1 h for antigen retrieval. After washing with PBS, the tissue sections were blocked with serum at RT and the blocked sections were incubated with a primary antibody against BTK antibody for 1 h. After washing with PBS, the sections were treated with H_2O_2 to block any endogenous peroxidase activity. The Envision Single Reagent (rabbit horseradish peroxidase) detection system (Dako, K4003) was used as a secondary antibody, and treatment was performed for 1 h. After development with 3,3-diaminobenzidine for 30 min, the sections were counterstained with hematoxylin (ab220365; Abcam) for 1 min and subjected to alcohol dehydration and clearing with xylene before mounting and sealing on slides.

Transient transfection

HEK293 cells were seeded on 6-well plates and transfected with pCMV3-N-Flag BTK WT, E41K, T474I, T474S, C481S, C481R, C481F, C481Y, C481T, and L528W plasmids using Lipofector-EZ Reagent (APTABIO, AB-LF-EX150) per the manufacturer's protocol with modifications. For each transfection, 1.5 µg of plasmid and 4 µL of transfection reagent were added

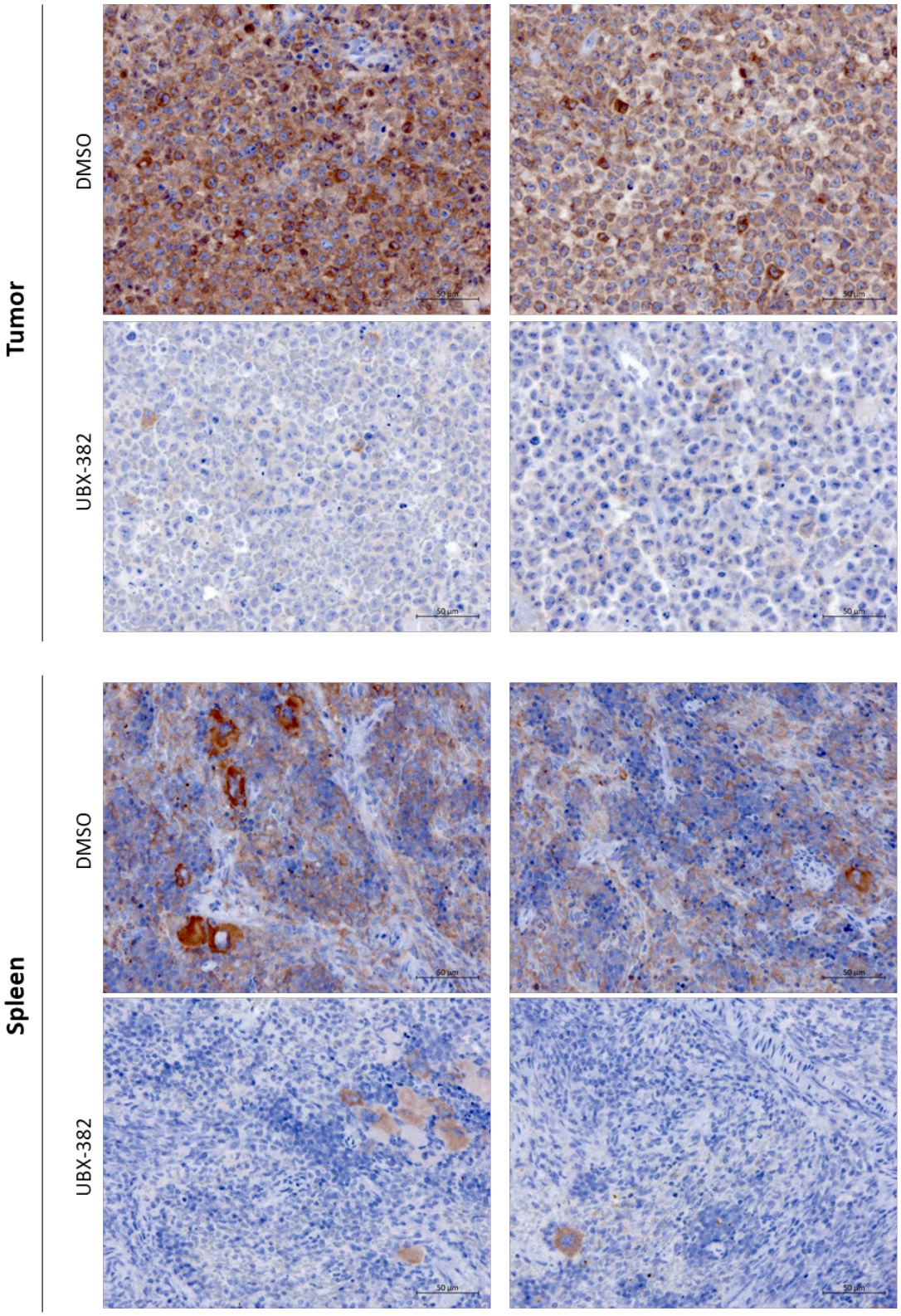
to 100 μL of a serum- and antibiotic-free medium. The mixture was incubated at RT for 15 min before adding it into the wells. The cells were incubated for 5 h before medium replacement. After 24 h, the transfected cells were reseeded onto a 12-well plate. Then, the cells were treated with UBX-382, ARQ-531, and MT-802 for 24 h. pCMV3-N-Flag BTK WT (Sino Biological, HG10578-NF) was purchased, whereas other BTK mutant vectors were generated by pCMV3-N-Flag BTK WT site-directed mutagenesis by Cosmo Genetech.

Supplementary Figure 1. Immunohistochemical study of BTK levels in TMD-8 tumor and spleen tissues treated with DMSO or UBX-382.

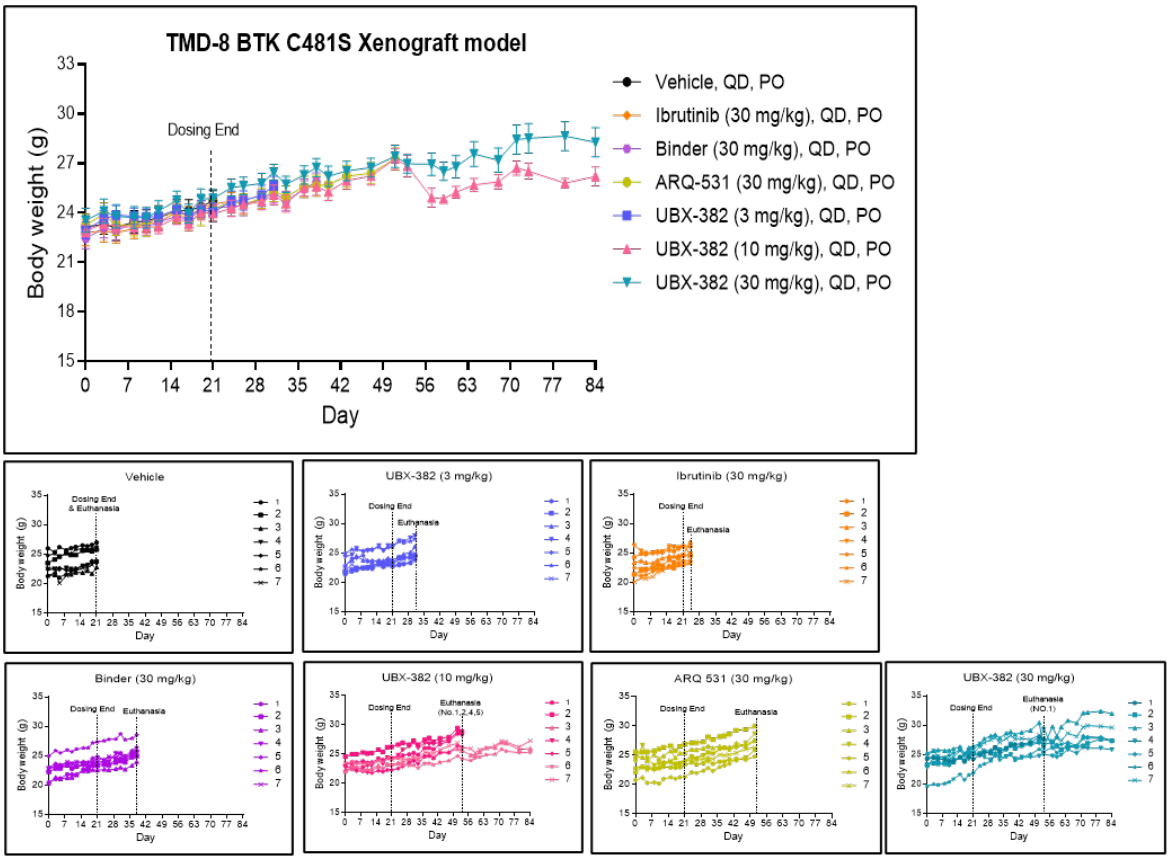
The immunohistochemical images are magnified images of those in Figure 4E.

Supplementary Figure 2. Curves of body weight in xenograft TMD-8 (BTK C481S)-bearing mice models

The mice were treated with vehicle, ibrutinib, Binder, ARQ-531, and UBX-382. Each drug was administered for 3 weeks, and the mice were euthanized when the tumor size reached an average of approximately 2000 mm3. Body weights were recorded 3 times a week, n=7 per group. Values are presented as mean ± SEM. Below data are expressed as an individual mouse in each group.



Supplementary figure 1



Supplementary figure 2