Method

Cell culture and treatment.

Ramos were obtained from the American Type Culture Collection (CRL1596). BTK-HiBiT Ramos was obtained from Promega. TMD8 WT and TMD8 C481S cell were shared by Dr. Jennifer Woyach at The Ohio State University. Both cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Cat. No. MT10040CV) supplemented with 10% fetal bovine serum (GE Healthcare, Cat. No. SH30071.03) and 1% Pen/Strep (Thermo Fisher Scientific, Cat. No. 15140163). HeLa cells (ATCC, Cat. No. CCL-2) were maintained in DMEM (Thermo Fisher Scientific, Cat. No. MT10013CV) with 10% fetal bovine serum and 1% Pen-Strep.

TR-FRET biochemical binding assays.

Time-resolved fluorescence resonance energy transfer (TR-FRET) assay was carried out to evaluate the binding of indicated compounds and BTK by competition with a BODIPY-FL labeled BTK tracer. The assay was performed in 16 μL assay buffer (50 mM Tris, pH7.5, 0.1% Triton X-100, 0.01% BSA, and 1 mM DTT) with 0.3 nM Tb-anti-His (Cisbio, Cat.No. 61HI2TLA), 2 nM His-BTK (SignalChem, Cat. No. B10–10H-10), 120 nM BTK-BODIPY tracer[1] and serial diluted compounds (10,000–0.64 nM, 5-fold dilutions) in opaque 384well plates. The assay mixtures were incubated at room temperature in dark for 120 min, unless specified otherwise, and the signals were collected using a PheraStar microplate reader to measure the fluorescence emission ratio (F520 nm/F490 nm) of each well. Raw data from the plate reader was used directly for analysis. The curve-fitting software GraphPad Prism was used to generate graphs and curves and to determine IC_{50} values.

Immunoblotting

Ramos and TMD8 WT and TMD C481S cells were seeded into the wells of six-well plates at the density of $5*10^5$ cells per mL in 2 mL of complete RPMI-1640 culture medium. After overnight adaptation, cells were treated with serially diluted compounds for 24 h. After completion of treatment, whole cell lysates for immunoblotting were prepared by pelleting cells at 4 °C and 200g for 5 min. The resulting cell pellets were washed once with ice cold PBS and lysed in 1x RIPA lysis buffer (Alfa Aesar, Cat. No. J62524) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher, Cat. No.78430). Lysates were centrifuged at 15,000g for 10 min at 4 °C and protein concentrations were assessed using BCA assay (Pierce, Cat. No.23225). Same amounts of protein (30 μg) for each sample were loaded onto sodium dodecyl sulfate-polyacrylamide gel, separated by electrophoresis (Bio-Rad) at 120 V for 1.5 h and transferred to PVDF membrane using a Transblot Turbo system (Bio-Rad). After blocking for 2 h at room temperature in 4% BSA-TBST, the membranes were immunoblotted with the specified primary antibodies at the dilution of 1:1000 in TBST (Cell Signaling Technology: anti-BTK Cat. No.8547 anti-β-actin Cat. No.4570) overnight at 4 °C, and the HRP-conjugated secondary antibodies (Cell Signaling, Cat. No.7074, 1:1000 in TBST) for 1 h at room temperature. Imaging was performed using the ECL Prime chemiluminescent Western blot detection reagents (Kindle Biosciences, Cat. No. R1100) by visualization of the blots with an Imager (Kindle Biosciences, Cat. No. D1001). All western blots were subsequently processed and quantified with Imager software ImageJ and protein level was normalized to the β-actin loading controls.

In vitro kinase assay.

BTK kinase activity was measured by PhosphoSens® Kinase Assay Kit (Assay Quant Technologies Inc., Cat.# CSKSAQT0101K). This assay was performed in 384-well, transparent flat bottom polystyrene microplates (Falcon REF 353961) at room temperature. Active recombinant full length His-BTK was purchased from SignalChem (Cat.# B10-10H-10). All the drugs (3 warhead control and 4 PROTACs) were dissolved in DMSO (10 mM). Duplicate drug concentration titrations as indicated in the figure were used to generate time course inhibition curves. Typical final concentrations of each reaction component are as follows: 1.25 nM BTK, and 10 μM PhosphoSens® Substrate, 50 mM HEPES, pH 7.5, 1 mM ATP, 1 mM TCEP, 0.01% Brij-35, 10 mM MgCl2, 1% glycerol, and 0.2 mg/mL BSA. Everything except for enzyme were mixed at room temperature and 20μl were dispensed into 384-well. 5μl enzyme (5x) in 20mM HEPES pH 7.5, 0.01% Brij-35, 5% glycerol and 1mg/ml BSA was immediately added into each well before collecting the fluorescence intensity readings (Ex 360 nm/Em 492 nm) for 120 min with 3 min interval in a BioTek Synergy H1 fluorescence microplate reader. The curves were globally fitted against all drug concentration using the following equation for each individual drug to obtain the kinact and KI value if it shows covalent binding behavior.

$$Y = (vs * X) + \left(\left(\frac{vi - vs}{kinact * \frac{I}{KI+I}}\right) * \left(1 - \exp\left(-\left(kinact * \frac{I}{KI+I}\right) * X\right)\right)\right) + Y0$$

, where Y is the RFU value, X is the elapsed time in second, I is the final drug concentration, vi and vs are the initial and final reaction velocity, Y0 is the background noise from time 0.

Intact protein mass-spectrometry

Intact proteins were analyzed on a Vanquish Neo UHPLC system (Thermo Scientific) equipped with a microflow setup. Separation was carried out on a NativePac OBE-1 (2.1 mm × 50 mm) SEC column (Thermo Scientific, Cat. No. 43803-052130) operated under isocratic conditions. The mobile phase consisted of 100 mM Ammonium Bicarbonate pH 8.0. The column was equilibrated at 17 μL/min and the gradient was maintained isocratic throughout the 8-min acquisition. Column compartment temperature control was off. The autosampler was operated at 7 °C with direct injection, fast loading enabled, and loop inline mode disabled.

MS analysis was performed on an Orbitrap Astral mass spectrometer (Thermo Scientific) operated in intact protein mode under high-pressure conditions. The electrospray ionization (NSI source) was used with the following parameters: spray voltage 1.8 kV (positive ion mode), ion transfer tube temperature 280 °C, RF lens 200%, and source fragmentation set to 120 V. Data were acquired in the Orbitrap mass analyzer at a resolution of 30,000 (at m/z 200) across a scan range of m/z 1000–8000. A custom AGC target of 3.0×10^6 ions (normalized 300%) was used, with a maximum injection time set manually. Ten microscans were averaged per spectrum. Data were collected in profile mode with advanced peak determination enabled. No lock mass was applied. The expected peak width was set at 30 s.

Raw intact protein MS data were processed using BioPharma Finder 5.1 (Thermo Scientific) with the *ReSpect* deconvolution algorithm. Source spectra were generated across the full chromatographic peak width using sliding windows algorithm with a target average spectrum width of 0.48 min. The deconvolution mass range was set to 74-80 kDa, with a deconvolution mass tolerance of 15 ppm and a relative abundance threshold of 5%. Signal-to-noise thresholding and smoothing were applied according to default vendor settings. Peak picking and isotope deconvolution were carried out with “Advanced Peak Determination” enabled. The resulting zero-charge intact mass spectra were exported for interpretation and figure generation.

Global Proteomics

Ramos cells were seeded in 96-well U-bottom plates at a density of 2 x 10^6 cells/mL in a volume of 100 µL. Twenty-four hours post-seeding, cells were treated in duplicate with a serial dilution of degrader compounds dissolved in DMSO, achieving the desired final concentrations. Control wells were treated with an equivalent volume of DMSO. After a 24-hour treatment period, cells were harvested by centrifugation. The supernatant was discarded, and the cell pellets were washed once with 1x PBS. Lysis was performed by incubating the pellets in 50 µL of lysis buffer (200 mM HEPES, 0.25% SDS, pH 8.5) supplemented with 1x Halt protease inhibitor cocktail (Thermo Scientific, 78440) and 10 U of benzonase (Sigma, E1014) per well. The plate was shaken at 800 rpm for 30 minutes at 37°C. For disulfide bond reduction, 5 µL of 50 mM dithiothreitol (DTT) was added to each well, followed by a 15-minute incubation at 37°C with shaking. Subsequent alkylation of cysteine residues was achieved by adding 14 µL of 100 mM iodoacetamide and incubating for 30 minutes at room temperature in the dark.

Proteins were captured from the lysate using the Single-Pot, Solid-Phase-enhanced Sample Preparation (SP3) method. To each well, 100 µg of SP3 beads (a 1:1 mixture of E3 and E7 carboxylic magnetic beads from Cytiva) were added. Protein binding was induced by the addition of 75 µL of 100% ethanol, followed by a 10-minute incubation at room temperature with shaking. The beads were then washed twice with 200 µL of 80% ethanol using a magnetic rack to immobilize the beads during washing. After a brief drying period, the captured proteins were subjected to on-bead digestion. Thirty microliters of a 12.5 ng/µL Trypsin/LysC mix (Promega, V5073) in 200 mM HEPES buffer were added to the beads. The digestion proceeded for 18 hours at 37°C with shaking at 1,000 rpm in a sealed plate.

Following digestion, the resulting peptides were acidified with 1 µL of formic acid. The samples were then desalted and purified using StageTips (CDS Analytical, 6091). The tips were first activated with 80% acetonitrile (ACN)/0.1% formic acid and equilibrated with 0.1% formic acid. The acidified peptide digests were loaded onto the tips, which were subsequently washed twice with 0.1% formic acid. Peptides were eluted using 100 µL of 80% ACN/0.1% formic acid. The eluted peptides were dried in a speed vacuum concentrator and subsequently resuspended in 30 µL of 5% ACN/0.1% formic acid. Peptide concentration was quantified using a fluorescent peptide assay (Pierce, 23290), and all samples were normalized to a uniform concentration across the plate before injection for LC-MS/MS analysis.

All data were collected on a Vanquish Neo UHPLC system coupled to an Orbitrap Astral mass spectrometer through an EASY-Spray source (Thermo Fisher Scientific). Between 300 ng and 1 µg of peptides were loaded onto a 300 µm × 5 mm trap column (Thermo Scientific, 174500) and separated on a 150 µm × 15 cm C18 column (Thermo Scientific, ES906). Peptides were eluted using a 23-minute gradient from 5-40% ACN containing 0.1% FA.

MS1 full scans were acquired in the Orbitrap from 0 to 23 min at a resolution of 240,000 (at m/z 200) across a 380–980 m/z range, with a normalized AGC target of 500% (5×10^6). Concurrently, data-independent acquisition (DIA) MS2 scans were performed using the Astral detector. The precursor range of 380–980 m/z was covered by 300 sequential isolation windows of 2 Th width. MS2 scans were collected from 150–2000 m/z with a normalized HCD collision energy of 25% and a normalized AGC target of 500% (5×10^4), all within a 0.6 s cycle time.

Raw files were converted into the mzML format using ThermoRawFileParser (v1.4.5) and searched with DIA-NN (v1.9.2). A spectral library was generated *in silico* from the reviewed UniProtKB *Homo sapiens* proteome (UP000005640, downloaded on Jan 7, 2025) and was refined based on the DIA data. Search parameters included a precursor m/z range of 380−980, fragment m/z range of 200−2000, and peptide lengths of 6-40 amino acids. Trypsin/P digestion rules were applied, allowing up to one missed cleavage. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine (max 2) was set as a variable modification. Mass accuracy was set to 15 ppm for MS1 and 20 ppm for MS2 fragments.

Protein groups were quantified using QuantUMS in high-precision mode, and a false discovery rate (FDR) of 1% was applied at both the peptide and protein levels. After filtering for common contaminants, the protein group quantification report was used for differential analysis and statistics using the limma model in R (v4.4.2), with Benjamini-Hochberg correction to control the FDR.

Nanoluc-based endpoint and protein degradation assays.

For endpoint assays, BTK-HiBiT or BTK-nLuc knock-in Ramos cells were plated in white opaque 96-well plate (Thermo Fisher) at a density of 20,000 cells per well in either 100 µL of phenol red-free RPMI 1640 medium (Ramos cells) or 100 µL of phenol red-free DMEM medium (Hela cells), both supplemented with 10% FBS. After settling down overnight, cells were incubated with serially diluted BTK degrader compounds with the concentrations used in the text for 24 h. For degradation kinetics experiments, bioluminescence was measured after degrader incubation at different time points (1, 2, 4, 8, 24, 48 hours). Nano-Glo luciferase assay substrate was 1:100 diluted in 2× nLuc lytic buffer (100 mM MES pH 6.0, 1 mM CDTA, 0.5% (v/v) Tergitol, 0.05% (v/v) Antifoam 204, 150 mM KCl, 1 mM DTT, and 35 mM thiourea) containing 200 nM LgBiT protein to make 2× nLuc substrate lytic buffer. 100 µL 2× buffer was subsequently added into each 96-well. After brief shaking and waiting 5 min for complete cell lysis and equilibration, the luminescence signals were collected.

In vivo PD assay.

Animal studies were conducted under protocol AN-6075, which was approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine and adhered to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Six-week-old female Balb/c mice (Jackson Laboratory) were used in all experiments and age-matched for consistency. Female mice were selected for their balanced representation and stable exploratory behavior. Mice were housed in the TMF Mouse Facility at Baylor College of Medicine under specific pathogen-free (SPF) conditions, with climate control and a 12-hour light/dark cycle. Fresh chow and water were provided ad libitum via an automated water system.

Following a one-week acclimation period, Balb/c mice (n=4) were administered either PSIRC3 (formulated in 30% PEG-400, 5% Tween-80, and 5% DMSO in deionized water) at a dose of 15 mg/kg or a corresponding vehicle via intravenous injection. Four hours post-administration, mice were sacrificed, and peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1083 density gradient medium (Sigma, Cat. No. 10831). Splenocytes were harvested using the Spleen Dissociation Kit (Miltenyi Biotec, Cat. No. 130-095-926). Both PBMCs and splenocytes were subsequently subjected to immunoblotting to assess BTK protein levels.

NanoBRET in-cell target engagement assay.

The target engagement assay for BTK was from Promega (BTK kit, Cat. No. N2500; CRBN kit, Cat. No. CS1810C136) and was performed according to the manufacturer's instructions with some modifications. Briefly, BTK-nLuc Ramos cells were resuspended in Opti-MEM medium (Life Technologies, Cat. No. 11058021) at the density of 2×10^5 cells per mL and were plated into 96-well plates (Corning, Cat. No. CLS3600). Cells were incubated with 1.0 μM (BTK) NanoBRET™ Tracer, the 3X Nano-Glo® Substrate with NanoLuc® extracellular inhibitor were added to cells and developed for 3 min at room temperature. then serially diluted unlabeled BTK PROTAC compounds were added with Pico 8, BRET signals were collected using a PheraStar microplate reader.

Ternary complex formation induced by PSIRC3.

The assay was performed in 10 μL assay buffer (50 mM Tris, pH7.5, 0.1% Triton X-100, 0.01% BSA, and 1 mM DTT) with 0.3 nM (400X from stock solution) Tb-anti-MBP (Revvity, Cat.No. 61HI2TLB), 5 nM MBP-BTK, 100 nM CRBN/His-DDB1ΔB and 200nM HIS Lite™ OG488-Tris NTA-Ni complex (AAT Bioquest, Cat No. 12615), serial diluted compounds (from 100 uM, 2-fold dilutions) in opaque 384 well plates： Incubated 5 nM MBP-BTK full length protein with Tb-anti-MBP for 30 minutes, incubated CRBN/His-DDB1ΔB with Tris-NTA-Ni for 30 minutes. After incubation, the two labeled protein solutions were mixed, and a mixture (10 μL/well) of the reagents was dispensed into a 384-well plate, then compound was dispensed with Pico 8. The assay mixtures were incubated at room temperature in dark for 3 min, unless specified otherwise, and the signals were collected using

a PheraStar microplate reader to measure the fluorescence emission ratio (F520 nm/F490 nm) of each well every 6 min. Raw data from the plate reader at 1h were used directly for analysis. The curve-fitting software GraphPad Prism 10 was used to generate graphs and curves.

MATLAB simulation

MATLAB (version R2022b) and the SimBiology package (v6.4) were used to build and simulate the PROTAC degradation model as shown in Fig.8A and 8B.

The covalent PROTAC degradation model was built based on the following 13 ordinary differential equations (ODEs):

$$d(T)/dt = -(k\_TP\_F * T * P - k\_TP\_R * TP) - ([k\_T - PL\_F] * PL * T - [k\_T - PL\_R] * TPL) \quad (1)$$

$$d(L)/dt = -(k\_PL\_F * P * L - k\_PL\_R * PL) - ([k\_TP - L\_F] * TP * L - [k\_TP - L\_R] * TPL) - ([k\_TP - L\_F] * [TP *] * L - [k\_TP - L\_R] * [TP * L]) + ([k\_TubP - L\_R] * TubPL - [k\_TP - L\_F] * TubP * L) + ([k\_TubP - L\_R] * [TubP * L] - [k\_TP - L\_F] * [TubP *] * L) \quad (2)$$

$$d(TP)/dt = (k\_TP\_F * T * P - k\_TP\_R * TP) - ([k\_TP - L\_F] * TP * L - [k\_TP - L\_R] * TPL) - (kinact * TP) \quad (3)$$

$$d(PL)/dt = (k\_PL\_F * P * L - k\_PL\_R * PL) - ([k\_T - PL\_F] * PL * T - [k\_T - PL\_R] * TPL) + ([k\_Tub - PL\_R\ ] * TubPL - [k\_T - PL\_F\ ] * Tub * PL) \quad (4)$$

$$d(TPL)/dt = ([k\_TP - L\_F] * TP * L - [k\_TP - L\_R] * TPL) + ([k\_T - PL\_F] * PL * T - [k\_T - PL\_R] * TPL) - (kinact * TPL) - (k\_ub * TPL) \quad (5)$$

$$d([TP *])/dt = (kinact * TP) - ([k\_TP - L\_F\ ] * [TP *] * L - [k\_TP - L\_R\ ] * [TP * L]) \quad (6)$$

$$d([TP * L])/dt = ([k\_TP - L\_F] * [TP *] * L - [k\_TP - L\_R] * [TP * L]) + (kinact * TPL) - (k\_ub * [TP * L]) \quad (7)$$

$$d([TubP * L])/dt = (k\_ub * [TP * L]) - ([k\_TubP - L\_R] * [TubP * L] - [k\_TP - L\_F] * [TubP *] * L) + (kinact * TubPL) \quad (8)$$

$$d(TubPL)/dt = (k\_ub * TPL) - ([k\_TubP - L\_R] * TubPL - [k\_TP - L\_F] * TubP * L) - ([k\_Tub - PL\_R] * TubPL - [k\_T - PL\_F] * Tub * PL) - (kinact * TubPL) \quad (9)$$

$$d(TubP)/dt = ([k\_TubP - L\_R] * TubPL - [k\_TP - L\_F] * TubP * L) - (k\_TP\_R * TubP - k\_TP\_F * Tub * P) - (k\_deg * TubP) \quad (10)$$

$$d(Tub)/dt = ([k\_Tub - PL\_R] * TubPL - [k\_T - PL\_F] * Tub * PL) + (k\_TP\_R * TubP - k\_TP\_F * Tub * P) - (k\_deg * Tub) \quad (11)$$

$$d([TubP *])/dt = ([k\_TubP - L\_R] * [TubP * L] - [k\_TP - L\_F] * [TubP *] * L) - (k\_deg * [TubP *]) \quad (12)$$

$$d(Tdeg)/dt = (k\_deg * [TubP *]) + (k\_deg * TubP) + (k\_deg * Tub) \quad (13)$$

… and the following 6 repeated assignments:

$$[k\_TP - L\_R\ ] = K\_PL * [k\_TP - L\_F\ ]/\ alpha \quad (14)$$

$$[k\_TubP - L\_R] = destab * [k\_TP - L\_R] \quad (15)$$

$$[k\_T - PL\_R] = K\_TP * [k\_T - PL\_F] / alpha \quad (16)$$

$$[k\_Tub - PL\_R] = destab * [k\_T - PL\_R] (17)$$

$$k\_PL\_R = K\_PL * k\_PL\_F (18)$$

$$k\_TP\_R = K\_TP * k\_TP\_F (19)$$

The non-covalent PROTAC degradation model was built based on the following 9 ordinary differential equations (ODEs):

$$d(T)/dt = -(k\_TP\_F * T * P - k\_TP\_R * TP) - ([k\_T - PL\_F] * PL * T - [k\_T - PL\_R] * TPL) (20)$$

$$d(L)/dt = -(k\_PL\_F * P * L - k\_PL\_R * PL) - ([k\_TP - L\_F] * TP * L - [k\_TP - L\_R] * TPL) + ([k\_TubP - L\_R] * TubPL - [k\_TP - L\_F] * TubP * L) (21)$$

$$d(TP)/dt = (k\_TP\_F * T * P - k\_TP\_R * TP) - ([k\_TP - L\_F] * TP * L - [k\_TP - L\_R] * TPL) (22)$$

$$d(PL)/dt = (k\_PL\_F * P * L - k\_PL\_R * PL) - ([k\_T - PL\_F] * PL * T - [k\_T - PL\_R] * TPL) + ([k\_Tub - PL\_R] * TubPL - [k\_T - PL\_F] * Tub * PL) (23)$$

$$d(TPL)/dt = ([k\_TP - L\_F] * TP * L - [k\_TP - L\_R] * TPL) + ([k\_T - PL\_F] * PL * T - [k\_T - PL\_R] * TPL) - (k\_ub * TPL) (24)$$

$$d(TubPL)/dt = (k\_ub * TPL) - ([k\_TubP - L\_R] * TubPL - [k\_TP - L\_F] * TubP * L) - ([k\_Tub - PL\_R] * TubPL - [k\_T - PL\_F] * Tub * PL) (25)$$

$$d(TubP)/dt = ([k\_TubP - L\_R] * TubPL - [k\_TP - L\_F] * TubP * L) - (k\_TP\_R * TubP - k\_TP\_F * Tub * P) - (k\_deg * TubP) (26)$$

$$d(Tub)/dt = ([k\_Tub - PL\_R] * TubPL - [k\_T - PL\_F] * Tub * PL) + (k\_TP\_R * TubP - k\_TP\_F * Tub * P) - (k\_deg * Tub) (27)$$

$$d(Tdeg)/dt = (k\_deg * TubP) + (k\_deg * Tub) (28)$$

… and the following 6 repeated assignments:

$$[k\_TP - L\_R] = K\_PL * [k\_TP - L\_F] / alpha (29)$$

$$[k\_TubP - L\_R] = destab * [k\_TP - L\_R] (30)$$

$$[k\_T - PL\_R] = K\_TP * [k\_T - PL\_F] / alpha (31)$$

$$[k\_Tub - PL\_R] = destab * [k\_T - PL\_R] (32)$$

$$k\_PL\_R = K\_PL * k\_PL\_F (33)$$

$$k\_TP\_R = K\_TP * k\_TP\_F (34)$$

Unless being scanned, the initial values of each species are set as below:

$T = 1e-7\ M,\ P = 1e-9\ M,\ L = 3e-8\ M$. All other species' initial values are 0.

Unless being scanned, the constant values are set as below:

$$kinact = 0.01\ s^{-1}, k\_ub = 0.01\ s^{-1}, k\_deg = 0.016\ s^{-1}, alpha = 1,$$

$$TP = 1e-5\ M, K\_PL = 1e-7\ M, destab = 1000$$

All the forward reaction velocity constants (k_XXX_F) are set as 1e5. The reverse reaction velocity constants (k_XXX_R) are determined by repeated assignment described above.

The simulation stop time was set at 86,400 seconds. ODE15s solver was used for all the simulations.

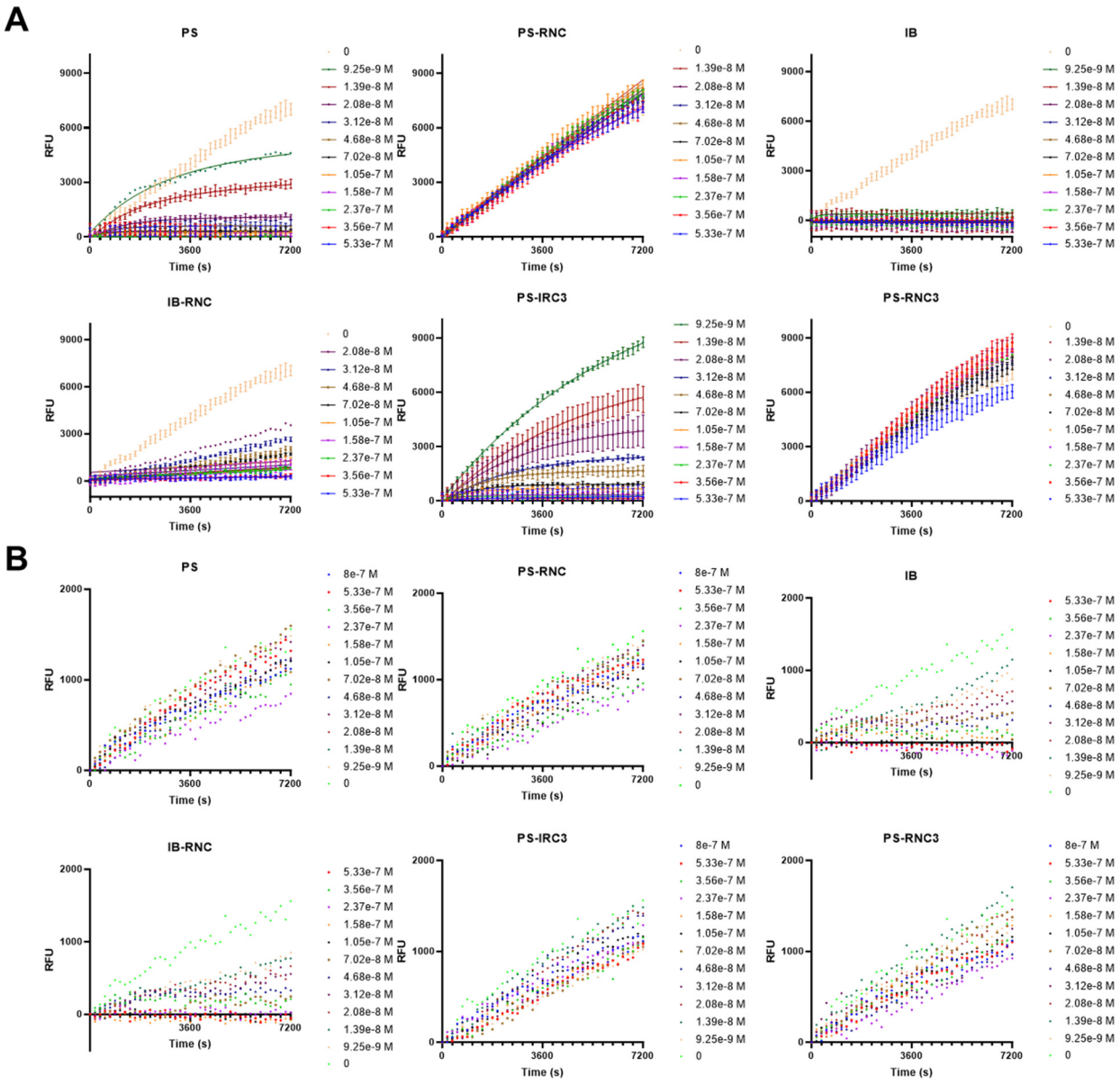


Figure S1. BTK Kinase Activity Assay Confirmed Poseltinib Series are Covalency-driven.

The biochemical WT-BTK and C481S-BTK inhibition (BTK Inhibition IC50) was measured using the kinase assay kit from AssayQuant Technologies Inc. (A) WT-BTK, (B) C481S-BTK.

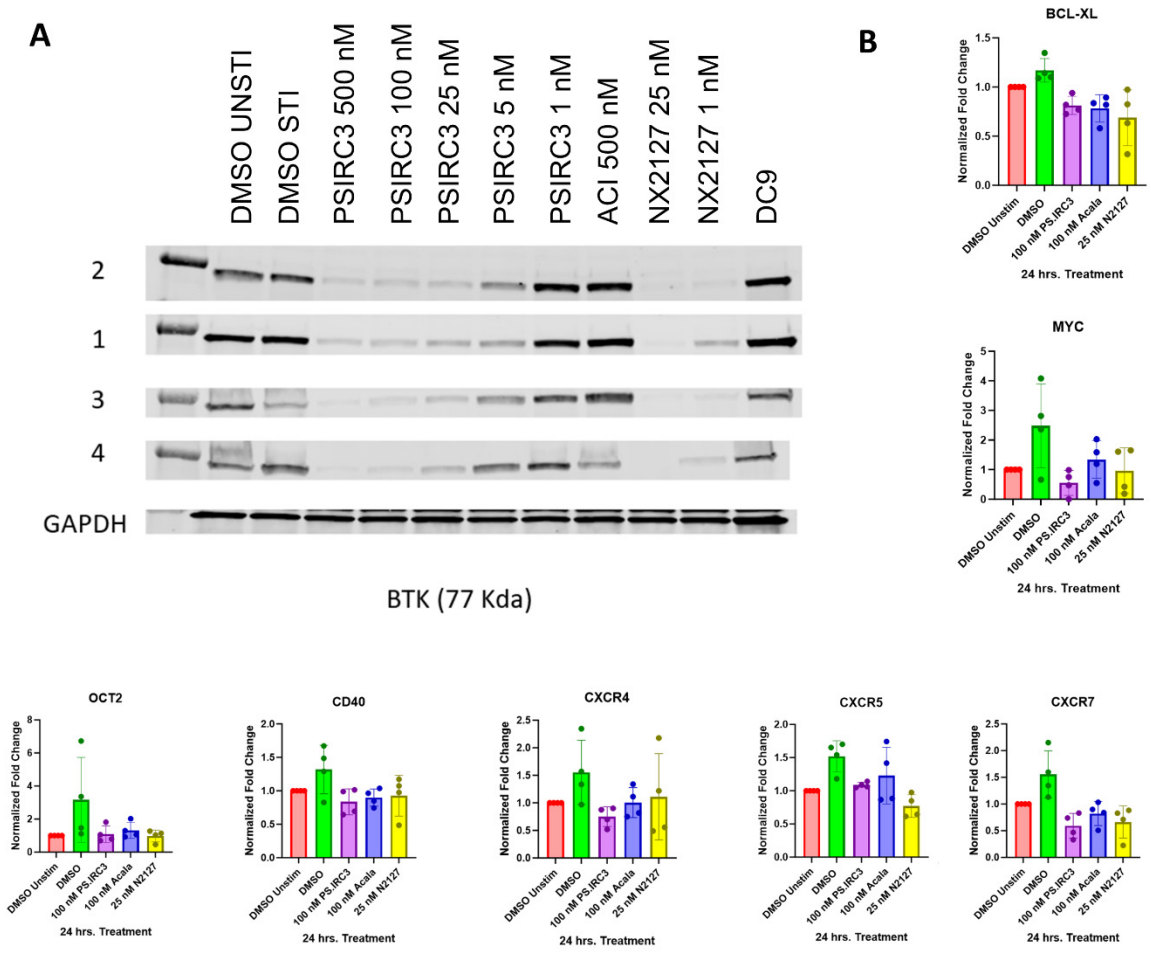


Figure S2. Characterization of BTK Degraders in CLL patient samples.

(A). PSIRC3 induced degradation of BTK BCR in CLL patient B cells (n=4) analyzed by western blot. **(B).** qRT-PCR analysis of BTK downstream signaling genes of CLL patient B cells (n=4) treated with PSIRC3 for 24 hours with Acalabrutinib and NX-2127 as positive controls.

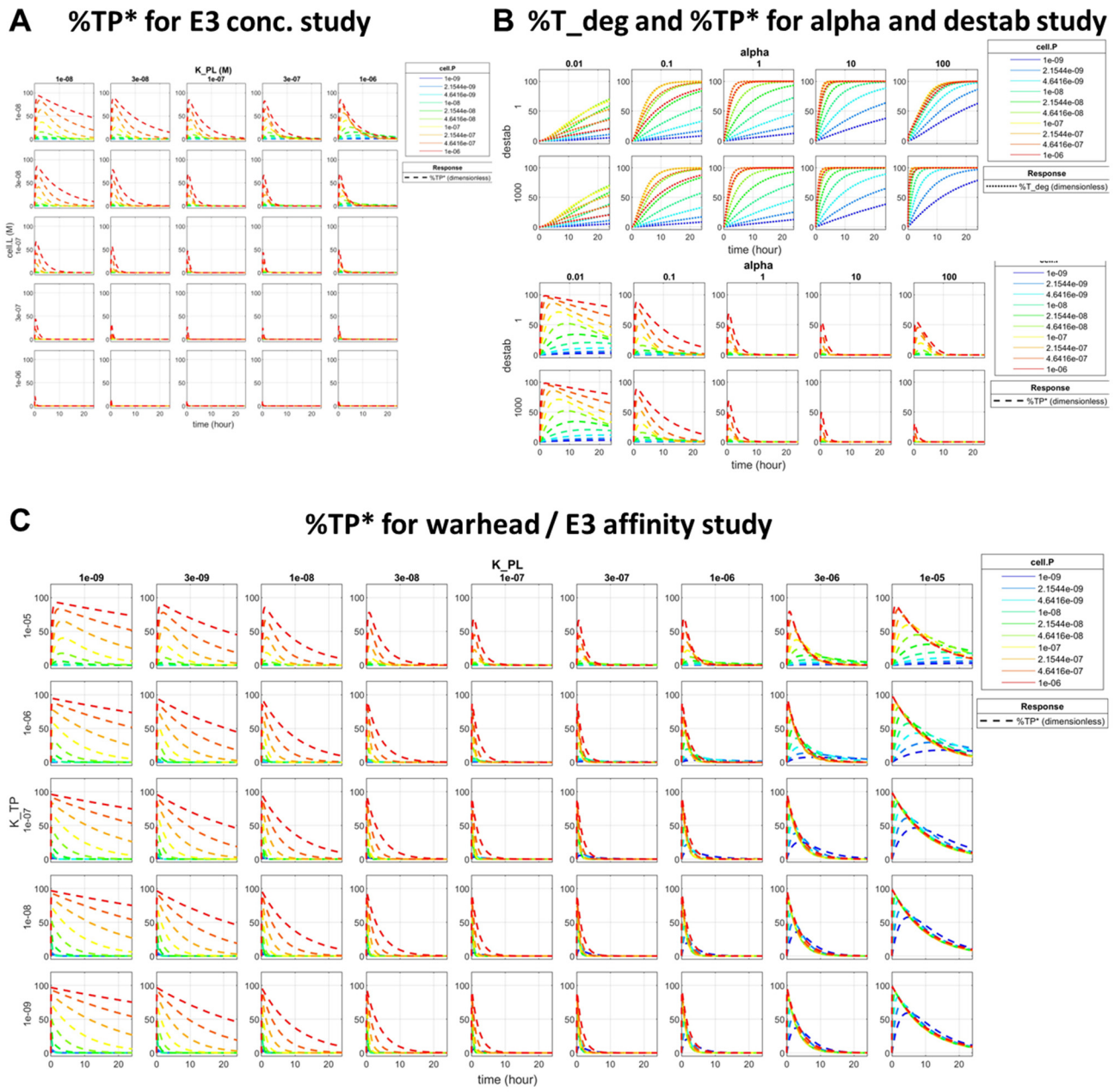


Figure S3. SimBio Modeling of Covalent PROTAC Degradation Event.

(A) The percentage of TP* (%TP*) associated with the experiment in Figure 8D. **(B)** The time-course simulation of %T_deg and %TP* with various alpha and destab values. **(C)** The percentage of TP* (%TP*) associated with the experiment in Figure 8C.

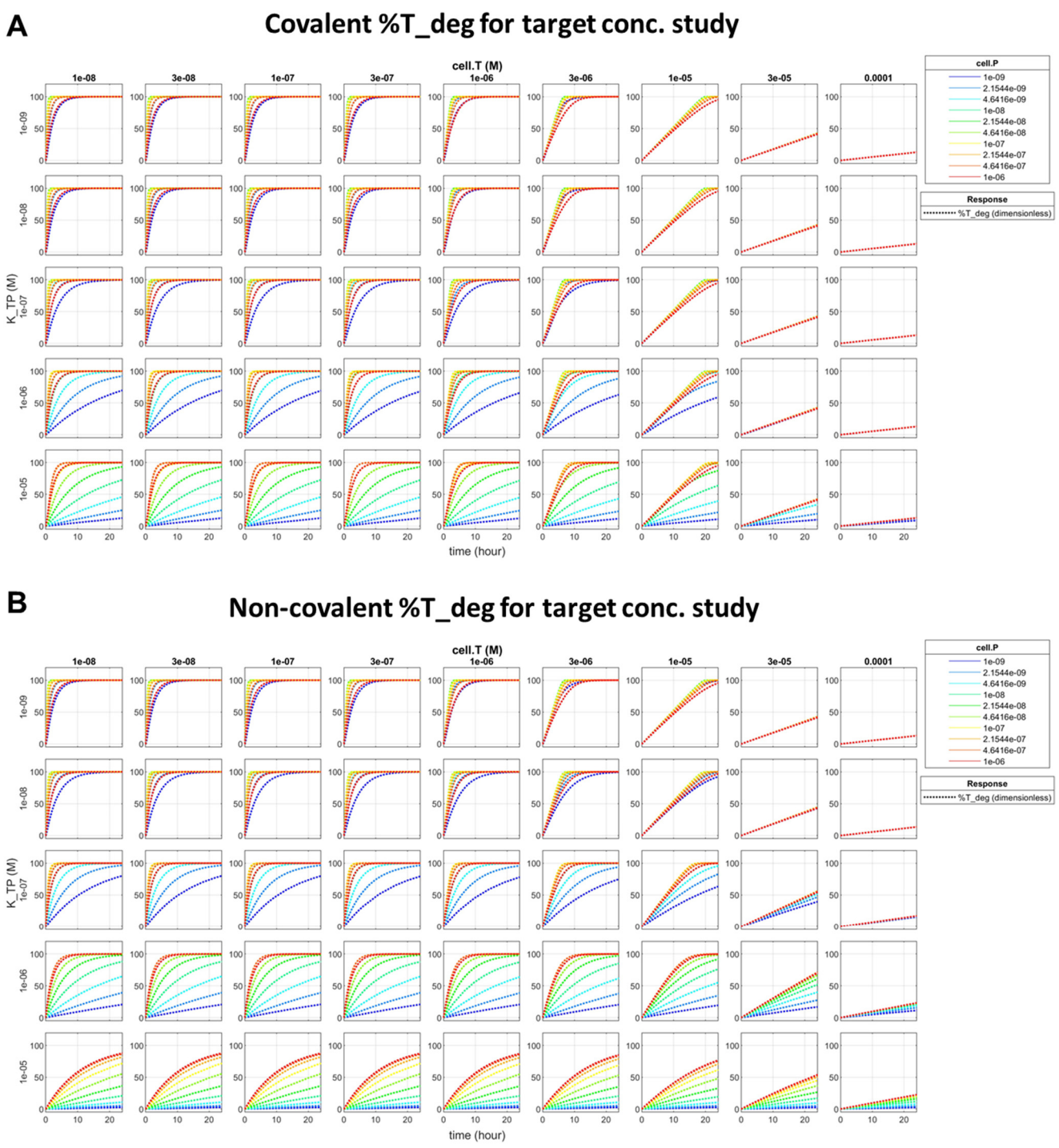
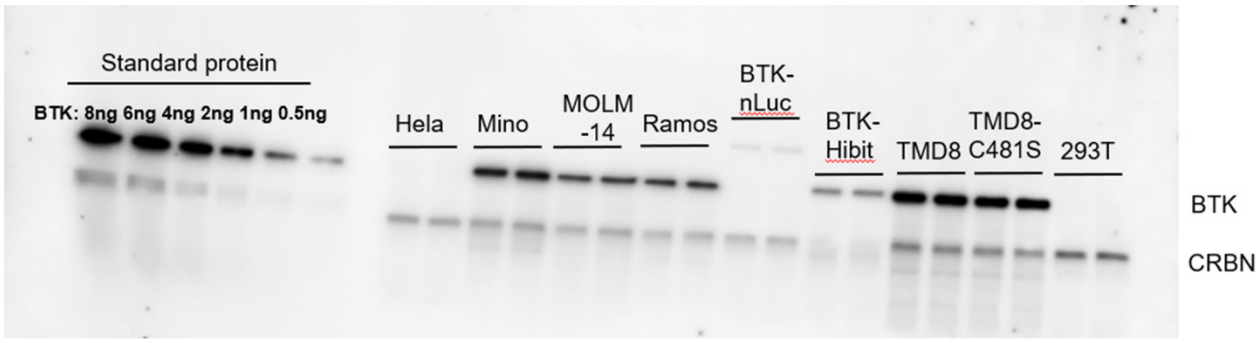


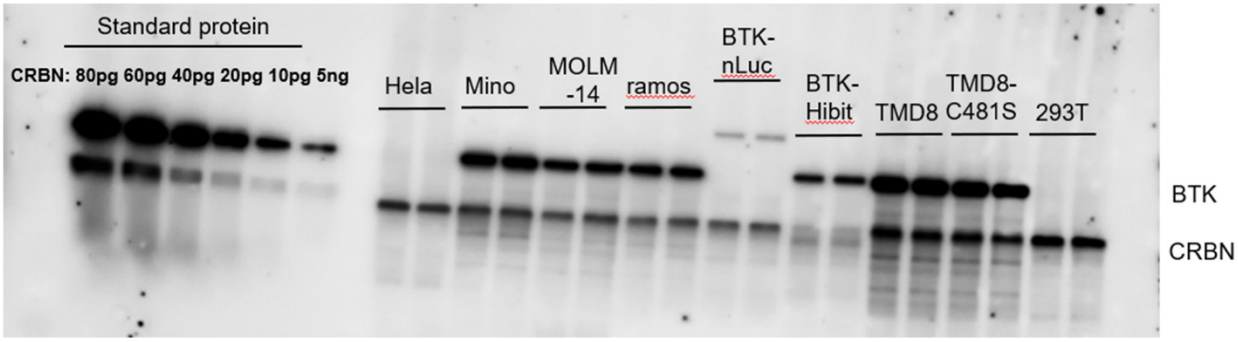
Figure S4. SimBio Modeling of Covalent or Non-covalent PROTAC Degradation Event.

The percentage of degraded target protein (%T_deg) with various concentrations of target protein (T) in **(A)** covalent or **(B)** non-covalent PROTAC model

A



B



C

	1M cell BTK amount/ng	1M cell CRBN amount/pg
HEK-293T	0	741
Hela	0	519
Mino	3.5	496
MOLM-14	2.4	455
Ramos	2.3	434
TMD8	3.9	724

Figure S5. Quantification of BTK and CRBN Protein Levels Across Cell Lines.

Quantitative Western blot analysis to determine the endogenous concentrations of BTK and CRBN. **(A)** BTK quantification using a 30s exposure against a standard protein curve. **(B)** CRBN quantification using a 2min exposure. **(C)** Table summarizing the absolute amounts of BTK (ng) and CRBN (pg) per 10^6 cells across various leukemia and solid tumor cell lines.

Data Availability

The mass spectrometry raw files for DIA proteomics have been deposited in the MassIVE dataset under accession number MSV000099557.

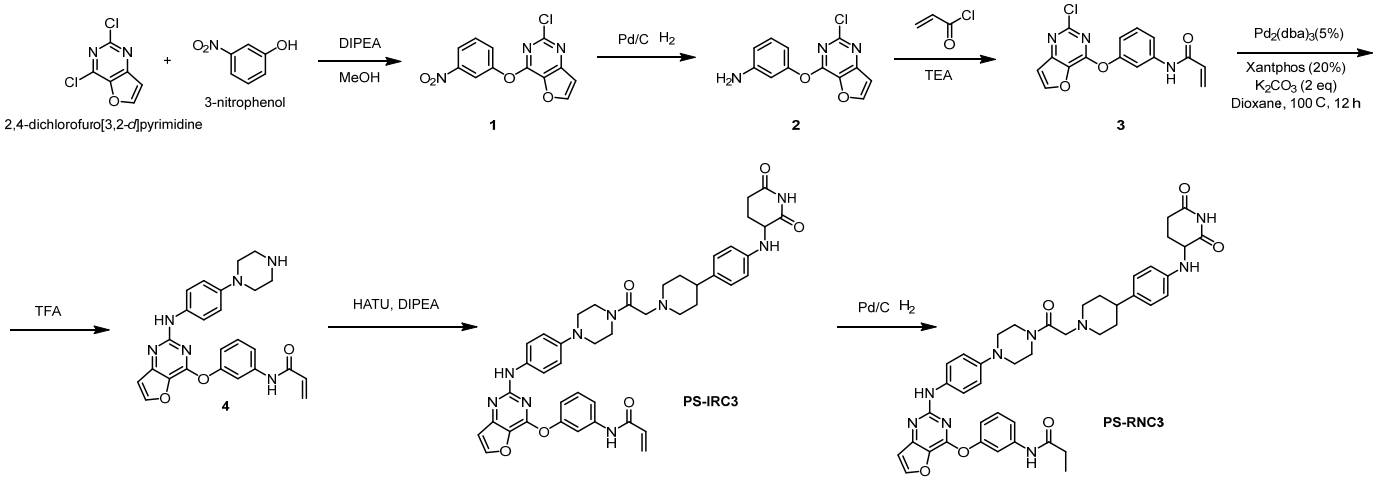
[https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?accession=MSV000099557].

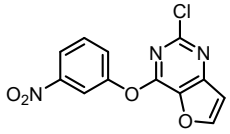
Chemistry

General Information: ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA) 600-MR spectrometer and are calibrated using residual undertreated solvent. Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are in Hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Flash chromatography was performed on a Smart Flash EPCLC W-Prep 2XY. ESI mass spectrometry was measured on an Agilent Mass Spectrometer.

Materials: All reagents were used as received from commercial sources without further purification, unless specified otherwise, or prepared as described in the literature.

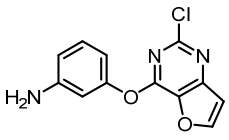
Compound Synthesis and Characterization



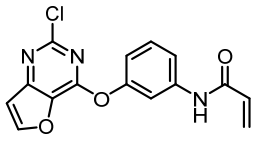


2-chloro-4-(3-nitrophenoxy)furo[3,2-d]pyrimidine (1) To a solution of 2,4-dichlorofuro[3,2-d]pyrimidine (10 g, 40.01 mmol, 1 *eq*) and 3-nitrophenol (1.67 g, 12 mmol 1.2 *eq*) in MeOH (80 mL) was added DIPEA (3.87 g, 3 eq), the mixture was stirred at 25°C for 12 hrs. The reaction mixture was added H_2O (100 mL) and then extracted with EtOAc (100 mL * 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (PE:EtOAc = 1:0 to 5:1). 2-chloro-4-(3-nitrophenoxy)furo[3,2-d]pyrimidine (2.35 g, 81% yield) was obtained as white solid which was confirmed by H NMR, C NMR and LC-MS. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.53 (d, *J* = 2.3 Hz, 1H), 7.21 (d, *J* = 2.2 Hz, 1H), 7.09 (t, *J* = 8.0 Hz, 1H), 6.56 – 6.34 (m, 3H). ^{13}C NMR (151 MHz, DMSO-

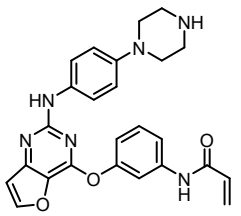
d_6) δ 157.03, 154.83, 154.08, 152.91, 152.48, 150.86, 133.61, 130.43, 112.31, 108.49, 107.86, 106.67. MS (ESI): m/z (%) 292.2 (M+H).



3-((2-chlorofuro[3,2-d]pyrimidin-4-yl)oxy)aniline (2) To a solution of 2-chloro-4-(3-nitrophenoxy)furo[3,2-d]pyrimidine (1.45 g, 5 mmol) in MeOH (120 mL) was added 10% Pd/C (40 mg), the mixture was stirred at room temperature for 12 hrs at 1 atm. H_2. When LC-MS indicated all starting material was converted to product, the reaction mixture was filtered and concentrated under reduced pressure. The residue was used without purification. 3-((2-chlorofuro[3,2-d]pyrimidin-4-yl)oxy)aniline (1.2 g, 92% yield) was obtained as light yellow solid which was confirmed by LC-MS. MS (ESI): m/z (%) 262.2 (M+H).

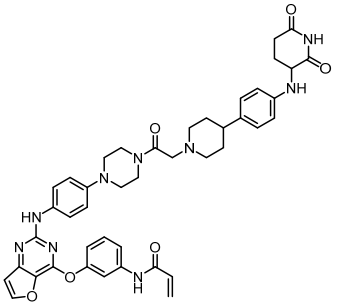


N-(3-((2-chlorofuro[3,2-d]pyrimidin-4-yl)oxy)phenyl)acrylamide (3) To a solution of 3 mmol of 3-((2-chlorofuro[3,2-d]pyrimidin-4-yl)oxy)aniline in anhydrous dichloromethane (DCM) was added triethylamine (3 equiv). The reaction mixture was cooled to 0 °C in an ice bath, followed by dropwise addition of acryloyl chloride (3.6 mmol, 1.2 equiv). The mixture was then allowed to warm to room temperature and stirred overnight. The reaction mixture was added H_2O (100 mL) and then extracted with EtOAc (100 mL * 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (PE:EtOAc = 1:0 to 5:1). N-(3-((2-chlorofuro[3,2-d]pyrimidin-4-yl)oxy)phenyl)acrylamide (711 mg, 75% yield) was obtained as white solid which was confirmed by H NMR, C NMR and LC-MS. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.43 (s, 1H), 8.59 (d, J = 2.2 Hz, 1H), 7.79 (t, J = 2.2 Hz, 1H), 7.58 – 7.53 (m, 1H), 7.46 (t, J = 8.1 Hz, 1H), 7.26 (d, J = 2.2 Hz, 1H), 7.09 (dd, J = 8.1, 2.3 Hz, 1H), 6.45 (dd, J = 17.0, 10.2 Hz, 1H), 6.28 (dd, J = 17.0, 1.9 Hz, 1H), 5.79 (dd, J = 10.2, 1.9 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.92, 157.26, 155.04, 153.85, 152.34, 151.99, 140.86, 133.55, 132.04, 130.53, 127.90, 117.59, 116.99, 112.76, 107.91. MS (ESI): m/z (%) 316.1 (M+H).

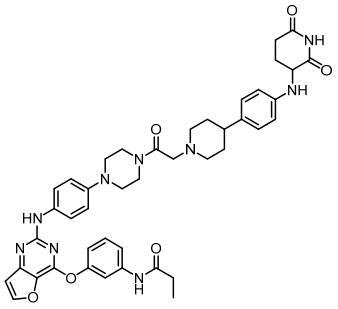


N-(3-((2-((4-(piperazin-1-yl)phenyl)amino)furo[3,2-d]pyrimidin-4-yl)oxy)phenyl)acrylamide (5) Compound **3** (2 mmol) and tert-butyl 4-(4-aminophenyl)piperazine-1-carboxylate (2.2 mmol) were dissolved in 2-Methyl-1-propanol (10 mL), 1-2 drops of trifluoroacetic acid was added. The mixture was heated to 90°C. After 12 hours, the mixture was cooled to room temperature and concentrated under reduced. The residue was purified with silica gel chromatography (DCM/Methanol = 20:1) to give crude

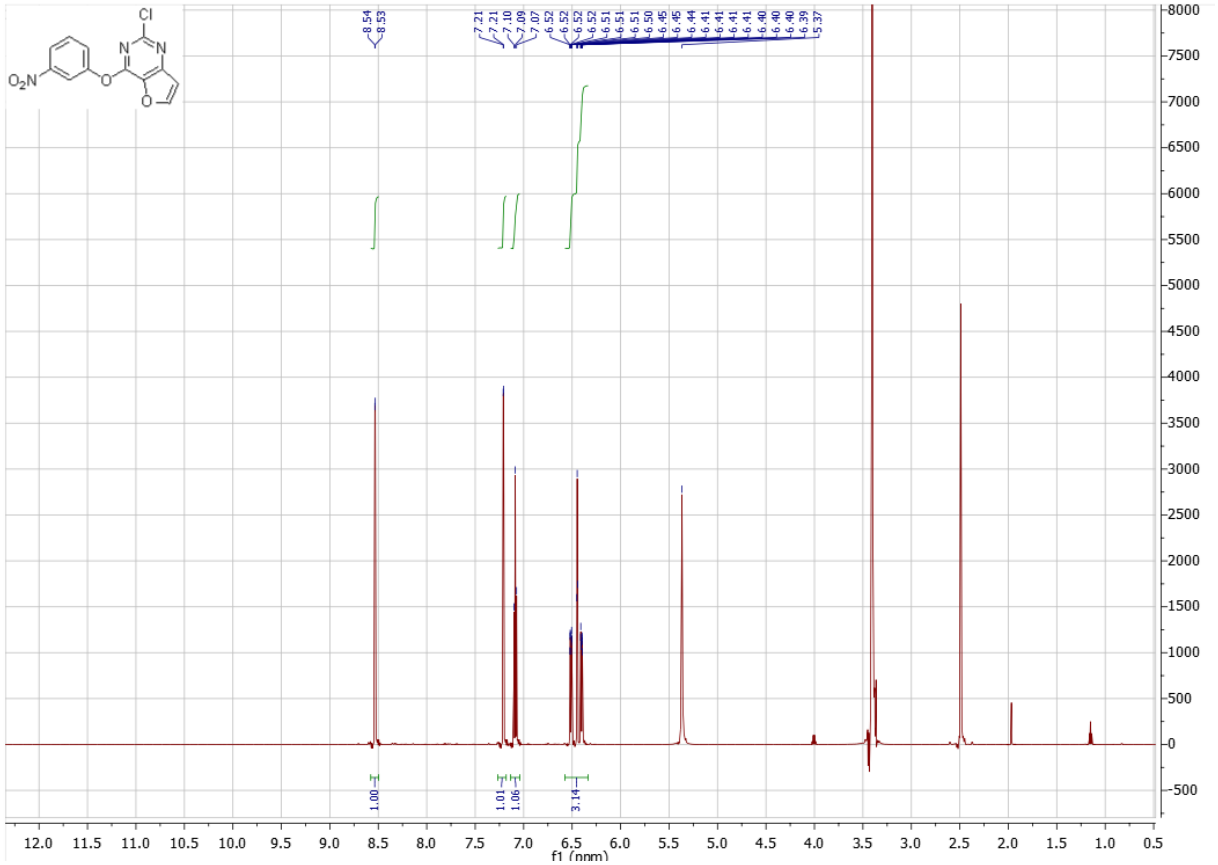
product. The crude product was stirred in 10% triacetic acid DCM solution for 30 minutes. After 30mins, the mixture concentrated under reduced. The residue was purified with silica gel chromatography (DCM/Methanol = 20:1 to 10:1). N-(3-((2-((4-(piperazin-1-yl)phenyl)amino)furo[3,2-d]pyrimidin-4-yl)oxy)phenyl)acrylamide (510 mg, 56% yield) was obtained as white solid which was confirmed by H NMR, fand LC-MS. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.43 (s, 1H), 9.17 (s, 1H), 8.77 (br, 1H), 8.29 (d, *J* = 2.2 Hz, 1H), 7.70 – 7.60 (m, 2H), 7.51 – 7.39 (m, 3H), 7.06 (ddd, *J* = 8.1, 2.4, 0.9 Hz, 1H), 6.94 (d, *J* = 2.2 Hz, 1H), 6.76 (d, *J* = 8.6 Hz, 2H), 6.44 (dd, *J* = 17.0, 10.2 Hz, 1H), 6.27 (dd, *J* = 17.0, 1.9 Hz, 1H), 5.79 (dd, *J* = 10.2, 1.9 Hz, 1H), 3.26 – 3.15 (m, 9H). MS (ESI): m/z (%) 457.1 (M+H).



N-(3-((2-((4-(4-(2-(4-(4-((2,6-dioxopiperidin-3-yl)amino)phenyl)piperidin-1-yl)acetyl)piperazin-1-yl)phenyl)amino)furo[3,2-d]pyrimidin-4-yl)oxy)phenyl)acrylamide (PSIRC3) In a 25 mL flask was added compound **5** (0.01 mmol), then 2-(4-(4-((2,6-dioxopiperidin-3-yl)amino)phenyl)piperidin-1-yl)acetic acid (0.015 mol), HATU (5.7 mg, 0.015 mmol) and DIPEA (6.4 mg, 0.05 mmol) and DMF (2 mL) was added. The mixture was stirred at room temperature for 4h. Then the reaction mixture was concentrated in vacuo and the residue was purified by prepHPLC with a reverse phase C18 column to afford the product. PSIRC3 (6.2 mg, 84% yield) was obtained as white solid which was confirmed by H NMR, C NMR and LC-MS. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.76 (s, 1H), 10.40 (s, 1H), 9.13 (s, 1H), 8.29 (d, *J* = 2.2 Hz, 1H), 7.71 – 7.60 (m, 2H), 7.45 (td, *J* = 8.1, 4.8 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 2H), 7.05 (ddd, *J* = 8.1, 2.4, 1.0 Hz, 1H), 6.98 – 6.90 (m, 3H), 6.73 (d, *J* = 8.6 Hz, 2H), 6.63 – 6.57 (m, 2H), 6.44 (dd, *J* = 16.9, 10.2 Hz, 1H), 6.27 (dd, *J* = 17.0, 1.9 Hz, 1H), 5.77 (dd, *J* = 10.2, 1.9 Hz, 1H), 5.62 (d, *J* = 7.5 Hz, 1H), 4.25 (ddd, *J* = 11.9, 7.5, 4.8 Hz, 1H), 3.71 (t, *J* = 5.1 Hz, 2H), 3.34 (d, *J* = 38.9 Hz, 3H), 3.08 – 2.87 (m, 8H), 2.73 (td, *J* = 12.1, 6.1 Hz, 1H), 2.64 – 2.54 (m, 2H), 2.44 – 2.25 (m, 2H), 2.17 – 1.99 (m, 3H), 1.96 – 1.75 (m, 1H), 1.69 (d, *J* = 11.3 Hz, 1H), 1.56 (qd, *J* = 12.5, 3.7 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 174.24, 173.65, 168.26, 163.87, 156.70, 156.63, 153.41, 153.09, 152.67, 146.44, 146.01, 140.72, 134.59, 133.99, 132.05, 130.47, 127.87, 127.40, 120.07, 117.31, 117.08, 116.80, 113.19, 107.38, 61.74, 54.15, 53.09, 50.55, 41.60, 40.95, 33.91, 31.15, 25.19. MS (ESI): m/z (%) 784.2 (M+H).



N-(3-((2-((4-(4-(2-(4-(4-((2,6-dioxopiperidin-3-yl)amino)phenyl)piperidin-1-yl)acetyl)piperazin-1-yl)phenyl)amino)furo[3,2-d]pyrimidin-4-yl)oxy)phenyl)propio-namide (PSRNC3) In a 25 mL flask, PSIRC3 (0.01 mmol), in MeOH (120 mL) was added 10% Pd/C (40 mg), the mixture was stirred at room temperature for 12 hrs at 1 atm. H_2. When LC-MS indicated all starting material was converted to product, the reaction mixture was filtered and concentrated under reduced pressure. PSRNC3 (5.9 mg,95% yield) was obtained as white solid which was confirmed by H NMR and LC-MS, From the H spectrum, all alkenyl groups are converted into ethyl groups. ^{1}H ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.76 (s, 1H), 10.10 (s, 1H), 9.13 (s, 1H), 8.28 (d, *J* = 2.2 Hz, 1H), 7.60 (t, *J* = 2.2 Hz, 1H), 7.57 – 7.54 (m, 1H), 7.40 (q, *J* = 8.5 Hz, 3H), 7.00 (dd, *J* = 8.2, 2.4 Hz, 1H), 6.98 – 6.90 (m, 3H), 6.74 (d, *J* = 8.7 Hz, 2H), 6.65 – 6.57 (m, 2H), 5.62 (d, *J* = 7.5 Hz, 1H), 4.25 (ddd, *J* = 11.9, 7.4, 4.8 Hz, 1H), 3.04 (t, *J* = 5.0 Hz, 2H), 2.99 – 2.86 (m, 4H), 2.73 (ddd, *J* = 17.5, 12.1, 5.4 Hz, 1H), 2.59 (t, *J* = 4.3 Hz, 1H), 2.57 – 2.54 (m, 0H), 2.39 (p, *J* = 1.9 Hz, 1H), 2.32 (q, *J* = 7.5 Hz, 2H), 2.09 (ddd, *J* = 13.7, 9.3, 3.1 Hz, 3H), 1.85 (s, 0H), 1.69 (d, *J* = 10.8 Hz, 2H), 1.62 – 1.51 (m, 2H), 1.07 (t, *J* = 7.6 Hz, 3H). MS (ESI): m/z (%) 786.2 (M+H).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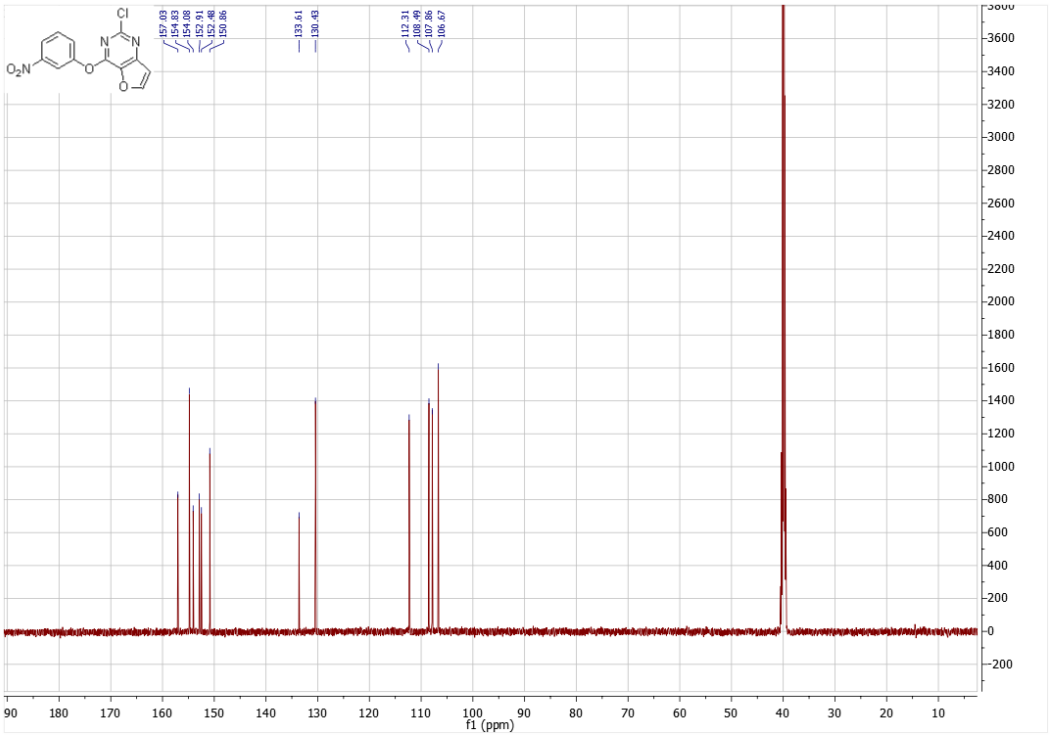
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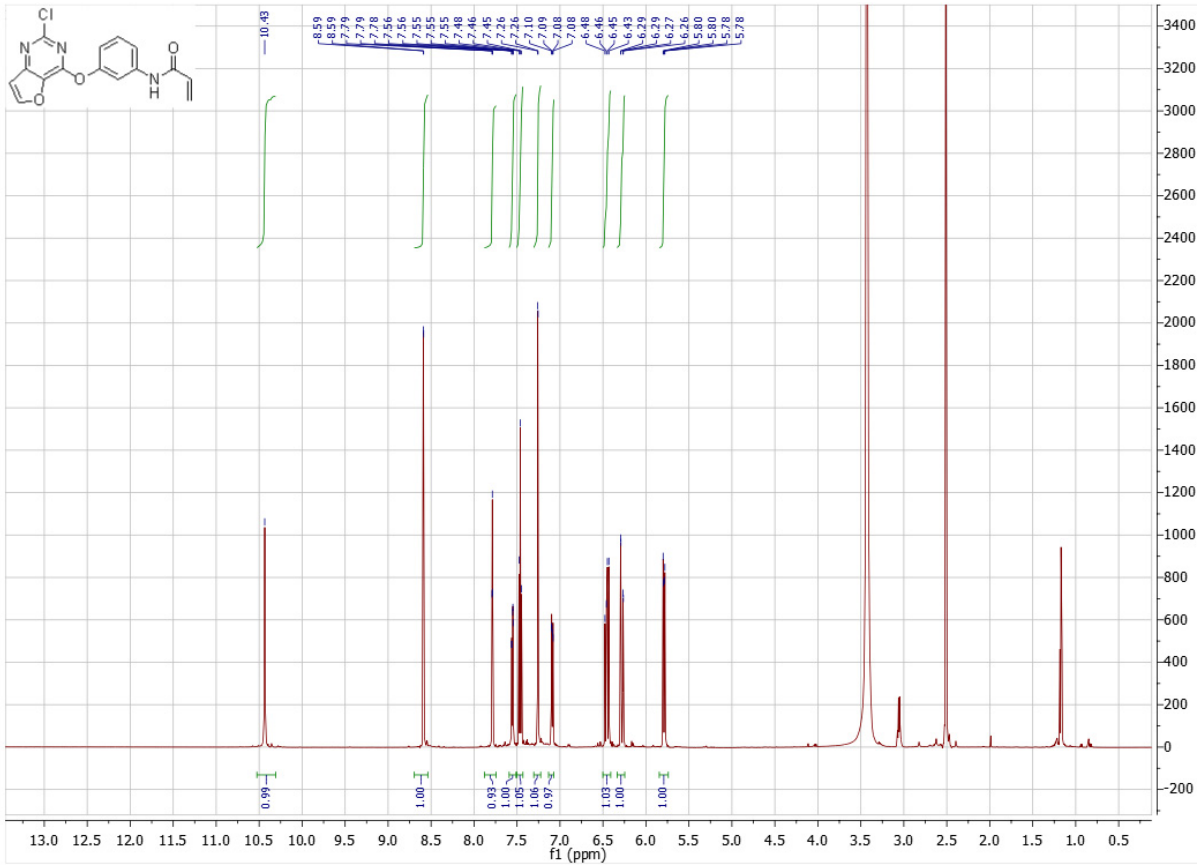
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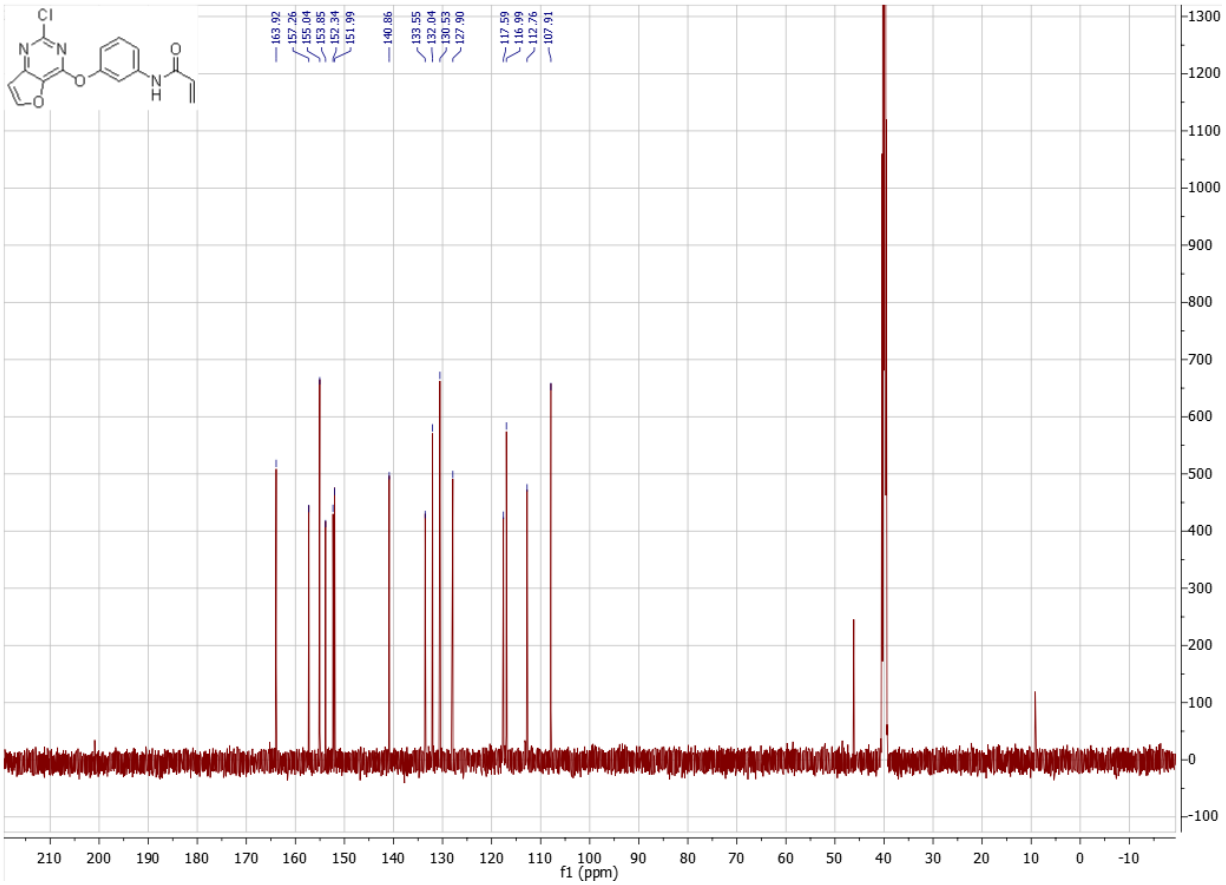
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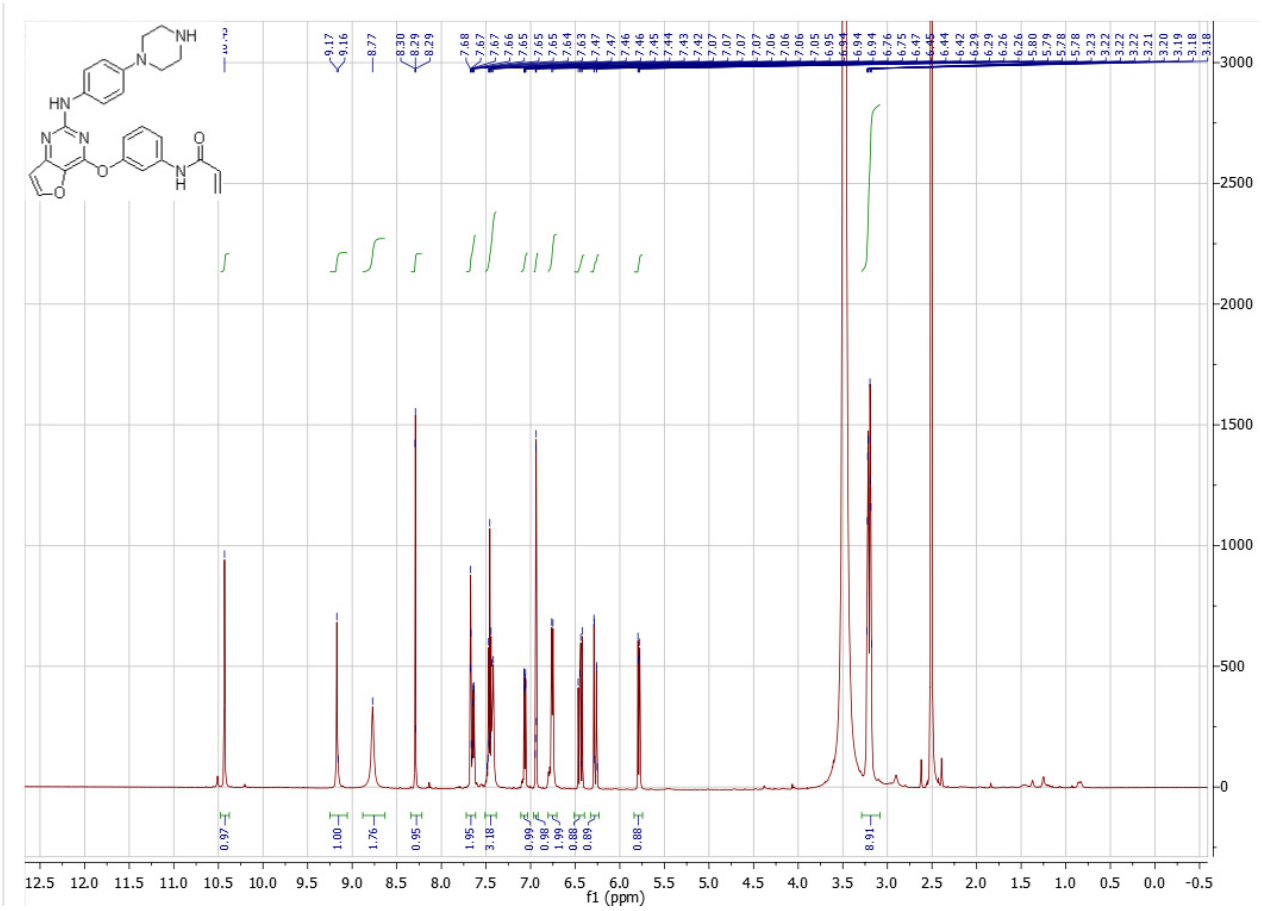
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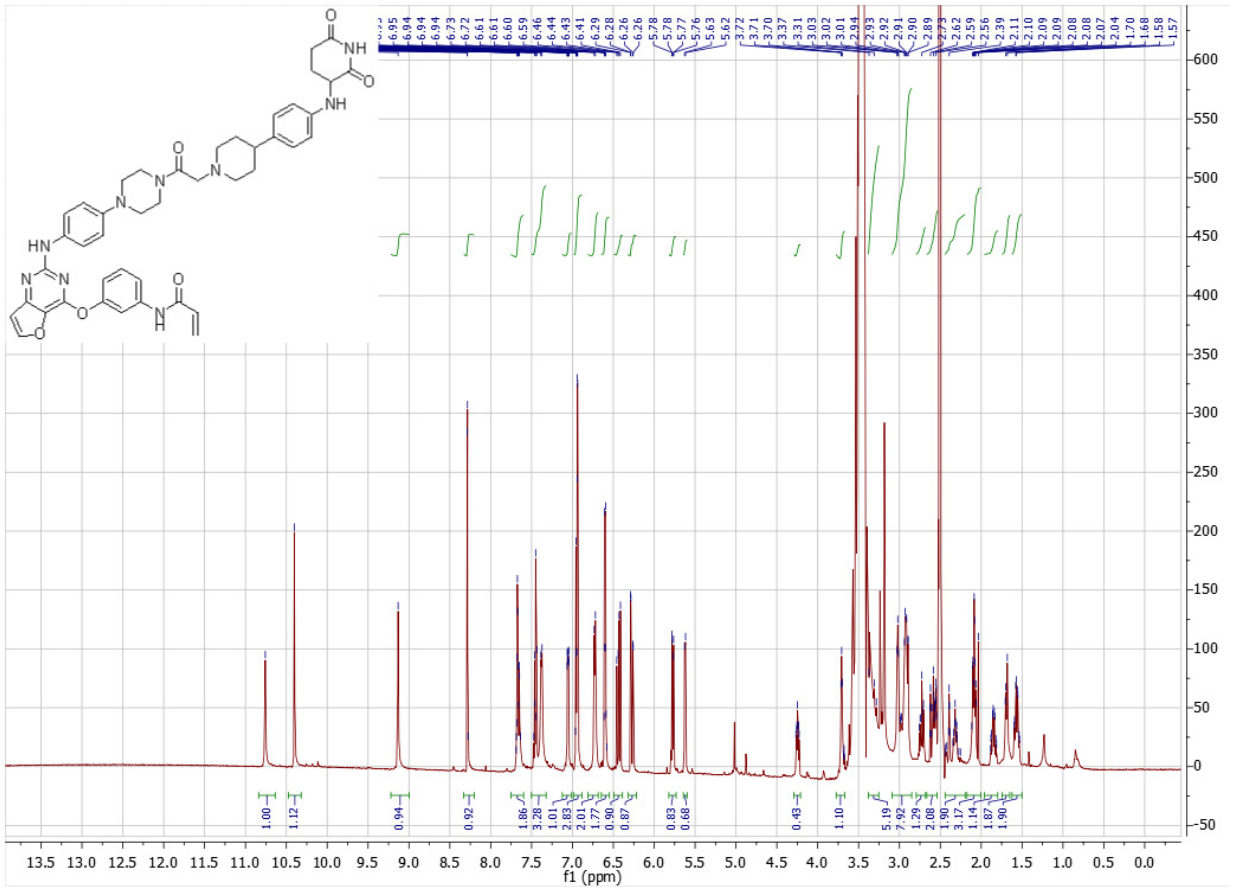
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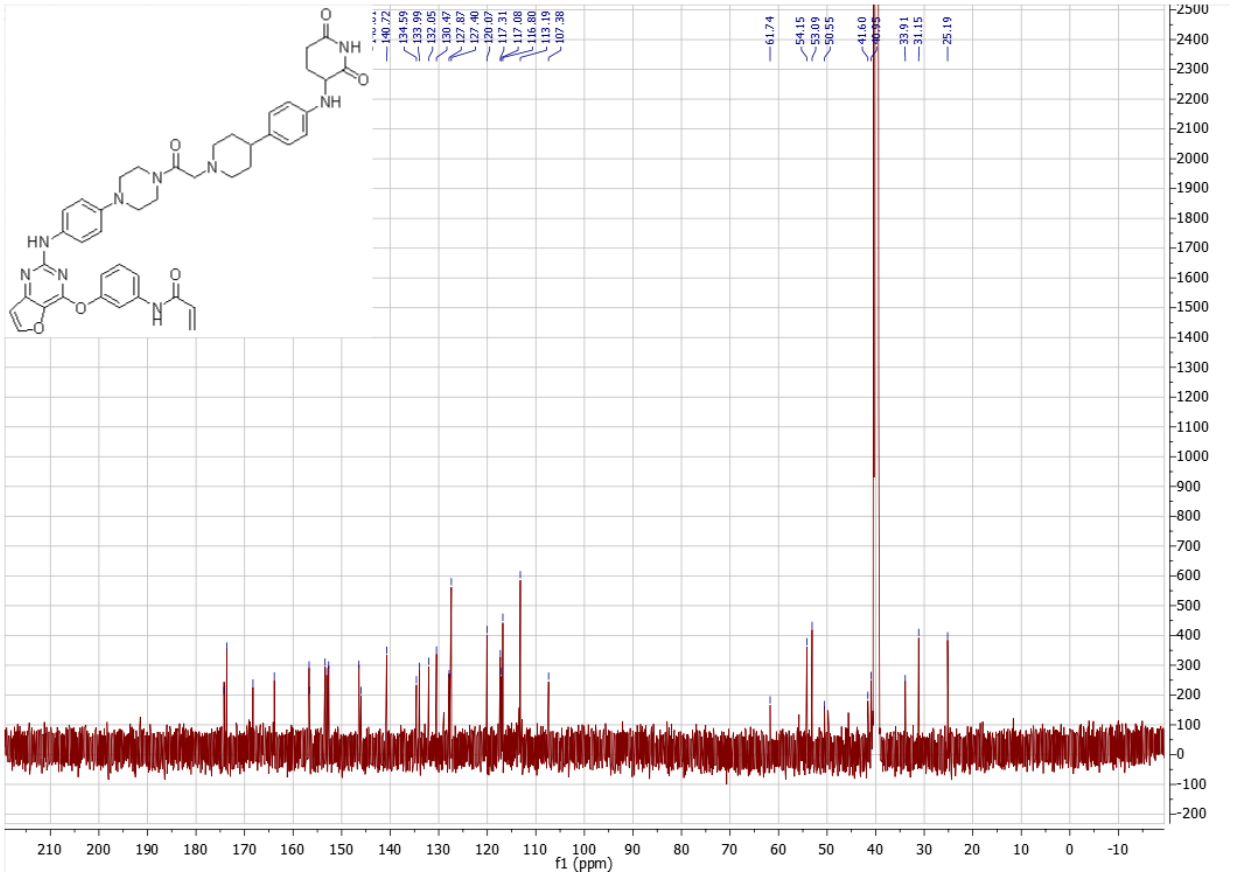
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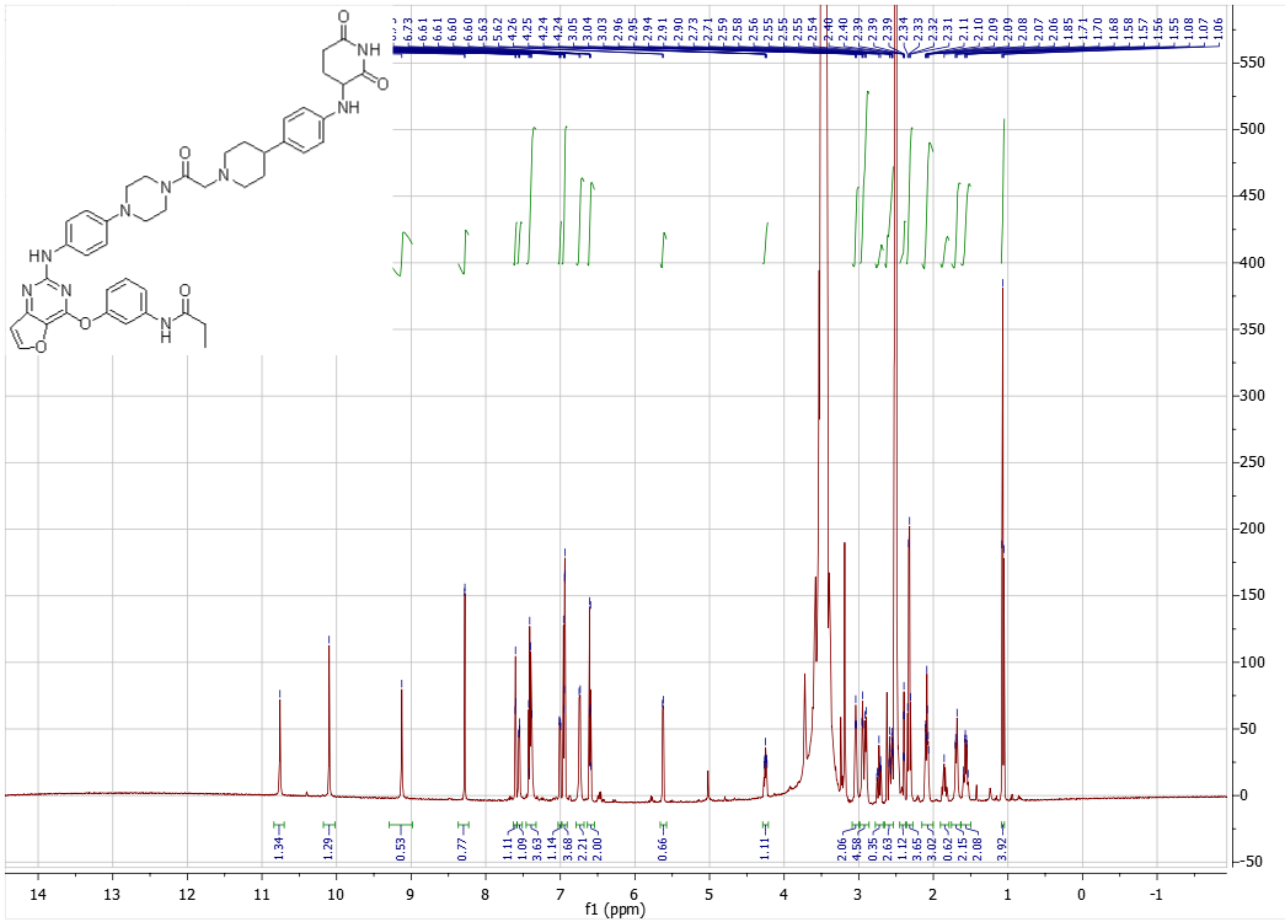
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