

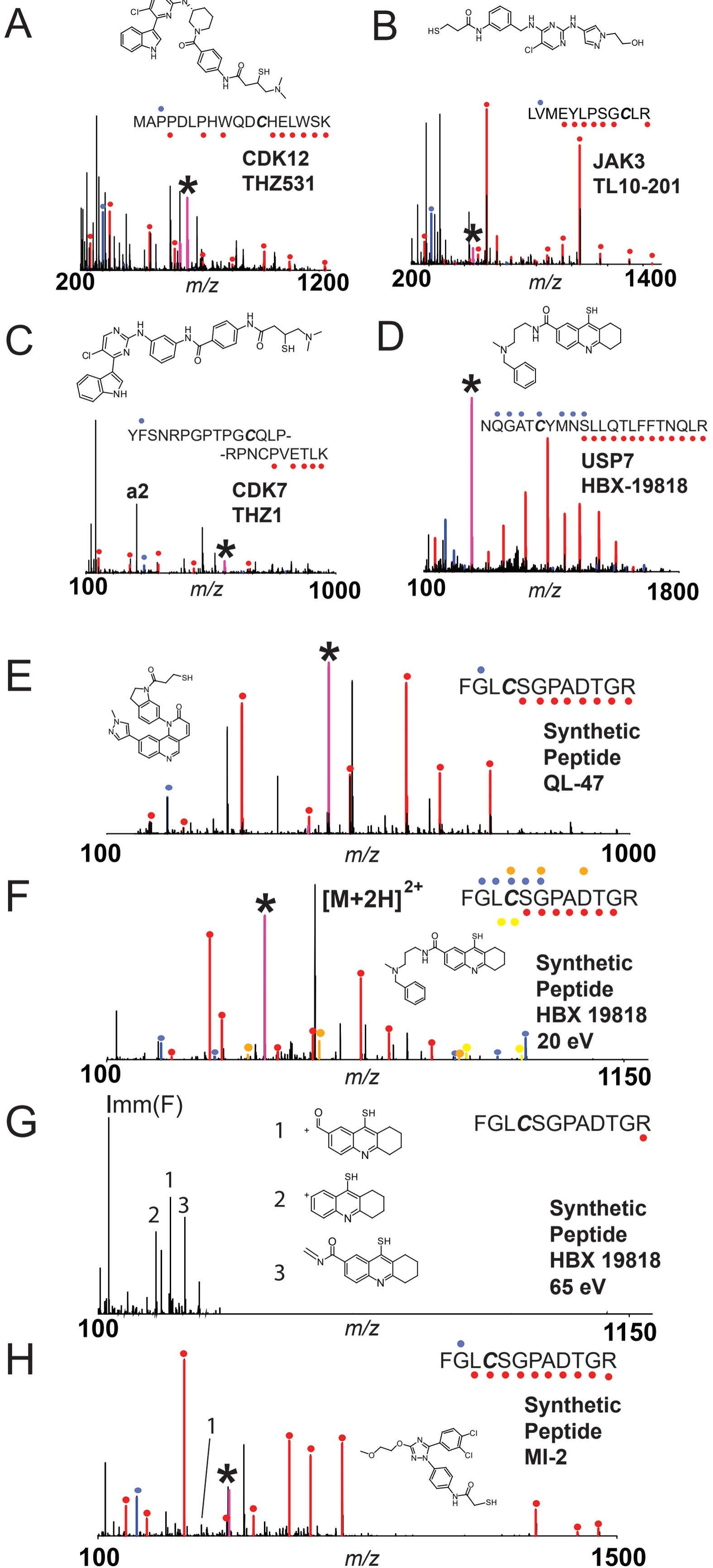
**Supporting Information for the Manuscript: “Leveraging Novel  
Fragmentation Pathways for Improved Identification and Selective  
Detection of Targets Modified by Covalent Probes”**

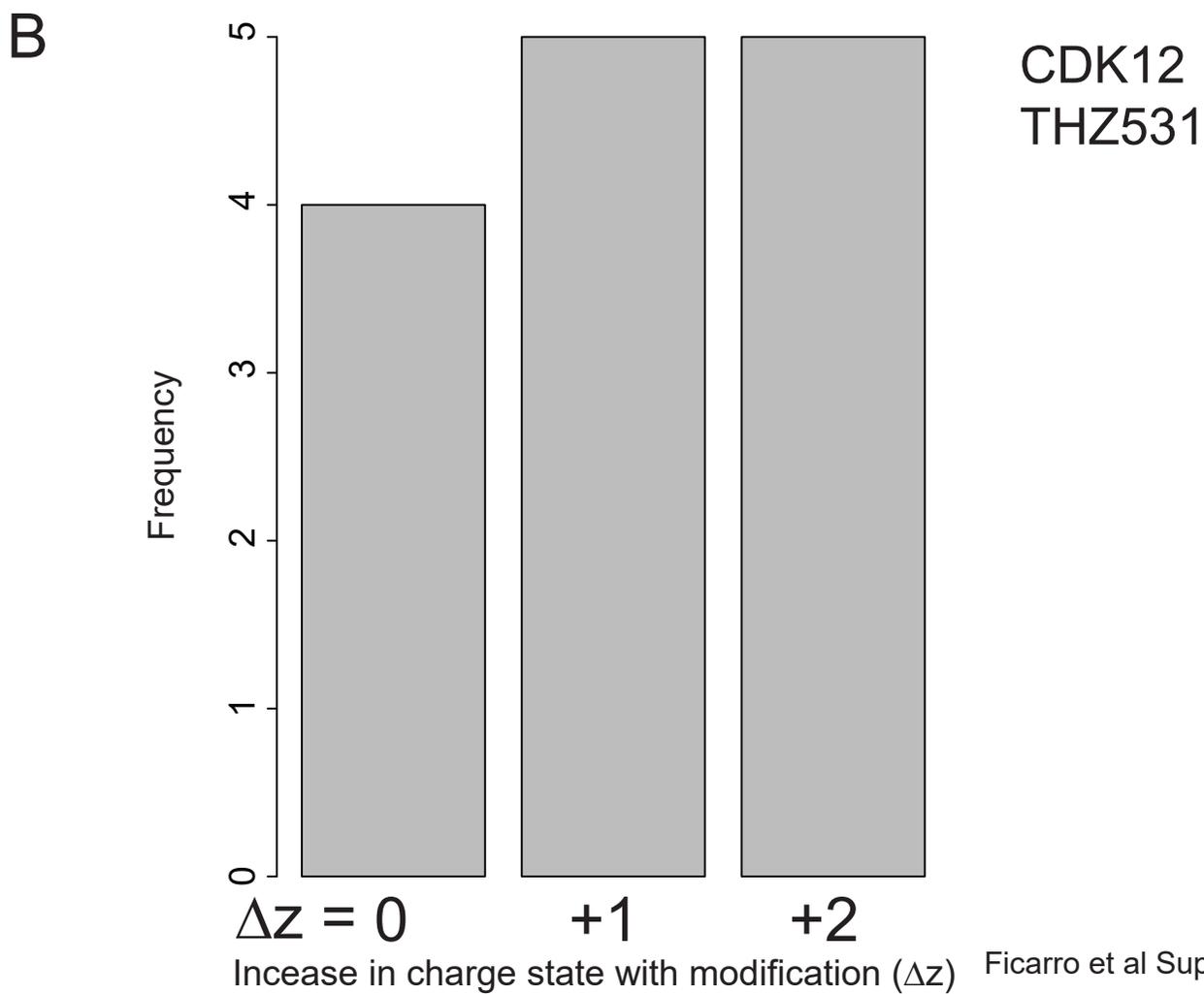
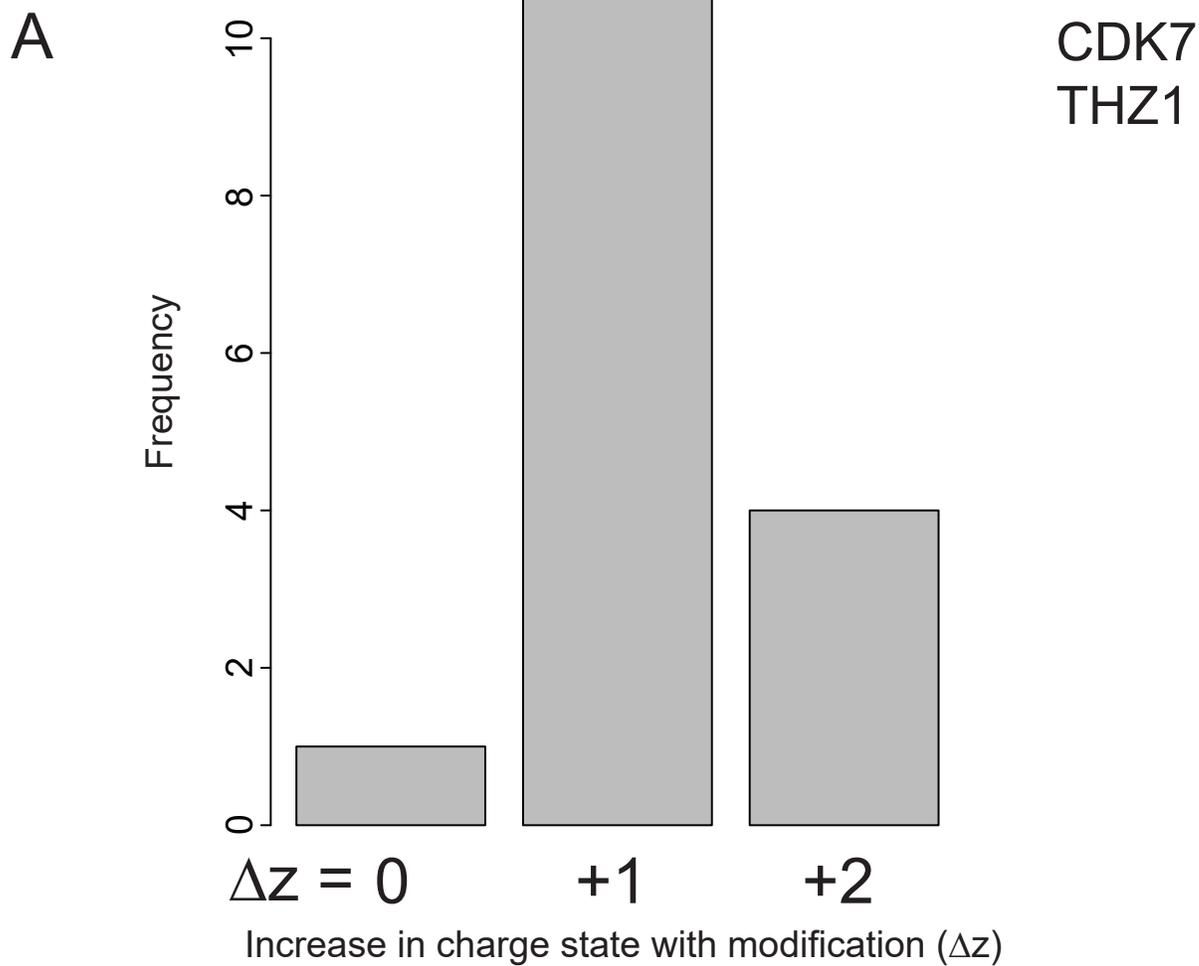
*Scott B. Ficarro<sup>1,2,3</sup>, Christopher M. Browne<sup>1,3</sup>, Joseph D. Card<sup>1,2</sup>, William M. Alexander<sup>1,2,3</sup>, Tinghu Zhang<sup>1,3</sup>, Eunyoung Park<sup>1,3</sup>, Randall McNally<sup>1,3</sup>, Sirano Dhe-Paganon<sup>1,3</sup>, Hyuk-Soo Seo<sup>1,3</sup>, Ilaria Lamberto<sup>1,3</sup>, Michael J. Eck<sup>1,3</sup>, Sara J. Buhrlage<sup>1,3</sup>, Nathanael S. Gray<sup>1,3</sup>, Jarrod A. Marto<sup>1,2,4,\*</sup>*

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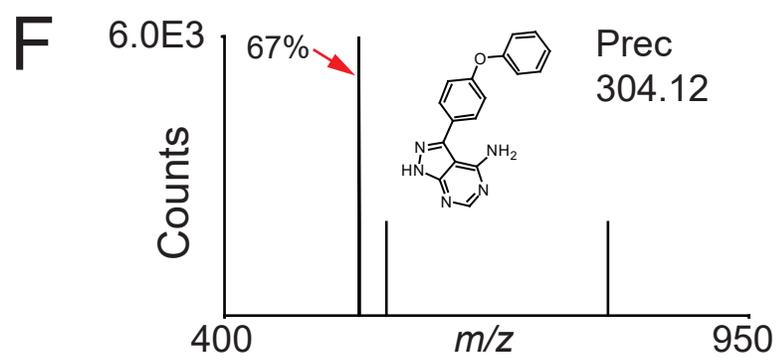
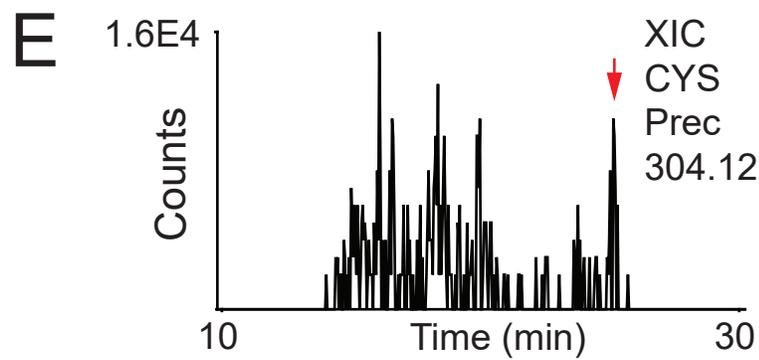
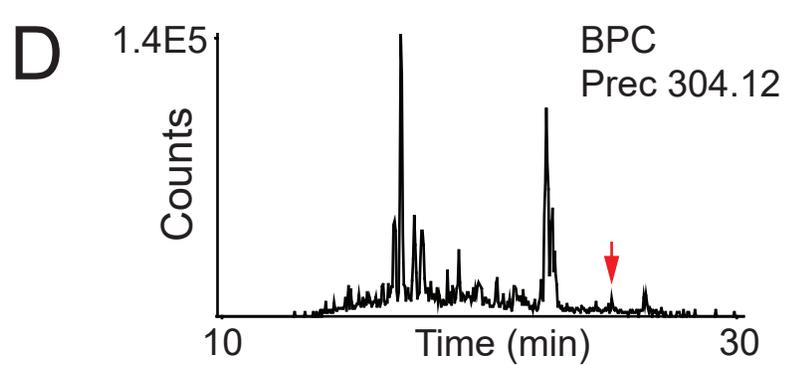
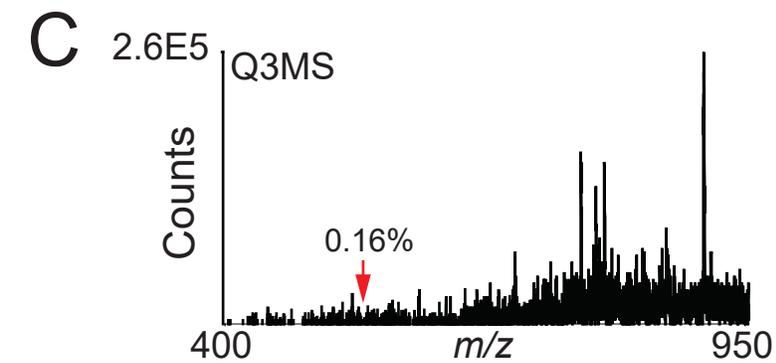
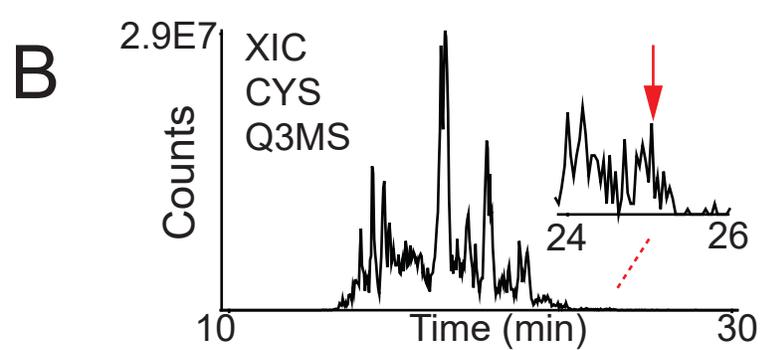
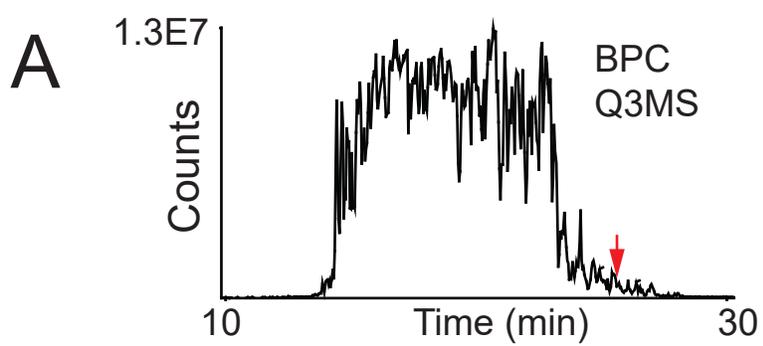
\*To whom correspondence should be addressed: Dr. Jarrod A. Marto, Department of Cancer Biology, Dana-Farber Cancer Institute, 360 Longwood Avenue, LC 2208, Boston, MA, 02115-6084, USA. Phone: (617) 632-3150 (office). Fax: (617) 582-4471. E-mail: jarrod\_marto@dfci.harvard.edu.

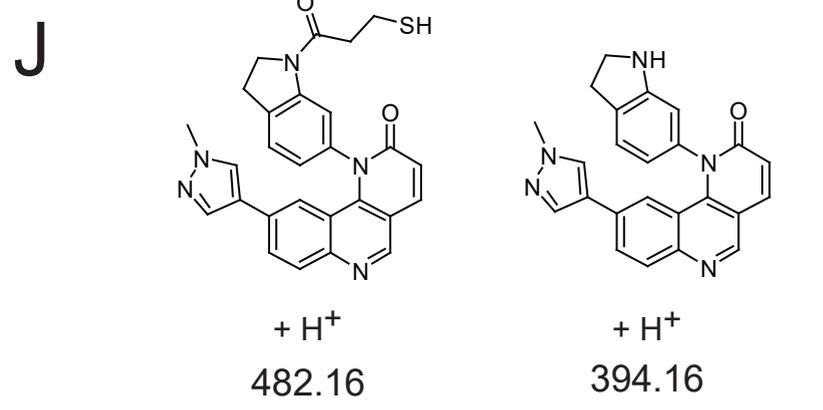
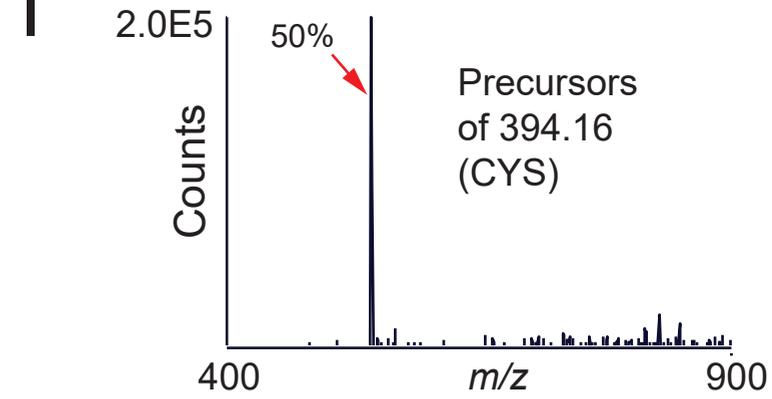
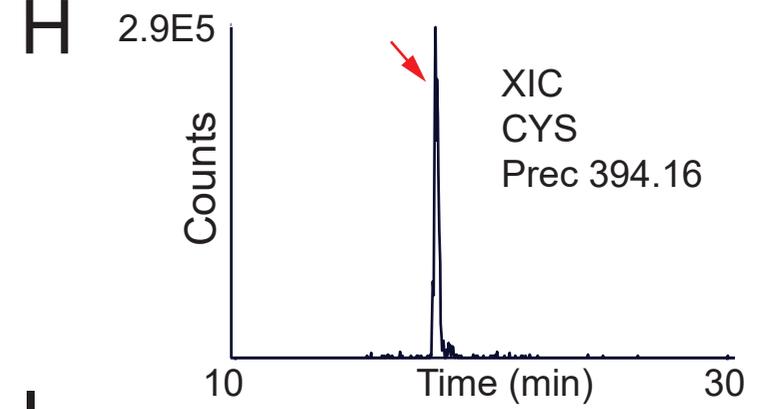
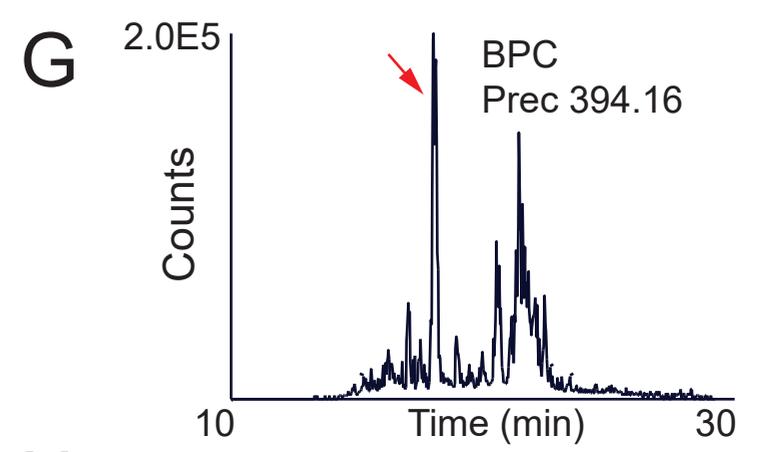
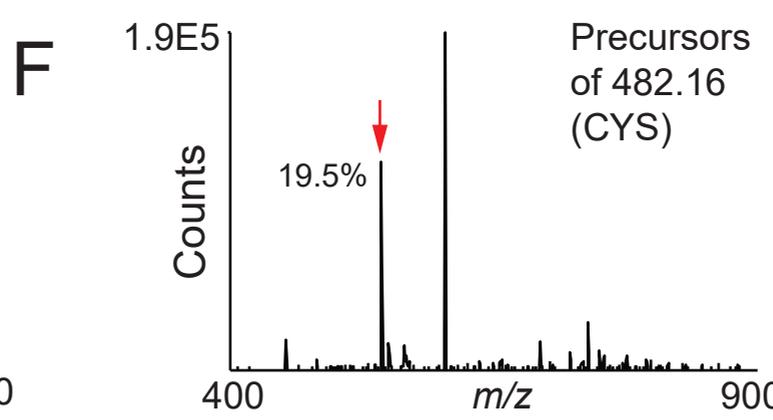
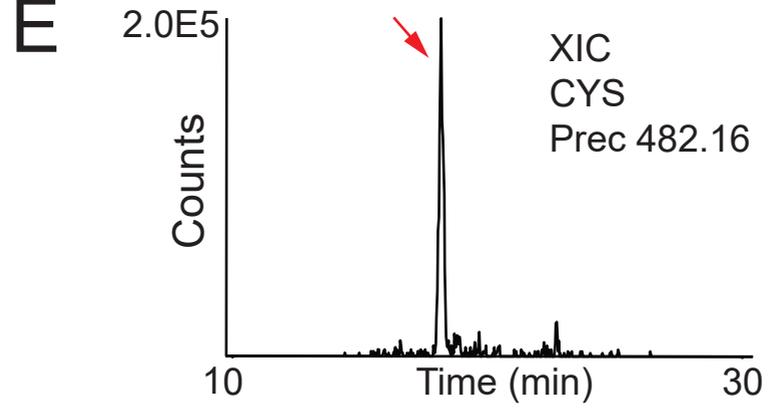
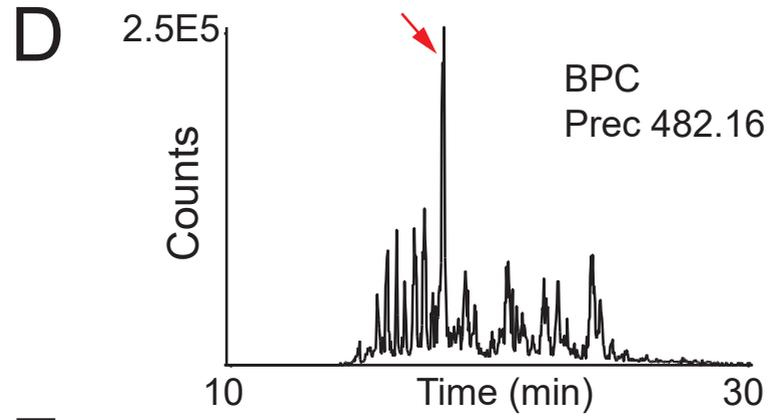
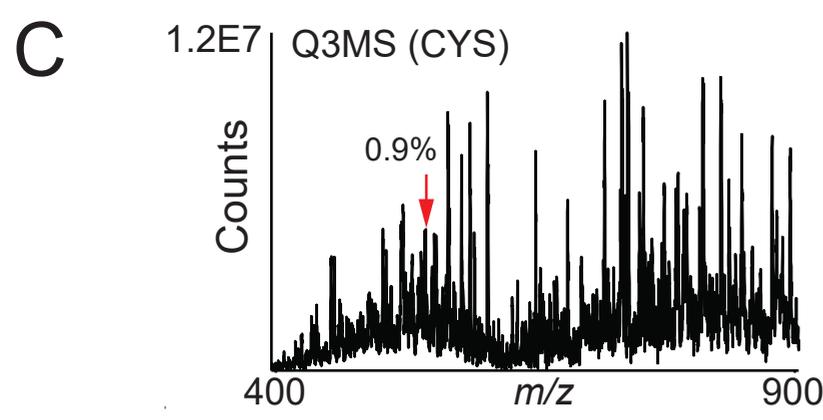
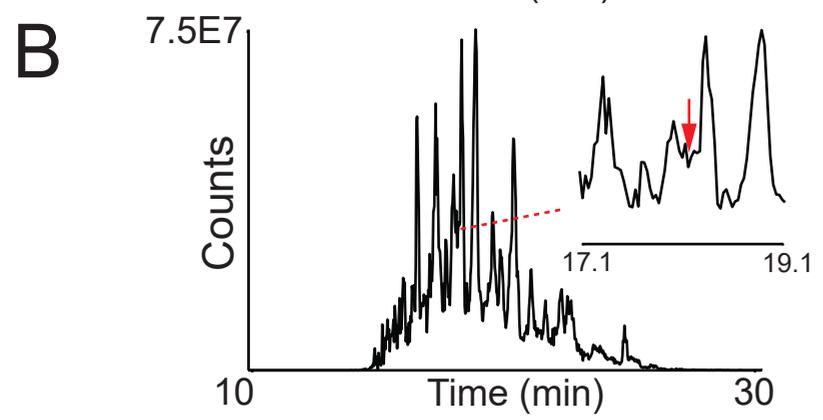
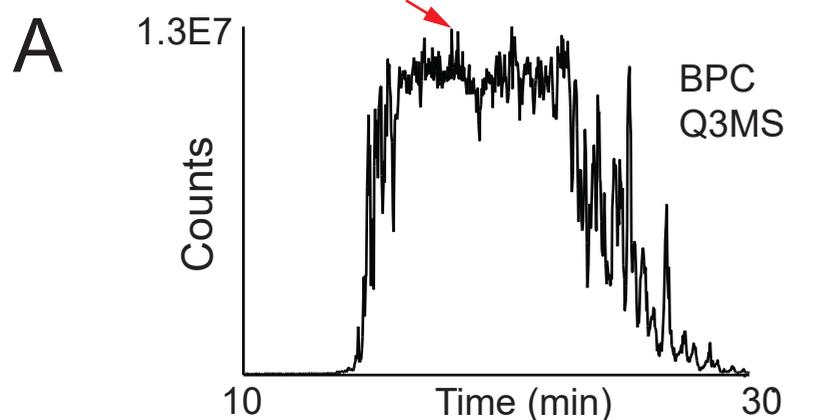
The following sections provide Supplemental Figures S1-S5, Supplemental Figure Legends, and Supplemental Table S1.











**Supplemental Figure S1.** Common fragmentation pathways are observed in MS/MS spectra of inhibitor conjugated peptides. MS/MS spectra for inhibitor target conjugates (A) THZ531 labeled CDK12 peptide; (B) TL10-201 labeled JAK3 peptide; (C) THZ1 labeled CDK7 peptide; (D) HBX-19818 labeled USP7 peptide. MS/MS spectra of the synthetic cysteine-containing peptide FGLCSGPADTGR conjugated to (E) QL-47; (F, G) HBX-19818; and (H) MI-2. Fragment ions containing the peptide N- (b-type) or C- (y-type) termini are indicated with blue and red glyphs above and below the peptide sequence. Modified cysteine residues are shown in bold, italic print. Thiolated ions are highlighted in purple and denoted with '\*'. (F) At a CE of 20 eV HBX-19818 modified peptide eliminates the inhibitor/thiol to produce an intense thiolated ion and a series of dehydroalanine containing b-/y-type ions (highlighted in orange/yellow). (G) At higher CE (65 eV) the HBX-19818 modified synthetic peptide produces an  $ib_1$  ion (1) and other structure-specific ions (2, 3). Imm(F) indicates the immonium ion of phenylalanine. (H) MI-2 modified peptide also shows a peak corresponding to amide bond cleavage ("1").

**Supplemental Figure S2.** Bar graph illustrating shift in charge state distribution observed after conjugation of reduced BSA peptides to (A) THZ1 (CDK7 inhibitor) or (B) THZ531 (CDK12 inhibitor) compared to the same peptides alkylated with iodoacetamide.

**Supplemental Figure S3.** Normalized fragment ion intensity vs. collision energy (CE) for inhibitor specific (thiolated and  $iy_1$ ) ions produced by MS/MS of triply charged synthetic cysteine-containing peptide FGLCSGPADTGR labeled with (A) TL10-201; (B) THZ1; (C) THZ531. For comparison, each plot includes profiles for b-/y-type ions produced by MS/MS of

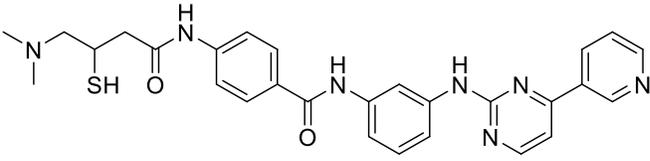
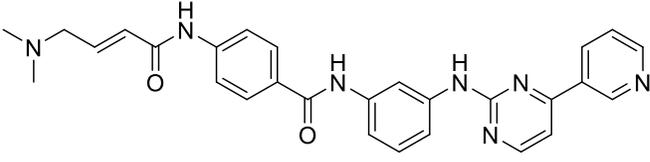
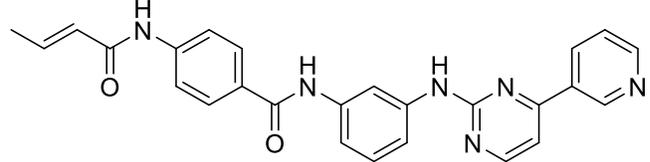
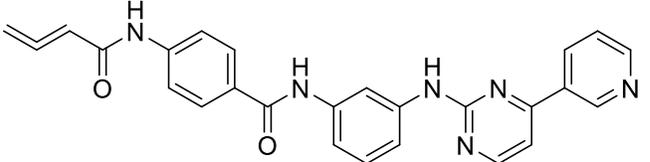
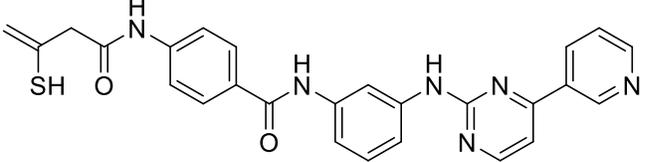
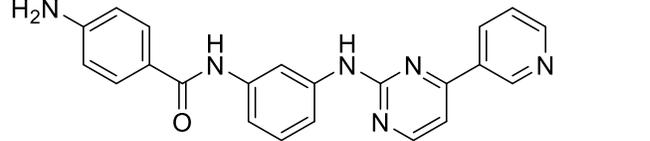
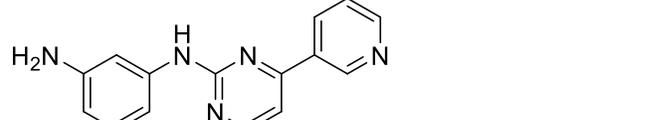
the unlabeled triply charged peptide. (D) Signal intensity as a function of collision energy for inhibitor-specific fragments recorded during MS/MS analysis of triply charged BTK peptide conjugated to JNK-IN-2 (JNK1 inhibitor). (E, F) Signal intensity as a function of collision energy for thiolated ions recorded during MS/MS analysis of doubly- or triply-charged FGLCSGPADTGR or BTK peptides conjugated to (E) TL10-201 (JAK3 inhibitor) or (F) JNK-IN-2 (JNK inhibitor).

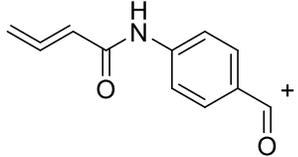
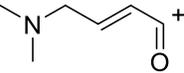
**Supplemental Figure S4.** A second dissociation pathway can be exploited to afford selective detection of an Ibrutinib labeled peptide by precursor ion mass spectrometry. (A) Base-peak chromatogram (BPC) and (B) extracted ion chromatogram (XIC; precursor +/- 0.5 Da) from Q3MS scans and (C) individual Q3 full-scan mass spectrum recorded during analysis of a synthetic cysteine-containing peptide labeled with Ibrutinib (FGLCSGPADTGR; indicated by CYS) and spiked into a mix of tryptic peptides derived from human myeloid K562 cells. Red arrow indicates the elution time (A and B) or  $m/z$  (C) for the labeled peptide. (Note: these data are generated from the same LC-MS/MS analysis as in Fig. 4, hence panels A-C are identical and included here for reference.) (D) Base-peak chromatogram and (E) XIC (precursor +/- 0.5 Da) from precursor ion spectra (precursors of 304.12, corresponding to alkylated amine cleavage; abbreviated as "Prec"), and individual precursor ion mass spectrum (F) recorded during the same LC-MS/MS analysis. Red arrow indicates the elution time (D and E) or precursor ion signal (F) for the Ibrutinib labeled peptide. (C, F) The % values in each panel represent the gas phase enrichment, calculated as the relative contribution of each ion (red arrow) as compared to the total ion current in that spectrum.

**Supplemental Figure S5.** Selective detection of a QL47-modified peptide using precursor ion scanning mass spectrometry. (A) Base-peak chromatogram (BPC) and (B) extracted ion chromatogram (XIC; precursor +/- 0.5 Da) from Q3MS scans and (C) individual Q3 full-scan mass spectrum recorded during analysis of a synthetic cysteine-containing peptide labeled with QL47 (FGLCSGPADTGR; indicated by CYS) and spiked into a mix of tryptic peptides derived from human myeloid K562 cells. Red arrow indicates the elution time (A and B) or  $m/z$  (C) for the labeled peptide. (D, G) Base-peak chromatograms; (E, H) XICs (precursor +/- 0.5 Da) from precursor ion spectra; and (F, I) individual precursor ion mass spectra recorded during the same LC-MS/MS analysis (D-F, precursors of 482.16, corresponding to the thiolated ion of QL47; G-I, precursors of 394.16, corresponding to the  $iy_1$  ion of QL47; abbreviated as "Prec"). Red arrow indicates the elution time (D, E, G, H) or precursor ion signal (F, I) for the labeled peptide. (C, F, I) The % values in each panel represent the gas phase enrichment, calculated as the relative contribution of each ion (red arrow) as compared to the total ion current in that spectrum. (J) Proposed structures and calculated masses of the QL47 thiolated (left) and  $iy_1$  (right) ions.

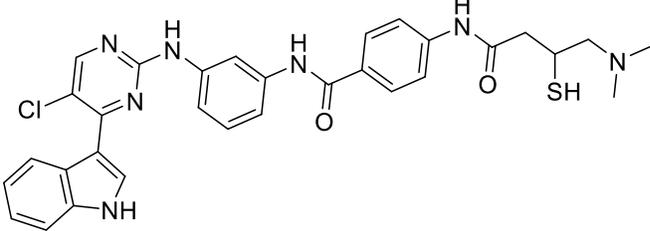
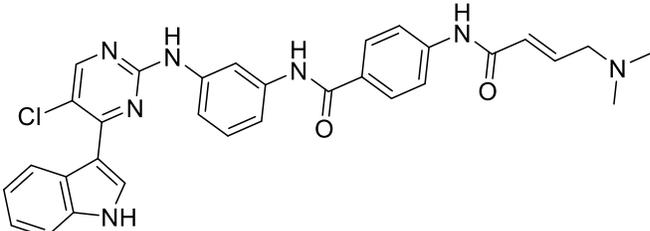
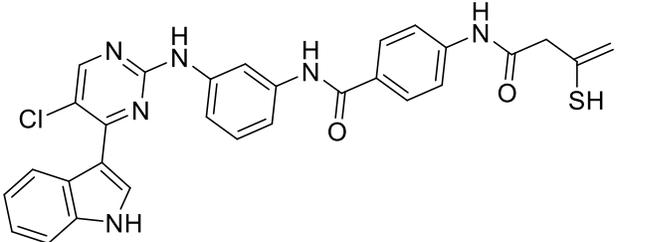
**Supplemental Table S1.** Inhibitor related ions produced by MS/MS of modified peptides.

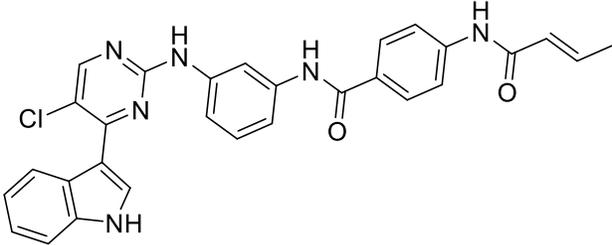
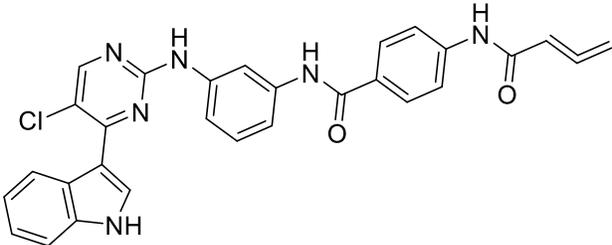
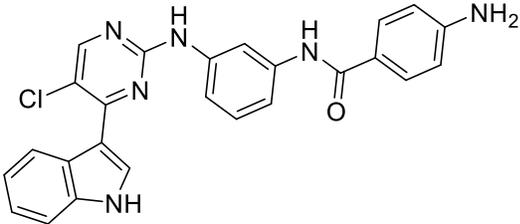
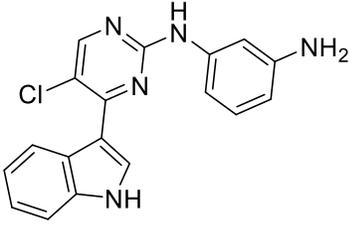
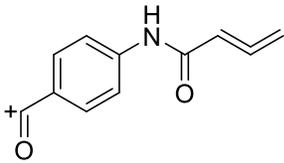
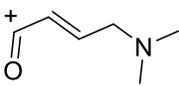
JNK-IN-2; Target = JNK1

ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C28 H29 N7 O2 S1	528.2176 (H+)
RMA (Retro-Michael addition)		C28 H27 N7 O2	494.2299 (H+)
RMA-DMAE-1 (Retro-Michael addition/dimethyl amine elimination type 1)		C26 H22 N6 O2	451.1877 (H+)
RMA-DMAE-2 (Retro-Michael addition/dimethyl amine elimination type 2)		C26 H20 N6 O2	449.1721 (H+)
TH-DMAE (Thiolated ion with dimethylamine elimination)		C26 H22 N6 O2 S1	483.1598 (H+)
iy1		C22 H18 N6 O1	383.1615 (H+)
iy2		C15 H13 N5	264.1244 (H+)

ib2 RMA-DMAE2 ib2 with retro-Michael addition- and type 2 dimethylamine elimination		C11 H8 N1 O2	186.0555
ib1		C6 H10 N1 O1	112.0762

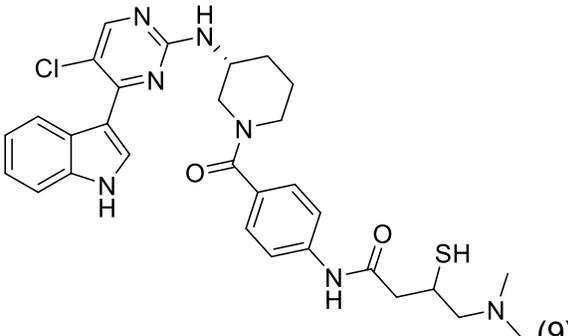
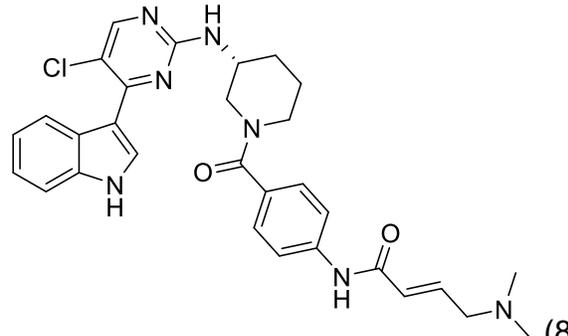
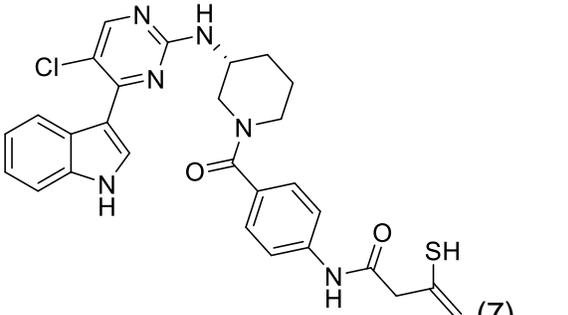
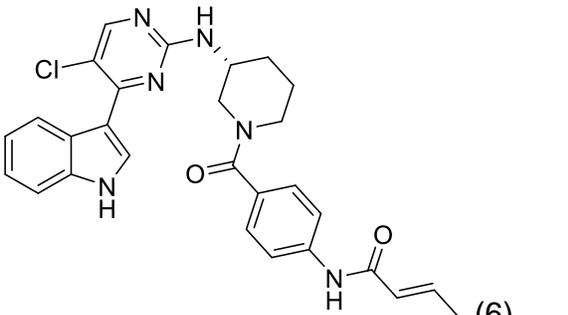
THZ1; Target = CDK7

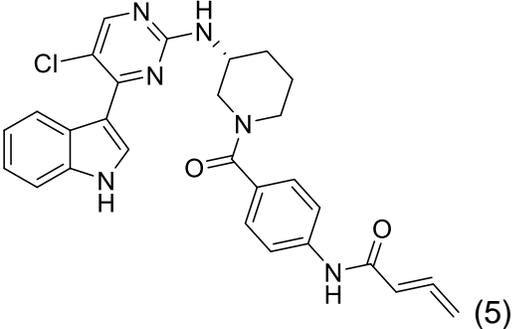
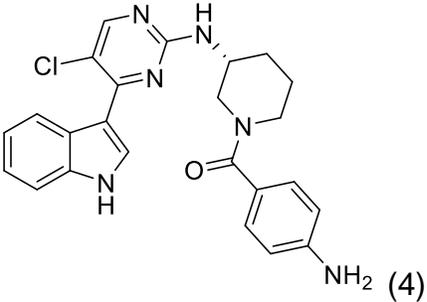
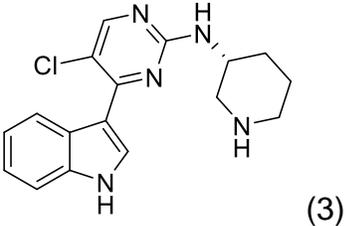
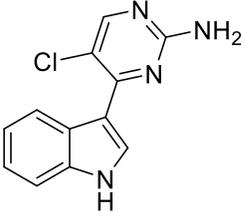
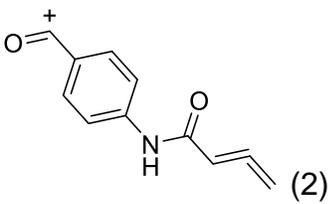
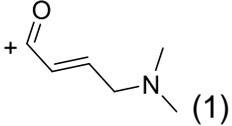
ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C31 H30 Cl1 N7 O2 S1	600.1943 (H+)
RMA (Retro-Michael addition)		C31 H28 Cl1 N7 O2	566.2066 (H+)
TH-DMAE (Thiolated ion with dimethylamine elimination)		C29 H23 Cl1 N6 O2 S1	555.1365 (H+)

<p>RMA-DMAE-1 (Retro-Michael addition/dimethyl amine elimination type 1)</p>		<p>C29 H23 Cl1 N6 O2</p>	<p>523.1644 (H+)</p>
<p>RMA-DMAE-2 (Retro-Michael addition/dimethyl amine elimination type 2)</p>		<p>C29 H21 Cl1 N6 O2</p>	<p>521.1487 (H+)</p>
<p>iy1</p>		<p>C25 H19 Cl1 N6 O1</p>	<p>455.1382 (H+)</p>
<p>iy2</p>		<p>C18 H14 Cl1 N5</p>	<p>336.1011 (H+)</p>
<p>ib2-RMA-DMAE-2 ib2 with retro- Michael addition and dimethylamine elimination type-2</p>		<p>C11 H8 N1 O2</p>	<p>186.0555</p>
<p>ib1</p>		<p>C6 H10 N1 O1</p>	<p>112.0762</p>

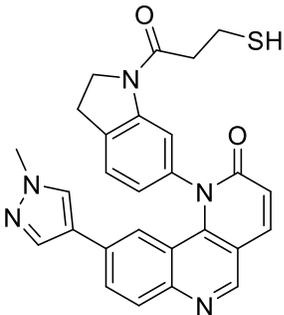
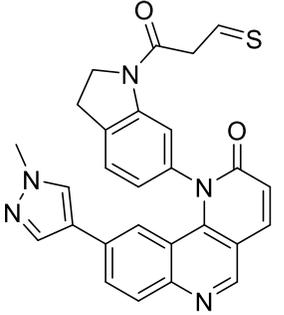
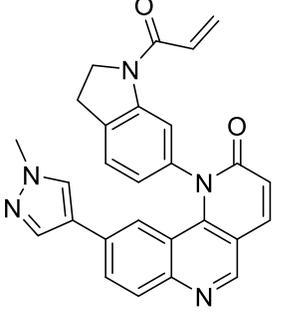
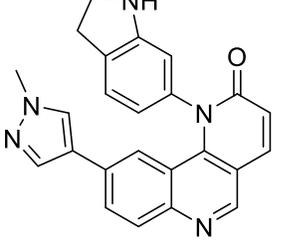
THZ531; Target = CDK12

\*, cleavage at alkylated amine only observed at high collision energies

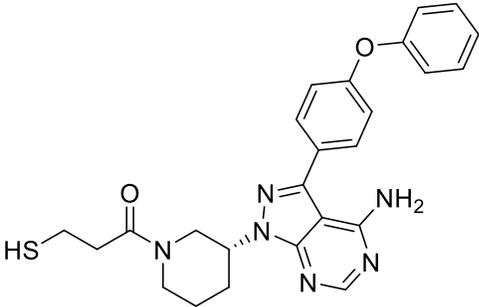
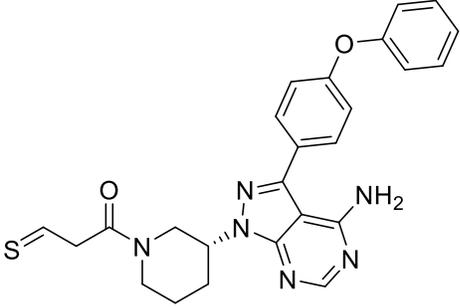
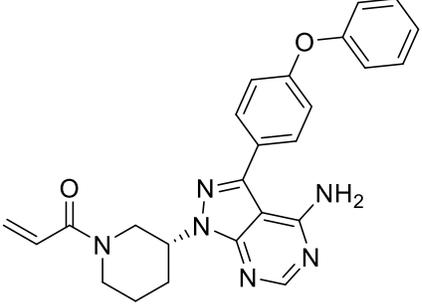
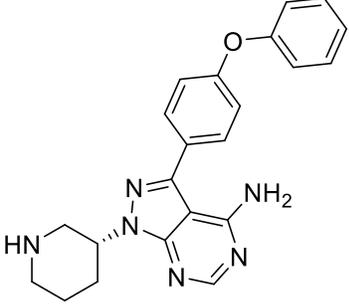
ION	PROPOSED STRUCTURE (1-9 corresponds to Figure 2A)	FORMULA	Ion m/z
TH (Thiolated ion)	 <p>(9)</p>	C30 H34 Cl1 N7 O2 S1	592.2256 (H+)
RMA (Retro-Michael addition)	 <p>(8)</p>	C30 H32 Cl1 N7 O2 H	558.2379 (H+)
TH-DMAE (Thiolated ion with dimethylamine elimination)	 <p>(7)</p>	C28 H27 Cl1 N6 O2 S1	547.1678 (H+)
RMA-DMAE-1 (Retro-Michael addition/dimethyl amine elimination type 1)	 <p>(6)</p>	C28 H27 Cl1N6 O2	515.1957 (H+)

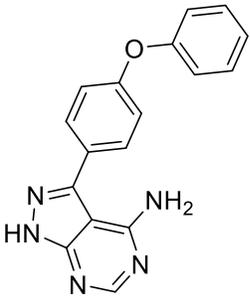
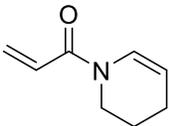
<p>RMA-DMAE-2 (Retro-Michael addition/dimethyl amine elimination type 2)</p>	 <p>(5)</p>	<p>C28 H25 Cl1 N6 O2</p>	<p>513.1800 (H+)</p>
<p>iy1</p>	 <p>(4)</p>	<p>C24 H23 Cl1 N6 O1</p>	<p>447.1695 (H+)</p>
<p>iy2</p>	 <p>(3)</p>	<p>C17 H18 Cl1 N5</p>	<p>328.1323 (H+)</p>
<p>Cleavage at alkylated amine</p>	 <p>* see note above</p>	<p>C12 H9 Cl1 N4</p>	<p>245.0589 (H+)</p>
<p>ib2-RMA-DMAE-2 ib2 with retro- Michael addition and dimethylamine elimination type-2</p>	 <p>(2)</p>	<p>C11 H8 N1 O2</p>	<p>186.0555</p>
<p>ib1</p>	 <p>(1)</p>	<p>C6 H10 N1 O1</p>	<p>112.0762</p>

QL-47; Target = BTK

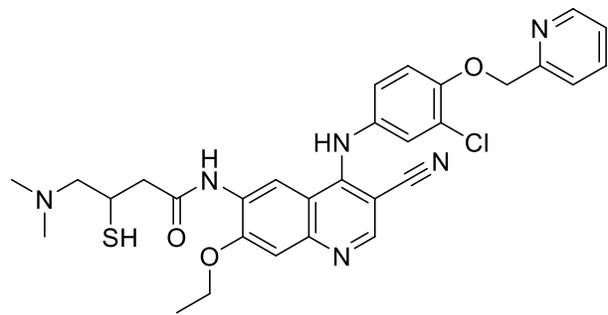
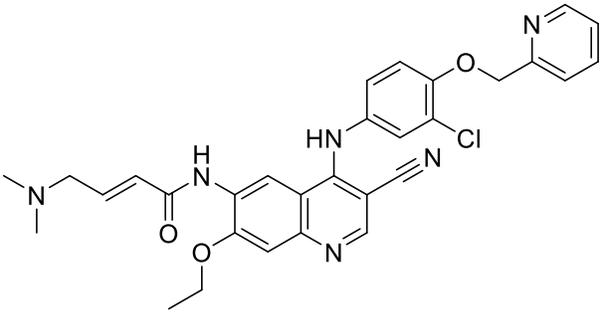
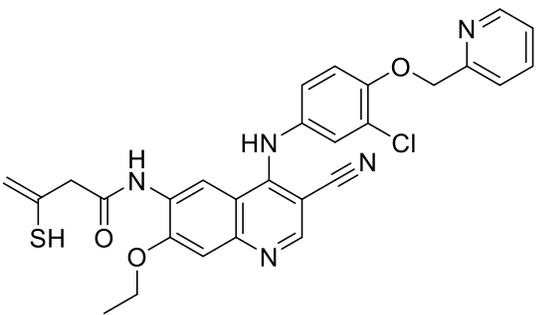
ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C27 H23 N5 O2 S1	482.1645 (H+)
TH-H2 (Thiolated ion with H2 elimination)		C27 H21 N5 O2 S1	480.1489 (H+)
RMA (Retro-Michael addition)		C27 H21 N5 O2	448.1768 (H+)
iy1		C24 H19 N5 O1	394.1662 (H+)

Ibrutinib; Target = BTK

ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)	 <p style="text-align: right;">(5)</p>	C25 H26 N6 O2 S1	475.1911 (H+)
Thiolated ion with H2 elimination	 <p style="text-align: right;">(4)</p>	C25 H24 N6 O2 S1	473.1754 (H+)
RMA (Retro-Michael addition)	 <p style="text-align: right;">(3)</p>	C25 H24 N6 O2	441.2034 (H+)
iy1	 <p style="text-align: right;">(2)</p>	C22 H22 N6 O1	387.1928 (H+)

Cleavage at alkylated amine	 <p style="text-align: center;">(1)</p>	C17 H13 N5 O1	304.1193 (H+)
RMA with alkylated amine cleavage		C8 H12 N1 O1	138.0913 (H+)

Neratinib; Target = EGFR/Her2

ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C30 H31 Cl1 N6 O3 S1	591.19396 (H+)
RMA (Retro-Michael addition)		C30 H29 Cl1 N6 O3	557.20624 (H+)
TH-DMAE (Thiolated ion with dimethylamine elimination)		C28 H24 Cl1 N5 O3 S1	546.13611 (H+)

RMA-DMAE-1 (Retro-Michael addition/dimethyl amine elimination type 1)		C28 H24 Cl1N5 O3	514.16404 (H+)
RMA-DMAE-2 (Retro-Michael addition/dimethyl amine elimination type 2)		C28 H22 Cl1N5 O3	512.14839 (H+)
iy1		C24 H20 Cl1 N5 O2	446.13783 (H+)
ib1		C6 H10 N1 O1	112.07624

TL10-201; Target = JAK3

ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C19 H22 Cl1 N7 O2 S1	448.13170 (H+)

Thiolated ion with H2 elimination		C19 H20 Cl1 N7 O2 S1	446.1160 (H+)
RMA (Retro-Michael addition)		C19 H20 Cl1N7 O2	414.14398 (H+)
iy1		C16 H18 Cl1 N7 O1	360.13341 (H+)
Cleavage at alkylated amine		C9 H11 Cl1 N6 O1	255.07556 (H+)

MI-2; Target = MALT1

ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C19 H18 Cl2 N4 O3 S1	453.05494 (H+)
Thiolated ion with H2 elimination		C19 H16 Cl2 N4 O3 S1	451.03929 (H+)

iy1		C17 H16 Cl2 N4 O2	379.07231 (H+)
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HBX-19818; Target = USP7

ION	PROPOSED STRUCTURE	FORMULA	Ion m/z
TH (Thiolated ion)		C25 H29 N3 O1 S1	420.21041 (H+)
Cleavage at alkylated amine		C18 H23 N3 O1 S1	330.1635 (H+)
Elimination product		C15 H14 N2 O1 S1	271.0900 (H+)
ib1		C14 H12 N1 O1 S1	242.06396
ia1 (loss of CO from ib1)		C13 H12 N1 S1	214.06904