An Activity-based Probe Targeting Non-catalytic, Highly Conserved Amino Acid Residues Within Bromodomains

Melissa D’Ascenzio, Kathryn Pugh, Rebecca Konietzny, Georgina Berridge, Cynthia Tallant, Shaima Hashem, Octovia P. Monteiro, Jason R. Thomas, Markus Schirle, Stefan Knapp, Brian D. Marsden, Oleg Fedorov, Chas Bountra, Benedikt M. Kessler, Paul E. Brennan

Submitted date: 16/10/2018 • Posted date: 17/10/2018
Licence: CC BY-NC-ND 4.0
Citation information: D’Ascenzio, Melissa; Pugh, Kathryn; Konietzny, Rebecca; Berridge, Georgina; Tallant, Cynthia; Hashem, Shaima; et al. (2018): An Activity-based Probe Targeting Non-catalytic, Highly Conserved Amino Acid Residues Within Bromodomains. ChemRxiv. Preprint.

Bromodomain-containing proteins are epigenetic modulators involved in a wide range of cellular processes, from physiological recruitment of transcription factors to pathological disruption of gene regulation and cancer development. Since the druggability of these acetyl-lysine reader domains was established, efforts were made to develop potent and selective inhibitors across the entire family. Here we report the development of a small molecule based approach to covalently modify recombinant and endogenous bromodomain-containing proteins by targeting a conserved lysine and a tyrosine residue in the variable ZA or BC loops. Moreover, the addition of a reporter tag, via copper-catalyzed alkyne azide coupling, to an alkyne handle on the probe allowed in-gel visualization and selective pull-down of the desired bromodomains using both recombinant and endogenous proteins.

File list (2)

Bromotriazine.pdf (587.67 KiB)  view on ChemRxiv  download file
Bromotriazine SI.pdf (3.23 MiB)  view on ChemRxiv  download file
An activity-based probe targeting non-catalytic, highly conserved amino acid residues within bromodomains

Melissa D’Ascenzio, Kathryn M. Pugh, Rebecca Konietzny, Georgina Berridge, Cynthia Tallant, Shaima Hashem, Octavia Monteiro, Jason R. Thomas, Markus Schirle, Stefan Knapp, Brian Marsden, Oleg Fedorov, Chas Bountra, Benedikt M. Kessler,* Paul E. Brennan*

Abstract: Bromodomain-containing proteins are epigenetic modulators involved in a wide range of cellular processes, from physiological recruitment of transcription factors to pathological disruption of gene regulation and cancer development. Since the druggability of these acetyl-lysine reader domains was established, efforts were made to develop potent and selective inhibitors across the entire family. Here we report the development of a small molecule based approach to covalently modify recombinant and endogenous bromodomain-containing proteins by targeting a conserved lysine and a tyrosine residue in the variable ZA or BC loops. Moreover, the addition of a reporter tag, via copper-catalyzed alkyne azide coupling, to an alkyne handle on the probe allowed in-gel visualization and selective pull-down of the desired bromodomains using both recombinant and endogenous proteins.

Chemical proteomics methodologies such as activity-based protein profiling (ABPP) have been developed in order to complement genetic manipulation and interrogate the proteome to capture the functional state of proteins in cells and tissues.[1] Through the use of covalent inhibitors, ABPP has been capable of providing quantitative readouts of the functional state of individual or multiple enzymes, taking into account allosteric, intrasteric, and post-translational control.[2] Furthermore, thanks to their broad spectrum of selectivity, ABPP probes have been used to screen prospective inhibitors against a wide panel of proteins and validate the engagement with their expected targets within the cellular environment.[3,4] Despite its widespread application to several different enzymatic families including kinases,[5] serine hydrolases,[6] and cysteine/threonine proteases,[7] only minor progress has been made with reference to epigenetic proteins. The value of developing activity-based probes directed against epigenetic modulators finds its rationale in the emerging potential of epigenetic proteins as therapeutic targets,[8] and it has been emphasized by recently reported successful attempts to use similar strategies in order to profile lysine methyl transferases,[9-11] or histone deacetylases.[12,13] However, a significant amount of the afore mentioned studies rely on photo-affinity labelling techniques for the development of efficient probes, mostly because of the lack of activated or easily targetable nucleophilic amino acid residues in the active sites of these proteins. While there are no reports of effective ABPP probes against native bromodomains (BRDs), Daguer et al. recently reported on the discovery of two small molecules that are able to engage with a fairly conserved cysteine residue at the back of the binding pocket when incubated with truncated bromodomains.[14] These small reader domains that specifically recognize the ε-N-acetylated (KAc) lysine mark on histone tails, have been identified as functional modules within at least 46 human proteins that are known to be involved in the regulation of a wide range of cellular events, from transcription factors recruitment to chromatin remodeling, and in the occurrence of several types of cancer.[15-17] Bromodomain-containing proteins constitute a class of epigenetic modulators that are particularly challenging to profile by ABPP, since they lack enzymatic activity and are deficient of activated amino acid residues in their ligand binding sites, but nevertheless of great interest. For this challenging task to be achieved, the new activity-based probe should be endowed with widespread activity across the BRD family, in order to target most of the 61 bromodomains encoded by the human proteome. Bromosporine (BSP) is currently the most broad spectrum inhibitor against the family of bromodomain binding proteins; it is characterized by in vitro K_\text{D} values in the nanomolar/low micromolar range, especially against the BET family, and proven cellular activity (IC50 < 1µM) against the first bromodomain of BRD4, BRD7, BRD9, CREBBP and CECR2.[18] Therefore, its scaffold was considered as a perfect starting point for the rational design of a novel broad spectrum activity-based BRD probe. Through a series of preliminary sequence alignment and docking studies, we observed that the methyl sulfonamide group of BSP pointed towards one of three lysine residues that are fairly conserved in the BC loop of BRDs belonging to different sub-families, and corresponded to residue 85, 90 or 91 in the first BRD of BRD4 (BRD4(1)) (Figure 1).[19]
As lysines are potentially nucleophilic amino acids that have been reported to be targetable by covalent labelling,\(^{[20,21]}\) we believed it would be possible to develop a novel BRD covalent probe by replacing the sulfonamide scaffold of BSP with a reactive warhead, and subsequently introduce a clickable alkyne handle on the solvent exposed ethyl carbamate moiety to allow tagged protein pull-down and in-gel visualization (Figure 2).

In the search for the most appropriate electrophile to use as a warhead for our probe, we found that Shannon et al. had reported the discovery of a dichlorotriazine reactive moiety that was capable of selectively labelling a range of lysine residues within the proteome of HeLa cells.\(^{[22]}\) A series of docking experiments suggested that the dichlorotriazine moiety could be well tolerated by the target proteins (Figure 1), and the synthesis of bromosporine was re-designed in order to include the selected warhead and the clickable handle (See Supporting Information).

The ability of the resulting probe, bromotriazine 2 (BTZ), to covalently modify BRDs characterized by the presence of a lysine residues on the variable BC loop, was tested by incubating the probe (30 µM) with recombinant BRDs (3 µM) in buffer for 1.5 hours at 37 °C. The formation of covalent adducts was detected by mass spectrometry (Figure 3). Truncated recombinant first(1) and second(2) bromodomain representatives of different BRD sub-families were used in a preliminary screen with the aim to define the covalent labelling potential of the newly synthesized probe: BRD4(1), BRD4(2), BRD3(2), BRD2(1), BRD2(2), TAF1(1), TAF1(2), TAF1L(1), BRD1, ATAD2 (Figure 3).

Moreover, a series of BRDs that did not contain a nucleophilic amino acid within reach were included in the study as controls: PB1(5), SMARCA2B, TIF1α, BRPF1B, and CREBBP. An accurate analysis of the data originated in this assay highlighted that the reactivity profile of BTZ (2) seemed to follow the activity profile observed with bromosporine, where higher affinity of the reversible probe corresponded to higher percentage of covalent modification by the irreversible probe, thus suggesting that the reversible binding played a pivotal role in driving the covalent labelling (Figure 3). In fact, bromotriazine efficiently labelled all the BETs (14-67%) and was particularly effective against BRD2(1). At the same time, bromodomains that were weakly interacting with bromosporine (IC\(_{50} \geq 1 \mu M\)) were only slightly modified (1-4%) by BTZ, despite presenting a targetable lysine residue in the correct position (GCN5L2, ATAD2, BRD1).
In order to confirm the correct binding of bromotriazine within the KAc site and provide evidence for the preference of the reactive warhead for lysine residues, recombinant BRDs were incubated with the probe before undergoing tryptic digestion and LC-MS/MS analysis.\(^{[23-24]}\) In the case of BRD4(1) and BRD3(2), this approach confirmed that the preferred sites of modification were in fact the predicted lysine 91 in BRD4(1) and the corresponding residue, lysine 336, in BRD3(2) (Figure 4; see Supplementary Information Section 6.1 - 6.2 for complete data).

These findings were compatible with the estimated binding mode of BTZ 2 in BRD4(1). Interestingly, the clearest covalent labelling was observed when BTZ was reacted with CECR2, a sub-family I BRD characterized by the presence of a tyrosine residue in place of the corresponding lysine 91 in BRD4(1). Surprisingly, BRD9, a IV BRD sub-family member, was covalently modified up to 77% after 1.5 hours incubation at 37 °C, despite that it lacked a lysine near the KAc binding site. Since repeated attempts to modify BRD9 resulted in consistently high percentages of labelling but no reliable data could be produced by LC-MS/MS peptide mapping, a crystallization experiment was set up in order to determine the exact site of interaction. The crystals obtained by seeding the purified fraction containing the modified protein showed a peculiar interlocked structure, in which one BTZ probe was located inside the binding site of one BRD, yet it was covalently linked to another tyrosine residue just outside of the binding pocket of another BRD in the crystallographic unit cell. (Figure 5 - Top and bottom left)

The resulting interlocked dimer is compatible with the previously reported 2:1 protein-peptide stoichiometry of BRD9 functional complexes.\(^{[25]}\) Moreover, the superimposition of the crystal structures of BTZ and bromosporine in the binding pocket of BRD9 highlighted the high similarity of the two binding modes (Figure 5 - Bottom right).

Based on this, we analysed all the BRDs amino acid sequences in order to identify those containing a tyrosine residue at the same position of the one targeted by BTZ 2 in BRD9 (Tyr106). Seven BRDs including PCAF, GCN5L2, TAF1 and TAF1L1 compiled with this requirement, and they also contain a targetable lysine in the BC loop.

The reactivity of BTZ against the former two BRDs was limited (9% and 0%, respectively), consistent with the low affinity of the non-covalent BSP for family I BRDs. However, labelling increased to 45% against TAF1(1) and TAF1(2), reaching 86% in the case of BRD7, a protein with high structural similarity to BRD9. These findings suggested that the presence of a tyrosine residue in proximity to the binding site could endow the corresponding BRD with higher reactivity against our probe when compared with lysine bearing BRDs.

To confirm this, we determined the kinetics of the covalent reaction between BTZ and BRDs, characterized by the presence of a targetable lysine (BRD4(1), BRD4(2), BRD3(2), BRD2(1)) or tyrosine (BRD9, CECR2) residue on the rim of the binding site. The reaction between a protein and its irreversible inhibitor should follow a second order kinetic model.\(^{[26]}\) However, the order of the reaction was reduced by applying the isolation method,\(^{[27]}\) where each BRD (1 μM) was incubated with a high concentration of BTZ (100 μM) and the decrease of unmodified protein was monitored over time with the RapidFire MS system. Each experiment was conducted in triplicate, as reported in Figure 6a for BRD4(1), and the average of the experimental values plotted as linear trends as shown in Figure 6b (for additional data see Supporting Information – Section 5). Since the experimental conditions did not allow the discrimination between reversible and irreversible binding to the kinetics of the process, a cumulative constant \(k_{\text{app}}\) was calculated for each reaction. Comparing the different \(k_{\text{app}}\) values confirmed the dramatic effect that changing the reactive amino acid had on the overall reaction rate (Figure 6b).

It should be highlighted that CECR2 \(k_{\text{app}} = 12.6 \times 10^4 \text{ s}^{-1}\) is characterized by the presence of two tyrosine residues, one in a position corresponding to Lys91 in BRD4(1) and the other one in a position corresponding to Tyr106 in BRD9. Taken together, we describe a broad spectrum probe that could covalently modify BRDs belonging to different sub-families.

**Figure 4.** Mapping the sites of covalent modification via LC-MS/M BTZ (2) was docked within the binding site of BRD4(1) (left) and then superimposed to the same pocket in BRD3(2) (right). Two lysines, K91 and K336, were found to be near the reactive dichlorotriazine warhead, thus suggesting they would be the expected site of covalent interaction. The predictions made by docking were near the reactive dichlorotriazine warhead, thus suggesting they would be the same pocket in BRD3(2) (right). Two lysines, K91 and K336, were found to be near the reactive dichlorotriazine warhead, thus suggesting they would be the expected site of covalent interaction. The predictions made by docking were subsequently confirmed by LC-MS/MS peptide mapping.

**Figure 5.** Mapping the sites of covalent modification via crystallographic techniques Crystal structure of the covalent complex formed by BTZ (2) and BRD9. The site of modification in this case was found to be a tyrosine (Tyr106) at the end of the AZ loop. The probe was reported to reversibly interact with the binding site of one BRD and form a covalent bond with the nucleophilic residue on an adjacent BRD, thus creating an interlocked dimer.
COMMUNICATION

Figure 6. BTZ binding kinetics against BRDs belonging to different sub-families a) BRD4(1) (1 μM) was incubated with BTZ (100 μM) and the amount of unmodified protein monitored over time as a function of the area (A) of the peak registered using a RapidFire low-injection QToF mass spectrometer. The experiment was run in triplicate in order to determine the kinetic rate constant of the overall process. b) Comparing the linear graphs obtained in the previous kinetic experiments allowed us to rank the relative reactivity of lysine (BRD4(1), BRD4(2), BRD3(2) and BRD2(1)) and tyrosine (BRD9 and CECR2) containing BRDs, indicating preferential binding of BTZ to CECR2

In addition, insightful information on the amino acid involved in the binding could be derived by co-crystallization or LC-MS/MS mapping. To validate the applicability of bromotriazine (2) as a chemical proteomics tool, recombinant BRD4(1) was incubated with the covalent probe and the obtained covalent complex subjected to a copper-catalyzed click chemistry reaction. When an azido-dye (Cyanine 5.5-IR dye) was used as the cycloaddition partner, the truncated protein appeared as a bright spot on polyacrylamide gel. When biotin azide was used in place of the azido-dye, BRD4(1) was successfully immobilized on Streptavidin-coated beads and then eluted from the resin by thermal denaturation. A covalent non-clickable analogue of bromotriazine (2b) was used in both experiments as a control since it lacked the alkyne functional handle necessary to react with the azide moiety on the dye or biotin tag (Figure 7).

Encouraged by these in vitro results, chemical proteomics experiments were conducted in K-562 chronic myelogenous leukemia cell line and in THP-1 monocytic cell line, as they both express BRD4 and BRD2 at a high endogenous level.[28] Promising results were obtained in particular after pull down experiments were conducted on the THP-1 cell line. THP-1 whole cell lysates were incubated with increasing concentrations of probe 2 (10, 25 and 100 μM) at 4 °C. Samples were taken at 1 h, 4 h, 8 h and 24 h; an additional time point was taken after 5 minutes of incubation in order to highlight any unspecific pull down that could be ascribed to the probe’s intrinsic reactivity more than to a genuine interaction with the desired target (Figure 8). Biotin azide was added to each sample after protein denaturation and Cu-AAC catalyzed click reaction was performed under previously reported conditions.[29] After excess reagents were removed by acetone precipitation, the total protein concentration of each sample was measured before submitting them to overnight incubation with streptavidin derivatized agarose beads. Isolated proteins were eluted from the beads using a denaturing buffer at 55 °C for 30 minutes, separated by SDS-PAGE and visualized by silver staining and Western blotting using anti-BRD4 and anti-BRD2 antibodies. Analysis of the silver stained and Western blots highlighted a direct correlation between the total amount of protein pulled down from the THP-1 lysates, the incubation time and the concentration of the probes. According to the silver stain and the subsequent Western blot against BRD4, BTZ (2) reacted quickly and in a non-selective, non-concentration dependent fashion as soon as it was added to the lysates (5 min). However, the direct relationship between probe concentration and amount of protein pulled down was reinstated after 4 hours of incubation. On the other hand, the Western blot against BRD2 showed a negligible, unselective pull down after 5 min of incubation, while a correlation between the amount of pull-down protein and probe concentration was maintained from 1 h to 24 h.

Figure 7. Fluorescent labelling and enrichment experiment conducted on recombinant BRD4(1) using BTZ (2) and its non-clickable analogue (2b). Recombinant BRD4(1) (1 mg/mL) was incubated with BTZ (100 μM) or its non-clickable analogue 2b (100 μM) for 1.5 hours at 37 °C. The obtained samples were reacted with an azido-dye (cyanine 5.5) (top) or biotin-azide (bottom) under copper-catalyzed alkyne azide cycloaddition (CuAAC) conditions, in order to selectively visualize the formed BRD4(1)-BTZ 2 covalent adduct under IR irradiation (top) or to pull down the obtained complex on Streptavidin beads (bottom).
The DMSO controls showed that no unspecific pull down occurred at any point in the case of BRD2, while a small amount of BRD4 was absorbed on the beads, and it was likely to contribute to the bands produced at the 5 min time point.

In a series of subsequent pull-down experiments aimed at discriminating between specific binding against non-specific absorption and reactivity, the formation of BRD-BTZ (2) covalent adducts was competed by preincubation of THP-1 whole cell lysates with 100 μM BTZ non-clickable analogue (2b) or 100 μM JQ1(+), a known BET bromodomains inhibitor. A biotin handle was added by click chemistry and the obtained samples were pulled down on NeutrAvidin beads. Although the MS/MS analysis of the competition experiments did not show a significant enrichment of BRDs pull down in the non-competed samples (See Supporting Information – Supplemental Figure 9), possibly because of the low concentration of probe required by the assay and the generally low levels of BRD pull down, these experiments highlighted a series of potentially interesting BTZ off-targets. In particular, Thymosin beta-4 (TMSB4X), a protein involved in cell proliferation and migration identified as a biomarker of malignancy in solid tumors, was enriched by treatment with BTZ (2) and selectively competed by pre-treatment with both 2b and JQ1(+), thus suggesting that BTZ (2) selectively bound and labeled this protein (See Supporting Information Section 1.6).

The initial attempts to selectively visualize the covalent BRD-BTZ adducts formed after incubation of K-562 cell lysates with BTZ (2) using Cy 5.5 dye proved to be extremely challenging, possibly due to the promiscuity of the probe (see Supporting Information - Figure 4).

However, the MS/MS analysis conducted on pull-down samples obtained by incubating non-transfected K-562 cell lysates with 5, 50, and 100 μM BTZ (2), respectively (See Supporting Information – Section 1.4), and then appending a biotin handle on the formed covalent complexes by CuAAC, showed a significant enrichment of BRDs and BRD associated proteins in the higher concentration samples, compared to the lowest concentration one (Figure 9).

In conclusion, we designed and synthesized a covalent probe, BTZ (2), against bromodomain containing proteins. Our results highlight the feasibility of selectively targeting non-catalytic amino acids on the rim of the ligand binding site by correctly orienting the chosen electrophilic moiety towards the desired nucleophilic residue, as recently reported by Zhao et al. in the case of protein kinases. In particular, an unprecedented selective labelling of non-catalytic and highly conserved lysines of purified bromodomains was reported. We also discovered the previously unreported ability of a 2,4-dichlorotriazine electrophile to label tyrosine residues and the surprising dimerization process induced by such covalent interaction with BRD9 that broadens the prospective for future design of covalent and selective inhibitors. This may apply not only to the bromodomain family, but to alternative cellular targets characterized by the presence of a lysine and/or tyrosine residue in the proximity of the binding site.

Acknowledgements

The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada, Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck KGaA Darmstadt Germany, MSD, Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and Wellcome [106169/ZZ14/Z]. We thank the Medical Research Council for
funding this research through the "Proximity to Industry" grant scheme and Dr. Suzanne Muller-Knapp for insightful conversations.

**Keywords:** Activity-based protein profiling, epigenetics, bromodomain containing proteins, covalent probes, chemical proteomics.

[1] Cravatt, B. F.; Wright, A. T. & Kozarich, J. W. *Annu. Rev. Biochem.* 2008, 77, 383–414.
[2] Li, N.; Overkleeft, H. S. & Florea, B. I. *Curr. Opin. Chem. Biol.* 2012, 16, 227–233.
[3] Niphovic, M. J. & Cravatt, B. F. *Annu. Rev. Biochem.* 2014, 83, 341–377.
[4] Adibekian, A.; Martin, B.; Chang, J.; Hsu, K.; Tsuboi, K.; Bachovchin, D.; Speers, A.; Brown, S.; Spencer, T.; Fernandez-Vega, V.; Ferguson, J.; Hodder, P.; Rosen, H. & Cravatt, B. Confirming Target Engagement for Reversible Inhibitors in Vivo by Kinetically Tuned Activity-Based Probes. *J. Am. Chem. Soc.* 2012, 134, 10345–10348.
[5] Lanning, B.; Whitby, L.; Dix, M.; Douhan, J.; Gilbert, A.; Hett, E.; Johnson, T.; Joslyn, C.; Kith, J.; Niessen, S.; Roberts, L.; Schnute, M.; Wang, C.; Holce, J.; Wei, B.; Whiteday, L.; Hayward, M.; Cravatt, B. Nat. Chem. Biol. 2014, 10, 760-767.
[6] Simon, G. M. & Cravatt, B. F. *J. Biol. Chem.* 2014, 289, 11051-11055.
[7] Li, N.; Overkleeft, H. S. & Florea, B. I. *Curr. Opin. Chem. Biol.* 2012, 16, 227–233.
[8] Davis; I., Vedadi, M. & Jin, J. *ChemMedChem* 2011, 6, 141–148.
[9] Luz, F.; Barron, R. W.; Harrington, R.; Bisch, O. & Golding, B. *Proc. Natl. Acad. Sci.* 2011, 108, 1069–1078.
[10] Lubin, A. S.; Rueda-Zubiaurre, A.; Matthews, H.; Baumann, H.; Fisher, F. R.; Morales-Sanfrutos, J.; Hadavizadeh, K. S.; Nardella, F.; Tale, E. W.; Baum, J.; Scherf, A. & Fuchter, M. J. *ACS Infect. Dis.* 2018, 4, 523–530.
[11] Konze, K.; Pattenden, S.; Liu, F.; Barsyte-Lovejoy, D.; Li, F.; Simon, J., Davis, I., Vedadi, M. & Jin, J. *ChemMedChem* 2014, 9, 549-553.
[12] Zhu, B.; Zhang, H.; Pan, S.; Wang, C.; Ge, J.; Lee, J. & Yao, S. *Chem. – Eur. J.* 2016, 22, 7824-7836.
[13] Schiedel, M.; Rumpf, T.; Karaman, B.; Lehoczky, A.; Gerhardt, S.; Ovldi, J.; Sippl, W.; Einsle, O. & Jung, M. *Angew. Chem. Int. Ed.* 2016, 55, 2252-2256.
[14] Allsop, J. P.; Zambrano-Castro, D.; Abegg, D.; Barmgarten, S.; Tallant, C.; Muller, S.; Adibekian, A. & Wissing, N. *Angew. Chem. Int. Ed.* 2015, 54, 6057–6061.
[15] Josling, G. A.; Selvarajah, S. A.; Petter, M. & Duffy, M. F. *Genes* 2012, 3, 320–343.
1. Chemical Proteomics Experiments .......................................................................................................................... 2
  1.1 In gel visualization of recombinant BRD4(1) using Cy 5.5 IR dye ........................................................................... 2
  1.2 Pull down of recombinant BRD4(1) using Biotin-azide ......................................................................................... 3
  1.3 Attempted in gel visualization of BRD4 in non-transfected K-562 cell lysates using Cy 5.5 IR dye .................. 4
  1.4 Pull down of BRD4 from non-transfected K-562 cell lysates ................................................................................. 6

  Western Blot – Anti-BRD4 and Anti-Biotin monoclonal antibodies ....................................................................... 6
  Sample Preparation for mass spectrometry analysis .................................................................................................. 7
  LC-MS/MS analysis ...................................................................................................................................................... 7

  1.5 Pull down of BRDs from non-transfected THP-1 cell lysates .................................................................................. 8
  Optimised procedure for chemical proteomics experiments: ..................................................................................... 8
  Time and Concentration Screening – WB BRD4 ........................................................................................................ 9
  Time and Concentration Screening – WB BRD2 .......................................................................................................... 9
  1.6 Quantitative Chemical Proteomics .......................................................................................................................... 10

2. Crystallographic Data .................................................................................................................................................. 12
  2.1 Materials and Methods ................................................................................................................................................ 12

  Cloning, protein expression and purification of bromodomains .................................................................................. 12
  2.2 Crystallographic data collection and refinement statistics ...................................................................................... 13

3. Docking Data .................................................................................................................................................................. 14
  3.1 BTZ (2) and BRD4(1) ................................................................................................................................................. 14

4. Synthetic Procedures and NMR Spectra ..................................................................................................................... 15
  Synthetic Scheme A ........................................................................................................................................................... 15
  Synthetic Scheme B ........................................................................................................................................................... 16
  3,6-Dichloropyridazin-4-amine (4) ................................................................................................................................. 17
  6-Chloro-3-hydrazinylpyridazin-4-amine (5) ................................................................................................................... 17
  6-Chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (6) ............................................................................................. 17
  Tert-butyl (6-chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (7). .............................................................. 17
  Tert-butyl (3-methyl-6-(4-methyl-3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (9) ......................... 18
  But-3-yn-1-yl carbobenochloridate (11) ...................................................................................................................... 18
  But-3-yn-1-yl (6-(3-nitro-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (12) ................. 19
  But-3-yn-1-yl (6-(3-amino-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (13) ............. 19
  But-3-yn-1-yl (6-((4,6-dichloro-1,3,5-triazin-2-yl) amino)-4-methylphenyl)-3-methyl-[1,2,4] triazolo [4,3-b]pyridazin-8-yl) carbamate hydrochloride (BTZ-2) .............................................................................................. 19
  Ethyl (6-(3-nitro-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (15) .......................... 20
  Ethyl (6-(3-amino-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (16) ......................... 21
  Ethyl (6-((4,6-dichloro-1,3,5-triazin-2-yl) amino)-4-methylphenyl)-3-methyl-[1,2,4] triazolo [4,3-b]pyridazin-8- yl)carbamate hydrochloride (2b) ...................................................................................................................... 21

NMR Spectra .................................................................................................................................................................... 22

5. Rapidfire Mass Spectrometry ........................................................................................................................................ 48
  5.1 Recombinant Bromodomains Sequences ................................................................................................................... 49
  5.2 Kinetic Experiments: .................................................................................................................................................. 52
    5.2.1 BTZ 100 µM and BRD4(1) 1 µM ............................................................................................................................ 52
    5.2.2 BTZ 100 µM and BRD4(2) 1 µM ............................................................................................................................ 54
    5.2.3 BTZ 100 µM and BRD3(2) 1 µM ............................................................................................................................ 56
    5.2.4 BTZ 100 µM and BRD2(1) 1 µM ............................................................................................................................ 58
    5.2.5 BTZ 100 µM and BRD9 1 µM ............................................................................................................................ 60
    5.2.6 BTZ 100 µM and ECECR 1 µM ............................................................................................................................ 62

6. LC-MS/MS Analysis ....................................................................................................................................................... 64
  6.1 BTZ & BRD4(1) – Lysine 91 ........................................................................................................................................ 66
  6.2 BTZ & BRD3(2) – Lysine 336 ..................................................................................................................................... 67
1. Chemical Proteomics Experiments

Unless otherwise specified, reagents, solvents, buffers and kits were purchased from Bio-Rad®, Sigma-Aldrich®, Thermofischer®, Lumiprobe® and used without any further purification and/or alteration. The reported procedures were adapted from previously published protocols.¹²

1.1 In gel visualization of recombinant BRD4(1) using Cy 5.5 IR dye.

Sample A: 3.8 µL of a 20 µM solution of Bromotriazine (2) in DMSO were added to 146 µL of a solution of the truncated form of BRD4(1) (1.0 mg/mL) in PBS to a final concentration of 0.5 µM.

Sample B: 3.8 µL of a 20 µM solution of BTZ-Non clickable analogue (2b) in DMSO were added to 146 µL of a solution of the truncated form of BRD4(1) (1.0 mg/mL) in PBS to a final concentration of 0.5 µM.

Samples A and B were incubated in an Eppendorf Thermomixer™ at 37°C shaking at 300 rpm for 1.5 hours. A 5 µL aliquot was collected from each sample, diluted to 10 µL and stored at -80°C. The remaining samples were then transferred into new tubes in 40 µL aliquots for the click reaction step. 1 µL of a 1.25 mM solution of Cy5.5 azido-dye (a molecule endowed with far red/near infrared emission) in PBS was added to samples Acetone P, A1 and B1 to a final dye concentration of approximately 25 µM. 1 µL of a 0.5 mM solution of Cy5.5 azido-dye in PBS was added to samples A2 and B2 to a final dye concentration of approximately 10 µM, and 1 µL of a 0.25 mM solution of Cy5.5 azido-dye in PBS was added to samples A3 and B3, to a final dye concentration of approximately 5 µM. 5 µL of 10% SDS were added and the solution mixed by vortexing before adding 5 µL of the catalyst mix containing 3 parts of TBTA (1.7 mM), 1 part of copper sulfate (50 mM) and 1 part of TCEP (50 mM). The obtained samples were incubated for 1.5 hour at 37°C shaking at 300 rpm. 0.5 µL of a 500 mM solution of EDTA were added and the samples precipitated by the addition of cold acetone (Acetone P) or by chloroform/methanol extraction (A1-A3 and B1-B3). The dried precipitated pellets were resuspended in 30 µL of 10%SDS and run on a polyacrylamide gel. The fluorescent gel was visualized using an Odyssey® CLx IR scanner (Supplemental Figure 1) and the protein content confirmed by Coomassie Brilliant Blue staining (Supplemental Figure 2).

Supplemental Figure 1. LI-COR assisted visualisation of the recombinant BRD4(1)-BTZ(2) complex tagged with CY 5.5 IR dye.

Supplemental Figure 2. Coomassie staining of the gel reported in Supplemental figure 1

¹ Mackinnon, A. L. & Taunton, J. Target Identification by Diazirine Photo-Cross-linking and Click Chemistry. Curr. Protoc. Chem. Biol. 2009, 1, 55-73.
² Hela, P. W.; Wright, M. H.; Thinon, E. & Tate E. W. Multifunctional protein labeling via enzymatic N-terminal tagging and elaboration by click chemistry. Nature Protocols 2012, 7, 105-107
1.2 Pull down of recombinant BRD4(1) using Biotin-azide.

Sample A: 12.5 µL of a 200 µM solution of Bromotriazine (2) in DMSO were added to 475 µL of a solution of the truncated form of BRD4(1) (0.5 mg/mL) in PBS to a final concentration of 5 µM.

Sample B: 12.5 µL of a 200 µM solution of BTZ-Non clickable analogue (2b) in DMSO were added to 475 µL of a solution of the truncated form of BRD4(1) (0.5 mg/mL) in PBS to a final concentration of 5 µM.

Sample C was treated as sample A until the click chemistry step.

Samples A-C were incubated in an Eppendorf Thermomixer™ at 37°C shaking at 300 rpm for 1.5 hours. The proteins were precipitated by incubating the samples in acetone for 2 hours at -26°C and the obtained pellets re-dissolved in 97.5 µL of PBS and 12.5 µL of 10% SDS via sonication. 2.5 µL of a 50 mM solution of Biotin-azide in PBS were added to Samples A and B, while only PBS was added to sample C. 12.5 µL of the catalyst mix containing 3 parts of TBTA (17 mM), 1 part of copper sulfate (500 mM) and 1 part of TCEP (500 mM) was added to each sample and the reaction incubated for 30 minutes at 37°C shaking at 300 rpm. 5 µL aliquot was removed at this point, 1 µL of Laemmli 4x was added and the aliquots were snap frozen and kept at -80°C (Input Samples to Affinity Precipitation). The remaining samples were precipitated by adding 1 mL of cold acetone to every sample and incubating the resulting suspension at -26°C overnight. The precipitated sediment was spun at 20000 x g for 10 minutes at 4°C, the supernatant was removed by aspiration and 0.5 mL of cold acetone were added. The samples were sonicated to break up and disperse the precipitated proteins and then returned to -80°C for 10 minutes. The supernatant was removed and the obtained pellets dried at the air before being redissolved in 50 µL of % SDS in PBS via sonication. The obtained solutions were diluted up by adding 0.5 mL of affinity purification buffer (50 mM HEPES, 100 mM NaCl, 1% NP-40). 20 µL aliquots were removed from each samples, added of 4 µL of Laemmli 4x and kept at -80°C (Efficiency of Precipitation and Resolubilization). 50 µL of a slurry of Streptavidin coated magnetic beads was added to each sample and they obtained suspension shaken at 700 rpm at room temperature for 2 hours. The beads were sedimented using a magnetic tube-holders, the supernatant was removed and 20 µL aliquots were kept at -80°C to be run on the gel as Flow Through After Incubation with Beads. The beads were washed twice (50 mM HEPES, 500 mM NaCl, 1% NP-40 in PBS) and eluted first using 50 µL of a 2 mM solution of free Biotin in 1 % NP-40 in PBS and then by boiling the streptavidin off at 90°C for 5 minutes. The samples were run on a pre-packed 4-12% polyacrylamide gel and stained using Coomassie Brilliant Blue staining solution.

Sample A, B and C were respectively:

A = Incubation with Bromotriazine (BTZ - 2) and CuAAC with azido-biotin

B = Incubation with Bromotriazine analogue without the clickable handle (2b) and CuAAC with azido-biotin

C = Incubation with Bromotriazine (1) and CuAAC without azido-biotin.

Supplemental Figure 3.

Coomassie staining of the gel obtained from the pull down of recombinant BRD4 covalently bond to BTZ(2) using Streptavidin coated magnetic beads. The extra bands appearing at higher molecular weights than the pure recombinant protein (15083 Da) were due to protein aggregation and precipitation induced after the incubation with the click reagents. In fact, LC-MS/MS analysis confirmed that all bands corresponded to recombinant BRD4(1).
1.3 Attempted in gel visualization of BRD4 in non transfected K-562 cell lysates using Cy 5.5 IR dye.

Sample A: 5 µL of a 20 µM solution of Bromotriazine (1) in DMSO were added to 195 µL of K-562 cell-lysates (1.0 mg/mL) in LBII (HEPES 50 mM, 10% glycerol, KCl 500 mM, TCEP 1 mM, NP40 1%, Roche® Protease Inhibitor cocktail), to a final concentration of 0.5 µM.

Sample B: 5 µL of a 20 µM solution of BTZ-Non clickable analogue (2b) in DMSO were added to 195 µL K-562 cell-lysates (1.0 mg/mL) in LBII, to a final concentration of 0.5 uM.

Samples A and B were incubated in an Eppendorf Thermomixer™ at 37°C shaking at 300 rpm for 1.5 hours. A 5 µL aliquot was collected from each sample, diluted to 20 µL and stored at -80°C (Protein before Incubation). 180 µL of both sample A and sample B were then transferred into new tubes for the click reaction step. Addition of 10% SDS was avoided at this point since it induced protein precipitation in LBII. 4 µL of a 0.25 mM solution of Cy5.5 azido-dye in PBS were added to both samples to a final dye concentration of approximately 5 µM. 20 µL of the catalyst mix containing 3 parts of TBTA (1.7 mM), 1 part of copper sulfate (50 mM) and 1 part of TCEP (50 mM) were added and the obtained samples were incubated for 1 hour at 37°C shaking at 300 rpm. 0.9 µL of a 5 mM solution of EDTA were added to half of the samples. Protein precipitation was performed in cold acetone or by chloroform/methanol extraction. The dried precipitated pellets were resuspended in 20 µL of 10%SDS and diluted to 80 µL with PBS. To half of the 15 µL aliquots for the gel, 1 µL of DTT was added and the obtained gel visualized using an Odyssey® CLx IR scanner.

**Supplemental Figure 4.** LI-COR scan of K-562 cell lysates treated with BTZ (2) and its non clickable analogue 2b. The gel showed selective labelling of samples incubated with the clickable compound, BTZ (2), over those incubated with compound 2b. However, the formation of smears and the evenly spread IR fluorescence indicated non-selective and unspecific binding of BTZ (2) to an extensive portion of the proteome.
1.4 Pull down of BRD4 from non-transfected K-562 cell lysates

Three different concentrations of Bromotriazine (2) were incubated with K-562 lysates (5mg/mL) in order to qualitatively and quantitatively study the changes protein pull down with the increasing concentration of probe. 

**Sample A1:** 10 µL of a 100 µM solution of Bromotriazine (2) in PBS (0.5% DMSO) were added to 200 µL of K-562 cell lysates (5 mg/mL) in LBI to a final concentration of 5 µM. 

**Sample A2:** 10 µL of a 1 mM solution of Bromotriazine (2) in PBS (5% DMSO) were added to 200 µL of K-562 cell lysates (5 mg/mL) in LBI to a final concentration of 50 µM. 

**Sample A3:** 10 µL of a 2 mM solution of Bromotriazine (2) in PBS (20% DMSO) were added to 200 µL of K-562 cell lysates (5 mg/mL) in LBI to a final concentration of 100 µM. 

**Control:** 10 µL of a 100 µM solution of Bromotriazine (2) in PBS (0.5% DMSO) were added to 200 µL of K-562 cell lysates (5 mg/mL) in LBI to a final concentration of 5 µM. 

Samples A1-A3 were incubated in an Eppendorf Thermomixer™ at 37°C shaking at 300 rpm for 1.5 hours. 10 µL aliquots were taken for the final gel run (Incubation with Bromotriazine). The lysates were divided into 100 µL aliquots and precipitated by adding cold acetone and incubating the samples for 2 hours at -26°C. The obtained pellets were re-dissolved in 20 µL of 10% SDS via sonication and diluted to 110 µL with PBS. 5 µL samples were taken from each tube to give a final volume of 10 µL for each concentration (Efficiency of Precipitation and Resolubilization). 2.5 µL of a 50 mM solution of Biotin-azide in PBS and 12.5 µL of the catalyst mix containing 3 parts of TBTA (17 mM), 1 part of copper sulfate (500 mM) and 1 part of TCEP (500 mM) were added to each sample, except from the control sample. The reactions were incubated for 1 hour shaking at 300 rpm, at 37 °C and room temperature respectively. 10 µL aliquots were taken at this point, 1 µL of Laemmli 4x was added and the aliquots snap frozen and kept at -80°C (Input Samples to Affinity Precipitation). 

The remaining samples were precipitated by adding 1 mL of cold acetone to each sample and incubating the resulting suspension at -26°C overnight. The precipitated sediment was spun at 20000 x g for 10 minutes at 4°C, the supernatant was removed by aspiration and 1 mL of cold acetone was added. Samples were sonicated to break up the pellets and disperse the precipitated proteins, they were then returned to -80°C for 10 minutes. The supernatant was removed and the obtained pellets dried at the air before being redissolved in 10 µL of 10% SDS and 10 10 µL of PBS via sonication. The obtained solutions were diluted up by adding 0.5 mL in affinity purification buffer (50 mM HEPES, 100 mM NaCl, 1% NP-40). 20 µL aliquots were removed from each samples, 4 µL of Laemmli 4x were added and kept at -80°C (Efficiency of 2nd Precipitation and Resolubilization). 175 µL of a slur of Streptavidin coated magnetic beads in affinity purification buffer was added to each sample and the obtained suspension incubated at 4 °C for 2 hours. The beads were sedimented using a magnetic tube-holders, the supernatant was removed and 20 µL aliquots were kept at -80°C to be run on the gel as Flow Through After Incubation with Beads. The beads were washed twice (50 mM HEPES, 500 mM NaCl, 1% NP-40 in PBS) and eluted by boiling the streptavidin off in Laemmli buffer at 90°C for 5 minutes. The samples were run on a pre-packed 4-12% polyacrylamide gel. The proteins were transferred on a membrane and blocked with 5-6% blocking agent in PBS for 15 minutes. The membrane was then incubated with an in house prepared anti-BRD4 (mouse) antibody at 4 °C (1/500), washed twice with PBST and incubated with a secondary antibody bearing an excitable Li-COR sensitive dye. Unfortunately, no band corresponding to full length BRD4 could be detected and even the low molecular weight bands at around 80 KDa, which could be truncated forms of BRD4, seemed to disappear after the click reaction step (Supplemental Figure 5). However, stripping the original membrane and blotting it against an anti-Biotin antibody demonstrated the efficiency of the click reaction. In this case, the amount of pull down on the beads was proportional to the concentration of Bromotriazine (2), despite no characteristic band corresponding to BRD4 could be identified (Supplemental Figure 5).
Western Blot – Anti-BRD4 and Anti-Biotin monoclonal antibodies:

- K-562 5 mg/mL (100 µL)

Supplemental Figure 5. First attempt to pull down BRD4 from non-transfected K-562 cell lysates using BTZ(2). The top membrane was incubated with anti-BRD4 antibodies and visualised using a secondary antibody bearing an excitable Li-COR sensitive dye. Since no BRD4 could be detected after the click reaction step, the membrane was subsequently stripped and blotted against biotin to investigate the efficiency of the click reaction – bottom membrane.

The proteomics analysis of the protein boiled off the beads, especially at 50 and 100 µM probe (2) concentration highlighted that almost 30% of pulled down proteins were nuclear proteins.
Sample Preparation for mass spectrometry analysis

After incubation of K-562 lysates with 100 µM, 50 µM, or 5µM BTZ (2), a biotin handle was appended to the covalent BTZ-target proteins adducts by CuAAC. Pull down of the covalent complexes was performed and the enriched extracts were subjected the gel-aided sample preparation (GASP) protocol as described previously (Fischer R et al., Proteomics 2015 Apr;15(7):1224-9, Sepil I et al., Molecular & Cellular Proteomics October 4, 2018, mcp.RA118.000831). In brief, samples were reduced with 50 mM DTT for ~ 20 minutes and subsequently mixed with an equal volume of 40% acrylamide/Bis solution (37.5:1. National Diagnostics), followed by a 30 min incubation at room temperature for cysteine propionamidylation. 5 l TEMED and 5 l 10% APS were added for acrylamide polymerization. Resulting gel plugs were shredded by centrifugation through a Spin-X filter insert without membrane (CLS9301, Sigma/Corning). Gel pieces were fixed in 40 % ethanol / 5 % acetic acid before two successive rounds of buffer exchange with 1.5 M Urea, 0.5 M Thiourea and 50 mM ammonium bicarbonate which were removed with acetonitrile. Immobilized proteins were digested with trypsin (Promega) overnight at 37°C. Peptides were extracted with acetonitrile, dried before desalting using Sola SPE columns (Thermo Fischer) and resuspended in 2% ACN, 0.1 % FA buffer. All samples were stored at -20°C until LC-MS/MS analysis.

LC-MS/MS analysis

Samples were analysed on a liquid chromatography tandem mass spectrometry (LC-MS/MS) platform consisting of a Dionex Ultimate 3000 UPLC system coupled to a Q-Exactive mass spectrometer (Thermo Fisher) essentially as described (Sepil I et al., Molecular & Cellular Proteomics October 4, 2018, mcp.RA118.000831).

Data processing and analysis

Processing of MS Data RAW files were imported into Progenesis QIP (v 3.0.6039.34628), and MS/MS spectra exported as mascot generic files (.mgf) files using the 200 most intense peaks without deconvolution for searching. Datasets were searched in Mascot (v2.5.1) using the following parameters: 10 ppm precursor mass accuracy, 0.05 Da fragment mass accuracy, Oxidation (M), Deamidation (N, Q) and Propionamide (K) as variable modifications, Propionamide (C) as a fixed modification, and two missed cleavage sites. We applied 1% FDR at peptide level (both search engines use a target-decoy method for FDR estimation) and an additional Mascot ion score cutoff of 20 before importing search results into Progenesis, where protein quantification was calculated using the Top3 method. We used Perseus software (v 1.6.0.2) to identify BTZ2 enriched proteins applying a student t test, and p values were corrected for multiple testing using permutation FDR with default settings in Perseus. The data was plotted as a volcano plot (Fig. 9) using 0.05 FDR and 0.1 S0.
1.5 Pull down of BRDs from non-transfected THP-1 cell lysates

Optimised procedure for chemical proteomics experiments:

THP-1 lysates were used in the proteomics experiments, since they constitutively express significant amounts of both BRD4 and BRD2. 30 mg of lysate was diluted to a final concentration of 4 mg/mL in base buffer (50 mM HEPES, 150 mM NaCl, 15 mM MgCl₂ and 5% Glycerol) and spun down at 18000rcf for 20 minutes at 4 °C. The supernatant was separated from the precipitate, Halt™ cocktail inhibitor and 10% NP40 (to final concentration of 0.4%) were added, together with the appropriate amount of compound 2 (in DMSO) to reach a final concentration of 10, 25 and 100 µM, respectively. The obtained samples were incubated at 4 °C up to 16 hours. 1 mL aliquots were taken from each solution at specific time points: 5 min, 1h, 4h, 8h, and 16h, snap frozen and preserved overnight. After the samples were gently thawed, 10% SDS was added to each tube to a final concentration of 1% SDS and the content of each tube vigorously mixed in order to denature the proteins and expose the alkyne moiety of the covalently bound probes. 25 µL of biotin azide 5M stock in DMSO was added, followed by 25 µL of the catalyst mix, which was previously prepared by adding 15 µL TBTA (1.7 mM), 50 µL of TCEP (50 mM), and 50 µL of copper sulfate (50 mM) to 0.135 mL of a 4:1 t-Butanol:DMSO solution. The obtained light green solutions were incubated at 37 °C for 2 hours in an Eppendorf ThermoMixer®. The protein content was precipitated on cold acetone overnight, centrifuged at 1250 rcf for 10 min at 4 °C, and the obtained pellets reconstituted in 150 µL of 1% SDS in base buffer. The complete re-dissolution of the proteins contained in the pellets was obtained by probe sonication (20 seconds cycles – 50% amplitude), after which the concentration of each sample was standardised by Pierce™ 660nm Protein Assay. An adequate amount of lysates, calculated in order to keep the total amount of sample submitted to the following step constant, was added to a slurry containing 50 mg of Agarose beads previously reconstituted in base buffer and 1% SDS. Samples were incubated overnight on a rocking platform at room temperature. The beads were then loaded on Mobicols and washed twice with 10% NP40 and 0.1% DTT in base buffer, followed by a single wash with 0.1% DTT. The excess buffer was removed from the columns by gentle centrifuge spin and the pull down proteins eluted in 72 µL of 2x LDS and 10 mM DTT by heating the samples at 55 °C for 30 minutes in an Eppendorf ThermoMixer®. 5 µL of a iodoacetamide (IAA) stock solution (200 mg/mL) was added and each sample run on three separate polyacrylamide gel, which were then submitted to silver staining (Supplemental Figure 6), following to the procedure reported in the Pierce™ Silver Stain Kit, and Western Blotting using anti-BRD4 (CSL BRD4 (E2A7X) Rabbit mAb #13440) and anti-BRD2 (CSL BRD2 (D89B4) Rabbit mAb #5848) monoclonal antibodies, respectively. Both WB where developed on photographic film after incubation with HRP-labelled secondary antibody (Supplemental Figures 7-8).

Supplemental Figure 6. Silver staining of the gels obtained by incubating THP-1 cell lysates with cmpd (2)
**Time and Concentration Screening – WB BRD4**

Supplemental Figure 7. WB developed on photographic film of BRD4 pull down from non-transfected THP-1 lysates treated with increasing concentrations of cmpd 2 and 6.

**Time and Concentration Screening – WB BRD2**

Supplemental Figure 8. WB developed on photographic film of BRD2 pull down from non-transfected THP-1 lysates treated with increasing concentrations of BTZ 2
1.6 Quantitative Chemical Proteomics on THP-1 cell lysates

THP-1 lysate generation

THP-1 lysates were generated as described previously. In brief, THP-1 cell pellets were thawed on ice and resuspended twice the cell pellet volume with ice cold lysis buffer (50 mM HEPES at pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, 0.8% NP-40, 1X HALT protease inhibitor (Peirce Biotechnology). The resuspended cell pellet subjected to dounce homogenization and was spun at 800 x g for 10 min at 4 °C. The resulting supernatant (S₀.₈) was saved, while the pellet was further lysed using via pressure cycling, subjected to salt extraction, and treatment with benzonase. This further processed lysate was combined with the previously saved S₀.₈ lysate and used as input material for affinity enrichment experiments.

Affinity enrichment and compound competition experiments in THP-1 lysates.

For each affinity enrichment condition, 1 mL of THP-1 lysate (5 mg) was preincubated with either DMSO or 100 uM competition ligand for 1 hr at 4 °C in binding assay buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 0.4% NP-40). The preincubated lysates, were then incubated with either DMSO or 1 uM BTZ for 1 hr at 4 °C. Probe-modified proteins were enriched, digested, subjected to isobaric labeling using TMT tags and sample analysis using nano-LC-MS/MS as previous described.⁴

Supplemental Figure 9: Quantitative Proteomics Analysis of Competition Experiments using BTZ (2), its non-clickable analogue (2b), and a known BRD inhibitor (JQ1(+)). THP-1 cell lysates were incubated with 1 µM BTZ(2) at 4°C after preincubation with 100 µM non-clickable analogue (2b) or 100 µM JQ1(+), respectively. The control sample was preincubated with DMSO. BRD2, BRD3, BRD4 and BRDT were neither significantly enriched nor competed and could be detected only after performing targeted MS/M5 experiments, indicating that the probe-labeled amount of BET family members was very low. Significant amounts of TMSB4X and HSPE1 were enriched and competed by pre-treatment with both 2b and JQ1(+), thus indicating that these proteins could be potential BTZ (2) off-targets. Dashed lines indicate 50% competition (y-axis) or 2 fold enrichment (x-axis), plotted protein fold changes are median renormalized as described in supplementary Table 1.
Supplementary Table 1: Legend for BTZ chemoproteomics data.

| PROTEIN_AC            | Representative Uniprot protein accession number |
|-----------------------|-----------------------------------------------|
| GENE_NAME             | Entrez gene name associated with Uniprot protein accession number |
| DESCRIPTION           | Protein description |
| PROTEIN_PROB          | ProteinProphet-derived protein probability |
| SPECTRUM_COUNT        | Number of total redundant Mascot-derived peptide spectrum matches for this protein |
| Q_PEP                 | Number of unique peptide sequences for this protein that were used for protein quantitation (not shared with any other protein sequence in list of identified proteins) |
| Q_SPEC                | Number of unique spectra matched to this protein that were used for quantitation (not shared with any other protein sequence in list of identified proteins) |
| LOG10FC_JQ1           | Log10 fold change 100 uM JQ1(+) over 1 uM BTZ |
| ADJ_PV_JQ1            | Adjusted P-value 100 uM JQ1(+) over 1 uM BTZ, proteins with Adj_Pvalue =1 were excluded from analysis |
| LOG10FC_JQ1 MEDIAN normalized | as plotted in supplemental Fig 9: Log10 fold change 100 uM JQ1(+) over 1 uM BTZ, renormalized using the median log10 fold change for all proteins for (Log10 fold change 100uM JQ1(+) over 1 uM BTZ) |
| LOG10FC_CMPD2B        | Log10 fold change 100uM Compound 2b over 1 uM BTZ |
| ADJ_PV_CMPD2B         | Adjusted P-value 100uM Compound 2b over 1 uM BTZ, proteins with Adj_Pvalue =1 were excluded from analysis |
| LOG10FC_CMPD2B MEDIAN normalized | as plotted in supplemental Fig 9: Log10 fold change 100uM Compound 2b over 1 uM BTZ, renormalized using the median log10 fold change for all proteins for (Log10 fold change 100uM Compound 2b over 1 uM BTZ) |
| LOG10FC_NOPROBE       | Log10 fold change 0 uM BTZ over 1 uM BTZ |
| ADJ_PV_NOPROBE        | Adjusted P-value 0 uM BTZ over 1 uM BTZ, proteins with Adj_Pvalue =1 were excluded from analysis |
| LOG10FC_NOPROBE MEDIAN normalized | as plotted in supplemental Fig 9: Log10 fold change 0 uM BTZ over 1 uM BTZ, renormalized using the median log10 fold change for all proteins for (Log10 fold change 0 uM BTZ over 1 uM BTZ) |

(3) Gower C.; Thomas J.; Harrington E.; Murphy J.; Chang M.; Cornella-Taracido I.; Jain R.; Schirle M.; Maly D. Conversion of a Single Polypharmacological Agent into Selective Bivalent Inhibitors of Intracellular Kinase Activity ACS Chem. Biol. 2015, 11, 121-131.

(4) Thomas, J.; Brittain, S.; Lipps, J.; Llamas, L.; Jain, R.; Schirle, M. A Photoaffinity Labeling-Based Chemoproteomics Strategy for Unbiased Target Deconvolution of Small Molecule Drug Candidates. Methods Mol Biol. 2017, 1647, 1-18.
2. Crystallographic Data

2.1 Materials and Methods

Cloning, protein expression and purification of bromodomains
cDNA encoding reported human bromodomains were cloned, expressed and purified as previously described.5

Co-purification of BRD9 with bromotriazine (2)
Purified BRD9 (UniProt Q9H8M2, residues 14 – 134) was mixed with BTZ (2) in a molar ratio of 1:1.5 M and leave the mixture 3 h at room temperature. A further purification step was carried out using a size exclusion chromatography column in 20 mM Hepes pH 7.5, 350 mM NaCl. The co-eluted peak fraction of the complex was pooled, buffer exchanged in 20 mM Hepes pH 7.5, 100 mM and concentrated up to 15.2 mg/ml.

Crystallization
Aliquots of purified BRD9-BTZ (2) were set up for crystallization using a mosquito® crystallization robot (TTP Labtech, Royston UK). Coarse screens were typically setup onto Greiner 3-well plates using three different drop ratios of precipitant to protein per condition (200+100 nl, 150+150 nl and 100+200 nl). Initial hits were optimized further scaling up the drop sizes. All crystallizations were carried out using the sitting drop vapor diffusion method at 293.15 K. Crystals were grown by mixing 200 nl of the protein with 100 nl of reservoir solution containing 0.1M ammonium citrate pH 7, 30% PEG3350. Diffraction quality crystals grew within a few days and were cryo-protected using the well solution supplemented with additional 20 % ethylene glycol and flash frozen in liquid nitrogen.

Data Collection and Structure solution
Crystallographic data was collected at Diamond beamline I03 at a wavelength of 0.9762 Å. Indexing and integration was carried out using XDS6 and scaling was performed with AIMLESS7. Initial phases were calculated by molecular replacement with PHASER8 using the apo BRD9 structure (PDB IDs 3HME). Initial models were built by ARP/wARP9 followed by manual building in COOT10. Refinement was carried out in REFMAC511. GRADE (global phasing)12 was used to generate compound coordinates and cif files. Thermal motions from individual monomers of the octamer within the asymmetric unit were analyzed using TLSIN (REFMAC), and hydrogen atoms were included in late refinement cycles. All model validations were carried out using MolProbity13. Data collection and refinement statistics can be found in Supplemental Table 1. The model and structure factors have been deposited with PDB accession code: 5O4B.pdb

(5) Filippakopoulos, P.; Picuda, S.; Mangos, M.; Keates, T.; Lambert, J. P.; Barysye--Lovejoy, D.; Felletar, I.; Volkm, R.; Muller, S.; Pawson, T.; Gingras, A. C.; Arrowsmith, C. H.; Knapp, S., Histone recognition and large-scale structural analysis of the human bromodomain family. Cell 2012, 149 (1), 214-231.
(6) Kabsch, W. (2010). XDS. Acta Cryst. D66, 125-132.
(7) P.R.Evans (2011). An introduction to data reduction: space-group determination, scaling and intensity statistics, Acta Cryst. D67, 282-292
(8) A.J. McCoy, R.W. Grosse-Kunstleve, P.D. Adams, M.D. Winn, L.C. Storoni, and R.J. Read. (2007). Phaser crystallographic software. J Appl Crystallogr 40, 658-674.
(9) Langer G., Cohen, SX., Lamzin, VS. and Perrakis, A. (2008). Automated macromolecular model building for X-ray crystallography using ARP/wARP version 7. Nat. Protoc. 3(7):1171-9.
(10) Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132.
(11) Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. Sect. D 53 240–255.
(12) O. S. Smart, T. O. Womack, A. Sharff, C. Flensburg, P. Keller, W. Paciorek, C. Vonrhein, and G. Bricogne. (2011) Grade v1.102. Cambridge, United Kingdom, Global Phasing Ltd., http://www.globalphasing.com.
(13) Chen VB, Arendall WB 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, and Richardson DC (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Cryst D66:12-21
2.2 Crystallographic data collection and refinement statistics

Supplementary Table 2

| PROTEIN ID | BRD9 |
|------------|------|
| Ligand ID  | BTZ (2) |
| Ligand Structure |

Data collection

| Space Group | P 2 2 1 2 1 |
| Cell Dimensions | a,b,c (Å) | 60.02 138.40 140.08 |
| | α, β, γ (º) | 90.00 90.00 90.00 |
| Resolutiona (Å) | 29.55 (1.95) |
| Unique Observations | 85016 (11992) |
| Completeness (%) | 98.7 (96.5) |
| Redundancy | 6.0 (6.0) |
| Rsym or Rmerge | 0.057 (0.682) |
| f|f| |
| Wavelength (Å) | 0.9762 |
| Phasing | MR |

Refinement

| Rworkc / Rfree (%) | 18.83 / 22.63 |
| Number of atoms | protein / other / solvent | 7617 / 48 / 285 |
| B-Factors (Å²) | protein / other / solvent | 39.17 / 53.89 / 39.94 |
| R.M.S.D. Bond (Å) | 0.009 |
| R.M.S.D. Angle (º) | 1.126 |
| Ramachandran statistics | Favored (%) | 99.54 |
| | Outliers (%) | 0 |
| PDB ID | 5O4B.pdb |

---

a Highest resolution outer shell (in Å) shown in parentheses.
b \( R_{sym} = \frac{\sum|I|}{\langle I \rangle} / \frac{\sum|I|}{\Sigma} \)
c \( R_{work} = \frac{\sum|Fobs|}{\Sigma} / |Fcalc| / \Sigma|Fobs| \) where Fobs and Fcalc are the observed and calculated structure factors, respectively.  
d \( R_{free} \) was calculated with 5% of the data excluded from the refinement calculation.  
MR = Molecular Replacement
3. Docking Data

Docking experiments were performed using the Molsoft L.L.C.® ICM-Pro software and the crystal structure of BRD4(1) (PDB code: 4OGI).

3.1 BTZ (2) and BRD4(1)

The poses generated by docking BTZ (2) in the binding pocket of BRD4(1) and the corresponding scores are shown in Supplementary Figure 9 and Table 3.

![Supplementary Figure 10. Docking poses of BTZ (2) in the binding pocket of BRD4(1)](image)

**Supplementary Table 3**

| Conf Num | L.  | Score2 | Score  | Strain   | ResConf  | Steric | Torsion | Electro | Hbond | Hydroph | Surface      |
|----------|-----|--------|--------|----------|----------|--------|---------|---------|-------|----------|--------------|
| 4        | 0   | -31.376064 | -35.890552 | 4.442969 | BRD4_rec_rec1:1 | -29.702127 | 3 | 5.99894 | -7.193427 | -7.804401 | 15.081486   |
| 5        | 0   | -30.69519 | -36.719933 | 6.024614 | BRD4_rec_rec1:1 | -30.039587 | 3 | 6.052549 | -7.210052 | -8.149517 | 14.968774  |
| 1        | -1  | -22.04709 | -30.86962 | 7.174852 | BRD4_rec_rec1:1 | -33.304332 | 3 | 9.684784 | -8.527765 | -7.97585 | 19.170577  |
| 3        | 0   | -21.00827 | -29.96883 | 6.952326 | BRD4_rec_rec1:1 | -32.900119 | 3 | 9.775489 | -8.721069 | -7.744049 | 19.140557  |
| 2        | 0   | -22.844649 | -28.989453 | 6.144604 | BRD4_rec_rec1:1 | -32.044748 | 3 | 10.3145 | -8.670201 | -7.750173 | 18.685289  |
4. Synthetic Procedures and NMR Spectra

Solvents were used as supplied from Fisher Scientific®, Acros Organics® or Sigma Aldrich® without further purification. Where mixtures of solvents are specified, the stated ratios are volume:volume. Unless otherwise indicated, all aqueous solutions used were saturated. Reagents were used directly as supplied by major chemical suppliers. Column chromatography was carried out using a Biotage Isolera™ Prime and pre-packed columns for flash chromatography. Analytical thin-layer chromatography was carried out on Sigma-Aldrich® silica gel or aluminium oxide on TLA aluminium foils with fluorescent indicator at 254 nm. Visualization was carried out under ultra-violet irradiation (254 nm). NMR spectra were recorded on a Bruker AV400 (1H: 400 MHz, 13C: 101 MHz). Chemical shifts are quoted in ppm, based on appearance rather than interpretation, and are referenced to the residual non-deuterated solvent peak. High resolution mass spectra were recorded on a QTof interfaced with a LC or a Rapidfire™ mass spectrometer.

The previously reported synthesis of bromosporine\(^{12}\) was optimised and adapted to produce compounds 2 and 2b, as shown in Scheme A and B.

**Synthetic Scheme A**

(12) Picaud, S.; Leonards, K.; Lambert, J.-P.; Dovey, O.; Wells, C.; Fedorov, O.; Monteiro, O.; Fujisawa, T.; Wang, C.-Y.; Lingard, H.; Tallant, C.; Nikbin, N.; Guetzoyan, L.; Ingham, R.; Ley, S. V.; Brennan, P.; Muller, S.; Samsonova, A.; Gingras, A.-C.; Schwaller, J.; Vassiliou, G.; Knapp, S. & Filippakopoulos, P. Promiscuous targeting of bromodomains by bromosporine identifies BET proteins as master regulators of primary transcription response in leukemia. *Sci. Adv.* **2016**, *2*, e1600760.
3,6-Dichloropyridazin-4-amine (4): In a sealed vial, 1.0 g (5.4 mmol) of 3,4,6-trichloropyridazine (3) were suspended in 10 mL of conc. ammonium hydroxide and heated up to 90 °C for 1 hour. The reaction was cooled down to room temperature, 10 mL of water were added and the precipitate was collected by filtration to give title compound as an off white solid (0.58 g, 65% yield).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ 6.83 (s, 1H, C(5)H), 7.16 (bs, 2H, NH$_2$);

$^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ 108.5 (C(5)H), 143.7 (C(Ar)), 146.3 (C(Ar)), 154.5 (C(Ar)).

6-Chloro-3-hydrazinylpyridazin-4-amine (5): In a sealed vial, 1.0 g (6.1 mmol) of 3,6-dichloropyridazin-4-amine (4) were suspended in 10 mL of hydrazine monohydrate and heated up to 90 °C for 3 hours. The reaction was cooled down to room temperature, poured on ice, and the resulting precipitate was collected by filtration to give title compound as a pale yellow solid (0.48 g, 49% yield).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ 4.26 (bs, 2H, NH$_2$), 6.28 (bs, 2H, NH$_2$), 6.39 (s, 1H, C(5)H), 7.30 (bs, 1H, NH);

$^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ 104.3 (C(5)H), 137.9 (C(Ar)), 147.1 (C(Ar)), 151.1 (C(Ar)).

6-Chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (6): In a round bottom flask, 3.0 g (19.0 mmol) of 6-chloro-3-hydrazinylpyridazin-4-amine (5) were suspended in 25 mL of conc. acetic acid and heated up to 90 °C for 3 hours. The reaction was cooled down to room temperature, poured on ice, and the resulting precipitate was collected by filtration. A solution of sat. sodium carbonate was added to the obtained solid and the resulting suspension stirred for half an hour at room temperature. The insoluble solid was filtered off to give title compound as an off white solid (2.7 g, 77% yield).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ 2.59 (s, 3H, CH$_3$), 6.12 (s, 1H, C(6)H), 7.90 (bs, 2H, NH$_2$);

$^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ 9.9 (CH$_3$), 94.0 (C(6)H), 139.8 (C(Ar)), 144.3 (C(Ar)), 147.2 (C(Ar)) 149.8 (C(Ar)).

Tert-butyl (6-chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (7): In a round bottom flask, di-tert-butyl dicarbonate (2.85 g, 13.1 mmol) and DMAP (0.13 g, 1.1 mmol) were added to a suspension of 6-chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (6) (2.0 g, 10.9 mmol) in 40 mL of THF at 0 °C. The reaction was allowed to warm up to room temperature over 3 hours. The solvent was evaporated, the crude oil absorbed on silica and purified by flash chromatography on silica gel (KP-Sil 25 g; 20:80 EtOAc : Cyclohexane) to give title compound as a white solid (2.2 g, 71% yield).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ 1.51 (s, 9H, 3 x CH$_3$), 2.65 (s, 3H, CH$_3$), 7.60 (s, 1H, C(6)H), 10.83 (bs, 1H, NH);

$^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ 9.9 (CH$_3$), 28.2 (3 x CH$_3$), 82.4 (C(CH$_3$)$_3$), 103.0 (C(6)H), 136.7 (C(Ar)), 138.9 (C(Ar)), 147.7 (C(Ar)) 150.0 (C(Ar)), 152.7 (C=O).
**Tert-butyl (3-methyl-6-(4-methyl-3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-yl) carbamate (9):** 4-methyl-3-nitrophenylboronic acid (8) (1.4 g, 7.0 mmol), Pd$_2$(dba)$_3$ (0.3 g, 5 mol%), XPhos (0.7 g, 10 mol%) and a solution of potassium phosphate (3.0 g, 14.1 mmol) in 6 mL of water were added to a stirring solution of tert-butyl (6-chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (2.0 g, 7.0 mmol) in 60 mL of degassed $n$-butanol. The reaction was heated up to 100 °C for 2h. The solvent was evaporated; the reaction was dissolved in EtOAc and filtered through Celite. The crude dark red oil was purified by flash column chromatography on silica gel (KP-Sil 50 g; 0 to 5% MeOH : dichloromethane). The obtained yellow gum was triturated with diethyl ether and filtered to give title compound as yellow solid (2.3 g, 85% yield).

**$^{1}$H-NMR (400 MHz, CDCl$_3$) $\delta$ 1.61 (s, 9H, 3 x CH$_3$), 2.71 (s, 3H, CH$_3$), 2.90 (s, 3H, CH$_3$), 7.53 (d, 1H, $J_{oc} = 8.0$ Hz C(12)H), 8.15 (dd, 1H, $J_o = 8.0$ Hz, $J_m = 2.0$ Hz, C(13)H), 8.22 (s, 1H, C(6)H), 8.51 (bs, 1H, NH), 8.65 (d, 1H, $J_m = 2.0$ Hz C(9)H);

**$^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ 5.2 (C$_3$H), 15.6 (C$_3$H), 23.3 (3 x CH$_3$), 78.7 (C(CH$_3$)$_3$), 95.3 (C(6)H), 118.7 (CH(Ar)), 126.5 (CH(Ar)), 128.7 (CH(Ar)) 129.7 (C(Ar)), 131.1 (C(Ar)), 134.0 (C(Ar)), 143.8 (C(Ar)), 145.0 (C(Ar)), 146.9 (C(Ar)), 148.7 (C=O).

3-methyl-6-(4-methyl-3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (10): trifluoroacetic acid (8.0 mL) was added to a solution of tert-butyl(3-methyl-6-(4-methyl-3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-yl) carbamate (9) in dichloromethane (100 mL). The reaction was stirred at room temperature for 2h. The solvent was removed in vacuum and the obtained solid suspended in a saturated solution of sodium carbonate and stirred for 30 minutes. The resulting suspension was filtered to give title compound as light yellow solid (0.74 g, 99% yield).

**$^{1}$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ 2.60 (s, 3H, CH$_3$), 2.70 (s, 3H, CH$_3$), 6.64 (s, 1H, C(6)H), 7.62 (bs, 2H, NH$_2$), 7.68 (d, 1H, $J_o = 8.1$ Hz C(12)H), 8.19 (dd, 1H, $J_o = 8.0$ Hz, $J_m = 2.0$ Hz, C(13)H), 8.49 (d, 1H, $J_m = 1.9$ Hz C(9)H);

**$^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ 10.1 (C$_3$H), 19.9 (C$_3$H), 92.0 (C(6)H), 122.8 (C(9)H), 131.6 (C(13)H), 134.0 (C(12)H) 134.8 (C(Ar)), 135.6 (C(Ar)), 140.3 (C(Ar)), 143.7 (C(Ar)), 147.5 (C(Ar)), 149.7 (C(Ar)), 152.4 (C(Ar)).

**But-3-yn-1-yl carbonochloridate (11):** Triphosgene (10.6 g, 35.7 mmol) was dissolved in 20 mL of anhydrous THF and the solution cooled down to 0°C. But-3-yn-1-ol (5.4 mL, 71.3 mmol) was diluted in 10 mL of dry THF and added dropwise over 2h at 0°C. The resultant mixture was warmed up to room temperature and stirred overnight. Anhydrous pyridine (8.6 mL, 107 mmol) was added dropwise to the solution over 1h at 0°C and then the reaction was warmed to room temperature and stirred for a further hour. Precipitated pyridinium hydrochloride salt was removed by filtration and the THF evaporated under vacuum to give title compound as an orange oil (5.8 g, quantitative yield).

**$^{1}$H-NMR (400 MHz, CDCl$_3$) $\delta$ 2.09 (t, 1H, $J = 2.7$ Hz, ≡CH), 2.67 (td, 2H, $J = 6.8$ Hz, $J = 2.6$ Hz, CH$_3$), 4.43 (t, 2H, $J = 6.8$ Hz, CH$_3$).
But-3-yn-1-yl \(6\)-(3-nitro-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (12): Triethylamine (0.3 mL, 2.1 mmol) was added to a suspension of 3-methyl-6-(4-methyl-3-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (0.3 g, 1.05 mmol) in dry dichloromethane (10 mL) at 0 °C. After 30 min but-3-yn-1-yl chloroformate (0.24 mL, 2.1 mmol) was added over 2 hours and the reaction mixture warmed up to room temperature and stirred overnight. This step was repeated until complete consumption of starting material. The reaction was absorbed on silica and purified by flash column chromatography (KP-Sil 25 g, 0 to 5% DCM/MeOH) to give the double carbamate as a brown oil (0.46 g, 0.96 mmol). The double carbamate was redissolved in 10 mL of methanol, potassium carbonate (0.29 g, 2.1 mmol) was added and the reaction stirred for 30 minutes at room temperature. Upon completion, the excess of potassium carbonate was filtered off, the filtrate was absorbed on silica and purified by flash column chromatography (KP-Sil 25 g; 0 to 2% MeOH : dichloromethane) to give title compound as light yellow solid (0.17 g, 43% yield).

\[
\begin{align*}
\text{H-NMR} & \ (400 \text{ MHz, DMSO-}d_6) \ \delta 2.62 \ (s, 3H, CH_3), 2.64 \ (td, 2H, J_1 = 6.5 \text{ Hz, } J_2 = 2.5 \text{ Hz, } CH_2), 2.77 \ (s, 3H, CH_3), 2.94 \ (t, 1H, J_1 = 2.7 \text{ Hz, } J_2 = 2.7 \text{ Hz, } \equiv CH), 4.31 \ (t, 2H, J_1 = 6.6 \text{ Hz, } CH_2), 7.73 \ (d, 1H, J_o = 7.7 \text{ Hz, } C(12)H), 8.15 \ (s, 1H, C(6)H), 8.23 \ (dd, 1H, J_o = 8.0 \text{ Hz, } J_m = 2.0 \text{ Hz, } C(13)H), 8.54 \ (d, 1H, J_m = 2.0 \text{ Hz, } C(9)H), 11.15 \ (bs, 1H, NH); \\
\text{C-NMR} & \ (100 \text{ MHz, DMSO-}d_6) \ \delta 10.0 \ (C(H_3), 19.0 \ (C(H_2), 19.9 \ (C(H_3), 64.0 \ (C(H_2), 73.3 \ (\equiv C(H), 81.1 \ (C(=O), 101.3 \ (C(6)H), 123.1 \ (C(9)H), 131.8 \ (C(13)H), 134.3 \ (C(12)H), 134.6 \ (C(13Ar), 135.6 \ (C(13Ar), 135.7 \ (C(13Ar), 139.3 \ (C(13Ar), 148.1 \ (C(13Ar), 149.8 \ (C(13Ar), 152.5 \ (C(13Ar), 154.0 \ (C=O). 
\end{align*}
\]

But-3-yn-1-yl \(6\)-(3-amino-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (13): In a sealed vial, iron powder (92 mg, 1.6 mmol) and ammonium chloride (354 mg, 6.6 mmol) were added to a solution of butyn-4-yl \(3\)-(3-methyl-6-(4-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (15) (210 mg, 0.5 mmol) in a mixture of IPA (6 mL) and water (3 mL). The reaction was stirred at 90 °C for 1 hour, cooled down to room temperature and evaporated. The crude material was dissolved in methanol and filtered through Celite®. The filtrate was absorbed on silica and purified by flash column chromatography (KP-Ultra 10 g; 0 to 5% MeOH : dichloromethane) to give title compound as light yellow solid (112 mg, 58% yield).

\[
\begin{align*}
\text{H-NMR} & \ (400 \text{ MHz, DMSO-}d_6) \ \delta 2.13 \ (s, 3H, CH_3), 2.62 \ (td, 2H, J_1 = 6.6 \text{ Hz, } J_2 = 2.7 \text{ Hz, } CH_2), 2.77 \ (s, 3H, CH_3), 2.94 \ (t, 1H, J_1 = 2.7 \text{ Hz, } J_2 = 2.7 \text{ Hz, } \equiv CH), 4.29 \ (t, 2H, J_1 = 6.6 \text{ Hz, } CH_2), 5.21 \ (bs, 2H, NH_2), 7.07-7.15 \ (m, 2H, 2 x C(Ar)H), 7.27 \ (d, 1H, J_m = 1.6 \text{ Hz, } C(6)H), 8.07 \ (s, 1H, C(6)H), 10.94 \ (bs, 1H, NH); \\
\text{C-NMR} & \ (100 \text{ MHz, DMSO-}d_6) \ \delta 10.0 \ (CH_3), 17.9 \ (CH_2), 19.0 \ (CH_2), 63.9 \ (CH_2), 73.3 \ (\equiv CH), 81.1 \ (C=CH), 101.8 \ (C(6)H), 112.3 \ (C(9)H), 115.2 \ (C(13)H), 124.4 \ (C(13Ar), 131.0 \ (C(12)H), 133.7 \ (C(13Ar), 134.9 \ (C(13Ar), 139.4 \ (C(13Ar), 147.7 \ (C(13Ar), 147.8 \ (C(13Ar), 154.0 \ (C(13Ar), 155.2 \ (C=O). 
\end{align*}
\]
But-3-yn-1-yl (6-(3-amino-4-methylphenyl)-3-methyl-[1,2,4] triazolo[4,3-\(b\)]pyridazin-8-yl) carbamate hydrochloride (BTZ-2): A stirred solution of cyanuric chloride 14 (42.3 mg, 0.23 mmol) in anhydrous tetrahydrofuran (7.5 mL) was cooled down to 0 °C. A suspension of but-3-yn-1-yl (6-(3-amino-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-\(b\)]pyridazin-8-yl)carbamate (13) (67 mg, 0.19 mmol) in 1.8 mL of anhydrous THF was added drop wise (1.2 mL/hour) and the resulting mixture stirred at 0 °C for 2 h. The solvent was evaporated to give title compound as orange solid (77.3 mg, 75% yield).

\[
\begin{align*}
\text{HNMR (400 MHz, DMSO-\(d_6\))} & \delta 2.30 (s, 3H, C\_CH\_3), 2.63 (td, 2H, \(J_1 = 6.6\) Hz, \(J_2 = 2.7\) Hz, C\_\(CH\_2\)), 2.76 (s, 3H, C\_CH\_3), 2.94 (t, 1H, \(J = 2.7\) Hz, ≡C\_\(CH\_2\)), 4.30 (t, 2H, \(J_1 = 6.6\) Hz, C\_\(CH\_2\)), 7.53 (d, 1H, \(J_o = 8.1\) Hz, C(12)\_\(H\)), 7.86 (dd, 1H, \(J_o = 8.0\) Hz, \(J_m = 2.0\) Hz C(13)\_\(H\)), 7.99 (d, 1H, \(J_m = 1.9\) Hz, C(9)\_\(H\)), 8.13 (s, 1H, C(6)\_\(H\)), 10.87 (bs, 1H, NH), 11.08 (bs, 1H, NH), 11.16 (bs, 1H, =N\_\(H\));
\end{align*}
\]

\[
\begin{align*}
\text{C-NMR (101 MHz, DMSO-\(d_6\))} & \delta 9.9 (C\_\(CH\_3\)), 18.3 (C\_\(CH\_3\)), 19.0 (C\_\(CH\_2\)), 64.2 (C\_\(CH\_2\)), 73.3 (≡C\_\(CH\_2\)), 81.1 (C\_\(\equivCH\)), 103.3 (C(6)\_\(H\)), 125.4 (C(9)\_\(H\)), 126.1 (C(13)\_\(H\)), 132.0 (C(12)\_\(H\)), 133.4 (C(\text{Ar})), 135.1 (C(\text{Ar})), 135.9 (C(\text{Ar})), 137.2 (C(\text{Ar})), 138.8 (C(\text{Ar})), 148.0 (C(\text{Ar})), 150.4 (C(\text{Ar})), 153.8 (C=O), 165.6 (C(\text{Ar})), 169.5 (C(\text{Ar})), 170.2 (C(\text{Ar})).\])
\end{align*}
\]

HRMS Calculated for C\(_{21}\)H\(_{17}\)Cl\(_2\)N\(_9\)O\(_2\) [M+1\(^+\)] = 498.0961 (35Cl), 500.0931 (37Cl); Observed [M+1\(^+\)] = 498.1024 (35Cl), 500.1009 (37Cl).

Ethyl (6-(3-nitro-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-\(b\)]pyridazin-8-yl)carbamate (15): Triethylamine (0.3 mL, 2.1 mmol) was added to a suspension of 3-methyl-6-(4-methyl-3-nitrophenyl)-[1,2,4]triazolo[4,3-\(b\)]pyridazin-8-amine (10) (300 mg, 1.05 mmol) in dry dichloromethane (10 mL) at 0 °C. After 30 min ethyl chloroformate (0.16 mL, 2.1 mmol) was added over 2 hours and the reaction mixture warmed to room temperature. This step was repeated until complete consumption of starting material. The reaction was quenched with 25 mL of water, filtered through a phase separator, absorbed on silica and purified by flash column chromatography (KP-Sil 10 g, 0 to 5% DCM/MeOH) to give a yellow solid. The crude mixture was redissolved in 6 mL of methanol, potassium carbonate (292 mg, xx mmol) was added and the reaction stirred for 30 minutes at room temperature. Upon completion, the excess of potassium carbonate was filtered off; the filtrate was absorbed on silica and purified twice by flash column chromatography (KP-Ultra 10 g; 0 to 2% MeOH : dichloromethane) to give title compound as orange solid (130 mg, 34% yield).

\[
\begin{align*}
\text{HNMR (400 MHz, CDCl}_3) & \delta 1.41 (t, 3H, J = 7.1 Hz, C\_\(CH\_2\)), 2.70 (s, 3H, C\_\(CH\_3\)), 2.89 (s, 3H, C\_\(CH\_3\)), 4.38 (q, 2H, J = 7.1 Hz, C\_\(\equivCH\)), 7.53 (d, 1H, \(J_o = 8.0\) Hz, C(12)\_\(H\)), 8.13 (dd, 1H, \(J_o = 8.0\) Hz, \(J_m = 2.0\) Hz C(13)\_\(H\)), 8.25 (s, 1H, C(6)\_\(H\)), 8.64 (d, 1H, J = 1.9 Hz, C(9)\_\(H\)), 8.83 (bs, 1H, NH);
\end{align*}
\]

\[
\begin{align*}
\text{C-NMR (101 MHz, CDCl}_3) & \delta 9.95 (C\_\(CH\_3\)), 14.3 (C\_\(CH\_3\)), 20.4 (C\_\(CH\_2\)), 62.9 (C\_\(CH\_2\)), 100.3 (C(6)\_\(H\)), 123.4 (C(9)\_\(H\)), 131.2 (C(13)\_\(H\)), 133.5 (C(12)\_\(H\)), 134.3 (C(\text{Ar})), 134.5 134.3 (C(\text{Ar})), 135.9 (C(\text{Ar})), 138.8 (C(\text{Ar})), 148.6 (C(\text{Ar})), 149.8 (C(\text{Ar})), 152.9 (C(\text{Ar})), 153.3 (C=O).
\end{align*}
\]

Due to the presence of rotamers and the weakness of the resulting carbon signals, the current spectrum is reported as a combination of \(_{13}\)C and APT spectra.
Ethyl (6-(3-amino-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (16): In a sealed vial, iron powder (60 mg, 0.05 mmol) and ammonium chloride (225 mg, 4.2 mmol) were added to a solution of ethyl (3-methyl-6-(4-nitrophenyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (15) (125 mg, 0.35 mmol) in a mixture of IPA (3 mL) and water (1.5 mL). The reaction was stirred at 90 °C for 2 hours, cooled down to room temperature and evaporated. The crude material was dissolved in methanol and filtered through Celite®. The filtrate was absorbed on silica and purified by flash column chromatography (KP-Ultra 10 g; 0 to 2% MeOH : dichloromethane) to give title compound as yellow solid (72 mg, 63% yield). 

\[ ^1H-NMR (400 MHz, DMSO-d_6) \delta 1.30 (t, 3H, J = 7.1 Hz, CH_3), 2.13 (s, 3H, CH_3), 2.73 (s, 3H, CH_3), 4.26 (q, 2H, J = 7.1 Hz, CH_2), 5.18 (bs, 2H, NH_2), 7.08 (dd, 1H, J_o = 7.07 Hz, J_m = 1.6 Hz, C(12)H), 7.11 (d, 1H, J_o = 7.11 Hz, C(11)H), 7.27 (d, 1H, J_m = 1.3 Hz, C(8)H), 8.09 (s, 1H, C(5)H), 10.78 (bs, 1H, NH); \]

\[ ^{13}C-NMR (100 MHz, DMSO-d_6) \delta 10.0 (C_H3), 14.8 (C_H3), 17.9 (C_H3), 62.1 (C_H2), 101.4 (C(6)H), 112.3 (C(9)H), 115.2 (C(13)H), 124.4 (C(Ar)), 131.0 (C(12)H), 133.7 (C(Ar)), 135.1 (C(Ar)), 139.4 (C(Ar)), 147.7 (C(Ar)), 147.8 (C(Ar)), 154.2 (C(Ar)), 155.2 (C=O). \]

Ethyl (6-(3-(4,6-dichloro-1,3,5-triazin-2-yl)amino)-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate hydrochloride (2b): A stirred solution of cyanuric chloride (14) (45 mg, 0.24 mmol) in anhydrous tetrahydrofuran (3 mL) was cooled down to 0 °C. A suspension of ethyl (6-(3-amino-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate (15) (67 mg, 0.19 mmol) in 1.2 mL of anhydrous THF was added drop wise (1.2 mL/hour) and the resulting mixture stirred at 0 °C for 2 h. The solvent was evaporated to give title compound as orange solid (106 mg, 99% yield). 

\[ ^1H-NMR (400 MHz, DMSO-d_6) \delta 1.30 (t, 3H, J = 7.1 Hz, CH_3), 2.30 (s, 3H, CH_3), 2.76 (s, 3H, CH_3), 4.27 (q, 2H, J = 7.1 Hz, CH_2), 7.53 (d, 1H, J_o = 8.1 Hz, C(12)H), 7.85 (dd, 1H, J_o = 8.0 Hz, J_m = 1.8 Hz, C(13)H), 7.98 (d, 1H, J_m = 1.5 Hz, C(12)H), 8.17 (s, 1H, C(6)H), 10.88 (bs, 1H, NH), 10.96 (bs, 1H, NH), 11.17 (bs, 0.5H, NH); \]

\[ ^{13}C-NMR (100 MHz, DMSO-d_6) \delta 9.96 (C_H3), 14.74 (C_H3), 18.23 (C_H3), 62.25 (C_H2), 101.94 (C(6)H), 125.60 (C(9)H), 126.13 (C(13)H), 132.01 (C(12)H), 133.65 (C(Ar)), 135.40 (C(Ar)), 135.89 (C(Ar)), 137.37 (C(Ar)), 148.02 (C(Ar)), 150.37 (C(Ar)), 154.08 (C=O), 154.32 (C(Ar)), 165.58 (C(Ar)), 169.59 (C(Ar)), 170.20 (C(Ar)); \]

HRMS Calculated for C_{19}H_{17}Cl_{2}N_{9}O_{2} [M+1]^+ = 474.0961 (^{35}Cl), 476.0931 (^{37}Cl); Observed [M+1]^+ = 474.1029 (^{35}Cl), 476.0997 (^{37}Cl).
NMR Spectra
## 5. Rapidfire Mass Spectrometry

Determining the selectivity of Bromotriazine against BRDs belonging to different families.

Reaction conditions: 30 µM probe, 3 µM recombinant BRD in buffer (20 mM HEPES, 200 mM NaCl). Incubations: 1.5 hours at 37 °C.

Each experiment was conducted in triplicates.

| Recombinant Bromodomain | Deconvoluted Area§ | Covalent Modification(%) |
|-------------------------|--------------------|--------------------------|
|                         | Modified BRD | SEM  | MW     | Unmodified BRD | SEM  | MW     | Total Area | Modified BRD | Unmodified BRD |
| BRD4(1)                 | 1114097      | 164682| 15545.60| 2977325       | 393624| 15083.88| 4091422     | 27.2         | 72.8         |
| BRD4(2)                 | 1446711      | 100901| 15498.52| 8496233       | 695755| 15036.63| 9942944     | 14.5         | 85.5         |
| BRD3(2)                 | 2284981      | 31519 | 13746.64| 8103269       | 191522| 13284.88| 10388250    | 22.0         | 78.0         |
| ATAD2A                  | 181088       | 10751 | 15892.62| 4338485       | 195511| 15430.95| 4519573     | 4.0          | 96.0         |
| SMARCA2B                | 0            | 0     |         | 7946558       | 247416| 14358.01| 7946558     | 0            | 100          |
| PB1(5)                  | 17124        | 17124 | 15088.26| 5085268       | 356467| 14626.36| 5102392     | 0.3          | 99.7         |
| CREBBP                  | 228574       | 62605 | 14669.46| 4738909       | 250376| 14207.89| 4967484     | 4.6          | 95.4         |
| TIF1a                   | 113262       | 2750  | 21839.29| 5913572       | 336762| 21378.04| 6026833     | 1.9          | 98.1         |
| CECR2A                  | 6180803      | 146286| 14171.91| 1462863       | 146286| 14171.91| 1462863     | 100          | 0            |
| BRD9                    | 82856        | 9069  | 15317.31| 10037190      | 399369| 14855.44| 10120047    | 0.8          | 99.2         |
| BRD7                    | 4221995      | 350698| 14689.06| 1249599       | 49637 | 14227.17| 5471594     | 77.1         | 22.9         |
| BRPF1B                  | 95674        | 5896  | 14149.19| 2977325       | 393624| 13687.16| 6901717     | 1.4          | 98.6         |
| BRD1                    | 2568623      | 230824| 13707.32| 426580        | 48093 | 13245.22| 2995203     | 85.7         | 14.2         |
| PCAF                    | 373811       | 9370  | 17077.48| 3966840       | 59891 | 16616.16| 4344791     | 8.7          | 91.3         |
| TAF1(1)                 | 488203       | 2930  | 18074.82| 594004        | 3071  | 17613.04| 1082207     | 45.1         | 54.9         |
| TAF1(2)                 | 501616       | 14676 | 18793.64| 610873        | 20235 | 18331.54| 1112489     | 45.1         | 54.9         |
| TAF1L(1)                | 201753       | 19454 | 17238.57| 1679168       | 149522| 16777.20| 1880921     | 10.7         | 89.3         |
| TAF1L(2)                | 398490       | 44659 | 18462.01| 1411734       | 144781| 18000.13| 1810223     | 22.0         | 78.0         |
| GCN5L2A                 | 0            | 0     |         | 1517711       | 716125| 15975.39| 1517711     | 0            | 100          |

§Deconvoluted Areas are reported as average values of three replicates.
### 5.1 Recombinant Bromodomains Sequences

Bromodomains were produced and purified as described previously.\(^\text{13}\)

| Recombinant Bromodomain | Amino acid sequence | MW |
|-------------------------|---------------------|----|
| BRD4(1)                 | SMNPPPETSNPKKRQTNTQLQYLLRVLKTLWIKHDFQAWPFQQPVDVAVKLNLPPDYKIKTPMDMGTTIKKRLENNYYWNQAECIQDFNTMFTNCYIYNNPKGDDVLMAMAEAEKLFQKINELPTEE | 15083 |
| BRD4(2)                 | SMKDVPDSQHQPAPEKSIVSEQKLCCSGLIKEMFAAKKHAAYAWFYKPVDFVEALGLHDYCDIIHKPMDSITKSKLAREYRDAQEFGADVRLMSFSNCKYNPPDHEVAMARKQDFVFEMRFAKMPDE | 15036 |
| BRD3(2)                 | SMGKLSHELRYCDSILREMLSKKHAYAWFYKPVDAEAEHELHDYHDIHKPMDSLTKRMDGREYDPADQGFAADVRLMSFNCYKNPPDHEVAMARKQDFVFEMRFAKMP | 13284 |
| ATAD2A                  | SMQEDTFRELRIFLNVTHLAIDKFRVFTKPVDPDEVYPDVTQIKPMDDLSSVSKIDLHKLYTLTKDYLDRLDIICSNALEVNPDRDPGDRLIRHRAELRTD TAYAIIKEELDEDFTELCEEIQESR | 15430 |
| SMARCA2B                | SMAEKLSPNPKLTKQMNAI DTVINYKDSGQLSEFIVQPLPSKELPELIKRPVDFFKKKIERINHKYRSLGQLEKVMILLCHNAQTFLNCQYEDSVLSQVFK SARKIAEEE | 14357 |
| PB1(5)                  | SMGRGSPKSKKSTDMPQQKLNVEYAEVKNTDKRGRRLAIFLRLPSRPSELTTYLTIKKPMDEMKEIRSHMMANKYQDISVMFDVLMFFNNAWYNRKT RSVYKFCALVLEFQETDVMQSL | 14626 |
| CREBBP                  | SMRRKIFKPELRQALMPTEALYRQDPSLPRQFPVDPQQLGIPDYFDIVKNPMDELSTIKRKLDTQYQEPYWVYDDVWLMMFINNAWYNRKT RSVYKFCALVLEFQETDVMQSL | 14207 |
| TIF1a                   | SMNEDWCACQNGGELLCEKCPKVFHLSCHYPTLTNFSGEWICTCRDLSKEVEYDCAOPSSHNEKKTGELVKTPIDKRCERR LFLYCEMSLAFDQPVLTPYDYYKIKPMDSLTIKRLQEDYESMYKPEDVAFRSLFQVCAEFNPSEVANAGIKLEFEELLNLYPSKG GLYNDIFEAQKIEWHE | 21377 |
| CEC2R2A                 | SMTRAFFDDDFTAMYKVLVDVKAHKDSWPFFLEPDESYPHYQIIPDDMSMKELNLGLGYCTKEEFVNDMKTFRNCRKYN GESSEYTMDSNLCRFRHMMKHPGED | 13708 |
| BRD1                    | SMRLTLPFFVLRLSVLDQQDKDPARIFAQPVSLKEVPDLHKHHPMFATMRKLEAQGYKLHFEEDFLIDNMCYNARDTVFY RAHAVRLDQPVGLVLRARRQDISGVEEMLHGPERS | 14855 |
| BRD9                    | SMKLGASAENSTPIQQLHELLFHRLQQLRKDHPHFGAFFVPVTAIPAPGYSIMKHHPDFGTMKDIFKIVANEYKSVTEFKADFKLMDCNAMTY NRDPDYYLKLKHLHAGFMMKSKKRELALKRSMS | 14227 |
| BRD9 His tag            | MHHHHHSSSGVDGTENLYFQSMKLKLSAENSTPIQQLHELFLQRLQQRKDPHFGAFFPVVTAIPAPGYSMMKHHPDFGTMKDIFKIVANE YKSVTEFKADFKLMDCNAMTYNRDPDYYLKLKHLHAGFMMKSKKRELALKRSMS | 16692 |
| BRPF1B                  | SMEMQLTLPFFLIRLRKTELQLEQKDNGTSFEPVPEPVDYLDHIKKPMDFFTMKNLEAYRNLFDDDFFEDFNLIVSNCLKYNAKDTIFY RAHAVRLDQPVGLVLRARRQDISGVEEMLHGPERS | 13687 |
| Protein | Sequence | Score |
|---------|----------|-------|
| BRD7    | SMEEVEQTPLQEALNQLMRQKPSAFFSFVTDPIAGYSMIKHPMDSTMKDKEQTRKNDQSEELKDNLKLMCTNAMIYKPE TYYKAAKKLHHGKMLKSLQERIQS | 13245 |
| PCAF    | MHHHHHSSSVGDLGTENLYFQSMGEKSEKEPRDLPQYLSTLKSILQQVKSHPQSAWFEMEPVKRTEAPGYEVIRFPMDKLMRTSLK NRYYSKLLFMADLVQRVTNCENKEYNPSEQEYKCANLIKEFFSKIKEGLID | 16616 |
| TAF1(1) | MHHHHHSSVGDLGTENLYFQSMRRTDPMVTLSSILESIINDMRDLPNTYPFHTPVNAKVVKDYKIITRPMODLQLQNLRENVKRLYPSR EEFEHLELIKNSATYNGPKHSLQTQSMQLDCEKLEKEDKLRKAINPLLDDDD | 17613 |
| TAF1(2) | MHHHHHSSVGDLGTENLYFQSMDDLDQQVAFSILDNIVTQKMMAVPSWPFHVNPKKFVDPPDVYKVIIVNPMDLETIRKNIKSHKHYQ SRESFLDQVLNANVSKYNGPSESQYTKTAQEVNNCYLTYDEHLTLQEDICTAEEAALAEALESLD | 18331.5 |
| TAF1L(1) | MHHHHHSSVGDLGTENLYFQSMVTLSSILESIINDMRDLAPTFPVNAKVVKDYKIITRPMODLQLQNLRENVKCLYPSREEFREHL ELIKNSATYNGPKHSLQTQSMQLDCEKLEKEDKLRKAINPLLDDDD | 16777 |
| TAF1L(2) | MHHHHHSSVGDLGTENLYFQSMQVAFSILDNIVTQKMMAVPSWPFHVNPKKFVDPPDVYKVIIVNPMDLETIRKNIKSHKHYQSRES FLDDQVNANVSKYNGPSESQYTKTAQEVNCYQTYDEHLTLQEDICTAEEAALAEALESLD | 18000 |
| GCNSL2A | MHHHHHSSVGDLGTENLYFQSMELKDQYTLNKLAAIQKSHPSAWFEPVKKSEAPDYEVIRFIPDLKTMTELRSRYYVTRK LFVADQVRVIANCREYNPPDSEYRCASALEKFFKLGLEDK | 15975 |

(13) Filippakopoulos, P.; Picaud, S.; Mangos, M.; Keates, T.; Lambert, J. P.; Barsyte-Lovejoy, D.; Felletar, I.; Volkmer, R.; Muller, S.; Pawson, T.; Gingras, A. C.; Arrowsmith, C. H.; Knapp, S., Histone recognition and large-
scale structural analysis of the human bromodomain family. *Cell* 2012, 149 (1), 214-231.
5.2 Kinetic Experiments:

Experimental conditions: 100 µM probe was incubated with 1 µM bromodomain in 20 mM HEPES and 200 mM NaCl at 37 ºC. At given time points (0, 5, 10, 20, 30, 60, 90 and 120 minutes), the solution was sampled (75 µL), purified through a quick solid phase extraction on C4 cartridge and analysed by Rapidfire 360™ MS interfaced with a QTOF. Blank experiments were Values are reported as absolute area of each mass peak.

5.2.1 BTZ 100 µM and BRD4(1) 1 µM

| Time (min) | Modified BRD | SEM | Unmodified BRD | SEM | Total Area | Control | Modified BRD | Unmodified BRD |
|------------|--------------|-----|----------------|-----|------------|---------|--------------|----------------|
| 0          | 36526.8      | 1766.3 | 2604013        | 76928.3 | 3188982  | 1.4     | 98.6         |
| 5          | 117009.3     | 4426.6 | 2181887        | 27340.2 | 3386286  | 5.1     | 94.9         |
| 10         | 160816.3     | 6195.6 | 1817258        | 71211.5 | 3274334  | 8.1     | 91.9         |
| 20         | 210887.3     | 4166.6 | 1658156        | 15019.7 | 2809483  | 11.3    | 88.7         |
| 30         | 338807.3     | 26646.0 | 1425869        | 52546.8 | 3199240  | 19.2    | 80.8         |
| 60         | 419799.0     | 15185.4 | 1160203        | 13123.3 | 3426590  | 26.6    | 73.4         |
| 120        | 457494.0     | 21260.8 | 898539.7       | 32427.7 | 3407567  | 33.7    | 66.3         |

§Values are reported as the average of three replicates.
Area values were converted into concentrations (µM) by assuming a direct proportionality between deconvoluted areas and concentrations. The average of deconvoluted areas registered during the control experiment is referred as the concentration of BRD at t=0 ([A₀] = 1 µM) as it doesn’t change during the experiment and it takes into account experimental errors. The concentration of unmodified bromodomain at different time points was measured with reference to this value, and so it was the natural logarithm of the ratio between the two (Ln ([A]/[A₀]).

| Time (min) | Deconvoluted Area | Ln ([A]/[A₀]) |
|------------|-------------------|---------------|
|            | Unmodified BRD    |               |
| 0          | 2456753           | -14.09        |
|            | 2639044           | -14.02        |
|            | 2716241           | -13.99        |
| 5          | 2217267           | -14.195       |
|            | 2128091           | -14.24        |
|            | 2200303           | -14.20        |
| 10         | 1922726           | -14.34        |
|            | 1847413           | -14.378       |
|            | 1681635           | -14.47        |
| 20         | 1660671           | -14.48        |
|            | 1630975           | -14.50        |
|            | 1682822           | -14.47        |
| 30         | 1326604           | -14.71        |
|            | 1445614           | -14.62        |
|            | 1505390           | -14.58        |
| 60         | 1155392           | -14.85        |
|            | 1184954           | -14.82        |
|            | 1140264           | -14.86        |
| 120        | 900107            | -15.10        |
|            | 841606            | -15.16        |
|            | 953906            | -15.04        |

Kapp (M⁻¹ s⁻¹)  SEM
0.0001284  0.0001387  0.0001343  2.98385E-06
### 5.2.2 BTZ 100 µM and BRD4(2) 1 µM

| Time (min) | Modified BRD[§] | SEM | Unmodified BRD | SEM | Total Area | Control | Modified BRD | Unmodified BRD |
|------------|-----------------|-----|----------------|-----|------------|---------|--------------|---------------|
| 0          | 53233.5         | 3911.545 | 5085796       | 333588.2 | 5139029.8  | 5406608 | 1.04         | 98.95         |
| 5          | 193647.3        | 20775.18  | 3719936       | 447929.2 | 3913583.7  | 5328213 | 4.95         | 95.05         |
| 10         | 249258.7        | 32747.87  | 449595.5      | 4440383.7 | 4786069    | 7.24    | 92.75        |               |
| 20         | 299186.0        | 35343.81  | 3191125       | 4493538.0 | 3138458.0  | 4779674 | 9.53         | 90.47         |
| 30         | 358939.7        | 42893.1   | 2839272       | 2839743.2 | 2623547.0  | 5076476 | 13.68        | 86.32         |
| 60         | 337781.7        | 42835.41  | 1679708       | 222426.4 | 2017489.7  | 4923649 | 16.74        | 83.26         |
| 120        | 331434.0        | 50953.44  | 1362977       | 212741.9 | 1694410.7  | 4860158 | 19.56        | 80.44         |

[§]Values are reported as the average of three replicates.
Area values were converted into concentrations (µM) by assuming a direct proportionality between deconvoluted areas and concentrations. The average of deconvoluted areas registered during the control experiment is referred as the concentration of BRD at t=0 ([A₀] = 1 µM) as it doesn't change during the experiment and it takes into account experimental errors. The concentration of unmodified bromodomain at different time points was measured with reference to this value, and so it was the natural logarithm of the ratio between the two (\( \text{Ln} ([\text{A}]/[\text{A₀}]) \)).

| Time (min) | Deconvoluted Area | Unmodified BRD | Ln ([A]/[A₀]) | Kapp (M⁻¹ s⁻¹) | SEM |
|-----------|-------------------|---------------|---------------|----------------|-----|
| 0         | 5432000           | 4418781       | 4114486       | -13.7372       | -13.9437 |
| 5         | 4610894           | 3193417       | 3355498       | -13.9011       | -14.2684 |
| 10        | 4090104           | 2758541       | 2724730       | -14.021        | -14.4148 |
| 20        | 3558287           | 2545943       | 2413586       | -14.1603       | -14.495  |
| 30        | 2860773           | 1991896       | 1941153       | -14.3784       | -14.7404 |
| 60        | 2123826           | 1479783       | 1435515       | -14.6763       | -15.0376 |
| 120       | 1788455           | 1148355       | 1152120       | -14.8482       | -15.2912 |

\( \text{Kapp} = 0.000151 \) \( \text{SEM} = 5.696E-06 \)
### 5.2.3 BTZ 100 µM and BRD3(2) 1 µM

| Time (min) | Modified BRD$^*$ | SEM | Unmodified BRD | SEM | Total Area       | Control       | Modified BRD | Unmodified BRD |
|------------|------------------|-----|----------------|-----|------------------|---------------|--------------|----------------|
| 0          | 81254.25         | 1999.449 | 6671653 | 233733 | 6752907.25       | 8153064       | 1.22         | 98.78          |
| 5          | 294984           | 6141.152 | 5597932 | 32074.5 | 5892916          | 7884086       | 5.00         | 94.99          |
| 10         | 448070           | 19321.28 | 4858693 | 305006.1 | 5306762.667      | 7118667       | 8.44         | 91.56          |
| 20         | 586922.7         | 24900.61 | 4694408 | 172933.8 | 5281330.667      | 7423659       | 11.11        | 88.89          |
| 30         | 950629           | 32019.02 | 4156861 | 154473.5 | 5107489.667      | 7379543       | 18.61        | 81.39          |
| 60         | 1251461          | 28693.38 | 3598428 | 106468.9 | 4849889.333      | 7395855       | 25.80        | 74.20          |
| 120        | 1469713          | 62007.74 | 3196837 | 134724.5 | 4666550          | 7445230       | 31.49        | 68.51          |

$^*$Values are reported as the average of three replicates.
Area values were converted into concentrations (µM) by assuming a direct proportionality between deconvoluted areas and concentrations. The average of deconvoluted areas registered during the control experiment is referred as the concentration of BRD at t=0 ([A₀] = 1 µM) as it doesn’t change during the experiment and it takes into account experimental errors. The concentration of unmodified bromodomain at different time points was measured with reference to this value, and so it was the natural logarithm of the ratio between the two (Ln ([A]/[A₀]).

| Time (min) | Modified BRD | Unmodified BRD | Ln ([A]/[A₀]) | Unmodified BRD |
|------------|--------------|---------------|---------------|---------------|
| 0          | 7069980      | 6684374       | -13.88        | -13.94        | -14.01 |
| 5          | 6191364      | 550911      | -14.01        | -14.13        | -14.22 |
| 10         | 5448994      | 4696750      | -14.14        | -14.29        | -14.43 |
| 20         | 5038357      | 4490928      | -14.22        | -14.33        | -14.55 |
| 30         | 4416571      | 4171916      | -14.35        | -14.41        | -14.77 |
| 60         | 3776372      | 3610742      | -14.51        | -14.55        | -15.07 |
| 120        | 3433840      | 3189349      | -14.60        | -14.68        | -15.29 |

Kapp (M⁻¹ s⁻¹) | SEM
---|---
0.0000894 | 0.0000876 | 0.0000945 | 2.0664E-06

0.0000894 0.0000876 0.0000945 2.0664E-06
### 5.2.4 BTZ 100 µM and BRD2(1) 1 µM

| Time (min) | Modified BRD* | SEM | Unmodified BRD | SEM | Total Area | Control | Covalent Modification(%) |
|------------|---------------|-----|---------------|-----|------------|---------|--------------------------|
| 0          | 81815.33      | 23270.16 | 938788       | 50315.13 | 1020603.33 | -       | 1.22 | 98.78 |
| 5          | 132810        | 1247.993 | 718016.3     | 5128.345 | 850826.33 | 1240639 | 5.00 | 94.99 |
| 10         | 165098.3      | 14815.35 | 557304       | 28132.34 | 722402.33 | 1938300 | 8.44 | 91.56 |
| 20         | 192926.7      | 7941.703 | 479827       | 672753.67 | 672753.67 | 1656247 | 11.11 | 88.89 |
| 30         | 230945.7      | 1777.656 | 308839       | 11339.18 | 539784.67 | 1661703 | 18.61 | 81.39 |
| 60         | 264628        | 7012.734 | 220126.3     | 11230.46 | 484754.33 | 1769332 | 25.80 | 74.20 |
| 120        | 283767        | 18529.09 | 136103.3     | 9030.167 | 419870.33 | -       | 31.49 | 68.51 |

*Values are reported as the average of three replicates.
Area values were converted into concentrations (µM) by assuming a direct proportionality between deconvoluted areas and concentrations. The average of deconvoluted areas registered during the control experiment is referred as the concentration of BRD at t=0 ([A₀] = 1 µM) as it doesn’t change during the experiment and it takes into account experimental errors. The concentration of unmodified bromodomain at different time points was measured with reference to this value, and so it was the natural logarithm of the ratio between the two (Ln ([A]/[A₀]).

| Time (min) | Deconvoluted Area  | Ln ([A]/[A₀]) |
|------------|---------------------|---------------|
|            | Unmodified BRD      | Unmodified BRD |
| 0          | 728101              | -14.64        |
| 5          | 711354              | -14.66        |
| 10         | 514492              | -14.92        |
| 20         | 610327              | -14.98        |
| 30         | 492100              | -15.05        |
| 60         | 286489              | -15.57        |
| 120        | 210491              | -15.88        |

Kapp (M⁻¹ s⁻¹) SEM
### 5.2.5 BTZ 100 µM and BRD9 1 µM

| Time (min) | Modified BRD* | SEM | Unmodified BRD | SEM | Total Area | Control | Modified BRD | Unmodified BRD |
|------------|---------------|-----|----------------|-----|------------|---------|--------------|----------------|
| 0          | 143217.25     | 701.11 | 2768218 | 205044 | 2911435.58 | 4212540 | 5.17         | 94.83          |
| 5          | 451175.00     | 12569.0 | 1676612 | 160157.4 | 1727787.33 | 3937281 | 21.20        | 78.80          |
| 10         | 599059.33     | 28274.96 | 1152637 | 128901.6 | 1751696.33 | 3754996 | 34.20        | 65.80          |
| 20         | 720230.67     | 46715.20 | 922279.7 | 98933.23 | 1642510.33 | 3796729 | 43.85        | 56.15          |
| 30         | 1021967.0     | 63061.07 | 491559.7 | 69587.42 | 1513526.67 | 4207068 | 67.52        | 32.48          |
| 60         | 911932.33     | 60404.46 | 239683.3 | 35682.94 | 1151615.67 | 3953821 | 79.19        | 20.81          |
| 120        | 813650.67     | 57026.19 | 131675.7 | 15728.55 | 945326.33 | 3814969 | 86.07        | 13.93          |

*Values are reported as the average of three replicates.
Area values were converted into concentrations (µM) by assuming a direct proportionality between deconvoluted areas and concentrations. The average of deconvoluted areas registered during the control experiment is referred as the concentration of BRD at t=0 ([A₀] = 1 µM) as it doesn’t change during the experiment and it takes into account experimental errors. The concentration of unmodified bromodomain at different time points was measured with reference to this value, and so it was the natural logarithm of the ratio between the two (Ln ([A]/[A₀]).

| Time (min) | Unmodified BRD | Unmodified BRD | Ln ([A]/[A₀]) | Ln ([A]/[A₀]) |
|-----------|----------------|----------------|---------------|---------------|
|           | Deconvoluted Area | Ln ([A]/[A₀]) |               |               |
| 0         | 3170817         | 2634483        | 2499355       | -14.04        | -14.22        | -14.27        |
| 5         | 1991469         | 1570176        | 1468192       | -14.50        | -14.74        | -14.80        |
| 10        | 1390391         | 1120083        | 947437        | -14.86        | -15.08        | -15.24        |
| 20        | 1118867         | 843439         | 804533        | -15.08        | -15.36        | -15.41        |
| 30        | 630328          | 431381         | 412970        | -15.65        | -16.03        | -16.07        |
| 60        | 295894          | 249658         | 173498        | -16.41        | -16.58        | -16.94        |
| 120       | 151423          | 143008         | 100596        | -17.078       | -17.13        | -17.49        |

Kapp (M⁻¹ s⁻¹) SEM
5.2.6 BTZ 100 µM and CECR2 1 µM

| Time (min) | Modified BRD* | SEM | Unmodified BRD | SEM | Total Area | Control | Covalent Modification(%) |
|------------|---------------|-----|----------------|-----|------------|---------|--------------------------|
| 0          | 291816.3      | 28245.24 | 2880204       | 128957 | 3172020.25 | 4754567 | 10.13 | 89.87 |
| 5          | 1117459       | 25024.41 | 709792.3      | 56518.17 | 1827251.667 | 3098162 | 61.16 | 38.84 |
| 10         | 1340677       | 35573.99 | 323764        | 23707.48 | 1664440.667 | 2811445 | 80.55 | 19.45 |
| 20         | 1353722       | 63716.98 | 149059.3      | 14036.28 | 1502781.667 | 2679638 | 90.08 | 9.92  |
| 30         | 1396766       | 64443.29 | 25539         | 4567.453 | 1422304.667 | 2870240 | 98.20 | 1.80  |
| 60         | 1366313       | 14702.47 | 0             | 0      | 1366312.667 | 2988766 | 100  | 0     |
| 120        | 1295672       | 36321.66 | 0             | 0      | 1295672.333 | 2886016 | 100  | 0     |

*Values are reported as the average of three replicates.
Area values were converted into concentrations (µM) by assuming a direct proportionality between deconvoluted areas and concentrations. The average of deconvoluted areas registered during the control experiment is referred to as the concentration of BRD at t=0 ([A₀] = 1 µM) as it doesn’t change during the experiment and it takes into account experimental errors. The concentration of unmodified bromodomain at different time points was measured with reference to this value, and so it was the natural logarithm of the ratio between the two (Ln ([A]/[A₀]).

| Time (min) | Unmodified BRD | Ln ([A]/[A₀]) |
|------------|----------------|---------------|
|            |                | Unmodified BRD |                |
| 0          | 2654396        | -13.99        |
|            | 3101033        | -13.83        |
|            | 2885183        | -13.91        |
| 5          | 817756         | -15.17        |
|            | 684807         | -15.34        |
|            | 626814         | -15.43        |
| 10         | 369702         | -15.96        |
|            | 310964         | -16.13        |
|            | 290626         | -16.20        |
| 20         | 177112         | -16.70        |
|            | 135948         | -16.96        |
|            | 134118         | -16.97        |
| 30         | 34575          | -18.33        |
|            | 22182          | -18.77        |
|            | 19860          | -18.88        |
| 60         | 0              | -            |
|            | 0              | -            |
|            | 0              | -            |
| 120        | 0              | -            |
|            | 0              | -            |

Kapp (M⁻¹ s⁻¹) SEM

0.0011647  0.0013071  0.001316  4.90174E-05

S63
6. LC-MS/MS Analysis

For the reaction of recombinant BRD proteins with probe, the following conditions were used: 30 µM probe was incubated for 1.5 hours at 37 ºC in the presence of 3 µM recombinant BRD (total amount of protein around 4 µg), in a buffer containing 20 mM HEPES and 200 mM NaCl, pH 7.4. For the reactions using BRD4(1) and BTZ 2, 100 µM probe was incubated for 1.5 hours at 37 ºC in the presence of 5 µM recombinant BRD4. Excess of probe material was removed by filtering the sample through a Micro Bio-Spin® column (Bio-Rad). For in-solution digestions, trypsin from bovine pancreas (1 µg/µL in 0.1 HCl, Promega) was reconstituted in 800 µL of 50 mM ammonium bicarbonate, and 4 µL of this solution were added to 100 µL of the 3 µM solution of BRD (+ probe). The solution was incubated overnight at 37 ºC. After the digestion was complete, the obtained peptides were purified by Sep-PAK C-18 solid-phase extraction according to the manufacturer’s instructions (Waters) and concentrated under vacuum and kept at -20 ºC until LC-MS/MS analysis as described previously (Fischer & Kessler, Proteomics, 2015). In brief, dried samples were reconstituted in 0.1% TFA in 1% Acetonitrile and analysed on Q Exactive mass spectrometer (Thermo) coupled with a Dionex Ultimate 3000 UPLC (Thermo). Samples were desalted online (PepMAP C18, 300µm x 5mm, 5 µm particle, Thermo) for 1 minute at a flow rate of 20 µl/min and separated on a nEASY column (PepMAP C18, 75 µm x 500 mm, 2 µm particle, Thermo) over 60 Minutes using a gradient of 2%-35% Acetonitrile in 5% DMSO/0.1% Formic acid at 250nl/min. Survey scans were acquired at a resolution of 70,000 @ 200m/z and the 15 most abundant precursors were selected for HCD fragmentation. Alternatively, samples were analysed using an Orbitrap Velos mass spectrometer (Thermo) coupled to a nAquity UPLC system (Waters). Briefly, samples were desalted online (Symmetry C18 column (180 µm x 20mm, 5 µm particle, Waters) for 5 minutes at a flow rate of 5µl/min, followed by separation on a BEH C18 column (75 µm x 250 mm, 1.7 µm particle, Waters) over 60 minutes using a gradient of 2%-40% Acetonitrile in 0.1% Formic acid at 250nl/min. Survey scans were acquired in the Orbitrap at a resolution of 60.000 @ 400m/z and the 20 most abundant precursors were selected for CID fragmentation. MS data were analysed using PEAKS software (version 7.5) as described.
Figure Legend: Mapping BRD-probe crosslinking sites by MS/MS. A) (top left panel) MS/MS spectrum of the BRD4(1) derived tryptic peptide 77-99 containing K91 that was found to be modified by BTZ (2) (+462.12 Da). Matching fragment ions are indicated in red (y-ions) and blue (b-ions). (top right panel) sequence coverage indicating the detection of a tryptic peptide containing modified K91 (in blue lines) as identified by PEAKS (red box). (lower left panel) Calculated MS/MS fragment ions (black) that match the experimentally observed y-ions (red) and b-ions (blue). (Lower right panel) BRD4(1) structure indicating a direct interaction between K91 and BTZ (2). B) (top left panel) MS/MS spectrum of the BRD3(2) derived tryptic peptide 327-346 containing K336 that was found to be modified by BTZ (2) (+462.12 Da). Matching fragment ions are indicated in red (y-ions) and blue (b-ions). (top right panel) sequence coverage indicating the detection of a tryptic peptide containing modified K336 (in blue lines) as identified by PEAKS (red box). (lower left panel) Calculated MS/MS fragment ions (black) that match the experimentally observed y-ions (red) and b-ions (blue). (Lower right panel) BRD3(2) structure indicating a direct interaction between K336 and BTZ (2).

(14) Fischer, R. & Kessler, B. M. Gel-aided sample preparation (GASP)—A simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells. Proteomics 2015, 15 (7), 1224-1229.
(15) Davis, S.; Charles, P. D.; He, L.; Mowlds, P.; Kessler, B. M & Fischer, R. Expanding Proteome Coverage with CHarge Ordered Parallel Ion aNalysis (CHOPIN) Combined with Broad Specificity Proteolysis. J. Proteome Res. 2017, 16, 1288-1299.
6.1 BTZ & BRD4(1) – Lysine 91

A  BRD4(1) - recombinant

### QE MS/MS BRD4(1) 5 uM – BTZ 100 uM

| #  | b     | b+H2O | b+NH3 | (b+2) | Seq  | y   | y+H2O | y+NH3 | (y+2) |
|----|-------|-------|-------|-------|------|-----|-------|-------|-------|
| 1  | 130.07| 120.08| 121.04| 69.53 | H    |     |       |       |       |
| 2  | 266.12| 248.11| 249.10| 133.56| Q    | 3129.48| 3111.47| 3112.45| 1565.24|
| 3  | 413.19| 396.18| 396.18| 207.10| F    | 3001.42| 2983.41| 2984.39| 1501.21|
| 4  | 484.23| 466.22| 467.20| 242.62| A    | 2854.35| 2836.34| 2837.33| 1427.68|
| 5  | 670.30| 652.30| 653.28| 355.66| W    | 2783.32| 2765.31| 2766.29| 1392.16|
| 6  | 767.35| 749.35| 750.31| 384.18| P    | 2597.24| 2579.23| 2580.21| 1299.12|
| 7  | 914.43| 896.41| 897.41| 457.72| F    | 2500.18| 2482.17| 2483.16| 1250.59|
| 8  | 1042.48| 1024.48| 1025.46| 521.74| Q    | 2353.12| 2335.10| 2336.09| 1177.06|
| 9  | 1170.54| 1152.54| 1153.50| 585.77| Q    | 2225.06| 2207.05| 2208.03| 1113.03|
| 10 | 1267.60| 1249.59| 1250.57| 634.30| P    | 2097.00| 2078.99| 2079.97| 1049.00|
| 11 | 1366.64| 1348.64| 1349.64| 683.83| V    | 1999.95| 1981.93| 1982.92| 1000.47|
| 12 | 1481.68| 1463.69| 1464.67| 741.35| D    | 1900.88| 1882.87| 1883.85| 959.94|
| 13 | 1552.69| 1534.72| 1535.71| 795.87| A    | 1785.85| 1767.84| 1768.82| 933.43|
| 14 | 1651.78| 1633.79| 1634.78| 826.40| V    | 1714.78| 1696.78| 1697.79| 887.11|
| 15 | 2242.02| 2224.01| 2224.09| 1121.50| K    | 1615.74| 1597.73| 1598.72| 808.36|
| 16 | 2256.10| 2238.09| 2238.07| 1178.05| L    | 1025.52| 1007.52| 1008.50| 513.27|
| 17 | 2469.14| 2451.13| 2452.12| 1235.07| N    | 912.44| 894.44| 895.41| 456.72|
| 18 | 2582.23| 2564.22| 2565.20| 1291.61| L    | 798.40| 780.38| 781.40| 399.70|
| 19 | 2679.28| 2661.27| 2662.25| 1340.14| P    | 665.31| 647.30| 668.29| 343.15|
| 20 | 2794.31| 2776.30| 2777.28| 1397.65| D    | 588.26| 570.25| 571.24| 294.63|
| 21 | 2957.37| 2939.36| 2940.34| 1479.19| Y    | 473.24| 455.23| 456.23| 237.12|
| 22 | 3120.43| 3092.42| 3093.41| 1560.72| Y    | 310.17| 292.16| 293.15| 155.59|
| 23 | K    | 147.11| 129.10| 130.09| 74.06| 1

S66
6.2 BTZ & BRD3(2) – Lysine 336

B ▪ BRD3(2) - recombinant

▪ Orbitrap MS/MS BRD3(2) 3 uM – BTZ 30 uM

| # | Immonium | b | b-H2O | b-NH | b (Z+) | Seq | y | y-H2O | y-NH | y (Z+) | # |
|---|---------|---|-------|------|--------|-----|---|-------|------|--------|---|
| 1 | 110.072 | 138.067 | 120.056 | 121.046 | 69.533 | H | 20 |
| 2 | 44.050 | 209.247 | 191.093 | 192.077 | 105.052 | A | 2653.205 | 2653.195 | 2653.185 | 2653.175 | 1371.103 | 19 |
| 3 | 44.050 | 280.141 | 262.228 | 263.234 | 140.570 | A | 2562.168 | 2564.157 | 2565.141 | 1291.584 | 18 |
| 4 | 136.076 | 443.323 | 425.308 | 426.177 | 221.102 | Y | 2511.131 | 2493.130 | 2494.104 | 1256.065 | 17 |
| 5 | 44.050 | 514.206 | 496.377 | 497.214 | 257.621 | A | 2348.068 | 2330.057 | 2331.041 | 1174.534 | 16 |
| 6 | 159.092 | 700.317 | 682.310 | 683.294 | 350.596 | W | 2277.031 | 2259.020 | 2260.030 | 1139.015 | 15 |
| 7 | 70.066 | 797.373 | 779.562 | 780.346 | 399.324 | P | 2099.951 | 2072.941 | 2073.924 | 1045.525 | 14 |
| 8 | 120.081 | 944.508 | 926.431 | 927.412 | 472.693 | F | 1993.898 | 1975.888 | 1976.871 | 997.429 | 13 |
| 9 | 136.076 | 1107.814 | 1089.495 | 1090.914 | 554.604 | Y | 1846.830 | 1828.820 | 1829.803 | 923.894 | 12 |
| 10 | 563.227 | 1697.719 | 1679.709 | 1680.693 | 899.667 | K | 1683.767 | 1665.756 | 1666.740 | 842.692 | 11 |
| 11 | 70.066 | 1794.772 | 1776.762 | 1777.745 | 897.433 | P | 1093.576 | 1075.570 | 1076.572 | 547.516 | 10 |
| 12 | 72.081 | 1893.841 | 1875.830 | 1876.814 | 947.420 | V | 996.671 | 978.653 | 979.643 | 498.750 | 9 |
| 13 | 88.040 | 2008.668 | 1989.857 | 1991.841 | 1004.748 | D | 897.433 | 892.928 | 890.904 | 449.216 | 8 |
| 14 | 44.050 | 2078.905 | 2061.894 | 2062.879 | 1040.669 | A | 762.396 | 764.877 | 765.377 | 391.702 | 7 |
| 15 | 102.035 | 2206.947 | 2190.937 | 2191.920 | 1104.974 | E | 711.579 | 693.533 | 694.540 | 356.422 | 6 |
| 16 | 44.050 | 2279.984 | 2262.974 | 2263.957 | 1140.944 | A | 550.349 | 564.362 | 565.356 | 291.772 | 5 |
| 17 | 86.097 | 2393.069 | 2375.058 | 2376.042 | 1197.034 | L | 511.353 | 493.157 | 494.333 | 256.234 | 4 |
| 18 | 102.055 | 2522.111 | 2504.101 | 2505.094 | 1261.556 | E | 398.145 | 380.227 | 381.176 | 199.602 | 3 |
| 19 | 86.097 | 2635.195 | 2617.185 | 2618.168 | 1317.614 | L | 269.121 | 251.202 | 252.134 | 135.680 | 2 |
| 20 | 110.072 | H | 156.077 | 138.066 | 139.050 | 78.531 | 1 |
