# PROTAC-Mediated Degradation of Bruton's Tyrosine Kinase is Inhibited by Covalent Binding

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**ABSTRACT:** The impact of covalent binding on PROTAC-mediated degradation of BTK was investigated through the preparation of both covalent binding and reversible binding PROTACs derived from the covalent BTK inhibitor ibrutinib. It was determined that a covalent binding PROTAC inhibited BTK degradation despite evidence of target engagement, while BTK degradation was observed with a reversible binding PROTAC. These observations were consistently found when PROTACs were employed that were able to recruit either IAP or cereblon E3 ligases. Proteomics analysis determined that use of a covalently bound PROTAC did not result in the degradation of covalently bound targets, whilst degradation was observed for some reversibly bound targets. This observation highlights the importance of catalysis on successful PROTAC-mediated degradation, and highlights a potential caveat for the use of covalent target binders in PROTAC design.

Protein degrading bifunctional molecules are emerging as a novel drug discovery strategy with the potential to offer pharmacologic control of biology that is not currently achievable with existing small molecule medicines.[1-3] Mechanistic hallmarks of proteolysis-targeting chimeras (PROTACs) include high cellular degradation potency ($DC_{50}$, the concentration at which 50% of substrate is degraded) high target selectivity, and extended pharmacodynamic duration of action that is dependent upon both drug pharmacokinetics and target protein resynthesis rate.[4, 5] The targeted protein degradation paradigm is driven by the ability of PROTACs to catalytically promote the degradation of a desired protein in an event-driven process. This contrasts with occupancy-driven pharmacology that is characteristic of traditional small molecule inhibitors.[3]

The catalytic mechanism of PROTAC-mediated protein degradation (Figure 1, blue arrows) has been speculated as being fundamental to the high degradation efficiency observed in both *in vitro* and *in vivo* models.[1] This catalytic paradigm creates opportunities to deliver novel medicines with very low doses.[3] However, in principle it may also be possible for a covalent binding PROTAC to act as a stoichiometric degrader, resulting in degradation of one protein molecule per PROTAC molecule (Figure 1, green arrows). To explore this hypothesis, the effect of covalent binding on PROTAC-mediated degradation of Bruton's tyrosine kinase (BTK) was tested. BTK was chosen as an exemplar protein target, as it has previously been shown to be degradable *via* the PROTAC approach.[6, 7, 8-10] It was envisioned that comparison of structurally similar covalent and non-covalent PROTACs that bind to BTK would elucidate whether the catalytic element of the PROTAC mechanism is important for the efficient degradation of this target. This is important because if stoichiometric PROTACs can be designed for less-tractable targets through the use of covalent target binders to enhance target binding affinity, then this would represent a novel approach for tackling the "undruggable" proteome.[11, 12]

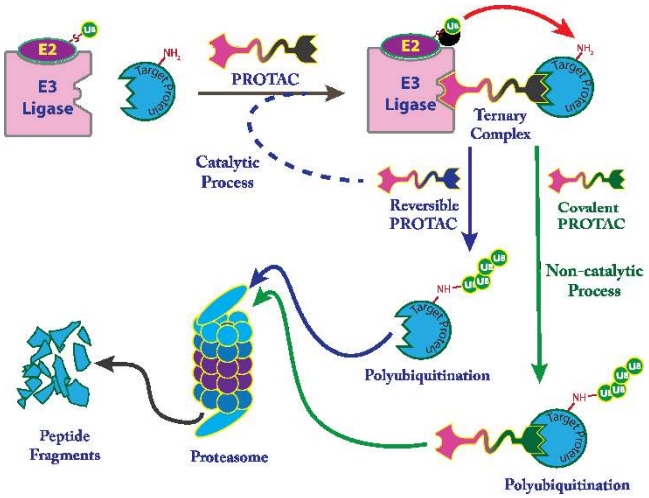



**Figure 1**. Proposed PROTAC mechanism for catalytic reversible PROTACs (blue) and non-catalytic covalent PROTACs (green).

Covalent-binding PROTACs have previously been shown to be functional target degraders. For example, HaloPROTACs containing a chloroalkane designed to covalently bind to a HaloTag target protein fusion have been used to investigate target degradability.[13] This method has been shown to induce degradation of specific fusion proteins by recruitment of the E3 ligases VHL[13] and IAP.[14] Additionally, ERK1/2 was degraded employing a covalent-binding moiety as the targeting element, although covalent binding to ERK1/2 was not confirmed.[15] Although illustrative, these reports do not employ a reversibly binding control compound, therefore the impact of covalent vs. reversible target binding on target degradation cannot be fully characterised.

The current study employs covalent BTK binding moieties chosen such that the covalent-binding element can be removed by formal reduction while still maintaining potent reversible BTK binding and inhibition.[16] This design element allows for a valid comparison of reversible and covalent PROTACs for this target.[10, 17] The covalent BTK inhibitor ibrutinib **1a** and reversibly binding analogue **1b**[16] were selected for elaboration into PROTACs **2** and **3**, respectively employing a moiety that binds and recruits E3 ligases from the inhibitor of apoptosis proteins (IAP) family.[18] The acrylamide moiety of ibrutinib **1a** was selected as a viable linkage point to the E3 ligase binder due to its solvent exposure in the co-crystal structure of BTK and ibrutinib **1a**,[19] and its use in fluorescent ligands or bioorthogonal probes which maintain BTK binding activity.[16, 18, 20] This resulted in ibrutinib-based PROTAC **2** and the corresponding reduced acrylamide analogue **3** as a reversible PROTAC control (**Figure 2**). PROTACs **2** and **3** were both observed to have biochemical inhibitory activity against BTK (**Supplementary Figure 1**). PROTAC **2** was found to covalently modify recombinant BTK *in vitro*, as evidenced by intact protein MS, whilst the saturated acrylamide analog **3** was not observed to covalently modify BTK, as expected (**Supplementary Figure 2**).

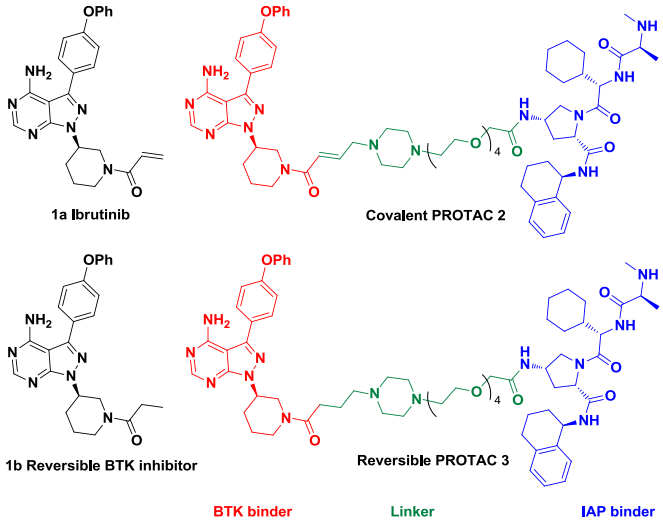



**Figure 2**: Ibrutinib **1a**, reversible BTK inhibitor **1b**, covalent BTK binding PROTAC **2**, and reversible BTK binding PROTAC **3**.

Both the covalently binding PROTAC **2** and the reversible PROTAC **3** were examined for their ability to cause BTK degradation. THP-1 cells were selected as IAP-mediated degradation of BTK had previously been observed in this cell line.[21] No BTK degradation was observed upon treatment of THP-1 cells with the covalent PROTAC **2** at increasing concentrations from 1 nM up to 10 μM for 16 h. (**Figure 3a**). In contrast, THP-1 cell treatment with the reversible PROTAC **3** caused concentration dependent reduction in BTK levels with an approximate $DC_{50}$ of 200 nM (**Figure 3b**). Ibrutinib itself (compound **1a**) was not found to induce BTK degradation (**Supplementary Figure 4**). BTK degradation caused by reversible PROTAC **3** was found to be time dependent in a time course experiment, whilst extended treatment with covalent PROTAC **2** did not result in a significant change to BTK protein levels (**Supplementary Figure 5**).

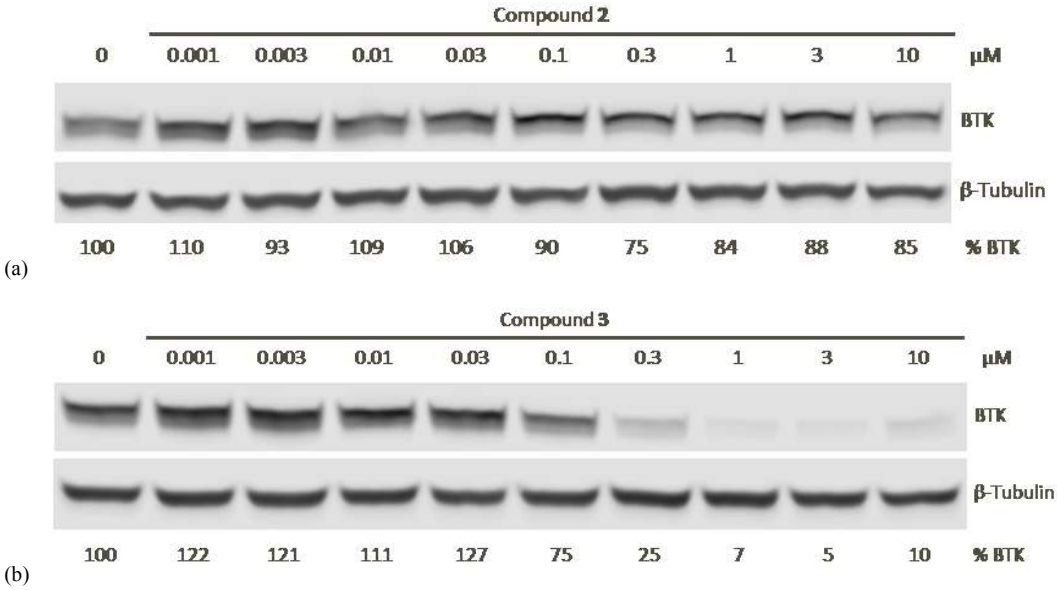



**Figure 3:** (a) Representative immunoblot of BTK following 16 h incubation with covalent PROTAC **2** at increasing concentrations (b) Representative immunoblot of BTK following 16 h incubation with reversible PROTAC **3** at increasing concentrations. Experiments were conducted in THP-1 cells with β-tubulin as a loading control (n = 3, see **Supplementary Figure 3**).

By appropriate selection of cell line, cellular target engagement can be assessed for both the target protein and the E3 ligase for PROTAC compounds **2** and **3**. Kinase domain occupancy of BTK has been shown to correlate with inhibition of BTK autophosphorylation at Y223 following BCR pathway stimulation.[18] Similarly, engagement of the recruited E3 ligase IAP can be assessed, since inhibition of cIAP1 causes its auto-ubiquitination and degradation.[22] THP-1 cells are not sensitive to BCR pathway stimulation, therefore this assessment was conducted in Ramos cells.[23] Following stimulation of the BCR pathway with α-IgM in Ramos cells and treatment with covalent PROTAC **2**, concentration dependent reduction of both pBTK Y223 and cIAP1 were observed suggesting engagement of both target protein and E3 ligase at concentrations above 30 nM, however no degradation of BTK was observed (**Figure 4**). Above concentrations of 300 nM, almost complete inhibition of pBTK Y223 was observed, suggesting full occupancy of the kinase domain. At this concentration, it would be expected that BTK degradation would occur in a stoichiometric manner, however there was no observed change to total BTK protein levels, suggesting the lack of BTK degradation is not due to the lack of PROTAC catalysis. For the reversible PROTAC **3**, cIAP1 levels were also reduced at concentrations greater than 30 nM, suggesting effective IAP target engagement and similar cellular penetration as the covalent binding PROTAC **2**. However, BTK autophosphorylation was reduced only at 300 nM suggesting weaker kinase binding as compared to **2**, as expected upon reduction of the acrylamide covalent binding moiety. Despite evidence of reduced BTK target binding, significant BTK degradation was observed upon treatment with the reversible PROTAC **3** at concentrations greater than 300 nM. Ibrutinib **1a** was again found to have no effect on total protein levels in Ramos cells (**Supplementary Figure 4**). The effect of covalent binding on induced BTK degradation was consistent across tested heterobivalent BTK-IAP PROTACs, and was not found to be linker length dependent (**Supplementary Figure 7**). Degradation of BTK was confirmed to be a result of IAP ligase family recruitment, since diastereomers of PROTACs **2** and **3** employing the inactive enantiomer of the parent IAP inhibitor did not degrade BTK at all concentrations tested (**Supplementary Figure 8**).

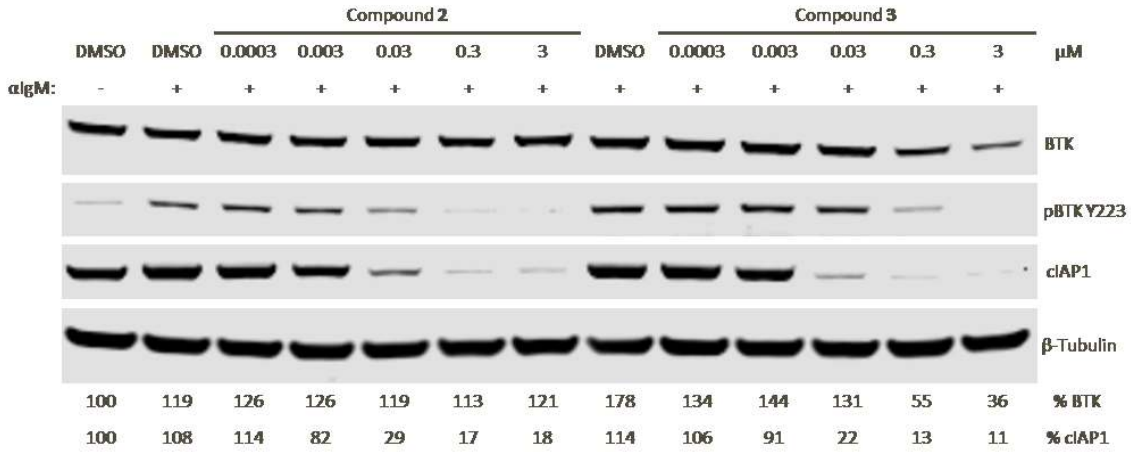
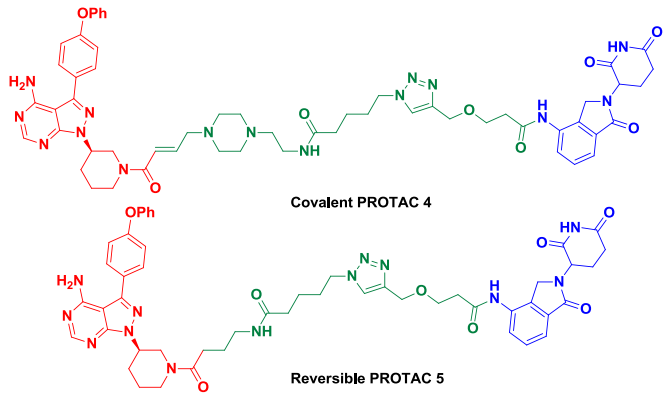


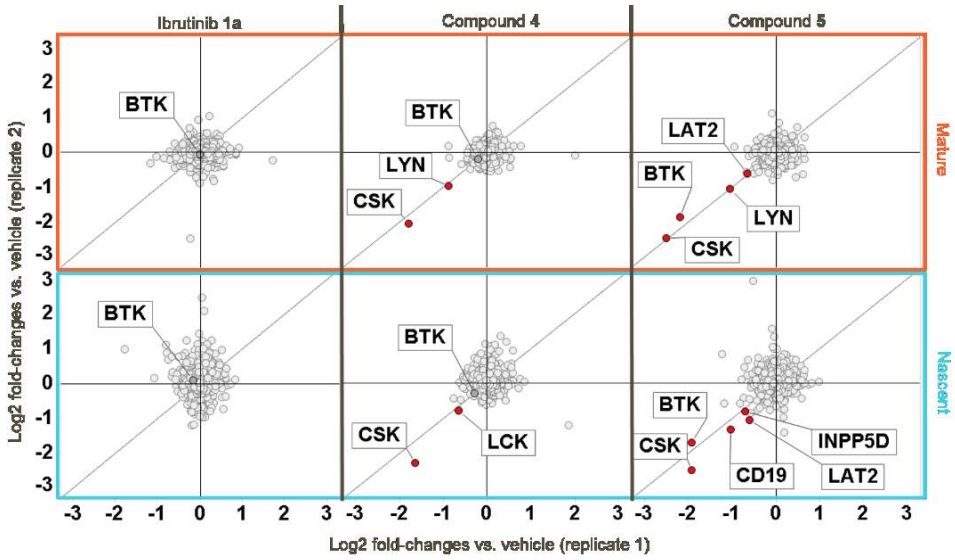

**Figure 4**: Representative immunoblots of BTK, pBTK Y223, cIAP1 following 16 h incubation in Ramos cells with PROTACs **2** or **3** following BCR pathway stimulation with α-IgM. Experiments were conducted in Ramos cells with β-tubulin as a loading control (n = 3, see **Supplementary Figure 6**).

To assess if the inability of covalent binding PROTAC **2** to degrade BTK was restricted to recruitment of IAP E3 ligases, ibrutinib-based PROTACs incorporating a cereblon binding motif were also prepared (**Figure 5A**). Multiplexed protein dynamics profiling experiments were carried out in Ramos cells with 24 h incubation of covalent and reversible cereblon-based PROTACs **4** and **5**, respectively.[24] This approach allows determination of differential effects of compound treatment on the proteome both on mature protein levels, and on *de novo* nascent protein synthesis. This allows detection of targets that are specifically degraded by a functional PROTAC, as these targets would show a reduction in the abundance of both mature and nascent protein levels, whereas downstream transcriptional or translational regulation will be detected by a reduction only in nascent protein levels. Neither ibrutinib **1a** nor the reversible BTK inhibitor **1b** showed any effect on either mature or nascent protein levels at 1 µM (**Figure 5B** and **Supplementary Figure 9**). Covalent PROTAC **4** showed no effect on BTK levels, but induced the degradation of CSK and LYN at 10 µM (concentration response presented in **Supplementary Figure 9**). Both CSK and LYN are SRC kinases reversibly bound by ibrutinib **1a**; both CSK and LYN lack the conserved cysteine present in the BTK kinase domain, suggesting that covalent PROTAC **4** can successfully degrade targets to which it binds with sufficient affinity in a non-covalent manner, though with some selectivity given the promiscuous nature of the warhead, demonstrated previously in our laboratories.[16] The reversible PROTAC **5** was observed to cause the degradation of BTK, as well as to induce the degradation of CSK, LYN and LAT2 at 10 µM (concentration response presented in **Supplementary Figure 9**). Additionally, downregulation of CD19 and INPP5D (SHIP1) expression was observed in the nascent protein pool, but not in the mature pool of proteins. Both LAT2 and CD19 have been reported as being directly involved in BCR signaling, and their translational regulation could plausibly be influenced by the degradation of BTK by PROTAC **5**.[25-27] Additionally, SHIP1 has been reported to play a role in phosphoinositol signaling upstream of BTK, and the translational regulation of SHIP1 may be influenced by the degradation of BTK by PROTAC **5**.[28] Alternatively, the role of LYN in SHIP1 tyrosine phosphorylation and activation could result in the translational downregulation of SHIP1. The protein degradation profile induced by both PROTAC **4** and **5** is concentration dependent, as reflected in BTK, CSK and LYN levels (**Figure 5C**; concentration response presented in **Supplementary Figure 9**), and demonstrates that the effect of covalency on degradation was reproducible between both IAPs and cereblon E3 ligases.

**A)**



B)

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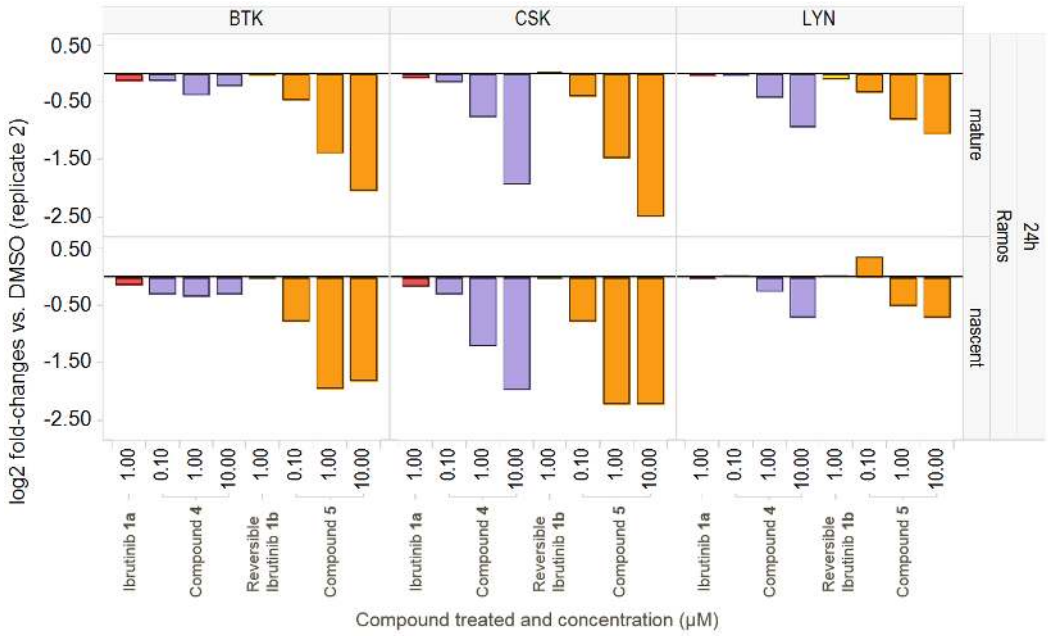


**Figure 5**: **A**) Cereblon PROTACs targeting BTK (PROTACs **4** and **5**). **B**) Multiplexed proteome dynamics profiling of Ramos cells upon PROTAC treatment. Ramos cells were incubated with either ibrutinib **1a** (1 µM), covalent PROTAC **4** (10 µM) or reversible PROTAC **5** (10 µM) for 24 h. Discrimination of mature and nascent proteins was enabled by a pulse SILAC approach. The graphs show fold change values ($log_2$ scale) of abundance compared to the vehicle control of replicate 2 over replicate 1 for each condition. Effects on mature proteins and nascent proteins are shown in the upper panels framed in orange and in the lower panels framed in blue, respectively. Proteins significantly regulated in at least one condition (fold change ≥ 33 % and p value ≤ 0.05) are marked in red. **C**) Multiplexed proteome dynamics profiling of Ramos cells upon compound treatment, showing fold change values ($log_2$ scale) for representative replicate 2 for BTK, CSK and LYN. Cellular treatment with ibrutinib **1a** or the reversible ibrutinib analogue **1b** (1 µM), compound **4** (0.1, 1 and 10 µM), and compound **5** (0.1, 1 and 10 µM).

The lack of PROTAC-mediated degradation of BTK observed with ibrutinib-derived covalent binding PROTACs **2** and **4** highlights a potential caveat for covalent-based approaches for the induction of targeted protein degradation, as our experiments show that irreversible target binding blocks IAP or cereblon-mediated degradation of this target. Potential explanations for this unusual effect may be that the covalently bound ligand blocks ubiquitin transfer onto a surface lysine required for degradation, or the covalent protein modification blocks the presentation of the intrinsically disordered region required for recognition by the proteasome.[29] In contrast, PROTACs that reversibly bind to BTK can induce the degradation of BTK, but may not induce the degradation of other kinases bound by the molecule, as suggested by the results observed with PROTAC **5**, and separately by Crews and Gray.[6, 7] This work suggests that despite literature evidence indicating that PROTAC molecules can successfully induce the degradation of covalently bound protein targets,[13, 14] different factors likely play a significant role in the degradation of covalently bound PROTAC targets as compared to reversibly bound PROTAC targets.

## ASSOCIATED CONTENT

### Supporting Information

Supporting information, including supplementary figures, materials and methods, and compound characterisation is available free of charge via the internet at http://pubs.acs.org

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### Author Contributions

±These authors contributed equally. C.P.T., H.L synthesised compounds and performed intact protein MS. L.D. synthesised compounds and performed proteomics study. L.D., M.B. conceptualized proteomics study and analysed results. Z.I.B., S.E.H. prepared immunoblots. H.D. managed biological outsourcing. M.M., I.E.D.S., W.J.K., G.A.B., J.D.H. provided supervisory support. C.P.T., H. L. prepared the manuscript.


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## ABBREVIATIONS

PROTAC, Proteolysis Targeting Chimera, IAP, inhibitor of apoptosis protein, XIAP, X-linked inhibitor of apoptosis protein, cIAP1, cellular inhibitor of apoptosis protein-1, BTK, Bruton's Tyrosine Kinase, VHL, Von Hippel-Lindau, ERK1/2, Extracellular signal–regulated kinases, BCR, breakpoint cluster region protein, CSK, C-terminal Src kinase, LAT2, Linker for Activation of T-cell Family Member2, CD19, Cluster of Differentiation 19, INPP5D, Inositol polyphosphate-5-phosphatase.

## REFERENCES


1. Tinworth, C. P.; Lithgow, H.; Churcher, I. Small molecule-mediated protein knockdown as a new approach to drug discovery. *Med. Chem. Commun.* **2016,** 7, 2206-2216.
2. Toure, M.; Crews, C. M. Small-Molecule PROTACS: New Approaches to Protein Degradation. *Angew. Chem. Int. Ed.* **2016,** 55, 1966-1973.
3. Churcher, I. Protac-Induced Protein Degradation in Drug Discovery: Breaking the Rules or Just Making New Ones? *J. Med. Chem.* **2018,** 61, 444-452.
4. Bondeson, D. P.; Mares, A.; Smith, I. E.; Ko, E.; Campos, S.; Miah, A. H.; Mulholland, K. E.; Routly, N.; Buckley, D. L.; Gustafson, J. L.; Zinn, N.; Grandi, P.; Shimamura, S.; Bergamini, G.; Faelth-Savitski, M.; Bantscheff, M.; Cox, C.; Gordon, D. A.; Willard, R. R.; Flanagan, J. J.; Casillas, L. N.; Votta, B. J.; den Besten, W.; Famm, K.; Kruidenier, L.; Carter, P. S.; Harling, J. D.; Churcher, I.; Crews, C. M. Catalytic in vivo protein knockdown by small-molecule PROTACs. *Nat. Chem. Biol.* **2015,** 11, 611-617.
5. Raina, K.; Lu, J.; Qian, Y.; Altieri, M.; Gordon, D.; Rossi, A. M. K.; Wang, J.; Chen, X.; Dong, H.; Siu, K.; Winkler, J. D.; Crew, A. P.; Crews, C. M.; Coleman, K. G. PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. *Proc. Natl. Acad. Sci. U. S. A.* **2016,** 113, 7124-7129.
6. Bondeson, D. P.; Smith, B. E.; Burslem, G. M.; Buhimschi, A. D.; Hines, J.; Jaime-Figueroa, S.; Wang, J.; Hamman, B. D.; Ishchenko, A.; Crews, C. M. Lessons in PROTAC Design from Selective Degradation with a Promiscuous Warhead. *Cell Chem. Biol.* **2018,** 25, 78-87.
7. Huang, H. T.; Dobrovolsky, D.; Paulk, J.; Yang, G.; Weisberg, E. L.; Doctor, Z. M.; Buckley, D. L.; Cho, J. H.; Ko, E.; Jang, J.; Shi, K.; Choi, H. G.; Griffin, J. D.; Li, Y.; Treon, S. P.; Fischer, E. S.; Bradner, J. E.; Tan, L.; Gray, N. S. A Chemoproteomic Approach to Query the Degradable Kinome Using a Multi-kinase Degrader. *Cell Chem. Biol.* **2018,** 25, 88-99.
8. Sun, Y.; Zhao, X.; Ding, N.; Gao, H.; Wu, Y.; Yang, Y.; Zhao, M.; Hwang, J.; Song, Y.; Liu, W.; Rao, Y. PROTAC-induced BTK degradation as a novel therapy for mutated BTK C481S induced ibrutinib-resistant B-cell malignancies. *Cell Res.* **2018,** 28, 779-781.
9. Zorba, A.; Nguyen, C.; Xu, Y.; Starr, J.; Borzilleri, K.; Smith, J.; Zhu, H.; Farley, K. A.; Ding, W.; Schiemer, J.; Feng, X.; Chang, J. S.; Uccello, D. P.; Young, J. A.; Garcia-Irrizary, C. N.; Czabaniuk, L.; Schuff, B.; Oliver, R.; Montgomery, J.; Hayward, M. M.; Coe, J.; Chen, J.; Niosi, M.; Luthra, S.; Shah, J. C.; El-Kattan, A.; Qiu, X.; West, G. M.; Noe, M. C.;

Shanmugasundaram, V.; Gilbert, A. M.; Brown, M. F.; Calabrese, M. F. Delineating the role of cooperativity in the design of potent PROTACs for BTK. *Proc. Natl. Acad. Sci. U. S. A.* **2018,** 115, E7285-E7292.
10. Buhimschi, A. D.; Armstrong, H. A.; Toure, M.; Jaime-Figueroa, S.; Chen, T. L.; Lehman, A. M.; Woyach, J. A.; Johnson, A. J.; Byrd, J. C.; Crews, C. M. Targeting the C481S Ibrutinib-Resistance Mutation in Bruton's Tyrosine Kinase Using PROTAC-Mediated Degradation. *Biochemistry* **2018,** 57, 3564-3575.
11. Huang, X.; Dixit, V. M. Drugging the undruggables: exploring the ubiquitin system for drug development. *Cell Res.* **2016,** 26, 484-98.
12. Dang, C. V.; Reddy, E. P.; Shokat, K. M.; Soucek, L. Drugging the 'undruggable' cancer targets. *Nat. Rev. Cancer* **2017,** 17, 502-508.
13. Buckley, D. L.; Raina, K.; Darricarrere, N.; Hines, J.; Gustafson, J. L.; Smith, I. E.; Miah, A. H.; Harling, J. D.; Crews, C. M. HaloPROTACS: Use of Small Molecule PROTACs to Induce Degradation of HaloTag Fusion Proteins. *ACS Chem. Biol.* **2015,** 10, 1831-1837.
14. Tomoshige, S.; Hashimoto, Y.; Ishikawa, M. Efficient protein knockdown of HaloTag-fused proteins using hybrid molecules consisting of IAP antagonist and HaloTag ligand. *Bioorg. Med. Chem.* **2016,** 24, 3144-8.
15. Lebraud, H.; Wright, D. J.; Johnson, C. N.; Heightman, T. D. Protein Degradation by In-Cell Self-Assembly of Proteolysis Targeting Chimeras. *ACS Cent. Sci.* **2016,** 2, 927-934.
16. Dittus, L.; Werner, T.; Muelbaier, M.; Bantscheff, M. Differential Kinobeads Profiling for Target Identification of Irreversible Kinase Inhibitors. *ACS Chem. Biol.* **2017,** 12, 2515-2521.
17. Farooqui, M. Z.; Valdez, J.; Martyr, S.; Aue, G.; Saba, N.; Niemann, C. U.; Herman, S. E.; Tian, X.; Marti, G.; Soto, S.; Hughes, T. E.; Jones, J.; Lipsky, A.; Pittaluga, S.; Stetler-Stevenson, M.; Yuan, C.; Lee, Y. S.; Pedersen, L. B.; Geisler, C. H.; Calvo, K. R.; Arthur, D. C.; Maric, I.; Childs, R.; Young, N. S.; Wiestner, A. Ibrutinib for previously untreated and relapsed or refractory chronic lymphocytic leukaemia with TP53 aberrations: a phase 2, single-arm trial. *Lancet Oncol.* **2015,** 16, 169-76.
18. Honigberg, L. A.; Smith, A. M.; Sirisawad, M.; Verner, E.; Loury, D.; Chang, B.; Li, S.; Pan, Z.; Thamm, D. H.; Miller, R. A.; Buggy, J. J. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl. Acad. Sci. U. S. A.* **2010,** 107, 13075-80.
19. Bender, A. T.; Gardberg, A.; Pereira, A.; Johnson, T.; Wu, Y.; Grenningloh, R.; Head, J.; Morandi, F.; Haselmayer, P.; Liu-Bujalski, L. Ability of Bruton's Tyrosine Kinase Inhibitors to Sequester Y551 and Prevent Phosphorylation Determines Potency for Inhibition of Fc Receptor but not B-Cell Receptor Signaling. *Mol. Pharmacol.* **2017,** 91, 208.
20. Liu, N.; Hoogendoorn, S.; van de Kar, B.; Kaptein, A.; Barf, T.; Driessen, C.; Filippov, D. V.; van der Marel, G. A.; van der Stelt, M.; Overkleeft, H. S. Direct and two-step bioorthogonal probes for Bruton's tyrosine kinase based on ibrutinib: a comparative study. *Org. Biomol. Chem.* **2015,** 13, 5147-57.
21. Harling, J. D.; Tinworth, C. P. Novel Compounds. WO2017/211924A1, 2017.
22. Sekine, K.; Takubo, K.; Kikuchi, R.; Nishimoto, M.; Kitagawa, M.; Abe, F.; Nishikawa, K.; Tsuruo, T.; Naito, M. Small Molecules Destabilize cIAP1 by Activating Auto-ubiquitylation. *J. Biol. Chem.* **2008,** 283, 8961-8968.
23. Nisitani, S.; Kato, R. M.; Rawlings, D. J.; Witte, O. N.; Wahl, M. I. *In situ* detection of activated Bruton's tyrosine kinase in the Ig signaling complex by phosphopeptide-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **1999,** 96, 2221-2226.
24. Savitski, M. M.; Zinn, N.; Faelth-Savitski, M.; Poeckel, D.; Gade, S.; Becher, I.; Muelbaier, M.; Wagner, A. J.; Strohmer, K.; Werner, T.; Melchert, S.; Petretich, M.; Rutkowska, A.; Vappiani, J.; Franken, H.; Steidel, M.; Sweetman, G. M.; Gilan, O.; Lam, E. Y. N.; Dawson, M. A.; Prinjha, R. K.; Grandi, P.; Bergamini, G.; Bantscheff, M. Multiplexed Proteome Dynamics Profiling Reveals Mechanisms Controlling Protein Homeostasis. *Cell* **2018,** 173, 260-274.e25.
25. Brdicka, T.; Imrich, M.; Angelisova, P.; Brdickova, N.; Horvath, O.; Spicka, J.; Hilgert, I.; Luskova, P.; Draber, P.; Novak, P.; Engels, N.; Wienands, J.; Simeoni, L.; Osterreicher, J.; Aguado, E.; Malissen, M.; Schraven, B.; Horejsi, V. Non-T cell activation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling. *J. Exp. Med.* **2002,** 196, 1617-26.
26. Callard, R. E.; Rigley, K. P.; Smith, S. H.; Thurstan, S.; Shields, J. G. CD19 regulation of human B cell responses. B cell proliferation and antibody secretion are inhibited or enhanced by ligation of the CD19 surface glycoprotein depending on the stimulating signal used. *J. Immunol.* **1992,** 148, 2983-7.
27. Xu, Y.; Fairfax, K.; Light, A.; Huntington, N. D.; Tarlinton, D. M. CD19 differentially regulates BCR signalling through the recruitment of PI3K. *Autoimmunity* **2014,** 47, 430-7.
28. Wisniewski, D.; Strife, A.; Swendeman, S.; Erdjument-Bromage, H.; Geromanos, S.; Kavanaugh, W. M.; Tempst, P.; Clarkson, B. A novel SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase (SHIP2) is constitutively tyrosine phosphorylated and associated with src homologous and collagen gene (SHC) in chronic myelogenous leukemia progenitor cells. *Blood* **1999,** 93, 2707-20.
29. Prakash, S.; Tian, L.; Ratliff, K. S.; Lehotzky, R. E.; Matouschek, A. An unstructured initiation site is required for efficient proteasome-mediated degradation. *Nat. Struct. Mol. Biol.* **2004,** 11, 830.