Recent Advances in PROTAC Technology towards BET Protein Targets for Cancer Treatment

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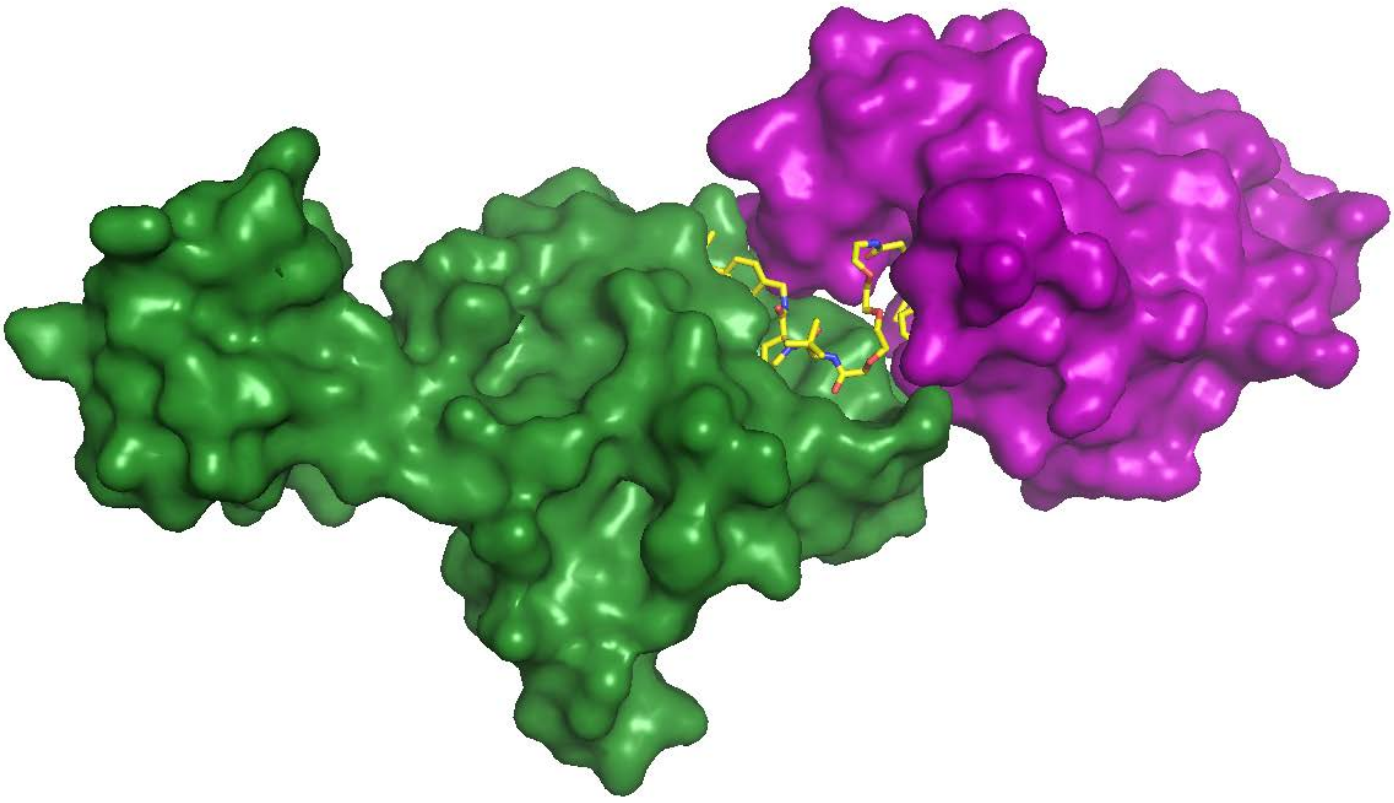

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Bromodomain and extraterminal proteins

In the last two decades it has been recognized that deregulation in epigenetics is as important in carcinogenesis as genetic mutation. [10a, b] Therefore the quest toward therapeutic targets has shifted to some extend toward epigenetic targets. One such example is the bromodomain and extraterminal protein family, or BET proteins which are chromatin readers. They recruit chromatin regulating enzymes, which in turn regulate gene expression. The BET proteins in the human proteome consist of BRD2, BRD3, BRD4 and the testis specific BRDT. These proteins share two distinct motifs, two bromodomains (henceforth called BD1 and BD2) in the amino-terminal region capable of recognizing acylated lysines and an extraterminal protein-protein interaction domain in the carboxy-terminal region.[10a, c-e] It has been extensively reported that BRD2 and BRD4 have a crucial role in cell cycle control and as such are essential for cell growth. BRD4 deregulation has been closely associated with numerous cancer diseases.[10a,f] Furthermore, inhibition of BRD4 decreases effective c-Myc protein levels, which has been reported to be elevated in various cancers and tumor cells. This has been extensively reviewed by Khan (2006).[11] This colloquium reports on the latest PROTAC developments using the BET protein family as a target, with its current advantages and drawbacks.

BET protein inhibitors

One of the first BET inhibitors was the JQ-1, named after Jun Qi, reported by Bradner's lab (2010).[12] Its target, BRD4, was later confirmed as a therapeutic target in acute myeloid leukemia in an RNAi screen in 2011.[13] Remarkable, only BRD4 of the BET proteins was evaluated as a target for acute myeloid leukemia, whilst JQ-1 has similar binding to BRD3 and slightly lower binding to BRD2 and BRDT. Inhibition of these BET family members may contribute to the observed biological effects of JQ-1. The discovery of JQ-1 was based on the observation made by Mitsubishi Tanabe Pharma Corporation in 2006 that the core structure of thienodiazepines has a good binding affinity towards BRD4. [14] JQ-1 showed some promising results, such as absence of adverse symptoms or weight loss and reduction of tumor growth when tested in mice. However, to be acceptable as a drug, improvement is desirable. For example, the described effectiveness is in tumor growth reduction, rather than tumor growth arrest or even tumor regression. Furthermore, the selectivity towards BRD4 specifically compared to other BET proteins is low, as its binding is similar to the binding with

BRD3 and only slightly better than binding with BRD2 and BRDT. This is to be expected as these binding sites are very much similar in all of these BET proteins. The binding affinity with Kd values for BRD4 of 50 nM and 90 nM for the first and second bromodomain respectively. An optimization study was performed by Wang's lab (2015).[1] The known BET protein inhibitors JQ-1, I-BET-762, I-BET-151 and OTX015 (Figure 1) were inspected and a similar motif in all of these was observed. For optimization they categorized these molecules into three parts: The head, the body and the tail.

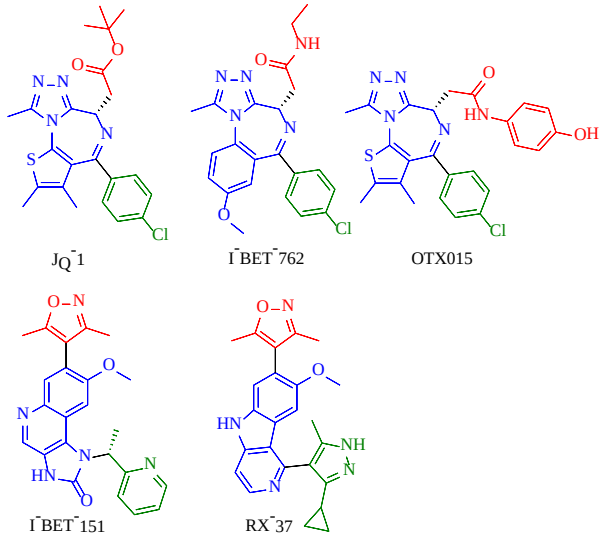

Figure 1: The known inhibitors and the optimized novel inhibitor developed by Wang's lab divided in 3 parts; the head in red, the body in blue and the tail in green.[1]

They performed optimization studies for these individual parts and discovered that the aza carbazole motif used as the body resulted in good binding affinities. They ultimately gained compound RX-37 with Ki binding affinities 3.2-24.7 nM for the BRD2, 3 and 4 proteins. It should be noted that the strongest binding was with BRD3, followed by BRD2 and lastly BRD4. Furthermore, their compound showed a moderate affinity of Kd = 670 nM for CREBBP protein, which could result in side effects in a therapeutic setting.

One major issue with BET protein inhibitor associated treatment is the robust and rapid accumulation of BRD4.[2,5] This results in moderate effects on downstream c-MYC suppression and proliferation. This finding is concerning, as it implies that the need for continuation of the BET inhibitor treatment is necessary not only to suppress active BRD4 enzyme, but also to keep the increased accumulated BRD4 protein levels from reactivating. The reactivating of BRD4 to express the c-MYC gene upon withdrawal of drug treatment was indeed confirmed by Crews' lab, by treating cells to 1.0 μM JQ-1 or OTX015 for 24 h, then the cells were washed out to remove the inhibitor and c-MYC production was confirmed within 4 h after the wash out. This finding may in part be the reason why drug resistance is developed in cancer cells upon prolonged exposure of BET inhibitors, the best example of this is the castration-resistant prostate cancer, or CRPC. Moreover, c-Myc levels were lowered, but never completely knocked out

when using BET inhibitors such as JQ-1 and OTX015, even when using up to 10-fold increased dosage. To combat this inhibitor induced increase of protein, the use of a new emerging technology has been employed.

PROTAC

PROTAC, meaning proteolysis-targeting chimeras is an emerging technology which was first described in Howley's lab in 2000.[15] This technology is based on using the ubiquitination machinery to degrade targeted enzymes. The molecules known as PROTACs are hetero-bifunctional compounds consisting of three parts; a target binding moiety, a ligase recruiting moiety and a linking-region to bridge the two functionalities. This makes the working mechanism of PROTACs unique compared to traditional drugs; rather than inhibiting the target enzyme to render it inactive, PROTACs initiate the degradation of the target proteins by using a ligase; the ligase initiates ubiquitination, marking the enzyme for degradation by the proteasome, depicted in figure 2. As such PROTACs inhibit their target as a tool to bring it in close proximity of a ligase for degradation, rather than having inhibition as its main mode of action. This eliminates the major issue of accumulation of inhibited BRD4 proteins described earlier. Another potential advantage is due to their degradation mode of action rather than inhibition; PROTACs have the potential to work catalytically. This could aid in a more acceptable drug due to the small dosages required in a therapeutic setting.

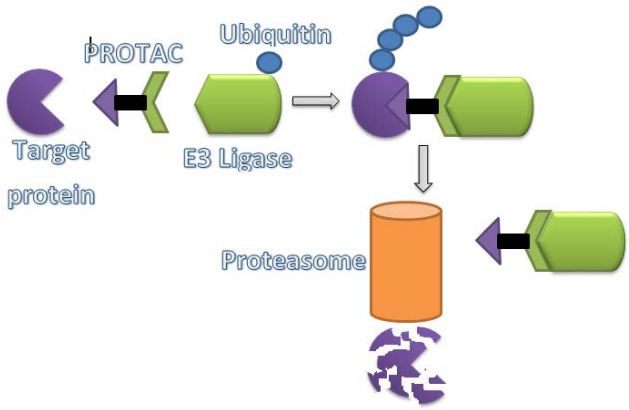

Figure 2: Simplified depicture of PROTAC mode of action; A PROTAC, made up of a target binder (purple), a linker (black) and a ligase recruiter (green). The ligase initiates the ubiquitination of the target protein brought in proximity by the PROTAC, which is then degraded by the proteasome.

PROTACs targeting BRD4

The labs of Crews, Ciulli and Bradner recognized the potential of PROTACs to target BRD4. They all made their own potent PROTAC in 2015, with Crews designing ARV-825, Ciulli with MZ1 and Bradner with dBET1.[2,3,4] All of these PROTACs use a similar BRD4 binding moiety based on either JQ-1 or OTX015. Crews and Bradner employ the thalidomide molecule which is known to bind cereblon, a protein that forms an E3 ligase complex that initiates ubiquitination.

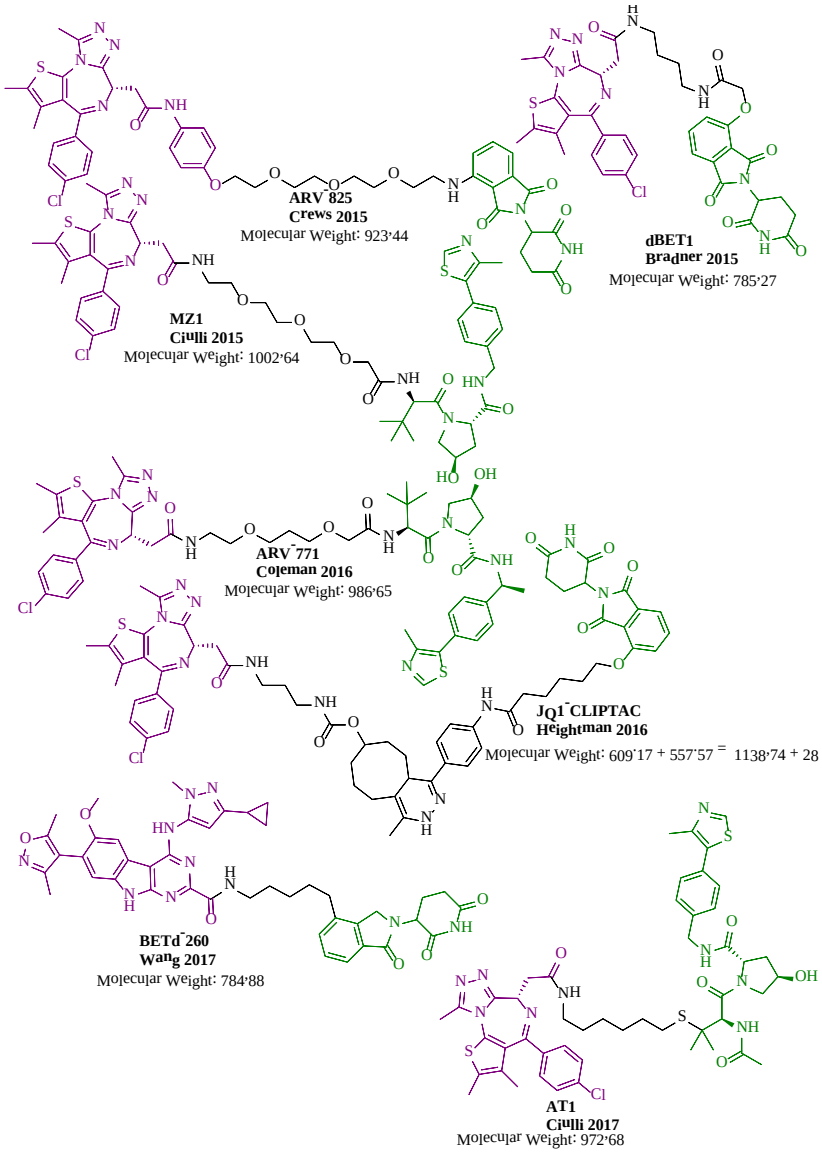

Figure 3: The PROTACs targeting the BET proteins reviewed in this colloquium. The BRD4 binder is depicted in purple, the ligase recruiter in green and the linker in black.[2-6, 8, 9]

Ciulli's lab employed VHL-1, an inhibitor for the von Hippel-Lindau protein which is an E3 ligase involved in the ubiquitination process. The main difference between these PROTACs is the length of the linker connecting the two binding moieties.

Ciulli's lab found MZ1 to have binding constant of 382 nM for BD1 and 120 nM for BD2. Ciulli found binding constants for JQ-1 of 49 nM for BD1 and 90 nM for BD2, consistent with Bradner's determined binding constants when they first synthesized JQ-1. Crews' lab found ARV-825 to have a binding affinity for BRD4 of 90 nM to BD 1 and 28 nM to BD 2. This is a 6 fold decrease with BD1 and 8 fold decrease with BD2 in binding affinity compared to OTX015, which they determined to have binding constants of 14 nM and 3.8 nM respectively. Surprisingly, when determining the binding constants for JQ-1, they found binding constants of 12 nM to BD1 and 10 nM for BD2. Although these binding affinities are lower than those of the original inhibitors, these PROTACs degraded BRD4 proteins rather than increasing its concentration; ARV-825 showed almost complete BRD4 degradation in Namalwa and CA-46 cells after overnight treatment at 1 nM concentration. MZ1 had complete degradation of BRD4 at 250 nM concentration after 8h in HeLa cells. Bradner tested dBET1 on MV4:11 cells and showed complete degradation of BRD4 after a 2h treatment of 250 nM dBET1. Bradner's lab noticed recovery of BRD4 levels after 24 h after dBET1 administering, which they presumed to be linked to the known instability of phthalimides. This last observation shows that more research needs to be conducted regarding the stability of PROTACs in

general. The effectiveness of PROTACs over inhibitors shows best when observing their impact on downregulated protein levels; Crews used Namalwa and Ramos cells for which they determined ARV-825 to reduce c-MYC suppression after overnight treatment at a concentration of 0.1 μM, whilst JQ-1 and OTX015 only showed some reduction of c-MYC concentration at 1 μM and significant reduction at 10 μM. They also tested the drug efficacy after wash-out, which can be compared to drug-withdrawal in a therapeutic setting. They found that when treating Namalwa cells for 24 h with 0.1 μM ARV-825, followed by three washes and then reseeding in fresh medium, BRD4 levels did not recover within 24 h, also c-MYC levels did not recover within the first 6 h, but after 24 h did recover to some extent. Ciulli treated HeLa cells with 1 μM MZ1 for 4 h followed by washing; they observed significant BRD4 recovery 20 h after the wash. MZ1 was the only PROTAC which had selectivity for BRD4 over BRD2 and BRD3, although no rationale was found for this preferred selectivity.

Further evaluation was done by Bradner's lab; they tested in vivo by treating tumor induced mice with 50 mg/kg of dBET-1.[4] This dBET-1 suppressed tumor growth significantly as depicted in Figure 4; additionally, they found that two weeks of treatment with dBET1 was well tolerated, as the mice showed insignificant loss of weight and normal blood counts. By these results, they decided to do a post mortem

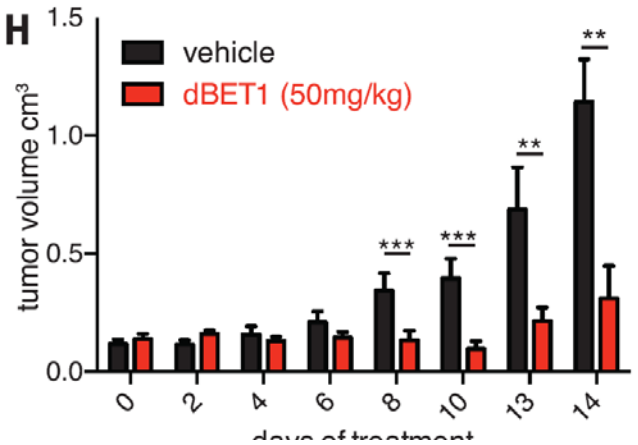

Figure 4: Tumor volume of vehicle-treated mice or mice treated with dBET1 (50 mg/kg) for 14 days. Reproduced from ref 4. Copyright Science (2015).

analysis of a leukemia model to see how many leukemia cells were left after a 19 day daily treatment with either a vehicle, JQ-1 or their compound dBET1. They found that the vehicle control had roughly 25% leukemic cells in the bone marrow, JQ-1 had roughly 20%, whilst dBET1 treated mice had less than 10% leukemic cells left in the bone marrow. These results show great potential, though more research should be conducted regarding binding potency, long term (side-) effects in vivo and stability of PROTACs.

Second Generation PROTAC

Inspired by these PROTACs, Coleman's lab designed a PROTAC aiming for stronger binding potency, called ARV-

771.[5] They claim that ARV-771 is 10 fold more potent than JQ-1, 100-fold more potent than OTX015 and 500 fold more potent than dBET1 in depleting c-MYC levels, but similarly potent to ARV-825. No comparison was made with MZ1 in this respect. They tested their PROTAC on CRPC mice xenografts with starting point of tumor volume of 250 mm^3 and found a tumor regression when using a 30 mg/kg treatment with ARV-771. Moreover, they found that 2 out of their 10 mice were devoid of any palpable tumor mass after 20 days of treatment and no treatment resulted in significant loss of body weight. However, they did find skin discoloration which suggested an overall deterioration of skin health, which they observed to be restored when taking a drug-holiday of 2-3 days.

CLIPTAC methodology

The PROTAC compounds described so far have one thing in common; they all have a high molecular weight and large size, especially ARV-825 and MZ1. This is highly undesirable as a drug, as this could lead to low cell permeability and solubility. To solve this issue Heightman's lab thought of the following; since PROTACs are made up of two functional moieties, they reasoned these moieties could be linked into one functional molecule within the cell, by the use of a bioorthogonal click reaction.[6] As such they developed two precursor compounds named JQ1-TCO and Tz-thalidomide based on the JQ-1 inhibitor and thalidomide, which they tagged with a *trans*-cyclo-octene (TCO) and a tetrazine (Tz) respectively. These functionalities are known to react in the inverse electron demand Diels-Alder cycloaddition, which is a fast, high yielding, bioorthogonal reaction, depicted in Figure 5.[16]

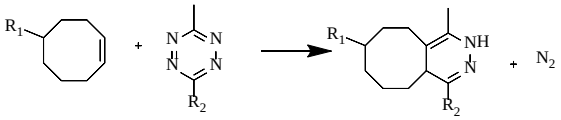

Figure 5: The fast, high yielding, biorthogonal inverse electron demand Diels-Alder cycloaddition reaction.[16]

The resulting CLIPTAC (from click derived PROTAC), had IC_{50} values of 9 nM for BD1 and 19 nM for BD2. They analyzed the ability of their CLIPTAC to degrade the BRD4 protein and its dependence of the E3 ligase. This was tested by first treating HeLa cells with JQ1-TCO for 18 h, followed by treatment with Tz-thalidomide for 18 h and *vice versa*. They found that indeed their CLIPTAC works by protein degradation via recruitment of the ligase by doing a control experiment using carfilzomib, a strong proteasome inhibitor, which resulted in rescued BRD4 levels. Furthermore they tested if their CLIPTAC was still functional if it was preformed outside of

the cell. This was done by treating HeLa cells with JQ1-CLIPTAC for 18 h, which resulted in no degradation of BRD4. This confirmed their presumption that their CLIPTAC has limited cell permeability and has to be formed within the cell in order to function. It should be noted that although their two precursors are smaller than the existing PROTACs targeting BET proteins, they are still quite large in size.

Extensive PROTAC assays

After these promising proof of concept results, Bhalla's lab has recently reported their more extensive assay and its findings about ARV-825 and ARV-771.[7] They confirmed that when using the PROTAC ARV-825 the depletion levels of c-Myc and other BRD4 upregulated proteins was sustained when tested 24 h after wash out of the drug, whilst for the inhibitor OTX015 the protein levels were restored. They also tested the in vivo anti secondary acute myeloid leukemia properties of ARV-771, the PROTAC made by Coleman's lab, compared to OTX015. Likewise they found that ARV-771 was more effective in reducing the secondary acute myeloid leukemia cells (or sAML cells) 7 days after engraftment by a bioluminescence assay of the sAML cells. Like Coleman's lab, they too found that treatment with ARV-771 gave less loss of body weight compared to OTX015. Most notable was their finding on the average survival rate of their sAML engrafted mice upon ARV-771 treatment. They did an assay whereby they engrafted mice with sAML cells. After 4 days they started treatment with either a vehicle, OTX015 or ARV-771. Then after one more week treatment was terminated. Within 19 days all vehicle treated mice died, after 25 days 40% of the OTX015 treated mice survived whilst for the ARV-771 treated mice 90% survived.

Rational structure-based PROTAC design

Wang's lab recognized the potential of PROTACs as well, thus using their optimized inhibitor RX-37 as a basis to design a new PROTAC.[8] They chose phthalimide to recruit cereblon as the ligase and then performed an optimization study to find the best linker length and linker composition to use in their final PROTAC. After finding their best cell growth inhibitor compounds they performed BET degradation studies to find the compound with the best BRD2, 3 and 4 degradation potency. Their best compound BETd-260 achieved IC_{50} inhibition of cell growth values of 51 pM in RS4;11 cell line and 2.3 nM in MOLM-13 acute leukemia cell lines. Furthermore, they found that their PROTAC had *in vivo* tumor regression with a dose of 5 mg/kg.

Moreover, no significant change in body weight was observed with BETd-260 treatment.

Ciulli's lab has most recently solved the crystal structure for their MZ1 PROTAC in a ternary complex with human VHL and BRD4 (PDB code: 5T35).[9] They found that the PROTAC induced protein-protein interactions between the VHL and BRD4, which resulted in strongly positive cooperativity, thus making the ternary complex highly populated compared to uncomplexed or binary complexed species. Based on this ternary structure they refined their PROTAC and thus designed AT1. AT1 showed impressive selectivity for BRD4 over BRD2 and BRD3 observed by AlphaLISA (an improved assay based on ELISA, meaning enzyme-linked immunosorbent assay). This was confirmed by treating HeLa cells to different concentrations of AT-1 for 24 h; at 1-3 μM BRD4 proteins were completely degraded, whilst BRD2 and BRD3 were still present. This is a good example of the power of structure guided design. It shows the necessity of information regarding structure conformation and interaction with both binding targets in a ternary complex, necessary for the ubiquitination process.

Outlook

In this report, the major recent developments with PROTACs targeting the BET proteins have been reviewed. It is interesting to see how the development here resembles that of any new technology, whereby the initial approach is mostly mix-and-match, followed by a rational design. The major limitations of these PROTACs are large sizes, limited stability and potential side effects. Many of these PROTACs employed thalidomide moieties to bind cereblon as the ligase. Thalidomides are infamous for causing birth defects when used by pregnant women,[17] which happened in Germany in 1957 where it was sold under the trade name Contergan. Thus more research should be conducted regarding these limitations, especially regarding side effects. Also better methods for rational PROTAC design are desirable, especially regarding what type of linker is used. The potential of protein degradation over inhibition should be evident by now, and it is intriguing to see which biological systems could benefit from this alternative approach. It is exciting to see where this technology applied to the BET protein family will lead to in a therapeutic setting. Lastly, the question is not if a marketable drug PROTAC will arise, but rather when.

References

Primary references

[1] X. Ran, Y. Zhao, L. Liu, L. Bai, C. Yang, B. Zhou, J.L. Meagher, K. Chinnaswamy, J.A. Stuckey and S. Wang, Structure-Based Design of γ‑Carboline Analogues as Potent and Specific BET Bromodomain Inhibitors, *J. Med. Chem.* **2015**, 58, 4927−4939
DOI: 10.1021/acs.jmedchem.5b00613
[2] J. Lu, Y. Qian, M. Altieri, H. Dong, J. Wang, K. Raina, J. Hines, J.D. Winkler, A.P. Crew, K. Coleman and C.M. Crews, Hijacking the E3 Ubiquitin Ligase Cereblon to Efficiently Target BRD4, *Chem Biol.* **2015**, 22(6), 755-763.
DOI:10.1016/j.chembiol.2015.05.009.
[3] M. Zengerle, K. Chan and A. Ciulli, Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4, *ACS Chem. Biol.* **2015**, 10, 1770−1777.
DOI: 10.1021/acschembio.5b00216
[4] G.E. Winter, D.L. Buckley, J. Paulk, J.M. Roberts, A. Souza, S. Dhe-Paganon, J.E. Bradner, Phthalimide conjugation as a strategy for in vivo target protein degradation, *Science.* **2015**, 348(6241), 1376-1381. DOI: 10.1126/science.aab1433.
[5] K. Rainaa, J. Lua, Y. Qiana, M. Altieria, D. Gordona, A.M.K. Rossia, J. Wanga, X. Chena, H. Donga, K. Siua, J.D. Winklera, A.P. Crewa, C.M. Crews and K.G. Coleman, PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer, *Proc Natl Acad Sci U S A.* **2016,** 113(26): 7124–7129.
DOI:10.1073/pnas.1521738113.
[6] H. Lebraud, D.J. Wright, C.N. Johnson and T.D. Heightman, Protein Degradation by In-Cell Self-Assembly of Proteolysis Targeting Chimeras, *ACS Cent. Sci.* **2016**, 2, 927−934.
DOI:10.1021/acscentsci.6b00280.
[7] D.T. Saenz, W. Fiskus, Y. Qian, T. Manshouri, K. Rajapakshe, K. Raina, K.G. Coleman, A.P. Crew, A. Shen, C.P. Mill, B. Sun, P. Qiu, T.M. Kadia, N. Pemmaraju, C. DiNardo, M. Kim, A.J. Nowak, C. Coarfa, C.M. Crews, S. Verstovsek and K.N. Bhalla, Novel BET protein proteolysis-targeting chimera exerts superior lethal activity than bromodomain inhibitor (BETi) against post-myeloproliferative neoplasm secondary (s) AML cells, *Leukemia*, **2017**, XXX, XXX-XXX. DOI: 10.1038/leu.2016.393.
[8] B. Zhou, J. Hu, F. Xu, Z. Chen, L. Bai, E. Fernandez-Salas, M. Lin, L. Liu, C. Yang, Y. Zhao D. McEachern, S. Przybranowski, B. Wen, D. Sun and S. Wang, Discovery of a Small-Molecule Degrader of Bromodomain and Extra-Terminal (BET) Proteins with Picomolar Cellular Potencies and Capable of Achieving Tumor Regression, *J. Med. Chem.* **2017**, XXX, XXX−XXX. DOI: 10.1021/acs.jmedchem.6b01816.
[9] M.S. Gadd, A. Testa, X. Lucas, K.H.Chan, W. Chen, D.J. Lamont, M. Zengerle and A. Ciulli, Structural basis of PROTAC cooperative recognition for selective protein degradation, *Nat. Chem. Biol.* **2017**, XXX, XXX−XXX.

Secondary references

[10] a) A.C. Belkina, G.V. Denis, BET domain co-regulators in obesity, inflammation and cancer, *Nat Rev Cancer.* **2014**, 12(7): 465–477. DOI:10.1038/nrc3256. b) K. Grønbaek, C. Hother, P.A. Jones, Epigenetic changes in cancer, *APMIS*, **2009**, 4, 229-249. c) B. Florence, D.V. Faller, You bet-cha: a novel family of transcriptional regulators, *Front. Biosci.* **2001**, 6, D1008-1018. d) S.Y. Wu, C.M. Chiang, The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation, *J. Biol. Chem.* **2007**, 282(18), 13141-13145. e) S.R. Haynes, C. Dollard, F. Winston, S. Beck, J. Trowsdale and I.B. Dawid, The bromodomain: a conserved sequence found in human, *Drosophila* and yeast proteins, *Nucl. Acids Res.* **1992**, 20(10): 2603. f) T. Kanno, Y. Kanno, R.M. Siegel, M.K. Jang, M.J. Lenardo and K. Ozato. Selective recognition of acetylated histones by bromodomain proteins visualized in living cells, *Mol Cell.* **2004**, 13(1), 33-43.

[11]S. Robson, S. Pelengaris and M. Khan, c-Myc and Downstream Targets in the Pathogenesis and Treatment of Cancer, *Rec. Pat. on Anti-Cancer Drug Disc.* **2006**, 1, 305-326. DOI:10.2174/157489206778776934.

[12]P. Filippakopoulos, J. Qi, S. Picaud, Y. Shen, W.B. Smith, O. Fedorov, E.M. Morse, T. Keates, T.T. Hickman, I. Felletar, M. Philpott, S. Munro, M.R. McKeown, Y. Wang, A.L. Christie, N. West, M.J. Cameron, B. Schwartz, T.D. Heightman, N. La Thangue, C.A. French, O. Wiest, A.L. Kung, S. Knapp and J.E. Bradner, Selective inhibition of BET bromodomains, *Nature.* **2010**, 468(7327): 1067–1073. DOI:10.1038/nature09504.

[13]J. Zuber, J. Shi, E. Wang, A.R. Rappaport, H. Herrmann, E.A. Sison, D. Magoon, J. Qi, K. Blatt, M. Wunderlich, M.J. Taylor, C. Johns, A. Chicas, J.C. Mulloy, S.C. Kogan, P. Brown, P. Valent, J.E. Bradner, S.W. Lowe & C.R. Vakoc, RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia, *Nature*, **2011**, 478, 524–528. DOI:10.1038/nature10334

[14][Patent]S. Miyoshi, S. Ooike, K. Iwata, H. Hikawa, K. Sugaraha, ANTITUMOR AGENT MITSUBISHI TANABE PHARMA CORPORATION, **2009**. p. 1-37.

[15]P. Zhou, R. Bogacki, L. McReynolds and P.M. Howley, Harnessing the Ubiquitination Machinery to Target the Degradation of Specific Cellular Proteins, *Mol. Cell*, **2000**, 6, 751-756 DOI: http://dx.doi.org/10.1016/S1097-2765(00)00074-5

[16] M.L. Blackman, M. Royzen, J.M. Fox, Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels−Alder Reactivity. *J. Am. Chem. Soc.* **2008**, 130, 13518−13519. DOI: 10.1021/ja8053805.

[17] K. Zwingenberger, S. Wnendt, Immunomodulation by thalidomide: systematic review of the literature and of unpublished observations. *J Inflamm.* **1995**, 96(46), 177-211.