Ubiquigent

CASE STUDY: PROTAC MZ1

PROTEOME*profiler*

Using the PROTEOME*profiler* assay to examine the proteomic changes in response to the PROTAC, MZ1

Background

In the decade or more since the Nobel prize was awarded for the discovery of "ubiquitin-mediated protein degradation", scientists have been interested in exploiting the ubiquitin system. There are two main strategic options for exploiting the system for therapeutic benefit:

- Direct targeting of ubiquitin system proteins, e.g., through the design of inhibitors of the proteasome complex, E1, E2 or E3 'enzymes', deubiquitinases (DUBs) or other elements of the signalling cascade.
- Hijacking the ubiquitin system through the design of PROTACs and related modalities.

Both approaches have received significant interest from the drug discovery community.

Hijacking the ubiquitin system to enable the precise targeting and degradation of specific proteins - especially those that remain "undruggable" by more traditional means - presents a myriad of therapeutic opportunities.

PROTACs are heterobifunctional compounds that - in their most reductive description - consist of two ligands connected by a linker; one ligand binds to the protein target of interest (PTI) while the other binds a component of an E3 ubiquitin ligase, thereby bringing the target protein and ligase into close proximity, to trigger the polyubiquitylation and subsequent selective degradation of the target protein via the proteasome complex. Exemplars of these agents have shown potent effects in cells and activity in vivo and some are undergoing clinical trials.

PROTACs

The degradation of the target protein(s) by PROTACs - and similar modalities such as molecular glues - offers several advantages over conventional occupancy-driven target engagement:

- The need for sub-stoichiometric amounts to achieve complete target degradation (the PROTAC only needs to bring the E3 ligase complex into close proximity to the target of interest in a manner that enables the target

to be ubiquitylated and then it can dissociate and engage with subsequent target molecules).

- Lower affinity ligands may be sufficient - in fact perhaps even advantageous - to achieve pharmacological effects; PROTACs can be effective at low occupancy due to "recycling".
- Prolonged duration of pharmacodynamic effect (restoration of target can only occur through de novo synthesis and only once the PROTAC has been metabolised or removed).
- Potential to achieve greater selectivity between closely related proteins due often to the requirement for multiple protein-protein interactions outside of the highly conserved domains.

Analysis of the PROTAC, MZ1, in the PROTEOME*profiler* assay

The BET family of proteins, including BRD2, BRD3, BRD4 and BRDT, are considered attractive targets in cancer and inflammation. Preliminary data from clinical trials of small molecule inhibitors of BET proteins offers important clinical validation that targeting this family is useful in the treatment of various types of cancer. These molecules are designed to bind the BET bromodomains and block the interaction of BET proteins with acetylated lysine residues on histone tails, thereby regulating gene expression.

However, one disadvantage of all BET inhibitors to date is their lack of selectivity towards the different BET family members, including the small molecule JQ-1. Potent inhibition of cancer cell growth and induction of apoptosis has been achieved with the PROTAC MZ1 consisting of JQ-1 linked to a VHL E3 ligase binding ligand. Surprisingly, selectivity for BRD4 over other family members was demonstrated with MZ1; structure-based design has enhanced the selectivity further in second generation MZ1 PROTAC analogues.

The PROTAC MZ1 was designed and characterised by the Ciulli group at the University of Dundee (Zengerle et al., 2015 ACS Chem Biol 10(8), 1770-7). We selected this publicly available PROTAC to demonstrate the utility of our PROTEOME*profiler*™ assay to characterise the proteomic response to PROTACs and similar such molecules.

Dose response and time-course of treatment with MZ1

In order to select the most appropriate samples for analysis in the MS experiment, we first conducted traditional analysis by Western blotting to examine the effects of MZ1 treatment on levels of BRDs in HeLa cells. HeLa cells were treated with 100 nM MZ1, or cisMZ1, a negative control compound that is structurally identical to MZ1 except for a reversed stereo-centre which renders it incapable of binding to VHL. Cells were collected at multiple time-points and the levels of BRD2, 3 and 4 were analysed by Western blotting using antibodies to each of the family members.

We next sought to characterise the dose response to MZ1. HeLa cells were treated with various concentrations of MZ1 for 24h. Samples were analysed by Western blotting as previously.

MZ1 treatment reduced the levels of BRD4 in HeLa cells in a time- (Figure 1) and dose- (Figure 2) dependent manner and with a high degree of selectivity over the other BET family proteins analysed. The levels of BRD2 and BRD3 were reduced to a much lesser extent (Figures 1 and 2) and at a higher compound concentration (Figure 2) compared to the effects on BRD4. As expected, cisMZ1 did not appreciably affect the levels of any of the BET family members (Figure 1).

Taken together, time and dose-response activity profiles of MZ1 reveal rapid and effective PROTAC-induced preferential degradation of

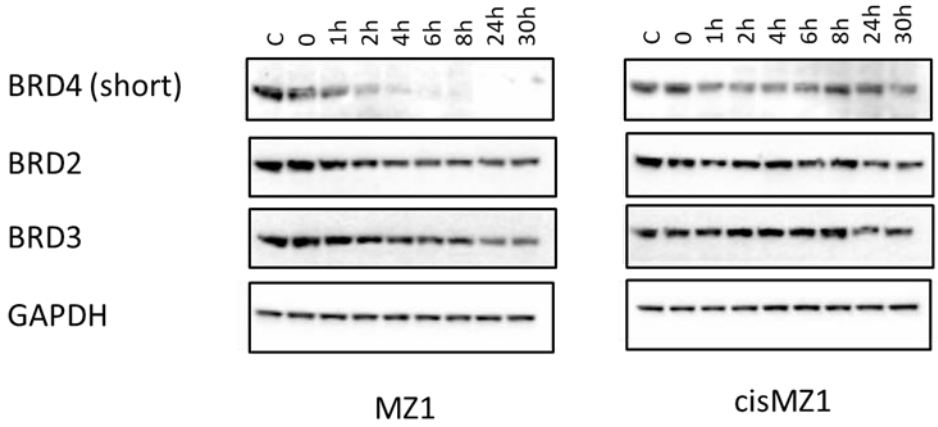

Figure 1: Time-dependent depletion of BRD4 by MZ1 in HeLa cells.

HeLa cells were treated with 100 nM MZ1 (left) or 100 nM cisMZ1 (right) for up to 30 h. Cells were harvested at the time-points indicated and lysates were analysed by Western blotting using antibodies specific to the individual BET family members. MZ1 selectively depletes BRD4, over BRD2 or BRD3; in contrast and as expected, the negative control compound, cisMZ1, has no appreciable effect on any of the BET family members. Note that there are two isoforms of BRD4; the "BRD4 long isoform" corresponds to the full-length transcript while the "BRD4 short isoform" corresponds to an alternative splicing variant, lacking exons 12-20. In this figure, only the short form Western blot data is shown since the long form was much less abundant.

BRD4 over BRD2 and BRD3. This establishes that the effect of MZ1 on BRD4 is consistent with a degradative MOA and replicates the finding of selectivity between BET family members reported in the literature.

The well-described hook-effect associated with heterobifunctional molecules such as PROTACs appears to be observed within these experiments; at high concentrations of MZ1, the degradation of BRD4 is diminished due to the formation of binary complexes consisting of MZ1-BRD4 and MZ1-VHL which compete for the formation of the degradative VHL-MZ1-BRD4 ternary complex (most clearly observed with the BRD4 short band in Figure 2).

Mechanistic insights into MZ1 treatment

In order to gain some mechanistic insight into the cellular effects of MZ1, key elements expected to be required for MZ1-mediated BRD4 degradation were targeted using chemical tools. Thus, the dependency of VHL binding, proteasome activity and E3 ligase activity (via neddylation) were all explored in HeLa cells via treatment with the following compounds: MZ1, cisMZ1 (incapable of binding to VHL), bortezomib (a proteasome inhibitor) or MLN4924 (inhibits NAE1 – NEDD8-activating enzyme 1 - and consequently Cullin Ring Ligase E3 ligase activity), alone or in combination, as indicated. Cells were collected after 24h and the levels of BRD2, 3 and 4 were analysed by Western blotting using antibodies that were selective for each of the family members (Figure 3).

As indicated, MZ1 was unable to trigger the degradation of BRD4 in the presence of an inhibitor of the proteasome or an inhibitor of NAE1 (thus inhibiting Cullin Ring Ligase (CRL) E3 activity) or if unable to bind to VHL (as demonstrated by cisMZ1). Thus, MZ1-mediated BRD4 degradation is dependent on a functional proteasome complex, CRL E3 ligase activity and VHL binding.

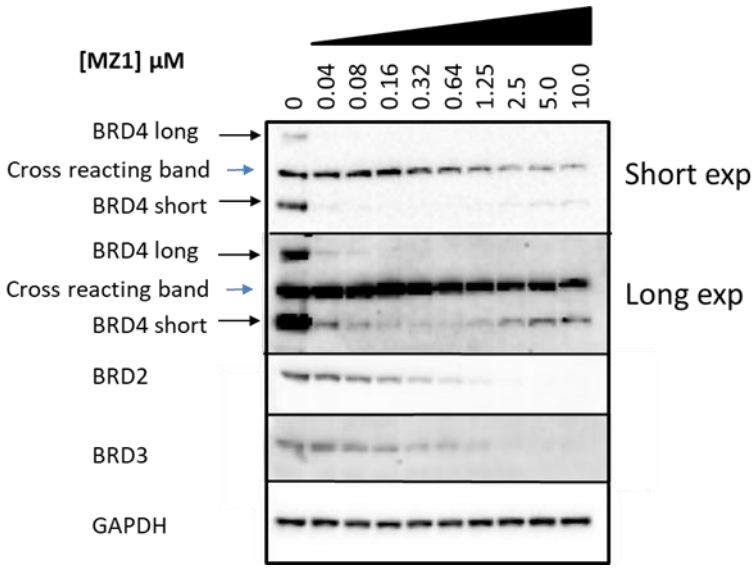

Figure 2: Potent and selective depletion of BRD4 by MZ1.

HeLa cells were treated with an MZ1 concentration range and cells were harvested at 24 hours. The levels of all family members were evaluated by Western blotting. Treatment of HeLa cells with 40 nM MZ1 leads to the complete ablation of BRD4 protein (as far as is detectable); the levels of BRD2 and BRD3 are also reduced in the presence of MZ1 but are not decreased to a similar extent as BRD4 until a much higher concentration of compound (> 1 µM)

Employing the PROTEOME*profiler* assay to examine the effects of MZ1 on the cellular proteome

The BET family of proteins recruit transcriptional regulatory complexes to acetylated chromatin thereby controlling specific networks of genes involved in cellular proliferation and cell cycle progression.

To further evaluate the range of effects of MZ1 in cells, and interrogate the dose response of MZ1, a PROTEOME*profiler* experiment was conducted in 11-plex format. Cells were treated with various concentrations of MZ1 and harvested after 24 hours. Analysis of the MS data was carried out in several different ways, in an unbiased manner.

Applying the most stringent criteria of accepting only unique peptides, a total of 7,552 proteins were quantified in the samples in this study.

Although we did not include replicate samples in this particular experiment, Principal Component Analysis (PCA) and hierarchical clustering was performed on the 11 samples to highlight whether there were appreciable differences between treated samples and the control sample.

PCA is a dimensionality-reduction method that is often used to reduce the dimensionality of large data sets, by transforming a large set of variables into a smaller one that still contains most of the information in the large set. The method permits an overview of the similarities between biological replicates (reproducibility) and differences between groups/treatments to be easily visualized within a proteomic dataset.

Figure 4 shows that, based on proteomic analysis, the individual samples which were derived from cells treated with different concentrations of MZ1 can be distinguished from one another and from the control sample.

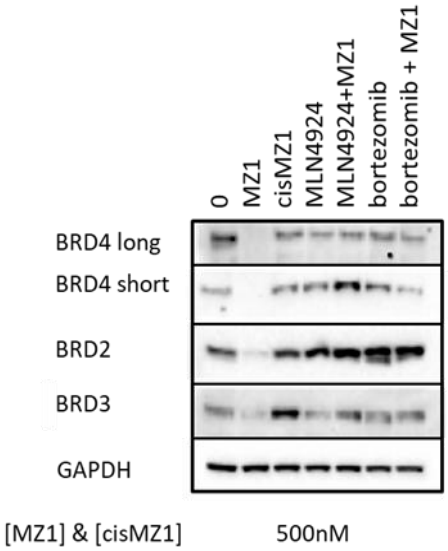

Figure 3: MZ1 degradation is dependent on MZ1-VHL binding, VHL E3 ligase and proteasome activity

HeLa cells were treated with MZ1 (500 nM), cisMZ1 (500 nM), bortezomib (500 nM; proteasome inhibitor) or MLN4924 (1 μM; NAE1 inhibitor) alone or in combination, as indicated above. N.B. MLN4924 and bortezomib were applied to cells for a 6h pre-treatment prior to the addition of MZ1. Cells were harvested 24h after the initiation of treatment, and the levels of BRD2, 3 and 4 were analysed by Western blotting using selective antibodies to each of the family members.

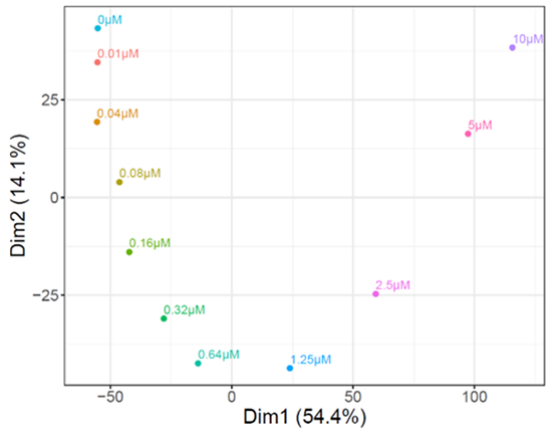

Figure 4: Principal Component Analysis (PCA) illustrates the separation of treated samples from one another and from the control sample.

The PCA analysis demonstrated that the concentration of MZ1 was clearly the principal source of variance in the dataset.

The output of the 11-plex proteomics experiment examining the impact of increasing concentrations of MZ1 can be examined by hierarchical clustering and visualized using a heatmap (Figure 5).

As with PCA, hierarchical clustering is performed in an unbiased manner and utilizes the relative abundance of each protein present in the sample to cluster together the samples most similar to one another.

A table was generated that listed the 7,552 proteins identified in the samples along with the fold changes in their abundance, relative to the control sample (data not shown). To pinpoint the proteins of most relevance to the mechanism of action of MZ1, a Spearman's correlation analysis was performed to identify those proteins that showed a perfect Spearman's correlation (ρ=-1/ ρ=+1) between MZ1 concentration and fold-change in protein abundance. In total, 46 proteins out of the total of 7,552 proteins had a perfect Spearman's correlation of ρ=-1 or ρ=+1 (Figure 6).

BRD4 was identified as the protein with the largest fold-change out of these 46 proteins; BRD4 levels were reduced by approximately 1.4 to 3.5-fold (≈log2FC -0.5 to -1.8) at the lowest concentrations of MZ1 (0.01 to 0.64 μM). SESN3 (sestrin 3) was also reduced in abundance by MZ1; in contrast, histone H2AZ1 and CCN1 were significantly increased in abundance in a dose-dependent manner by MZ1.

Interestingly a connection between BRD4 and SESN3 (a protein involved in maintaining physiological concentrations of intracellular reactive oxygen species) has previously been reported. In BRD4 knockdown cells, oxidative stress response genes were found to be significantly enriched. Similarly, knockdown of BRD4 or treatment with the BRD4 inhibitor JQ-1 resulted in decreased reactive oxygen species (ROS) production and increased cell viability

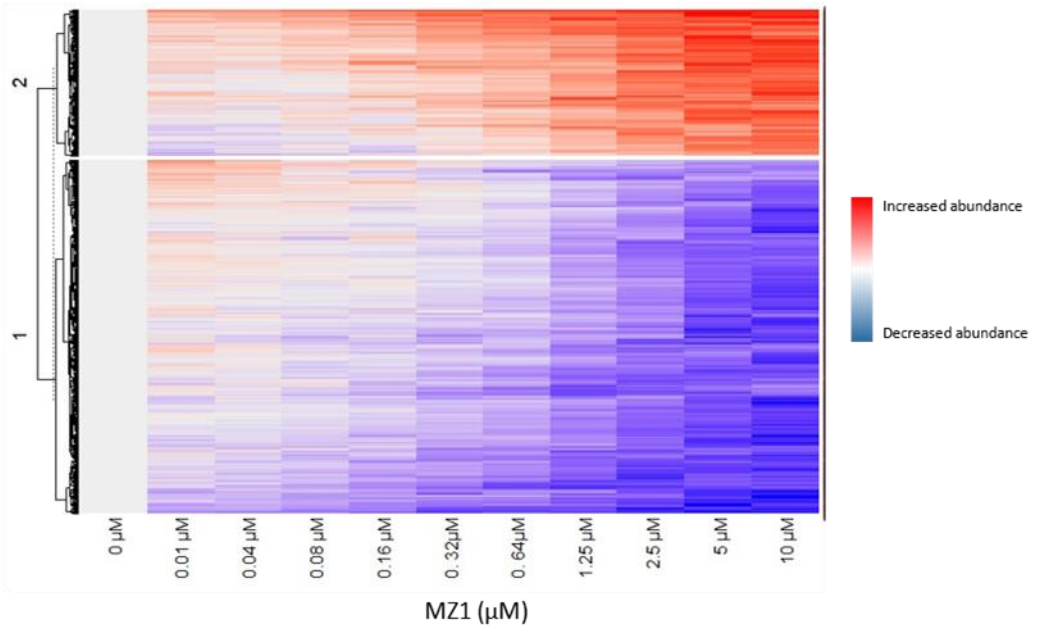

Figure 5: Hierarchical clustering heatmap of MZ1 dose-response treatment based on changes in the abundance of the entire proteome.

Data was scaled and normalised to the control; the abundance of all proteins in the control sample is shown as white/pale grey. Increases or decreases in abundance (compared to the control sample) were shown on a gradient of red (increased) to blue (decreased); proteins that had equivalent abundance between the treated and control samples are shown in grey. The hierarchical clustering analysis reveals two main groups of proteins: proteins that are negatively regulated by MZ1 (bottom section, branch 1 of the dendrogram) and proteins that are positively regulated by MZ1 (top section, branch 2 of the dendrogram). Clearly the number of proteins whose abundance changes and the extent to which they change increases in an MZ1 dose-dependent manner.

under H_2O_2 exposure. In an extension to this study, the authors examined genes within prostate tissue that showed correlations with BRD4 expression; 21 genes had positive correlations to BRD4 in prostate cancer, including three that were involved in the maintenance of physiological concentrations of intracellular ROS: SESN3 (sestrin 3), HDAC6 (histone deacetylase 6), and KEAP1 (Hussong et al (2014) Cell Death & Disease 5, e1195). Thus, the concomitant reduction in BRD4 and SESN3 seen in the dose response conducted here is perhaps not surprising.

Next, we focused on the impact of MZ1 on the expression of all BRD family members to address the question of selectivity (Figure 7). The graph indicates that the BRD family member showing the most significant dose-dependent decrease in expression in response to increasing concentrations of MZ1 is BRD4. BRD3 also decreases with increasing concentrations of MZ1 but to a lesser extent, with a maximum of 2.8x fold decrease (log2FC = -1.5 at 10 µM MZ1) versus the 6.9x maximum fold decrease observed in BRD4 (log2FC = -2.8 at 10 µM MZ1), thereby mirroring the results found by Western blotting.

As indicated, a total of 7,552 proteins were identified in this PROTEOME*profiler* experiment. These proteins were collected into 'bins' according to the change in abundance in response to MZ1 treatment (Figure 8). This bar chart illustrates the impact of MZ1 treatment on the entire proteome and highlights the selectivity of this agent in affecting a relatively small number of proteins. At very high concentrations of MZ1, a greater number of proteins appear to be changed in abundance. It is unclear whether this wider impact on the

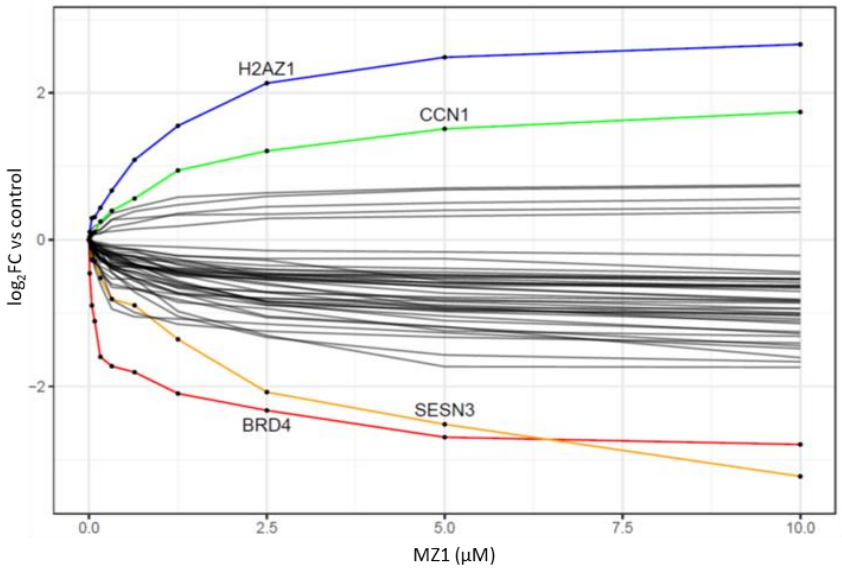

Figure 6: Graph presenting the fold change of all proteins with Spearman's correlation of ρ=1 or -1.

Several proteins were identified as having a p-value indistinguishable from 0, and a rho (ρ) value of 1 or -1, these were plotted on a line graph demonstrating the correlation of the abundance of these proteins with the concentration gradient. The majority of proteins which passed these stringent filters were negatively associated with MZ1 concentration, i.e their abundance was decreased in a dose-dependent manner (with increasing concentrations of MZ1). Four proteins stood out from this analysis, two which correlated negatively with MZ1 concentration (BRD4 and SESN3) and two which correlated positively (H2AZ1 and CCN1).

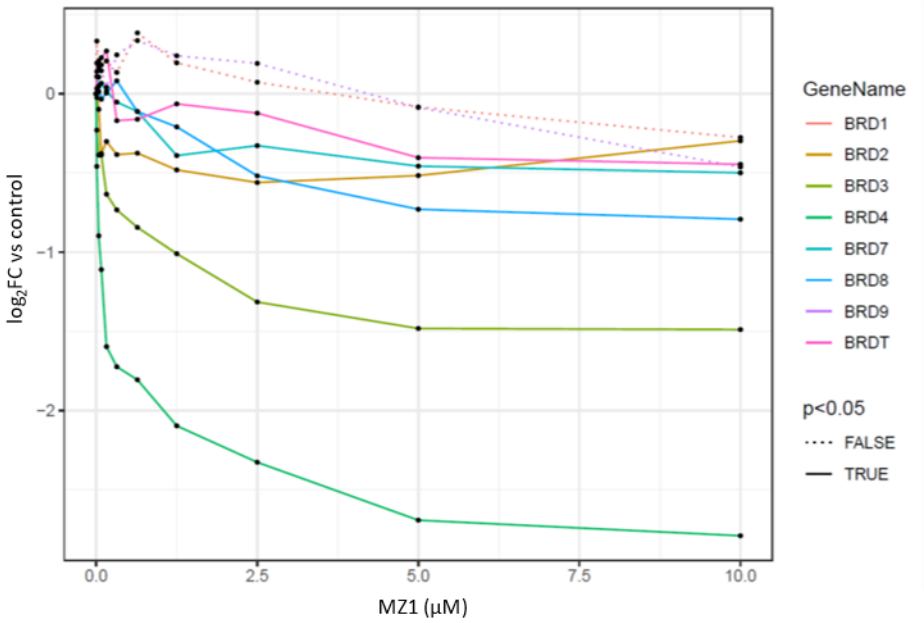

Figure 7: Graph presenting the fold change in abundance of all BRD family members identified in the MS analysis.

Fold changes in the abundance of all detected BRD family proteins were plotted against MZ1 concentration, demonstrating that the majority of the BRD family members correlated significantly (p<0.05) and negatively with the concentration gradient of MZ1, with the exception of BRD1 and BRDT (p>0.05). Nevertheless, the selectivity observed in Western blots was also observed by MS.

proteome is mechanism-based or due to a loss of selectivity at high concentrations (off-target effects). Additional experiments interrogating proteomic changes over time could aid in the interpretation of this data.

For the next set of data analysis, a single concentration of MZ1 was selected to interrogate which other proteins were affected by MZ1 and to begin to understand the biological response to the PROTAC, beyond the primary target of BRD4 (Figure 9). Based on the PCA analysis shown above, we focused on the 0.64 µM MZ1 treatment since it displays the largest separation from the control sample in both dimensions of the PCA plot.

The vast majority of proteins (90%) show less than 1.3x fold-change in abundance comparing MZ1 treatment with the DMSO control (bins ranging from -0.4 to 0.4). Note that BRD4 is one of very few proteins showing a decrease in expression at a relatively modest concentration of MZ1.

In addition to the above analysis, a candidate protein approach was taken within this dataset to investigate known downstream targets of BRD4 that have been disclosed in the literature (Figure 10).

The dependence of MYC and p21 expression on BRD4 is well characterised. BRD4 activates Myc transcription which in turn represses the transcription of the cell cycle inhibitor, p21 (CDKN1A) leading to the stimulation of cell cycle progression. Although it is reported in the literature that JQ-1, the small molecule inhibitor which releases BRD4 from chromatin and reduces MYC transcription has no effect on MYC protein stability, MZ1, which degrades BRD4, has the paradoxical effect of decreasing MYC transcription but increasing MYC stability. In Zengerle et al., 2015, MYC mRNA was decreased at 12h by MZ1 but by 24h, levels had recovered. Similarly, in the analysis of the dataset presented

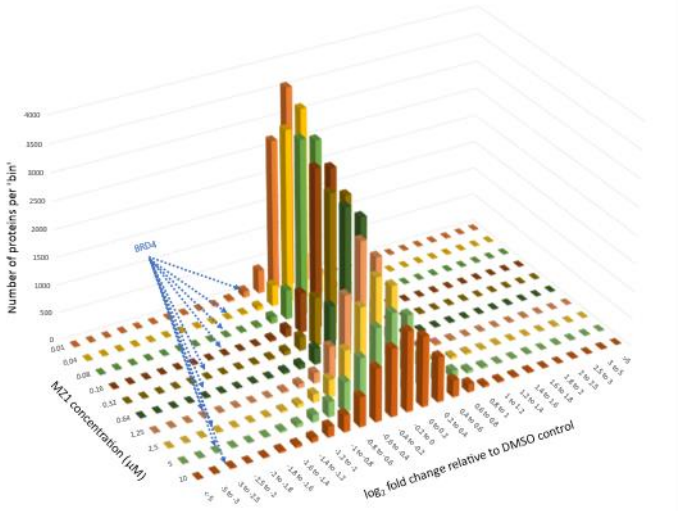

Figure 8: MS proteomic profile across the MZ1 dose response.

Distribution of protein fold change across each MZ1 treatment vs control. Proteins with similar relative abundance (ratios) at a given MZ1 concentration were grouped into 'bins' so that the proteins within the dataset are distributed across 28 bins. BRD4 is highlighted; the ratio of BRD4 in MZ1-treated samples to the control sample decreased in a dose-responsive manner.

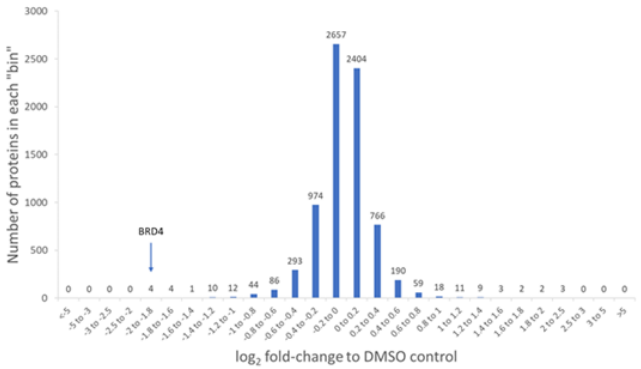

Figure 9: Histogram presenting the proteins up and downregulated at 0.64 µM MZ1

Distribution of protein fold-change at 0.64 µM MZ1 treatment vs control. Proteins with a similar relative abundance at 0.64 µM MZ1 were grouped into 'bins' so that the proteins within the dataset are distributed across 28 bins. BRD4 abundance is in the bin with the largest observed fold-change reduction at this MZ1 concentration.

here (also captured at 24h), MYC levels appeared to be unchanged/only modestly changed at a level similar to BRD2 by MZ1 at concentrations lower than 5 µM. In addition, p21 (CDKN1A) which was shown to be significantly upregulated at the mRNA level in Zengerle et al ((2015) ACS Chem Biol 10(8), 1770-7) was also upregulated several fold at the protein level in the experiment shown here.

In order to understand the inter-relationship between the observed proteomic changes and to pinpoint which changes may be a direct consequence of degrading the primary target of MZ1, two types of analysis were performed: STRING network analysis and Ingenuity Pathway analysis (IPA).

The protein interaction analysis and Gene Ontology Network analysis aid in focusing findings from large proteomics datasets and potentially identifying relevant subnetworks that are responding to treatment as well as which molecular functions or biological processes are enriched in the dataset.

IPA Upstream Regulator Analysis can be used to identify which upstream regulators are activated or inhibited to explain the increased or decreased protein abundances in the dataset. Knowledge of this regulatory cascade can help understand the biological activities occurring in the tissues or cells under study or the effect of specific compound treatments. Similarly, IPA Mechanistic Network analysis enables the discovery of plausible sets of connected upstream regulators that can work together to elicit the changes observed in a dataset.

Both of these analyses were applied to the dataset for 0.64 µM MZ1 relative to the DMSO control and are shown below.

Firstly, a STRING network analysis was conducted on this PROTEOME*profiler* data set (Figure 11). The STRING network analysis measures the probability of a list of proteins being related as opposed to consisting of a random list of proteins. As indicated previously, a total of 46 proteins reported an MZ1 concentration effect curve with a perfect Spearman's rank correlation (ρ=-1/ρ=+1) (including BRD4). The STRING analysis examines the 46 proteins that are

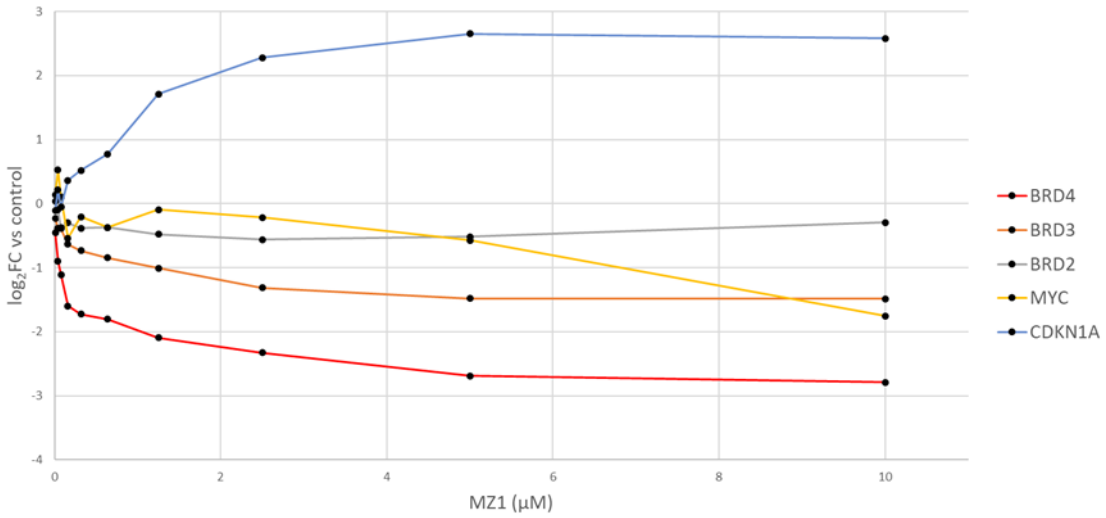

Figure 10: Graph presenting fold-change in abundance of selected proteins

The observed changes of protein abundance in response to MZ1 for BRD4, BRD2, BRD3, MYC and CDKN1A (p21) are consistent with the published literature.

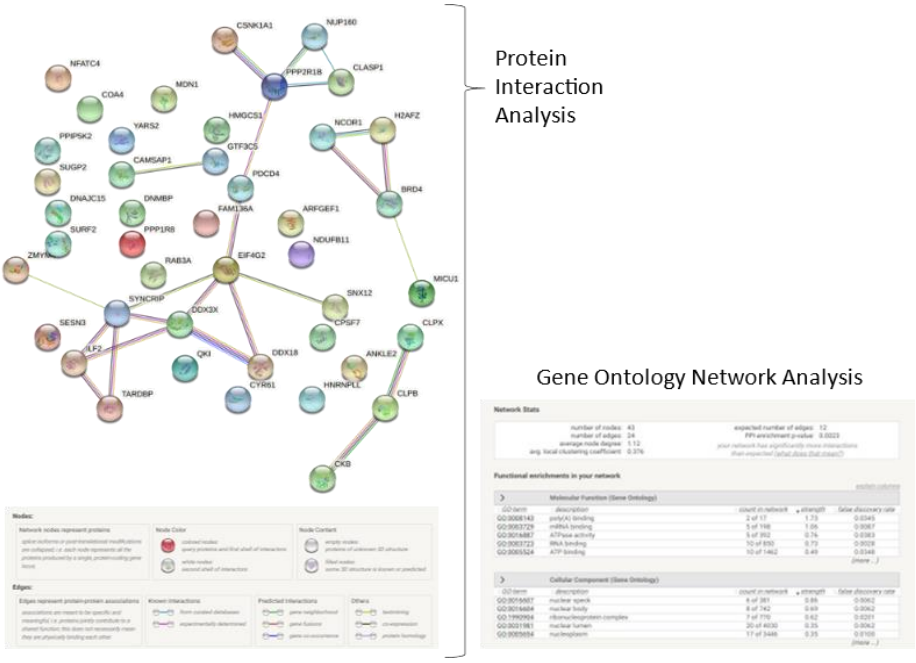

Figure 11: Gene ontology and protein interaction analysis of proteins up and downregulated by MZ1 with Spearman's correlation of ρ=1 or -1

STRING network analysis of the 46 proteins reported with a perfect Spearman's rank correlation (ρ=-1/ ρ=+1) with the MZ1 concentration effect curve (including BRD4) reveals this network has significantly (p=0.0023) more interactions than a random list of proteins of similar size. Gene Ontology Network Analysis highlights specific molecular functions and cellular localization that are also significantly enriched in this network

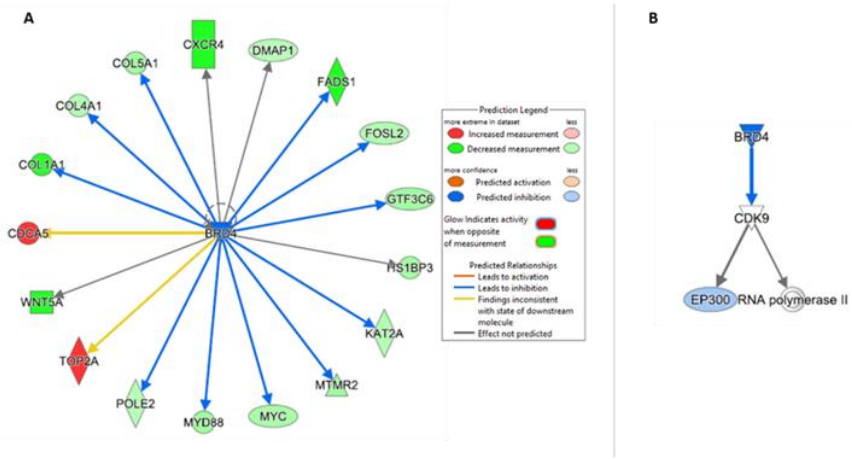

Figure 12: Ingenuity® Pathway Analysis (IPA, QIAGEN®) predicts BRD4 as an upstream regulator of observed protein changes (A) and proposes a mechanism of cascade activation (B)

A) p-values obtained by Spearman's Rank Correlation were utilised with Log2FC calculated from the datasets for 0.64 µM MZ1 over the DMSO control. Filters of p<0.01 and increases or decreases of greater than 1.2x were applied to the analysis. IPA analysis identified BRD4 as a potential upstream regulator (p = 4.93E-3) and predicted that BRD4 was inhibited in the experimental condition (z = -2.653), in association with 17 downstream molecules.

B) In this dataset, the IPA Mechanistic Network analysis proposes a pathway of BRD4 to CDK9, and CDK9 to EP300 and RNA polymerase II as explanatory for the changes observed in up to 56 significantly changed proteins.

correlated to the MZ1 dose-response and queries whether none, some, or the majority of these proteins have connections with one other. A total of 43 of those hits could be mapped against the database and were indicated to be at least partially biologically connected.

Using the abundance fold-change at 0.64 µM MZ1 and Spearman's correlation p-values, after applying a stringent statistical cut-off (up or down FC > 1.2x and p<0.01), the IPA analysis predicts that a down-regulation of BRD4 could explain the fold-change of 17 downstream proteins, which match the experimentally measured decrease of BRD4 in the dataset (Figure 12A). An additional type of analysis, the Mechanistic Network analysis, proposes that a pathway connecting BRD4 down-regulation/inhibition to CDK9 and further downstream to EP300 and RNA polymerase II can explain changes observed in up to 56 significantly changed proteins (Figure 12B).

As discussed above, the BRD family serve as epigenetic readers of histone acetylation and can recruit transcriptional regulator complexes such as positive transcription elongation factor (p-TEFb; consisting of CDK9/cyclin T1) to chromatin, which in turn activates RNA Pol II through phosphorylation of the C-terminal domains Ser2 and Ser5 for transcriptional extension, mediating transcription of BRD4 transcriptional programs (Taniguchi (2016) Int. J. Mol. Sci. 17(11): 1849). The findings of the unbiased analysis of the MS dataset presented here therefore appears to be very much consistent with the published literature.

The Protein Interaction Network and Gene Ontology Network analyses in conjunction with Ingenuity Pathway Analyses provide powerful tools to evaluate the proteome-wide effects of compounds/treatments and their impact on molecular and cellular processes.

Conclusions

As illustrated in this report, we have utilized the unbiased, flexible and cost-effective PROTEOME*profiler* assay to confirm the primary target(s) of the PROTAC molecule, MZ1, and to examine the MOA of the molecule by evaluating changes in the global proteome in response to treatment. The study has yielded important information about the impact on the target protein(s) as well as highlighting additional activities of the compound. The platform can be applied to agents of any type, including PROTACs, molecular glues, DUB inhibitors, other small molecules, biologics, or stimuli of any type in a disease-relevant setting. Other important applications of the platform, in addition to MOA determination, include across target identification and validation, and the identification of biomarkers for clinical application.

We look forward to discussing how PROTEOME*profiler* can accelerate and enhance your drug discovery programme

Contact us: services@ubiquigent.com