DUB*profiler*-Cell™

A single, flexible platform to identify and validate cellular DUB targets, and determine the cellular DUB target engagement and selectivity of novel compounds

Overview

DUB*profiler*-Cell™ supports the development of novel deubiquitylase (DUB) enzyme inhibitors by revealing the 'active DUBome' in a given cell-line or tissue, and by reporting the target engagement of test compounds to that 'active DUBome'. The DUB*profiler*-Cell™ assay utilises activity-based probes (ABPs) to capture active DUBs (i.e. DUBs with reactive catalytic site cysteine residues) within a cell-line or tissue of interest. By pre-incubating lysates or live cells with test compounds, the assay can be used to evaluate target engagement by compounds, including those with either covalent or non-covalent mechanisms of action. Employing both Western blotting and multiplexed tandem mass spectrometry (MS) the assay can be configured to evaluate compounds at either single or multiple concentrations (reporting relative EC_{50} values) examining one, several or all active DUBs in the sample.

The DUB*profiler*-Cell™ platform has been extensively optimised and validated for detecting target engagement to any active DUB by either covalent or non-covalent inhibitors with proof of concept data available in respect of compounds selective for two example DUBs; USP7 and USP30 (important targets in oncology and neurodegeneration, respectively).

DUB*profiler*-Cell™ can be accessed as part of a Collaborative Drug Discovery project or as a discreet fee-for-service offering.

Registered office: Ubiquigent Ltd, Dundee University Incubator, Dundee Technopole, James Lindsay Place, Dundee, DD1 5JJ, UK
Company number: SC362461; VAT number: 991 1953 88 Version 1.0

Main Applications

DUB target identification and validation

The DUB*profiler*-Cell™ platform can be employed as part of your target identification / validation approaches to reveal which DUBs are expressed – and active – in any given cell or tissue type and under any given circumstance. Customers can supply us with disease versus healthy tissue, an isogenic cell line pair, or cells stimulated under different conditions. Using Western blotting and MS, we can report on the relative abundance of individual DUBs of interest or the entire active DUBome in your samples to reveal novel disease-relevant DUB targets.

Determination of inhibitor-DUB in-cell target engagement

With the small molecule chemical tractability of DUBs demonstrated by a number of groups through the development of high affinity and selective inhibitors (supported by the DUB*profiler*™ service) it is now becoming increasingly important to determine the in-cell DUB target engagement profile of these molecules as drug discovery programmes progress towards the clinic. DUB*profiler*-Cell™ provides for the "single point" (single concentration) screening of many compounds, e.g. to establish a rank order of their potency, or analysis of a full compound dilution series to establish relative EC_{50} values against selected DUBs or the entire MS-detectable active DUBome of any cell line or tissue.

Key Features

- Physiologically relevant - use unmodified, disease-relevant cells or tissues; assess compound potency and specificity against endogenous DUBs.
- Informative - ability to address one or two DUBs or the entire active DUBome; unbiased analysis by multiplexed MS.
- Flexible - either combine multiple compounds in one analysis to rank according to potency or determine EC_{50} values for individual compounds against all endogenous DUBs to address potency and selectivity.
- Efficient - generate EC_{50} curves simultaneously across multiple DUBs with different orders of magnitude of abundance due to the large dynamic range of the MS platform employed.
- Robust - probe binding conditions optimised for each compound and validated prior to preparation of MS samples; aliquot of MS samples verified by Western blotting prior to submission to ensure validity of data.

Registered office: Ubiquigent Ltd, Dundee University Incubator, Dundee Technopole, James Lindsay Place, Dundee, DD1 5JJ, UK
Company number: SC362461; VAT number: 991 1953 88 Version 1.0

Options

DUB*profiler*-Cell™ can be configured to analyse multiple cell lines or tissues in a single experiment for elucidation and comparison of the active DUBome between the selected samples, e.g. between diseased and healthy tissue. For compound target engagement, the platform is similarly flexible and will accommodate either 1-2 concentrations of multiple compounds or multiple concentrations of a single compound, up to a maximum of 8 test samples per experiment.

A typical project workflow is shown in Figure 1; customers may select individual modules to comprise their project depending on their needs. Customers are advised to begin with the PBL (Probe Binding Lysate) module, in which activity probe binding conditions are optimised for future experiments. A large number of compounds can be examined at this stage at a single or limited number of concentrations to ensure that assay conditions are suitable for all compounds of interest. Optimal conditions are determined by Western blotting using antibody(s) to the DUB(s) of interest. The optional ASV (Antibody Selection and Validation) module tests and validates up to two antibodies for each DUB of interest.

In the DSL (Dilution Series Lysate) module, a dilution series of each compound of interest is examined using conditions informed by the PBL module. The customer may choose the top concentration and dilution series, e.g. half-log, 2-fold etc. Again, as with the PBL module, analysis is performed by Western blotting. This is also a good opportunity to select a small number of related DUBs to examine any potential off-target engagement, if desired. From the analysis of the ratio of probe-bound DUB to "free" DUB, compound-target engagement EC_{50} values can be reported for any compound against any DUB that is examined.

Customers may then continue to a full analysis of all active DUBs in their cell line or tissue of choice by exploiting the benefits of MS. A more detailed description of the experimental workflow for the MS module is described in the next section.

Customers may also choose to repeat the probe binding optimisation and compound dilution series experiments in the context of live cells (Probe Binding Cells 'PBC' and Dilution Series Cells 'DSC' modules, respectively) to address the cell permeability of their compounds and compound behaviour in a live cell setting.

In addition, if customers are interested in substituting compound treatments with analysing different cell lines, or different stimuli, normal versus disease tissue, or any other variation, we would be very happy to discuss individual requirements and adapt our workflow accordingly.

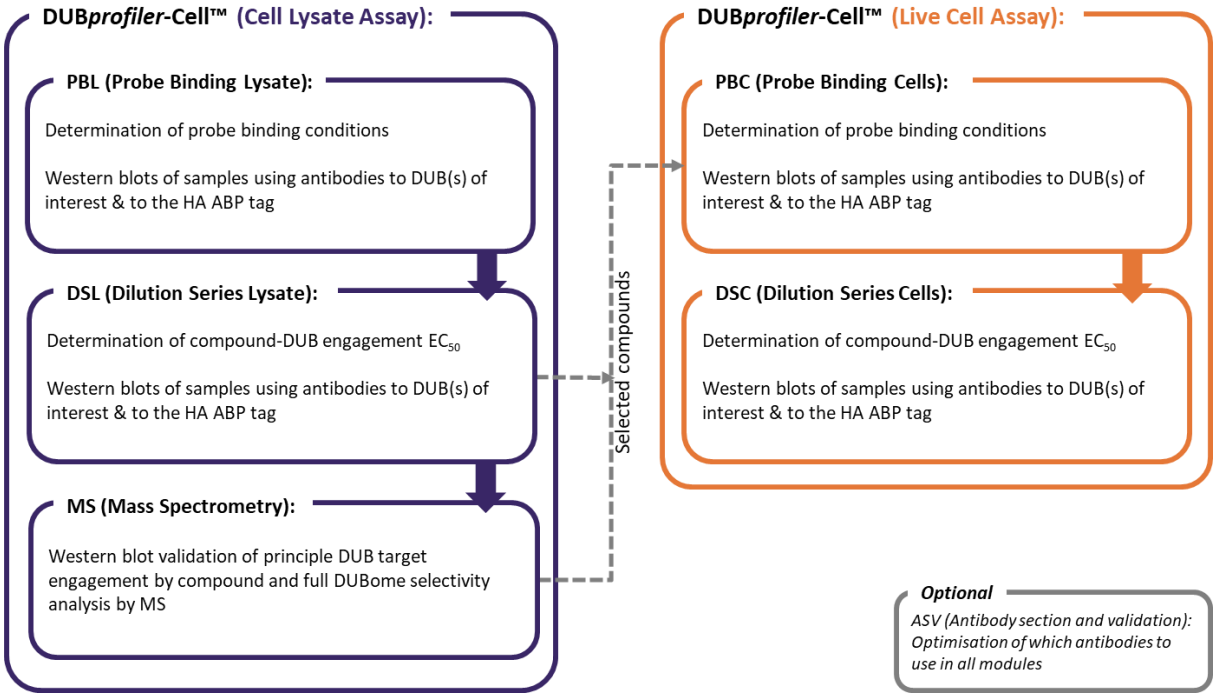

Figure 1: DUB*profiler*-Cell™ project modules and suggested workflows

MS Assay Workflow Summary

A schematic representation of the MS experimental workflow is presented in Figure 2.

- Test compounds are incubated with cell lysate to enable engagement with their DUB target(s).
- Following compound incubation, the ABP is added to engage the active DUBome (sub-proteome) in a covalent manner. The DUBome is then enriched via immunoprecipitation (IP) using an anti-HA antibody (the ABP carries an HA tag).
- ABP-labelled samples are probed with antibodies by Western blotting to visualize the effect of the compounds on ABP target(s) engagement and confirm the quality of the samples, prior to processing for MS.
- The enriched, immunoprecipitated samples (in the presence or absence of compound treatment) are enzymatically digested for MS analysis.
- Offline fractionation of samples prior to LC-MS/MS analysis is performed to reduce sample complexity and improve the detection of low abundance targets.
- Prevention of ABP engagement to a specific DUB(s) due to test compound pre-binding to the DUB(s) is reported as a ratio relative to the no compound control samples for each protein identified by MS.
- Where multiple concentrations of a single compound are used, a target-engagement EC_{50} may be provided for each DUB that is detected, thus reporting compound selectivity.

As part of the validation of the MS workflow, it was important to establish whether it was possible to measure prevention of probe binding to any DUB that was identified by MS, regardless of its abundance.

In order to do this, lysates were incubated with increasing concentrations of the untagged version of the activity probe, to mimic a pan DUB inhibitor. Lysates were analysed according to the workflow in Figure 2 and the data generated is presented in Figure 3.

The data indicates that it is possible to measure the impact of compounds on probe-binding to DUBs, irrespective of their abundance so long as the DUB is detectable by MS via the workflow described in Figure 2. In the light of this validation, the EC_{50} values for compound-DUB engagement are reported for any DUB that is detected in both of the positive control samples (plus ABP) in the MS experiment.

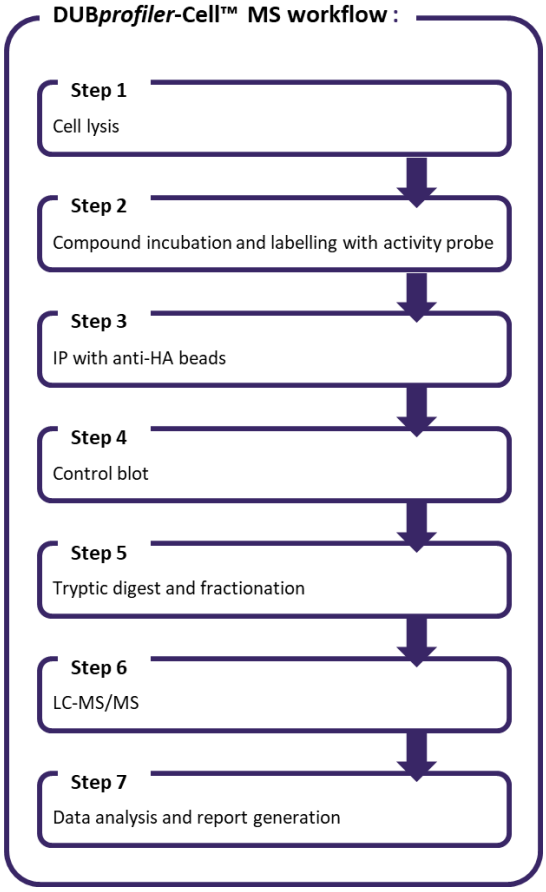

Figure 2: DUB*profiler*-Cell™ mass spectrometry workflow

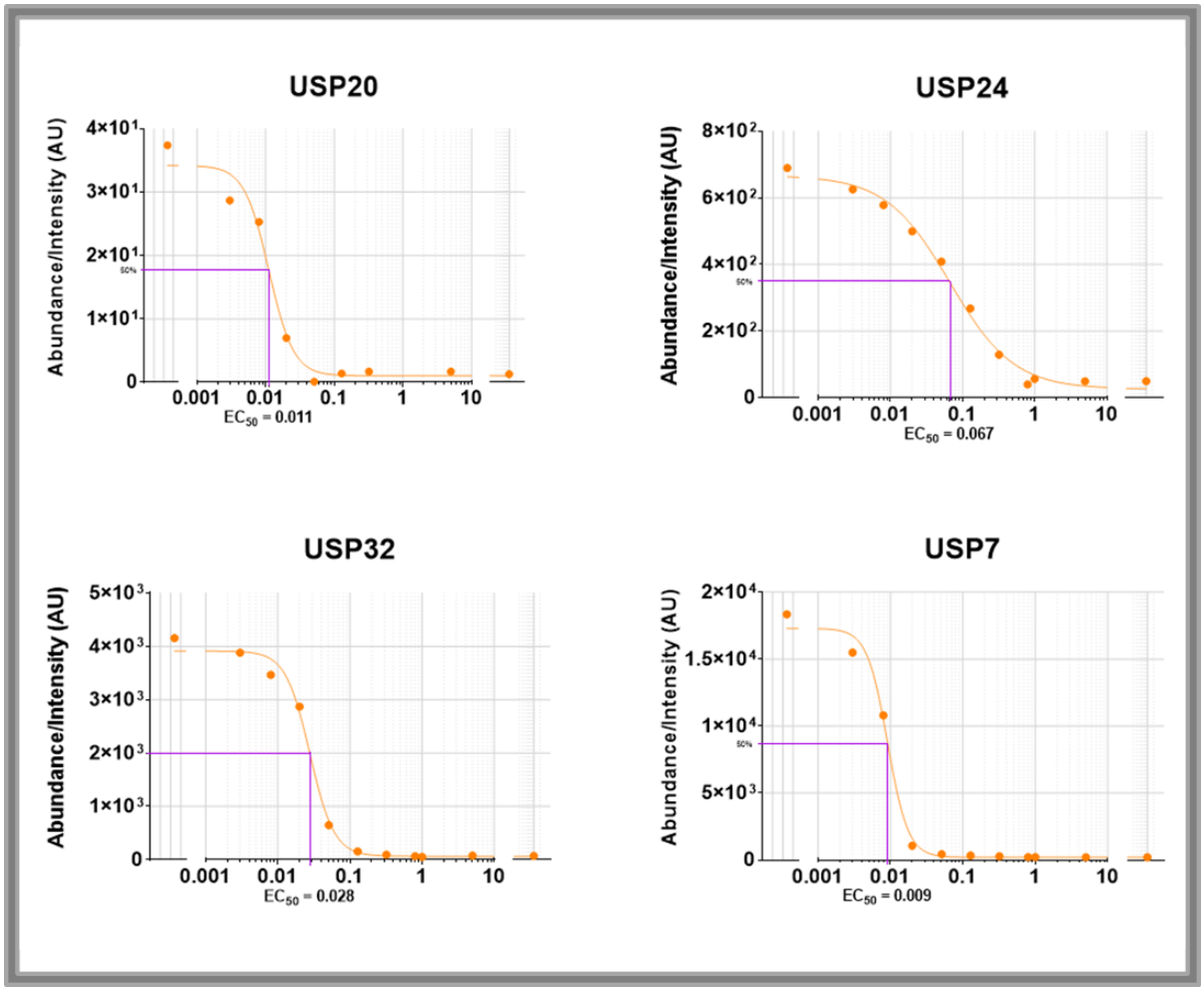

Figure 3: DUB*profiler*-Cell™ large dynamic range. Prevention of probe binding by compound can be monitored, irrespective of protein abundance. Lysates were pre-incubated with increasing concentrations of untagged ABP lacking the reporter tag (to simulate a pan-DUB inhibitor compound) and prevented binding of tagged ABP in a dose-responsive manner. EC_{50} values can be calculated from proteins with significant differences in abundance (almost 3 orders of magnitude; intensities ranging from ≈ 40 to 20,000).

Compound Submission and Study Design

Accessing DUB*profiler*-Cell™ is as easy as accessing any of Ubiquigent's DUB*profiler*™ services. Study designs are discussed and agreed with each customer upfront to ensure that any specific requirements are met. Once agreed, customers are then asked to complete one of our compound submission forms confirming the following:

- Test compound name(s)
- The mechanism of action of the test compound(s) e.g. - covalent / non-covalent or unknown (this information will be used to inform the compound incubation and probe optimisation conditions in Modules PBL and/or PBC).
- The identity of the primary DUB target(s) of the test compound(s) and whether the customer can supply a validated antibody(s) to the target(s).
    - Where a validated antibody(s) is available, the customer will be asked to provide the antibody(s) and their conditions for use in Western blotting experiments.
    - If the customer is unable to supply a validated antibody(s), Ubiquigent can validate antibodies supplied by the customer within the ASV module.
- Type of Assay:
    - Lysate-based or live cell
    - Western blot readout only or MS analysis (which incorporates a pre-MS Western blotting validation step)
- Choice of samples for the 8 data-points:
    - Single point: Up to 8 data points (e.g. 4 compounds x 2 replicates at a single concentration or 4 compounds at 2 concentrations with no replicates; 8 compounds, single concentration, no replicates etc)
    - EC_{50}: 8 data-points; choose appropriate dilution series (2-fold linear, ½ log, ¼ log etc)
- Maximum assay screening concentration of compound(s)
- Compounds should be supplied in 100% DMSO at 100x the maximum screening concentration
- Identity of the cell line. The assay has been validated using MCF7 cells, but customers may provide another cell line if preferred; for example, where there is a wish to correlate compound-target engagement with functional efficacy and/or a pharmacodynamic (PD) marker(s). Please note that where customers do not wish to include compound treatments, and instead wish to submit multiple untreated samples (such as diseased vs healthy tissue), it will be necessary to discuss appropriate controls for the experiment.

The Process

Once the study design has been finalised, the submission form, test compound(s) and any antibodies, cell pellets or cell lines being supplied, should be sent to Ubiquigent.

Once the above materials have been received, Ubiquigent will schedule the study and provide regular progress updates.

Once completed, a fully annotated data report will be sent to the customers with an offer to arrange a post project meeting to discuss the results.

Compound-target engagement data will be reported as percentage prevention of probe binding relative to the no compound control for all detected and quantified DUBs. In the EC_{50} version of this assay, the individual compound-target engagement EC_{50} values (where data reaches an EC_{50}) for all detected and quantified DUBs will be reported.

Proof of Concept and Assay Validation

Covalent and Non-Covalent Inhibition of USP7

Background

FT671 and FT827 are potent and selective USP7 inhibitors developed by FORMA Therapeutics. Consistent with USP7 target engagement in cells, FT671 was shown to cause degradation of the USP7 substrate MDM2, leading to re-activation of p53 and inhibition of tumour growth in mice (Turnbull *et al*., 2017).

Ubiquigent screened these compounds in our standard DUBprofiler™ *in vitro* assay against the Ubiquitin-Rhodamine110 substrate and established the selectivity of both compounds for USP7.

DUB*profiler*™ - an *in vitro* Ubiquitin-Rhodamine110 cleavage assay - establishes the selectivity of the two USP7 inhibitors FT671 and FT827, kindly supplied by FORMA Therapeutics; each dot represents one of 45 DUB enzymes (data generated at Ubiquigent and published in Turnbull *et al*. Nature 550, 481-486, 2017)

FT671 is a non-covalent inhibitor of USP7, while FT827 features a vinylsulfonamide moiety that covalently modifies the catalytic Cys223 of USP7 and therefore inhibits the enzyme in a covalent manner. These compounds were selected for evaluation in DUB*profiler*-Cell™ to provide proof-of-principle data that the assay is capable of reporting target engagement by compounds with either a covalent or non-covalent mechanism of action.

Lysate-Based Western Blot and MS Analysis

Lysates were prepared from MCF7 cells and pre-incubated with a full concentration range of each compound, followed by the ABP. Using the DUB*profiler*-Cell™ PBL module, parameters were optimised according to the covalent or non-covalent mechanism of action of each compound. Lysates were examined by Western blotting using a validated anti-USP7 antibody. The DUBome of lysates for MS analysis were first enriched using the HA tag on the probe prior to MS analysis. The data shown in Figures 4 and 5 demonstrates the level of agreement between the two assay formats – western blotting and MS – and highlights the added value of the MS readout.

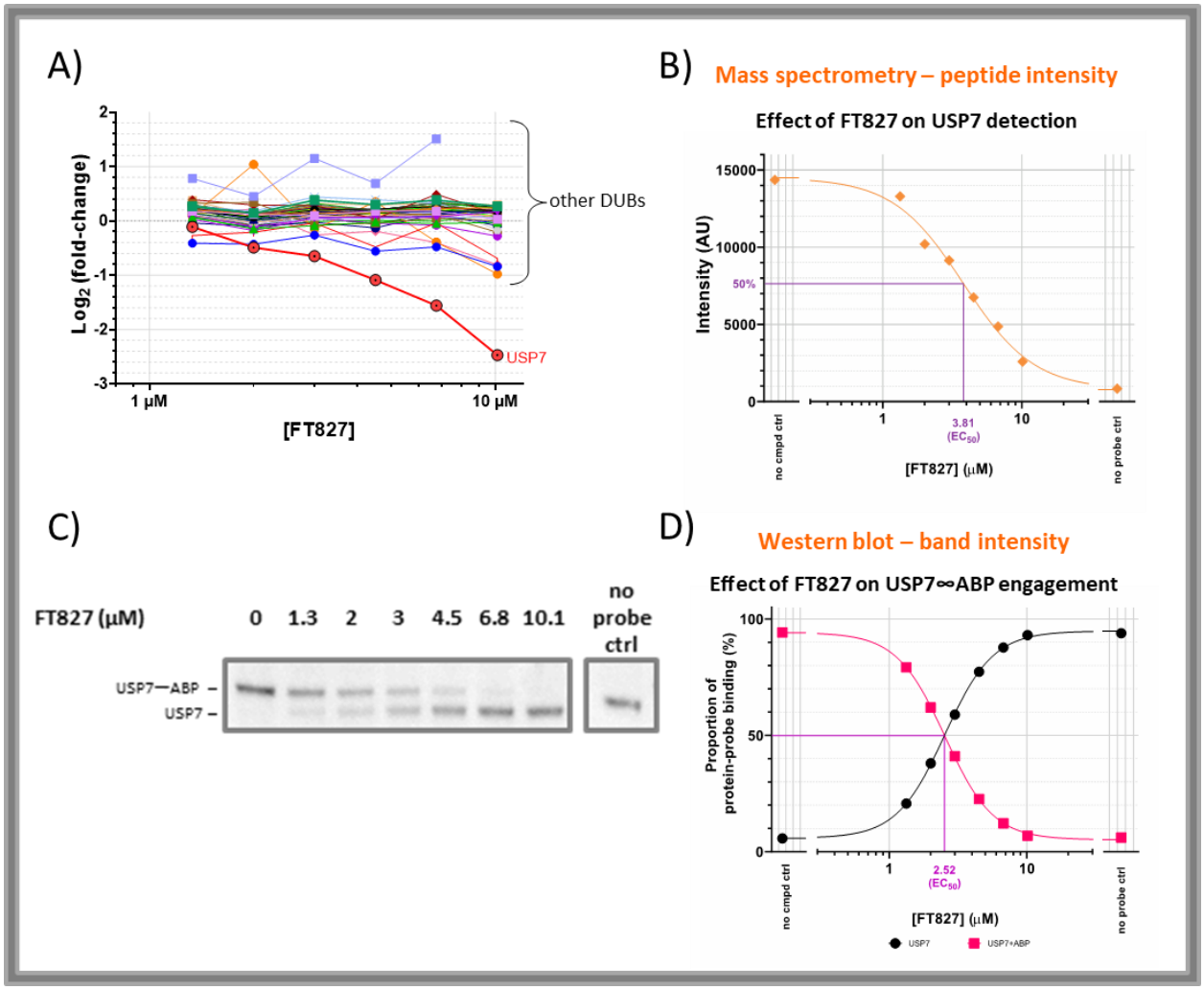

Figure 4: DUB*profiler*-Cell™ reports target engagement by covalent DUB inhibitors. FT827, a covalent inhibitor of USP7 prevents the binding of the activity-based probe to USP7 in a dose-dependent and highly selective manner (A). Estimated EC_{50} values based on MS data (MS module analysis; B) correlate with data obtained by target-specific Western blot detection (DSL module analysis; C and D).

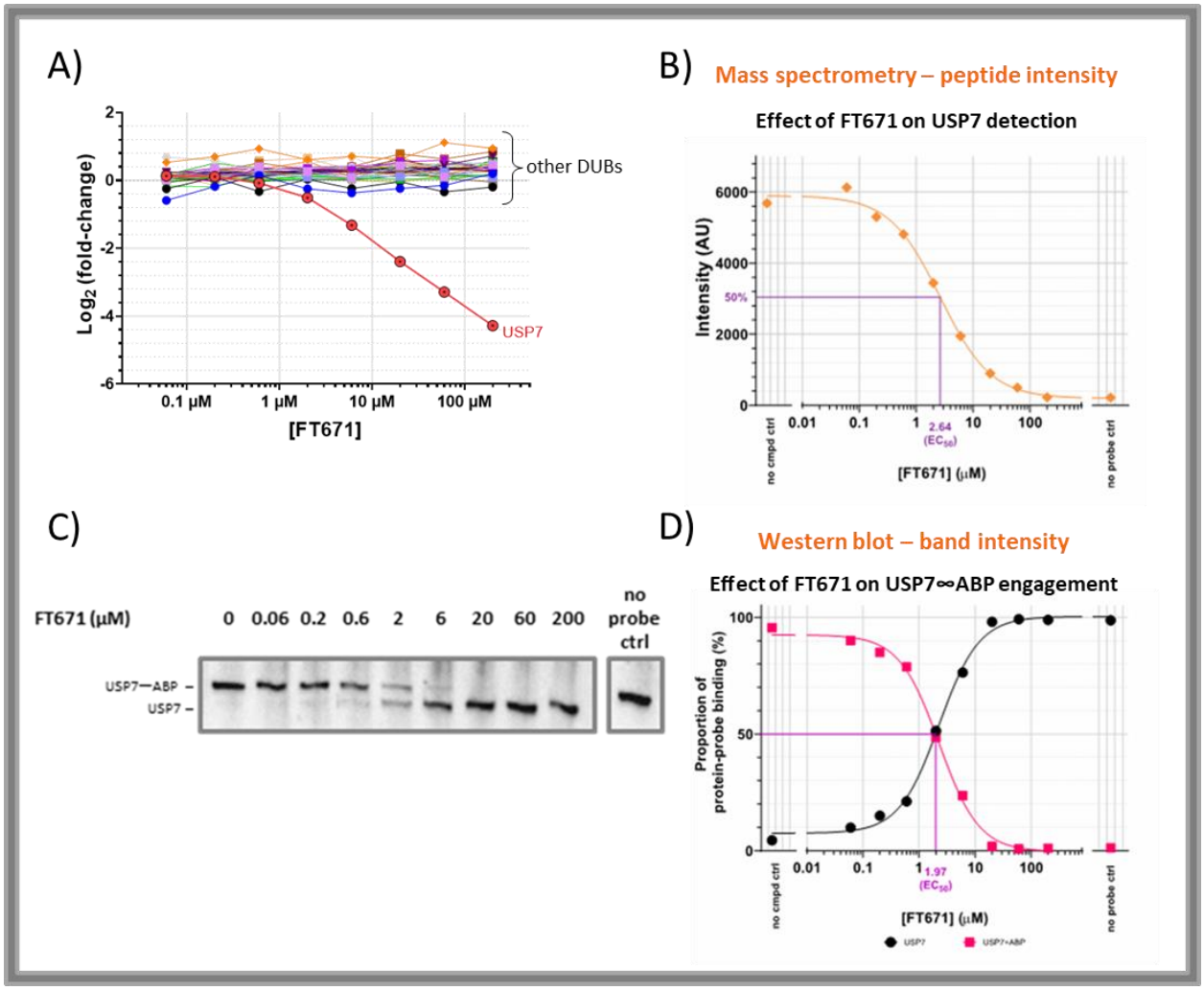

Figure 5: DUB*profiler*-Cell™ reports target engagement of non-covalent DUB inhibitors. FT671, a non-covalent inhibitor of USP7 prevents the binding of the activity-based probe to USP7 in a dose-dependent and highly selective manner (A). Estimated EC_{50} values based on MS data (MS module analysis; B) correlate with data obtained by traditional targeted WB detection (DSL module analysis; C and D).

Live Cell Analysis

MCF7 cells were seeded and treated with various concentrations of FT827 or FT671 (or DMSO) for 6 hours. Cell pellets were collected, and lysates prepared from each sample; samples were then incubated with the activity probe and analysed by Western blotting (Figure 6).

The data demonstrates that the incubation of live cells with test compounds can prevent the binding of the ABP to the compounds' target DUB (Figure 6A) in a dose-dependent manner, as was previously demonstrated in lysates treated with compounds *in vitro* (Figures 4 and 5). The ability to do so, in a therapeutically relevant cell line, allows for target engagement to be correlated with biomarkers of response or phenotypic readouts, such as induction of p53 (Figure 6B). It also has the benefit of demonstrating cell permeability of the compounds of interest and allows the compounds to engage with DUBs in their endogenous environment in the presence of relevant binding partners and in their natural subcellular location(s). We believe the ability to conduct treatments in live cells and analyse probe binding is a very powerful new addition to our DUB*profiler*-Cell™ platform.

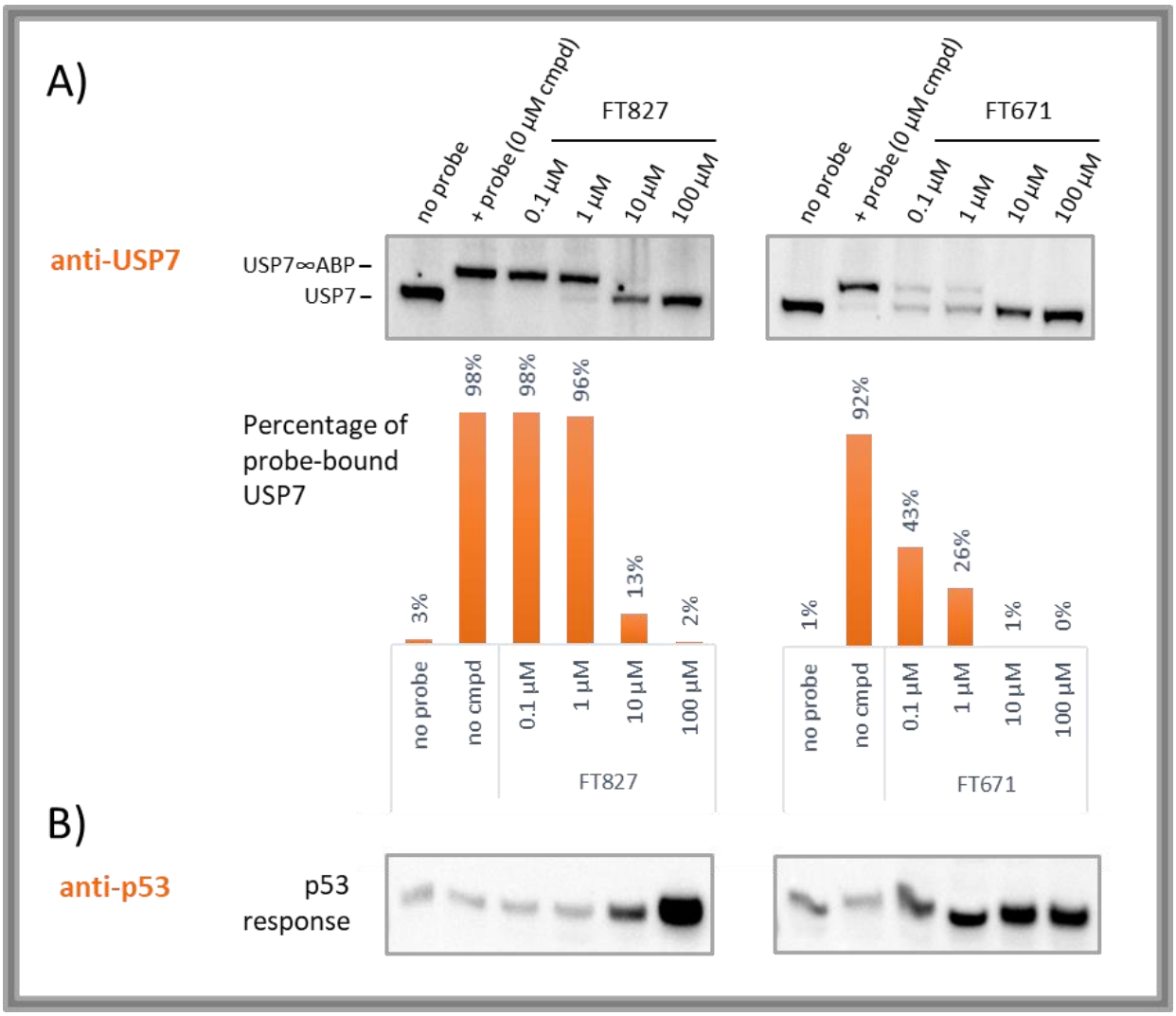

Figure 6: Demonstration of compound target engagement and achievement of predicted pharmacodynamic endpoint in live cells, conducted in the same experiment. A) Both USP7 inhibitors prevent probe binding in the DUB*profiler*-Cell™ live cell assay; the bar graphs indicate the percentage of probe binding, which is reduced in lysates prepared from live cells treated with either compound in a dose-dependent manner. B) In addition, consistent with the predicted cellular outcome of inhibition of USP7, p53 levels are increased in a dose-dependent manner at concentrations of compound that reduce or prevent probe binding.

Using Activity-Based Probes (ABPs) to identify and measure changes in the DUBome

ABPs are small molecule tools that can be used to monitor the activity of specific classes of enzymes. ABPs have three components: a reactive electrophile for covalent modification of the enzyme active site, a linker or a specificity group for directing probes to specific enzymes, and a reporter for visualizing or enriching for enzymes bound to the probe.

For DUBs, many different probe architectures have been reported with different selectivity toward individual DUBs. Probes using a molecule of Ubiquitin (Ub) as the specificity motif have been the more popular ones[1,2,3,4], with a Cysteine-reactive electrophilic group such as a vinyl sulfone (VS), vinyl methyl ester (VME) or propargylamide (PA) replacing the C-terminal glycine residue. However, di-Ubiquitin ABPs mimicking the different poly-Ub linkages recognised by DUBs have also been generated and studied[5], and Ward *et al* [6] have reported on the synthesis of a cell-permeable ABP that is reactive with a number of DUBs. Since their introduction, DUB ABPs have become a valuable tool in drug discovery through the identification of new DUB families, characterisation of DUB inhibitor selectivity and investigating changes in DUB activity in response to exogenous stimuli.

Refs: 1. Borodovsky *et al.*, EMBO J. 20(18):5187–5196, 2001; 2. Borodovsky *et al.*, Chemistry & Biology, Vol. 9:1149–1159, 2002; 3. Borodovsky *et al.*, Chembiochem. 6(2):287-91, 2005; 4. Hemelaar *et al.*, J Proteome Res. 3(2):268-76, 2004; 5. McGouran *et al.*, Chem Biol. 20(12):1447-55, 2013; 6. Ward *et al.*, ACS Chem Biol. 11(12):3268-3272, 2016

Mass Spectrometry: Enabling an unbiased readout of compound-target engagement

The introduction of separation and analytical strategies such as multidimensional liquid chromatography of peptides coupled to high-performance tandem MS has led to a dramatic increase in the depth and breadth of sampling of a given proteome. The ability to identify the spectrum of proteins interacting with a small molecule at an early stage in the drug discovery process can support decision-making as compounds progress and may even lead to the repositioning of existing drugs by the identification of additional targets. For selectivity assessment the compound of interest is typically used as a competitor over a range of concentrations in a lysate of a disease-relevant cell line or tissue. The affinity of the compound to all members of the target class is determined by quantifying the (reduced) amount of proteins captured by the ABP. More specifically, inhibition curves of ABP binding are obtained, from which apparent compound-target engagement EC_{50} values can be calculated. This is a very powerful approach because proteins are assayed under physiological conditions (e.g. use of relevant cell line or tissue; proteins at endogenous expression levels and in their native structural form and modification status). In addition, the multiplexing capability of MS for protein identification and quantification provides ranked affinities of a compound engagement against all members of the MS detectable target class in one experiment.

Contact

For further information about DUB*profiler*-Cell™ or any of Ubiquigent's capabilities please contact us via the following address:

services@ubiquigent.com

- For access to Ubiquigent's capabilities on a fee-for-service basis
- For access to our biology and/or chemistry platforms to support a Collaborative Drug Discovery programme
- To discuss access to Ubiquigent's novel DUB inhibitors