Identification of covalent fragment binding sites by proteolytic digestion and High-Resolution LCMS

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Introduction

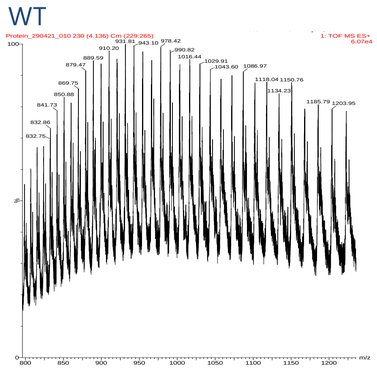
Covalent inhibitors of proteins are an ever growing field of interest with the number of publications on the topic tripling in the last decade leading to several new irreversible drugs currently both on the market and under development. Historic irreversible inhibitors have focused on adding covalent functionality to existing optimised noncovalently binding compounds. This functionality increases the duration of action improving efficiency requiring a lower dose and hence increasing patient compliance by reducing frequency of dosing. As such the interest in this area has driven the development of techniques making it possible to perform novel hit to lead identification though high throughput screening of covalent fragment libraries against a target protein.

At Domainex we have developed LC-MS workflows to identify covalent fragment screening hits and confirm they bind and their stoichiometry. Further to this we are always looking for ways to increase the information we can gain from any assay. Hence, we have developed further protease digest work flow in tandem with intact mass analysis to identify hits stoichiometry and confirm the site of binding through peptide mapping.

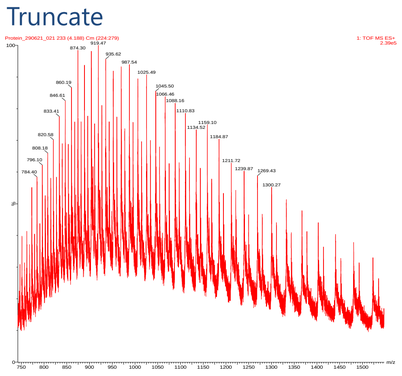
Protein Preparation

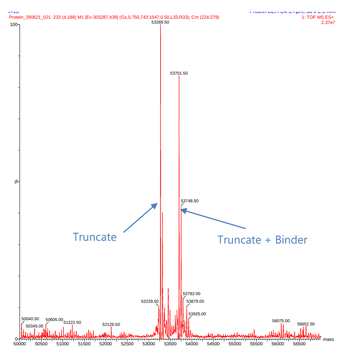
Initial analysis was undertaken on the wild type (WT) protein of interest using a previously validated method on a Waters G2-XS QToF. This gave reasonable chromatography and deconvoluted to the expected mass. While the protein analysis was sufficient for analysing the single species, once the binders were added and multiple species were created, the signal to noise ratio (S/N) became too low to distinguish individual peaks. As the protein was relatively large it was theorised that a smaller protein would give a higher S/N. To this end the truncated version of the protein was investigated and the S/N was much higher and individual peaks were identifiable even in complex mixtures.

WT



Truncate

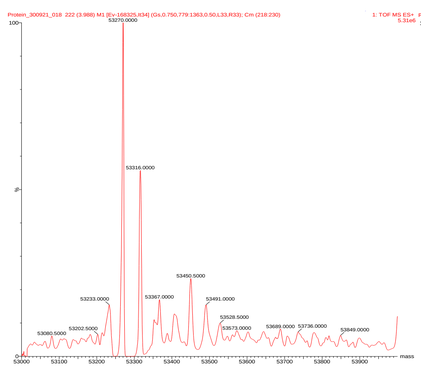




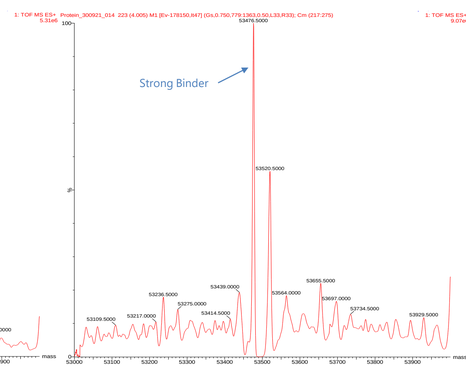
Library Screen

The Domainex acrylamide library was screened using the optimised binding conditions with 5 fragments per well (1% DMSO). These samples were analysed on a Waters G2-XS QToF, utilising the chromatography from a Waters Acquity UPLC Protein BEH C4 300 Å 1.7 µM, 2.1 mm x 50 mm on a Waters Acquity H-Class Plus Bio. To ensure multiple binders could be identified in each pool, fragments were selected using an automated process to give >5 Da difference between fragments in each pool. This generated 10 strong binder hits from the library of 73, with 13 weaker binders also identified. The compound pools were investigated further to ensure the hits were repeatable as singletons. Hits were confirmed by singleton analysis (1% DMSO).

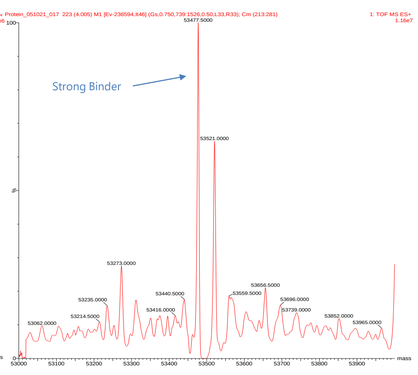
+DMSO



5 Fragment cassettes



Singleton Confirmation



Optimising Digestion Conditions

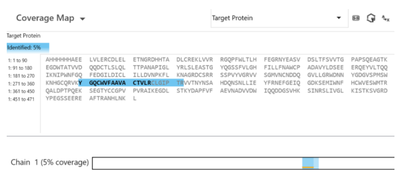
Following the singleton analysis samples were digested using a standard method of Iodoacetamide end capping of remaining reduced Cys, denaturation using 8M Urea or Guanidine chloride and Trypsin protease incubation over night. These conditions reliable provided 99% peptide mapping coverage for the selected target protein. Building upon this capability we have now developed a screening cascade using various other proteases for both difficult to digest target proteins and to assist in more exact location of binding site.

Binding Site Identification

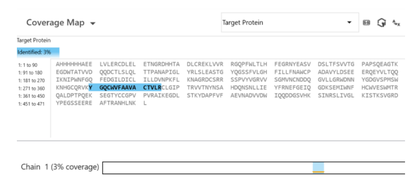
Digested samples were analysed on a Waters G2-XS QToF, utilising the chromatography from a Waters Acquity UPLC Peptide CSH C18 130 Å 1.7 µm, 2.1 mm x 150 mm on a Waters Acquity H-Class Plus Bio. Using Waters UNIFI peptide mapping software, combinations of filters can selectively narrow down the list of identified peptides to a high confidence. Domainex covalent fragment library was imported into UNIFI as amino acid modifications, the software can then filter peptide sequences which have been modified by a binder with high confidence. In the figure below we show that on incubation and digestion with no binder we observe no modified peptides. When comparing this with incubations of fragments of Cluster A and B we can see where the binders have bound on the peptide sequence (highlighted in blue)

No Binder

Cluster A Fragment



Cluster B Fragment

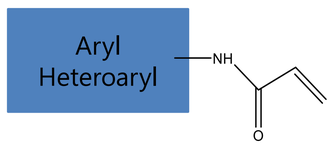


By utilising Waters MSe fragmentation data a finger print for each of the candidate peptides are generated, increasing identification confidence. MSe fragmentation data is also used to identify the exact Cys residue on the peptide sequence where the modifier is bound. The software will automatically assign the fragment fingerprint and showing the exact binding site of each modification in the display below

Conclusions

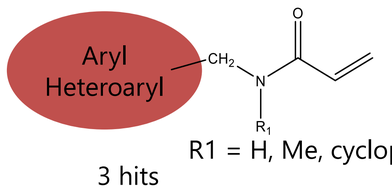
Strong binders fell into one of the following three clusters.

Cluster A



5 hits

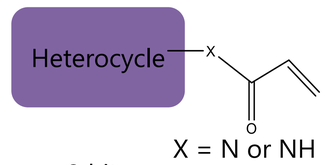
Cluster B



R1 = H, Me, cyclopropyl

3 hits

Cluster C



X = N or NH

2 hits

From the initial intact protein analysis strong binders fell into 3 clusters representing potentially interesting starting points for further elaboration & delivery of interesting novel binders lacking peptidic character. Using the protein digest workflow, we have identified 3 clusters that have an affinity for the same binding site. Applications of these workflows can allow for the high throughput screening of various libraries followed by binding site confirmation of different binder characteristics to ensure the same site of action.

Next steps

- Carry forward pools of compounds screened against intact mass and assess whether strength of binding can be determined from the MS response of the associated peptide.
- Assess capability to identify binding of reversible covalent inhibitors and the changes produced at the binding site.
- Develop native size exclusion mass spec methodology for the possible identification of noncovalent reversible inhibitors using similar intact mass workflow.

Contact

If you would like to learn more about applying our drug-discovery platforms, please contact:
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