LC-MS based covalent fragment screening strategy

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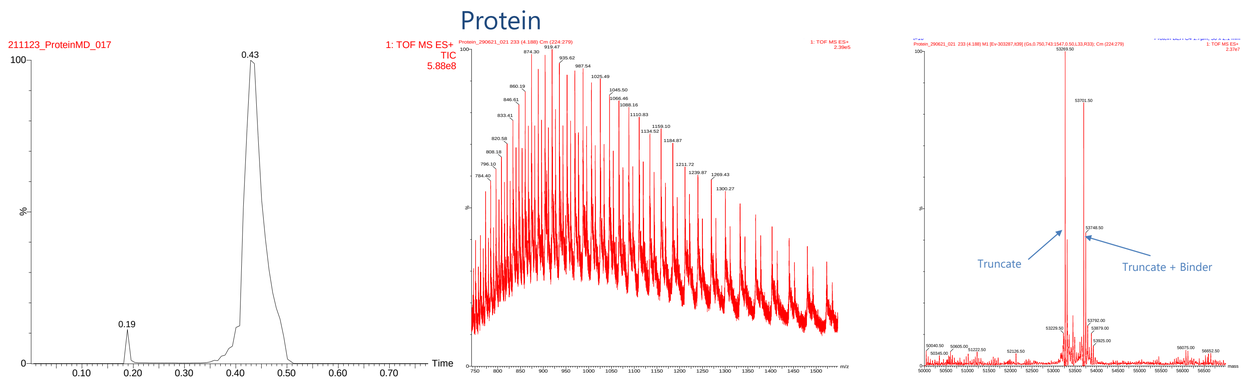
Introduction

Covalent inhibitors of proteins are an ever-growing field of R&D interest with the number of publications on the topic tripling in the last decade and new irreversible drugs on the market and several under development. Historic irreversible inhibitors have been developed by adding covalent functionality to existing optimised noncovalently binding compounds. This functionality Imparts advantages such as prolonged duration of action, improved potency and high selectivity for the target of interest. 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 a strategy to identify selective covalent fragments utilising three LCMS assays; Intact mass covalent fragment screening to assess protein binding and stoichiometry, counter screen of hits with glutathione to assess covalent reactivity and binding site identification by digest and LCMS peptide mapping to confirm selectivity of target site.

Protein MS analysis

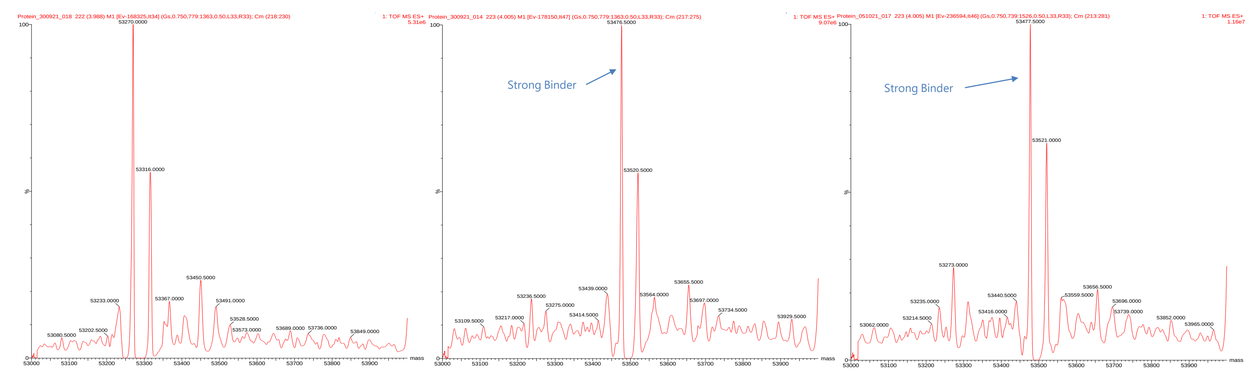
Initial analysis of the protein of interest will be carried out on a Waters G2-XS QToF. Using rapid chromatography and deconvoluted to the expected mass. The protein will be investigated, and the S/N determined prior to compound screening.



Library Screen

Domainex can screen single compounds or in pools of 5 per well (1% DMSO). These samples will then be analysed on a Waters G2-XS QToF, utilising the chromatography from a Waters ACQUITY UPLC Protein BEH C4 VanGuard Pre-column, 300Å, 1.7 µm, 2.1 mm X 5 mm on a Waters Acquity H-Class Plus Bio. To ensure multiple binders can be identified in each pool, fragments are selected using an automated process to give >5 Da difference between fragments in each pool.

+DMSO | 5 Fragment cassettes | Singleton Confirmation



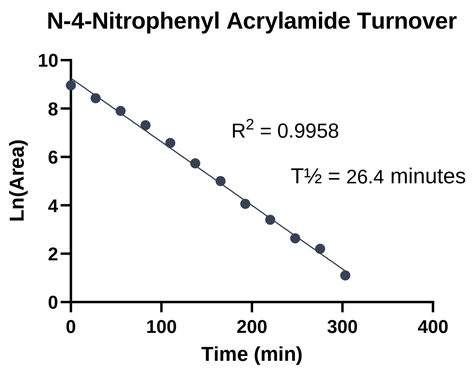
Glutathione Counter screen

Compound concentration	1.67 µM
Final solvent concentration	<0.2% DMSO
Positive control	N-nitrophenyl acrylamide
Incubation duration	6 hours*
Analysis	LCMS with UV-DAD
Compound requirements	50 µL of 10 mM DMSO stock or 1 mg of solid material
Deliverables	half-life ($t_{1/2}$), presence of GSH adduct (Y/N), PBS stability (%)

Hits from the covalent screen can be assessed for Glutathione (GSH) reactivity.

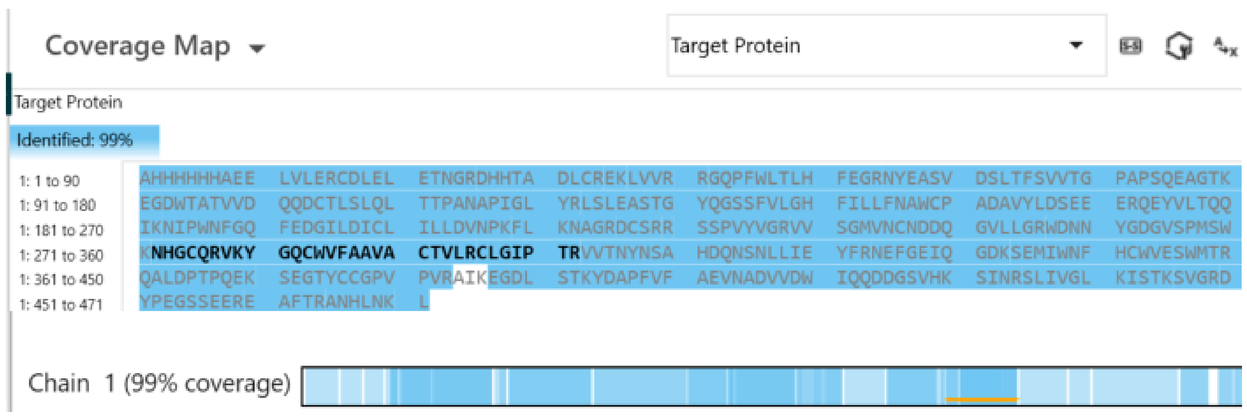
Test compounds are incubated with GSH in the presence of ethylenediaminetetraacetic acid (EDTA) at 37 ° C over a period of 6 hours. The reactivity of compound with GSH is assessed in real time using Ultra-High Performance Liquid Chromatography (UHPLC) connected to a mass spectrometer. The stability of test compound in PBS over the course of the run is also assessed. The GSH reactivity assay can be performed using single test compounds or as pools of 5* for increased capacity.

Compounds found to have high reactivity ($T_{1/2}$<100 minutes) are unlikely to exhibit the right properties and can be discarded. Example data shown below



Binding Site Identification - Digest

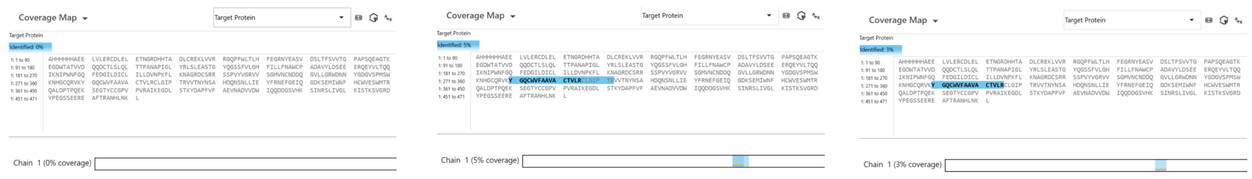
Selected hits from covalent screen can be digested using a standard or optimized digestion method that uses Iodoacetamide to end cap reduced Cys, Urea or Guanidine chloride for denaturation and protease incubation over night.



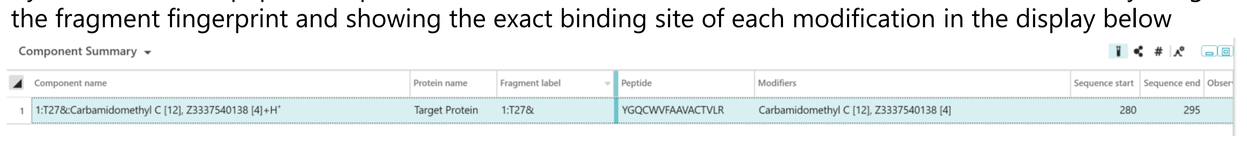
Binding Site Identification- LC-MS

Digested samples are analysed on a Waters G2-XS QToF, utilising the chromatography from a Waters CORTECS Premier T3 Column, 1.6 µm, 2.1 mm x 100 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. Selected covalent fragments can be 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 A and B we can see where the binders have bound on the peptide sequence (highlighted in blue)

No Binder | Fragment A | Fragment B



By utilising Waters MSe fragmentation data a 'fingerprint' 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



	Component name	Protein name	Fragment label	Peptide	Modifiers	Sequence start	Sequence end	Observ
1	1:T27&:Carbamidomethyl C [12], Z3337540138 [4]+H+	Target Protein	1:T27&	YGQCWVFAAVACTVLR	Carbamidomethyl C [12], Z3337540138 [4]	280	295	

Conclusions, Timelines and Next steps

Domainex can provide a fast covalent library screening approach to generate high quality stoichiometric data over a large chemical space screening as many as 2000 fragments in 3 weeks. Building upon this we can generate GSH counter screen data providing insight into intrinsic reactivity of compounds against cysteine moieties. Using the protein digest workflow, we can identify the binding site and the selectivity for this 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.

Screening Assay	No. of Compound per batch	Timeline
Covalent Library Screen	1000-2000 compounds	<3 weeks
GSH Counter Screen	Up to 400 compounds	<3 weeks
Binding site identification	Up to 50	<3 weeks

Next Steps

- Binding Kinetics assessment through K_{inact}/Ki
- Assess non-covalent binders using SEC chromatography in tandem with RP (affinity selection MS)

Contact

If you would like to learn more about our drug discovery platforms, please contact: enquiries@domainex.co.uk