Identification of novel hit chemotypes by Covalent Fragment Screening and Binding Site Identification using proteolytic digestion and High-Resolution LCMS

Domainex
Enrich your Medicines Pipeline

Jake Stewart, Sam Braidley, Scott Martin, Andrew Ratcliffe, Roland Schindler, Michael Knaggs, Ray Boffey and Anna Hopkins

Domainex Ltd, Chesterford Research Park, Little Chesterford, Saffron Walden, CB10 1XL

Introduction

Interest in covalent based inhibitors within both industry and academia has gained momentum through the approval of several such irreversible drugs in the cancer area. Notable examples include the EGFR inhibitors afatinib (Gilotrif) and osimertinib (Tagrisso) or BTK inhibitors ibrutinib (Imbruvica) and acalabrutinib (Calquence). Targeted covalent inhibitors carry the potential advantages of prolonged duration of action, improved potency and high levels of selectivity for the target of interest.

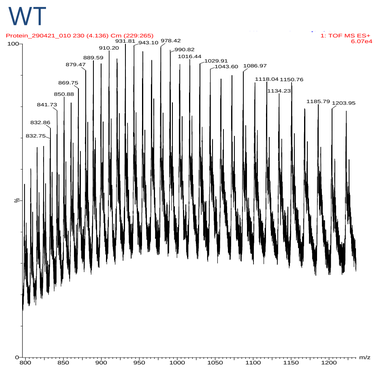
Fragment based screening has been a successful hit discovery approach for reversible inhibitors in providing better chemical space coverage and higher probability of binding due to lower molecular weight complexity. One of the challenges of fragment based screening is the requirement for sensitive biophysical detection methods due to the weak binding affinity of fragment hits. In addition, in the absence of crystallography, rationalization of which functional groups within the fragment are driving target binding is often unknown. The screening of covalent fragments looks to address these limitations, given covalent binders are easy to detect by mass spectrometry and the dominant interaction is unambiguous.

To this end Domainex has investigated a covalent fragment screen of a cysteine containing protein which has been implicated in the pathogenesis of several diseases including cancer, fibrosis and neurodegenerative diseases. Although potent cysteine based covalent inhibitors against the target protein are known in the literature, the majority have evolved from peptidic starting points and consequently retain a significant degree of peptidic character. To our knowledge no covalent fragment screen has been applied to this particular protein.

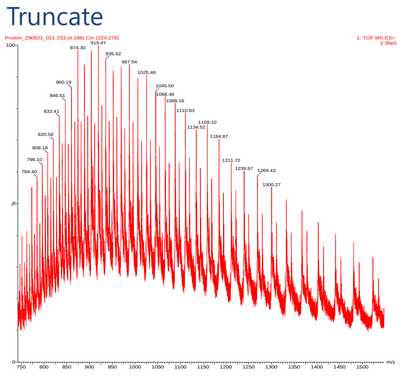
Protein Preparation

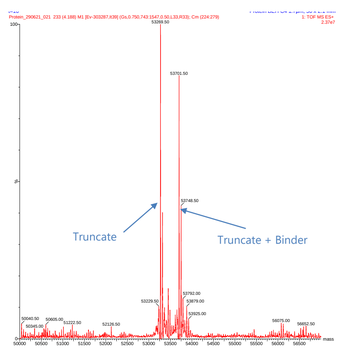
Initial analysis was undertaken on the wild type (WT) protein of interest using a previously validated method on a Waters G2-XS QToF. This gave reasonable chromatography and deconvoluted to the expected mass. While the protein analysis was sufficient for analysing the single species, once the binders 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

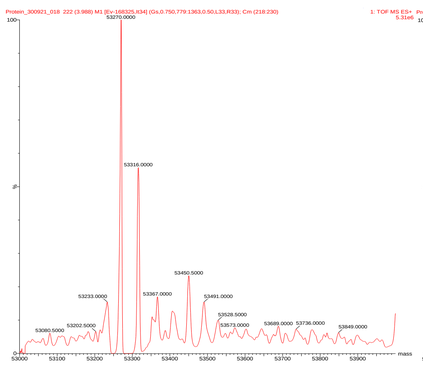




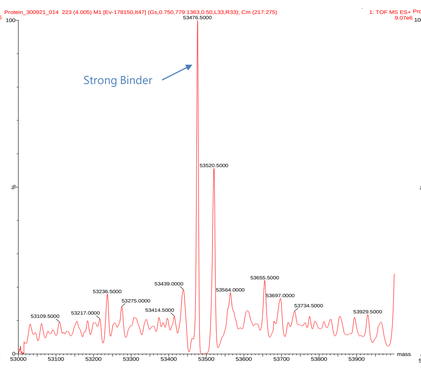
Library Screen

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).

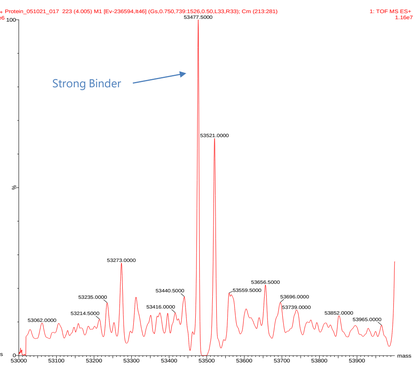
+DMSO



5 Fragment cassettes

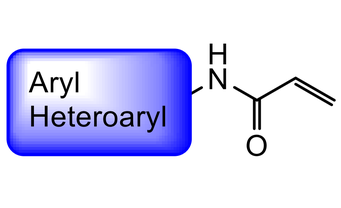


Singleton Confirmation



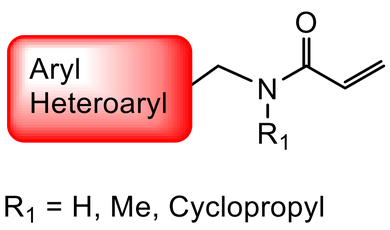
Strong binders fell into one of the following three clusters with 1:1 fragment to protein stoichiometry.

Cluster A



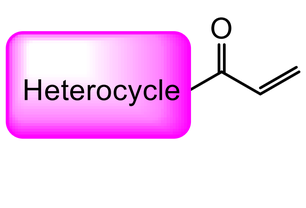
5 hits

Cluster B



3 hits

Cluster C



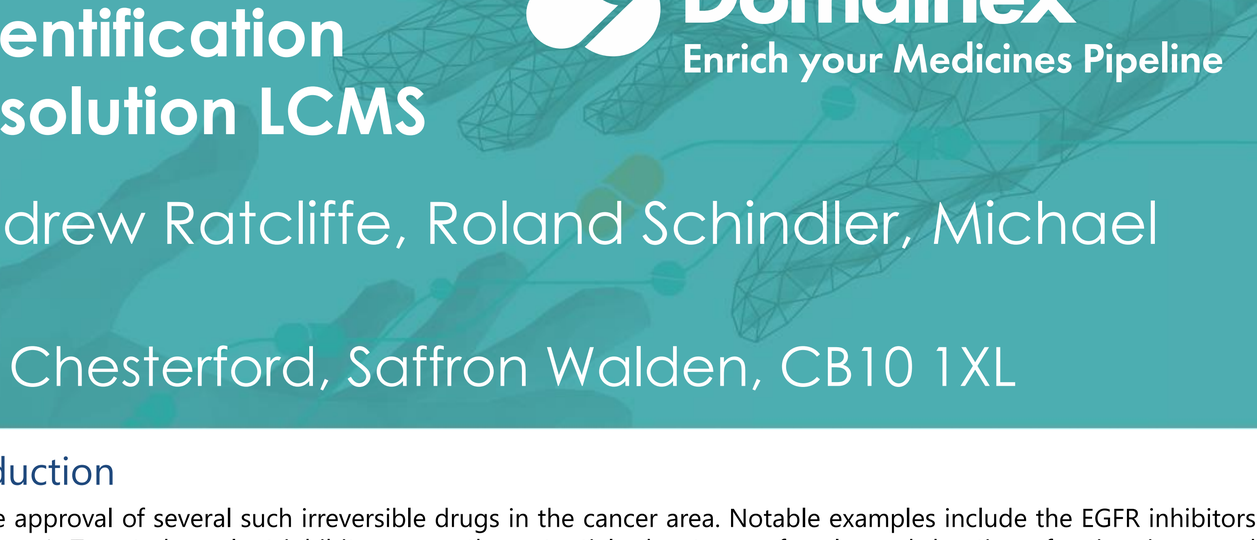
2 hits

R_1 = H, Me, Cyclopropyl

To confirm that the fragments bound at the same cysteine as the known peptidic covalent inhibitors a protein digest workflow in tandem with intact mass analysis was undertaken.

Optimising Digestion Conditions

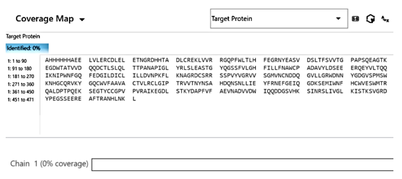
Following the singleton analysis samples were digested using a standard method of Iodoacetamide capping of remaining reduced cysteines, denaturation using 8M Urea or Guanidine chloride and Trypsin protease incubation over night. These conditions reliably provided 99% peptide mapping coverage for the 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 establishing an exact location of the binding site.



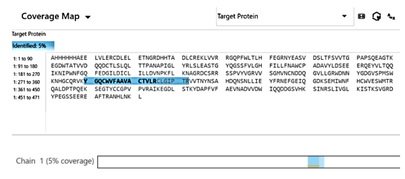
Binding Site Identification

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 that 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 observe where the binders have bound on the peptide sequence (highlighted in blue)

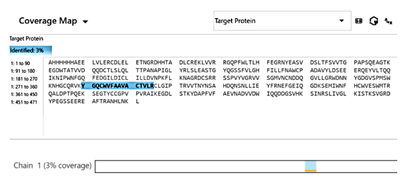
No Binder



Cluster A Fragment



Cluster B Fragment



By utilising Waters MSe fragmentation data a fingerprint for each of the candidate peptides are generated, increasing identification confidence. The software will automatically assign the fragment fingerprint and show the exact binding site of each modification (see below). Using this approach confirmed that the same cysteine as for known peptidic covalent inhibitors was modified by exemplars from clusters A, B and C (data not shown)

Component Summary

	Component name	Protein name	Fragment label	Peptide	Modifiers	Sequence start	Sequence end	Observ
1	1:T278:Carbamidomethyl C [12], Z3337540138 [4]+H+	Target Protein	1:T278:	YGQCWVFAAVACTVLR	Carbamidomethyl C [12], Z3337540138 [4]	280	295	

Conclusions

- Using a mass spectrometry based platform a covalent fragment screen identified 3 distinct clusters of novel fragments that bound the protein in a 1:1 stoichiometry.
- Using a protein digest and peptide mapping workflow exemplars from the 3 fragment clusters were shown to bind to the same cysteine as known peptidic covalent inhibitors.

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:
enquiries@domainex.co.uk

domainex.co.uk