Covalent Fragment Screen using QToF analysis

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Introduction

Interest in covalent based inhibitors within both industry and academia has gained momentum through the approval of a number of 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.

Historically one of the most frequent approaches to discovering new covalent inhibitors has relied upon incorporation of an electrophile into an already optimised reversible inhibitor. Although virtual screening of covalent libraries has been utilized, its deployment is most effective when a crystal structure (or high quality model) of the target of interest is available. Owing to concerns about promiscuous activity, HTS campaigns seeking to identify covalent inhibitors are generally avoided.

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 of 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.

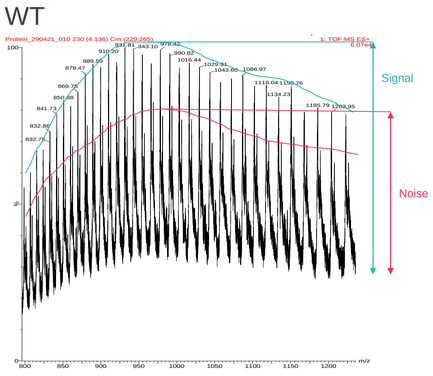
To this end Domainex has investigated a covalent fragment screen of a cysteine containing protein which has been implicated in the pathogenesis of several diseases including cancer, fibrosis and neurodegenerative diseases. Although potent cysteine based covalent inhibitors against the target protein 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

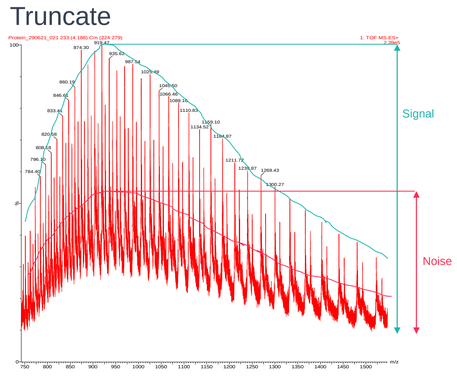
Initial analysis was undertaken on the wild type (WT) protein of interest using a previously validated method on a Waters G2-XS QToF. This gave reasonable chromatography and deconvoluted to the expected mass. A set of 5 literature based fragments were selected to explore the limits of the binding assay; one <10μM binder, two 10-50μM binders, one >1500μM binder and one predicted inactive compound, a saturated acrylamide.

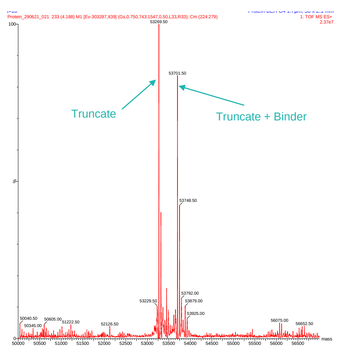
While the protein analysis was sufficient for analysing the single species, once the binders were added and multiple species were created, the signal to noise ratio (S/N) became too low to distinguish individual peaks. As the protein was relatively large it was theorised that a smaller protein would give a higher S/N. To this end the truncated version of the protein was investigated and the S/N was now much higher and individual peaks were identifiable even in complex mixtures.

WT



Truncate





Optimisation of Binding Conditions

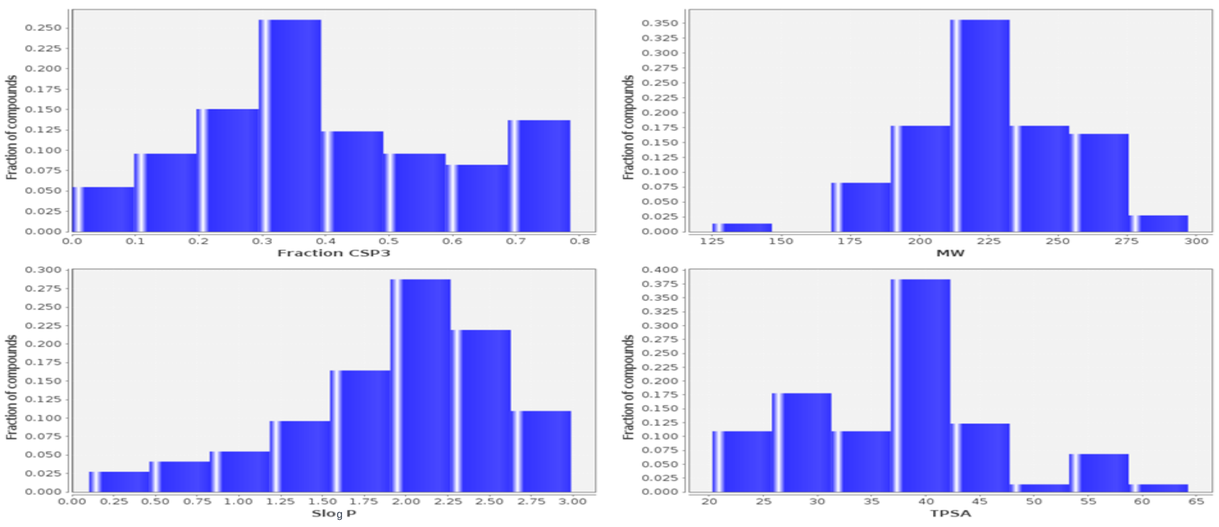
Once a suitable method for MS analysis had been identified, the next step was to optimise conditions to observe binding. All experiments were conducted at 2μM protein, which was found to be a good compromise between signal intensity and supply of protein during the preliminary studies. The key variables effecting rate of binding investigated were concentration of compound (relative to protein), temperature and length of time allowed before sampling. Using the 5 fragments selected, analysis was conducted at four different ratios of concentration ranging from equimolar to 100xmolar. No binding was observed for the non-binder in any experiment, nor was binding observed for the >1500μM binder.

Experiments were also conducted, with sequential sampling over 2 hours + a 16 hour timepoint at high and low temperature. While the high temperature allowed for faster binding, over the course of the experiment the protein degraded to the point where it could not be cleanly deconvoluted to the parent masses and generated multiple species. The low temperature was much slower, with only the <10μM binder showing significant binding after 2 hours, however at the 16 hour timepoint sufficient binding was observed for the two 10-50μM binders.

As the intention was to perform a library screen, the 5 fragments were then submitted as a cassette at a concentration which appeared suitable from the singleton studies. This resulted in decreased levels of binding for all fragments whereby the <10μM binder, which had gone to completion overnight as a singleton, had only bound about $^{1}/_{3}$ of the available protein. Cassette sampling was tested further at higher concentrations, which gave better responses for all previously observed binders and so higher concentrations were used in the subsequent library screen.

Domainex Library Design

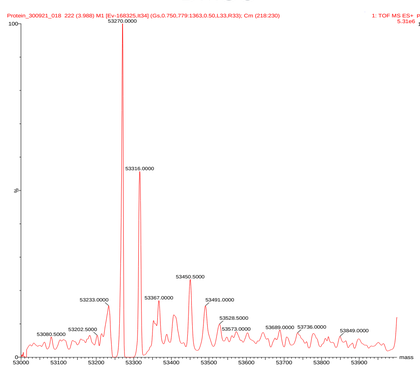
A small library of acrylamide based fragments [n=73] was assembled using several computational descriptors and K-mean clustering to impart a high degree of 3D shape and diversity within the library.



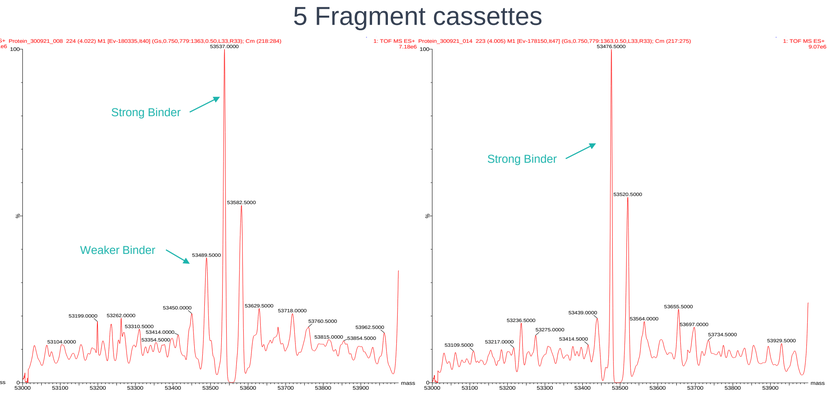
Library Screen

The Domainex acrylamide library was screened using the optimised binding conditions with 5 fragments per well (5% 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, 2.1mm x 50mm 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 the widest amu between fragments in each pool. This generated 10 strong binder hits from the library of 73, with 13 weaker binders also identified.

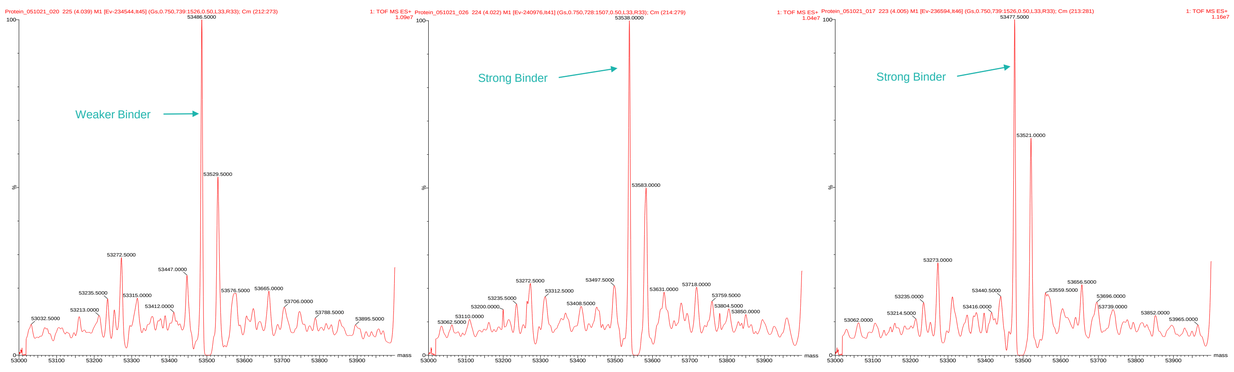
+DMSO



5 Fragment cassettes



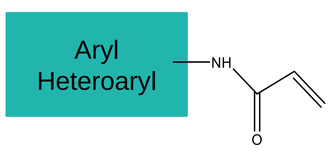
The compound pools were investigated further to ensure the hits were repeatable as singletons. Hits were confirmed by singleton analysis (1% DMSO).



Conclusions and Next Steps

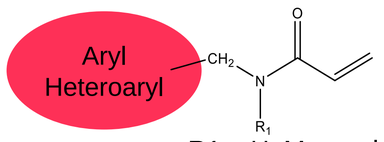
Strong binders fell into one of the following three clusters.

Cluster A



5 hits

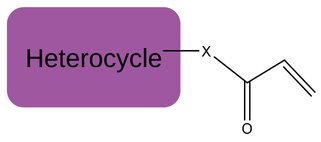
Cluster B



R1 = H, Me, cyclopropyl

3 hits

Cluster C



X = N or NH

2 hits

All 3 clusters represent potentially interesting start points for further elaboration & delivery of interesting novel binders lacking peptidic character. This work will be continued by running trypsin digest experiments on the bound samples to identify where on the protein each fragment binds and assess if there are differences between the binding sites of each cluster identified.

Domainex welcomes interest from any potential collaborators, industrial or academic. If you would like to learn more about applying our drug-discovery platform to other targets, please contact:

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