Search for Transglutaminase 2 (TG2) covalent inhibitors using Domainex's covalent fragment library, and a LC-MS based covalent fragment screening platform

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Introduction

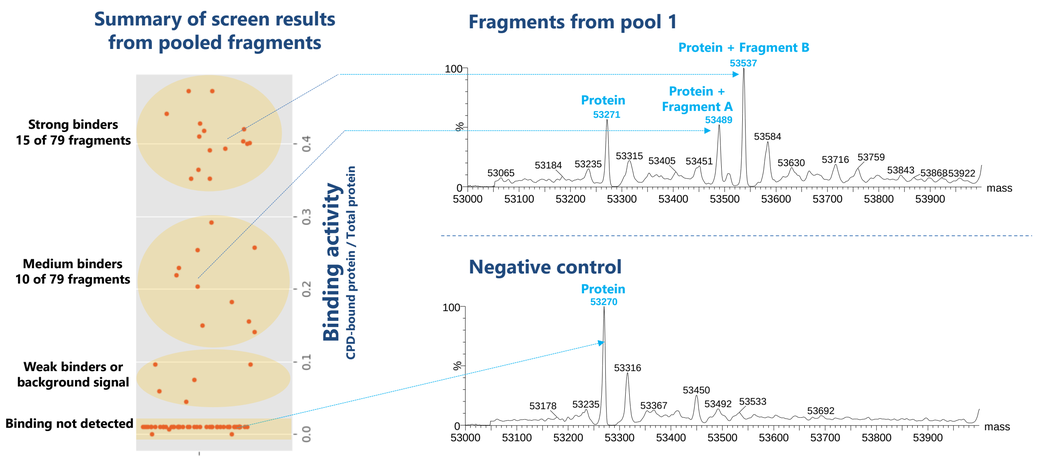
Recent advances in the tools to support discovery/design of covalent inhibitor drugs and the success of block buster drugs such as Osimertinib and Ibrutinib has led to increased interest in 'electrophile-first' covalent drug discovery. Covalent fragment-based screening by intact protein mass spectrometry (MS) has been shown to be a powerful tool, demonstrated by the discovery of KRAS(G12C) inhibitors [1]. Additional assays to support covalent fragment-based screening, including assessing warhead reactivity via a GSH assay and binding site identification by proteolytic digestion and peptide mapping further optimise hits. Covalent inhibition is time-dependent, so the preferred measure of potency is the second-order rate constant kinact/Ki, rather than IC50.

Here we describe a case study assessing covalent fragments against Transglutaminase 2 (TG2) protein (a protein that is implicated in the pathogenesis of several diseases, including cancer, fibrosis and neurodegenerative diseases), which to our knowledge has not been done before.

Covalent Screening by MS

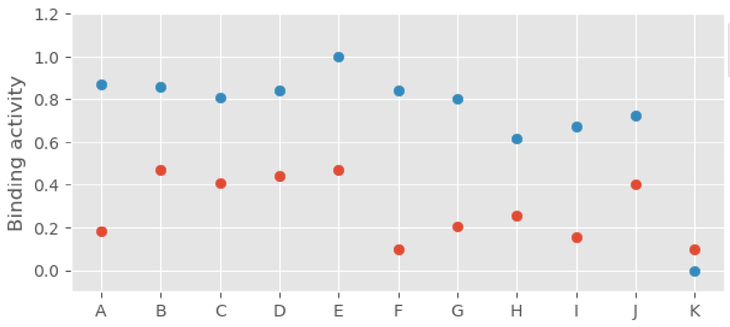
Initial screening of pooled fragments

- Following method optimisation, TG2 was incubated with pools of fragments for 18 hours at +4ºC and covalent binding was measured by rapid intact LC-MS under denatured condition.
- Fragment pools were prepared using an automatic sample handler, fragments were pooled into groups of 5 with >5 Da mass difference.
- LC-MS data were acquired on 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. Data of up to 800 pools can be acquired per night.
- LC-MS data were analysed and visualized by an in-house developed data processing pipeline, which can process 4 000 LC- HRAMS data files within 20 minutes.



Selecting individual fragments to validate hits

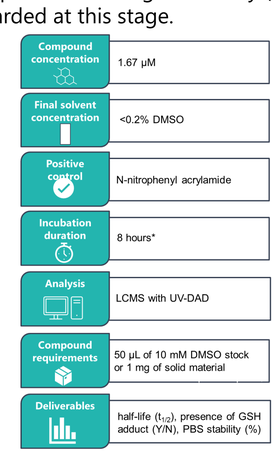
- Example data of fragment bindings in individual runs vs pooled runs
- Side-by-side data comparisons were performed by an in-house developed rapid data processing pipeline.



- Most fragments in the singleton validation showed higher binding activity, due to the absence of competition from other fragments
- Fragment K did not show binding during the singleton validation, suggesting the binding identified in pooled screen was a background signal (false positive).

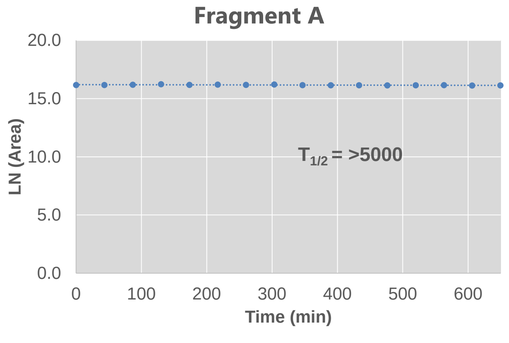
Glutathione Counter screen

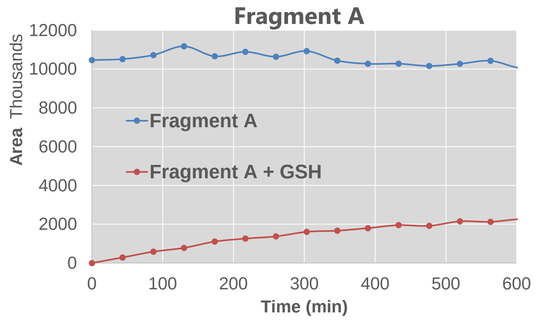
- Hits from the covalent screen were assessed for Glutathione (GSH) reactivity in real time by LC-UV-MS
- Compounds with high reactivity ($T_{1/2}$<100 minutes) are unlikely to exhibit desirable properties and were discarded at this stage.



Fragment	$T_{1/2}$	PBS Stability (% remaining)
A	>5000	110
B	>5000	117
C	2923	109
D	>5000	110
E	>5000	114
F	1023	114
G	1118	113
H	148	110
I	>5000	106
J	473	114

- Multiple fragments show relatively high binding with low GSH reactivity
- All 10 strong binder hits had a ½ life >100 minutes and were taken forward for binding site identification by proteolytic digestion, followed by peptide mapping.
- Exemplar data for fragment A is shown below

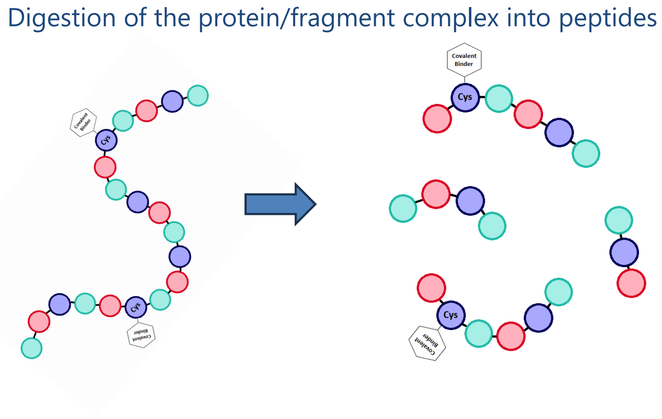




Binding Site Identification

- Fragments were individually incubated with TG2 for 18 hours at +4ºC
- The samples were then denatured with urea, reduced with TCEP, alkylated with IAA and digested overnight at + 37ºC with trypsin

Digestion of the protein/fragment complex into peptides



LC-HRAMS analysis

Peptide mapping software identifies the peptide where fragment A is bound

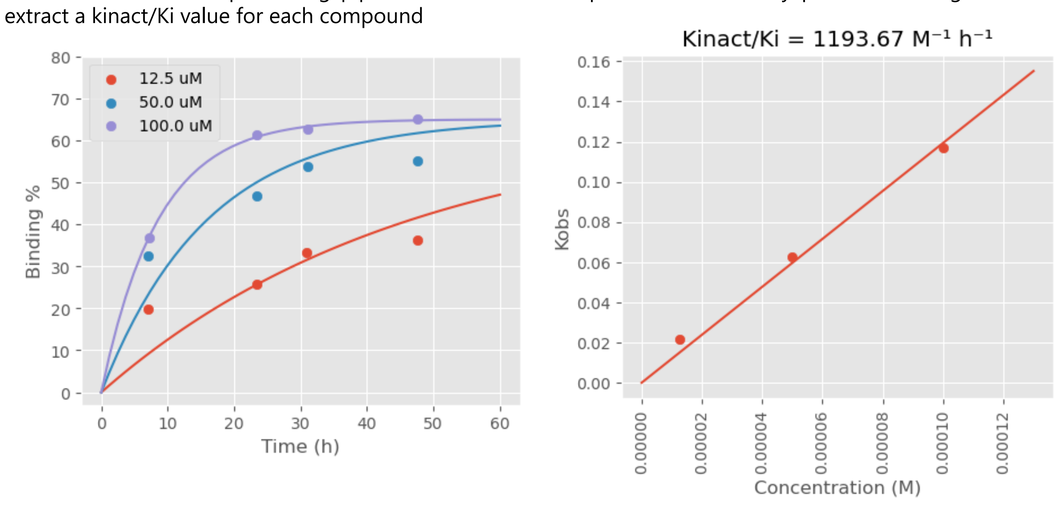
AHHHHHHAEE	LVLERCDLEL	ETNGRDHHTA	DLCREKLVVR	RGQPFWLTLH	FEGRNYEASV	DSLTFSVVTG	PAPSQEAGTK
EGDWTATVVD	QQDCTLSLQL	TTPANAPIGL	YRLSLEASTG	YQGSSFVLGH	FILLFNAWCP	ADAVYLDSEE	ERQEYVLTQQ
IKNIPWNFGQ	FEDGILDICL	ILLDVNPKFL	KNAGRDCSRR	SSPVYVGRVV	SGMVNCNDDQ	GVLLGRWDNN	YGDGVSPMSW
KNHGCQRVKY	**GQCWVFAAVA**	**CTVLR**CLGIP	TRVVTNYNSA	HDQNSNLLIE	YFRNEFGEIQ	GDKSEMIWNF	HCWVESWMTR
QALDPTPQEK	SEGTYCCGPV	PVRAIKEGDL	STKYDAPFVF	AEVNADVVDW	IQQDDGSVHK	SINRSLIVGL	KISTKSVGRD
YPEGSSEERE	AFTRANHLNK	L					

MS/MS data is used by the software to identify the exact binding site within the peptide

- Binding site data was successfully generated for all 10 fragments
- All fragments were shown to bind to the target cysteine (residue 277) of TG2

Kinact/Ki

- Covalent inhibition is time-dependent, so the preferred measure of potency is the second-order rate constant kinact/Ki, rather than IC50
- TG2 was incubated with a fragment at different concentrations for different periods of time. The binding percentage at each time point was measured by LC-MS.
- An in-house fast data processing pipeline has been developed to automatically plot the binding data and extract a kinact/Ki value for each compound



Conclusions

- Domainex has developed a MS based covalent platform which successfully identified several fragments as potential leads for optimisation against TG2.
- GSH counter screen of positive hits can be used to triage out highly reactive warheads
- Proteolytic digestion and peptide mapping determine the binding site of selected hits
- K_{inact}/K_I was successfully determined for selected hits and can be used in combination with GSH reactivity to optimise compound potency during the hit to lead stage
- Applications of these workflows allows for the high throughput screening of various libraries against a target protein, as well as supporting decisions during of the optimisation process.

Future Development

- Evaluate a LC-MS based covalent fragment screening platform for alternative binding sites, beyond cysteine.

Contact

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