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Establishment of a High-Throughput Screening and Characterization Platform for Covalent Inhibitor Drugs

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PURPOSE

Covalent inhibitors contain specific functional groups that can form covalent bonds with the corresponding sites of key mediating proteins related to diseases[1], causing their conformational changes, inhibiting their activity and losing their pathological functions. The covalent bond with the target protein is more persistent and can achieve complete target occupancy at relatively low concentrations. Therefore, covalent inhibitors have the advantages of prolonged efficacy, reduced dosing frequency and higher selectivity. Covalently bound drugs are a rapidly developing discipline in the field of drug discovery. We have established a high-throughput screening and characterization platform for covalent binding drugs to provide technical support for drug researchers.

METHOD

High-throughput screening platform based on differential scanning fluorescence (DSF)

Application: High-throughput initial screening (96 or 384 plate)

Screening and characterization platform based on intact protein

Application: Screening candidate compounds, confirm covalent binding or not and calculate binding ratio

Characterization platform based on peptide mapping

Application: Confirm the specific binding site of small molecule to the target protein

Figure 1 . Covalent drug screening and characterization platforms

We take KRAS^{G12C} protein and its inhibitor sotorasib as an example. First, the differential scanning fluorescence (DSF) method was established for high-throughput screening of covalent inhibitors. By using programmed temperature increase, the protein unfold at high temperature and the fluorescent dye will bind with the exposed hydrophobic part. The data will be fitted with the Boltzmann equation to calculate the melting temperature (T_m) of the protein. Second, after the target protein and candidate drug binding, the shift of molecular weight can be used to determine whether a covalent bond has been formed by HRMS. Finally, peptide mapping method using high-resolution mass spectrometry was established to identify which amino acid was binding with the candidate drug.

RESULT

Intact Protein (HRMS) - Screening and Characterization Platform

Mass spectrometry is widely used in covalent drug discovery. When compounds bind to the target protein, the protein's molecular weight increases, allowing for precise measurement and monitoring of irreversible covalent bonds using HRMS. The method is simple to develop, offers high throughput and demonstrates excellent reproducibility. As shown in Figure 2, we take KRAS^{G12C} and its inhibitor sotorasib as an example. The molecular weight shift can be observed after incubation. The mass shift is exactly the molecular weight of sotorasib. Under this incubation condition, KRAS^{G12C} fully combined with sotorasib with a binding rate of 100%. LC-HRMS can monitor the binding and calculate the binding ratio.

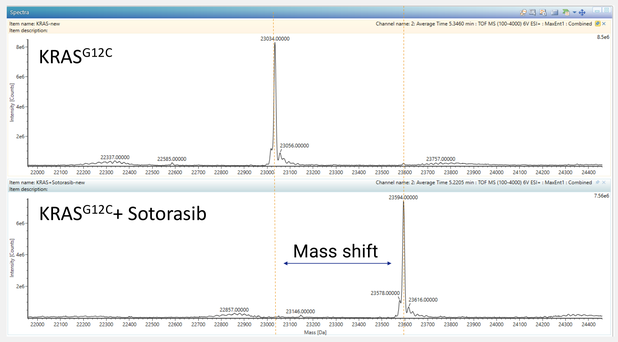

Figure 2. Intact protein result by using LC-HRMS

Differential Scanning Fluorimetry (DSF) - High Throughput Screening Platform

While MS methods are widely used for screening covalent drugs, increasing interest in the field has led to the exploration of additional high-throughput strategies to evaluate more compounds in less time. Differential scanning fluorimetry (DSF) is a method to track the folding state and thermal stability of proteins[2]. DSF is commonly used in protein thermal stability studies, protein-ligand interaction studies protein stabilizers, and inhibitor screening studies. The principle of DSF is that as the temperature rises, proteins unfold, exposing hydrophobic regions that bind fluorescent dyes, resulting in an enhanced fluorometric signal. The data is fitted to the Boltzmann equation to calculate the melting temperature (Tm) of the protein. Tm is defined as the temperature at which 50% of the protein sample is folded and 50% is unfolded[2]. DSF is often utilized to detect the binding of small molecule ligands to target proteins[3]. In covalent drug screening, DSF can also monitor changes in protein thermal stability before and after binding.

KRAS^{G12C} and sotorasib result shows that ΔTm was observed from the melt curve plot. As shown in Figure 3, the blue line shows the state of the target protein. The purple line shows the state of the target protein binding to drug candidates. The difference in melting temperature between the two lines is expressed as (ΔTm) which can be used to evaluate whether the drug candidates bind to the target protein. The DSF method offers advantages such as low protein sample requirements, high throughput and rapid analysis times, making it suitable for high-throughput drug candidate screening and target discovery.

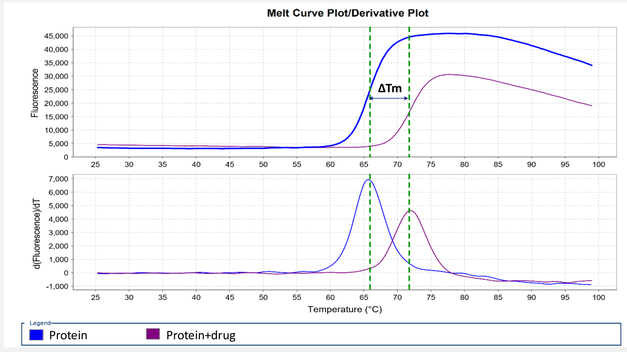

Figure 3. Covalent binding of drugs induces protein melting temperature (Tm) migration

Peptide Mapping (HRMS) - Binding Characterization Platform

Peptide mapping is a key technique for determining the primary structure (amino acid sequence) of proteins and is commonly employed in the quality control of protein and peptide drugs[4]. Peptide mapping relies on HRMS and exhibits high sensitivity to identify end blocking or modifications in samples. In the screening stage of covalent drugs, peptides are analyzed by HRMS to identify which amino acid sites covalently bind to the protein.

Figure 4 shows peptide mapping results of KRAS^{G12C} protein and its inhibitor sotorasib. After trypsin digestion, the sequence coverage reached 100%. Sotorasib covalently binds to cysteine in the peptide LVVVGACGVGK, with a retention time of 58.4 minutes and a confidence score of 100.

Level	No.	Identification	Peptide Sequence	Modification	Site	Delta (ppm)	Confidence Score
Component	3838	1:L25-K35 = 1000.5739m(-C31+Sotorasib)	LVVVGACGVGK	Sotorasib	-C31	-0.11	100.0

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
KRAS^{G12C}	1506	24.1%	100.0%	100.00%

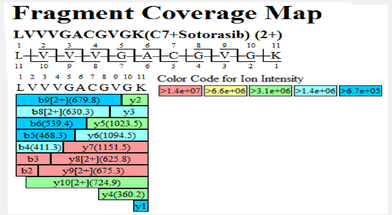

Figure 4. Peptide mapping result of KRAS^{G12C} after covalent binding with sotorasib

CONCLUSION(S)

In this work, we have established high-throughput screening and characterization platform based differential scanning fluorescence, intact protein and peptide mapping analysis which is applicable to covalent inhibitors. The platforms are showed high-throughput, accuracy, stability and rapidity.

REFERENCES

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