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Strategy to follow-up and validate hits from phenotypic screening by cellular thermal shift assay coupled with mass spectrometry (CETSA-MS)

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Master thesis, Analytical Chemistry

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INTRODUCTION

Phenotypic screening is a widely used approach in drug discovery to identify small molecules exhibiting desirable effects in disease models. However, a significant challenge is to determine the targets of these phenotypic screen hits. This leads to the need of efficient target deconvolution strategies to understand their mode of action and off-target effects, which would accelerate drug development in addition [1, 2].

One recent technology used for target deconvolution is the cellular thermal shift assay (CETSA). This assay works on the principle that heating lead to protein denaturation and denatured proteins start to aggregate, what decreases the solubility. Combined with the knowledge that the denaturation temperature (melting temperature) of a protein is shifted upon compound binding, the interaction can be investigated by quantifying the soluble part of a sample at different temperatures [3]. Now, if the CETSA technique is connected to mass spectrometry (MS) bottom-up proteomics, it enables the investigation of interactions between a compound and thousands of proteins in one experiment. For this reason, CETSA-MS is a powerful and efficient assay for target deconvolution [4].

CONCEPT

The main objective of the master thesis was to develop a workflow to identify and validate targets for phenotypic screen hits, based on CETSA-MS. To develop the workflow, compound Y, a phenotypic screen hit, served as model compound. The workflow consists of the following steps:

1. Target identification for compound Y by dose-response CETSA-MS.
2. Melting curve determination of target by CETSA-MS.
3. Compound Y target confirmation by targeted CETSA-MS.
4. Investigating influence of compound Y on target activity by enzymatic assay.

Beside the CETSA-MS experiments, an enzymatic assay was performed, to investigate the influence of compound Y on protein X activity, as CETSA only detect the binding to a target but contains no information about the effect of a compound on the target activity [5]. Now, the combination of CETSA-MS with the enzymatic assay leads to a powerful strategy to follow-up and validate hits from phenotypic screening.

RESULTS

In the first experiment, a dose-response CETSA-MS was performed to identify the target of compound Y. As shown in Fig. 1, kinase X showed a significant thermal stabilization while treated with compound Y. In addition, kinase X shows a positive dose-response to the concentration of compound Y. This identified kinase X as target of compound Y.

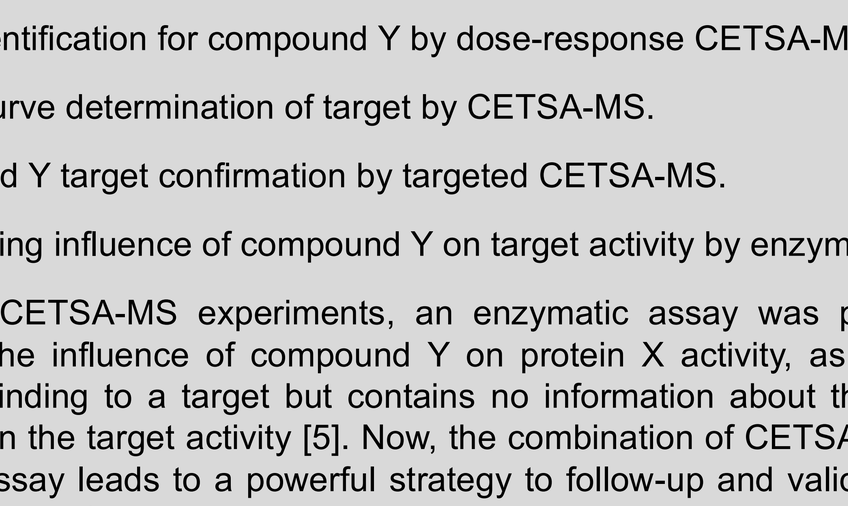

Fig. 1: Dose-response CETSA-MS results of the compound Y at 1 µM, 10 µM, 30 µM, and 100 µM (1 % DMSO) vs. the DMSO samples (1 %), identified kinase X as target.

After target identification, the melting curve of kinase X was determined. In Fig. 2, two melting curves of kinase X are depicted, one under the influence of compound Y (blue) and one without (red). The shift in the melting curve and the increase of the kinase X melting temperature due to compound Y further confirmed that compound Y targets kinase X. Additionally, the temperatures with the highest assay window were identified, which will be used for the subsequent targeted CETSA-MS experiment.

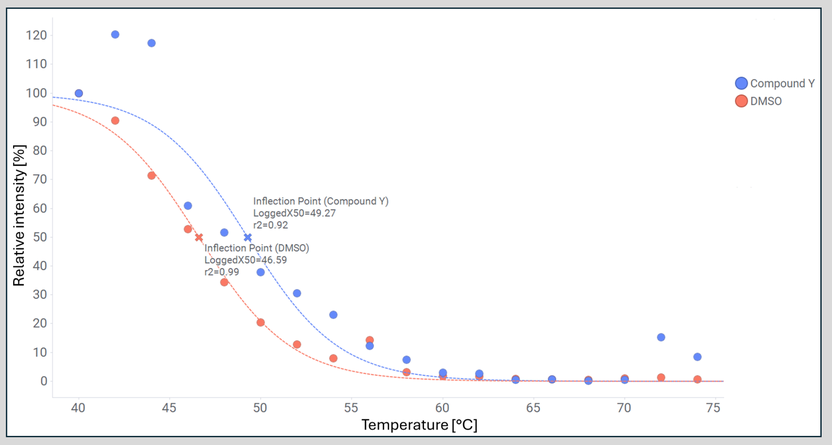

Fig. 2: Melting curve of kinase X under the influence of compound Y (blue) and without compound Y (red). The detected melting point is 49.27 °C (R^2 = 0.92) for the compound treated and 46.59 °C (R^2 = 0.99) for the DMSO samples.

The targeted CETSA-MS experiment was conducted at 50 °C, 52 °C, and 54 °C. This experiment compared compound Y with compound Z (another compound from phenotypic screening), an inactive compound with a very similar structure to compound Y, and a commercial inhibitor of kinase X. This comparison confirmed the compound-Y induced thermal stabilization of protein X again.

In a final step, an enzymatic assay was performed with the same substances used in the targeted CETSA-MS. The b-value in Fig. 3 represents the increase in the fluorescence signal of the enzymatic assay over time, which depends on the activity of kinase X. That means, the larger the b-value, the greater the activity of kinase X. As the b-value for compound Y is smaller compared to the positive control (without compound), a weak but significant inhibition of kinase X by compound Y is detected, which validated kinase X as target of compound Y.

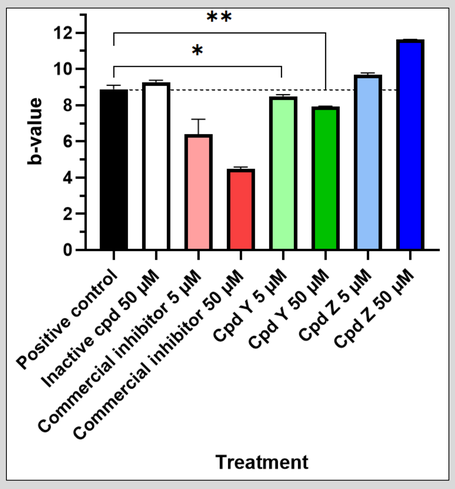

Fig. 3: b-values from enzymatic assay. The significance was tested with a two-tailed t test for Compound Y at 50 µM and 5 µM compared to the positive control. Both showed a significant lower b-value (** p-value < 0.01, *p-value < 0.05).

Conclusion

In this work, a CETSA-MS-based strategy, to identify and validate targets of phenotypic screen hits, was successfully developed on the example of compound Y. In a first dose-response CETSA-MS experiment, kinase X was identified as target. The subsequent kinase X melting curve determination was performed to select the temperatures with the highest assay window for targeted CETSA-MS, where kinase X was confirmed as target. In a final step, kinase X was validated as target of compound Y by an enzymatic assay, which proved the inhibition of kinase X by compound Y.

This strategy may find application in future projects and can significantly accelerate the process of target identification and validation in drug discovery, which further enhances the understanding of compound-target interactions and facilitates drug development.

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