Covalent inhibitors: how we can find them in an early stage of a drug discovery program

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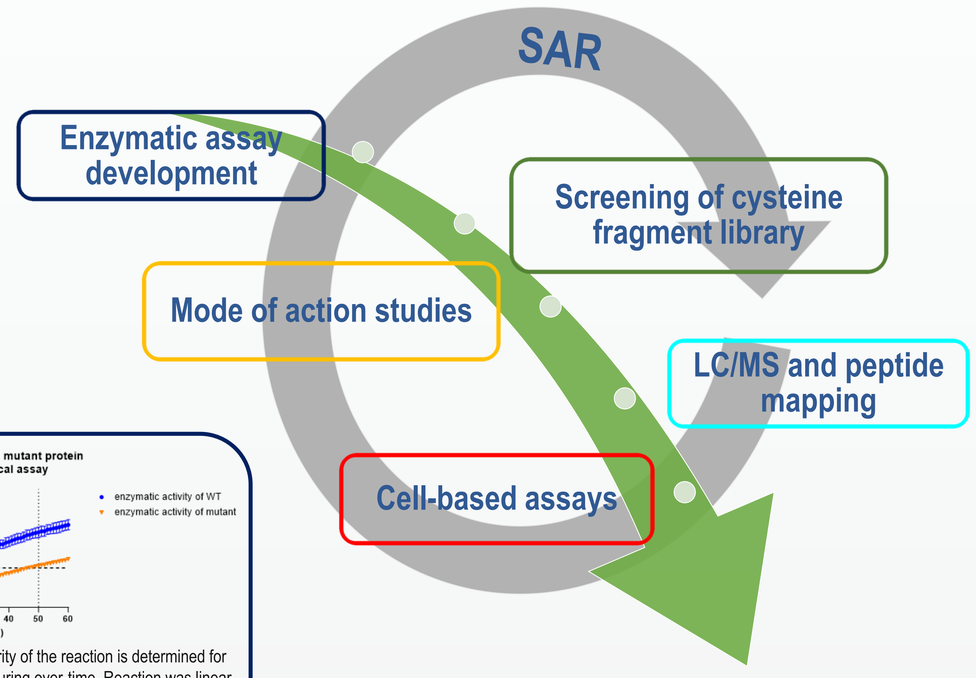
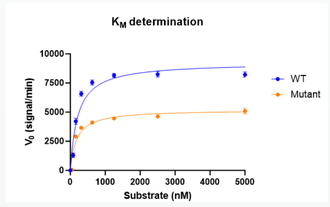
Background

Over the past ten years, advances in covalent drug discovery have led to the successful development and approval of drugs, including covalent inhibitors of BTK, Mutant EGFR, KRAS (G12C) and SARS-CoV-2 Mpro. Nowadays, covalent inhibitors are no longer identified, as in the past, by serendipity: on the contrary, well-defined multidisciplinary approaches have been established that can pave the way to the successful identification of the compounds. Multiple drug discovery programs focused on the development of first-in-class covalent inhibitors are currently ongoing at IRBM. In this poster, we describe the assay cascade that we established to identify novel covalent pharmacophores against an undisclosed target. The starting point was a homogenous assay used to monitor the enzymatic activity of the target protein and suitable to screen covalent fragment libraries. A mutant variant of the protein with the target cysteine residue converted into alanine was prepared: as the enzymatic activity of the mutant protein was only <50% lower than the WT, an appropriate and target-relevant counter screen assay was developed. Confirmation of the binding specificity of the hit compounds was conducted by LC/MS analysis and peptide mapping. Biochemical characterization of the more interesting molecules (i.e. time-dependency assay, progression curve to measure the Kobs) followed along with a cell-based target engagement assay are included in the screening cascade. This approach led to the identification and characterization of hits and the transition to the hit expansion phase with an ongoing SAR.



Enzymatic assay development

Biochemical assay following enzymatic activity of the protein was developed to identify and characterize covalent inhibitors. The WT and mutant protein showed similar affinity for the substrate (K_M of 212.9 nM and 177.4 nM, respectively) as reported in Fig.1. The processivity of the mutant instead was <50% lower that the WT. The enzymatic reaction is linear up to 20 minutes for the WT and 50 minutes for the mutant protein (Fig.2). These final assay conditions were used to screen the cysteine fragment library and to characterize the hits.



Enzyme	KM (nM)	Vmax (RFU/min)
WT	212.9	9256
mutant	177.4	5208

Figure 1. The Michaelis-Menten constant (K_M) was measured for the WT and mutant enzyme. The graph showed the plot of the V_0 measured at different time points and in presence of the substrate titration. Data are the mean of two independent experiments. In table the K_M and V_{max} were measured.

Figure 2. The linearity of the reaction is determined for both enzymes measuring over-time. Reaction was linear up to 20 minutes for WT and 50 minutes for the mutant protein using the substrate at K_M value.

Screening of cysteine fragment library

A cysteine focused fragment library was used to identify covalent inhibitors. As shown in Fig.3, we clearly observed that several compounds were more active on WT protein than on mutant protein. As covalent compounds were time-dependent, the potency of selected compounds were measured varying the pre-incubation time between enzyme and compound (30 minutes Fig.4A and 240 minutes Fig.4B). As expected, we observed that inactive or weak inhibitor at shorter pre-incubation time showed a potency jump when pre-incubation was increased. The same compounds continued to show weak inhibition on mutant protein (Fig.5).

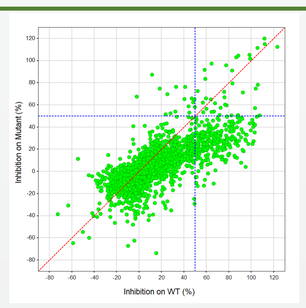


Figure 3. Cysteine fragment library compounds were screened at 100 µM and with 240 minutes of pre-incubation between enzyme and compounds. In the plot reported the correlation between the percent of inhibition on WT and mutant protein is reported. Compounds with % I > 50 on WT protein were selected for potency determination.

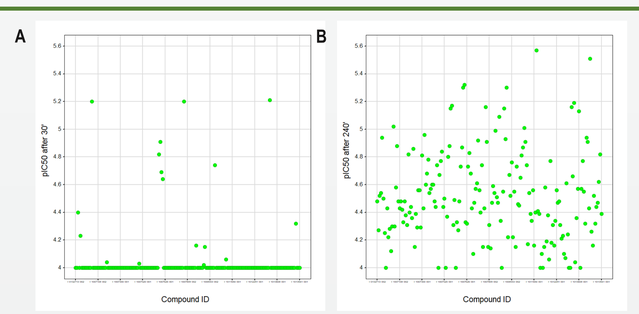


Figure 4. Potencies (pIC50) of hits compounds was measured at two pre-incubation time for WT protein: at 30 minutes (A) and at 240 minutes (B).

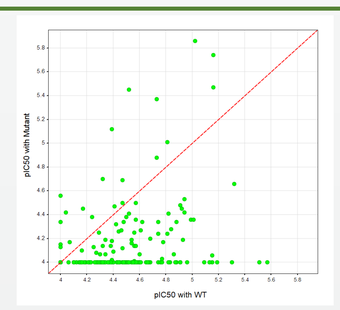
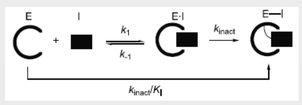


Figure 5. Potencies (pIC50) of the confirmed hits on the WT vs mutant proteins. Potency was measured at 240 minutes of pre-incubation time. In red 1:1 correlation

Mode of action studies

The IC50 shift is only the first step of the characterization of these inhibitors. The Fig.6 showed the behavior of the most active compound: the molecule confirmed to be more potent increasing the pre-incubation time with the enzyme and it was active only on the WT protein. As the IC_{50} value for a covalent compound vary with the pre-incubation times, the estimation of the rate of covalent modification is required to support the SAR. For this reason, we measured K_{inact}/K_I using the progression curve as reported in Fig.7 and 8. K_{inact}/K_I is a second-order rate constant describing the efficiency of covalent bond formation resulting from the potency (K_I) of the first reversible binding event and the maximum potential rate (K_{inact}) of inactivation.



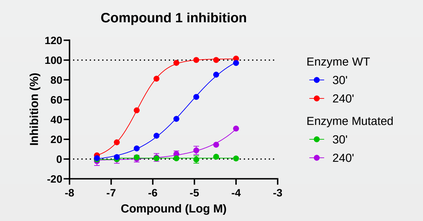


Figure 6. Active compound showed as a typical covalent behavior, being more potent increasing the pre-incubation time with the enzyme. The same compound was instead inactive on mutant protein.

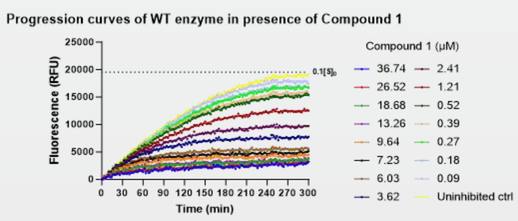


Figure 7. Progression curve for the most active compound. The enzymatic activity was measured over time in presence of a compound titration.

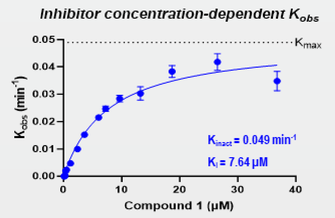
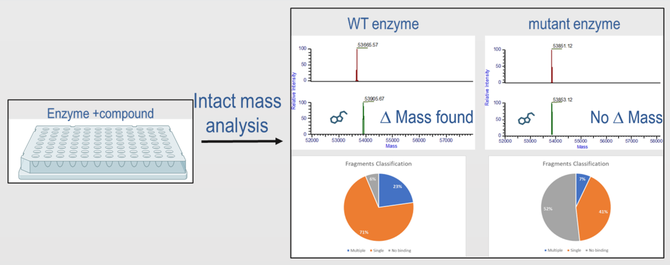


Figure 8. Direct determination of the kinetic parameters of the covalent compound. The Kobs measured in the progression curve assay was used to determine Kinact and KI.

LC/MS and peptide mapping

The presence of the irreversible bond was confirmed using the intact mass. Each molecule covalently bounded causes a corresponding increase in protein mass, allowing the determination of stoichiometry and a relative abundance of the modification. The identification of the amino acid directly linked to the fragments can be performed with an approach known as peptide mapping. This methodology is based on enzymatic hydrolysis of the protein and protein-ligand complex, peptide separation and detection by tandem mass spectrometry (MS/MS).

From our screening, the compounds can be divided in three categories: compounds with multiple binding, compounds with single binding and compounds with no binding (Fig.9). As expected, the number of compounds in these categories varied when intact mass was run on mutant protein: in particular, we observed an increase of compound classified as no binders. For the best compounds, the peptide mapping was carried out. The compounds with the following behaviors were selected for further investigation: a clear covalent binding only on wt protein with 1:1 stoichiometry and with position of the binding confirmed using peptide mapping.



Best performers	% Binding vs WT Intact Mass	% Binding vs mutant Intact Mass
1	100	0
2	100	0
3	100	0
4	100	0
5	90	0

Figure 9. Workflow used for intact mass analysis. Clear Δ mass with single binding was found for 71% of tested compounds on wt protein. Among that, the more interested compounds did not show binding on mutant protein (52%). For the most active compounds, peptide mapping was carried out to confirm the binding on the specific cysteine.

Target engagement cell-based assay

The HiBiT Thermal Shift Assay (BiTSA) was selected to determine the target engagement of the inhibitors in the cellular environment (Fig.10). Cell line contained our target protein tagged at its native genomic loci with HiBiT was used. Figure 11 showed the Melting Temperature (Tm) measured in intact cells (Fig.11A) and lysates (Fig.11B) for our target. Compounds are under testing, in dose-response at fixed Tm.

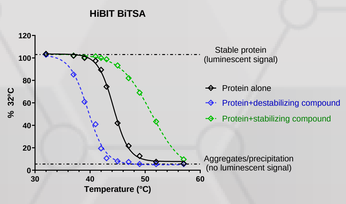


Figure 10. Scheme showed the measurement of target engagement using BiTSA approach. Compound that binds HiBiT-target protein can induces protein stabilization or aggregation. Luminescence signal is used to follow the protein folding.

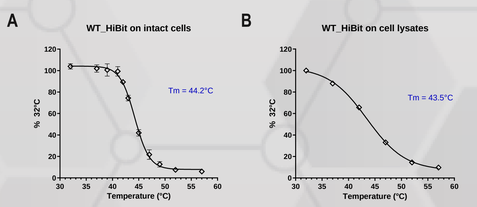


Figure 11. Determination of the Melting temperature for the HiBiT-target protein on intact cells (A) and on cell lysates (B). The first approach can be used for the cell permeable compounds. The second approach can be used for all the hits independently of their permeability.

Antiproliferative effect on responsive cell-lines

We know that the inhibition of our targeted protein impairs the viability of responsive cell lines, while no effect is observed for non-responsive cell line. The Cell-Titer Glo was used to evaluate the effect of selected compounds and results are reported in Figure 12A and 12B. The two covalent compounds showed activity on responsive cell line in µM range with a potency around 3-digit nM in the biochemical assay. These results are promising, and further optimization of molecule are ongoing to reach a more potent effect in cells.

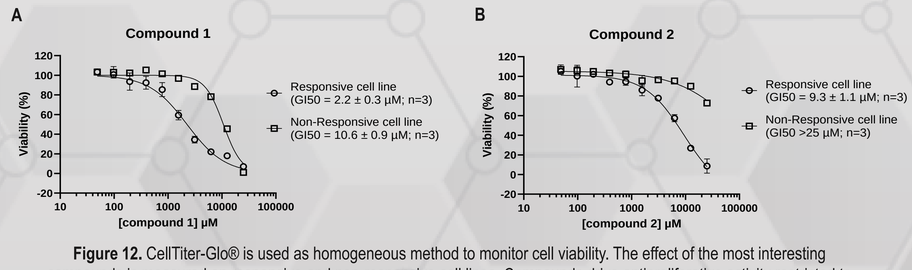


Figure 12. CellTiter-Glo® is used as homogeneous method to monitor cell viability. The effect of the most interesting compounds is measured on responsive and non-responsive cell lines. Compounds drive antiproliferative activity restricted to responsive cell model.

Conclusions

At IRBM, we are working on the discovery of new covalent selective inhibitors able to bind a single cysteine of our target protein. A screening cascade was fashioned including:

- Biochemical assay development on wt and mutant protein
- Screening of focused cysteine library
- Hit confirmation through the analysis of kinetic parameters
- Quantitative analysis by LC/MS approach
- Develop and use of cell-based assays

This assay funnel allow us to identify and characterize candidates for progression to the hit-to-lead studies for this specific target, but the same approach can be adopted to other targets.

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