Screening for inhibitors of the ubiquitination regulator and anticancer target CSN5 using a HTS fluorescence polarization method

U . Hassiepen, M.D. Jones, R. Assenberg, I. Bechtold, C. Logel, M. Renatus, B. Martoglio
Novartis Pharma AG, Switzerland

- **Determine the activity of a de-neddylating protease in an HTS compatible format**
- **Use of a fluorophore with a long fluorescence lifetime to extend the assay window**
- **PHERAstar® enables measurement due to speed and accuracy**

Introduction

The COP9 signalosome (CSN) catalyzes the de-neddylation of cullin-RING E3 ubiquitin ligases (CRLs) turning the ligase into the inactive state. Literature evidence suggests that trapping CRLs in the inactive state will keep selected tumor suppressors at elevated levels. Therefore, the inhibition of CSN's proteolytic subunit CSN5 represents a potential novel modality for the treatment of cancer (Fig. 1).
A biochemical protease assay to probe the de-neddylating activity of CSN was developed in order to identify by high-throughput screening (HTS) chemical starting points for drug discovery.

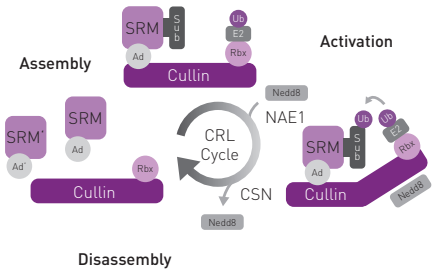


Fig. 1: Regulation of CRL by cullin neddylation and de-neddylation. Ad, adaptor protein; SRM, interchangeable substrate recognition module; Sub, substrate. Figure adapted from Schlierf et al.[1]

Assay Principle

A major complication for establishing an appropriate enzyme activity assay was the fact that CSN5 is only proteolytically active in the context of the CSN complex. In addition, CSN-catalyzed de-neddylation requires neddylated cullin complexes as substrate (i.e., CRNB-DDB1-DDB2-Rbx-Nedd8). Hence, both the enzymatically active protease and the corresponding substrate are multi-domain protein complexes. As a consequence, the assay had to be adapted for detecting the release of the 8 kDa ubiquitin-like protein Nedd8 from an approx. 270 kDa CRL complex (Fig. 2a).

As assay readout, we applied fluorescence polarization (FP), which relies on rotational movement of molecules in solution. Large molecules rotate slower than small molecules. Therefore, fluorophores tightly bound to small molecules depolarize emission light stronger than those bound to larger molecules. In order to optimize the assay window for the protease activity assay with respect to the expected mass changes, we selected the fluorophore PureTime-22 (PT22) as the FP probe. PT22 is an acridone dye exhibiting a remarkable long fluorescence lifetime (FLT) of 22 ns. Since the FP value depends on both, the molecular mass of the protein and the FLT of the probe, the use of the long FLT probe PT22 offers an obvious advantage over the use of frequently used probes such as Alex Fluor® 488, which have a FLT in the range of only 3-4 ns. The long FLT of the PT22 probe significantly increases the window of the FP assay when proteins or protein complexes of high molecular mass are under investigation (Fig. 2b).

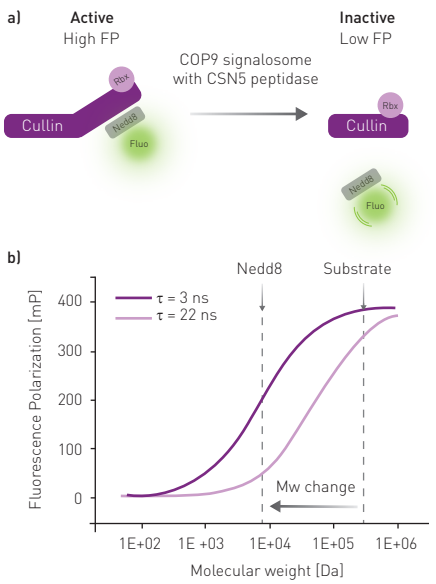


Fig. 2: Schematic representation of the CSN activity assay. (a) Assay principle (b) Graphical representation of the relationship between the FP value, the molecular mass and the fluorescence lifetime of the FP probe.

Materials & Methods

- CliniPlates (ThermoScientific) 384 well black
- Cul4A E3 ligase modified with fluorophore-labeled NEDD8[1] (fluorophore PT22, GE Healthcare, TTP Labtech)
- CSN complex[1]
- PHERAstar® *FS*, BMG LABTECH

Experimental procedure
The CSN enzyme solution (12.5 µl, final concentration 150 pM) was added to 0.25 µl of test compound to

Rev. 04/2017
Keywords: **HTS, NEDD8, Posttranslational modification, SDE**

preincubate at room temperature for 1 h. The reaction was started by addition of the Cul-NEDD8-PT22 substrate (12.5 µl, final concentration 150 nM)[1].
For the comparative TR-FRET assay a terbium labelled anti-His antibody was used in combination with an Alexa 488-coupled substrate. Please contact BMG LABTECH for further information.

Instrument settings

	Fluorescence Polarization	TR-FRET
Optic settings	*FP 540 590 590 optic module* Excitation: 540 Emission: (parallel and perpendicular) at 590	*LanthaScreen optic module* Excitation: 337 Tb emission: 488 Alexa488 emission: 520
	Focus and gains adjusted before measurement, target mP 75	Integration start: 70 µs Integration time: 500 µs
	Top optic	Top optic
General settings	10 flashes (flash lamp)	10 flashes (flash lamp)
	0.1 s settling time	0.1 s settling time
Measure-ment mode	Kinetic parameters: Plate mode, 60 s cycle time, 240 cycle number or endpoint	Endpoint measurement
Shaking	5 s before first cycle, double orbital 500 rpm	5 s before first cycle, double orbital 500 rpm

Results & Discussion

The PT22 fluorophore with a comparatively long fluorescence lifetime of 22 ns was employed to monitor the proteolytic activity of CSN5 resulting in the release of PT22-NEDD8 from the Cul4A ubiquitin ligase. The proteolytic activity results in a decrease of the FP value. With increasing concentrations of CSN5, the FP value decreased more rapidly. The overall assay window of 130 mP is sufficient for both, screening and subsequent inhibitor validation and characterization activities[2].

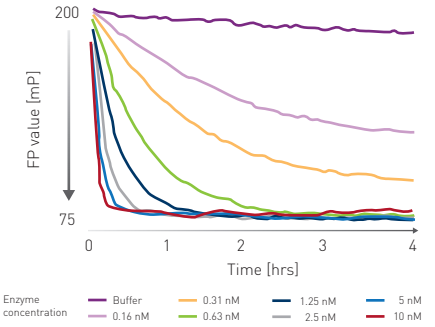


Fig. 3: Progress curves for the de-neddylating activity of CSN5 at different concentrations.

When used for hit finding and during the hit-lead and lead-optimization phase, the FP assay performed well compared to a previously established TR-FRET activity assay (Fig 4). The TR-FRET assay employed a Tb chelate introduced via streptavidin-biotin system and an Alexa488 fluorophore bound to the Nedd8 corresponding donor-acceptor pair. We witnessed that many highly potent CSN5 small molecule inhibitors tested during the later stage of the drug discovery project interfered with the TR-FRET but not with the FP assay readout.

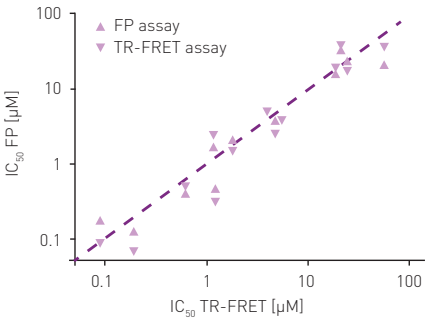


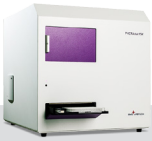
Fig. 4: Correlation plot of IC_{50} values of selected CSN5 inhibitors determined by the FP and TR-FRET assays. FP and TR-FRET measurements were done 60 and 40 min after substrate addition, respectively.

Conclusion

The fluorescence polarization assay presented here allows to screen for inhibitors of the CSN5, a metalloprotease regulating the activity of ubiquitin ligases and implicated in cancer. The assay window was optimized by choosing an FP probe with a remarkable long fluorescence lifetime, because the FP value depends on both, the molecular mass of the protein and the fluorescence lifetime of the probe. The high sensitivity of the PHERAstar reader together with the simultaneous detection of both emission channels enabled for fast FP measurements. Consequently, this allowed for running the assay in a continuous manner, i.e., without quenching the protease activity prior to plate reading, and hence for a lean and economic workflow in HT screening and inhibitor characterization.

References

1. A. Schlierf *et al.* (2016) *Nat. Commun.* **2016; 7**: 13166.
2. J. Woelcke and U Hassiepen (2009) *CRC Press* 2009, Taosheng Chen



PHERAstar® *FSX*

*The PHERAstar *FSX* is the newest PHERAstar reader.