Critical use of HTRF technologies at multiple steps in the drug discovery of deubiquitylating enzyme inhibitors

**Introduction**

The ubiquitin proteasome system (UPS) has emerged as a growing source for novel anti-cancer therapeutics. Inhibitors of a number of enzymes in the UPS have been successfully translated into clinical development (e.g., Proteasome, E1, E3, and p97). However, to date, deubiquitylating enzymes (DUBs) lag behind in terms of progress. MISSION therapeutics has identified a number of DUBs as attractive targets for a several therapeutic areas, including oncology and neurodegeneration.

A broad drug discovery platform combining unique biochemical, cellular, biophysical and structural assays has been developed at MISSION to identify and optimise potent and selective DUB inhibitors. Homogenous time-resolved fluorescence (HTRF) technologies play a central role in the MISSION drug discovery program. Assays were designed at various stages of the screening cascade, from initial hit identification in biochemical assays, to cellular target engagement and finally to target engagement in animal tissues.

HTRF was benchmarked against alternative technologies such as fluorescence intensity, fluorescence polarization and luminescence in biochemical assays. High throughput cellular assays were designed for over 20 DUBs and have demonstrated critical data for both target engagement, selectivity and reversibility in hit validation and lead optimisation phases. Biomarker assays were also used to assess and monitor pharmacodynamic pathways modulated by various DUB targets. The challenges and advances in using HTRF technologies to demonstrate DUB target engagement in early drug discovery as well as in pre-clinical evaluation are also presented here.

**MISSION: targeting DUBs in disease**

- DUBs involved in the DNA damage response for synthetic lethality applications
- DUBs involved in tumour resistance to ‘standard-of-care’ e.g. platinums
- Oncogene DUBs driving tumorigenesis
- Non-oncology DUBs with high disease linkages (e.g. infection, neurodegeneration)

**Biochemical assays**

- Biochemical assays are used as a primary screen to identify DUB inhibitors
- Substrates preferentially contain ubiquitin linked via an isopeptide bond to a fluorescent label
- Compounds with sub μM IC₅₀ have been identified for > 15 DUB targets
- Biochemical assays are used to investigate the selectivity of compounds

**On-target cellular assays for exogenous DUBs**

- On-target cellular activity: principles of the activity probe assay
  - Good correlation between WB and HTRF AP assays
  - Up to 80 IC₅₀ per day
  - Less expensive than WB
  - >20 HTRF assays developed so far
- Comparison between HTRF and FP IC₅₀ for FLAG-UCHL1
  - Good correlation between FP and HTRF AP assays for UCHL1
  - Target accessibility, cell permeability and toxicity are tested simultaneously
  - Vital information for hit to lead optimisation
  - Allosteric inhibitors can be identified
- Reversibility of compounds for FLAG-USP30ΔN
  - HTRF AP assays can be used to assess compound reversibility
  - Allows the rapid progression of compounds with the desired properties
- Validation of single point screening campaigns for FLAG-USP11 and FLAG-USP2A
  - Cellular HTRF AP assays can be used for single-point screening
  - Fewer false positives and non desirable compounds (e.g. fluorescent, toxic or non cell permeable) identified
  - Comparison between biochemical IC₅₀ and cellular HTRF AP SPS are shown on the left for two DUB targets

**On-target cellular assays for endogenous DUBs**

- HTRF AP assays were used to monitor target engagement of endogenous DUBs in cancer cells and mouse tissues
- HTRF AP assays can be used for assessment of target engagement in bioprisms from PK/PD/efficacy animal studies
- Such assays open the possibility to confirm target engagement in patient samples

**Conclusion**

HTRF assays have been successfully integrated into the MISSION Therapeutics technology platform at all stages of the drug-discovery process. These assays have provided critical information about initial hits, target engagement in cells and in animal tissues. HTRF technology has proved to be vital for decision-making processes for the design-make-test cycle in the drug discovery of DUB inhibitors.