Establishment of Assay Systems for Drug Development Against the Oncogenic Phosphatase WIP1

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Introduction

The serine/threonine phosphatase WIP1 (PPM1D) is a key negative regulator of the DNA damage response (DDR, see below Fig.1) and a recognized oncogene, frequently amplified or truncated in various cancers including breast, ovarian, neuroblastoma, and glioblastoma. Its inhibition restores DDR signaling, offering a promising therapeutic strategy. Despite initial efforts, including the development of the allosteric inhibitor GSK2830371 (see Fig.2), clinical translation has been limited.

We report the establishment of a comprehensive assay platform to support drug development targeting WIP1. Biochemical assays were optimized to finally use fluorescein diphosphate (FDP) for enabling robust activity profiling. Cellular mechanism-of-action assays were developed to monitor phosphorylation changes in WIP1 substrates, with pS15-p53 ELISA in U2OS cells selected for medium-throughput iterative compound profiling. Phenotypic assays demonstrated compound efficacy in 2D and 3D proliferation models, with leukemic cell lines showing pronounced sensitivity.

Combination studies revealed strong synergy between WIP1 inhibitors and MDM2 antagonists (e.g., Nutlin3a), supporting a dual-targeting approach for p53 pathway reactivation.

Assay systems were successfully applied to identify WIP1 inhibitor ANV0175, which also proved active in vivo in an neuroblastoma hollow fiber assay as reported for GSK2830371.

Fig.1: WIP1: nullifies p53 tumor suppressor function

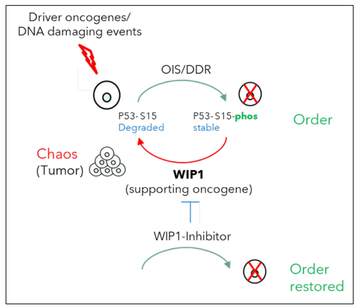
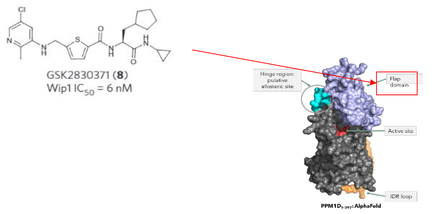


Fig.2: Benchmark inhibitor GSK2830371 inhibits WIP1 through allosteric inhibition



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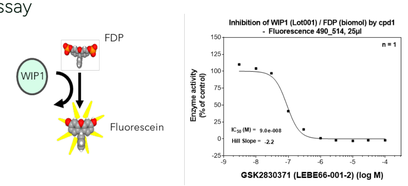
Results (Biochemical Assays)

Different approaches were tested to analyze WIP1 activity

1. Fluorescein Diphosphate (FDP)
2. Malachite green with p53 H2AX, p38-MAPK phosphopeptides
3. TR-FRET assay for substrate pT180-P38 MAPK

Fig.3: FDP WIP1 assay: was chosen as 1. line activity assay



Method:
The assay uses fluorescein diphosphate (FDP) as a substrate. FDP is a non-fluorescent molecule. WIP1 dephosphorylates FDP converting it into fluorescein monophosphate (FMP) and eventually into fluorescein. Recombinant WIP1 was provided by ZoBio, Leiden, NL.

Results (Cellular MoA Assays)

Cellular effects of WIP1 inhibition using reference inhibitor GSK283937:

1. Phosphorylation status of direct WIP1 substrates:
   1. P53 -> pS15
   2. H2AX -> pS139
   3. p38 MAPK -> pT180/Y182
2. In different cells: MCF-7, U2OS
3. Detection: Western blotting

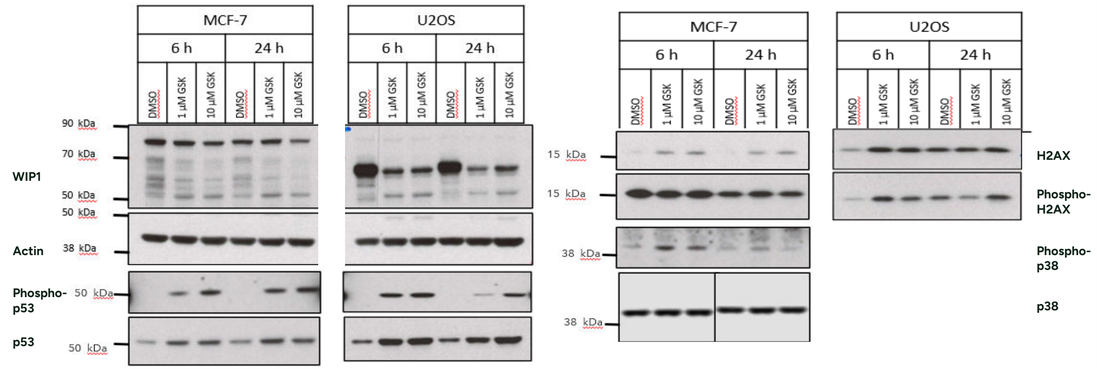
Both cell lines responded with conc-dependent increase of phosphorylation of all substrates analyzed, but also total protein was affected. Most robust effects were observed with U2OS and p53-pS15.

Method:

Indicated cells were seeded and next day treated with indicated concentration and duration.

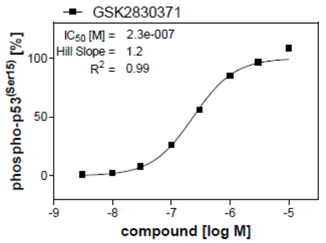
Cells were lysed, lysates separated by SDS-PAGE, blotted and detected with specific antibodies (CST).

Fig.4: Focus on P53-pS15 in U2OS chosen as best candidate for ELISA set up



Results (Cellular ELISA)

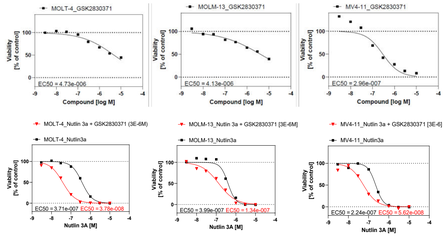
Fig.5: A p53-pS15 ELISA with U2OS lysates for high throughput analysis of WIP1 inhibitors



Method:
Cells were seeded and next day treated with indicated concentrations for 90 min, lysed and lysates analyzed by ELISA (CST) in highly reproducible manner. The IC50 was determined using the sigmoidal slope regression in the software GraphPad Prism. Activity observed for GSK2830371 corresponded well to biochemical and literature data.

Results (Proliferation)

Fig.6: WIP1 inhibition reduces leukemic cells growth, and potentiates activity of mdm2-Inhibitor Nutlin 3a



Method:
Cells were seeded and treated with GSK2830371alone for 7 days (A.) or with Nutlin 3a +/- 1E-6M GSK for 3 days (B.) before CellTiterGlo (Promega) cell viability analysis.

Application: Identification of WIP1 Inhibitor AN00175

Cyclic screening performed with the assay suite:

Start material: Compounds selected on SPR-binding data

- Frequency: biweekly
- Assays
  - 1x Biochemical WIP1 activity assay
  - 1x Cellular WIP1 phosphor S15-p53 ELISA
  - 1x Cellular MV4;11 proliferation
- Assay format: IC50 determination
  - No of cpds up to 28
  - No. of cycles 9

Lead Compound profile (AN00175):

- Allosteric inhibitor
- IC50 in biochemical assay (~50 nM)
- Cellular efficacy IC50 phospho-S15-p53 (< 200 nM)

Lead compound efficacy

- Combination studies show strong synergy with:
  - MDM2 inhibitors
  - Inhibitors of the p38/MAPK pathway
  - Drugs targeting the DDR pathway
  - Chemotherapeutics
- Successful in vivo PoC studies in combination with DNA damage inducing chemotherapy
- In vivo PoC in neuroblastoma hollow fiber model (see below)

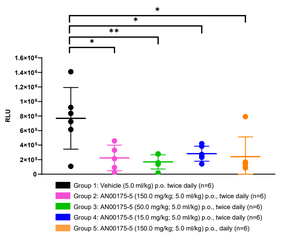
Lead compound status

- No off-target activity against related phosphatases and broader panel
- Modulate downstream targets of PPM1D (e.g., CHK1/2, p53)
- Initial biomarkers identified (e.g. p21)
- CEREP panel: no flags
- Well tolerated up to 150mg/kg dosing in mice both alone and in combination
- IP filed

In Vivo PoC for AN00175 in Neuroblastoma

Fig.7: ANV00175 not only shows potent activity in the p53-pS15 assay (data not shown) but also on neuroblastoma cell line SHSY5Y in the Hollow fiber assay, an in-Vivo proliferation assay.

Method:
SHSY5Y neuroblastoma cells were inoculated into hollow fibers, and fibers were implanted subcutaneously into NMRI-nude mice. After randomization 3 days post-implantation, the indicated treatment regiment was performed for 15 days, before fibers were explanted and cell numbers determined by CellTiterGlo.
Results are in-line with those reported for the GSK2830371 Lit: Sci Rep.2016 dec;19(6):38011



Summary

We have successfully established an integrated assay suite for the development of WIP1 phosphatase inhibitors, on biochemical, cellular, and in vivo level.

Application of these methodologies enabled us to provide critical support of client driven drug development with iterative screening and lead optimization, culminating in the identification of a novel potent allosteric WIP1 inhibitor with nanomolar activity and promising preclinical efficacy. These findings support WIP1 as a viable target in oncology and provide a foundation for future clinical development.

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