Detailed mechanistic understanding of ACR-2316, a novel, clinical-stage WEE1/PKMYT1 inhibitor, rationally designed for superior single-agent activity through potent activation of CDK1, CDK2, and PLK1 using Acrivon's Generative Phosphoproteomics AP3 platform

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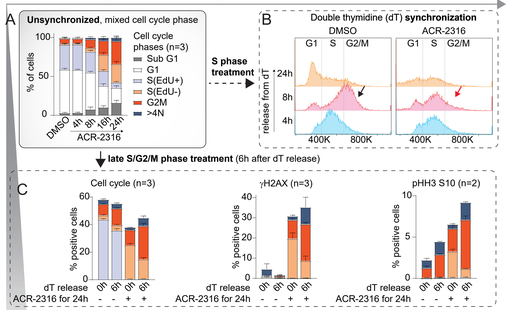
INTRODUCTION

WEE1 and PKMYT1 are essential kinases that safeguard genomic integrity by regulating the cyclin-dependent kinases CDK1 and CDK2. ACR-2316 is a potent, selective WEE1/PKMYT1 inhibitor rationally designed using Acrivon's proprietary Generative Phosphoproteomics-driven precision medicine platform (Acrivon Predictive Precision Proteomics, or AP3) and advanced from initial lead to clinical dosing in less than 15 months. ACR-2316 was uniquely designed, optimized, and advanced by AP3 to deliver complete tumor regression and pro-apoptotic tumor cell death through potent activation of the desired downstream signaling pathways, specifically not only of CDK1 and CDK2, but also PLK1 to ensure efficient mitotic catastrophe. The role of on-target CDK1/2 activation in mediating sensitivity to ACR-2316 was validated by CDK1/2 inhibitor (CDK1/2i)-mediated reversal of drug efficacy and the DNA damage phenotype. We leveraged AP3 Generative Phosphoproteomics, to map and quantify ACR-2316-regulated CDK1/2 substrates providing an unbiased understanding of drug action. The identified, dose-dependently regulated phospho-sites included key mediators of DNA damage sensing and repair, which were verified *in vivo* in human cancer cell derived xenograft models, and were reversed by CDK1/2i. In concordance with this DNA damage response, double-strand break (DSB) mapping via chromatin immunoprecipitation sequencing (ChIP-seq) identified unique intronic regions which were susceptible to ACR-2316-induced DSBs, and multiplexed immunofluorescence revealed a large ACR-2316-induced increase in the number of mitotic aberrations in human cancer cells, compared to control or single-agent WEE1 or PKMYT1 inhibitors alone.

These results demonstrate how Acrivon's AP3 Generative Phosphoproteomics platform can, in a completely unbiased manner, reveal signaling pathways associated with drug efficacy, and mechanistically support the superior potency and activity of ACR-2316 which we observe in comparison to current clinical WEE1 or PKMYT1 inhibitors.

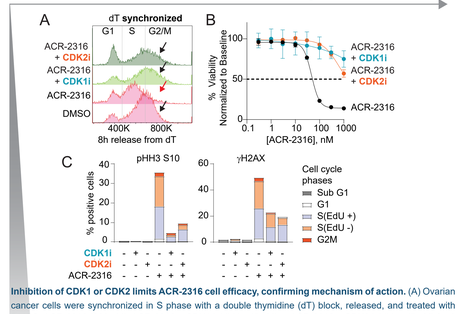
DNA DAMAGE, MITOTIC ABERRATION AND DEATH OF CANCER CELL LINES BY ACR-2316 IS DEPENDENT ON CDK1/2 ACTIVITY

ACR-2316 arrests cancer cells in S and G2/M phase, promoting DNA damage and premature mitotic entry



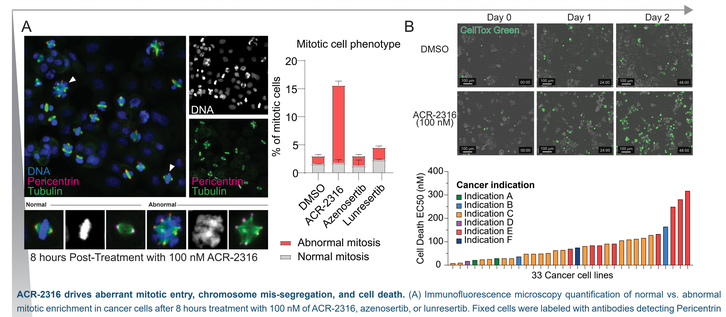
(A) Unsynchronized ovarian cancer cells were treated with DMSO or ACR-2316 (200 nM), and cell cycle distribution was evaluated by flow cytometry using propidium iodide (PI) and 5-ethynyl-2'-deoxyuridine (EdU) staining. (B) Ovarian cancer cells were synchronized in S phase using a double thymidine (dT) block, released and treated with DMSO or ACR-2316 (200 nM) for 4, 8 or 24 hr before PI staining. (C) dT-synchronized ovarian cancer cells were released for 6 hours, treated with DMSO or ACR-2316 (200 nM), and analyzed by flow cytometry for cell cycle distribution (PI and EdU), DNA damage (γH2AX) or mitotic progression (pHH3 S10).

CDK1 and CDK2 mediate most of ACR-2316 activity



Inhibition of CDK1 or CDK2 limits ACR-2316 cell efficacy, confirming mechanism of action. (A) Ovarian cancer cells were synchronized in S phase with a double thymidine (dT) block, released, and treated with DMSO, ACR-2316 (200 nM), ACR-2316 + CDK1 inhibitor (5 μM) or ACR-2316 + CDK2 inhibitor (5 μM) for 8 hours and evaluated by flow cytometry using PI staining. (B) Ovarian cancer cells were treated with DMSO, ACR-2316 (as shown), ACR-2316 + CDK1 inhibitor (5 μM) or ACR-2316 + CDK2 inhibitor (5 μM) for 6 days and cell viability was assessed by CellTiterGlo assay. (C) Unsynchronized ovarian cancer cells were treated with DMSO, ACR-2316 (200 nM), ACR-2316 + CDK1 inhibitor (5 μM) or ACR-2316 + CDK2 inhibitor (5 μM) for 4 hours, followed by flow cytometry analysis for cell cycle (PI + EdU), pHH3 S10 or γH2AX.

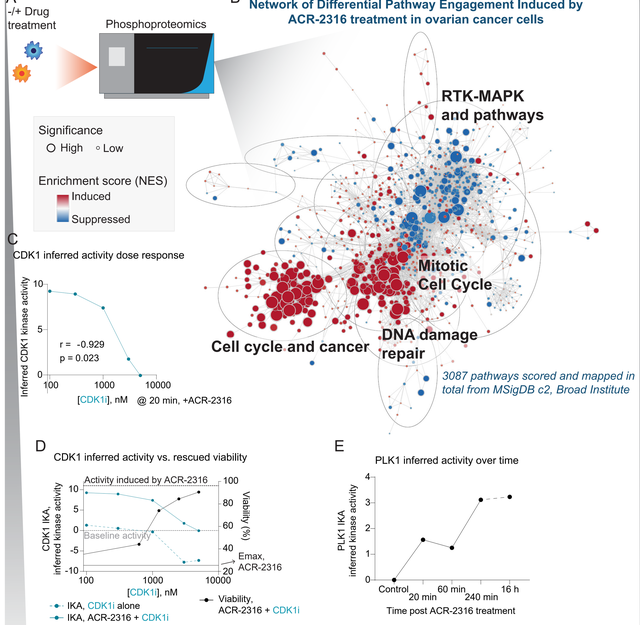
Defective DNA replication and premature mitotic entry induced by ACR-2316 result in aberrant mitosis and cancer cell death across cell lines and indications



ACR-2316 drives aberrant mitotic entry, chromosome mis-segregation, and cell death. (A) Immunofluorescence microscopy quantification of normal vs. abnormal mitotic enrichment in cancer cells after 8 hours treatment with 100 nM of ACR-2316, azenosertib, or lunresertib. Fixed cells were labeled with antibodies detecting Pericentrin (Magenta) and Tubulin (Green), and were stained with DAPI (Blue) to visualize DNA. (B) Upper panel: live cell imaging using CellTox Green cytotoxicity dye shows cell death initiation within 1 day of ACR-2316 treatment and near-complete loss of viability by Day 2. Lower panel: cell lines representing various cancer types were stained with live/dead cell dyes to enable live cell imaging. Cells were treated with increasing concentrations of ACR-2316 and cell growth was monitored every 4 hours for 72 hours. A summary of ranked potencies is shown.

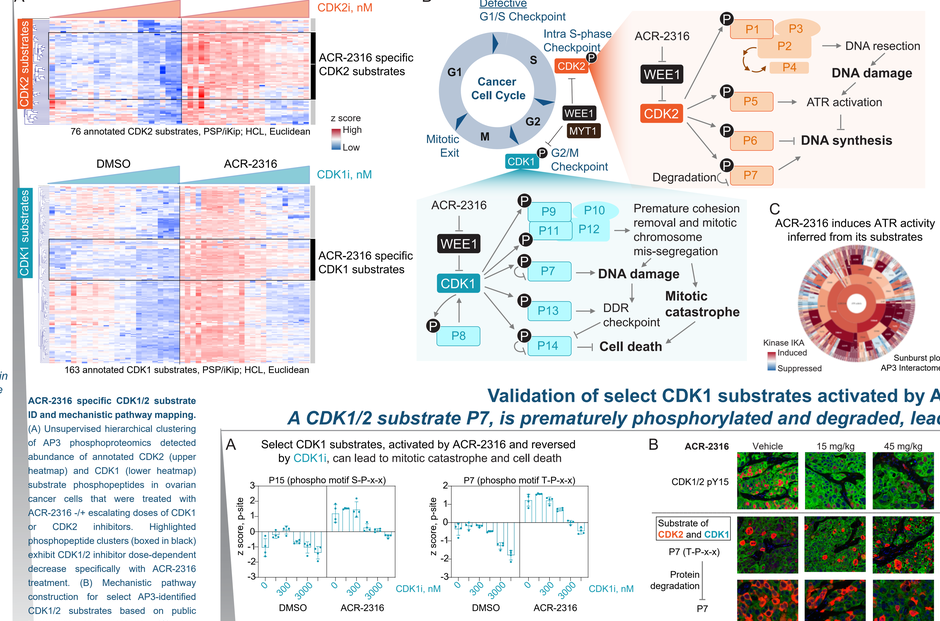
ACRIVON'S PREDICTIVE PRECISION PROTEOMICS PLATFORM IDENTIFIES ACR-2316 EFFICACY-ASSOCIATED SUBSTRATES OF CDK1/2 KINASES

Acrivon's Predictive Precision Proteomics (AP3) unbiasedly identifies signaling pathways associated with ACR-2316 efficacy



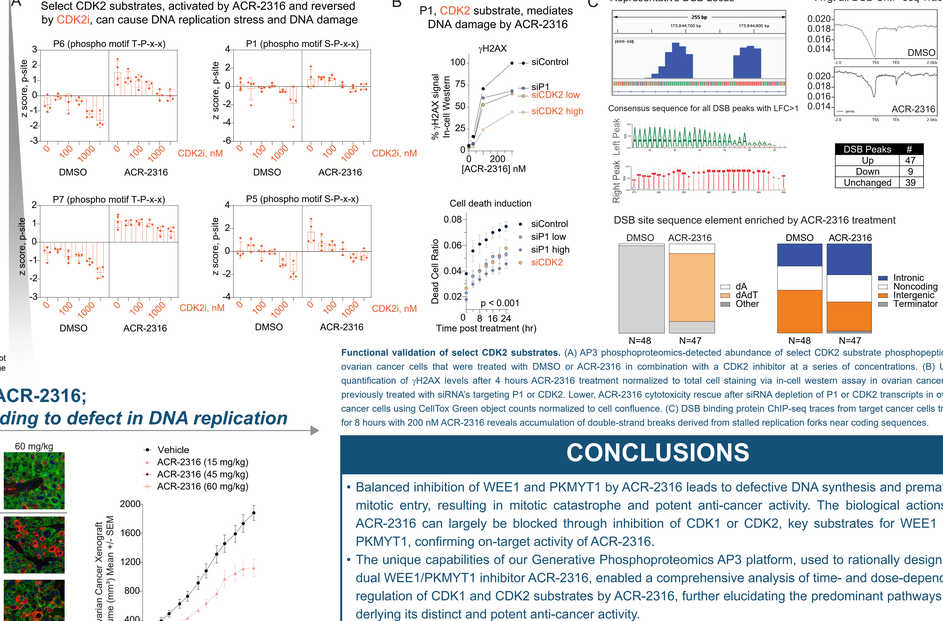
ACR-2316 treatment enriches for cell cycle and mitotic pathway activation that are in turn altered by CDK1/2 inhibitor. (A) Simplified workflow of AP3 drug-tailored phosphoproteomics profiling. (B) ACR-2316 induced phosphorylation pathway activity relating to cell cycle and mitotic pathways. The AP3 derived phosphoproteomes of ACR-2316 vs. DMSO treated human ovarian cancer cells were analyzed via dose dependency (20-200-1000 nM), pathway enrichment, and network construction. (C-E) Kinase activity inference (IKA) was derived from phosphoproteomics with a statistical method leveraging publicly annotated kinase substrates across hundreds of kinases. (C) CDK1 kinase activity inference across a series of CDK1i doses in combination with ACR-2316 treatment. (D) Overlay of CDK1 inferred kinase activity and cell viability across series of CDK1i doses -/+ ACR-2316. (E) PLK1 inferred kinase activity at 20 min, 60 min, 240 min, and 16 h post ACR-2316 treatment.

AP3 phosphoproteomics reveals CDK1 and CDK2 substrates responsible for ACR-2316 activity, enabling mechanistic pathway construction



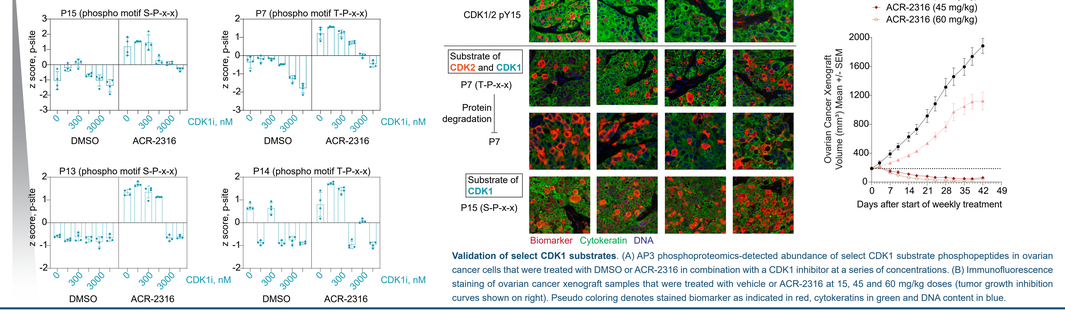
ACR-2316 specific CDK1/2 substrate ID and mechanistic pathway mapping. (A) Unsupervised hierarchical clustering of AP3 phosphoproteomics detected abundance of annotated CDK2 (upper heatmap) and CDK1 (lower heatmap) substrate phosphopeptides in ovarian cancer cells that were treated with ACR-2316 -/+ escalating doses of CDK1 or CDK2 inhibitors. Highlighted phosphopeptide clusters (boxed in black) exhibit CDK1/2 inhibitor dose-dependent decrease specifically with ACR-2316 treatment. (B) Mechanistic pathway construction for select AP3-identified CDK1/2 substrates based on public knowledge and literature. (C) ATR inferred kinase activity regulated by ACR-2316 treatment across Acrivon proprietary AP3 experiments. Activity inference derives from a statistical method leveraging publicly annotated substrates.

Validation of select CDK2 substrates activated by ACR-2316; *Activation of the CDK2 substrates by ACR-2316 provokes over-resection and DNA damage*



Functional validation of select CDK2 substrates. (A) AP3 phosphoproteomics-detected abundance of select CDK2 substrate phosphopeptides in ovarian cancer cells that were treated with DMSO or ACR-2316 in combination with a CDK2 inhibitor at a series of concentrations. (B) Upper, quantification of γH2AX levels after 4 hours ACR-2316 treatment normalized to total cell staining via in-cell western assay in ovarian cancer cells previously treated with siRNA's targeting P1 or CDK2. Lower, ACR-2316 cytotoxicity rescue after siRNA depletion of P1 or CDK2 transcripts in ovarian cancer cells using CellTox Green object counts normalized to cell confluence. (C) DSB binding protein ChIP-seq traces from target cancer cells treated for 8 hours with 200 nM ACR-2316 reveals accumulation of double-strand breaks derived from stalled replication forks near coding sequences.

Validation of select CDK1 substrates activated by ACR-2316; *A CDK1/2 substrate P7, is prematurely phosphorylated and degraded, leading to defect in DNA replication*



Validation of select CDK1 substrates. (A) AP3 phosphoproteomics-detected abundance of select CDK1 substrate phosphopeptides in ovarian cancer cells that were treated with DMSO or ACR-2316 in combination with a CDK1 inhibitor at a series of concentrations. (B) Immunofluorescence staining of ovarian cancer xenograft samples that were treated with vehicle or ACR-2316 at 15, 45 and 60 mg/kg doses (tumor growth inhibition curves shown on right). Pseudo coloring denotes stained biomarker as indicated in red, cytokeratins in green and DNA content in blue.

CONCLUSIONS

- Balanced inhibition of WEE1 and PKMYT1 by ACR-2316 leads to defective DNA synthesis and premature mitotic entry, resulting in mitotic catastrophe and potent anti-cancer activity. The biological actions of ACR-2316 can largely be blocked through inhibition of CDK1 or CDK2, key substrates for WEE1 and PKMYT1, confirming on-target activity of ACR-2316.
- The unique capabilities of our Generative Phosphoproteomics AP3 platform, used to rationally design the dual WEE1/PKMYT1 inhibitor ACR-2316, enabled a comprehensive analysis of time- and dose-dependent regulation of CDK1 and CDK2 substrates by ACR-2316, further elucidating the predominant pathways underlying its distinct and potent anti-cancer activity.
- ACR-2316 has advanced into the clinic significantly ahead of schedule and is currently in a Phase 1 clinical trial in subjects with selected high unmet need solid tumor types predicted sensitive to ACR-2316 using AP3-based indication finding.
- Early data from the first two dose levels show approximate dose proportionality based on pharmacokinetic analyses and drug target engagement in human PBMCs using Acrivon's clinical mass spectrometry-based AP3 profiling. Finally, clinical activity with significant tumor shrinkage and shrinkage of metastatic lesions has been observed already at dose level 3, which is 2 dose levels lower than the anticipated MTD.
- Acrivon anticipates reporting initial ACR-2316 clinical Phase 1 data in the second half of 2025.