FEN1 nuclease inhibitor using a novel metal-binding pharmacophore synergizes with PARP, USP1, PARG, ATR inhibitors

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Abstract

Flap endonuclease 1 (FEN1) is a structure-specific metallonuclease that cleaves 5' DNA flaps during replication and repair. FEN1 is an attractive target for development of anticancer therapeutics because it is overexpressed in many tumor types and has a large number of synthetic lethality partners including PARP and genes in Homologous Recombination (HR) pathway.

Utilizing an innovative library of metal-binding pharmacophores (MBPs) and a fragment-based drug discovery approach we identified a novel FEN1-selective chemical scaffold represented by BSM-1516 (IC50 of 7 nM and 460 nM in biochemical assays for FEN1 and EXO1, respectively; FEN1 cellular thermal shift target engagement assay EC_{50} of 24 nM).

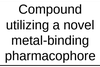
In clonogenic assay, BRCA2-deficient DLD1 cells were ~15-fold more sensitive to FEN1 inhibition than their isogenic BRCA2-wild-type counterparts (EC_{50} of 350 nM and 5 μM, respectively), showed G2/M phase cell cycle arrest and accumulated chromatin-bound RPA32, a marker of ssDNA.

Examination of proteins on replication forks by iPOND-SILAC-MS in the presence of BSM-1516 revealed rapid enrichment of Okazaki fragment maturation (OFM) proteins FEN1/PCNA/LIG1, PARP1/2 enzymes, poly(ADP-ribose) binders (e.g. CHD1L) and alternative OFM pathway repair proteins XRCC1 and LIG3.

FEN1-inhibitor-anchored CRISPR screen revealed that in addition to HR pathway inactivation, genomic perturbations in EXO1, USP1 and PARP1 genes sensitized cells to FEN1 inhibition. Combination of BSM-1516 with inhibitors of USP1, PARP1/2, PARG and ATR was strongly synergistic in vitro. BSM-1516 enhanced antiproliferative effect of PARP1/2 inhibitors by up to 100-fold (an effect not observed in normal fibroblasts) and led to robust activation of ssDNA break repair markers: phospho-RPA2(Ser33) and chromatin-bound RPA2.

In vivo PK studies showed that BSM-1516 had oral bioavailability of 40% and $T_{1/2}$ of 2.9 hr in mice. Safety of BSM-1516 was assessed in vivo in mice at a daily dose of 120 mg/kg PO and 90 mg/kg IP qd for 7 days and revealed no signs of hematological toxicity. These collective data support further in vivo testing in PD and efficacy studies either as a single agent or in combination with PARPi.

Biochemical assays: Improved potency and greater selectivity of BSM-1516 compared to historic FEN1 inhibitors

Company	Structure	ID	FEN1 IC_{50} (μM)	EXO1 IC_{50} (μM)	FEN1 vs. EXO1 selectivity (fold)
Astra Zeneca		Cmpd 4	0.21	0.72	3.4
Athersys		Cmpd 8	0.13	0.41	3.2
Ideaya		Cmpd 12	2.7	14	5.2
Blacksmith Medicines	Compound utilizing a novel metal-binding pharmacophore	BSM-1516	0.007	0.46	65

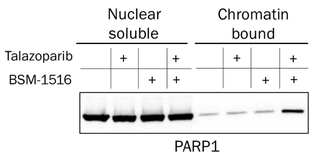
Potency of BSM-1516 and reference compounds against FEN1 and EXO1 (members of RAD2 family of structure-specific metallonucleases) was measured in enzymatic assays. These fluorescence-based assays were adapted from van Pel et al., PLOS Genetics 2013 and utilized a flap-containing synthetic substrate carrying a fluorophore on the 5' flap and a quencher at the opposite end of the DNA oligonucleotide.

Cellular Target Engagement and Mechanism of Action

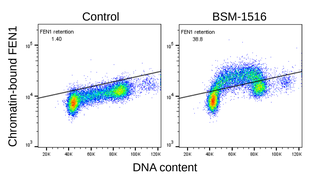
BSM-1516 stabilizes FEN1 and increases its chromatin retention in S phase accompanied by Talazoparib-mediated PARP1 trapping

Company	ID	CETSA EC_{50} (μM)
Astra Zeneca	Cmpd 4	1.2
Athersys	Cmpd 8	0.21
Ideaya	Cmpd 12	>100
Blacksmith Medicines	BSM-1516	0.024

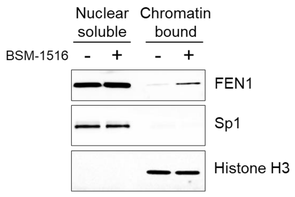
Cellular thermal shift assay (CETSA™) was performed using InCELL Pulse™ target engagement assay (DiscoverX) in stably transfected HEK293 cells expressing FEN1 catalytic domain with C-terminal ePL tag.



Western blot analysis of endogenous chromatin-bound PARP1 in DLD1 cells treated with indicated inhibitors for 3h and then fractionated.

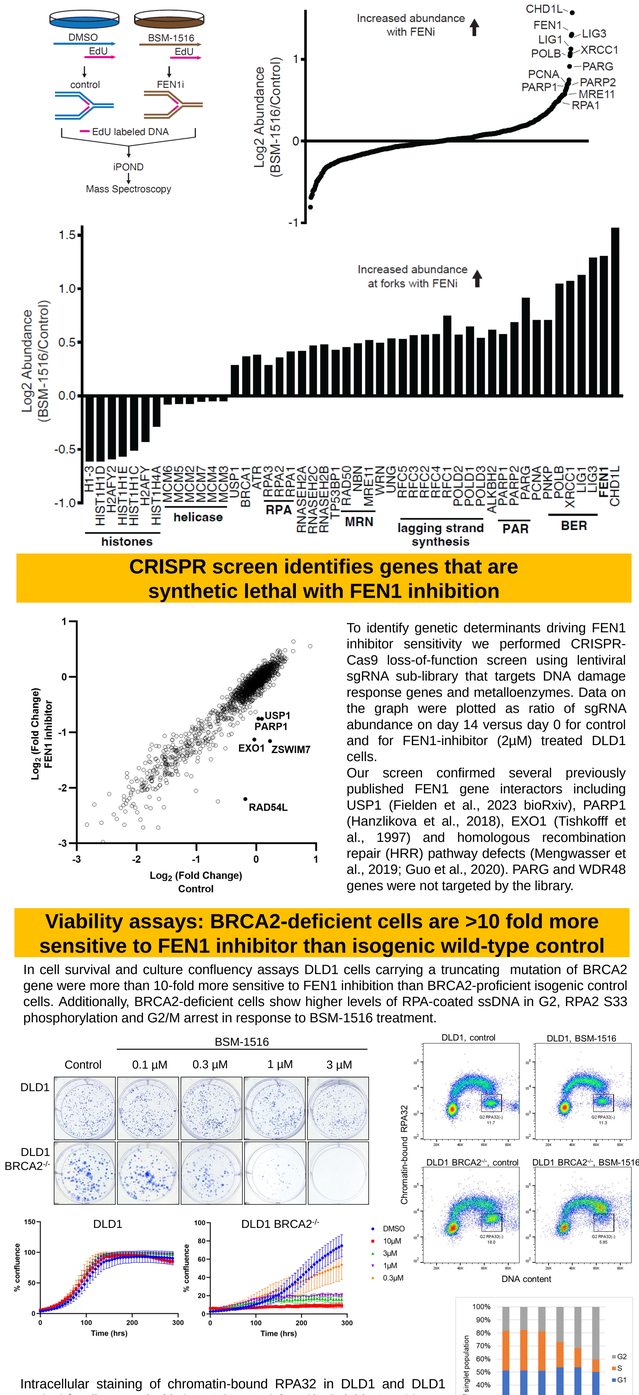


Intracellular staining of endogenous chromatin-bound FEN1 in DLD1 cells treated with 3μM BSM-1516 for 16h. Soluble FEN1 was extracted with detergent prior to fixation and subsequent analysis by flow cytometry.



Western blot analysis of endogenous chromatin-bound FEN1 in S phase. DLD1 cells were arrested by double thymidine block, released for 3h into fresh media with or without 3μM BSM-1516 and then fractionated.

iPOND-SILAC-MS



CRISPR screen identifies genes that are synthetic lethal with FEN1 inhibition

To identify genetic determinants driving FEN1 inhibitor sensitivity we performed CRISPR-Cas9 loss-of-function screen using lentiviral sgRNA sub-library that targets DNA damage response genes and metalloenzymes. Data on the graph were plotted as ratio of sgRNA abundance on day 14 versus day 0 for control and for FEN1-inhibitor (2μM) treated DLD1 cells.

Our screen confirmed several previously published FEN1 gene interactors including USP1 (Fielden et al., 2023 bioRxiv), PARP1 (Hanzlikova et al., 2018), EXO1 (Tishkoff et al., 1997) and homologous recombination repair (HRR) pathway defects (Mengwasser et al., 2019; Guo et al., 2020). PARG and WDR48 genes were not targeted by the library.

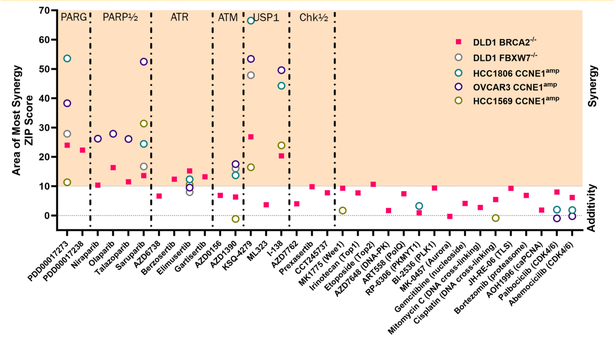
Viability assays: BRCA2-deficient cells are >10 fold more sensitive to FEN1 inhibitor than isogenic wild-type control

In cell survival and culture confluency assays DLD1 cells carrying a truncating mutation of BRCA2 gene were more than 10-fold more sensitive to FEN1 inhibition than BRCA2-proficient isogenic control cells. Additionally, BRCA2-deficient cells show higher levels of RPA-coated ssDNA in G2, RPA2 S33 phosphorylation and G2/M arrest in response to BSM-1516 treatment.

Intracellular staining of chromatin-bound RPA32 in DLD1 and DLD1 BRCA2$^{-/-}$ cells treated with 3μM BSM-1516 for 16h. Soluble RPA32 was extracted with detergent prior to fixation and subsequent analysis by flow cytometry.

Pharmacological inhibition of FEN1 by BSM-1516 resulted in G2/M cell cycle arrest of BRCA2–deficient DLD1 cells.

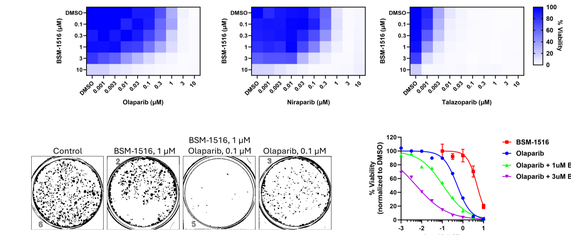
BSM-1516 synergizes with inhibitors of USP1, PARP, PARG, and ATR in BRCA2$^{-/-}$ and CCNE1^{amp} cell models

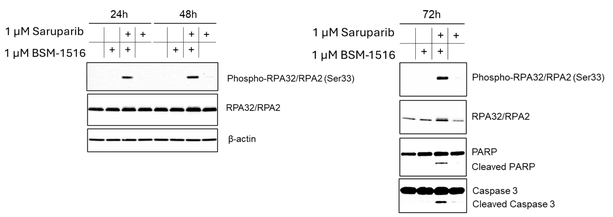


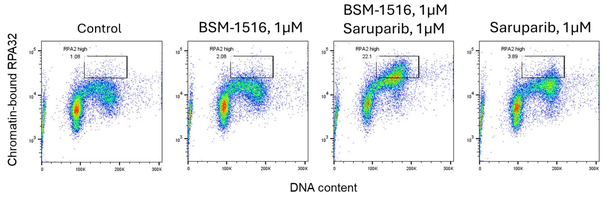
To explore BSM-1516 combination potential we performed combination treatment screen with a panel of DNA damage inhibitors and agents. Cell lines were assayed in 6x10 combination half-log dose-response matrix using CellTiter-Glo® 2.0 Cell Viability Assay with media change every 3 days. Synergy scores were calculated using Synergy Finder.

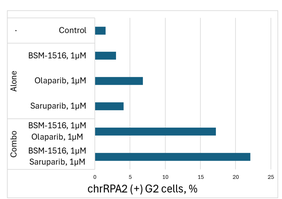
BSM-1516 / PARP inhibitor combination synergy in ovarian CCNE1^{amp} cancer model

The growth inhibition effect by a combination of BSM-1516 with PARP inhibitors was evaluated in high-grade serous ovarian carcinoma (HGSOC) Cyclin E1-amplified cell line NIH:OVCAR3 using CellTiter-Glo® 2.0 Cell Viability and clonogenic cell survival assays. Additionally, dual inhibition of FEN1 and PARP led to robust activation of ssDNA break repair markers: phospho-RPA2(Ser33) and chromatin-bound RPA2.







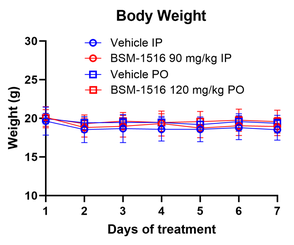


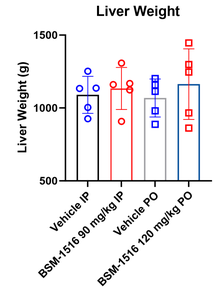
Chromatin-bound RPA2/RPA32 in NIH:OVCAR3 cells treated with BSM-1516, PARP inhibitors alone or in combinations for 24h was analyzed by flow cytometry after detergent extraction, fixation and intracellular staining.

7-day repeat dose tolerability study in mice

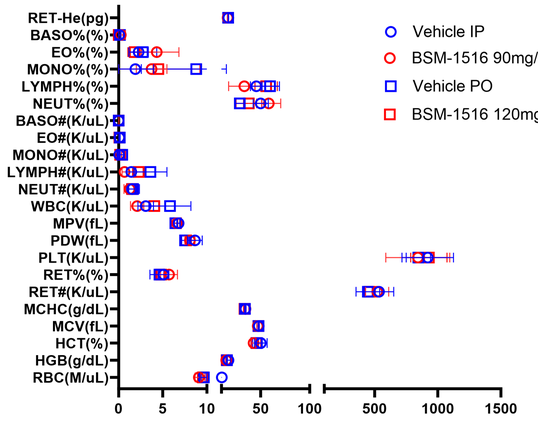
No clinical observations in 7-day tolerability study of BSM-1516.

Biochemistry analyses showed no changes in alanine transaminase, aspartate transaminase, alkaline phosphatase, creatinine and phosphorus; bilirubin was not detected.



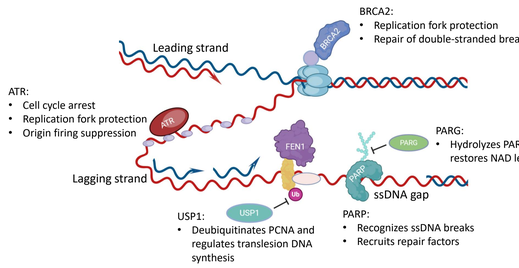


Procyte Analysis



Summary

- Blacksmith Medicines FBDD approach for metalloenzymes identified a series of cell active FEN1-selective inhibitors that use a novel metal-binding pharmacophore.
- Pharmacologic inhibition of FEN1 is synthetic lethal with defects in HRR and synergizes with inhibitors of USP1, PARP, PARG, ATR and Chk1.
- BSM-1516 exhibits favorable *in vivo* PK properties and is well tolerated in mice in a 7-day study.



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