In vitro and *in vivo* Biology Characterization of Two Novel Inhibitors Targeting the Different Domains of DNA Polymerase Theta (Polθ)

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Introduction

DNA polymerase theta (Polθ) is a polymerase A enzyme that mediates DNA repair through microhomology-mediated end joining (MMEJ) and contains both a helicase-like and a polymerase domain.

Polθ is an attractive therapeutic target in BRCA-deficient cancers that have defects in their DNA repair pathways. Despite the development of several Polθ inhibitors, a direct comparison of the biological effects of compounds targeting the different Polθ domains has not yet been reported.

Here, we compare the *in vitro* and *in vivo* biological profiles of two novel Polθ inhibitors generated through a collaboration between Pharmaron and NewBay Pharma: one targeting the DNA polymerase (POL) domain and the other targeting the helicase-like (HEL) domain.

Methods

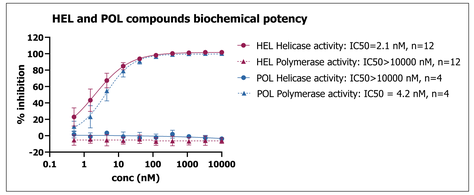
- The two compounds HEL and POL were compared in biochemical assays (ATPase activity and PicoGreen polymerase assays) as well as in a cellular colony formation assay (CFA) using a BRCA2-deficient cell line (DLD1), which are sensitive to disruption of DNA repair.
- The functional effects of compounds HEL and POL were determined using MMEJ and DNA damage assays.
- The synergistic effect of HEL and POL compounds in combination with a PARP inhibitor was tested in a CellTiter-Glo luminescent cell viability assay.
- Compound HEL was further characterized for selectivity, pharmacokinetics (PK), and *in vivo* efficacy.

Conclusion

- Both HEL and POL inhibitors show strong biochemical potency, consistent with domain selectivity.
- In all cellular and functional assays relevant to homologous recombination deficiency (HRD), the HEL inhibitor shows superior potency, greater induction of DNA damage, and stronger synergy with PARP inhibition.
- HEL demonstrates favorable PK, high selectivity, and robust *in vivo* efficacy in BRCA-deficient xenografts.
- Together, these results support preferential targeting of the Polθ helicase-like domain for synthetic-lethal therapeutic strategies.

1

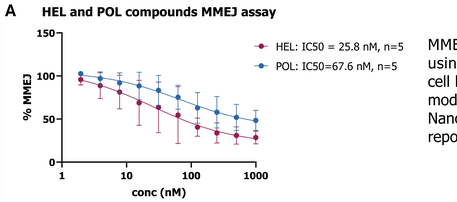
HEL and POL Compounds Exhibit Similar Biochemical but Different Cellular Potencies



Evaluation of the potency of HEL and POL compounds.
(A) HEL and POL compounds exhibit low nanomolar potency in helicase (ATPase) and PicoGreen polymerase activity assays, respectively.
(B) The HEL inhibitor is about 60-fold more potent than POL inhibitor in a cellular CFA assay in DLD1 BRCA2-/- cells, despite similar physicochemical properties.

2

HEL Inhibitor is More Potent in Functional Assays



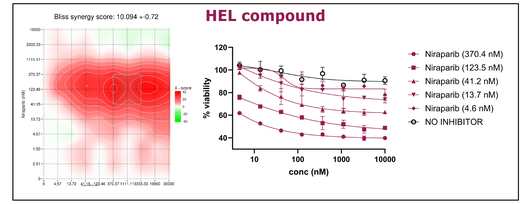
MMEJ was detected using a stable HEK293 cell line expressing a modified version of the NanoLuc luciferase reporter.

DNA damage was detected via quantification of micronuclei formation, a downstream marker of genomic instability in BRCA2-/- cells.
* p <0.05; ** p <0.01

Assessment of MMEJ and DNA damage.
(A)HEL has a 3-fold higher potency than POL in reducing the frequency of MMEJ events.
(B)HEL has a 200-fold higher potency in inducing micronuclei formation compared to POL.

3

HEL Compound Shows Stronger Synergy with PARP Inhibition



Assessment of synergistic effects in BRCA2-/- cells. Cell viability assays in BRCA2-/- cells demonstrate that HEL exhibits markedly higher synergy with Niraparib than POL. Comparable synergy was also observed in BRCA1-deficient MDA-MB-436 cells (data not shown).

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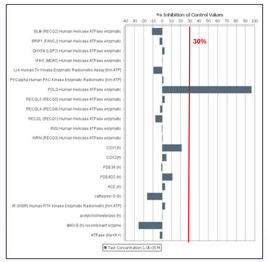
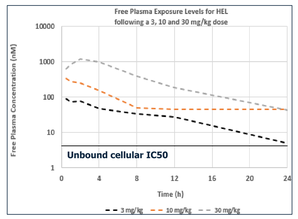
HEL Compound is Highly Selective for Polθ and has a Favorable PK Profile





Safety profile. No off-target activities for compound HEL observed against 22 helicases (Eurofins Discovery) at the tested concentration of 10 uM.

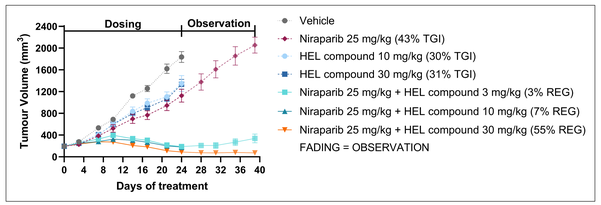
Species	Clearance (mL/min/kg)	Volume (L/kg)	Half-Life (hrs)	Bioavailability (F%)
Mouse	11.8 (9% Liver blood flow)	2.4	2.4	100
Rat	6.7 (9% Liver blood flow)	1.0	3.6	52
Dog	1.8 (3% Liver blood flow)	0.7	5.3	100

PK profile of HEL compound.
- Low clearance in mouse, rat, and dog
- Good oral bioavailability (52–100%)
- Moderate half-life enabling free plasma concentrations to be maintained above cellular IC_{50} for 24 hours at doses > 3 mg/kg.

5

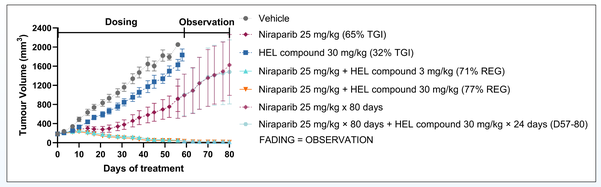
HEL Compound Drives Tumor Regression in BRCA-Deficient Xenograft Models

DLD1 BRCA2 -/-



All compounds dosed PO, daily (QD); No body weight changes observed throughout the study.

MDA-MB-436 BRCA1- deficient



***In vivo* characterization of compound HEL in a DLD1 BRCA2-/- and BRCA1-deficient MDA-MB-436 mouse xenograft models.** 55% tumor regression in DLD1 BRCA2-/- xenografts at 30 mg/kg when combined with Niraparib; 70% tumor regression in MDA-MB-436 BRCA1-deficient xenografts at 3 mg/kg when combined with Niraparib.

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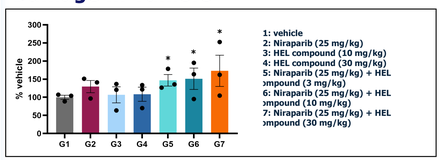
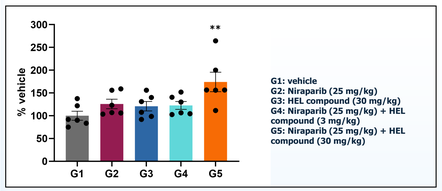
HEL + PARP Inhibition Increases DNA Damage in Tumors



All compounds dosed PO, daily (QD); Dosing conducted for 3 days. * p <0.05; ** p <0.01



Levels of phosphorylated γH2AX, a biomarker for dsDNA breaks in tumor tissues. Combination treatment with HEL and Niraparib significantly elevates phosphorylated γH2AX levels in tumor tissue (relative to G1).