Abstract 2476

Preclinical assessment of the PARP inhibitor rucaparib in homologous recombination deficient prostate cancer models

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BACKGROUND

Rucaparib is a small molecule inhibitor of poly(ADP-ribose) polymerase (PARP)-1, PARP-2 and PARP-3, and was recently approved for the treatment of patients with deleterious *BRCA* mutation (germline and/or somatic) associated advanced ovarian cancer who have been treated with two or more chemotherapies. PARP inhibitors have demonstrated clinical activity in patients with *BRCA1*, *BRCA2*, and *ATM* mutant metastatic castration resistant prostate cancer (mCRPC),[1] and recent studies have demonstrated that greater than 20% of mCRPC patients may have an alteration in an HR repair gene.[2] However, limited preclinical validation of PARP inhibitors in prostate cancer cell lines has been reported to date, since no homozygous *BRCA* mutant nor HR deficient prostate cell lines have been identified. In this study, we modelled the functional inactivation of DNA repair genes using siRNA and CRISPR/Cas9, and examined the impact on PARP inhibition in prostate cancer cell lines.

METHODS AND MATERIALS

- Cytotoxicity assays: 600-1000 cells per well were seeded in 384-well plates. The next day, rucaparib or enzalutamide (MedChem Express) was added and the cells were incubated for 6 days. For androgen receptor (AR) studies, 10% charcoal stripped serum (CSS) with 1 nM DHT was used in phenol red-free media. Cell viability was measured using CellTiter-Glo (Promega).
- siRNA transfections: 0.3×10^6 DU145 and PC-3 cells (ATCC) per well were seeded in 6-well plates. The next day, 25 nM of siRNA (Dharmacon) and DharmaFECT1 reagent (Dharmacon) were added, and cells were used for qRT-PCR and cytotoxicity assays the following day.
- qRT-PCR analysis: RNA was extracted with PureLink RNA kit (Invitrogen), and reverse transcribed with SuperScript III (Invitrogen). qPCR was performed with Taqman Assays (Applied Biosystems) using the ViiA7 Real-Time PCR system (Applied Biosystems).
- *ATM* and *BRCA2* knockout clones: 0.2×10^6 22Rv1 cells (ATCC) per well were seeded in a 6-well plate. The next day, cells were transfected with Fugene 6 (Promega) and 1 µg *ATM* or *BRCA2* CRISPR plasmid (Santa Cruz Biotechnology), and selected with 2 µg/mL puromycin. Single cell clones were generated by limiting dilution and expansion.
- *ATM* and *BRCA2* sequencing: DNA was isolated using a PureLink DNA kit (Invitrogen), and 200 ng DNA was PCR amplified with KOD Master Mix (EMD Millipore) using the manufacturer's recommended parameters. The PCR product was purified using QIAquick PCR Purification kit (Qiagen) and Sanger sequencing was performed (MCLAB).

	PCR Forward Primers	PCR Reverse Primers	Sequencing Primers
ATM	5'- TCGGCCAGAGAACAGTTTATC	5'- TCTTCCCATTGTCACCTGTTC	5'- CTGCTGGTCTGAACCTCTTT
BRCA2	5'- GCAAAGACCCACATTGGAAAG	5'- TGCTTCAAACTGGGCTGAAC	5'- AGCAAACGCTGATGAATGTG

- ATM and BRCA2 immunoblot: RIPA buffer (Sigma) and NE-PER Nuclear Reagents (Pierce) were used to generate cellular and nuclear lysates. Three or 60 µg of nuclear or total protein, respectively, was loaded onto Tris-acetate gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Detection was performed with antibodies for ATM (Abcam), BRCA2 (Millipore), and vinculin (CST). Blots were imaged on the Odyssey Fc (LI-COR Bioscience).
- Immunofluorescence: 4×10^4 cells were seeded in 8-chamber slides, two days later cells were treated with 10 µM rucaparib or DMSO, and the following day stained with RAD51 and γH2AX (Abcam), and Alexa Fluor 488 and 594 (Invitrogen) using methods previously described.[3]

Fig. 1 Rucaparib has limited activity in homologous recombination (HR) proficient prostate cancer cell lines

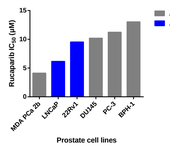
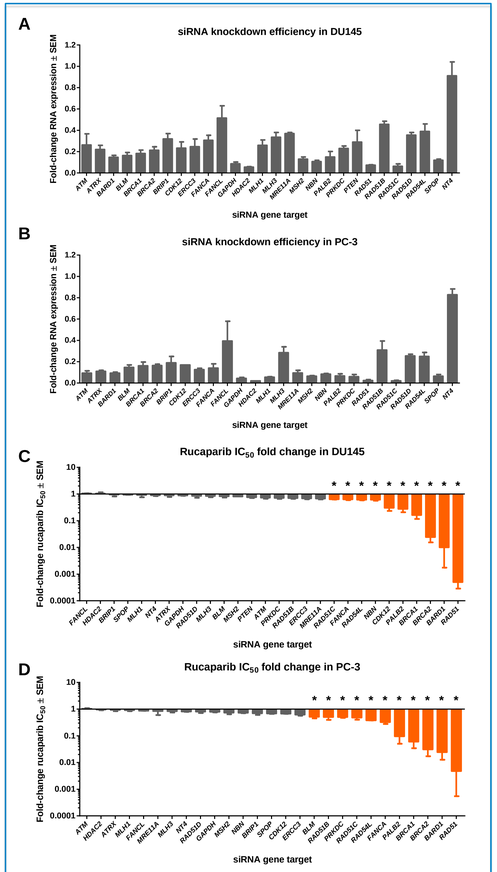


Table 1. Rucaparib IC$_{50}$ in prostate cell lines

Cell line	*BRCA* status	Rucaparib IC$_{50}$ (µM)
MDA PCa 2b	Wild type	4.09
LNCaP	*BRCA2*$^{+/-}$	6.13
22Rv1	*BRCA2*$^{+/-}$	9.50
DU145	Wild type	10.17
PC-3	Wild type	11.34
BPH-1	Wild type	13.00

- Rucaparib has limited activity in commercially available prostate cancer cell lines (Fig.1). Four of these cell lines have wild type *BRCA*, while two are heterozygous *BRCA2* mutated without loss-of-heterozygosity (LOH) of the other allele, thus all of the prostate cell lines tested are HR proficient (Table 1).

Fig. 2 siRNA knockdown of DNA repair genes increases rucaparib cellular cytotoxicity



- siRNA knockdown of DNA repair genes was performed in DU145 and PC-3 cells. Knockdown efficiency was determined by qRT-PCR 24 hours after transfection. RNA levels were expressed at an average of 0.23-fold and 0.13-fold of the levels observed in untransfected DU145 and PC-3, respectively (Fig. 2A, 2B), and scrambled siRNA control (NT4) showed no change.
- Rucaparib cytotoxicity was evaluated in siRNA transfected cells, and the results are reported as fold change in IC$_{50}$ normalized to the untransfected control (Fig. 2C, 2D). The knockdown of several genes, including *BARD1*, *BRCA1*, *BRCA2*, *PALB2*, and *RAD51*, significantly (*p<0.05) increased the sensitivity to rucaparib.

Fig. 3 *BRCA2* knockout increases rucaparib sensitivity in 22Rv1 prostate cancer cell line

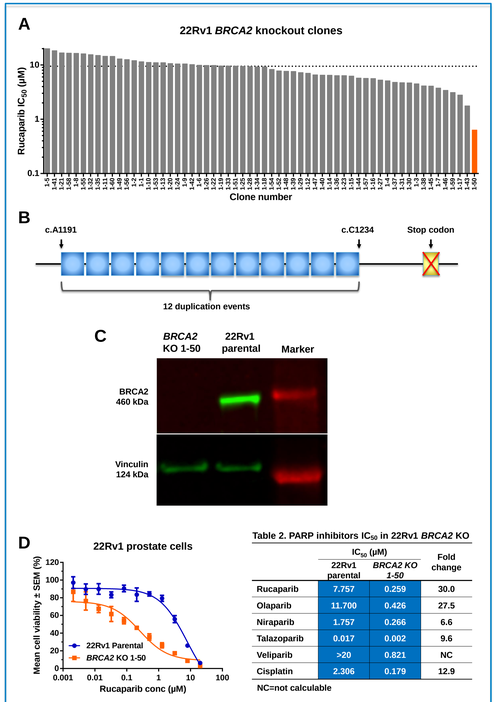


Table 2. PARP inhibitors IC$_{50}$ in 22Rv1 *BRCA2* KO

	IC$_{50}$ (µM)		Fold change
	22Rv1 parental	*BRCA2* KO 1-50	
Rucaparib	7.757	0.259	30.0
Olaparib	11.700	0.426	27.5
Niraparib	1.757	0.266	6.6
Talazoparib	0.017	0.002	9.6
Veliparib	>20	0.821	NC
Cisplatin	2.306	0.179	12.9

NC=not calculable

- CRISPR was used to knockout (KO) *BRCA2* in 22Rv1 PCa cells, a human cell line derived from a xenograft tumor (CWR22) after castration-induced regression and relapse. Clones were screened in a cytotoxicity assay as a functional approach to identify clones with *BRCA2* inactivation. Clone 1-50 was the most sensitive to rucaparib treatment (orange bar) and was selected for further analysis (Fig. 3A).
- Genomic DNA sequencing of clone 1-50 showed 44 nucleotides were replaced with 12 duplication events of differing *BRCA2* exon 10 sequences totaling 297 nucleotides (blue boxes). The mutation c.1191_1234delins297 caused a shift in the reading frame, generating a premature stop codon (yellow box) (Fig. 3B).
- Immunoblot of nuclear enriched lysate demonstrated that clone 1-50 lacked the full-length BRCA2 protein, whereas the 22Rv1 parental had BRCA2 expression (Fig. 3C).
- Cytotoxicity assays verified the initial screening results, confirming an increase in rucaparib potency in the *BRCA2* knock-out clone 1-50 as compared to the parental 22Rv1 cell line. The IC$_{50}$ in the parental and clone 1-50 cells was 7.757 and 0.259 µM, respectively, representing a 30-fold increase in sensitivity to rucaparib in the *BRCA2* knockout cells (Fig. 3D). Similar increases in sensitivity were also observed with cisplatin and other PARP inhibitors, including olaparib, niraparib, talazoparib, and veliparib (Table 2).

Fig. 4 Increased rucaparib activity observed in *ATM* knockout 22Rv1 prostate cancer cell line

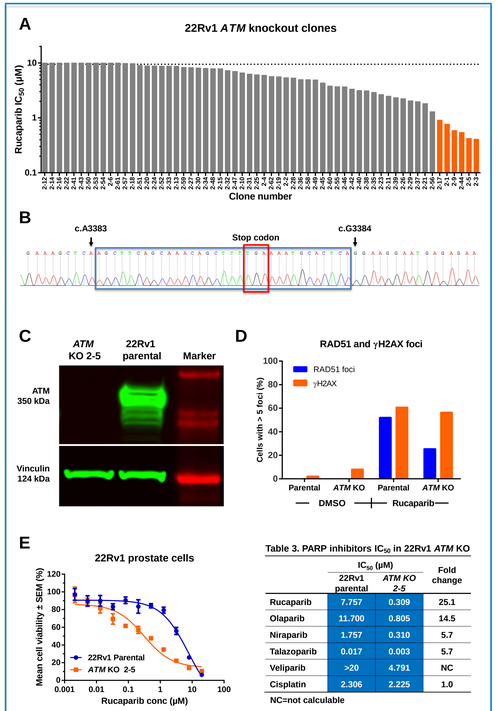


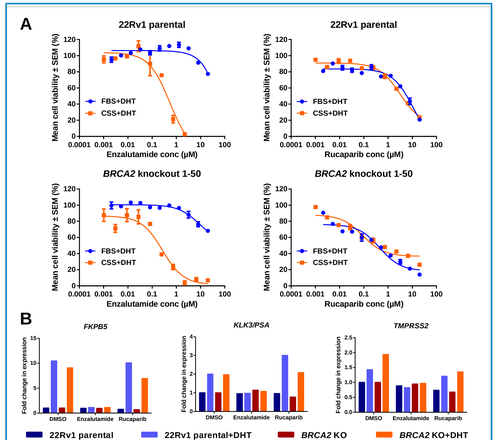
Table 3. PARP inhibitors IC$_{50}$ in 22Rv1 *ATM* KO

	IC$_{50}$ (µM)		Fold change
	22Rv1 parental	*ATM* KO 2-5	
Rucaparib	7.757	0.309	25.1
Olaparib	11.700	0.805	14.5
Niraparib	1.757	0.310	5.7
Talazoparib	0.017	0.003	5.7
Veliparib	>20	4.791	NC
Cisplatin	2.306	2.225	1.0

NC=not calculable

- CRISPR technology was used to knockout *ATM* in 22Rv1 PCa cells. Cytotoxicity assays were used to screen clones as a functional selection to identify clones with *ATM* inactivation. Several clones were observed to have greater sensitivity to rucaparib (orange bars) including clone 2-5, and were selected for further analysis (Fig. 4A).
- Genomic DNA sequencing of *ATM* KO clones with increased sensitivity to rucaparib showed mutations in *ATM* sequences. However, only clone 2-5 had a biallelic mutation that was easily resolved, a 33 nucleotide insertion c.3383_3384ins33 (p.Q1128_E1129insTASANSF*), resulting in an in-frame stop codon (Fig. 4B).
- Immunoblot results confirmed that clone 2-5 lacked the full-length ATM protein, whereas the 22Rv1 parental had ATM expression (Fig. 4C). Reduced HR repair as measured by RAD51 foci was observed in clone 2-5 treated with 10 µM rucaparib for 24 hours as compared to the 22Rv1 parental cell line (Fig. 4D).
- The IC$_{50}$ in the 22Rv1 parental and *ATM* KO clone 1-50 were 7.757 and 0.309 µM respectively, demonstrating a 25-fold increase in sensitivity to rucaparib (Fig. 4E). Similar increases in sensitivity were also observed with other PARP inhibitors, including olaparib, niraparib, talazoparib and veliparib, but not with cisplatin (Table 3).

Fig. 5 PARP inhibition by rucaparib does not impact androgen receptor signaling in prostate cancer cell lines



- Dihydrotestosterone (DHT) induced AR mediated proliferation in 22Rv1 parental and *BRCA2* KO 1-50 cells selectively in CSS media. Proliferation was inhibited by enzalutamide, whereas rucaparib had no impact on cell growth (Fig. 5A).
- PARP inhibition has been reported to impact the expression of genes involved in AR signaling.[4] The 22Rv1 parental and *BRCA2* KO 1-50 cell lines both showed upregulation of *FKBP5*, *KLK3*, and *TMPRSS2* with 3 nM DHT treatment, and downregulation to baseline with 5 µM enzalutamide. However, rucaparib (5 µM) had no impact on DHT induced gene expression in the 22Rv1 parental and *BRCA2* KO 1-50 cell lines (Fig. 5B). DHT, enzalutamide and rucaparib treatment did not change the expression of the housekeeping genes *ALAS1*, *GAPDH*, and *GUSB* (data not shown).

CONCLUSIONS

- Rucaparib has potent activity in homologous recombination deficient prostate cancer cell lines.
  - In cytotoxicity assays using siRNA knockdown of individual genes, rucaparib potency increased with knockdown of *BARD1*, *BRCA1*, *BRCA2*, *FANCA*, *PALB2*, *RAD51*, *RAD51C*, and *RAD54L* in both DU145 and PC-3; and with *BLM*, *CDK12*, *NBM*, *PRKDC*, and *RAD51B* in at least 1 cell line.
- Enhanced rucaparib sensitivity was observed in 22Rv1 prostate cells engineered by CRISPR to knockout *ATM* or *BRCA2* expression.
- PARP-1, PARP-2, and PARP-3 inhibition by rucaparib does not interfere with androgen receptor signaling in prostate cancer cell lines.

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