Pharmacological ascorbate induces ‘BRCAness’ and enhances the effects of Poly(ADP-Ribose) polymerase inhibitors against BRCA1/2 wild-type ovarian cancer

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Received March 5, 2019; Accepted September 24, 2019

DOI: 10.3892/ol.2020.11364

Abstract. The promise of poly(ADP-ribose) polymerase inhibitors (PARPis) in the management of epithelial ovarian cancer (EOC) is hampered by the limited clinical activity against BRCA wild-type or homologous recombination-proficient EOC. In order to decrease the resistance and increase the efficacy of PARPis, combination treatments of pharmacological ascorbate and PARPis in preclinical BRCA wild-type EOC models were investigated. The cytotoxicity of pharmacological ascorbate, olaparib and veliparib in a panel of BRCA1/2 wild-type EOC cell lines were measured using MTT assays. Poly(ADP-ribose) levels were quantified using chemiluminescent ELISA. The expression of proteins involved in DNA damage and DNA double-strand breaks (DSBs) repair pathways were assessed by western blotting. The in vivo efficacy of pharmacological ascorbate, olaparib and their combination was evaluated in an intraperitoneal xenograft mouse model of BRCA1/2 wild-type EOC. Pharmacological ascorbate induced H2O2-dependent cytotoxicity in BRCA1/2 wild-type EOC cells. SHIN3 and OVCA1R5 cells were resistant to olaparib and veliparib treatment; however, the combination of ascorbate with olaparib or veliparib significantly enhanced cell death. Pharmacological ascorbate enhanced the effects of olaparib or veliparib by downregulating the expression of BRCA1, BRCA2 and RAD51. Consequently, the combination of pharmacological ascorbate and olaparib potently enhanced DNA DSBs and significantly decreased tumor burden, ascites volume and the number of tumor cells in ascites in mice bearing BRCA1/2 wild-type ovarian cancer xenografts. The combination of pharmacological ascorbate and PARPis may be a promising therapeutic approach worth clinical investigation in patients with BRCA wild-type or PARPi-resistant EOC.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal type of gynecological malignancy and ranks as the 5th leading cause of cancer-associated mortality among women in the USA (1), accounting for 22,240 new cases and 14,070 deaths in 2018 (2). Despite intensive treatment options, including debulking surgery, platinum and taxane-based chemotherapy and targeted therapy, such as poly(ADP-ribose) polymerase inhibitors (PARPis), angiogenesis inhibitors and immunotherapy agents, the overall 5-year survival rate for all types of EOC is relatively low (47.4%) and has remained stagnant for >2 decades (3). Additionally, ~60% of patients with EOC possess distant metastases at initial diagnosis, and the 5-year survival for these patients is considerably lower at 26% (2). There are a number of reasons for these poor survival outcomes, including the absence of reliable and accurate screening tests and limited effectiveness of current chemotherapies (1,2). Cumulative toxicity, cross-resistance to chemotherapies and compromised quality of life are additional serious clinical challenges for patients with EOC. Therefore, developing more effective and less toxic therapeutic strategies that target the fundamental vulnerabilities of EOC is required to improve patients' outcomes and quality of life.

PARPis are a new class of oncology drugs that are transforming the management of EOC (4). PARPis exert anti-cancer properties by trapping PARP on DNA at the sites of single-strand breaks, which leads to DNA repair defects and the generation of DNA DSBs that require homologous recombination (HR) mediated by BRCA1, BRCA2 and other proteins (such as ATM, ATR, RAD51, CHK1 and FANCA) (5-7). Therefore, BRCA1/2 mutant or HR-deficient cells are exceptionally sensitive to PARP inhibition (7-9), and the combination of two genetic deficiencies (e.g., BRCA1/2 and PARP) leads to synthetic lethality in cancer cells. Based on the promising clinical efficacy and the manageable toxicity profile of PARPis in patients with advanced EOC (10-13), three
PARPi (olaparib, rucaparib and niraparib) were approved by the U.S. Food and Drug Administration, either as a mono-
therapy (olaparib and rucaparib) for women with heavily
pretreated germline BRCA-mutated (gBRCAm) EOC or as a
maintenance therapy (olaparib, rucaparib and niraparib) for
women with platinum-sensitive recurrent EOC regardless of
BRCA or HR-deficiency (HRD) status. In addition, a recent
Phase III multicenter study (NCT01844986) (12) revealed that
olaparib maintenance monotherapy significantly improved
progression-free survival times in women newly diagnosed
with advanced ovarian cancer who harbored a BRCA1/2
mutation (14). However, new challenges have arisen for PARPi
therapy. Mutations in BRCA1 or BRCA2 occur in <20% of
patients with EOC (16% germline and 4% somatic) (15,16).
The majority of patients with EOC are BRCA1/2 wild-type
carriers who respond much less favorably to PARPi, limiting
the clinical efficacy and utility of PARPi. Additionally,
although several clinical studies have shown that some
non-gBRCAm or patients with HRD negative cancer can
benefit from PARPi (13,17), developing effective predic-
tion tools independent of HRD is difficult, due to the lack of
predictive biomarkers, which makes patient selection chal-
lenging. The combination of a PARPi and chemotherapy
have yet to show significant clinical benefits, and enhanced
myelosuppression, as the main dose-limiting toxicity, has been
observed (18,19), which may limit future combinatorial use of
these two types of therapy. As such, developing novel PARPi
combination therapies with a broad efficacy and low toxicity
is one potential direction for improving treatment of patients
with EOC.

Previously, it has been shown that ascorbate (vitamin C)
when used in high intravenous doses (IVC), has potential as
a therapeutic agent for the treatment of a variety of different
types of cancer (20-25). High-dose IVC, in contrast to oral
doses, establishes pharmacological concentrations in the
millimolar range in tissues, and selectively kills cancer cells
by generating H2O2 in the extracellular fluid, while leaving
healthy cells unharmed (21-23). The exquisite selectivity of
pharmacological ascorbate suggests a low toxicity of IVC treat-
ment. Multiple early phase clinical trials in patients with solid
or hematological malignancies, where IVC was used alone or
in combination with conventional chemotherapies or radiation
therapy, demonstrated that IVC was safe, well tolerated and
did not increase the toxicities of standard therapies (20,25).
The authors of the present study first demonstrated a notable
decrease in chemo-associated toxicities by adding IVC to
standard carboplatin/paclitaxel chemotherapy in patients with
stage III or IV EOC (20). In addition, the preliminary clinical
benefits in prolonged relapse time and/or tumor responses by
adding IVC to standard chemo- or radiation therapy has been
demonstrated (20,25).

By generating H2O2, pharmacological ascorbate damages
DNA and preferentially kills cancer cells (20,29). Therefore,
it was hypothesized that the combination of pharmacological
ascorbate and PARPi may enhance DNA repair deficiency,
and thus enhance the therapeutic effect of either agent alone
against EOC, regardless of BRCA status. In the present study,
the DNA damage response (DDR) induced by pharmaco-
logical ascorbate in ovarian cancer cells bearing wild-type
BRCA1/2 was characterized, and the efficacy and feasibility of
the combination treatment of pharmacological ascorbate and
the PARPi, olaparib, in preclinical models of EOC harboring
wild-type BRCA were investigated.

Materials and methods

Cell culture and reagents. Human EOC cell lines OVCAR8
and SHIN3 were kindly provided by Dr Peter Eck (University
of Manitoba, Manitoba, Canada) and OVCAR3, OVCAR5,
OVCAR10, SKOV3, A2780 and HIO-80 (an immortalized,
nontumorigenic human ovarian epithelium cell line) were
kindly provided by Dr Thomas Hamilton, or were derived by
Dr Andrew K. Godwin, both of the Fox Chase Cancer Center
(Philadelphia, USA). SHIN3 cells were maintained in DMEM
supplemented with 10% FBS, both (Sigma-Aldrich; Merck
KGaA) and 1% penicillin-streptomycin. HIO-80 was cultured
in M199/MCDB105 medium (1:1, v/v) containing 4% FBS,
insulin (0.3 U/ml) and 2 mM L-glutamine. The remaining
cell lines were cultured in PRMI-1640 medium supplemented
with 10% FBS and 1% penicillin-streptomycin. All cells were
cultured at 37°C with 5% CO2 and 85-95% humidity. Cell
line authentication was carried out by the Clinical Molecular
Oncology Laboratory of University of Kansas Medical Center
(Kansas, USA) using multiplex short tandem repeat DNA
profiling.

L-Ascorbic Acid (Thermo Fisher Scientific, Inc.) was
prepared as 1 M stock solutions in sterile water, with sodium
hydroxide added drop-wise to adjust the pH to 7.0. Aliquots
were stored at -80°C and thawed for single use. Catalase
(Sigma-Aldrich; Merck KGaA) was prepared in distilled water
at 10,000 units/ml, and was used at a working concentration
of 600 units/ml. Olaparib and veliparib were obtained from
Selleck Chemicals and were prepared in dimethyl sulfoxide
(DMSO) and diluted with cell culture media to working
concentrations, for the in vitro experiments, for the in vivo
experiments, olaparib was dissolved in PBS containing 10%
2-hydroxy-propyl-betacyclodextrin (Sigma-Aldrich; Merck
KGaA). All other reagents and chemicals were obtained from
Thermo Fisher Scientific, Inc., unless specifically indicated.

BRCA1/2 mutation analysis. The BRCA1/2 wild-type status
was reported previously (30) for all the EOC cell lines used in
the present study except SHIN3. The genomic DNA of SHIN3
cells was extracted using a Blood & Cell Culture DNA Mini
kit (Qiagen GmbH). The largest and functionally most impor-
tant exon (exon 11) of both BRCA1 (3,630 bp) and BRCA2
(5,018 bp) was amplified from the genomic DNA template
using PCR as previously described (31). The PCR amplicons
were submitted to Genewiz, Inc. for DNA sequencing. The
primer sequences are provided in Table S1. The thermocy-
cling conditions and Taq enzyme used were as previously
described (31). DNA sequences were analyzed using the
DNASTAR analysis package (version 8.1; DNASTAR, Inc.).
Both nucleic acid and amino acid sequences were aligned
using BioEdit (version 7.2) (32).

MTT assay. Cells were seeded at a density of 1×10^4 cells per
well in a 96 well plate, and incubated overnight. Cells were
then exposed to a serial dilution of ascorbate (0-3.5 mM),
olaparib (0-1,000 µM in SHIN3 cells; 0-800 µM in OVCAR5
cells) and veliparib (0-1,000 µM in SHIN3 cells; 0-800 µM in OVCAR5 cells), or treatment combinations and incubated for 24 or 48 h. In the drug combination groups, either olaparib or veliparib was added 15 min prior to ascorbate treatment. Following treatment, the culture medium was replaced with fresh, drug-free medium, and cells were incubated with MTT for 4 h. Formazan crystals were dissolved using DMSO and the absorbance at 492 nm was measured on a Synergy™ 4 Hybrid microplate reader (BioTek Instruments, Inc.).

The half maximal inhibitory concentration (IC₅₀) was determined using a non-linear regression analysis to fit the data to the log₁₀ [inhibitor] compared with a normalized response with a variable slope model.

Different concentrations of ascorbate (ranging from 0-5 mM) were used to avoid drawing conclusions from a single particular concentration. Concentration at IC₅₀ or a concentration range including the IC₅₀ were used. If the treatment time was <48 h, concentrations >IC₅₀ were used, with additional multiple concentrations including at least one close to or lower than the IC₅₀. The in vitro concentration ranges used in the present study are easily achievable in patients by intravenous ascorbate infusion (26).

Poly(ADP-ribose) (PAR) level measurement. PAR levels were measured using a HT PARP in vivo Pharmacodynamic assay II (Trevigen, Inc.), and normalized to the protein contents. Protein concentrations of cell lysates were measured using a Pierce bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.).

Western blot analysis. Cells were lysed in ice-cold radioimmunoprecipitation buffer (Thermo Fisher Scientific, Inc.), supplemented with cOmplete™ Mini Protease Inhibitor Cocktail Tablets (Sigma-Aldrich, Merck KGaA) and Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.). Protein concentration was determined using the Bradford Protein Assay Kit (Bio-Rad, Inc.). A total of 60 µg protein/lane was resolved on the 4-20% Mini-PROTEAN TGX™ Precast gels (Bio-Rad, Inc.) and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Inc.). The membranes were blocked using 5% skim milk in TBST (20 mM Tris_HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h at 4°C, followed by incubation at 4°C overnight with specific antibodies against H₂AX (1:500; Cell Signaling Technology, Inc.; cat. no. 7076); p-H₂AX(ser139) (1:1,000; Cell Signaling Technology, Inc.; cat no. 9718); ATM (1:1,000; Cell Signaling Technology, Inc.; cat. no. 2873); p-ATM(ser1981) (1:500; Cell Signaling Technology, Inc.; cat. no. 13050); BRCA1 (1:1,000; Cell Signaling Technology, Inc.; cat no. 14823); BRCA2 (1:1,000; R&D Systems, Inc.; cat. no. MAB2476); RAD51 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 8875); Ku70 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 4588); Ku80 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 2,180); p-DNA-PKcs(Thr2660) (1:350; Thermo Fisher Scientific, Inc.; cat. no. PA5-12913); DNA-PKcs (1:4,000; Santa Cruz; cat. no. sc-9051); β-actin (1:5,000; Thermo Fisher Scientific, Inc.; cat. no. MA5-15739); or vinculin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 13901). Target proteins were visualized using horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (1:5,000; Cell Signaling Technology, Inc.; cat. no. 7,074) or HRP-conjugated horse-anti-mouse IgG (1:5,000; Cell Signaling Technology, Inc.; cat. no. 7076) for 1 h at room temperature with Pierce™ ECL Plus Western blotting substrate (Thermo Fisher Scientific, Inc.). Each western blot analysis was performed. b-actin or vinculin were used as the loading controls. In vivo xenograft mouse model. All procedures were performed in accordance with a protocol (ACUP #2018-2443) approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center (Kansas, USA). The intraperitoneal (i.p.) tumor xenografts were established via i.p. injection of 2 x 10⁶ SHIN3 cells suspended in 200 µl PBS in fifty 4-6-week-old female athymic NCr-nu/nu mice (20-25 g body weight; National Cancer Institute). A total of 2 weeks after cell injection, mice were randomly grouped as follows: i) Control group; i.p. injection of saline solution osmotically equivalent to ascorbate twice daily and olaparib's solvent (PBS containing 10% 2-hydroxy-propyl-betacyclodextrin) once daily in volumes equivalent to the olaparib treated group; ii) ascorbate group, i.p. injection of ascorbate at 4 g/kg twice daily; iii) olaparib group, i.p. injection of olaparib at 50 mg/kg once daily; and iv) combination of ascorbate and olaparib, which were prepared and administered in the same manner as individual drug treatments. After 25 days of treatment, all mice were euthanized by CO₂ inhalation in a closed chamber (20% volume/min) followed by bilateral thoracotomy as approved in the protocol, and gross necropsy was performed with tumor weights and ascites volumes measured, and the number of tumor cells in the ascetic fluids counted. The total tumor burden of each mouse was indicated as total tumor weight at the end of experiment. The liver, kidney and spleen from each group were subjected to histopathological analysis using hematoxylin and eosin (H&E) staining, on 4-µm tissue sections with an automated procedure, as previously reported (33).

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.). Multiple comparisons between groups were performed using a one-way ANOVA with a post-hoc Turkey's test with a family-wise error rate of 0.05. Adjusted P<0.05 was considered to indicate a statistically significant difference.

Results

Pharmacological ascorbate induces H₂O₂-dependent cytotoxicity in BRCA1/2 wild-type EOC cells. Exon 11 is the largest and most functionally important exon. To the best of our knowledge, the current study was the first to sequence the BRCA1 and 2 genes (both of exon 11) of SHIN3 cells. As indicated in Fig. S1, a single variant of A→G at codon 349 of BRCA1 was detected, which is not known to have a functional outcome or be associated with breast or ovarian cancer. In addition, two variants were detected at codon 2660 A→G and codon 4560 G→C, in exon 11 of BRCA2, which do not result in amino acid changes. The present results indicate that no functional mutations were detected in exon 11 of BRCA1 and 2 in SHIN3 cells. A panel of BRCA1/2 wild-type human ovarian cancer cell lines (A2780, OVCA10, OVCA3, OVCA5, SKOV3, OVCA8 and SHIN3) (34), and an immortalized, non-tumorigenic human ovarian epithelium cell line (HIO-80) were then screened for sensitivity to ascorbate. As presented...
in EOC cells (20) and PARPi impair DNA damage repair, it was hypothesized that the combination of pharmacological ascorbate and PARPi may enhance DNA repair deficiency and improve therapeutic efficacy against EOC. In order to verify this hypothesis, the effects of the combination treatment of pharmacological ascorbate and PARPi olaparib or veliparib in SHIN3 and OVCAR5 cells were determined. Treatment with olaparib (20 µM) or veliparib (20 µM) for 24 and 48 h, respectively, minimally affected the cell viability of SHIN3 and OVCAR5 (Fig. 2A and B). The combined treatment of pharmacological ascorbate with either olaparib or veliparib significantly decreased cell viability compared with either single drug treatment and vehicle control, in both SHIN3 and OVCAR5 cells (Fig. 2A and B). PAR levels were significantly increased by pharmacological ascorbate in SHIN3 cells compared with the control, as early as 15 min (P<0.05; Fig. 3), suggesting that PARP was activated as a cellular response to the H\textsubscript{2}O\textsubscript{2}-induced DNA damage mediated by ascorbate. Treatment with olaparib significantly decreased PAR levels in the presence of ascorbate compared with the control (P<0.05; Fig. 3), suggesting that PARP-mediated DNA repair was inhibited. Taken together, these results suggest that when PARP activity is

Pharmacological ascorbate in combination with PARPi synergistically inhibits the growth of BRCA1/2 wild-type EOC cells. Since ascorbate-induced H\textsubscript{2}O\textsubscript{2} damage to DNA in EOC cells (20) and PARPi impair DNA damage repair, it was hypothesized that the combination of pharmacological ascorbate and PARPi may enhance DNA repair deficiency and improve therapeutic efficacy against EOC. In order to verify this hypothesis, the effects of the combination treatment of pharmacological ascorbate and PARPi olaparib or veliparib in SHIN3 and OVCAR5 cells were determined. Treatment with olaparib (20 µM) or veliparib (20 µM) for 24 and 48 h, respectively, minimally affected the cell viability of SHIN3 and OVCAR5 (Fig. 2A and B). The combined treatment of pharmacological ascorbate with either olaparib or veliparib significantly decreased cell viability compared with either single drug treatment and vehicle control, in both SHIN3 and OVCAR5 cells (Fig. 2A and B). PAR levels were significantly increased by pharmacological ascorbate in SHIN3 cells compared with the control, as early as 15 min (P<0.05; Fig. 3), suggesting that PARP was activated as a cellular response to the H\textsubscript{2}O\textsubscript{2}-induced DNA damage mediated by ascorbate. Treatment with olaparib significantly decreased PAR levels in the presence of ascorbate compared with the control (P<0.05; Fig. 3), suggesting that PARP-mediated DNA repair was inhibited. Taken together, these results suggest that when PARP activity is

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inhibited, pharmacological ascorbate significantly potenti-
ates cell death, potentially through enhanced DNA damage
in BRCA1/2 wild-type EOC cells.

Pharmacological ascorbate inhibits HR repair of DNA
DSBs in BRCA1/2 wild-type EOC cells. As pharmacological
ascorbate induced $\text{H}_2\text{O}_2$ and caused DNA damage in cancer
cells (20), the effects of pharmacological ascorbate on DDR
in BRCA1/2 wild-type EOC cells was determined in the
present study, with a focus on the HR and non-homologous
end joining (NHEJ) signaling pathways. Treatment with
pharmacological ascorbate for 2 and 6 h both notably
increased p-H2AXSer139 levels in both SHIN3 and OVCAR5
cells, a marker of DNA DSBs (Fig. 4). Expression of BRCA1
was downregulated in both tested cell lines 2 h post-ascorbate
treatment and further decreased 6 h post treatment (Fig. 4).
Notably, the expression of BRCA2 and RAD51 was slightly
increased in SHIN3 cells 2 h post treatment, followed
by decreases 6 h post treatment (Fig. 4), suggesting that
pharmacological ascorbate transiently activated HR repair
machinery as part of the stress response, and then inhibited
the HR repair machinery. Similar patterns of changes in
the expression of BRCA2 and RAD51 were observed in
OVCAR5 cells (Fig. 4). Together, these data suggest that
pharmacological ascorbate inhibited HR DNA DSBs repair
pathway by decreasing the expression of BRCA1, BRCA2
and RAD51 in BRCA1/2 wild-type EOC cells.

The NHEJ pathway was also investigated. The data
revealed that pharmacological ascorbate treatment minimally
affected the expression of Ku70 and Ku80 in SHIN3 cells
within the first 2 h. After 6 h of treatment, a dose-dependent
decrease in the expression of Ku70 and Ku80 was observed
following ascorbate treatment (Fig. 4). Ascorbate treat-
ment also decreased the expression of Ku70 and Ku80 in a
dose-dependent manner in OVCAR5 cells at either 2 or 6 h
of treatment (Fig. 4). Ascorbate treatment increased the levels
of $\text{p-DNA-PKcsThr2609}$, a product of activated NHEJ, in SHIN3
and OVCAR5 cells after of 2 h treatment as part of the stress
responses. After 6 h, $\text{p-DNA-PKcsThr2609}$ levels were decreased
in OVCAR5 cells, but not in SHIN3 cells (Fig. 4). However,
the expression of total DNA-PKcs was decreased by ascorbate
in a dose-dependent manner in SHIN3 cells after both 2 and
6 h treatment. In OVCAR5 cells DNA-PKcs was first upregu-
lated (2 h) and then downregulated (6 h) following ascorbate
treatment (Fig. 4). These data suggest that the regulation of
pharmacological ascorbate on the NHEJ DNA repair proteins
(Ku70, Ku80 and DNA-PKcs) was cell-line dependent with an
overall tendency of inhibition.

Combination of pharmacological ascorbate and olaparib
enhances DNA DSBs. As presented in Fig. 5A, treatment with
ascorbate alone or olaparib alone increased the expression
of p-ATMSer1981, an early marker of oxidative DNA damage,
and the expression of p-H2AXSer139, a marker of DNA DSBs,
in a dose-dependent manner in SHIN3 cells treated for 2 h.
The combination treatment of ascorbate and olaparib further
enhanced the expression of p-ATMSer1981 and p-H2AXSer139
compared with both drugs alone. Treatment with catalase
decreased the levels of p-ATMSer1981 and p-H2AXSer139 when
it was added prior to treatment with olaparib and ascorbate,
suggesting that catalase protects cells from oxidative DNA damage. Similar observations were also observed in OVCAR5 cells (Fig. S2).

In addition, the time-dependent expression levels of p-ATM^{Ser1981} and p-H2AX^{Ser139} were detected following incubation with 3 mM ascorbate in SHIN3 cells (Fig. 5B). Consistently, the combination treatment of ascorbate and olaparib further increased the expression of p-ATM^{Ser1981} and p-H2AX^{Ser139} compared with either treatment alone in SHIN3 cells. Cells were treated with the drug combinations at the indicated concentrations for 2 h. (B) Representative western blotting images demonstrate the time course of p-ATM^{Ser1981} and p-H2AX^{Ser139} levels with Asc + olaparib, respectively, in SHIN3 cells. Cells treated with the different drug combinations at the indicated concentrations were collected at the indicated time points. Experiments were performed twice independently. Vinculin served as a loading control. Asc, ascorbate.

Figure 4. Pharmacological ascorbate induced DNA double stranded breaks and inhibited homologous repair in BRCA1/2 wild-type epithelial ovarian cancer cells, while regulating non-homologues end joining repair in a cell line-dependent manner. SHIN3 and OVCAR5 cells were treated with Asc for either 2 or 6 h. Representative western blots from two independent experiments are presented. β-actin served as a loading control. Asc, ascorbate.

Figure 5. Combination treatment of pharmacological ascorbate and olaparib enhanced DNA double stranded breaks. (A) Representative western blotting images presenting treatment with Asc in combination with olaparib leads to enhanced expression of p-ATM^{Ser1981} and p-H2AX^{Ser139} compared with either treatment alone in SHIN3 cells. Cells were treated with the drug combinations at the indicated concentrations for 2 h. (B) Representative western blotting images demonstrate the time course of p-ATM^{Ser1981} and p-H2AX^{Ser139} levels with Asc + olaparib, respectively, in SHIN3 cells. Cells treated with the different drug combinations at the indicated concentrations were collected at the indicated time points. Experiments were performed twice independently. Vinculin served as a loading control. Asc, ascorbate.
with either treatment alone. All treatments were well tolerated and did not result in weight loss (Fig. 6D). H&E staining demonstrated no pathological changes in the livers, kidneys or spleens of the animals in all treatment groups, suggesting that ascorbate, olaparib and the combination treatment were of low toxicity at the tested concentrations (Fig. 6E).

Discussion

BRCA1/2 mutations occur in <20% of patients with EOC, and it is hypothesized that this mutation limits the clinical efficacy of PARPis (15,16). However, PARPis are used in certain patients with EOC regardless of BRCA status (36,37). Currently, three PARPis are used as standard treatment for EOC, and two of these, olaparib and rucaparib, are used for treatment of recurrent BRCA mutant ovarian cancer, as well as maintenance therapy in platinum-sensitive relapsed EOC regardless of BRCA or HRD status (36,37). Niraparib, the third clinically used PARPi, is indicated for maintenance, and used irrespective of BRCA status (38). There is an unmet clinical need to decrease the resistance and enhance the efficacy of PARPis.

In order to expand the applicability and increase the efficacy of PARPis, the addition of pharmacological ascorbate to treatment with PARPis in BRCA wild-type EOC models was assessed for several reasons: i) A previous study demonstrated that pharmacological ascorbate decreased the toxicities of conventional chemotherapies (20); ii) pharmacological ascorbate produces peroxide and damages DNA, thus could work synergistically with an inhibitor of the DNA repair machinery, as presented in the present study; and iii) ascorbate influenced the homologous recombination pathway, and thus could decrease resistance to PARPis, also demonstrated in the present study. Therefore, the present study highlights the potential of combining pharmacological ascorbate and PARPis and how it may benefit a broader population of patients with EOC, even with wild-type BRCA. A clinical proof-of-concept study is required as the next step to further investigate this potential.

The present study demonstrates the preclinical efficacy and feasibility of combining pharmacological ascorbate and PARPis for treating BRCA wild-type ovarian cancer. In vitro, the combination synergistically induced the death of BRCA wild-type EOC cells; in vivo, the combination treatment significantly decreased tumor and ascites burdens without inducing any toxicity. The present study demonstrates that pharmacological ascorbate potentiates the therapeutic efficacy of olaparib in BRCA wild-type EOC by inducing HR deficiency or a ‘BRCAness’ phenotype. Therefore, the combination used in the present study is a novel and potentially promising therapeutic option for treating patients with EOC, particularly those who do not respond to PARPis alone. Such a strategy could be applied to a variety of heterogeneous and hard-to-treat malignancies, including breast, pancreatic and prostate cancer, where BRCA1, BRCA2 or other HR repair proteins are instrumental in the repair of DNA DSBs and the potential of PARPis has not yet been fully exploited (39).
Consistent with previous studies (22,29,40), the data in the present study demonstrated that treatment with pharmacological ascorbate resulted in the production of H$_2$O$_2$, which damages DNA, leading to PARP activation, and this was impaired by the PARPis. The oxidative stress induced by pharmacological ascorbate caused excessive DNA DSBs in BRCA1/2 wild-type EOC cells within the first 6 h of treatment. The concentration and time ranges are clinically relevant to those of i.v. ascorbate infusion (43). Pharmacological ascorbate is selectively lethal to cancer cells, but not normal cells, which is partially attributed to the increased intracellular levels of reactive oxygen species (ROS) and the decreased ability to metabolize H$_2$O$_2$ in cancer cells compared with normal cells (41,42). The ROS-induction mechanism of high dose ascorbate provides the first rationale for combining ascorbate with a PARPi.

Ascorbate inhibited DNA repair enzymes, which provides another rationale for combined treatment with PARPi. HR and NHEJ are the two primary DNA DSBs repair pathways in eukaryotic cells (43). HR is a template-directed DNA repair with high-fidelity, which is crucial for the maintenance of both telomere integrity and genomic stability; whereas NHEJ is an error-prone DNA repair process, which does not use a comple-

tentary template and can introduce deleterious mutations during repair (43). The results of the present study suggest that pharmacological ascorbate suppressed the expression of HR repair proteins BRCA1, BRCA2 and RAD51, leading to HR deficiency. In addition, ascorbate influences the NHEJ pathway, impeding both HR and NHEJ pathways. Patel et al (44) and Do et al (45) reported that with PARP inhibition, HR-deficient cancer cells upregulated the NHEJ as an alternative DNA repair pathway. As pharmacological ascorbate impedes both HR and NHEJ, adding it to a PARPi can further promote genomic instability and enhance cytotoxicity.

Patients with EOC with wild-type BRCA are considerably less responsive to PARPis compared with carriers of germ-line or somatic BRCA mutations (13,17,46). Consistent with the clinical observations, the present study demonstrated that BRCA wild-type EOC cells are not sensitive to olaparib, both in vitro and in vivo. Addition of pharmacological ascorbate to olaparib overcomes the resistance to olaparib and significantly decreased tumor burden. These results highlight the potential of a novel clinical solution for patients with EOC who do not benefit from PARPis alone.

It is well-documented that high-dose IVC is well tolerated with minimal toxicity in humans (26-28,47). A previous Phase I/II clinical trials in patients with EOC (20) and pancreatic cancer (40), together with other trials (25,27,28), have consistently demonstrated that adding IVC to standard treatments (chemotherapy or radiation therapy) is safe, well tolerated, feasible and potentially effective. Addition of IVC to carboplatin and paclitaxel chemotherapy substantially decreased side-effects in patients with stage III or IV EOC (20). Potential survival benefits of IVC were reported in combination with chemotherapy or radio-chemotherapy in the treatment of pancreatic cancer, glioblastoma multiforme and advanced-stage non-small cell lung cancer (25,28). Several randomized Phase II trials evaluating the efficacy of IVC are underway (48).

A limitation of the present study lies in the cell line-derived xenograft, which may not be representative of the heterogeneous nature of tumors in patients. Due to the idiosyncratic character-

istics of different tumors from different patients, the present study now warrant testing this novel drug combination in multiple patient-derived xenograft mouse models, which will provide more relevant data for future clinical trials. Nevertheless, the results of the present study highlight new opportunities for the translational studies of pharmacological ascorbate in combination with PARPis for treating patients with EOC, regardless of BRCA or HRD status.

In conclusion, the present study demonstrated that the combination treatment of pharmacological ascorbate with PARPis had the potential to provide therapeutic benefits to patients with ovarian cancer who do not respond to PARPis alone. The advantages of this combination therapy lie in the potentially broad applicability, improved efficacy and low toxicity.

Acknowledgements

The authors would like to thank Dr. Peter Eck (University of Manitoba; Manitoba, Canada) for providing the SHIN3 cell line, Ms. Min Yang (University of Kansas Medical Center; Kansas City, USA) for helping to generate part of the western blotting data, Dr. Kishore Polireddy and Dr. Thuy-Vy Do (University of Kansas Medical Center; Kansas City, USA) for assisting with exploratory experiments, Dr. Chunhua Li and Dr. Nan He (University of Kansas Medical Center; Kansas City, USA) for DNA sequencing and sequence analysis, and the staff of the Clinical Molecular Oncology Laboratory of University of Kansas Medical Center for cell line authentication.

Funding

The present study was financially supported by a bridging grant from the University of Kansas Research Institute (Kansas, USA), and a grant from the University of Kansas Endowment provided by the GR’s Foundation, Mosby Lincoln Foundation, and Donlan Foundation (Kansas, USA).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

QC, AKG, JAD and YM conceived and designed the study. YM, PC and DK developed the methodology, performed the experiments and collected data. YM, PC, QC and DK analyzed, computed and interpreted the data. YM wrote the manuscript. YM, QC, AKG, JAD, PC and DK reviewed and revised the manuscript. JAD, AKG and QC provided administrative, technical and material support. QC supervised the study.

Ethics approval and consent to participate

Not applicable.
Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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