Molecular Cancer Research

Supplementary Information

for

Pharmacological Ascorbate Primes Pancreatic Cancer Cells for Death by Rewiring Cellular Energetics and Inducing DNA Damage

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Supplementary Fig. S1. There is differential susceptibility to P-AscH⁻ across cell lines. Pancreatic cancer (MIA PaCa-2, PANC-1 and 339 cells) or normal pancreatic cells (H6c7 cells) were treated with varying concentrations of P-AscH⁻ for 1 h and cells viability was assessed with a clonogenic survival assay. We observed that there is a wide range for the sensitivity to P-AscH⁻ across cell lines. Normal pancreatic cells appear to be more resistant to P-AscH⁻ in comparison to cancer cells. Cell viability is expressed as survival fraction, as a percentage. The amount of P-AscH⁻ required to reduce clonogenic survival to 50% (ED₅₀) was estimated for each on the basis of the dose-response curves. ED₅₀ values are listed in Supplementary Table S1. (n = 3 biological replicates, with 3 technical replicates in each biological replicate; mean ± SEM).
**Supplementary Fig S2. Bolus addition of hydrogen peroxide depletes intracellular ATP and NAD⁺ in MIA PaCa-2 and PANC-1 cells.** Cells were treated with a bolus of H₂O₂ (MIA PaCa-2, 14 pmol cell⁻¹; PANC-1, 20 pmol cell⁻¹), incubated for 1 h, and then intracellular levels of ATP as well as NAD⁺ were determined. **(A, B)** Exposure to a bolus of H₂O₂ resulted in decreases in ATP and NAD⁺ availability in both cell lines. These results are parallel to results from exposure to P-AscH⁻, supporting a contribution of H₂O₂ in cytotoxicity of P-AscH⁻ (**n** = 3; mean ± SEM; **** **p** < 0.0001 vs. untreated control).
Supplementary Fig. S3. Pharmacological ascorbate depletes intracellular NADH in MIA PaCa-2 cells, but not in PANC-1, 339, and H6c7 cells. Cells were treated with varying amounts of ascorbate (0 – 40 pmol cell⁻¹) or a bolus of H₂O₂ (MIA PaCa-2, 14 pmol cell⁻¹; PANC-1 and H6c7, 20 pmol cell⁻¹) for 1 h and then intracellular levels of NADH were determined. (A – D) The loss of NADH availability following exposure to P-AscH⁻ or H₂O₂ was observed only in MIA PaCa-2 cells, not in PANC-1, 339 or H6c7 cells (n = 3; mean ± SEM; **** p < 0.0001, *** p < 0.001 vs. untreated control).
Supplementary Fig. S4. Pharmacological inhibition of PARP1 by olaparib enhances the cytotoxicity of P-AscH− in MIA PaCa-2 cells. MIA PaCa-2 cells were pre-treated with olaparib for 18 h followed by 1 h treatment of P-AscH− + olaparib. The amount of olaparib was 500 fmol cell⁻¹ (25 μM) and the amount of P-AscH− was 7 pmol cell⁻¹. Cell viability was determined with a clonogenic survival assay. Pre-treatment with a higher concentration of olaparib increases the cytotoxicity of P-AscH− in MIA PaCa-2 cells. Clonogenic survival of MIA PaCa-2 cells is expressed as relative survival fraction. DMSO was used as the vehicle control for olaparib (n=3, each of the biological replicates consists of three technical replicates, mean ± SEM; ** p < 0.01, **** p < 0.0001 vs. untreated control; †† p < 0.01, †††† p < 0.0001 vs. P-AscH− + olaparib).
Supplementary Fig. S5. A combination of P-AscH⁻ and prexasertib synergistically inhibits cell proliferation of MIA PaCa-2 cells. MIA PaCa-2 cells were treated with varying concentrations of prexasertib (18 h), or P-AscH⁻ (1 h), or prexasertib + P-AscH⁻ with a constant ratio for the two drugs. The doses of prexasertib were 50, 100, 200, 400, and 800 amol cell⁻¹; while the doses of P-AscH⁻ were 0.625, 1.25, 2.5, 5, and 10 pmol cell⁻¹; keeping a constant ratio of prexasertib to P-AscH⁻ for these experiments of 1:12,500. After treatment, drugs were removed and cell proliferation was then assessed 48 h later with the MTT assay. The potential synergistic interaction for prexasertib + P-AscH⁻ was evaluated with the Combination Index (CI) of Chou and Talalay [1]. The plot of CI values vs. fraction of affected cells (Fa) demonstrates the synergistic effects of P-AscH⁻ and prexasertib (CI < 1). The CI values of P-AscH⁻ and prexasertib range from 0.16 to 0.87. These data are from three independent experiments. Each biological replicate consists of three technical replicates.
Supplementary Table S1. Normal pancreatic cells are more resistant to P-AscH⁻ than pancreatic cancer cells.

<table>
<thead>
<tr>
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<th>ED$_{50}$ (pmol cell$^{-1}$)\textsuperscript{a}</th>
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<tr>
<td>MIA PaCa-2</td>
<td>6.3 ± 0.7</td>
</tr>
<tr>
<td>PANC1</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>339</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>H6c7</td>
<td>15.2 ± 1.1</td>
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\textsuperscript{a} ED$_{50}$ = effective dose of ascorbate that caused 50% clonogenic cell death. ED$_{50}$ values were estimated from the data of Supplementary Fig. S1 and the uncertainties are reported as standard error.

Comparing ED$_{50}$ values demonstrates that pancreatic cancer cells are more sensitive to P-AscH⁻ than normal pancreatic cells. For pancreatic cancer cells in this study, MIA Pa-Ca-2 cells are the most sensitive cells to treatment with P-AscH⁻, while PANC-1 and 339 cells are relatively resistant to treatment. These results strongly support the safety and efficacy of P-AscH⁻ in the treatment of cancer.
**Supplementary Table S2.** The ED$_{50}$’s for depletion of ATP and NAD$^+$ correlate with $k_{cell}$ for the removal of H$_2$O$_2$.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cell}$ ($10^{-12}$ s$^{-1}$ cell$^{-1}$ L$^{-1}$)$^a$</th>
<th>ED$_{50}$ for reduction of ATP (pmol cell$^{-1})^b$</th>
<th>ED$_{50}$ for depletion of NAD$^+$ (pmol cell$^{-1})^b$</th>
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<tr>
<td>MIA PaCa-2</td>
<td>1.1</td>
<td>7.9 ± 1.2</td>
<td>8.1 ± 0.7</td>
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<tr>
<td>H6c7</td>
<td>3.7</td>
<td>34.8 ± 2.7</td>
<td>13.5 ± 0.9</td>
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<tr>
<td>PANC-1</td>
<td>5.1</td>
<td>47.6 ± 9.4</td>
<td>22.7 ± 2.8</td>
</tr>
<tr>
<td>339</td>
<td>5.4</td>
<td>42.9 ± 6.2</td>
<td>33.2 ± 4.7</td>
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$^a$ $k_{cell}$ was obtained from [2].

$^b$ ED$_{50}$ of P-AscH$^+$ for depletion of ATP and NAD$^+$ were estimated from Fig 2A-D; the uncertainties are reported as standard error.
## Supplementary Table S3. The gene target and primer pairs used for QPCR.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
<th>References</th>
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<td>12.2 kb region of the DNA polymerase beta gene (accession number L11607)</td>
<td>CCTGGAGTAGGAACAAAAAA TTGCTG</td>
<td>CATGTCACCCACCTGGAC TCTGCAC</td>
<td>[8]</td>
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<tr>
<td>8.9 kb mitochondria fragment</td>
<td>TTTCATCATGCGGAGATGT TGGATGG</td>
<td>TCTAAGCCTCCTATTCG AGCCGA</td>
<td>[8]</td>
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<tr>
<td>220 bp mitochondria fragment</td>
<td>CCCCCAAAACCCCATTACTAAACCA</td>
<td>TTTCATCATGCGGAGATGTGGATGG</td>
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Extended Materials and Methods

Cell culture

MIA PaCa-2 and PANC-1 human pancreatic adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA). MIA PaCa-2 and PANC-1 cells were cultured in Dulbecco’s modified eagle medium (DMEM) with high glucose from Invitrogen (Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units mL⁻¹)/streptomycin (100 µg mL⁻¹) at 37 °C, 5% CO₂. The patient-derived pancreatic cancer cells, 339 cells, were obtained from the Medical College of Wisconsin surgical oncology tissue bank (Milwaukee, WI). 339 cells were cultured in DMEM nutrient mixture F12 (Ham) medium (Millipore Sigma, Burlington, MA), supplemented with 6% FBS, 0.1% epidermal growth factor (EGF) human recombinant, 0.4% bovine pituitary extract, 4% hydrocortisone, 0.014% insulin human recombinant, and GlutaMAX™ at 37%, 5% CO₂. In addition to pancreatic cancer cell lines, we also used H6c7 cells, a human non-tumorigenic, immortalized pancreatic ductal epithelial cell line. The genotypic and phenotypic characteristics of H6c7 cells are similar to normal pancreatic ductal epithelial cells [3]. Hence, these cells are widely used as a model for normal pancreatic ductal epithelial cells in preclinical studies. H6c7 cells were cultured in keratinocyte serum-free media from ThermoFisher Scientific (Waltham, MA), supplemented with epidermal growth factor (5 ng mL⁻¹), bovine pituitary extract (50 μg mL⁻¹) and penicillin (100 units mL⁻¹)/streptomycin (100 µg mL⁻¹) at 37 °C, 5% CO₂.

As appropriate, confirmation of all cell lines was accomplished by IDEXX-RADIL. The genetic profiles of cell stocks were compared to the genetic profiles of cell lines that are available in the DSMZ STR database. This ensures that they do not match any other reported profiles in the DSMZ database.

Generation of PARP1 CRISPR/Cas9 knockout cells

In order to knockout PARP1, CRISPR/Cas9 plasmid DNA, encoding PARP1-specific non-coding guide RNA, Cas9 nuclease and green fluorescent protein (GFP) were used to transfect cells. With the GFP reporter system, the transfected cells were selected with fluorescence-activated cell sorting (FACS) techniques.

MIA PaCa-2 or PANC-1 cells were seeded into 6-well plates at 150,000 cells in 3.0 mL of standard DMEM media per well. Cells were cultured at 37 °C, 5% CO₂ for 24 h. Standard medium was replaced with 3.0 mL of antibiotic-free DMEM prior to transfection. After media replacement, cells were then transfected with a mixture of PARP1 CRISPR/Cas9 knockout plasmid for 24 h. The mixture of PARP1 CRISPR/Cas9 knockout plasmid consists of 1 - 3 µg of PARP1 CRISPR/Cas9 knockout plasmid DNA (Santa Cruz Biotechnology, Dallas, TX; sc-400046), 5 μL of transfection reagent (sc-395379; Santa Cruz Biotechnology, Dallas, TX) and transfection medium (sc-108062; Santa Cruz Biotechnology, Dallas, TX). After 24 h of transfection, cells were grown in standard DMEM for an additional 48 h. To create a single monoclonal cell line with PARP1 knockout, the GFP-positive cells were selected by using BD FACSAria Fusion flow cytometer (Becton Dickinson); single cells were sorted into individual wells of a 96-well plate. The cell lines with bi-allelic or mono-allelic PARP1 knockouts were determined with western blot analysis. Using similar procedures, control cells were generated by transfecting parental cell lines with control CRISPR/Cas 9 plasmid (sc-418922; Santa Cruz Biotechnology, Dallas, TX).
Treatment with P-AscH

L-Ascorbic acid was obtained from Macron Fine Chemicals. Stock solutions of L-ascorbic acid (1.00 M) were prepared in Nanopure® Type 1 water (18 QM) under Argon. The pH of stock solution was adjusted to 7.0 with 10.0 M NaOH. The concentrations of ascorbate were further confirmed with UV/Vis spectroscopy, $\varepsilon_{265} = 14.5 \text{mM}^{-1}\text{cm}^{-1} [4]$. Stock solutions were stored at 4 °C in sealed borosilicate glass tubes with minimum headspace.

Cells were seeded into appropriate culture containers at indicated cell density; 1 set of dishes for treatment and reserving 1 dish for determining cell number. Prior to exposure to ascorbate, the number of cells in each set of dishes/wells was determined using a hemocytometer. This cell number and volume of medium present during treatment was used to determine the initial dose in units of mol cell$^{-1} [2, 5]$. The formation of H$_2$O$_2$ due to oxidation of ascorbate is dependent on the pH of the growth medium. Hence, an exchange of medium was done prior to treatment with ascorbate to minimize differences in pH and thereby differences in the flux of H$_2$O$_2$ between experiments. After replacement with fresh DMEM medium, cells were then incubated with ascorbate at 37 °C for 1 h, unless specified elsewhere. For control experiments, medium was replaced with fresh DMEM, as done with cells exposed to P-AscH.

Treatment with catalase

Bovine catalase was used to investigate the contribution of H$_2$O$_2$ in the cytotoxicity of P-AscH. Bovine catalase (Millipore Sigma, Burlington, MA) was prepared in water to produce a stock solution of 100 U μL$^{-1}$ and stored at 4 °C. Cells were incubated with bovine catalase at a concentration of 200 U mL$^{-1}$ immediately prior to the treatment with P-AscH.

Treatment with Olaparib

Olaparib (inhibitor of PARPs) was used to study the contribution of PARPs to the cytotoxic mechanism of P-AscH. Olaparib (Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO (Thermo Fisher Scientific, Waltham, MA) to produce a 30 mM stock solution. Aliquots of olaparib were stored frozen at -20 °C. Cells were pre-treated with olaparib for 18 h following by treatment with P-AscH + olaparib. Control cells were incubated with an equivalent amount of DMSO (vehicle control). One additional dish was used to determine cell density at the time of treatment so the initial dose of olaparib in units of mol cell$^{-1}$ could be determined [5].

Treatment with Prexasertib

Prexasertib (inhibitor of Chk1) was used to study the influence of Chk1 on the cytotoxicity of P-AscH. Prexasertib (MedChemExpress, Monmouth Junction, NJ) was dissolved in DMSO to produce 10.0 – 100 μM stock solutions. Aliquots of prexasertib were stored frozen at -20 °C. Cells were pre-treated with prexasertib for 18 h followed by treatment with P-AscH + prexasertib. Control cells were incubated with an equivalent amount of DMSO (vehicle control). One additional dish was used to obtain a cell count so that the initial dose of prexasertib could be determined in units of mol cell$^{-1} [5]$. 

Clonogenic survival assay

The clonogenic survival assay is an in vitro approach that is commonly used to determine the reproductive ability of single cells to form colonies following exposure to xenobiotics or a biophysical challenge [6]. Hence, we used this assay to evaluate the effectiveness of treatment with P-AscH against pancreatic cancer cells.
Cells were seeded into multiple 60 mm² culture dishes at 100,000 cells per dish in 4.0 mL medium. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO₂ before exposure to experimental conditions. For ascorbate treatment, cells were exposed at indicated concentrations for 1 h. For bovine catalase experiments, cells were incubated with catalase at indicated concentration immediately prior exposed to ascorbate. For olaparib experiments, cells were pre-treated with olaparib for 18 h then exposed to P-AscH⁺ + olaparib for 1 h. For prexasertib experiments, cells were pre-treated with prexasertib for 18 h then exposed to P-AscH⁺ + olaparib for 1 h.

Following treatment, exposure medium was removed; cells were trypsinized, counted using a Moxi Z Mini Automated Cell Counter (ORFLO Technologies, Ketchum, ID), and seeded in triplicate into 6-well plates at 300 cells per well in 4.0 mL of their respective medium. For H6c7 cells, irradiated MIA PaCa-2 cells (30 Gy; $2 \times 10^5$ cells well⁻¹) were used as feeder layer to support clonogenicity. Plates were then incubated for 7–14 days at 37 °C, 5% CO₂. Surviving colonies were fixed with 70% ethanol for 5 min and stained with Coomassie Blue for 30 min. Colonies were counted as a cluster of at least 50 cells. The plating efficiency and surviving fraction were calculated [6]; plating efficiency (PE) = ((average of number of colonies counted) / (number of cells plated)) x 100; clonogenic survival fraction (SF) = ((PE of treated sample) / (PE of untreated control)).

The effective dose of P-AscH⁺ that caused 50% clonogenic cell death (ED₅₀) for each cell line was calculated from dose-response curves using GraphPad Prism 7.0d (GraphPad Software, La Jolla, CA).

**Measurement of intracellular ATP**

Cells were seeded into multiple culture dishes (60 mm x 15 mm) at 250,000 cells per dishes in 4.0 mL media. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO₂. For ascorbate treatment, cells were exposed at indicated concentrations for 1 h. For bovine catalase experiments, cells were incubated with catalase added immediately prior to being exposed to ascorbate. For treatment with olaparib, cells were pre-treated with olaparib for 18 h, then exposed to P-AscH⁺ + olaparib for 1 h.

After treatment, cells were trypsinized, and re-suspended with PBS. Intracellular ATP was then determined with CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WA) according to the manufacturer’s recommendation. Briefly, 50,000 cells in 100 μL of PBS were added to experimental wells of 96-well white opaque-bottom plate. Then, an equal volume of CellTiter-Glo reagent was directly added to lyse the cells and initiate the luminescence reaction. After 10 min, luminescence was detected using SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA). The luminescent signal is directly proportional to the amount of ATP present. Standard curves for each experiment were generated by using serial dilutions of ATP stock solutions (0 – 1000 μM). The ATP concentration in a sample was calculated from the corresponding standard curve and then further transformed to an average intracellular concentration by using the cell number, as determined using a hemocytometer, and cell volume, as measured with a Moxi Z Mini Automated Cell Counter (ORFLO Technologies, Germany). The effective dose of P-AscH⁺ that resulted in a 50% reduction of intracellular ATP for each cell line was calculated from dose-response curves using GraphPad Prism 7.0d (GraphPad Software, La Jolla, CA).
**Measurement of intracellular NAD\(^+\) and NADH**

The cell culture conditions and treatments were similar to those used for the measurement of ATP. After treatment, levels of NAD\(^+\) and NADH in samples were measured with the NAD/NADH-Glo\textsuperscript{TM} Assay (Promega, Madison, WA) according to the manufacturer’s recommendations. This luminescent-based assay takes advantage of the differential stability of NAD\(^+\) and NADH in acidic conditions. Generally, NAD\(^+\) is stable in acidic conditions, while NADH is stable in basic solution [7]. Hence, the luminescence signals from acid-treated samples are proportional to the level of NAD\(^+\) in the sample. Luminescence signals from samples in base solutions are proportional to the level of NADH in the sample.

Briefly, cell suspensions (25,000 cells, 50 μL) were lysed directly with 50 μL of base solution (0.2 M NaOH with 1% dodecyltrimethylammonium bromide). Lysed samples were then divided equally (50 μL) into two tubes; one tube for acid treatment (measurement of NAD\(^+\)) and another tube for base treatment (measurement of NADH). For NAD\(^+\) measurements, lysed samples (50 μL) were treated with 0.4 M HCl (25 μL) and heated at 60 °C for 15 min. Acid-treated samples were incubated at room temperature for 10 min, then Trizma base solution (25 μL) was added to neutralize the acid. For NADH measurement, lysed samples in base solution (50 μL) were heated at 60 °C for 15 min. Base-treated samples were incubated at room temperature for 10 min, then HCl/Trizma solution (50 μL) was added to neutralize the base.

After preparation, neutralized samples (50 μL; 12,500 cells total) were added into 96-well white plates with opaque bottoms. Then, 50 μL of the luciferin-based detection reagent was directly added to sample to initiate the luminescence reaction. After 30 min, luminescence was determined using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA). Standard curves for each experiment were generated by serial dilutions of NAD\(^+\) and NADH stock solutions (0 – 400 nM final concentration). The intracellular NAD\(^+\) and NADH concentrations of samples were calculated from the corresponding standard curve and then further transformed to an intracellular concentration by using the cell number, as determined using a hemocytometer, and cell volume, as measured with a Moxi Z Mini Automated Cell Counter (ORFLO Technologies, Germany). The effective dose of P-AscH\(^-\) that resulted in a 50% depletion of NAD\(^+\) for each cell line was calculated from dose-response curves using GraphPad Prism 7.0d (GraphPad Software, La Jolla, CA).

**Genomic isolation and measurement of DNA damage with quantitative PCR (QPCR)-based approach**

Cells were seeded into multiple culture dishes (100 mm x 20 mm) at 500,000 cells per dish in 10.0 mL of medium. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO\(_2\). For treatment with ascorbate, cells were exposed to ascorbate at indicated concentrations for 1 h. After a 1-h treatment, genomic DNA was isolated using Blood & Cell Culture DNA Mini Kit (Qiagen, Valencia, CA) as described by the manufacturer’s instructions. Isolation of genomic DNA by this technique has been demonstrated to be suitable for QPCR-based measurement of both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) damage without a separate step for mitochondrial DNA purification [8]. The QPCR method to quantitate DNA damage is based on the principle that various types of lesions on DNA can impede progression of DNA polymerase. If equal amounts of DNA from different biological samples are amplified under identical PCR conditions, DNA with a greater degree of damage will amplify to a lesser extent when compared to DNA with less damage [8]. Hence, the amount of PCR amplification is inversely proportional to lesion frequency within DNA samples.
Prior to QPCR, concentrations of total cellular DNA were quantified using an Implen Nanophotometer P-330 (Implen Inc., Germany) at 260 nm. QPCR was performed in a 2720 Thermal Cycle (Applied Biosystems, Foster City, CA) with LA PCR Kit, Version 2.1 (Clontech Laboratories, Mountain View, CA). The total volume of reaction was 50.0 μL, containing 15.0 ng (for nDNA assay), or 5.0 ng (for mtDNA assay) of total genomic DNA, 1X LA PCR buffer II (Mg²⁺ plus), 400.0 μM dNTP mixture, 0.4 μM primers and 2.5 units of Takara LA Taq. The oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The primer nucleotide sequences used for amplification of nDNA and mtDNA are listed on Supplementary Table S3. The 12.2 kb region of the DNA polymerase beta gene was used to probe for nDNA lesions. The PCR conditions were as follows: an initial denaturation at 94 °C for 2 min followed by 26 cycles of denaturation at 94.5 °C for 25 s, primer extension at 68 °C for 13 min (for nDNA) or 20 cycles of denaturation at 94 °C for 25 s, primer extension at 68 °C for 10 min 30 s (for mtDNA). A final extension at 72 °C was performed for 10 min at the end of the PCR cycle. Fifty-percent controls, containing half of the amount of undamaged DNA, were used as quality control for each PCR to validate that PCR reaction had been terminated within the exponential phase. The PCR amplicons were quantified by fluorescence measurement with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The specificities of the PCR reactions were confirmed with agarose gel electrophoresis. Mitochondrial DNA amplification of each sample was normalized with relative mitochondrial DNA copy number by standardizing to the amplification of small mitochondrial fragment (220 bp). DNA lesion frequencies were calculated as previously described [8, 9, 10], by the formula λ = -ln (A_D/A_Ct), where λ = lesion frequency per fragment, A_D = amplification of treatment, A_Ct = amplification of control.

Measurement of cellular metabolic flux

Seahorse Bioscience XF Instrumentation (Agilent, Santa Clara, CA) is a powerful technology that allows high-throughput measurement of cellular metabolic flux, e.g. rate of oxygen consumption (OCR; reflecting oxidative phosphorylation), and rate of extracellular acidification (ECAR; informing on rate of glycolysis). Briefly, cells were seeded into Seahorse 96-well XF Cell Culture Microplate V3-PET (Agilent, Santa Clara, CA) at 10,000 cells per well in 200 μL media. Each group of samples in an individual experiment included 10 technical replicates with 2 additional wells for determining cell number at time of exposure to a xenobiotic. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO₂. After a 48-h incubation period, cells were exposed to ascorbate (50 pmol cell⁻¹) for 1 h. Following treatment, cells were washed gently with media; pre-warmed XF Base Medium Minimal DMEM (Agilent, Santa Clara, CA), containing 25 mM glucose, 2 mM Glutamax™ (Thermo Fisher Scientific, Waltham, MA) and 1 mM sodium pyruvate, with the pH of media being approximately 7.4 and buffer capacity determined prior to each assay; OCR and ECAR were then measured using the Seahorse XF96 analyzer with the standard mitochondrial stress test [11]. ECAR was converted to PPR using buffer capacity of the assay medium and the Seahorse XF96 software. For the mitochondrial stress test, oligomycin (2.5 μM final), FCCP (0.3 μM final) and rotenone with antimycin A (10 μM final each) were sequentially injected after determining the resulting OCR and ECAR of each addition. At the end of experiments, cell densities (cells L⁻¹) from representative wells (at least 5 wells) were determined with a hemocytometer. Using the average of cell density, OCR and ECAR were then normalized per cell for control and treatment. The basal OCR were calculated from the parameters obtained from the mitochondrial stress test; OCRbasal = OCRATP + OCRprotonleak. The basal ECAR was calculated from an average of first three readings of ECAR.
Immunofluorescence

Cells were seeded on sterile round coverslips (15 mm) in 12-well plates at a density of 100,000 cells per well in duplicate. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO₂. To investigate formation of PAR, cells were exposed to ascorbate at indicated concentrations for 30 min. For bovine catalase experiments, cells were incubated with catalase at indicated concentration immediately prior exposed to ascorbate.

After treatment, cells were washed twice with culture media, fixed on coverslips with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS (room temperature; 30 min), and incubated with permeabilizing agent, 0.2% Triton X-100 in PBS (room temperature; 15 min). Following permeabilization, coverslips were washed with PBS (3 x 5 min), blocked with blocking solution (5% normal goat serum (Electron Microscopy Sciences, Hatfield, PA) + 5% bovine serum albumin in PBS) for 30 min, incubated with mouse monoclonal anti-PAR (1:100 in PBS; Santa Cruz Biotechnology, Dallas, TX; Cat. no. sc-56198) at 4 °C for 18 h, and washed with PBS (3 x 5 min). Coverslips were subsequently incubated in dark with Alexa Fluor 488-conjugated goat anti-mouse polyclonal secondary antibody (1:200 in PBS; Thermo Fisher Scientific, Waltham, MA; Cat. no. A-11017) for 2 h at room temperature. Fixed and stained cells were then washed with PBS (3 x 5 min) and incubated with TO-PRO-3 (1:1000 in PBS; 15 min; Thermo Fisher Scientific, Waltham, MA; Cat. no. T3605) for nuclear staining. After final washing with PBS, coverslips were mounted face-down onto microscope slide using Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired with laser scanning microscope Zeiss LSM 510 with x63 objective lens (Carl Zeiss AG, Germany). All microscopic parameters were kept constant across samples. At least three different areas were imaged per samples.

Western blot analysis

Cells were seeded into multiple culture dishes (100 mm x 20 mm) at 500,000 cells per dish in 10.0 mL media. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO₂. For ascorbate treatment, cells were exposed to ascorbate for 1 h, unless specified otherwise. For bovine catalase experiments, cells were incubated with catalase immediately prior to exposure to P-AscH⁺. For prexasertib treatment, cells were pre-treated with prexasertib for 18 h then exposed to P-AscH⁺ + prexasertib for 1 h. For olaparib treatment, cells were pre-treated with olaparib for 18 h then exposed to P-AscH⁺ + prexasertib for 1 h, unless specified otherwise.

After treatment, media were removed; cells were washed with PBS and allowed to recover in fresh media for 1 h before harvesting, unless specified otherwise. Cells were then harvested by scraping on ice and lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA) with protease inhibitor cocktail (cOmplete™ Mini; Millipore Sigma, Burlington, MA) and phosphatase inhibitor cocktail (PhosSTOP™; Millipore Sigma, Burlington, MA). Prior to electrophoresis, the protein concentrations were determined in the supernatant of whole cell lysates using a modified Lowry assay (DC™ protein assay; Bio-Rad, Hercules, CA). Equal amounts of protein lysates (20.0 – 50.0 μg) were separated by 4 - 20% precast polyacrylamide gel (Bio-Rad, Hercules, CA) and then electrotransferred to Immobilon PVDF membrane (Millipore Sigma, Burlington, MA). Membranes were blocked in 5% nonfat milk in PBST for 1 h, and then incubated with primary antibody overnight at 4 °C; rabbit monoclonal anti-catalase (1:1000; Cell Signaling Technology, Danvers, MA; Cat. no. 12980), mouse monoclonal anti-PAR (1:500, EMD Millipore, Cat. no. AM80), rabbit polyclonal anti-PARP1 (1:1000, Cell signaling, Cat. no. 9542S), mouse monoclonal anti-Chk1 (1:1000; Cell Signaling Technology, Danvers, MA; Cat. no. 2360), rabbit monoclonal anti phosphor-Chk1 (Ser345) (1:1000; Cell Signaling Technology, Danvers, MA; Cat. no. 2348), and rabbit monoclonal anti-phospho-Histone H2AX
(Ser139) (1:2000; Cell Signaling Technology, Danvers, MA; Cat. no. 9718). Actin and alpha-tubulin (loading control) were detected with rabbit polyclonal anti-actin (1:10,000, Millipore sigma, Cat. no. A2103), or mouse monoclonal anti-alpha tubulin (1:50000, Proteintech, Cat. no. 66031-1), respectively. Horseradish peroxidase-conjugated goat anti-rabbit (1:10,000; Millipore Sigma, Burlington, MA; Cat. no. AP307P) or goat anti-mouse (1:10,000; Millipore Sigma, Burlington, MA; Cat. no. AP308P) was used as a secondary antibody. The signal was developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA).

For xenograft tissues, tissues were homogenized in 500 μL of PhosphoSafe Extraction Reagent (Millipore Sigma, Burlington, MA) containing protease inhibitor cocktail (cOmplete™ Mini; Millipore Sigma, Burlington, MA). The conditions for western blot analysis were similar to cell lysates from in vitro experiment.

**MTT assay and synergy calculation**

The MTT assay (thiazolyl blue tetrazolium bromide) is a colorimetric approach that is widely used to assess cell proliferation and metabolic activity. This assay relies on the ability of mitochondrial oxidoreductase activity to convert the tetrazolium dye MTT into its insoluble formazan [12]. In this present study, the MTT assay was used to evaluate the potential synergistic interaction of P-AscH and prexasertib. Briefly, cells were seeded into 96-well plate at 10,000 cells per well in 200 μL media. Each group of samples in an individual experiment included 3 technical replicates with 2 additional wells for determining cell number at time of exposure to a xenobiotic. Cells were cultured in their respective media for 48 h at 37 °C, 5% CO₂ before exposure to experimental conditions. For ascorbate treatment, cells were exposed to ascorbate for 1 h. For prexasertib treatment, cells were treated with prexasertib for 18 h. For prexasertib + P-AscH combination, cells were pre-treated with prexasertib for 18 h then exposed to P-AscH for 1 h.

Following treatment, exposure media were removed; cells were washed twice with PBS and incubated in 200 μL fresh media for additional 48 h. After 48-h period, cells were incubated in dark with 100 μL MTT solution (1 mg/mL in serum-free media; Millipore Sigma, Burlington, MA) for 3 h at 37 °C. At the end of the incubation, supernatants were aspirated and the resulting formazan crystals were dissolved in 100 μL DMSO. The absorbance of formazan at 540 nm was determined using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA).

To evaluate synergistic effects of P-AscH and prexasertib, the combination index (CI) was calculated using method of Chou and Talalay with five drug concentrations at a fixed-dose ratio; CI < 1, synergism; CI = 1, additive effect; CI > 1 antagonism [1]. The data were analyzed using Compusyn software (ComboSyn, Inc., Paramus, NJ).

**Murine xenograft models**

Thirty-day-old athymic nude mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The nude mice protocol was reviewed and approved by the Animal Care and Use Committee of The University of Iowa. Animals were allowed to acclimate in the unit for 1 week before any manipulations were performed. MIA PaCa-2 human pancreatic tumor cells (2 to 3) x 10⁶) were delivered subcutaneously into each flank region of nude mice with a 1-mL tuberculin syringe equipped with a 25-gauge needle. The tumors were allowed to grow until they reached between 3 mm and 4 mm in greatest dimension (3 weeks) before experimental treatment began. Once tumors were established, mice were treated intraperitoneally with
ascorbate (4 g kg\(^{-1}\)) or saline (NaCl at 1 M) twice daily for 5.5 days. On day-5.5, mice were euthanized by CO\(_2\) asphyxiation. Tumors were then harvested for experimental analyses.

**Statistical analysis**

One-way ANOVA followed by Tukey’s post hoc was used to examine statistical differences between means for multiple comparisons. All means were calculated from at least three independent experiments, with error bars representing SEM. Western blot analyses were performed with at least two biological replicates. Data are expressed as mean ± SEM. Test of statistical significance were performed using GraphPad Prism 7.0d (GraphPad Software, La Jolla, CA).
References


