

Omeprazole Enhances Olaparib-Associated Cytotoxicity in Gastric Cancer Cells: Metabolic and Transcriptional Responses

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Abstract: Gastric cancer remains a major therapeutic challenge, and the clinical activity of poly(ADP-ribose) polymerase inhibitors such as olaparib may be limited in tumors without established DNA-repair vulnerabilities. This study investigated the effects of combining olaparib with the proton-pump inhibitor omeprazole in AGS and MKN45 gastric cancer cells. Cell viability and cytotoxicity were evaluated using MTS and LDH assays. Oxidative DNA-damage-associated signal, gene expression, lactate production, intracellular ATP, glucose uptake, and Caspase-3 activity were assessed using ELISA-based, quantitative PCR, and colorimetric methods. Under the tested *in-vitro* conditions, omeprazole enhanced the olaparib-associated reduction in MTS signal and increased LDH release. Combination-index analysis indicated concentration- and cell-line-dependent interactions: all tested AGS combinations had CI values below 1, although the lowest AGS combination was close to additivity, whereas the lowest tested MKN45 combination was antagonistic and the remaining tested MKN45 combinations had CI values below 1. Combination treatment was associated with an increased 8-OHdG signal, reduced lactate production, decreased intracellular ATP levels and glucose uptake, and altered expression of BRCA1, RAD51, GLUT1/SLC2A1, HK2, LDHA, MCT4/SLC16A3, p21/CDKN1A, and p16/CDKN2A. Increased Caspase-3 activity was consistent with enhanced apoptosis-associated signaling. The observed changes in BRCA1 and RAD51 transcript levels suggest a potential modulation of DNA-repair-related responses, although their functional significance remains to be established. Overall, these findings support further investigation of the olaparib–omeprazole combination as a hypothesis-generating strategy in gastric cancer models, with future studies required to clarify its molecular basis and translational relevance.

Keywords: Gastric cancer, Olaparib, Omeprazole, PARP inhibition, Metabolic perturbation, Transcriptional response.

INTRODUCTION

Gastric cancer remains a major global health problem and is among the most frequently diagnosed malignancies and important causes of cancer-related mortality worldwide [1-3]. Its biological heterogeneity includes distinct molecular and clinical subgroups, including chromosomal instability, microsatellite instability, genomic stability, and Epstein–Barr virus-associated disease [4]. This heterogeneity contributes to differences in treatment response and underscores the need for biomarker-informed therapeutic strategies. Despite advances in surgery, systemic therapy, and molecular classification, outcomes remain limited for many patients with advanced or metastatic disease [2, 3].

Poly(ADP-ribose) polymerase inhibitors, including olaparib, are important agents in the treatment of selected tumors with defects in DNA-repair pathways. Their antitumor activity is associated with interference in the repair of DNA lesions and increased replication-associated stress, particularly in cells with limited capacity to maintain genome integrity [5-8]. Clinical studies in ovarian, breast, pancreatic, and prostate

cancers have shown that the activity of these agents is influenced by tumor type, treatment setting, and the biological context of DNA repair [9-13]. In gastric cancer, however, the clinical benefit of olaparib-based treatment has been more limited in unselected populations, emphasizing the need to understand treatment response and to identify combination strategies that can be evaluated in appropriate experimental models [9, 10].

Proton-pump inhibitors, including omeprazole, are widely used for acid-related gastrointestinal conditions. In addition to their established pharmacological effects on gastric acid secretion, these agents have been investigated in experimental cancer models because of possible effects on intracellular pH regulation, metabolism, stress responses, and treatment sensitivity [14-17]. Previous studies have reported that proton-pump inhibitors may influence cancer-cell survival and responses to anticancer agents, although the magnitude and mechanism of these effects appear to depend on the drug, concentration, cellular model, and experimental conditions [15-17]. These observations provide a rationale for examining omeprazole as a potential modifier of olaparib-associated responses in gastric cancer cells, while also requiring cautious interpretation of concentration-dependent *in-vitro* findings.

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Cancer-cell metabolism is closely linked to proliferation, stress adaptation, and treatment response. Increased reliance on aerobic glycolysis can be accompanied by changes in glucose transport, lactate production, ATP availability, and the expression of metabolic regulators such as GLUT1, HK2, LDHA, and MCT4 [18-21]. Metabolic measurements can therefore provide useful information about treatment-associated cellular responses. However, changes in metabolic activity may also influence viability assays that depend on cellular reduction or energy metabolism. For this reason, metabolic readouts should be interpreted together with cytotoxicity-associated, DNA-damage-associated, apoptosis-associated, and transcriptional measurements rather than being treated as direct evidence of a single causal pathway.

Changes in the expression of DNA-repair-related genes may provide information about the cellular response to treatment. BRCA1 and RAD51 are involved in homologous-recombination biology [5, 6, 22], but transcript abundance alone does not establish protein abundance, protein activity, pathway competence, or the overall DNA-repair phenotype of a cell. Similarly, changes in p21 and p16 transcripts may reflect altered cell-cycle regulation or a stress-associated transcriptional response but do not, by themselves, establish cellular senescence [23].

The present study investigated whether omeprazole modifies the response of AGS and MKN45 gastric cancer cells to olaparib under *in-vitro* conditions. The study evaluated treatment-associated changes in MTS signal, LDH release, 8-OHdG-associated oxidative DNA-damage signal, BRCA1 and RAD51 transcript levels, glycolysis-related gene expression, lactate production, intracellular ATP, glucose uptake, and Caspase-3 activity. AGS and MKN45 cells were used as experimental gastric cancer models rather than as definitive representatives of a clinically established DNA-repair category. The results are interpreted as associations between combination treatment and measured cellular readouts. In particular, transcript-level changes in BRCA1 and RAD51 are considered DNA-repair-related transcriptional observations, while the broader molecular and translational implications of the combination remain to be determined in future studies.

MATERIALS AND METHODS

Cell Lines and Culture Rationale

The human gastric adenocarcinoma cell lines AGS and MKN45 were selected for this study to represent

distinct clinical and molecular profiles of gastric cancer. AGS cells were derived from a primary gastric tumor, whereas MKN45 cells, derived from a liver metastasis, represent a poorly differentiated, highly aggressive phenotype with high metastatic potential. Both cell lines were used to investigate treatment-associated responses to olaparib and omeprazole. These cell lines were kindly provided by Assistant Professor Dr. Ceyda Okudu, Department of Molecular Biology and Genetics, Faculty of Engineering and Natural Sciences, Istanbul Atlas University. Both cell lines were maintained in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (10,000 U/mL; Gibco). Cultures were incubated in a humidified incubator (Thermo Scientific Heracell VIOS 160i) at 37°C with 5% CO₂. Mycoplasma contamination was routinely ruled out every 30 days using the MycoAlert PLUS Detection Kit (Lonza, Basel, Switzerland). To preserve the original phenotypic and genotypic characteristics, all experiments were conducted using cells between passages 5 and 20.

Chemical Reagents and Preparation

Olaparib (AZD2281, >99% purity) was obtained from Selleckchem (Houston, TX, USA). Omeprazole (pharmaceutical grade, >98% purity) was purchased from Sigma-Aldrich. Primary stock solutions were prepared in molecular biology grade DMSO (Sigma-Aldrich) at concentrations of 10 mM for olaparib and 100 mM for omeprazole. These stocks were filtered through 0.22 µm PTFE syringe filters, aliquoted, and stored at -80°C in the dark. Working solutions were freshly prepared by diluting the stocks in RPMI-1640 medium immediately prior to each experiment. The final concentration of DMSO in all treatment and control groups was strictly maintained at 0.1% (v/v) to minimize potential vehicle-associated effects.

Cell Viability Assay

The treatment-associated viability/metabolic signal was assessed using the MTS-based CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were seeded in 96-well clear-bottom microplates (Greiner Bio-One, Kremsmunster, Austria) at a density of 5.0×10^3 cells per well in 100 µL of complete medium. After a 24-hour attachment period, the medium was replaced with 100 µL of medium containing olaparib at concentrations of 0, 1, 2, 5, 10, or 20 µM, with or without omeprazole at a concentration of 40 µM. The omeprazole concentration

was selected based on preliminary *in-vitro* dose-finding experiments and its limited monotherapy-associated effect under the tested conditions; it should not be interpreted as a clinically validated exposure. Following 48 hours of treatment, 20 μ L of MTS reagent was added to each well. After an additional 2-hour incubation at 37°C, absorbance was recorded at 490 nm using a Bio-Rad iMark microplate reader. The resulting signal was interpreted as a treatment-associated viability/metabolic readout rather than as a direct measure of cell number or proliferation.

Evaluation of Combination Effects

The interaction between olaparib and omeprazole was evaluated using the median-effect principle of Chou and Talalay. MTS-derived viability-associated signals were used to generate dose–effect curves for olaparib alone and in combination with a fixed concentration of omeprazole (40 μ M). Combination-index (CI) values were calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) according to the Chou–Talalay method [24]. CI values below 1, equal to 1, and above 1 were interpreted as synergistic, additive, and antagonistic interactions, respectively. Because the MTS assay reflects cellular metabolic activity, the CI values were interpreted as dose-specific interactions based on the measured viability-associated signal and not as evidence of a specific molecular mechanism.

Quantification of LDH Release and Cytotoxicity

To assess treatment-associated membrane damage and cytotoxicity, lactate dehydrogenase (LDH) release was measured using the colorimetric Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). After the 48-hour treatment period, 100 μ L of cell-free supernatant was collected and transferred to a fresh 96-well plate. The LDH catalyst and dye solution were added, and the plate was incubated for 30 minutes at room temperature in the dark. The reaction was stopped by adding 50 μ L of stop solution, and absorbance was measured at 490 nm. Total LDH release was determined by lysing a separate set of control cells with 1% Triton X-100. Cytotoxicity was calculated as follows:

$$(\text{Experimental LDH} - \text{Spontaneous LDH}) / (\text{Maximum LDH} - \text{Spontaneous LDH}) \times 100.$$

Assessment of the 8-OHdG-Associated Oxidative DNA-Damage Signal

The treatment-associated oxidative DNA-damage-associated signal was assessed by measuring 8-

hydroxy-2'-deoxyguanosine (8-OHdG) levels. Genomic DNA was extracted from treated cells using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). A 5- μ g aliquot of DNA was enzymatically digested into single nucleosides using nuclease P1 and alkaline phosphatase (Cayman Chemical). The resulting samples were analyzed using a competitive 8-OHdG ELISA kit (Cayman Chemical, Cat# 589320). Absorbance was measured at 450 nm, and 8-OHdG concentration was calculated from a standard curve and expressed as pg per μ g of total DNA.

RNA Extraction and Quantitative Real-Time PCR (qPCR)

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured, and 1 μ g of RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20 μ L. Quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR System using PowerUp SYBR Green Master Mix. The amplification protocol consisted of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Amplification specificity was assessed by melting-curve analysis. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH. Each sample was analyzed using the technical replicate design described in the Statistical Analysis section. Primer sequences, amplicon lengths, and calculated melting temperatures are provided in Table 1.

Metabolic Profiling

Extracellular Lactate Secretion Assay

To assess treatment-associated changes in extracellular lactate levels, L(+)-lactate was measured in conditioned medium using a colorimetric Lactate Assay Kit (Sigma-Aldrich, Cat# MAK064). Following the 48-hour treatment period, 1.0 mL of culture supernatant was collected and centrifuged at 1,000 $\times$ g for 5 minutes at 4°C to remove cellular debris. Samples were then filtered through 10-kDa molecular-weight-cutoff spin filters (Amicon Ultra-0.5, Merck Millipore) to remove proteins that could interfere with the enzymatic reaction. A 50- μ L aliquot of the deproteinized sample was transferred to a 96-well plate and mixed with 50 μ L

Table 1: Primer Sequences Used for Quantitative RT-PCR

Gene	Primer Direction	Sequence (5'–3')	Product Length (bp)	Tm (°C)
GAPDH	Forward- Reverse	TCTCTGCTCCTCCTGTTCGA GCGCCCAATACGACCAAATC	122	59.96 59.97
BRCA1	Forward- Reverse	CACAGCCACTACTGACTGCA AGAGTGAGAGGAGCTCCCAG	104	59.97 60.03
RAD51	Forward- Reverse	TAAGTCCAGCTTGGCCAAGG AGTAGCTGGGACTACAGGCA	98	59.96 59.96
GLUT1/SLC2A1	Forward- Reverse	ACTCATGACCATCGCGCTAG GGACCCTGGCTGAAGAGTTC	150	59.97 60.04
HK2	Forward- Reverse	CCCGGGAAAGCAACTGTTTG ACCGGTGTTGAGAAGCTCTG	136	59.97 59.97
LDHA	Forward- Reverse	CCAACATGGCAGCCTTTTCC TCACGTTACGCTGGACCAAA	149	60.04 59.90
MCT4/SLC16A3	Forward- Reverse	ATAGCATTCTAGCAGGGGC ACATTTTCAGAGGCCACCCTG	90	59.96 59.96
p21	Forward- Reverse	TCTAGGAGGGAGACACTGGC TGTCTGACTCCTGTTCCGC	109	60.03 59.97
p16/CDKN2A	Forward- Reverse	CACCGCTTCTGCCTTTTCAC ATGAAGTCGACAGCTTCCGG	119	60.04 60.11

of reaction mixture containing 46 μ L of Lactate Assay Buffer, 2 μ L of Lactate Enzyme Mix, and 2 μ L of Lactate Probe. The reaction was incubated at room temperature (25°C) for 30 minutes in the dark. Absorbance was measured at 450 nm using a Bio-Rad iMark microplate reader. Absolute lactate concentrations were calculated using an L(+)-lactate standard curve ranging from 0 to 10 nmol per well and normalized to the total protein concentration of the corresponding cell lysate. This assay was used to characterize treatment-associated extracellular lactate levels and was not interpreted as a direct measurement of complete glycolytic flux.

Intracellular ATP Quantification

Intracellular ATP levels were quantified using the ATP Colorimetric Assay Kit (Abcam, Cat# ab83355). Cells were harvested by trypsinization, washed twice with ice-cold PBS, and lysed in 100 μ L of ATP Assay Buffer. To prevent the rapid degradation of ATP by endogenous ATPases, the samples were immediately deproteinized by adding 4M perchloric acid (PCA) to a final concentration of 1M. Following a 5-minute

incubation on ice, the samples were centrifuged at 13,000 $\times$ g for 2 minutes at 4°C. The resulting acidic supernatant was carefully collected and neutralized by adding 2M KOH until the pH reached 6.5–8.0. The precipitated potassium perchlorate was removed by a second centrifugation step. A 50 μ L aliquot of the neutralized sample was mixed with 50 μ L of the ATP Reaction Mix (containing ATP Assay Buffer, ATP Converter, ATP Developer, and ATP Probe). The reaction was incubated at room temperature for 30 minutes in the dark. In this assay, ATP is phosphorylated by the converter to produce a product that reacts with the developer and probe to form a stable colorimetric product. Absorbance was recorded at 570 nm. ATP levels were calculated from a standard curve (0–10 nmol/well) and expressed as nmol per mg of total protein.

Glucose Uptake Measurement

A 2-deoxyglucose-based colorimetric assay was used to measure the glucose uptake-associated signal in treated cells using the Glucose Uptake Colorimetric Assay Kit (Abcam, Cat# ab136955). Cells were seeded

in 96-well plates and, upon reaching approximately 80% confluence, were serum-starved in 100 μ L of RPMI-1640 containing 0.5% FBS for 4 hours. Cells were then washed three times with PBS and incubated in 100 μ L of Krebs–Ringer phosphate–HEPES (KRPH) buffer containing 2% BSA for 40 minutes. Subsequently, 10 mM 2-deoxyglucose (2-DG) was added, and cells were incubated for 20 minutes at 37°C. The reaction was terminated by washing the cells three times with ice-cold PBS. Cells were lysed in 20 μ L of Extraction Buffer and heated at 85°C for 40 minutes to degrade endogenous NADP and inactivate enzymes. Following neutralization with 20 μ L of Neutralization Buffer, samples were incubated with the enzymatic reaction mixture, which converts accumulated 2-deoxyglucose-6-phosphate into NADPH. The resulting NADPH signal was detected using a coupled recycling reaction and colorimetric probe, and absorbance was measured at 412 nm. Glucose uptake-associated values were calculated using a 2-DG6P standard curve and normalized to total protein content determined by the BCA assay. The result was interpreted as a treatment-associated glucose uptake readout rather than as a direct measurement of transporter protein activity.

Caspase-3 Activity Assay

Caspase-3 enzymatic activity was measured as an apoptosis-associated readout using the Caspase-3 Colorimetric Assay Kit (Sigma-Aldrich, Cat# CASP3C). The assay is based on spectrophotometric detection of p-nitroaniline at 405 nm following cleavage of the Ac-DEVD-pNA peptide substrate.

Statistical Analysis and Data Visualization

Statistical analyses and data visualization were performed using GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, CA, USA). Each experiment consisted of three independent biological replicates ($n = 3$), with three technical replicates performed for each condition. Technical replicate wells were averaged within each biological experiment before statistical analysis, and the biological experiment was treated as the statistical unit. Data are presented as mean $\pm$ standard deviation (SD), and individual biological replicate values are displayed as individual points in the revised figures. Differences among groups were analyzed using one-way or two-way analysis of variance (ANOVA), as appropriate, followed by Tukey's multiple-comparisons test. A two-sided p value < 0.05 was considered statistically significant. Statistical significance is indicated as

follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

RESULTS

Omeprazole Modifies the Olaparib-Associated MTS Viability/Metabolic Signal

To identify a fixed omeprazole concentration for the subsequent combination experiments, AGS and MKN45 gastric cancer cells were exposed to increasing concentrations of omeprazole for 48 hours. Omeprazole produced limited monotherapy-associated effects at lower concentrations. At 40 μ M, the MTS-derived signal was $92.5 \pm 2.4\%$ of the control value in AGS cells and $91.2 \pm 2.8\%$ in MKN45 cells (Figure 1A). A more pronounced reduction in the MTS-derived signal was observed at higher concentrations. Based on the limited monotherapy-associated effect observed at 40 μ M, this concentration was selected for the subsequent combination experiments. This *in-vitro* concentration should not be interpreted as a clinically validated exposure.

AGS and MKN45 cells were then treated with increasing concentrations of olaparib in the absence or presence of 40 μ M omeprazole. In AGS cells, olaparib monotherapy produced a concentration-associated reduction in the MTS-derived viability-associated signal, with an estimated IC₅₀ of approximately 9.2 μ M. In the presence of 40 μ M omeprazole, the estimated olaparib IC₅₀ decreased to approximately 2.8 μ M (Figure 1B). In MKN45 cells, the combination curve was lower than the olaparib-alone curve across the tested olaparib concentrations (Figure 1C). These findings indicate an enhanced treatment-associated reduction in the MTS-derived signal in the presence of omeprazole. Because the MTS assay is influenced by cellular metabolic activity, these results were interpreted as viability-associated/metabolic responses rather than as a direct measurement of cell number or a specific molecular mechanism.

Quantitative Assessment of Combination Effects

The interaction between olaparib and omeprazole was quantitatively evaluated using the Chou–Talalay combination-index method. MTS-derived dose–effect data were analyzed using a non-constant-ratio design, in which olaparib concentrations were varied from 1 to 20 μ M while omeprazole was maintained at a fixed concentration of 40 μ M. Combination-index values were calculated for each tested olaparib–omeprazole dose pair. Because the analysis was based on MTS-

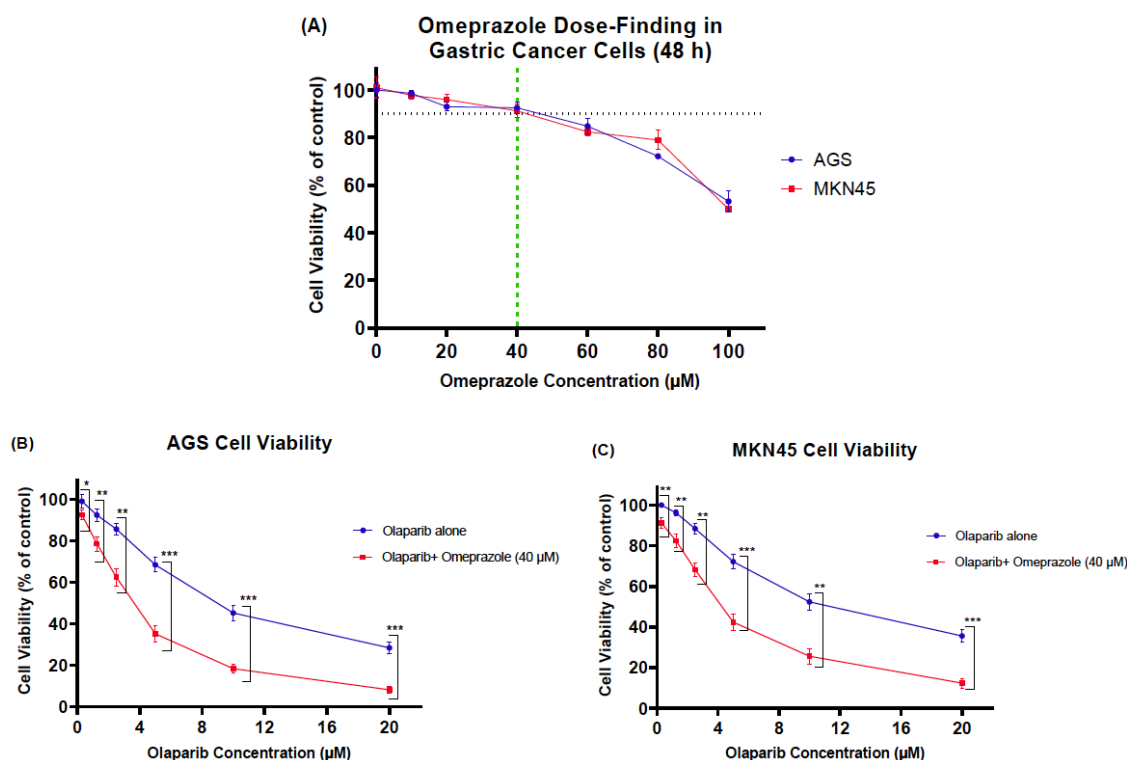


Figure 1: Dose-finding and olaparib-associated MTS viability/metabolic signal in gastric cancer cells. **(A)** MTS-derived signal following treatment with increasing concentrations of omeprazole (0–100 μM) in AGS and MKN45 cells indicating the 90% viability threshold (black dotted line) and selected dose (40 μM , green dashed line). **(B)** Olaparib dose-response curves in the absence or presence of 40 μM omeprazole in AGS cells. **(C)** Corresponding olaparib dose-response curves in MKN45 cells. Data represent mean $\pm$ SD ($n=3$) from three independent biological experiments. Technical replicate wells were averaged within each biological experiment before statistical analysis. Each point represents one independent biological experiment. Asterisks indicate significance (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

derived viability-associated signals, the CI values were interpreted as dose-specific drug interactions under the tested *in-vitro* conditions.

In AGS cells, the calculated CI values were 0.96390, 0.82577, 0.86808, 0.68042, and 0.64037 at 1, 2, 5, 10, and 20 μM olaparib, respectively. All CI values were below 1, indicating synergistic interactions according to the Chou–Talalay interpretation. The 1 μM olaparib plus 40 μM omeprazole combination produced a synergistic interaction close to additivity (CI = 0.96390). The 2 and 5 μM combinations showed slight/mild synergistic interactions (CI = 0.82577 and 0.86808, respectively), whereas the 10 and 20 μM combinations showed moderate synergistic interactions (CI = 0.68042 and 0.64037, respectively).

In MKN45 cells, the interaction profile differed from that observed in AGS cells. The CI values were 1.32819, 0.70123, 0.70186, 0.51797, and 0.61577 at 1, 2, 5, 10, and 20 μM olaparib, respectively, in the presence of 40 μM omeprazole. The 1 μM combination showed antagonism (CI = 1.32983). The 2 and 5 μM combinations had CI values close to the slight/mild-synergism threshold (CI = 0.70497 and 0.70186,

respectively). The 10 and 20 μM combinations showed moderate synergistic interactions, with CI values of 0.51797 and 0.61577, respectively. Thus, unlike AGS cells, MKN45 cells showed an antagonistic response at the lowest tested olaparib concentration, followed by synergistic interactions at higher concentrations.

Considering the magnitude of the treatment-associated response and the need to retain a measurable dynamic range for the subsequent endpoint analyses, the combination of 10 μM olaparib and 40 μM omeprazole was selected for the remaining experiments. This treatment condition was subsequently used to assess the 8-OHdG-associated signal, LDH release, gene-expression changes, extracellular lactate, intracellular ATP, glucose uptake, and Caspase-3 activity.

Overall, the CI results indicated concentration- and cell-line-dependent interactions. Antagonism was observed at the lowest tested MKN45 combination, whereas CI values below 1 were observed for the remaining tested MKN45 combinations and for all tested AGS combinations. The findings should not be interpreted as evidence of a specific molecular

Table 2: Chou–Talalay Combination-Index Analysis of Olaparib and Omeprazole in AGS and MKN45 Cells

Cell line	Olaparib (μM)	Omeprazole (μM)	Fraction affected (Fa)	Combination index (CI)	Interpretation
AGS	1	40	0.18	0.96390	Synergistic interaction; close to additivity
AGS	2	40	0.30	0.82577	Synergistic interaction; slight/moderate synergism
AGS	5	40	0.48	0.86808	Synergistic interaction; slight/moderate synergism
AGS	10	40	0.69	0.68042	Synergistic interaction; moderate synergism
AGS	20	40	0.82	0.64037	Synergistic interaction; moderate synergism
MKN45	1	40	0.15	1.32819	Antagonistic interaction
MKN45	2	40	0.35	0.70123	Synergistic interaction; close to slight/mild-synergism threshold
MKN45	5	40	0.49	0.70186	Synergistic interaction; slight/mild synergism
MKN45	10	40	0.68	0.51797	Synergistic interaction; moderate synergism
MKN45	20	40	0.75	0.61577	Synergistic interaction; moderate synergism

Note: CI values represent concentration-specific interactions calculated from MTS-derived viability-associated signals under the tested *in-vitro* conditions. The interaction was not uniformly synergistic across all concentrations and cell lines. CI < 1 indicates a synergistic interaction, CI = 1 indicates an additive interaction, and CI > 1 indicates an antagonistic interaction according to the Chou–Talalay interpretation. Using the descriptive classification applied in this study, synergism is further classified as: mild synergism: (0.7–0.9), moderate synergism (0.3–0.7), strong synergism (0.1–0.3), and very strong synergism (<0.1) [25]. All CI values were calculated from MTS-derived viability-associated signals in AGS and MKN45 cells.

mechanism or as a direct measure of complete cell death, because the analysis was based on an MTS-derived viability-associated signal.

Treatment-Associated 8-OHdG Signal and LDH Release

To characterize treatment-associated oxidative DNA-damage-associated and cytotoxicity-associated responses, the accumulation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a critical marker of oxidative DNA damage was quantified. In both AGS and MKN45 cells, the combination of olaparib and omeprazole led to an increase in the 8-OHdG-associated signal. Specifically, in AGS cells, 8-OHdG levels increased to 35.4 ± 2.5 pg/μg DNA, representing a nearly 3-fold increase over olaparib monotherapy (12.5 ± 1.2 pg/μg DNA) and an 8-fold increase over the control (4.2 ± 0.5 pg/μg DNA) (Figure 2A). A comparable trend was observed in MKN45 cells, where the combination group reached 28.6 ± 2.2 pg/μg DNA (Figure 2A). In AGS cells, LDH release reached $42.8 \pm 3.2\%$, while MKN45 cells showed $36.4 \pm 3.1\%$ release, both representing an increase relative to the corresponding treatment groups, compared to

monotherapies (Figure 2B). These findings support an association between the combination treatment and increased membrane-damage-associated cytotoxicity under the tested conditions. LDH release does not, by itself, establish the complete extent of cell death or long-term loss of proliferative capacity.

Treatment-Associated Changes in DNA-Repair-Related, Metabolic, and Cell-Cycle-Associated Transcripts

Quantitative PCR was used to examine treatment-associated changes in BRCA1, RAD51, GLUT1/SLC2A1, HK2, LDHA, MCT4/SLC16A3, p21/CDKN1A, and p16/CDKN2A transcript levels. Combination treatment was associated with reduced BRCA1 and RAD51 mRNA levels in both cell lines. In AGS cells, BRCA1 and RAD51 transcript levels decreased by approximately 72% and 79%, respectively, in the combination group. In MKN45 cells, the corresponding reductions were approximately 68% and 76% (Figure 3A, B). Combination treatment was associated with altered BRCA1 and RAD51 transcript levels, together with changes in other DNA-repair-related transcripts, in both gastric cancer cell lines.

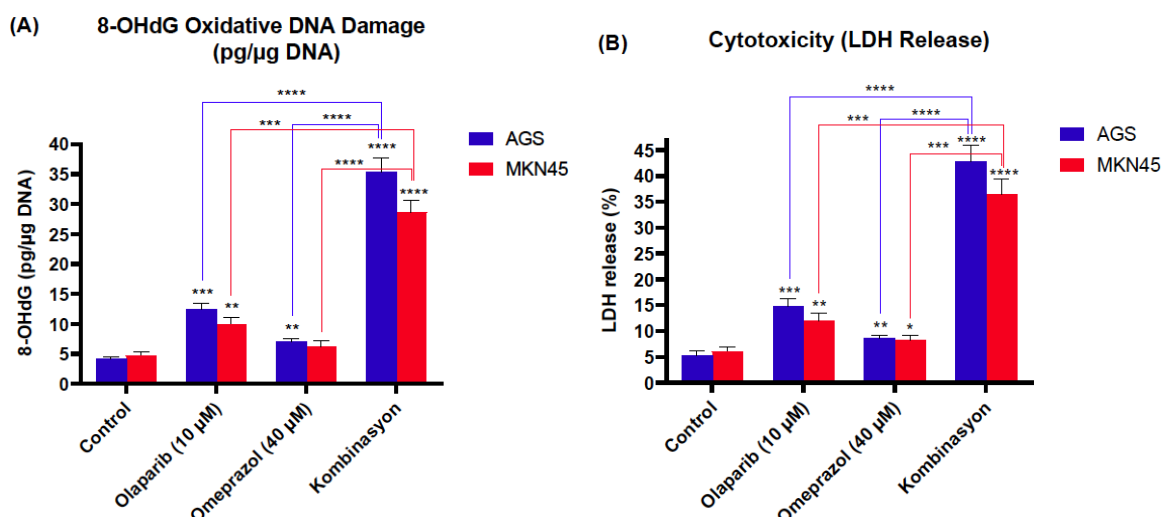


Figure 2: Treatment-associated 8-OHdG signal and LDH release in AGS and MKN45 cells. **(A)** 8-OHdG levels measured using a competitive ELISA and expressed as pg/ μ g DNA. **(B)** LDH release expressed as a percentage of maximum LDH release. Data represent mean $\pm$ SD ($n=3$) from three independent biological experiments. Technical replicate wells were averaged before statistical analysis, and individual biological replicate values are shown as points. Asterisks indicate significance relative to control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

These findings describe a treatment-associated transcriptional response, and their broader biological significance requires further investigation.

Combination treatment was also associated with changes in the expression of GLUT1/SLC2A1, HK2, LDHA, and MCT4/SLC16A3 in both cell lines. The largest reported change among these transcripts was an approximately 75% reduction in MCT4/SLC16A3 expression in AGS cells (Figure 3A, B). In addition, p21/CDKN1A and p16/CDKN2A transcript levels increased following combination treatment. p21/CDKN1A expression increased approximately 6.45-fold in AGS cells and 5.84-fold in MKN45 cells (Figure 3A, B). These results indicate treatment-associated transcriptional changes in genes related to DNA repair, metabolism, and cell-cycle regulation. The p21/CDKN1A and p16/CDKN2A findings represent cell-cycle-associated transcriptional changes and do not, by themselves, establish cellular senescence.

Treatment-Associated Changes in Metabolic Readouts

Colorimetric assays were used to examine extracellular lactate production, intracellular ATP levels, and glucose uptake. In AGS cells, extracellular lactate levels decreased from 425.6 ± 18.4 nmol/mg in the control group to 136.2 ± 12.8 nmol/mg in the combination-treatment group (Figure 4A). A comparable reduction was observed in MKN45 cells, in which lactate levels reached 128.4 ± 11.5 nmol/mg following combination treatment (Figure 4A).

Combination treatment was also associated with an approximately 62% reduction in intracellular ATP levels in both AGS and MKN45 cells compared with the respective control groups (Figure 4B). In addition, glucose uptake-associated values decreased by approximately 55% in both cell lines (Figure 4C). Together, these findings indicate treatment-associated changes in extracellular lactate, intracellular ATP, and glucose uptake under the tested conditions. Because these measurements can be influenced by cell number and overall cellular metabolic activity, they do not demonstrate that metabolic changes caused the observed cytotoxicity-associated or DNA-damage-associated responses.

Caspase-3 Activity Following Combination Treatment

Caspase-3 activity was measured as an apoptosis-associated enzymatic readout. Combination treatment increased Caspase-3 activity approximately 6.8-fold in AGS cells and 5.9-fold in MKN45 cells compared with the respective control groups (Figure 5). These findings are consistent with enhanced apoptosis-associated signaling under the tested conditions. Caspase-3 activity alone does not establish that apoptosis was the exclusive or predominant mode of cell death and does not quantify the complete proportion of affected cells.

DISCUSSION

The present study examined whether omeprazole modifies the response of AGS and MKN45 gastric

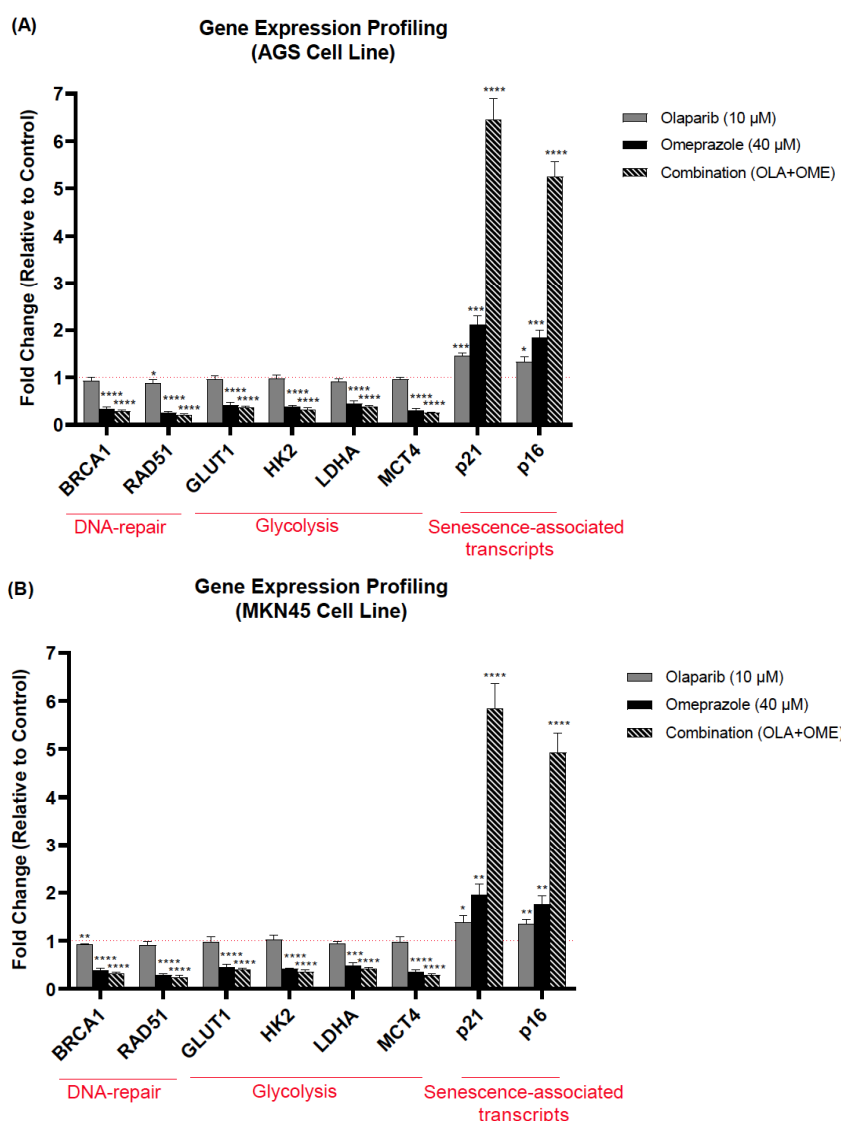


Figure 3: Treatment-associated changes in DNA-repair-related, metabolic, and cell-cycle-associated transcripts in AGS and MKN45 gastric cancer cells. **(A)** AGS cells and **(B)** MKN45 cells were treated with olaparib, omeprazole, or their combination. Quantitative real-time PCR was used to measure the transcript levels of BRCA1, RAD51, GLUT1/SLC2A1, HK2, LDHA, MCT4/SLC16A3, p21/CDKN1A, and p16/CDKN2A. Expression values were normalized to GAPDH and are presented relative to the corresponding control group using the 2- $\Delta\Delta C_t$ method. Data are shown as mean $\pm$ SD from three independent biological experiments ($n = 3$). Technical replicate wells were averaged within each biological experiment before statistical analysis. Each individual point represents one independent biological experiment. Asterisks indicate statistically significant differences relative to the corresponding control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

cancer cells to olaparib. Under the tested *in-vitro* conditions, the combination produced a greater reduction in the MTS-derived viability-associated signal than olaparib alone and was accompanied by changes in LDH release, 8-OHdG-associated signal, lactate production, intracellular ATP, glucose uptake-associated signal, transcript levels, and Caspase-3 activity. The combination-index analysis indicated concentration- and cell-line-dependent interactions. All tested AGS combinations had CI values below 1, although the 1 μ M combination was close to additivity. In MKN45 cells, antagonism was observed at the lowest tested combination, whereas the remaining

tested combinations had CI values below 1. Thus, the interaction was not a uniform property of the olaparib–omeprazole combination. The response was therefore dose- and model-dependent rather than a uniform property of the drug pair.

The gastric-cancer PARP-inhibitor literature provides an important context for interpreting these observations. Reviews of PARP inhibitors in gastric cancer emphasize substantial molecular heterogeneity and the difficulty of translating DNA-damage-response biomarkers from experimental models into clinical selection strategies [26]. In a study of 12 gastric cancer

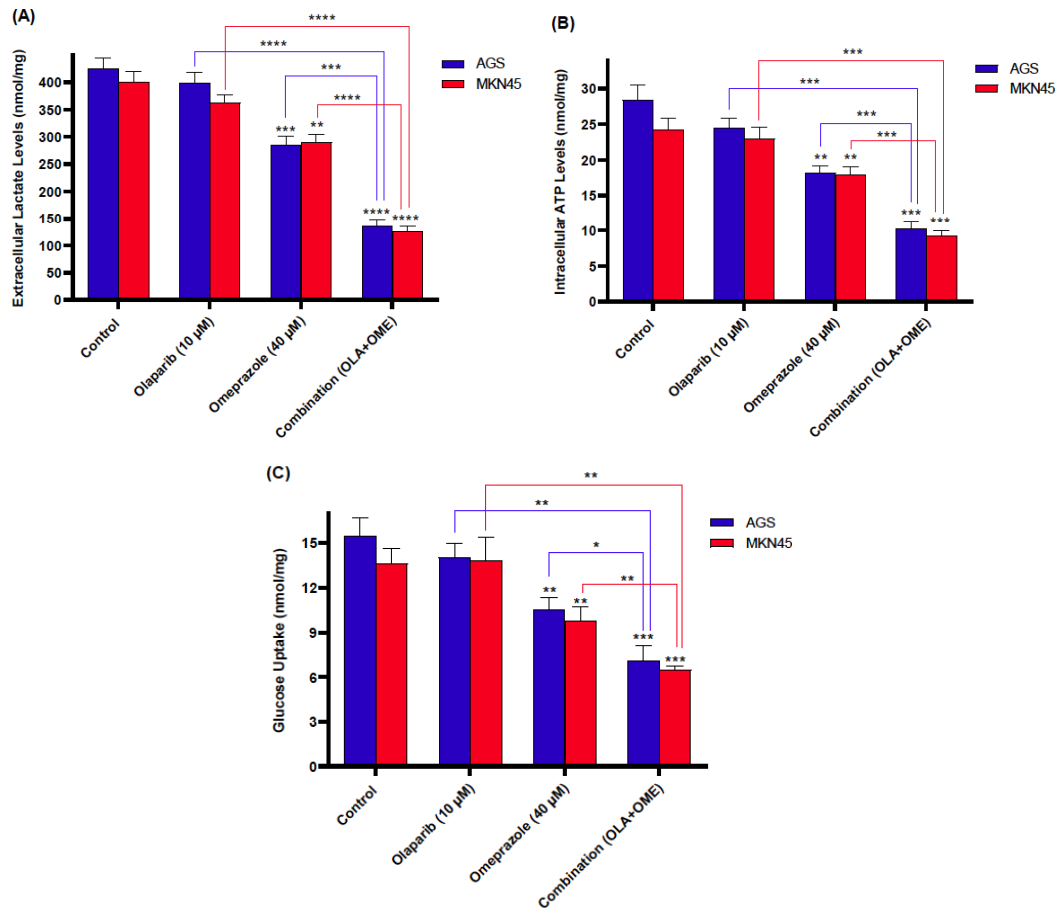


Figure 4: Treatment-Associated Metabolic Readouts. **(A)** Extracellular lactate levels, **(B)** intracellular ATP concentrations, and **(C)** glucose uptake rates in AGS and MKN45 cells following 48 hours of treatment with Control, Olaparib (10 µM), Omeprazole (40 µM), or the Combination. Data represent mean $\pm$ SD ($n=3$). Asterisks indicate significance relative to control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Technical replicate wells were averaged before statistical analysis, and individual biological replicate values are shown as points. These measurements represent treatment-associated metabolic readouts and were not interpreted as evidence of a single causal metabolic mechanism.

cell lines, Shi *et al.* identified MTA2 as a determinant associated with olaparib sensitivity and supported this association using protein-level, replication-stress, and *in-vivo* experiments [27]. Their work is informative for the present study because it shows that olaparib sensitivity can vary substantially among gastric cancer models. However, it also illustrates the difference between a mechanistically validated biomarker study and the present hypothesis-generating analysis, which measured treatment-associated transcript changes and phenotypic readouts but did not perform protein-level or functional DNA-repair validation.

A related comparison is provided by Lim *et al.*, who used three-dimensional gastric cancer patient-derived cell models and a high-throughput combination-screening platform [28]. Their results demonstrated that drug interactions can vary according to patient-derived molecular characteristics and that responses observed in conventional cell lines may not represent the full heterogeneity of gastric tumors. The present work used

two-dimensional AGS and MKN45 cultures, which allowed controlled comparison of the olaparib–omeprazole condition but did not reproduce tumor architecture, stromal interactions, or patient-specific heterogeneity. The differences between the two approaches support the need to test the present combination in additional gastric cancer cell lines, three-dimensional cultures, and patient-derived models.

The clinical experience with olaparib in gastric cancer is also mixed. In the phase 3 GOLD trial, olaparib plus paclitaxel did not significantly improve overall survival in either the overall population or the prespecified ATM-negative population [10]. By contrast, the phase 1/2 NC110066 study evaluated olaparib with ramucirumab in previously treated metastatic gastric and gastroesophageal-junction adenocarcinoma and reported an objective response rate of 14% among evaluable patients, with median progression-free survival of 2.8 months and median overall survival of

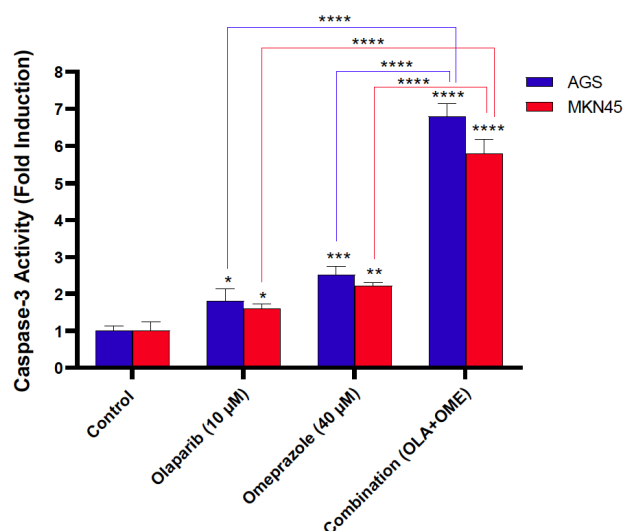


Figure 5: Caspase-3 activity following olaparib and omeprazole treatment. Caspase-3 enzymatic activity was measured using a colorimetric assay in AGS and MKN45 cells. Technical replicate wells were averaged before statistical analysis, and individual biological replicate values are shown as points. Increased Caspase-3 activity is interpreted as an apoptosis-associated readout and not as evidence that apoptosis was the only cell-death pathway involved. Data are presented as mean $\pm$ SD from three independent biological experiments. Asterisks indicate significance relative to control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

7.3 months [29]. The latter study used a different combination partner, a clinical dosing schedule, patient tumors, and radiographic endpoints; it therefore cannot be directly compared with the present cell-culture data. Nevertheless, together with the negative GOLD result, it indicates that the effect of olaparib in gastric cancer is strongly influenced by combination partner, disease setting, biomarker definition, and model selection. Outside gastric cancer, clinically meaningful olaparib benefit has been established in selected biomarker-defined settings such as germline BRCA-mutated metastatic pancreatic cancer [30]. These clinical examples reinforce that an *in-vitro* response in two cell lines should be regarded as preliminary and should not be interpreted as evidence of patient benefit.

The choice of omeprazole is supported by a broader preclinical literature on proton-pump inhibitors as treatment modifiers. Luciani *et al.* reported that PPI pretreatment increased the sensitivity of melanoma, adenocarcinoma, and lymphoma models to cisplatin, 5-fluorouracil, and vinblastine; their experiments included measurements of pH-related parameters and a melanoma xenograft model [31]. In gastric cancer, Feng *et al.* reported that omeprazole pretreatment increased the inhibitory effects of 5-fluorouracil, cisplatin, and paclitaxel in AGS and HGC-27 cells and associated the response with FTO/m6A/mTORC1/

DDIT3 and autophagy-related findings [32]. The present study is similar to Feng *et al.* in using AGS cells and omeprazole as a treatment modifier, but differs in the partner drug, exposure design, endpoints, and molecular measurements. In particular, the present experiments did not test the FTO/m6A/mTORC1/DDIT3 pathway; therefore, the mechanism described by Feng *et al.* cannot be assigned to the current olaparib response.

Other tumor models show why PPI effects should not be assumed to be universal. In colorectal cancer, pantoprazole increased 5-fluorouracil sensitivity in HT29 and RKO cells and enhanced tumor-growth inhibition in xenografts, whereas retrospective clinical associations differed between FOLFOX and CapeOx regimens [33]. In chemoresistant epithelial ovarian cancer models, omeprazole increased the effects of paclitaxel or cisplatin preferentially in resistant cell lines and xenografts; that study directly examined V-ATPase expression and intracellular pH [34]. These results are directionally compatible with the concept that a PPI can modify drug response, but the drugs, tumor types, resistance states, and measured mechanisms differ from the current study. Reviews similarly describe both potential treatment-sensitizing effects and unresolved clinical issues surrounding PPI use in oncology [35-37]. Thus, the present data support further investigation of omeprazole as an experimental treatment modifier, but they do not establish that pH regulation, V-ATPase activity, drug retention, or a particular signaling pathway caused the observed effects.

The fixed 40 μ M omeprazole concentration was selected after dose-finding experiments showed limited monotherapy-associated effects under the tested conditions. Olaparib was varied from 1 to 20 μ M, producing a non-constant-ratio design. The 10 μ M olaparib plus 40 μ M omeprazole condition was then used for the remaining endpoint experiments because it produced a substantial but incomplete response in both cell lines while retaining a measurable dynamic range for secondary assays. The 20 μ M condition produced strong inhibition in some measurements, but a more extensive reduction in the MTS-derived signal could reduce the ability to distinguish treatment-associated changes across downstream endpoints. The selected condition should therefore be considered an experimental working condition rather than an optimized clinical dose or a universally superior drug ratio.

The Chou–Talalay method provides a quantitative framework for describing combination effects, but the

interpretation of CI values depends on the single-agent dose–effect curves, dose design, response model, data preprocessing, and biological variability [38, 39]. In the present analysis, omeprazole was fixed at 40 μ M and olaparib was varied across five concentrations. AGS CI values ranged from 0.64037 to 0.96390. In MKN45 cells, the lowest combination was antagonistic (CI = 1.32983), while the remaining CI values ranged from 0.51797 to 0.70497. It is more accurately described as concentration- and cell-line-dependent interaction, with predominantly synergistic estimates at the higher tested combinations. Because the omeprazole monotherapy curve in the reported CompuSyn analysis was based on only two dose–effect points, the omeprazole median-effect parameters and dose-reduction indices should be considered exploratory. Additional dose matrices replicated single-agent curves, and response-surface approaches would provide a stronger assessment of the interaction [38, 39].

The MTS and LDH results should also be interpreted in relation to assay biology. Tetrazolium assays such as MTS measure metabolic or enzymatic activity associated with viable cell number, and their signal can be affected by cell density, nutrient availability, pH, and treatment-related metabolic changes [40]. Chan *et al.* demonstrated that MTS and ATP measurements can diverge from direct cell counts in a compound- and cell-line-dependent manner [41]. More recently, Coyle *et al.* showed that pentose-phosphate-pathway-associated metabolism can alter tetrazolium reduction and complicate the interpretation of cytotoxicity results [42]. Accordingly, the present MTS data are best described as changes in a viability-associated metabolic signal, while the LDH results provide evidence of treatment-associated membrane damage. Together, the two assays support a broader cytotoxicity-associated response, but neither directly determines cell number, long-term clonogenic survival, or the relative contribution of specific cell-death pathways.

The metabolic measurements can be compared with established methods for evaluating glycolysis. Glucose uptake and extracellular lactate production are useful indicators of glucose utilization, but they do not account for all possible fates of glucose-derived carbon and should be interpreted with attention to cell number and normalization [43]. The present combination treatment was associated with lower lactate production, lower intracellular ATP, lower glucose-uptake-associated signal, and altered expression of

GLUT1/SLC2A1, HK2, LDHA, and MCT4/SLC16A3. These findings are compatible with treatment-associated metabolic perturbation, but they do not constitute a direct measurement of glycolytic flux. Direct extracellular-flux analysis, isotope tracing, transporter or enzyme activity assays, and carefully normalized cell-number measurements would be required to determine whether a defined metabolic pathway was causally altered. The broader cancer-metabolism literature also emphasizes that tumors can differ in their dependence on glycolysis, oxidative metabolism, glutamine, and other nutrient sources [18, 20, 21].

The 8-OHdG-associated signal provides a further phenotypic comparison with oxidative DNA-damage literature. 8-OHdG is widely used as a biomarker of oxidative DNA damage, but measurements can be influenced by DNA isolation, hydrolysis, normalization, and assay-specific analytical factors [44, 45]. The present increase therefore indicates an association with oxidative DNA-damage-related change under the tested conditions.

The qPCR findings showed treatment-associated changes in BRCA1, RAD51, GLUT1/SLC2A1, HK2, LDHA, MCT4/SLC16A3, p21/CDKN1A, and p16/CDKN2A transcripts. The BRCA1 and RAD51 results are consistent with altered DNA-repair-related transcriptional responses [27].

The increase in Caspase-3 activity is consistent with enhanced apoptosis-associated signaling after combination treatment. However, Caspase-3 activity alone does not establish that apoptosis was the exclusive or predominant mode of cell death. The finding is therefore best integrated with the LDH and MTS results as part of a broader cytotoxicity-associated response.

Collectively, the findings indicate that omeprazole modifies the olaparib-associated response of AGS and MKN45 gastric cancer cells in a concentration- and cell-line-dependent manner. The combination was associated with reduced MTS-derived viability-associated signal, increased LDH release and Caspase-3 activity, altered 8-OHdG-associated and metabolic readouts, and treatment-associated changes in DNA-repair-related, metabolic, and cell-cycle-related transcripts. Further studies using protein-level analyses, independent functional assays, three-dimensional gastric cancer models, organoid or patient-derived cultures, and xenograft models would be valuable for determining the reproducibility, biological

significance, and translational relevance of these findings.

CONCLUSION

Under the tested *in-vitro* conditions, the olaparib–omeprazole combination was associated with greater treatment-related effects than olaparib alone in AGS and MKN45 gastric cancer cells. However, the interaction was concentration- and cell-line-dependent: antagonism was observed at the lowest tested MKN45 combination, whereas CI values below 1 were observed for the remaining tested MKN45 combinations and for all tested AGS combinations. These findings should therefore not be interpreted as evidence of uniform synergism and require further validation in additional experimental models.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTIONS

N.T.P. designed the study, performed the experiments, analyzed the data, and wrote the manuscript.

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