Title

Synergistic effect of olaparib with combination of cisplatin on PTEN deficient lung cancer cells

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Abstract (234 words < 250)

Poly (ADP-ribose) polymerase (PARP) enzyme plays a key role in the cellular machinery responsible for DNA damage repair. PTEN is a tumor-suppressor gene deactivating PI3K downstream of EGFR signaling. We hypothesize that PTEN deficient lung cancer cells suppressed DNA damage signaling and that the absence of PTEN can sensitise these cells to a concurrent treatment of a DNA-damaging agent (cisplatin) and a PARP inhibitor (olaparib). To investigate the effect of olaparib and cisplatin on PTEN deficient lung tumors, two EGFR mutant (deletion in exon19) NSCLC cell lines, PC-9 (PTEN wild type) and H1650 (PTEN loss) were used. We transfected intact PTEN gene into H1650 cells (H1650PTEN+) and knocked down PTEN expression in the PC-9 cells (PC-9PTEN-) using shRNA. Combination of cisplatin with olaparib showed a synergistic effect in vitro according to the combination index in H1650 cells. Restoration of PTEN in the H1650 cells decreased sensitivity to the combination. Ablation of PTEN in PC-9 cells increased sensitivity to olaparib and cisplatin. We also examined the effectiveness of cisplatin and olaparib in a xenograft model using H1650 and PC-9PTEN- cells. The combination of cisplatin with olaparib was more effective than each agent individually. This effect was not observed in a xenograft model using H1650PTEN+ and PC-9 cells. Mechanistic investigations revealed that PTEN-deficiency caused reductions in nuclear
RAD51 and RPA focus formation, phosphorylated Chk1 and Mre11. Thus, genetic inactivation of PTEN led to suppression of DNA repair.
Introduction

Poly(ADP-ribose) polymerase (PARP) inhibitor is one of the most promising new therapeutic approaches to cancers, either as a single agent or in combination with other DNA-damaging agents including radiation therapy (1). When PARP is inhibited, single-strand breaks (SSBs) degenerate to more lethal double-strand breaks (DSBs) that require repair by homologous recombination (HR). Therefore, cells that are deficient in HR are highly susceptible to PARP inhibitors (2-4), and this finding has been clinically validated (5-7).

As many cancer chemotherapeutic drugs and radiation therapy cause DNA damage, tumor cells defective in DNA repair pathways are predicted to be sensitive to their effects (8). Indeed, cell lines deficient in BRCA1 and BRCA2 have been shown to be sensitive to the DNA cross-linking agents cisplatin and mitomycin C (9, 10), the topoisomerase inhibitor etoposide (11), and oxidative DNA damage (12).

PARP1 has been suggested to be involved in base excision repair and SSB repair (13). Moreover, PARP-1 was reported to bind to DNA damages induced by platinum compounds, suggesting a direct role of PARP-1 in the repair of such damages (14, 15). The exquisite sensitivity of these cells to the PARP inhibitor olaparib (AZD2281), alone or in combination with cisplatin, provides strong support for olaparib as a novel targeted
therapeutic against BRCA-deficient cancers (16). Olaparib alone and in combination with carboplatin greatly inhibit growth in BRCA2-mutated ovarian serous carcinoma (17). The exquisite sensitivity of BRCA1 or BRCA2 mutant cells to PARP inhibitors forms the rationale behind clinical trials that are now assessing the potential of these agents (18). The preliminary results from these clinical trials are promising, with favorable toxicity and sustained tumor responses to the drug (19).

Mutations in the phosphatase and tensin homolog (PTEN) gene and loss of PTEN expression have both been associated with a wide range of human tumors (20). Approximately 2% to 9% of non-small cell lung cancer (NSCLC) tumors are considered to have PTEN loss. PTEN loss and epidermal growth factor receptor (EGFR) mutation co-occurred in 1 out of 24 EGFR mutant patients with lung adenocarcinoma (21, 22), and a recurrent gross-mutation of the PTEN gene is identified in lung cancer with deficient DNA DSB repair. In other studies, 24% of early NSCLC samples lacked PTEN expression, which correlated with PTEN promoter methylation (23) and PTEN protein expression was reduced or lost in 74% of lung tumors, and was associated with low or aberrant TP53 staining (24). In a later study, PTEN has novel nuclear functions, including transcriptional regulation of the RAD51 gene, whose product is essential for HR repair of DNA breaks (25, 26). McEllin et al. reported that loss of PTEN in
astrocytes resulted in increased sensitivity to $N$-methyl-$N^\prime$-nitro-$N$-nitrosoguanidine, a functional analogue of temozolomide, and PARP inhibitor, due to inefficiently repaired (27).

We hypothesized that PTEN deficient lung cancer cells suppressed DNA damage signaling and investigated whether the absence of PTEN could sensitize these cells to a concurrent treatment of cisplatin and olaparib.

Materials and methods

Cell lines

Cells were cultured at 37°C with 5% CO₂ in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. H1650 is a lung adenocarcinoma cell line with co-occurrence of an EGFR mutation (in-frame deletion in exon 19) and homozygous deletion of PTEN. PC-9 is a lung adenocarcinoma cell line having the same in-frame deletion mutation of EGFR with wild type PTEN. PTEN transfected into H1650 cells (H1650PTEN+) and PTEN expression knocked down in the PC-9 cells (PC-9PTEN-) using shRNA, were established in our laboratory (28). H1299 and A549 are NSCLC lines with wild type EGFR and wild type PTEN. PC-3 is a prostate cancer cell line with PTEN loss (Supplemental Fig. 1). PC-9 was obtained from Immuno-Biological
Laboratories. H1650, H1299, A549 and PC-3 were purchased from the American Type Culture Collection (Rockville, MD). The Mre11 expression vector was kindly provided by Drs. Kenshi Komatsu and Junya Kobayashi (Kyoto University). The expression vector was transfected to H1650 cells using Lipofectamine 2000 (Invitrogen, Carlsband, CA) according to the manufacturer’s protocol.

**Sensitivity test**

Antiproliferative activity was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in terms of 50% inhibitory concentration (IC$_{50}$) values. Briefly, the cells were plated on 96-well plates at a density of 1,000-3000 cells per well, and continuously exposed to each drug for 144 h. Each assay was performed in triplicate or quadruplicate and IC$_{50}$ values were expressed as mean ± standard deviation.

**Design for drug combination**

The constant-ratio design for the combination assay is highly recommended as it allows the most efficient data analysis (29). The multiple drug effect analysis of Chou and Talaly, based on the median-effect principle, was used to calculate the combined drug effect (30). H1650, H1650$^{PTEN^+}$, PC-9, PC-9$^{PTEN^-}$, H1299, A549 and PC-3 cells were seeded in triplicate in 96-well plates and were treated with cisplatin and olaparib at
the indicated doses. After simultaneous exposure of the cells to two drugs for 144 h, growth inhibition was determined using an MTT assay and the multiple drug effect analysis (Supplemental method). Computer programs based on the median-effect plot parameters and combination index (CI) equation have been used for data analysis in the present study (31). Experiments were repeated in triplicate.

**Immunoblotting analysis**

Cells were exposed to cisplatin (10 μM) or/and olaparib (20 μM) for 6 h. The cells 6 h after irradiation at a dose of 10 Gy using a Hitachi MBR-1520-R irradiator (150 kV; 20 mA; filter: 0.5-mm aluminum and 0.1-mm copper) were also used. They were lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerolphosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate containing protease inhibitor tablets (Roche Applied Sciences GmbH)]. Proteins were separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies followed by detection with enhanced chemiluminescence plus (GE Healthcare Biosciences).

**Reagents and antibodies**

Olaparib and cisplatin were kindly provided by AstraZeneca and Nippon
Kayaku Kogyo Co., Ltd., respectively. Rabbit antisera against Akt, phosphorylated (p)Akt (Ser473) (D9E), PTEN, pChk1 (Ser345), Mre11 (31H4) and β-actin were purchased from Cell Signaling Technology. Rabbit antiserum against RPA70 was purchased from Abcam. Mouse antisera against Chk1 (G-4) was purchased from Santa Cruz Biotechnology.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tissue blocks from the samples were cut to a thickness of 5 μm, placed on glass slides, then deparaffinized in xylene and graded alcohol for 10 min. The antigen was incubated in 10 mM sodium citrate buffer, pH 6.0, for 10 min in a 95 °C water bath. The sections were then blocked for endogenous peroxidase with 0.3% hydrogen peroxide in methanol. The slides were rinsed with TBS containing 0.1% Tween 20 and the sections were blocked with goat normal serum for 60 min. The sections were incubated with 1:200 dilution of cleaved caspase-3 (Asp175) (5A1E) (Cell Signaling) antibody overnight at 4 °C. The sections were amplified using biotinylated anti-rabbit antibodies and avidin–biotinylated horseradish peroxidase conjugate for 10 min (LSAB™ 2 Kit, Dako Cytomation Cytomation) then reacted with 3,3′-diaminobenzidine. Finally, the sections were counterstained with hematoxylin.
Cleaved caspase-3 expression was scored as positive if >10% of the tumor cells exhibited cytoplasmic staining (32).

**Immunofluorescence staining**

We fixed cells 8 h after exposure to cisplatin (10 μM) or/and olaparib (20 μM) or treatment with 10 Gy. Cells cultured on glass coverslips fixed with 4% formaldehyde, permeabilized in phosphate-buffered saline (PBS)-0.25% Triton X-100. For DNA damage and repair analyses, cells were stained with 1:50 dilution of rabbit polyclonal anti-RAD51 (Santa Cruz Biotechnology) and 1:500 dilution of mouse monoclonal anti-\(\gamma\)H2AX (Millipore) for 2 h at room temperature. Cells were washed with PBS and incubated for 30 min at room temperature with either Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen) secondary antibody for RAD51 or \(\gamma\)H2AX, respectively. Nuclei were visualized by staining with DAPI. For quantification of RAD51 and \(\gamma\)H2AX foci, at least 100 cells from each group were visually scored. Cells showing more than five foci were counted as positive for \(\gamma\)H2AX or RAD51. These slides were examined under a fluorescence microscope (BIOREVO BZ-9000; Keyence).

**Xenograft model**

Female athymic mice at 7 wk of age were purchased from Japan Charles River.
Co. All mice were provided with sterilized food and water and housed in a barrier facility under a 12-h light/dark cycle. Cells (1 x 10^6) were injected bilaterally subcutaneously into the backs of 7 week-old female athymic mice. At 10 days after injection, mice were randomly assigned to 4 groups (five mice per group) that received either vehicle, 5 mg/kg/week of cisplatin, 50 mg/kg/day of olaparib, and 5 mg/kg/week of cisplatin plus 50 mg/kg/day of olaparib. Vehicle and olaparib were administered once a day, 5 times a week by intraperitoneal injection, and cisplatin was administered once a day, once a week by intraperitoneal injection. Tumor volume (width^2 x length/2) and body weight were determined periodically. Tumor volume was expressed as mean ± standard deviation. After the completion of the treatment, all mice per group were sacrificed and the tumor specimens were obtained for analysis. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research.

Results

Olaparib synergizes cisplatin in PTEN deficient lung cancer cells.

Synergy between PARP inhibitors and platinum drugs was expected (14-15) in
triple negative breast cancer and BRCA2 ovarian cancer cells (8, 17). We expected that combination of cisplatin with olaparib showed a synergistic effect in *PTEN* deficient lung cancer cell lines. Cells were treated either with 10 μM cisplatin and 10 to 50 μM olaparib or 20 μM cisplatin and 20 to 100 μM olaparib. Combination of cisplatin with olaparib showed a synergistic effect according to the CI in the H1650 cells (Fig. 1A, 1B; Table 1). CIs were 0.23, 0.20, 0.57 and 0.29 when concentration ratios of cisplatin and olaparib were designed to be molar ratios of 1:1, 1:2, 1:3 and 1:5, respectively. Restoration of *PTEN* in the H1650 cells decreased sensitivity to olaparib and cisplatin (CI >1) (Fig. 1C, 1D). Ablation of *PTEN* in PC-9 cells increased sensitivity to olaparib and cisplatin (CI <1), and PC-9 cells decreased sensitivity to olaparib and cisplatin (CI >1). Our results showed that *PTEN* deficient lung cancer cell lines, H1650 and PC-9<sup>PTEN</sup>-, exhibited synergism for all combinations of olaparib doses, whereas H1650<sup>PTEN</sup>+ and PC-9 cells exhibited antagonistic effects for most dose combinations. A synergistic effect was also shown in the PC-3 cells, whereas antagonistic effects at most dose combinations were shown in H1299 and A549 cells (Supplemental Table 1).

Sensitivity of cisplatin or olaparib monotherapy is shown in Table 2. IC<sub>50</sub> values of olaparib in H1650 and H1650<sup>PTEN</sup>+ cells were 15.47 ± 6.8 μM and 50.83 ± 7.7 μM, respectively. *PTEN*-restored H1650 became resistance to olaparib (*P* < 0.05). On
the other hand, the IC$_{50}$ values of olaparib in PC-9 and PC-9$_{PTEN^{-}}$ cells were 5.88 ± 1.4 μM and 6.52 ± 6.7 μM, respectively. PC-9$_{PTEN^{-}}$ cells didn’t confer sensitization to olaparib alone. IC$_{50}$ values of cisplatin or olaparib alone in PC-3, H1299 and A549 cells are shown in supplemental Table 2.

**PTEN inactivation suppresses DNA damage signaling**

Oncogenic activation of Akt frequently results from loss PTEN expression or function leads to suppression of DNA damage signaling (33). In this study, immunoblotting assay revealed that H1650 and PC-9$_{PTEN^{-}}$ cells exhibited much higher levels of pAkt than H1650$_{PTEN^{+}}$ and PC-9 cells, respectively (Fig. 2A). In addition, pChk1 was not overexpressed in both H1650 and PC-9$_{PTEN^{-}}$ cells despite of drug treatment. pChk1 was expressed after irradiation; however, the increase was less in PTEN deficient lung cancer cells compared with their counterparts (Fig. 2B). Meanwhile, PTEN deficient lung cancer cells exhibited lower levels of Mre11 compared with their counterparts (Fig. 2C). To address how lower expression levels of Mre11 affects the synergism between cisplatin and olaparib, Mre11 expression vector was transfected into H1650 cells, and a stable transformant in which Mre11 was overexpressed was obtained (designated H1650$_{Mre11^{+}}$ cells) (Supplemental Fig. 2). In this cell line, CIs were 0.76, 0.91, 0.97 and 0.77 when concentration ratios of cisplatin
and olaparib were designed to be molar ratios of 1:1, 1:2, 1:3 and 1:5, respectively in H1650Mre11+ (Supplemental Table 3). Although these CI values were somewhat elevated compared to those observed in original H1650 cells, these results indicated that lower levels of Mre11 alone could not be the sole reason for the synergism.

On the other hand, PTEN has other nuclear functions, including transcriptional regulation of the RAD51 gene, whose product is essential for HR repair of DNA breaks (25, 26). Replication protein A (RPA) is displaced from single stranded DNA by RAD51 to initiate HR (33). In this study, we investigated whether the formation of RAD51 and RPA foci was reduced in PTEN deficient lung cancer cell lines. Subcellular localization of RAD51, γH2AX and RPA is shown in Fig. 3A and in supplemental Fig. 3A. PTEN deficiency resulted in significant reduction in RAD51 and RPA focus formation after drug-exposure or γ irradiation compared to H1650PTEN+ cells (P < 0.05) although γH2AX was similarly increased in both cells (Fig. 3B, Supplemental Fig. 3B).

**Effectiveness of the cisplatin with olaparib in a xenograft model**

We examined xenograft tumors to determine the effectiveness of the cisplatin with olaparib in PTEN deficient lung cancer cells in vivo. H1650 and H1650PTEN+ xenograft tumors grew at almost same rate. The immunostaining of cleaved caspase-3 is shown in Fig. 4A. The combination of cisplatin and olaparib induced significant higher
positive cells than other groups in H1650 xenografts ($P < 0.05$). The positive cells were 43 ± 3% for cisplatin plus olaparib, 10.6 ± 1.1% for cisplatin alone, 16 ± 3.6% for olaparib alone and 3 ± 2% for vehicle. However, the combination did not display synergistic effect in H1650$^{PTEN^+}$ xenografts. The positive cells were 10 ± 2.6% for cisplatin plus olaparib, 17.3 ± 2.0% for cisplatin alone, 6.6 ± 0.5% for olaparib alone and 4.6 ± 0.5% for vehicle (Fig. 4A).

Treatment with cisplatin plus olaparib significantly suppressed growth of the H1650 tumors compared with cisplatin alone, olaparib alone, and the untreated controls (Fig. 4B). The tumor sizes (mm$^3$) at day 22 were 124.8 ± 33.1, 351.8 ± 150.9, 413.1 ± 66.0, and 847.8 ± 98.5, respectively. H1650$^{PTEN^+}$ xenograft tumors did not show significant response to the combination of cisplatin and olaparib compared with cisplatin alone, olaparib alone, and the untreated controls. The tumor sizes (mm$^3$) at day 22 were 467.8 ± 103.0, 373.9 ± 113, 524.5 ± 145.7, and 801.4 ± 113.2, respectively.

Next, we examined whether the combination of cisplatin with olaparib in PC-9 and PC-9$^{PTEN^+}$ xenografts was effective or not. Cleaved caspase-3 expressions in PC-9$^{PTEN^+}$ xenografts treated with the combination displayed a significant higher number of positive cells than those of other groups (44.3 ± 4.0% for the combination, 7.3 ± 3.2% for cisplatin, 15.3 ± 1.5% for olaparib, 1.0 ± 1.7% for vehicle). In PC-9
xenografts, there were no differences among four groups: 10.6 ± 2.0% for the combination, 7.3 ± 1.5% for cisplatin, 8.3 ± 1.5% for olaparib, 4.0 ± 1.7% for vehicle (Fig. 4A). Western blotting also indicated that the combination of cisplatin with olaparib seemed to induce more cleaved-caspase 3 expressions than other treatments in H1650 and PC-9<sup>PTEN-</sup> xenografts, but not in PC-9 and H1650<sup>PTEN+</sup> xenografts (Supplemental Fig. 4).

In the PC-9<sup>PTEN-</sup> xenograft model, cisplatin plus olaparib inhibited tumor growth than other treatment (152.6 ± 8.06 mm<sup>3</sup> for the combination, 336.6 ± 45.7 mm<sup>3</sup> for cisplatin, 411.2 ± 67.2 mm<sup>3</sup> for olaparib, 774.3 ± 95.8 mm<sup>3</sup> for vehicle). PC-9 xenograft tumors did not show significant response to the combination compared with cisplatin alone, olaparib alone and the untreated controls (279.1 ± 69.5 mm<sup>3</sup>, 254.2 ± 42.2 mm<sup>3</sup>, 296.0 ± 57.5 mm<sup>3</sup>, and 642.3 ± 133.2 mm<sup>3</sup>, respectively) (Fig. 4B).

In toxicity evaluation, all the treatment animals did not show substantial loss of body weight (>10%) and the addition of olaparib to cisplatin did not significantly increase weight loss compared with cisplatin single agent treatment (data not shown).

**Discussion**
We demonstrated here that *PTEN* deficient lung cancer cell lines suppressed DNA damage signaling, and were sensitive to the combination of olaparib with cisplatin. Synergy between PARP inhibitor and platinum drugs was expected in triple negative breast cancer and BRCA2 ovarian cancer cells (8, 17). In other study, addition of PARP inhibitor after alkylating agent, demethyl sulfate, treatment increased SSB levels indicating ongoing repair even at this late time point (34). Recently evidence suggested that *PTEN* was important for the maintenance of genome stability (25, 26). The HR impairment caused by *PTEN* deficiency sensitized tumor cells to potent inhibitors of the DNA repair enzyme, both *in vitro* and *in vivo* (35). Our results were in agreement with their studies. We showed that xenograft tumors bearing *PTEN* deficient lung cancer cells were sensitive to the combination of cisplatin with olaparib, although this effect was not observed in a xenograft model using *PTEN* wild type cells (Fig. 4B). However, it is possible that the results may be cell specific or different effect of PTEN loss on HR capacity because *PTEN* deficient prostate cancer cells had only mild PARP inhibitor and DNA damaging agent’s sensitivity (36).

Drug interaction between cisplatin and olaparib in *PTEN* deficient lung cancer cells has not been elucidated. Our investigation revealed that *PTEN*-deficiency caused a reduction of pChk1 (Fig. 2A) and decreased drug or radiation-induced nuclear RAD51
and RPA focus formation (Fig. 3, Supplemental Fig. 3). Oncogenic activation of Akt frequently resulted from loss of PTEN expression or function (37). How PTEN loss affects DNA damage signaling should be clarified. H1650 and PC-9PTEN+ cells exhibited much higher levels of pAkt than H1650PTEN+ and PC-9 cells, respectively. Activation of Chk1 after irradiation was attenuated in PTEN deficient cells (Fig. 2B). Chk1 has a critical role in maintaining genomic stability by delaying S- and G2-phase progression of cells containing DNA damage to allow time for repair before to mitosis and, the DSBs that arise when Chk1 is inhibited are apparently related to a specific S-phase role whereby Chk1 suppresses aberrant initiation of DNA replication that wound generate DNA lesions (38). Chk1 is reported to require for HR repair and Chk1-depleted cells failed to form RAD51 nuclear foci after exposure to hydroxyurea (39). McEllin and colleagues showed a significant decrease in mRNA expression on RAD51B, C, and D, reduced HR-mediated repair in PTEN null astrocytes (27). Meanwhile, Xu et al. observed PTEN knockdown in HCT116 cells attenuated Mre11, which was a key gene in HR repair of DSBs (33). In addition, Fraser et al. reported PARP inhibitor sensitivity associated with a defect in Mre11 expression (36). Our observation that PTEN deficient lung cancer cells exhibited lower levels of Mre11 compared with their counterparts was in agreement with their studies. In order to investigate the effect of Mre11 itself on the
synergy, we examined the combination effect of olaparib with cisplatin using H1650^{Mre11+} cells. Unexpectedly, restored levels of Mre11 did not suppress the phenotypes observed in PTEN-deficient cells in this study (Table 1, Supplemental Table 3). Further investigations should be required to clarify whether and to what extent the molecular events including Chk1, Mre11 and RAD51 are responsible for the synergic effect. Interestingly, Shen and colleagues recently showed that PTEN is important for maintaining basal levels of transcription of the RAD51 gene in mouse embryonic fibroblasts (25). Although there were no significant differences of RAD51 and RPA levels by Western blotting (Supplemental Fig. 5), the formation of RAD51 and RPA foci was reduced in PTEN deficient lung cancer cells (Fig. 3, Supplemental Fig. 3). As RPA binds to single stranded DNA, the RPA focus could be a marker for end resection at the double stranded DNA ends (33). Thus, inactivation of PTEN might lead to suppression of DNA damage signaling, leading to the lower levels of end resection and, hence, less RPA focus formation. As shown above, reduced levels of Mre11 alone could not provide a sufficient explanation for this, though Mre11 is involved in the molecular mechanisms of the end resection (40).

A number of clinical trials to treat triple negative breast cancer, metastatic melanoma, malignant glioma, advanced colorectal cancer, ovarian cancer, and lung
cancer are now underway to test the efficacy of PARP inhibitors or PARP inhibitors in combination with DNA damaging agents (41). Later phase II studies using olaparib established proof of concept of selectively killing of HR-deficient breast cancer and ovarian cancer cells with BRCA1 or BRCA2 mutations, resulting in a substantial clinical benefit with minimal toxicity (42-44). DNA repair biomarkers from multiple DNA repair pathways on treatment response and cancer survival offers opportunity to evaluate patient tumor samples and determine their status of DNA repair pathways prior to and during therapy for individual patients. In recent years, our understanding of how to treat NSCLC has undergone a paradigm shift by the identification of EGFR mutations (45-46) and EML4–ALK translocation (47). In BRCA1-deficient lung cancer, PARP inhibition induced BAX/BAK-independent synthetic lethality (48). Knowledge of the status of multiple DNA repair profiling of patients and may discriminate patients with likelihood to respond to PARP inhibitors.

We hypothesized that tumor cell with HR deficiency (such as PTEN mutated cancer cells) were hypersensitive to PARP inhibitors combination with cisplatin, resulting in killing of tumor cells based on the synthetic lethality principle. A major important solution to these barriers is to build biomarker testing into patient tumor identification, and to use the biomarker panels during treatment. The combination of
cisplatin with olaparib in PTEN deficient lung tumors might be further pursued in clinical trials.

Disclosure of Potential Conflicts of Interest

N. Takigawa and K. Kiura; Honoraria from speakers bureau, AstraZeneca. The other authors disclosed no potential conflicts of interest.

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Figure legends

Figure 1
Combination index and surviving fraction of H1650 and H1650\textsuperscript{PTEN+} cells treated with cisplatin in combination with olaparib simultaneously for 144 h.

Synergistic effects in H1650 cells were shown, designating to be molar ratios of 1:2 (A) and 1:5 (B). Antagonistic effects in H1650\textsuperscript{PTEN+} cells were shown, designating to be molar ratios of 1:2 (C) and 1:5 (D).

Figure 2
PTEN inactivation and DNA damage signaling.
A: PTEN loss activated Akt and suppressed pChk1. pChk1 was not overexpressed in both H1650 and PC-9\textsuperscript{PTEN-} cells (\textit{PTEN} deficient lung cancer cells) despite of drug treatment. B: pChk1 was expressed after irradiation; however, the increase was less in \textit{PTEN} deficient lung cancer cells compared with their counterparts. C. \textit{PTEN} deficient lung cancer cells exhibited lower levels of Mre11 compared with their counterparts.
Figure 3

PTEN inactivation and expression of RAD51 and γH2AX.

A: Subcellular localization of RAD51 and γH2AX in H1650 or H1650<sup>PTEN+</sup> cells after exposure of olaparib (20 μM) or irradiation (10 Gy). B: PTEN deficiency resulted in significant reduction in RAD51 focus formation after exposure of cisplatin (10 μM) or/and olaparib (20 μM), or irradiation (10 Gy) compared to H1650<sup>PTEN+</sup> cells (P< 0.05) although γH2AX was similarly increased in both cells.

Figure 4

A: Cleaved-caspase-3 staining (×400) of tumor sections from each treatment group in H1650, H1650<sup>PTEN+</sup>, PC-9 and PC-9<sup>PTEN−</sup> cells, included as graphical representation.

B: Growth of xenograft tumors. Growth curves of H1650, H1650<sup>PTEN+</sup>, PC-9 and PC-9<sup>PTEN−</sup> cells xenograft tumors in animals receiving the indicated drugs (50 mg/kg/day of olaparib, 5 mg/kg/week of cisplatin, and both agents) or vehicle i.p were compared using Student's t test. Bars, SD.
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Recombination Repair in Astrocytes: Implications for Glioblastoma Therapy with Temozolomide or Poly(ADP-Ribose) Polymerase Inhibitors. Cancer Res 2010;70:5457-64.


35. Mendes-Pereira AM, Martin SA, Brough R, et al. Synthetic lethal targeting of PTEN mutant cells with PARP


Figure 1

A. H1650
Concentration ratio (molar) of cisplatin to olaparib 1:2

B. H1650
Concentration ratio (molar) of cisplatin to olaparib 1:5

C. H1650PTEN+
Concentration ratio (molar) of cisplatin to olaparib 1:2

D. H1650PTEN+
Concentration ratio (molar) of cisplatin to olaparib 1:5
Figure 2

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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pChk1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chk1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>β-actin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C</th>
<th>H1650</th>
<th>H1650^PTEN+</th>
<th>PC-9</th>
<th>PC-9^PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mre11</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>β-actin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 3

A H1650 No treatment
RAD51 γ-H2AX DAPI

H1650 PTEN+
No treatment
RAD51 γ-H2AX DAPI

H1650 PTEN+
Olaparib
RAD51 γ-H2AX DAPI

H1650 PTEN+
IR
RAD51 γ-H2AX DAPI

B

RAD51 γ-H2AX

Foci-positive cells (%)

Olaparib − + * +
Cisplatin − + *

*P < 0.05 (t test)
Table 1. Combination index

<table>
<thead>
<tr>
<th>Concentration ratio (molar) of cisplatin to olaparib</th>
<th>1:1</th>
<th>1:2</th>
<th>1:3</th>
<th>1:5</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1650</td>
<td>0.23</td>
<td>0.20</td>
<td>0.57</td>
<td>0.29</td>
</tr>
<tr>
<td>H1650&lt;sup&gt;PTEN+&lt;/sup&gt;</td>
<td>1.76</td>
<td>1.59</td>
<td>1.77</td>
<td>1.07</td>
</tr>
<tr>
<td>PC-9</td>
<td>4.38</td>
<td>5.16</td>
<td>10.8</td>
<td>0.55</td>
</tr>
<tr>
<td>PC-9&lt;sup&gt;PTEN−&lt;/sup&gt;</td>
<td>0.43</td>
<td>0.40</td>
<td>0.66</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Combination index (CI) according to various concentration ratios of cisplatin and olaparib in each cell line was described. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.
Table 2. Drug sensitivity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cisplatin (μM)</th>
<th>olaparib (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1650</td>
<td>2.12 ± 0.72</td>
<td>15.47 ± 6.8</td>
</tr>
<tr>
<td>H1650&lt;sup&gt;PTEN+&lt;/sup&gt;</td>
<td>1.65 ± 0.97</td>
<td>50.83 ± 7.7</td>
</tr>
<tr>
<td>PC-9</td>
<td>0.21 ± 0.019</td>
<td>5.88 ± 1.4</td>
</tr>
<tr>
<td>PC-9&lt;sup&gt;PTEN-&lt;/sup&gt;</td>
<td>0.42 ± 0.10</td>
<td>6.52 ± 6.7</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD of 50% inhibitory concentration (IC<sub>50</sub>) of the drug.

H1650<sup>PTEN+</sup>: PTEN-restored H1650; PC-9<sup>PTEN-</sup>: PTEN-ablated PC-9; * P < 0.05
Molecular Cancer Research

Synergistic effect of olaparib with combination of cisplatin on PTEN deficient lung cancer cells

Daisuke Minami, Nagio Takigawa, Hiromasa Takeda, et al.

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