Original article

# Anti-tumor and anti-cancer stem cell activity of a poly ADP-ribose polymerase inhibitor olaparib in breast cancer cells

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**Abstract**

**Background:** Although the poly ADP-ribose polymerase (PARP) inhibitor olaparib is known to have a potent anti-tumor activity in BRCA-related breast cancer cells, a limited number of preclinical and clinical studies have shown anti-tumor activity of olaparib in BRCA-naïve breast cancer. We have investigated anti-tumor activity of olaparib in breast cancer cell lines derived from patients with non-familial sporadic breast cancer.

**Methods:** Effects of olaparib alone or in combination with five different chemotherapeutic agents on cell growth, cell cycle progression, apoptosis and proportion of cancer stem cells using the mammosphere assay and CD44/CD24/ESA cell surface marker assay were investigated in a panel of six sporadic breast cancer cell lines. The ERK phosphorylation was also investigated to elucidate action mechanisms of olaparib.

**Results:** Olaparib inhibited the growth of two estrogen receptor (ER)-positive and HER2-negative breast cancer cell lines and two ER-negative and HER2-negative breast cancer cell lines (the 50% growth inhibitory concentrations were 1.3 - 3.0μM) associated with the G2/M accumulation and induction of apoptosis. In contrast, two HER2-positive cell lines were resistant to olaparib. Interestingly, olaparib significantly decreased the proportion of putative cancer stem cells in either sensitive or resistant cell lines. In addition, olaparib increased the expression of p-ERK. Combined treatments of olaparib with a MEK inhibitor U0126 completely suppressed the expression of p-ERK. These treatments also inhibited the G2/M accumulation and apoptosis induction by olaparib. Among five chemotherapeutic agents commonly used for breast cancer treatment, only an irinotecan metabolite SN38 showed additive anti-tumor activity with

olaparib. Importantly, the combined treatment enhanced an increase in the G2/M accumulation and apoptosis induction as well as a decrease in the proportion of cancer stem cells.

**Conclusions:** This study has indicated for the first time that the PARP inhibitor olaparib has substantial anti-tumor and anti-cancer stem cell activity in breast cancer cell lines of a non-familial origin. Up-regulation of p-ERK might explain, at least in part, anti-tumor and anti-cancer stem cell activity of olaparib. A combined treatment of olaparib with irinotecan might be effective in the treatment of non-BRCA-related breast cancer.

**Keywords:** PARP inhibitor, olaparib, breast cancer, SN38, cancer stem cells

## Introduction

In hereditary breast cancer associated with the germ-line mutation of BRCA1 or BRCA2, harboring loss of protein expression and/or dysfunction of BRCA1 or BRCA2, the homologous recombination machinery to repair DNA double-strand breaks is frequently impaired. Preclinical studies have shown that inhibition of poly ADP-ribose polymerase (PARP), which plays important roles in repairing DNA single-strand breaks, provide so-called synthetic lethality and apoptosis to tumor cells bearing loss or dysfunction of BRCA1 or BRCA2 [1, 2]. In addition, recent clinical studies have revealed that a PARP inhibitor olaparib has remarkable anti-tumor activity in breast or ovarian cancer associated with BRCA1 or BRCA2 germ-line mutations [3-5].

Although it is well known that olaparib has a potent anti-tumor activity in BRCA1 or BRCA2-related breast cancer cells, a limited number of preclinical studies have shown anti-tumor activity of olaparib in BRCA1 or BRCA2-naïve breast cancer cell lines [6]. Therefore, we have investigated anti-tumor activity of olaparib in a panel of breast cancer cell lines derived from patients with non-familial sporadic breast cancer in this study.

Recent preclinical and clinical studies have suggested that breast cancer stem cells play critical roles in tumor growth, invasion, metastasis and resistance to cytotoxic agents and radiation [7]. Thus, anti-cancer stem cell activity of olaparib was also investigated. Furthermore, it has been suggested that combined treatments of agents harboring anti-cancer stem cell activity with conventional chemotherapeutic agents may provide tumor eradication or prolonged tumor response to experimental tumors built with cancer stem cells and non-stem cells [8-10]. Therefore, we also examined combined anti-tumor and anti-cancer stem cell activity of olaparib with

chemotherapeutic agents commonly used in the treatment of breast cancer.

## Materials and Methods

### Breast cancer cell Lines and culture conditions

The KPL-1, KPL-3C and KPL-4 breast cancer cell lines were established in our laboratory [11-13]. The MDA-MB-231 cell line was provided by late Dr. Robert B. Dickson (Lombardi Cancer Research Center, Georgetown University Medical Center, Washington DC, USA). The MDA-MB-157 and BT-474 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines used were reported to derive from metastatic breast cancer patients of non-familial origin [11-16].

According to our previous study [17], the KPL-1 and KPL-3C cell lines were categorized as the estrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negative subtype, the BT-474 cell line as the ER-positive and HER2-positive subtype, the KPL-4 cell line as the ER-negative and HER2-positive subtype, and the MDA-MB-231 and MDA-MB-157 cell lines as the triple negative subtype. Overexpression of HER1, cytokeratin5/6 and vimentin was also observed in the MDA-MB-231 and MD-MB-157 cell lines. These findings indicate that MDA-MB-231 and MDA-MB-157 cells were classified as the basal B subtype [18-20].

All cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium (D-MEM, Sigma Co, St. Louis, MI, USA) supplemented with 10% fetal bovine serum (FBS, MBL Co, Nagoya, Japan).

### Reagents

Olaparib was kindly provided by AstraZeneca Global Research-based Biopharmaceutical Company (London, UK). Etoposide, doxorubicin, paclitaxel, SN38 and cisplatin were purchased from Sigma Co.

**Cell proliferation analysis**

To investigate the effects of olaparib and/or chemotherapeutic agents on cell growth, breast cancer cells (1-5 × $10^4$ cells per well) were seeded in 24-well plates (SB Medical, Tokyo, Japan) and grown in D-MEM supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere for two days. After washing with phosphate-buffered saline (PBS, Nissui Co., Tokyo, Japan), the cells were treated with D-MEM supplemented with 10% FBS plus indicated concentrations of olaparib and/or chemotherapeutic agents for three days. In the combination treatment, the cells were treated with D-MEM supplemented with 10% FBS plus 2.0 μM olaparib and indicated concentrations of the respective chemotherapeutic agent for three days. After the treatments, the cells were harvested and counted with a Coulter counter (Coulter Electronics, Harpenden, UK). Reproducibility was confirmed in at least two separate experiments.

To evaluate the antitumor effects of combined treatments, a combination index based on the 50% inhibitory concentration ($IC_{50}$) was calculated according to the following formula: combination index = $IC_{50}$ with combined treatment/$IC_{50}$ with single treatment. Combination index < 0.5 was considered evidence of additive interaction [21].

Appropriate concentrations of chemotherapeutic agents for anti-tumor experiments were selected according to the $IC_{50}$s of the agents obtained from our previous study [17].

**Cell cycle and apoptosis analyses**

Breast cancer cells (0.3 – 1.5 × $10^5$ cells per well) were seeded in 6-well plates (SB Medical, Tokyo, Japan) and grown in D-MEM supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere for two days. After washing with PBS, the cells were treated with D-MEM supplemented with 10% FBS plus indicated concentrations of olaparib and/or chemotherapeutic agents for three days. In the combination treatment, the cells were treated with D-MEM supplemented with 10% FBS plus 2.0 μM olaparib and indicated concentrations of the respective chemotherapeutic agent for three days. After washing with PBS, the cells were treated with D-MEM supplemented with 10% FBS plus indicated concentrations of olaparib and/or chemotherapeutic agents for three days.

To investigate the effects of agents on cell cycle progression, harvested cells were stained with propidium iodide using a CycleTest Plus DNA Reagent kit (Becton Dickinson, San Jose, CA, USA). The percentages of apoptotic cells were stained with an Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's recommendations. Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson), and the DNA histogram was analyzed using a CELLQuest version 1.2.2 (Becton Dickinson).

**Mammosphere assay**

Breast cancer cells (0.3 – 1.5 × $10^5$ cells per well) were seeded in 35mm-dishes (SB Medical) and grown in D-MEM supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere for two days. After washing with PBS, the cells were incubated with

D-MEM supplemented with 10% FBS plus indicated concentrations of olaparib and/or SN38 for three days. After washing with PBS, the cells were incubated with D-MEM supplemented with 10% FBS without any agent for three days. Then, the cells were dispersed, and single cell suspension (1 x $10^4$ cells/ well) were incubated in the MammoCult™ basal medium (Stem Cell Technologies Co., Tokyo, Japan) supplemented with 10% MammoCult™ proliferation supplements (Stem Cell Technologies Co.) plus heparin (Stem Cell Technologies Co.) and hydrocortisone (Stem Cell Technologies Co.) in the non-adhesive 6-well plates (CORNING Co., NY, USA) for seven days. Mammosphere colonies more than 60μm in size were counted with an Olympus phase-contrast microscope [22].

**CD44/CD24/ESA cell surface marker assay**

Breast cancer cells (0.3 – 1.5 × $10^5$ cells per well) were seeded in 6-well plates (SB Medical) and grown in D-MEM supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere for two days. After washing with PBS, the cells were incubated with D-MEM supplemented with 10% FBS plus indicated concentrations of olaparib and/or SN38 for three days.

To analyze cell surface markers, the dispersed cells were treated with three fluorescene-labeled antibodies, PE conjugated anti-CD24 antibody (clone G44-26, Becton Dickinson), FITC conjugated anti-CD44 antibody (clone ML5, Becton Dickinson）, PerCP-Cy5.5 conjugated anti-EpCAM antibody (clone EBA-1, Becton Dickinson). Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson), and analyzed using CELLQuest Software version 1.2.2 (Becton Dickinson). Cells showing CD44-positive, CD24-negative or low and EpCAM

(ESA)-positive were recognized as cancer stem cells [23].

**Western blot analysis**

The MDA-MB-231 and KPL-3C cells were treated with 2μM olaparib plus minus 10 or 20 μM U0126 (Promega, Madison, WI, USA), respectively. After the treatments for 0, 1, 12 and 24 hours, the cells were collected and eluted by the elution buffer (100μl RIPA buffer, 1μM EDTA, 1μM Halt Protease and Phosphatase Inhibitor Cocktail, Thermo, Waltham, MA, USA). The supernatant was collected and centrifuged at 4 ℃ by 15,000 rpm. After adjusting the amount of protein to 5μg per sample using BCA Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), electrophoresis is performed in 10% SDS-PAGE gel (Bio-Rad Laboratories), and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). After blocking the membrane with 1% albumin/T-TBS (Tris-buffered saline with 0.1% [v/v] Tween-20), treatments with primary antibodies and secondary antibodies were performed. As primary antibodies, anti-p-ERK (E-4, sc-7383, 1:1,000), anti-ERK1 (K-23, sc-94, 1:2,000) and anti-β-actin (ACTBD11B7, sc-81178, 1:2,000) monoclonal antibodies were used. As secondary antibodies, anti-goat anti-mouse IgG-horseradish peroxidase (HRP) (sc-2055, 1:1,000) and anti-goat anti-rabbit IgG-HRP (sc-2054, 1:2,000) were used. All antibodies were purchased from Santa Cruz Co. (CA, USA). Chemiluminescence was made by the ELC kit (GE Healthcare Japan, Tokyo, Japan) and analyzed with the ChemiStage CC chemiluminescence detection-16 (KURABO, Japan). Both ERK1 and β-actin were used as the internal control [24].

**Statistical analysis**

All values are expressed as the mean ± SE. ANOVA analysis with StatView computer software (ATMS Co., Tokyo, Japan) was used to compare the differences between two groups. A two-sided P value less than 0.05 was considered statistically significant.

## Results

### Anti-tumor activity of olaparib

The $IC_{50}$s of olaparib were 1.27 ± 0.09 μM and 1.97 ± 0.52 μM for the KPL-1 and KPL-3C cell lines of the ER-positive and HER2-negtive subtype, respectively. Those were 2.95 ± 0.10 μM and 1.93 ± 0.13 μM for the MDA-MB-157 and MDA-MB-231 cell lines of the basal B subtype, respectively. In contrast, those were much higher for the BT-474 and KPL-4 cell lines expressing a high level of HER2 (Figure 1).

### Effects of olaparib on the cell cycle progression and apoptosis

Olaparib significantly increased the percentage of cells at the G2/M phase in the MDA-MB-231 and KPL-3C cells, respectively. The percentages of cells at the G2/M phase were 21.5 ± 0.2% for the control cells, 29.9 ± 0.4% for 2 μM olaparib-treated cells (P = 0.0002) and 30.7 ± 0.3% for 5 μM olaparib-treated cells (P = 0.0001) in the MDA-MB-231 cells (Figure 2A). The percentages of cells at the G2/M phase were 18.2 ± 0.7% for the control cells, 29.1 ± 0.5% (P = 0.0425) for 2 μM olaparib-treated cells and 52.2 ± 3.8% (P = 0.0018) for 5μM olaparib-treated cells in the KPL-3C cells (Figure 2B). In contrast, olaparib did not significantly increase the percentage of cells at

the G2/M phase in the olaparib-resistant BT-474 cells.

Olaparib dose-dependently increased the percentage of apoptotic cells in both cell lines. The percentages of apoptotic cells were 5.6 ± 0.2% for the control cells, 10.5 ± 0.1% for 2 μM olaparib-treated cells ($P = 0.0004$) and 15.6 ± 0.2% for 5 μM olaparib-treated cells ($P < 0.0001$) in the MDA-MB-231 cells (Figure 3A). The percentages of apoptotic cells were 2.5 ± 0.4% for the control cells, 10.3 ± 0.3% for 2 μM olaparib-treated cells ($P = 0.0003$) and 14.8 ± 0.0% for 5 μM olaparib-treated cells ($P < 0.0001$) in the KPL-3C cells (Figure 3B). Olaparib slightly increased the percentage of apoptotic cells in the BT-474 cells (Figure 3C).

**Effects of olaparib on ERK phosphorylation**

Recent experimental studies have revealed a close relationship between the ERK signaling pathway and PARP activity, which is unrelated to DNA damage [25, 26]. Additionally, some biological agents have been reported to promote a sustained ERK phosphorylation and induce a G2/M cell cycle accumulation and apoptosis [24, 27, 28], changes in the expression levels of p-ERK were investigated after the treatment with olaparib and/or a MEK inhibitor U0126 to elucidate action mechanisms of olaparib.

Olaparib increased the expression level of p-ERK at 1 hour and 12 hours after the start of the treatment in the MBA-MB-231 cells. In contrast, olaparib increased it at 12 hours and 24 hours after in the KPL-3C cells. U0126 completely suppressed the up-regulation of p-ERK expression in both cell lines (Figure 4). To test whether the up-regulation of p-ERK expression by olaparib causes the G2/M cell cycle accumulation and apoptosis induction, changes in the cell cycle progression and apoptosis after the treatment with olaparib plus minus U0126 were also investigated. As

expected, U0126 completely suppressed the G2/M cell cycle accumulation and apoptosis induction by olaparib in the MDA-MB-231 cells (Figure 5).

**Effects of olaparib on the proportion of cancer stem cells**

Olaparib significantly decreased the proportion of $CD44^+/CD24^{-\text{ or low}}/ESA^+$ cells in the MDA-MB-231 cells. The proportions were 2.9 ± 0.2% for the control cells, 1.7 ± 0.2% for 2 μM olaparib-treated cells (P = 0.0139) and 1.2 ± 0.1% for 5 μM olaparib-treated cells (P = 0.0052), respectively. Olaparib also slightly decreased the proportion of $CD44^+/CD24^{-\text{ or low}}/ESA^+$ cells in the KPL-3C cells. The proportions were 14.1 ± 2.2% for the control cells, 12.5 ± 0.3% for 2 μM olaparib-treated cells (P = 0.4656) and 11.0 ± 0.6% for 5 μM olaparib-treated cells (P = 0.1956), respectively. Interestingly, olaparib slightly decreased the proportion of $CD44^+/CD24^{-/low}/ESA^+$ cells in the olaparib-resistant BT-474 cells. The proportions were 11.5 ± 1.0% for the control cells, 7.6 ± 1.8% for 2 μM olaparib-treated cells (P = 0.1436) and 4.6 ± 1.3% for 5 μM olaparib-treated cells (P = 0.0397), respectively (Figure 6).

Similarly, olaparib significantly decreased the number of mammospheres formed by all cell lines tested (Figure 7). The numbers of mammospheres/1,000 seeded cells were 62.5 ± 2.5 for the control cells, 45.0 ± 3.0 for 2 μM olaparib-treated cells (P = 0.0191) and 42.5 ± 2.5 for 5 μM olaparib-treated cells (P = 0.0132), respectively, in the MDA-MB-231 cells. The numbers were 361.0 ± 1.0 for the control cells, 119.5 ± 1.5 for 2 μM olaparib-treated cells (P < 0.0001) and 83.5 ± 3.5 for 5 μM olaparib-treated cells (P < 0.0001), respectively, in the KPL-3C cells. Interestingly, olaparib also slightly decreased the numbers of mammospheres/1,000 seeded cells in the olaparib-resistant BT-474 cells. The numbers were 77.5 ± 2.5 for the control cells, 65.5 ± 0.5 for 2 μM

olaparib-treated cells (P = 0.0426) and 58.5 ± 3.5 for 5 μM olaparib-treated cells (P = 0.0126), respectively.

**Anti-tumor activity of olaparib in combination with chemotherapeutic agents**

To investigate combined anti-tumor activity of olaparib with chemotherapeutic agents, the MDA-MB-231 and KPL-3C cells were treated with 2μM olaparib plus minus indicated concentrations of five different chemotherapeutic agents. Combination indexes were calculated as described in the Material and Methods and shown in Table 1.

A significant additive anti-tumor activity was observed in the combination of olaparib with SN38 in the MDA-MB-231 cells (the combination index = 0.24 ± 0.04, Figure 8). Non-significant but close to significantly additive interactions were also observed in the combination of olaparib with etoposide for the MDA-MB-231 cells, and that of olaparib with doxorubicin for the KPL-3C cells (the combination indexes were 0.51 ± 0.02 and 0.52 ± 0.01, respectively).

**Anti-tumor and anti-cancer stem cell activity of olaparib combined with SN38**

To investigate effects of a combined treatment of olaparib with SN38 on cell cycle progression, induction of apoptosis and the proportion of cancer stem cells, the MDA-MB-231 cells were treated with olaparib plus minus 0.25 nM SN38 for three days.

This combination significantly enhanced a G2/M cell cycle accumulation more than each agent alone. The percentages of cells at the G2/M phase were 7.6 ± 0.2% in the control cells, 8.6 ± 0.4% in 2 μM olaparib-treated cells, 14.6 ± 0.3% in 0.25 nM

SN38-treated cells and 29.3 ± 0.2% in the combination (olaparib alone vs. combination, P < 0.0001; SN38 alone vs. combination, P < 0.0001, Figure 9A). Similarly, this combination significantly enhanced apoptosis induction more than each agent alone. The percentages of apoptotic cells were 5.9 ± 0.5% in the control cells, 10.5 ± 0.1% in 2 μM olaparib-treated cells, 9.8 ± 0.3% in 0.25 nM SN38-treated cells and 16.4 ± 0.3 in the combination (olaparib alone vs. combination, P = 0.0001; SN38 alone vs. combination, P < 0.0001, Figure 9B).

Based on the CD44/CD24/ESA cell surface assay, the proportion of $CD44^{+}/CD24^{-\text{ or }low}/ESA^{+}$ putative cancer stem cells was decreased by olaparib alone or olaparib/SN38 combination, but it was not changed by SN38 alone (Figure 10A). The proportions were 3.1 ± 0.1% in the control cells, 2.4 ± 0.2% in olaparib-treated cells (P = 0.0409, compared with control), 3.4 ± 0.3% in SN38-treated cells (P = 0.3460, compared with control) and 2.0 ± 0.3% in the combined treated cells (P = 0.0117, compared with control).

Similarly, the number of mammospheres was decreased by olaparib alone or olaparib/SN38 combination, but it was not changed by SN38 alone (Figure 10B). The numbers of colonies/1,000 seeded cells were 57.5 ± 2.5 in the control cells, 37.0 ± 3.0 in olaparib-treated cells (P = 0.0344, compared with control), 61.0 ± 9.0 in SN38-treated cells (P = 0.6763, compared with control) and 20.0 ± 5.0 in the combination (P = 0.0085, compared with control).

**Discussion**

Preclinical and clinical studies have shown that PARP inhibitors have potent anti-tumor activity in breast cancer cells harboring genetic mutations in *BRCA1* or *BRCA2* gene

[1-5]. Here, we studied the anti-tumor activity as well as anti-cancer stem cell activity of a PARP inhibitor olaparib in a panel of six cell lines derived from patients with breast cancer of non-familial origin. When we started this study, we expected that the PARP inhibitor olaparib may have potent anti-tumor activity in breast cancer cells of the triple negative subtype bearing BRCA1 loss or dysfunction. Unexpectedly, olaparib provided substantial anti-tumor activity to ER-positive/HER2-negative breast cancer cell lines as well as triple negative/basal B breast cancer cell lines (Figure 1). The $IC_{50}$ of olaparib for the cell lines were less than 2 μM. Based on the pharmacokinetic data in a phase Ⅰ trial [3], 2 μM olaparib is achievable in the patient blood. However, according to an *in vitro* experiment, the $IC_{50}$s of olaparib were reported to be close to 0.1 μM in BRCA-mutated mammary tumor cells [29]. The $IC_{50}$s of olaparib were much higher in breast cancer cell lines tested in this study than those in the BRCA-mutated mammary tumor cells. These findings indicate that olaparib may work by another mechanism rather than by so-called synthetic lethality in sporadic non-hereditary breast cancer cells.

According to the new sub-classification of triple negative breast cancer advocated by Lehmann BD et al., the $IC_{50}$s of olaparib were 14 μM for the BRCA1-related MDA-MB-436 cell line of the basal-like subtype and higher than 100 μM for the MDA-MB-231 and MDA-MB-157 of the mesenchymal stem-like subtype used in this study, respectively [6]. The $IC_{50}$s of olaparib for these cell lines are much higher in their report than those in this study. This discrepancy may be derived from differences in assay procedures how to treat the cells and to evaluate cell growth. Otherwise, the $IC_{50}$s of olaparib for non-BRCA-related cancer cell lines were closer to those obtained from this study [30-32].

To elucidate the mechanism of action of olaparib, we first examined the effects of olaparib on cell cycle progression and apoptosis. Olaparib dose-dependently induced

a G2/M cell cycle accumulation and apoptosis in olaparib-sensitive breast cancer cell lines of non-familial origin. Interestingly, olaparib unexpectedly inhibited the growth of breast cancer cell lines of not only triple negative subtype but also ER-positive/HER2-negative subtype. These findings suggest that anti-tumor activity of olaparib may be driven not only by so-called synthetic lethality but also by other mechanisms. In addition, to further explore the molecular mechanism of olaparib, sustained phosphorylation of ERK was investigated based on the reports showing that some anti-tumor agents cause sustained phosphorylation of ERK associated with G2/M cell cycle accumulation and apoptosis [24, 27, 28]. Although ERK phosphorylation plays an important role in the Ras/Raf-MEK-ERK signaling pathway, which is activated by various growth factors and regulates cell growth, the ERK pathway also has a pro-apoptotic function in apoptosis induced by DNA-damaging agents [26]. Our preliminary results have shown that olaparib significantly induces ERK phosphorylation and that a combined treatment of olaparib with a MEK inhibitor U0126 completely suppresses it and inhibits a G2/M cell cycle accumulation and induction of apoptosis by olaparib (Figures 4 and 5). These findings suggest that ERK phosphorylation induced by olaparib may, at least in part, play a role in anti-tumor activity of olaparib.

Recent preclinical studies have shown combined treatments of olaparib with a DNA-damaging agent cisplatin synergistically enhance anti-tumor activity in BRCA-related cancer cell lines [29, 33]. These findings prompted us to investigate combined treatments of olaparib with cytotoxic agents commonly used in the treatment of breast cancer. Unexpectedly, only a combined treatment of olaparib with an irinotecan metabolite SN38 but not cisplatin showed a more than additive anti-tumor activity in the MDA-MB-231 cell line of the triple negative subtype. It has been shown that SN38 exerts anti-tumor activity mainly associated with inhibiting type Ⅰ DNA

topoisomerase action and inducing apoptosis in target cells [34]. It is conceivable that anti-tumor activity of olaparib in non-BRCA-related breast cancer cell lines is not simply derived from so-called synthetic lethality. The mechanisms of actions causing an additive interaction of olaparib and SN38 are largely unknown so far. Otherwise, a recent *in vivo* study has shown that showed additive anti-tumor activity of olaparib with a topoisomerase Ⅰ inhibitor topotecan in the BRCA1;p53-dificient mouse mammary tumor model [35].

Cancer stem cells consisting of a small proportion of cancer have been suggested to play important roles in tumor growth, invasion, metastasis and resistance to cytotoxic and radiation therapies [7]. Preclinical and clinical studies have shown that cytotoxic agents such as anthracyclines and taxanes increased the proportion of $CD44^{+}/CD24^{-/low}$ or aldehyde dehydrogenase-positive putative breast cancer stem cells but anti-HER2 agents such as trastuzumab and lapatinib decreased them [36-40]. These remnant cancer stem cells may cause tumor recurrence after successful clinical response to certain therapies. It should be noted that olaparib alone dose-dependently reduced the proportion of putative cancer stem cells measured by either the mammosphere assay or the CD44/CD24/ESA cell surface marker assay in this study (Figures 7 and 8). Mechanisms of action of olaparib responsible for the reduction in the proportion of breast cancer stem cells remain to be elucidated. Recent research data including data in this study have suggested that 1) there is a close relationship between the ERK signaling pathway and PARP activity, which is unrelated to DNA damage [25, 26], 2) PARP1 inhibition may modulate ERK signaling pathway such as sustained ERK phosphorylation (Figure 4) and may damage the ability of self-renewal and/or survival of cancer stem cells. Interestingly, a recent study has also reported that icaritin, a prenylflavonoid derivative, induced a sustained phosphorylation of ERK and more

potently inhibited the growth of breast cancer stem cells [24].

Preclinical studies have shown that anti-tumor agents attacking both cancer stem cells and non-cancer stem cells may provide a longer progression-free survival or cure of cancer to the patients [8-10]. Therefore, we investigated anti-cancer stem cell activity of olaparib plus conventional cytotoxic agents. Although a chemotherapeutic agent SN38 alone showed anti-tumor activity without a decrease in the proportion of cancer stem cells, a combined treatment of olaparib and SN38 showed an enhanced antitumor activity with a decrease in the proportion of cancer stem cells (Figures 9 and 10). These findings suggest that a combined treatment of olaparib with SN38 may provide stronger and long-lasting antitumor activity in the treatment of breast cancer. Clinical studies are needed to clarify the additive interaction of olaparib with irinotecan in the near future.

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**References**

1. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al: Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434:917-21.
2. Ashworth A: A synthetic lethal therapeutic approach: poly(ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. J Clin Oncol. 2008;26:3785-90.
3. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, et al: Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med. 2009;361:123-34.
4. Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, et al: Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet. 2010;376:235-44.
5. Gelmon KA, Tischkowitz M, Mackay H, Swenerton K, Robidoux A, Tonkin K, et al: Olaparib in patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triple-negative breast cancer: a phase 2, multicentre, open-label, non-randomised study. Lancet Oncol. 2011;12:852-61.
6. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al: Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 2011;121:2750-67.
7. Kakarala M, Wicha MS: Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. J Clin Oncol. 2008;26:2813-20.
8. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K: Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res. 2009;69:7507-11.

9. Zhou J, Zhang H, Gu P, Margolick JB, Yin D, Zhang Y: Cancer stem/progenitor cell active compound 8-quinolinol in combination with paclitaxel achieves an improved cure of breast cancer in the mouse model. Breast Cancer Res Treat. 2009;115:269-77.
10. Iliopoulos D, Hirsch HA, Struhl K: Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. Cancer Res. 2011;71:3196-201.
11. Kurebayashi J, Kurosumi M, Sonoo H: A new human breast cancer cell line, KPL-1 secretes tumour-associated antigens and grows rapidly in female athymic nude mice. Br J Cancer 1995;71:845-53.
12. Kurebayashi J, Kurosumi M, Sonoo H: A new human breast cancer cell line, KPL-3C, secretes parathyroid hormone-related protein and produces tumours associated with microcalcifications in nude mice. Br J Cancer 1996;74:200-7.
13. Kurebayashi J, Otsuki T, Tang CK, Kurosumi M, Yamamoto S, Tanaka K, et al: Isolation and characterization of a new human breast cancer cell line, KPL-4, expressing the Erb B family receptors and interleukin-6. Br J Cancer 1999;79:707-17.
14. Cailleau R, Young R, Olivé M, Reeves WJ Jr: Breast tumor cell lines from pleural effusions. J Natl Cancer Inst. 1974;53:661-74.
15. Young RK, Cailleau RM, Mackay B, Reeves WJ: Establishment of epithelial cell line MDA-MB-157 from metastatic pleural effusion of human breast carcinoma. In Vitro. 1974;9:239-45.
16. Lasfargues EY, Coutinho WG, Redfield ES: Isolation of two human tumor epithelial cell lines from solid breast carcinomas. J Natl Cancer Inst 1978;61:967-78.
17. Kurebayashi J, Kanomata N, Moriya T, Kozuka Y, Watanabe M, Sonoo H:

Preferential antitumor effect of the Src inhibitor dasatinib associated with a decreased proportion of aldehyde dehydrogenase 1-positive cells in breast cancer cells of the basal B subtype. BMC Cancer. 2010;10:568.

18. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al: Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 2004;10:5367-74.
19. Prat A, Perou CM : Mammary development meets cancer genomics. Nat Med. 2009;15:842-4.
20. Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, et al: Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One. 2009;4:e6146.
21. Kurebayashi J, Nukatsuka M, Nagase H, Nomura T, Hirono M, Yamamoto Y, et al: Additive antitumor effect of concurrent treatment of 4-hydroxy tamoxifen with 5-fluorouracil but not with doxorubicin in estrogen receptor-positive breast cancer cells. Cancer Chemother Pharmacol 2007;59:515-25.
22. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al: In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. 2003;17:1253-70.
23. Fillmore CM, Kuperwasser C: Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res. 2008;10:R25.
24. Guo Y, Zhang X, Meng J, Wang ZY: An anticancer agent icaritin induces sustained activation of the extracellular signal-regulated kinase (ERK) pathway and inhibits growth of breast cancer cells. Eur J Pharmacol. 2011;658:114-22.

25. Cohen-Armon M, Visochek L, Rozensal D, Kalal A, Geistrikh I, Klein R, et al: DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. Mol Cell. 2007;25:297-308.
26. Cohen-Armon M: PARP-1 activation in the ERK signaling pathway. Trends Pharmacol Sci. 2007;28:556-60.
27. Hsu YL, Kuo PL, Lin LT, Lin CC: Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells. J Pharmacol Exp Ther. 2005;313:333-44.
28. Shin SY, Hyun J, Yu JR, Lim Y, Lee YH: 5-Methoxyflavanone induces cell cycle arrest at the G2/M phase, apoptosis and autophagy in HCT116 human colon cancer cells. Toxicol Appl Pharmacol. 2011;254:288-98.
29. Evers B, Drost R, Schut E, de Bruin M, van der Burg E, Derksen PW, et al: Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin. Clin Cancer Res. 2008;14:3916-25.
30. Weston VJ, Oldreive CE, Skowronska A, Oscier DG, Pratt G, Dyer MJ, et al: The PARP inhibitor olaparib induces significant killing of ATM-deficient lymphoid tumor cells in vitro and in vivo. Blood. 2010;116:4578-87.
31. Takahashi M, Koi M, Balaguer F, Boland CR, Goel A: MSH3 mediates sensitization of colorectal cancer cells to cisplatin, oxaliplatin, and a poly(ADP-ribose) polymerase inhibitor. J Biol Chem. 2011;286:12157-65.
32. Huehls AM, Wagner JM, Huntoon CJ, Geng L, Erlichman C, Patel AG, et al: Poly(ADP-Ribose) polymerase inhibition synergizes with 5-fluorodeoxyuridine but not 5-fluorouracil in ovarian cancer cells. Cancer Res. 2011;71:4944-54.
33. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, et

al: High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. Proc Natl Acad Sci U S A. 2008;105:17079-84.

34. Catley L, Tai YT, Shringarpure R, Burger R, Son MT, Podar K, et al: Proteasomal degradation of topoisomerase I is preceded by c-Jun NH2-terminal kinase activation, Fas up-regulation, and poly(ADP-ribose) polymerase cleavage in SN38-mediated cytotoxicity against multiple myeloma. Cancer Res. 2004;64:8746-53.
35. Zander SA, Kersbergen A, van der Burg E, de Water N, van Tellingen O, Gunnarsdottir S, et al：Sensitivity and acquired resistance of BRCA1;p53-deficient mouse mammary tumors to the topoisomerase I inhibitor topotecan. Cancer Res. 2010;70:1700-10.
36. Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, et al: Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst. 2008;100:672-9.
37. Korkaya H, Paulson A, Iovino F, Wicha MS: HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene. 2008;27:6120-30.
38. Tanei T, Morimoto K, Shimazu K, Kim SJ, Tanji Y, Taguchi T,, et al: Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. Clin Cancer Res. 2009;15:4234-41.
39. Calcagno AM, Salcido CD, Gillet JP, Wu CP, Fostel JM, Mumau MD, et al: Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. J Natl Cancer Inst. 2010;102:1637-52.
40. Lee HE, Kim JH, Kim YJ, Choi SY, Kim SW, Kang E, et al: An increase in cancer

stem cell population after primary systemic therapy is a poor prognostic factor in breast cancer. Br J Cancer. 2011;104:1730-8.

**Table 1**

Combination indexes of olaparib with five different cytotoxic agents in two breast cancer cell lines sensitive to olaparib

| | Doxorubicin | Etoposide | Cisplatin | Paclitaxel | SN38 |
|---|---|---|---|---|---|
| KPL-3C | 0.52±0.01 | 0.84±0.06 | 0.67±0.34 | 1.25±0.04 | 0.85±0.01 |
| MDA-MB-231 | 0.72±0.01 | 0.51±0.02 | 0.74±0.22 | 1.07±0.03 | 0.24±0.04 |

**Figure Legends**

**Figure 1**

Growth inhibitory curves of olaparib in a panel of six breast cancer cell lines of non-familial origin. The cells were treated with the indicated concentration of olaparib for three days. The values were the means±SEs. -◆-, KPL-1 cells; -■-, KPL-3C cells; -▲-, BT-474 cells; -□-, KPL-4 cells; -✳-, MDA-MB-157 cells; and -●-, MDA-MB-231 cells.

**Figure 2**

Effects of olaparib on cell cycle progression in the MDA-MB-231 cells (A) and KPL-3C cells (B). The cells were treated with 2 or 5 $\mu$M of olaparib for three days. Percentages of cells at each cell cycle phase were analyzed as described in the Materials and Methods. The values at each cell cycle phase were the means±SEs. White bars represent the values for the control cells. Grey bars represent the values for 2 $\mu$M olaparib-treated cells. Black bars represent the values for 5 $\mu$M olaparib-treated cells. *, $P < 0.05$; **, $P < 0.01$.

**Figure 3**

Effects of olaparib on apoptosis induction in the MDA-MB-231 cells (A), KPL-3C cells (B) and BT-474 cells (C). The cells were treated with 2 or 5 $\mu$M of olaparib for three days. Percentages of apoptotic cells were analyzed as described in the Materials and Methods. The values were the means±SEs. White bars represent the values for the control cells. Grey bars represent the values for 2 $\mu$M olaparib-treated cells. Black bars represent the values for 5 $\mu$M olaparib-treated cells. *, $P < 0.05$; **, $P < 0.01$.

**Figure 4**

Western blot analyses for p-ERK, ERK and β-actin in the MDA-MB-231 cells (A) and KPL-3C cells (B). The cells were treated with 2 μM olaparib plus minus 10 or 20 μM U0126 for indicated duration of time.

**Figure 5**

Effects of olaparib and U0126 on cell cycle progression (A) and apoptosis induction (B) in the MDA-MB-231 cells. The cells were treated with 2 μM olaparib plus minus 10 μM U0126 for three days. Percentages of cells at each cell cycle phase and apoptotic cells were analyzed as described in the Materials and Methods. The values were the means±SEs. White bars represent the values for the control cells. Light grey bars represent the values for 2 μM olaparib-treated cells. Dark grey bars represent the values for 10 μM U0126-treated cells. Black bars represent the values for cells treated with both agents. *, $P < 0.05$; **, $P < 0.01$.

**Figure 6**

Effects of olaparib on the proportion of $CD44^+/CD24^{-\text{ or low}}/ESA^+$ putative cancer stem cells in the MDA-MB-231 cells (A), KPL-3C cells (B) and BT-474 cells (C). The cells were treated with 2 or 5 μM of olaparib for three days. Percentages of $CD44^+/CD24^{-\text{ or low}}/ESA^+$ cells were analyzed as described in the Materials and Methods. The values were the means±SEs. White bars represent the values for the control cells. Grey bars represent the values for 2 μM olaparib-treated cells. Black bars represent the values for 5 μM olaparib-treated cells. *, $P < 0.05$; **, $P < 0.01$.

**Figure 7**

Effects of olaparib on the number of mammospheres/1,000 seeded cells in the MDA-MB-231 cells (A), KPL-3C cells (B) and BT-474 cells (C). The cells were treated with 2 or 5 $\mu$M of olaparib for three days. Mammospheres were analyzed as described in the Materials and Methods. The values were the means±SEs. White bars represent the values for the control cells. Grey bars represent the values for 2 $\mu$M olaparib-treated cells. Black bars represent the values for 5 $\mu$M olaparib-treated cells. *, $P < 0.05$; **, $P < 0.01$.

**Figure 8**

Growth inhibitory curves of SN38 plus minus olaparib in the MDA-MB-231 cells. The cells were treated with the indicated concentrations of SN38 plus minus 2 $\mu$M olaparib for three days. The values were the means±SEs. ○-○, SN38 alone; and ●-●, SN38 and olaparib.

**Figure 9**

Effects of olaparib and SN38 on cell cycle progression (A) and apoptosis induction (B) in the MDA-MB-231 cells. The cells were treated with 2 $\mu$M olaparib plus minus 0.25 nM SN38 for three days. Percentages of cells at each cell cycle phase and apoptotic cells were analyzed as described in the Materials and Methods. The values were the means±SEs. White bars represent the values for the control cells. Light grey bars represent the values for olaparib-treated cells. Dark grey bars represent the values for SN38-treated cells. Black bars represent the values for cells treated with both agents. *, $P < 0.05$; **, $P < 0.01$.

**Figure 10**

Effects of olaparib and SN38 on the proportion of $CD44^{+}/CD24^{-\text{ or low}}/ESA^{+}$ putative cancer stem cells (A) and number of mammospheres/1,000 seeded cells (B) in the MDA-MB-231 cells. The cells were treated with 2 $\mu$M olaparib plus minus 0.25 nM SN38 for three days. The cell surface marker assay and mammosphere assay were performed as described in the Materials and Methods. The values were the means±SEs. White bars represent the values for the control cells. Light grey bars represent the values for olaparib-treated cells. Dark grey bars represent the values for SN38-treated cells. Black bars represent the values for cells treated with both agents. *, $P < 0.05$; **, $P < 0.01$.

**Figure 1**

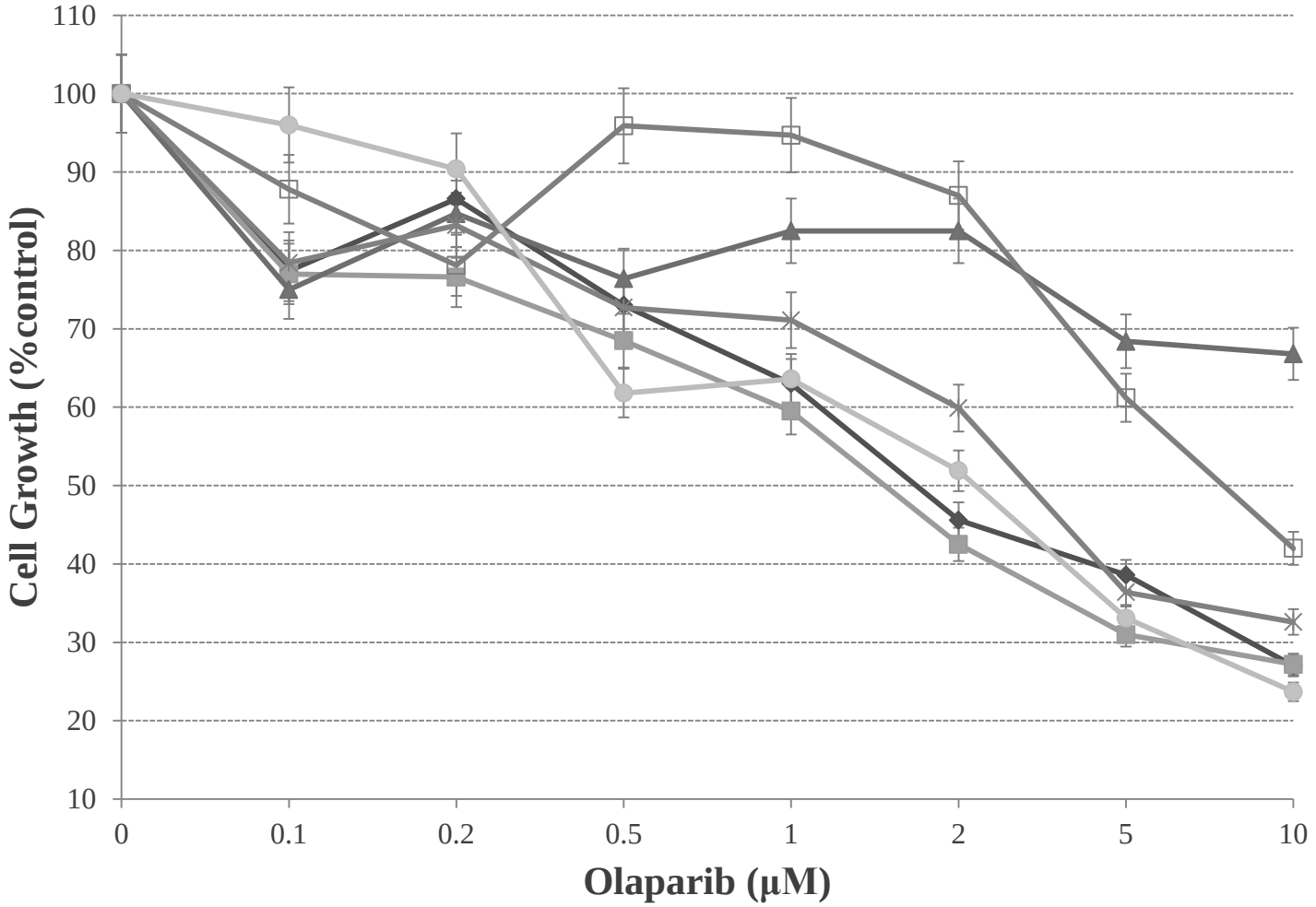

110
100
90
80
70
60
50
40
30
20
10
Cell Growth (%control)
0
0.1
0.2
0.5
1
2
5
10
Olaparib (μM)

**Figure 2A**

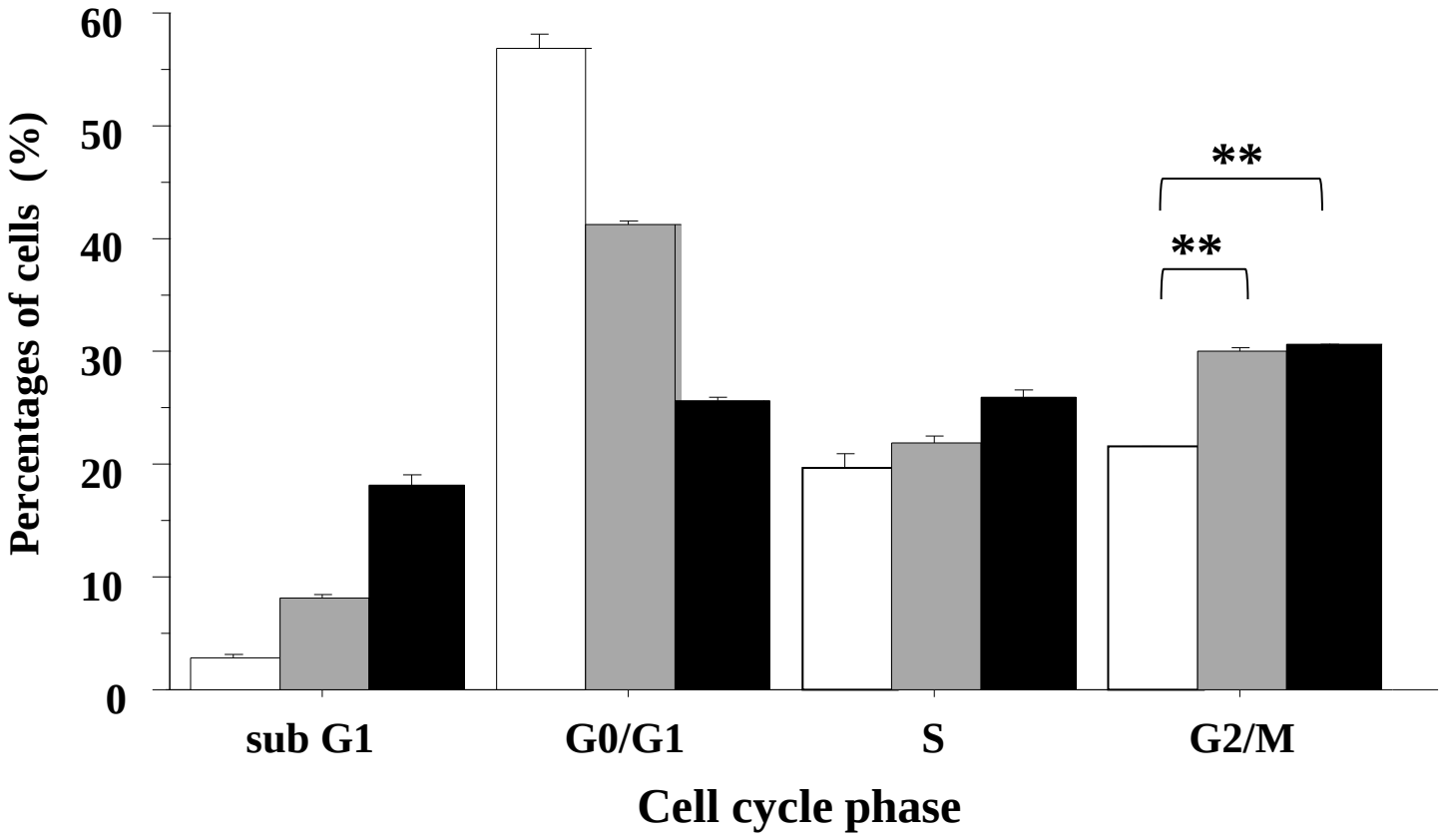

60
50
40
30
20
10
0
Percentages of cells (%)
**
**
sub G1
G0/G1
S
G2/M
Cell cycle phase

**Figure 2B**

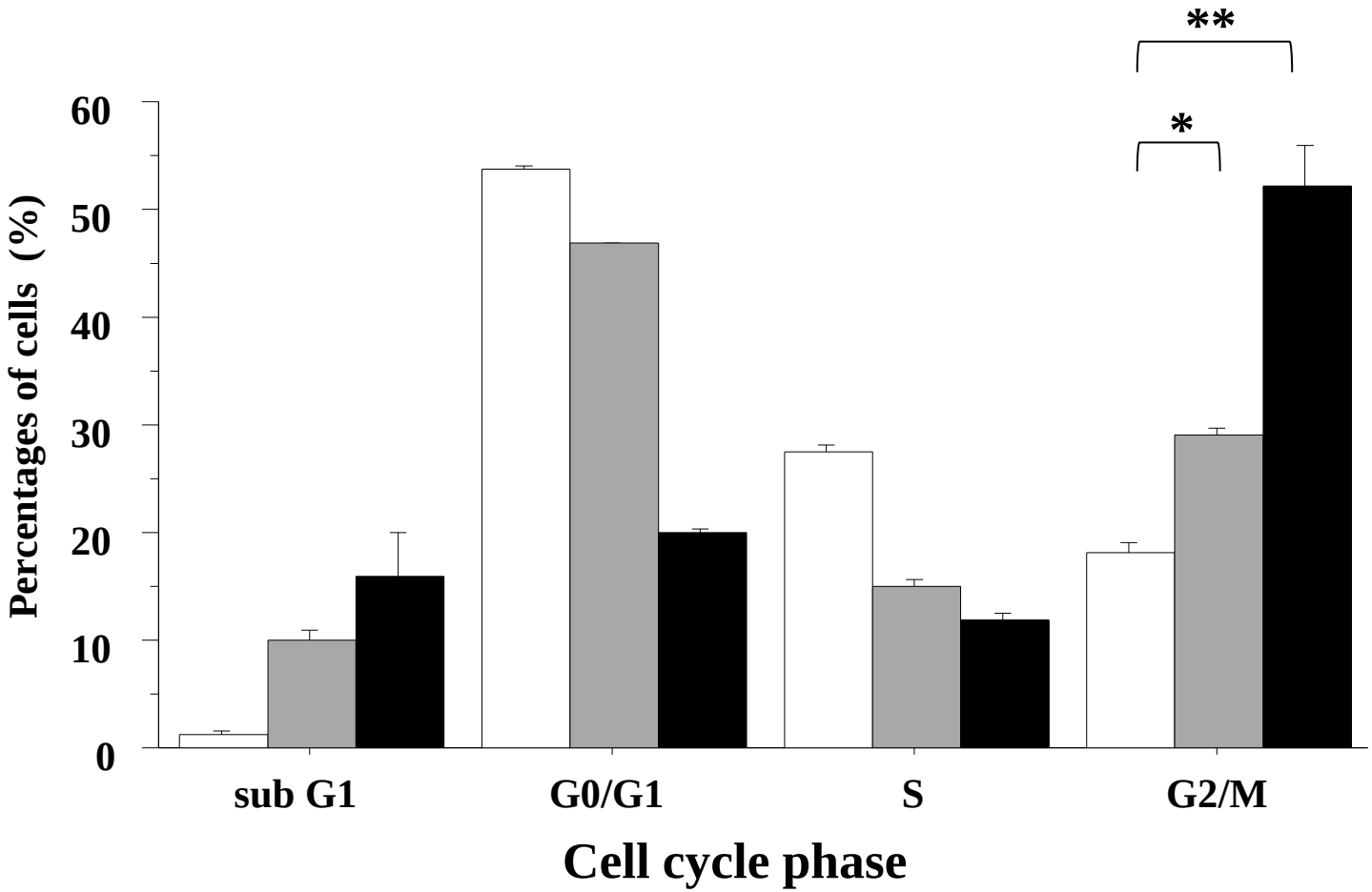
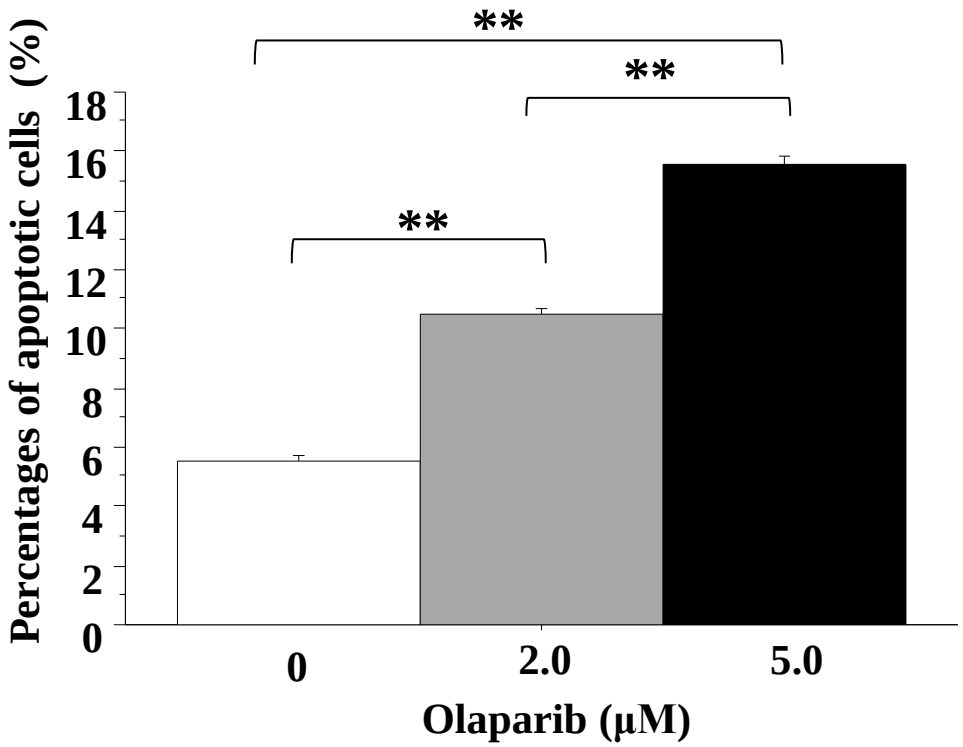

**
*
60
50
40
30
20
10
0
Percentages of cells (%)
sub G1
G0/G1
S
G2/M
Cell cycle phase

**Figure 3A**


**
**
**
Percentages of apoptotic cells (%)
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16
14
12
10
8
6
4
2
0
0
2.0
5.0
Olaparib (μM)

**Figure 3B**

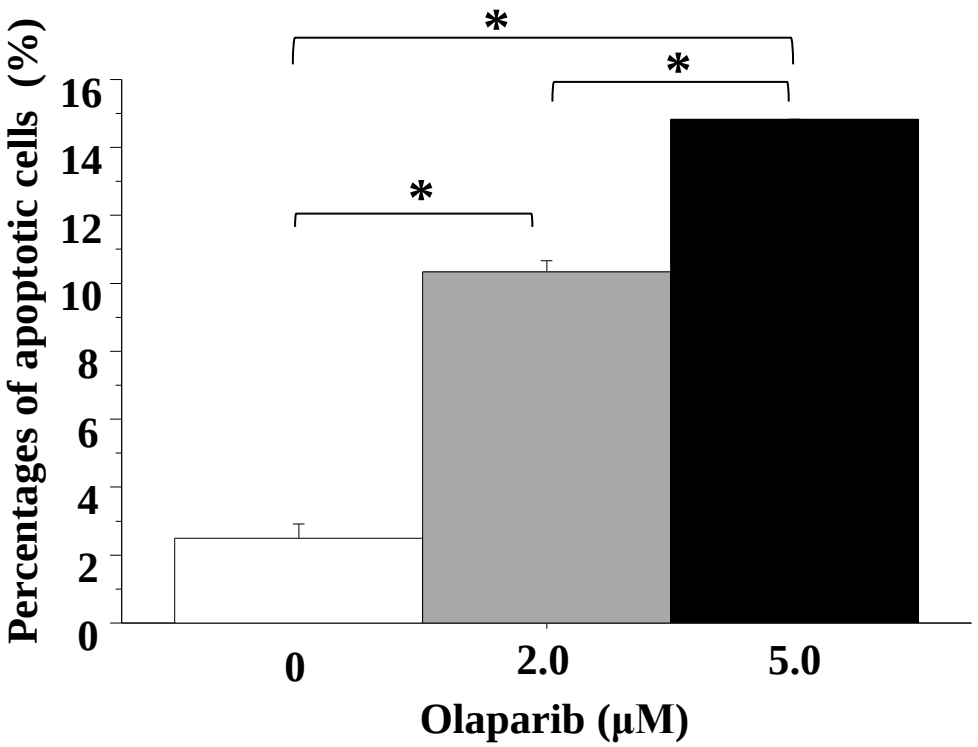

*
*
*
Percentages of apoptotic cells (%)
16
14
12
10
8
6
4
2
0
0
2.0
5.0
Olaparib (μM)

**Figure 3C**

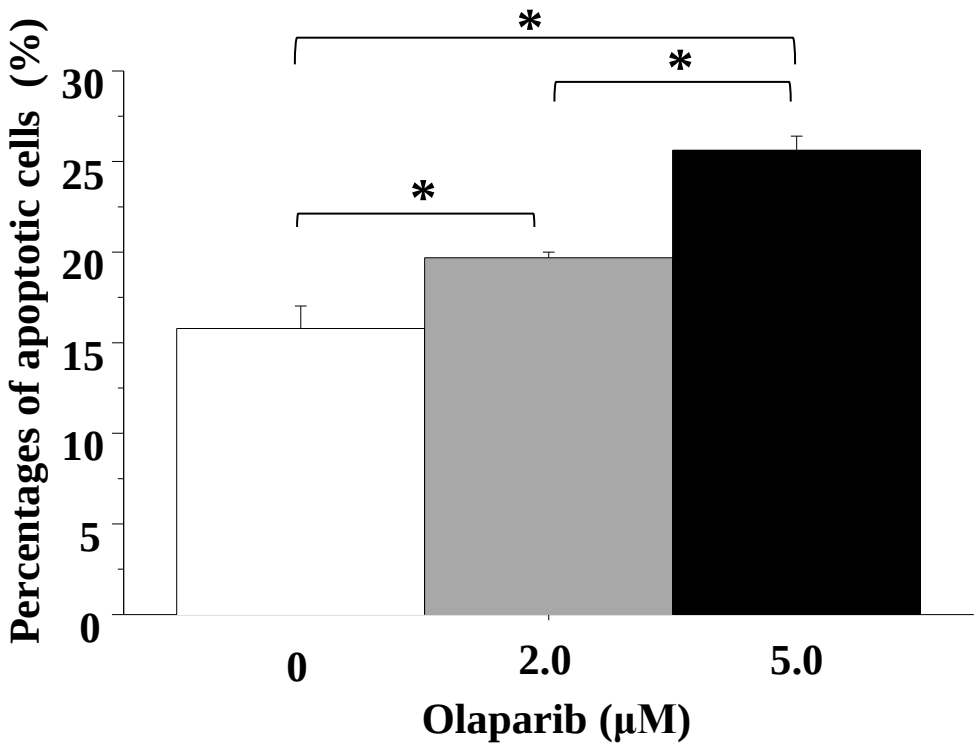

*
*
*
Percentages of apoptotic cells (%)
30
25
20
15
10
5
0
0
2.0
5.0
Olaparib (μM)

**Figure 4A**

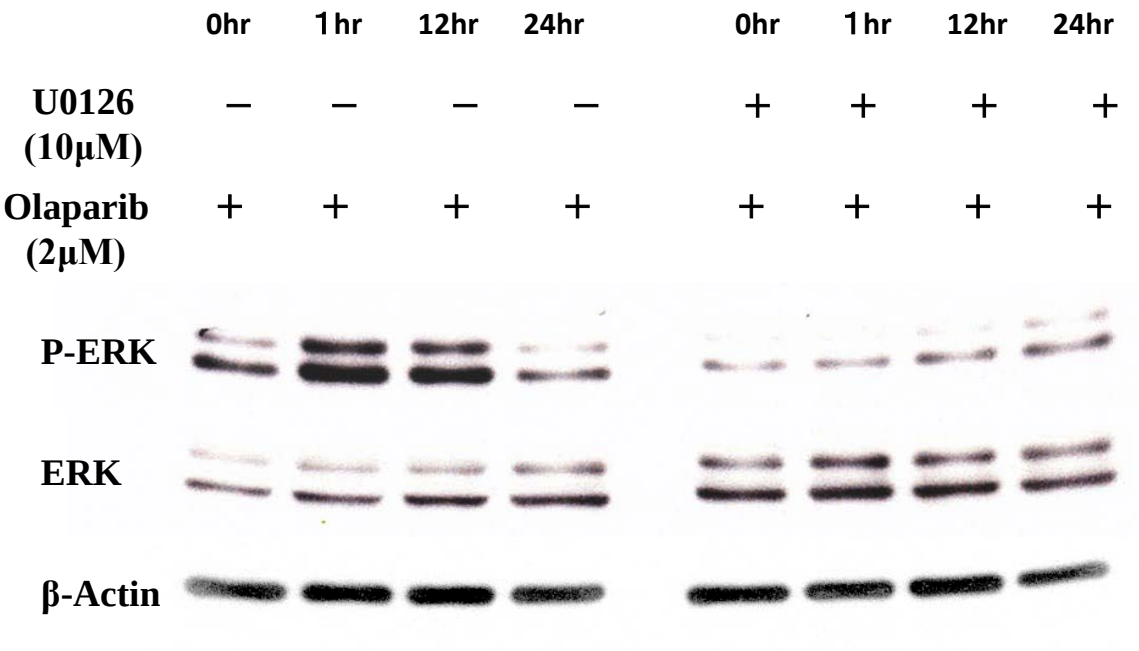

0hr
1hr
12hr
24hr
0hr
1hr
12hr
24hr
U0126 (10μM)
− − − − + + + +
Olaparib (2μM)
+ + + + + + + +
P-ERK
ERK
β-Actin

**Figure 4B**

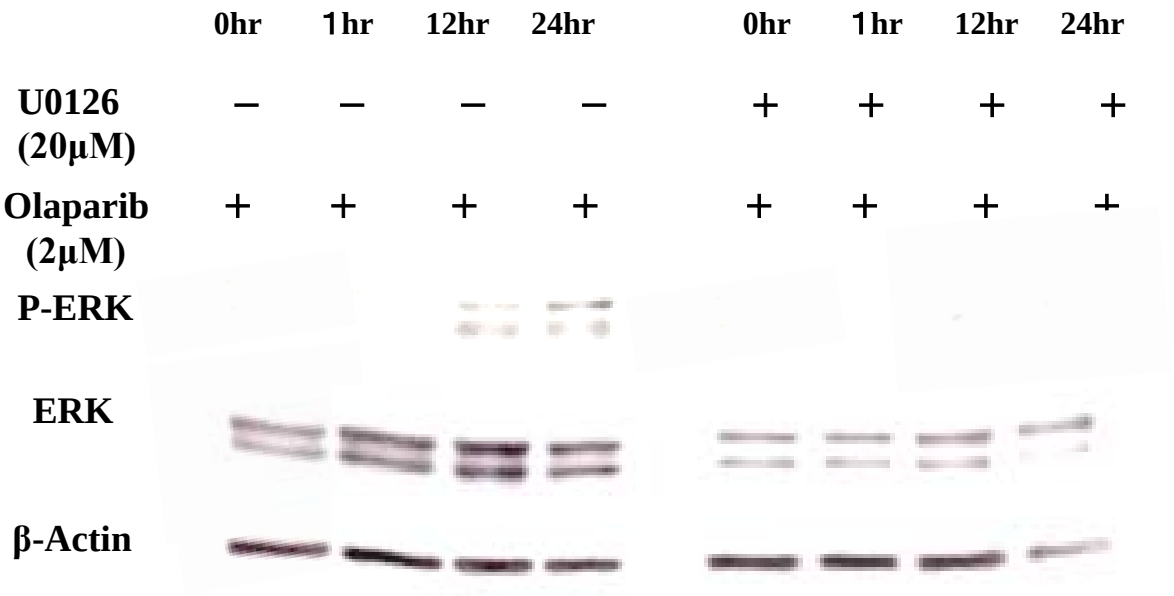

0hr
1hr
12hr
24hr
0hr
1hr
12hr
24hr
U0126 (20μM)
− − − − + + + +
Olaparib (2μM)
+ + + + + + + +
P-ERK
ERK
β-Actin

**Figure 5A**

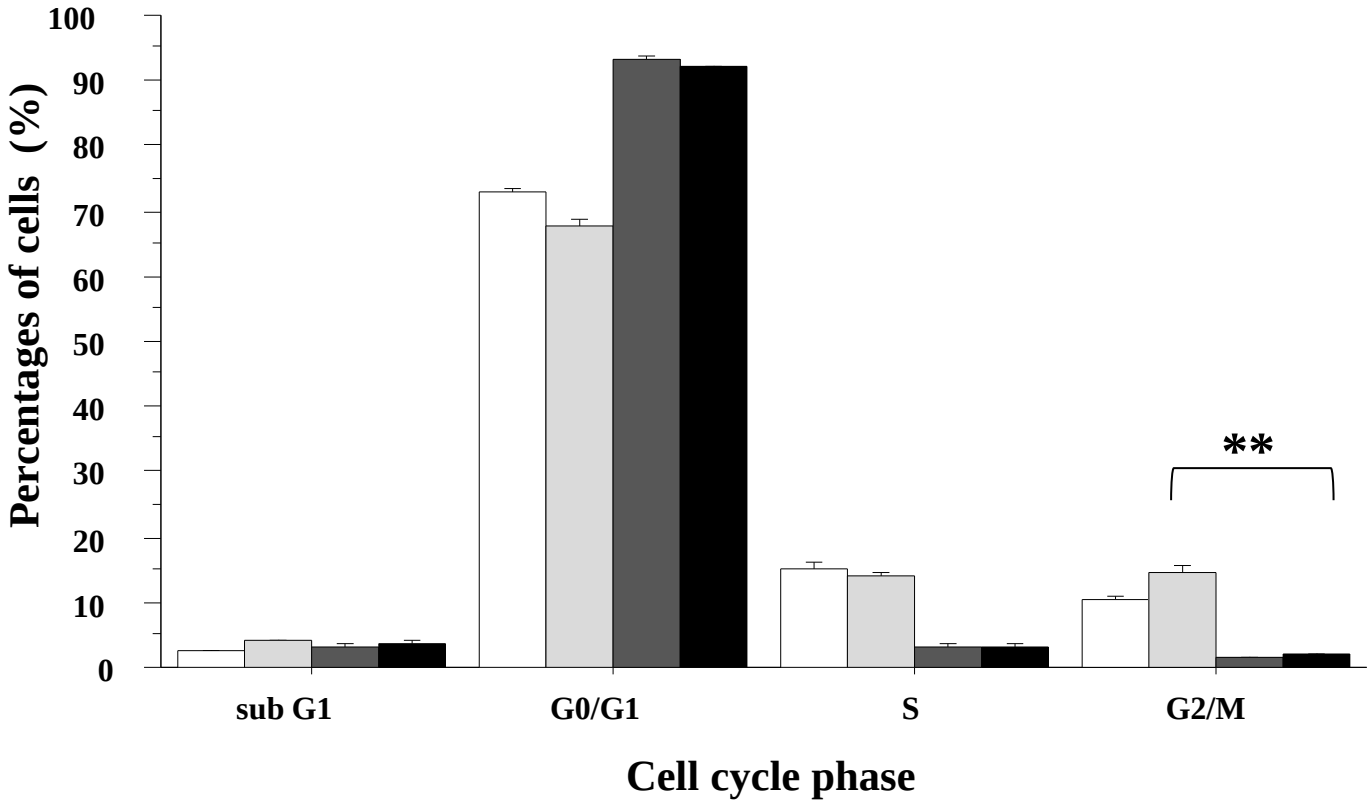
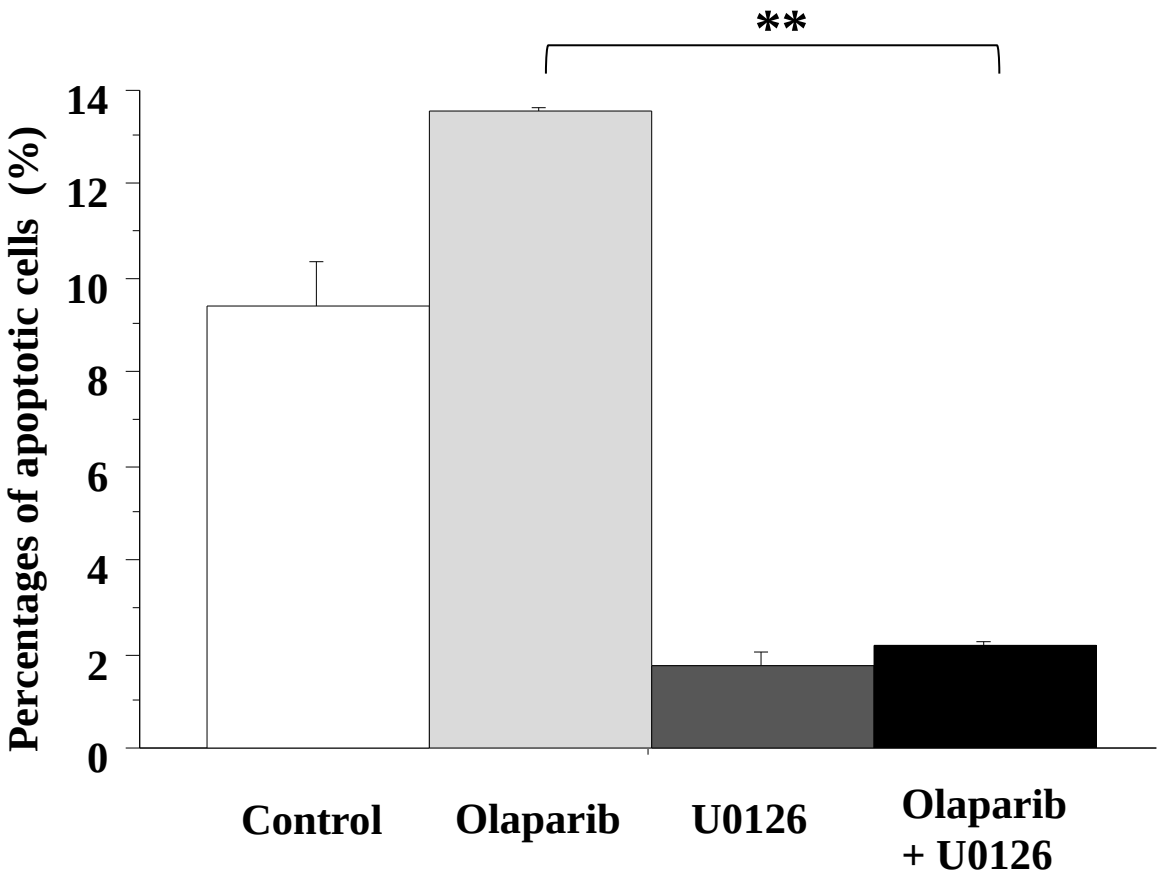

Percentages of cells (%)
100
90
80
70
60
50
40
30
20
10
0
**
sub G1
G0/G1
S
G2/M
Cell cycle phase

**Figure 5B**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Percentages of apoptotic cells (%)
0
2
4
6
8
10
12
14
Control
Olaparib
U0126
Olaparib
+ U0126

**Figure 6A**

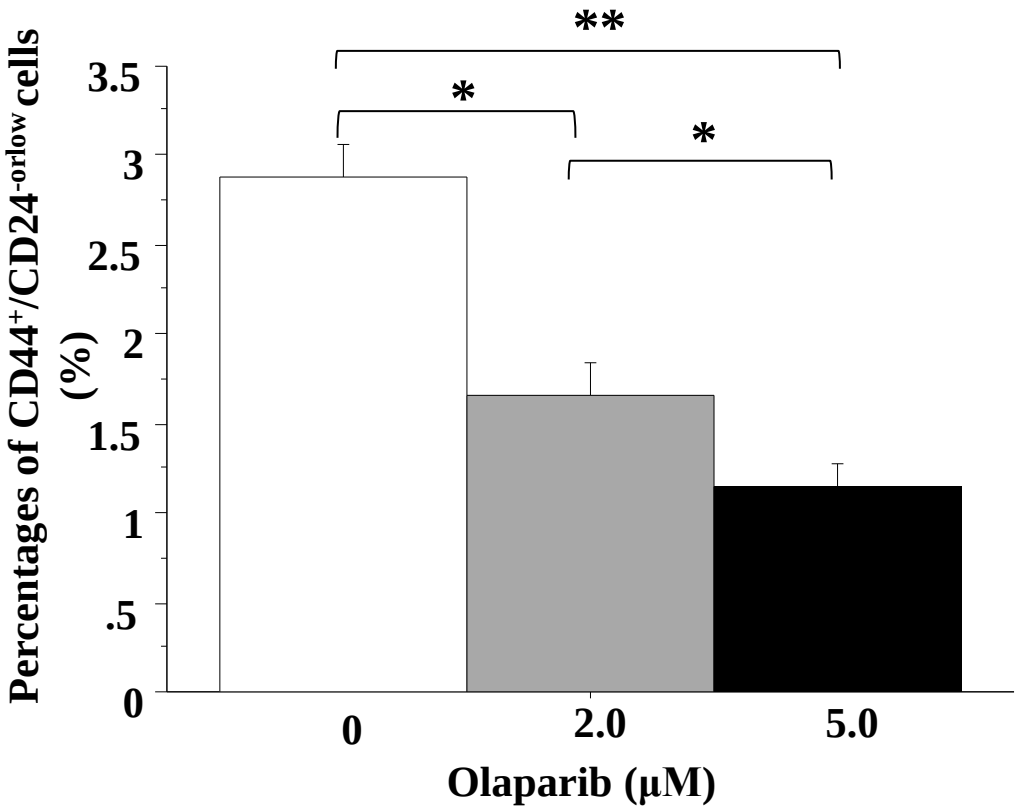

**
*
*
3.5
3
2.5
2
1.5
1
.5
0
Percentages of CD44+/CD24-orlow cells (%)
0
2.0
5.0
Olaparib (μM)

**Figure 6B**

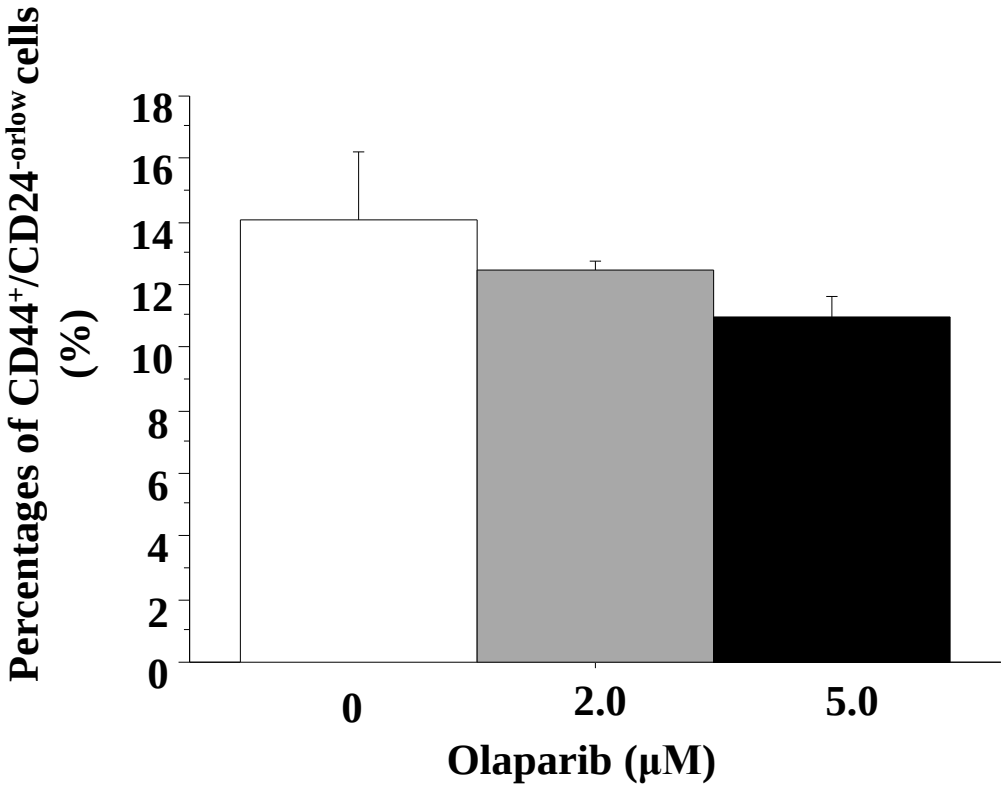
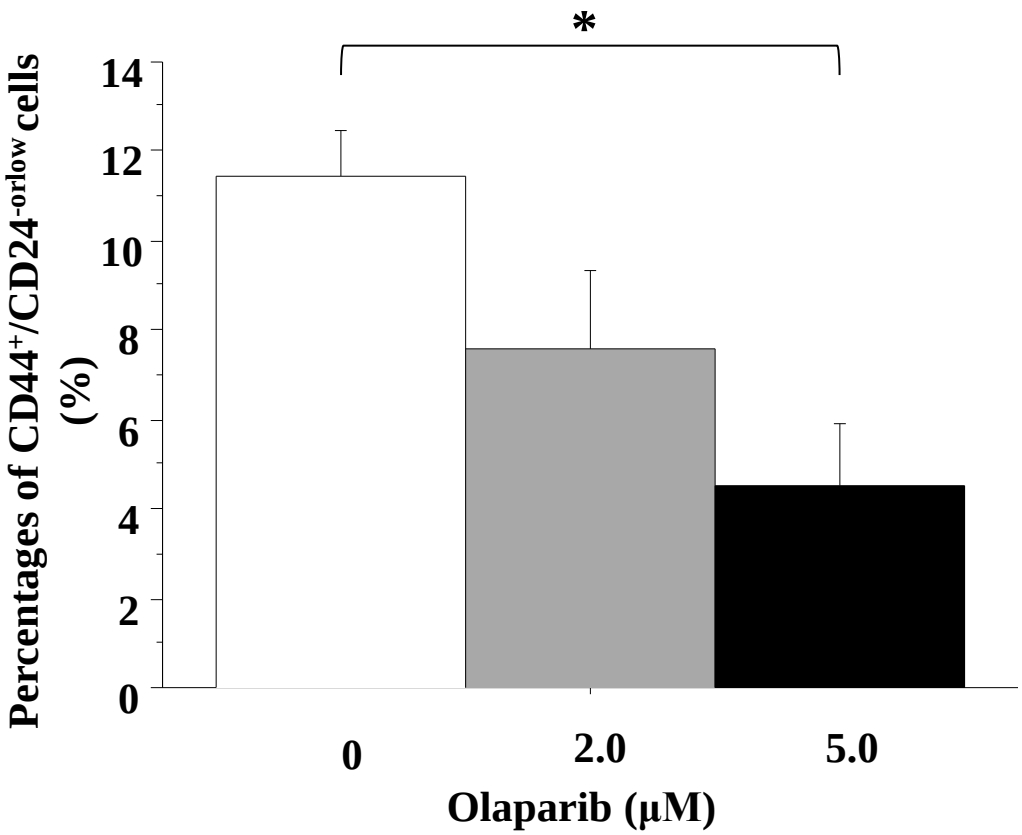

Percentages of CD44+/CD24-orlow cells (%)
18
16
14
12
10
8
6
4
2
0
0
2.0
5.0
Olaparib (μM)

**Figure 6C**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Percentages of CD44+/CD24-orlow cells (%)
14
12
10
8
6
4
2
0
0
2.0
5.0
Olaparib (μM)

**Figure 7A**

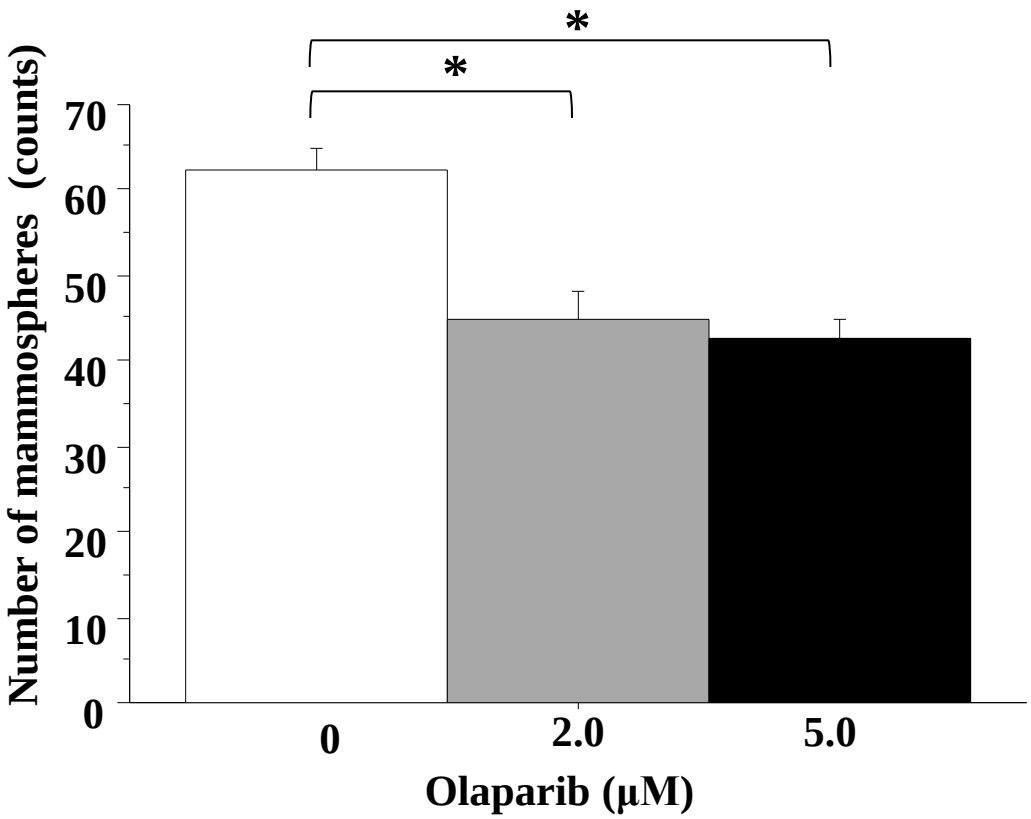

*
*
Number of mammospheres (counts)
70
60
50
40
30
20
10
0
0
2.0
5.0
Olaparib (μM)

**Figure 7B**

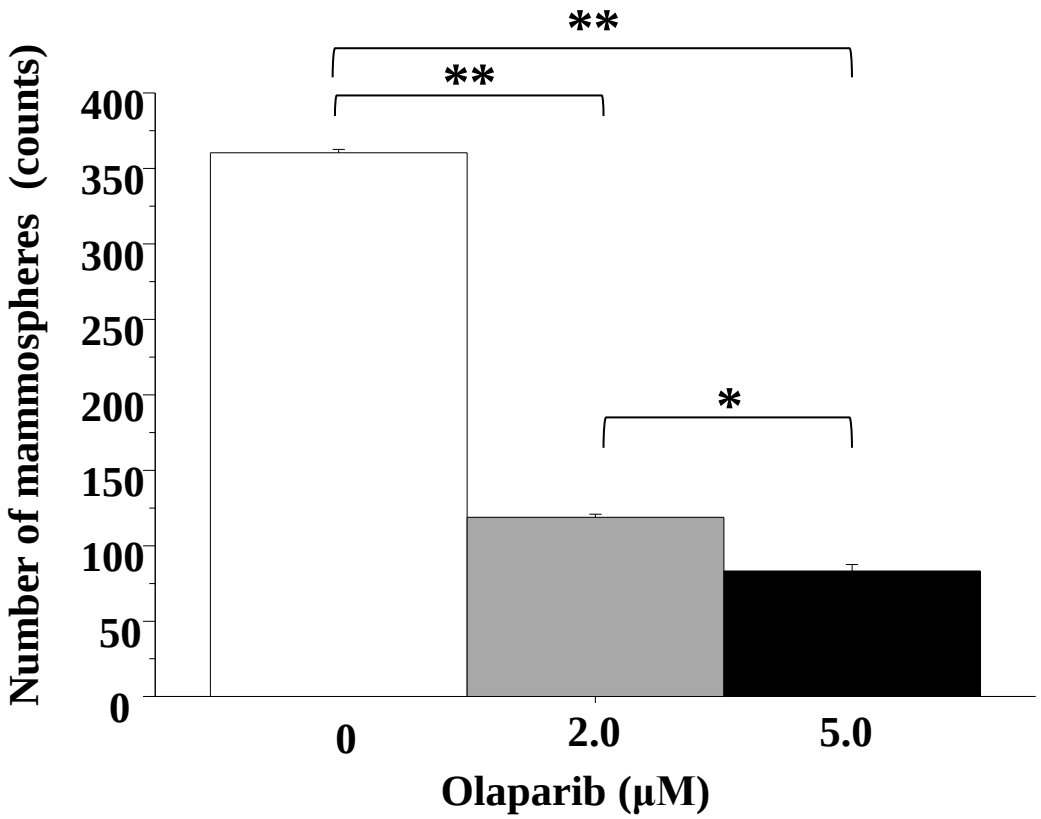

**
**
*
Number of mammospheres (counts)
400
350
300
250
200
150
100
50
0
0
2.0
5.0
Olaparib (μM)

**Figure 7C**

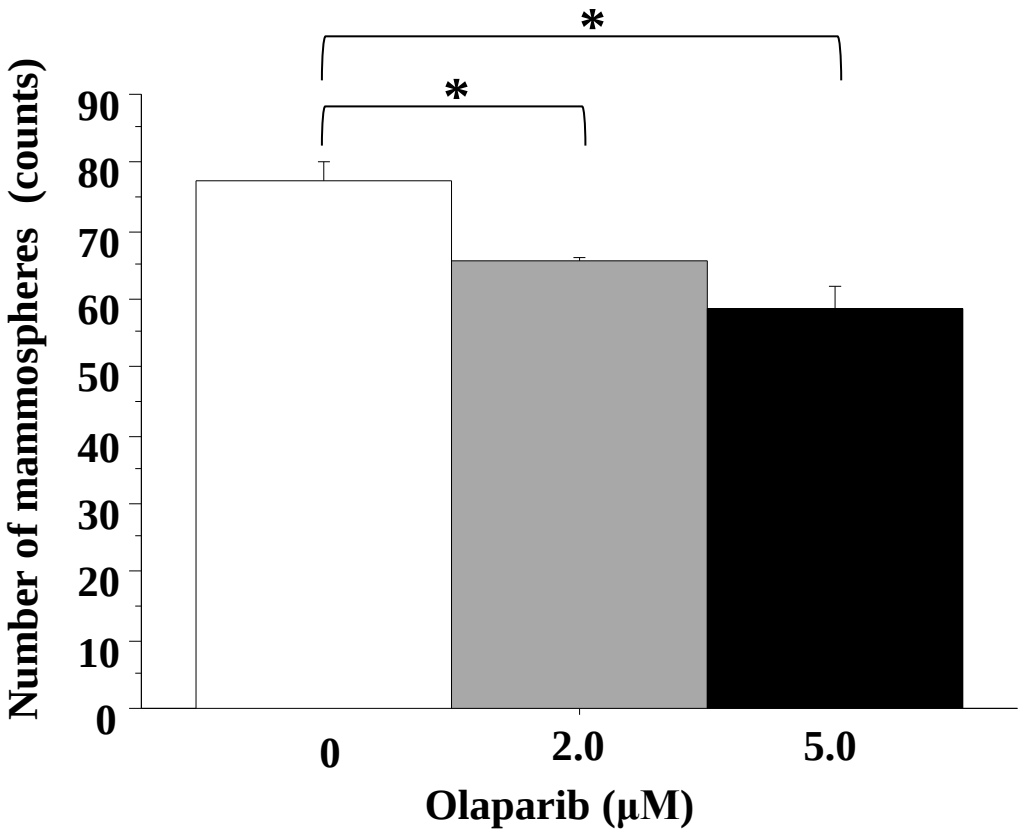

*
*
Number of mammospheres (counts)
90
80
70
60
50
40
30
20
10
0
0
2.0
5.0
Olaparib (μM)

**Figure 8**

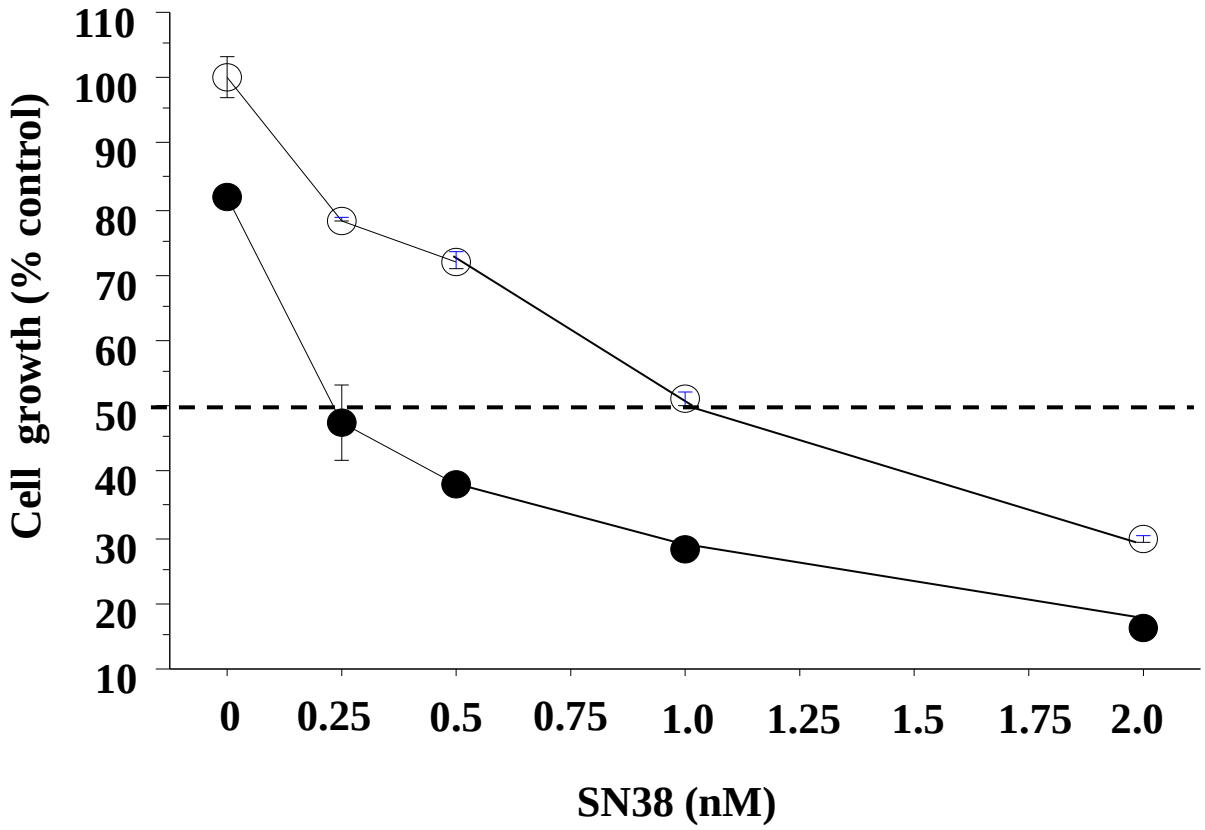

Cell growth (% control)
110
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
0.75
1.0
1.25
1.5
1.75
2.0
SN38 (nM)

**Figure 9A**

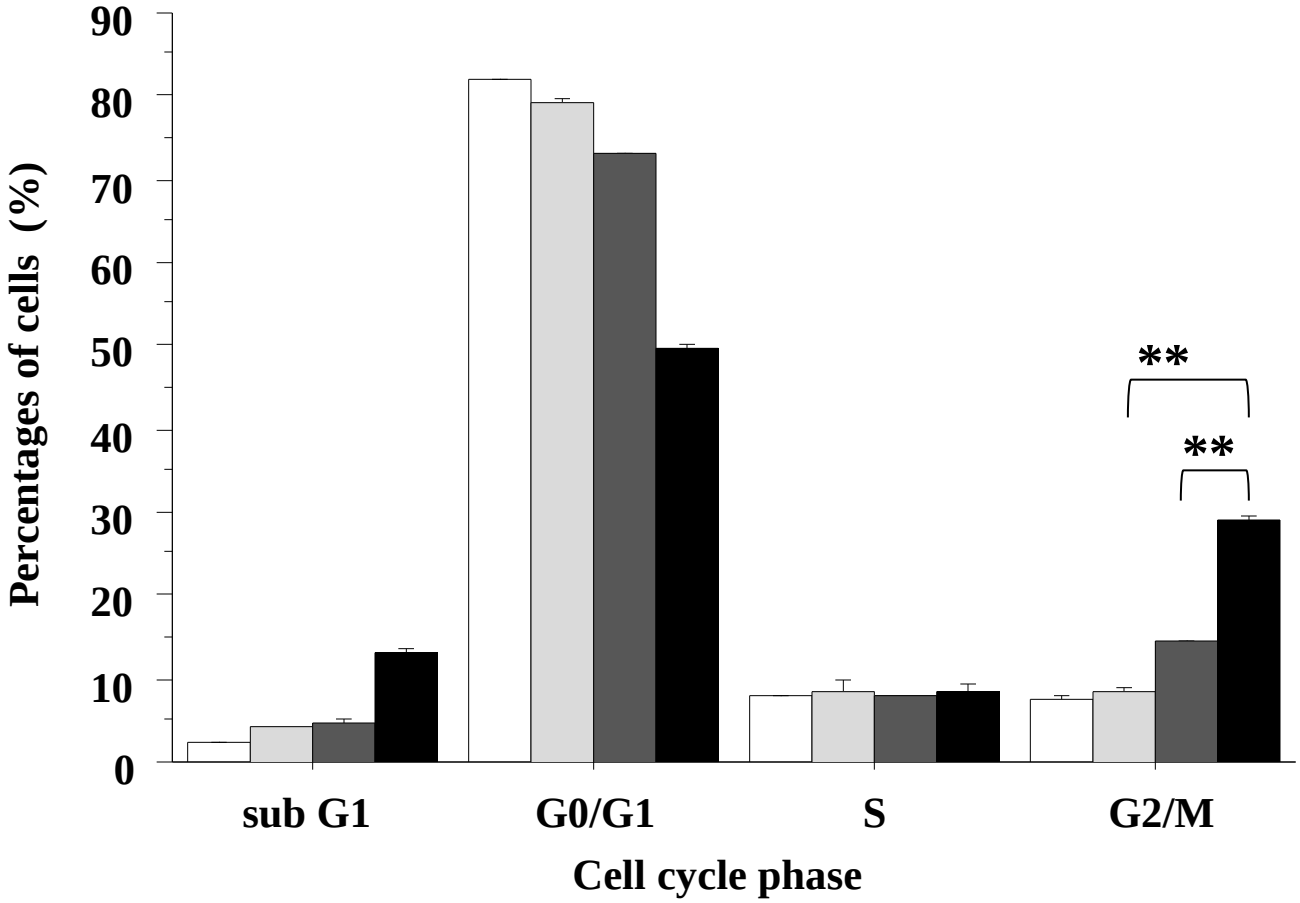

Percentages of cells (%)
90
80
70
60
50
40
30
20
10
0
**
**
sub G1
G0/G1
S
G2/M
Cell cycle phase

**Figure 9B**

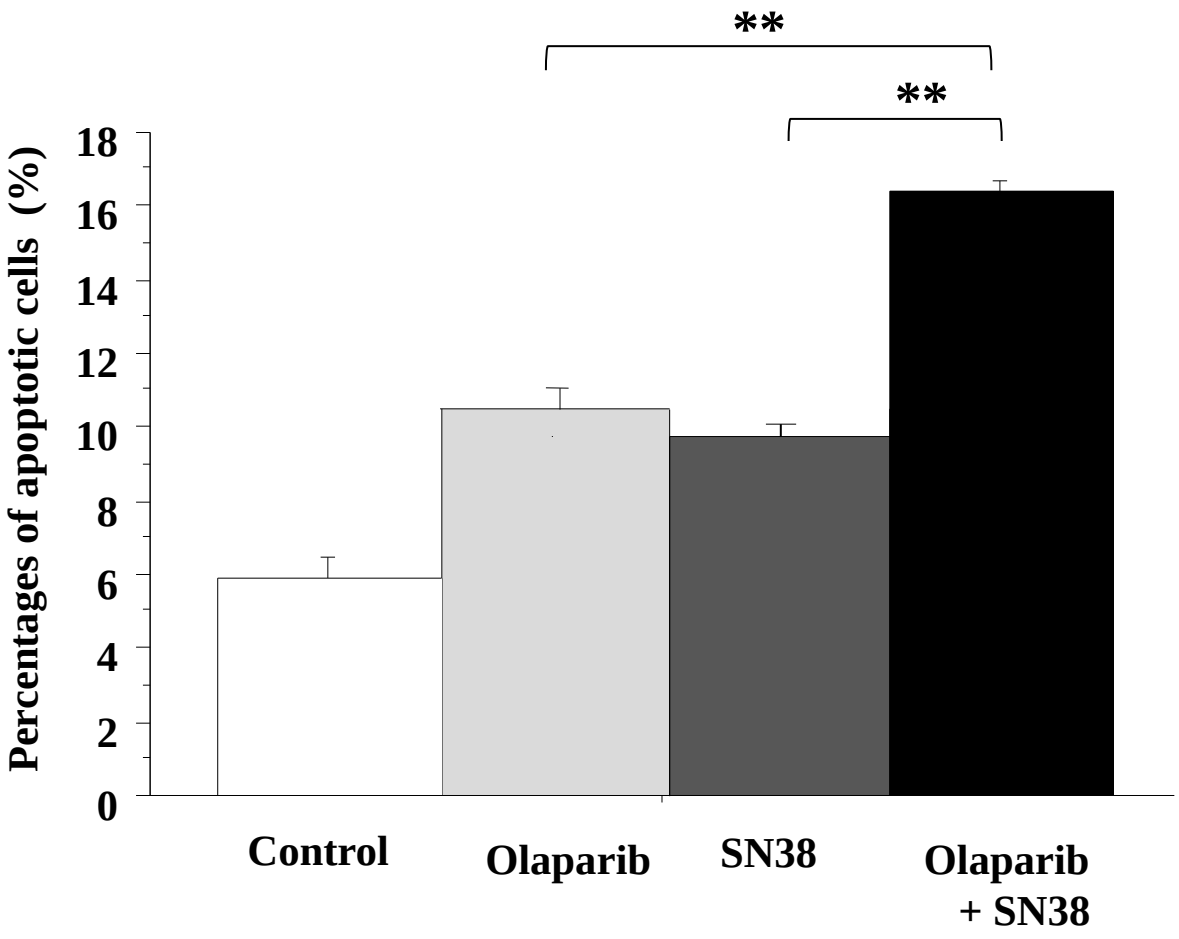

**
**
Percentages of apoptotic cells (%)
18
16
14
12
10
8
6
4
2
0
Control
Olaparib
SN38
Olaparib
+ SN38

**Figure 10A**

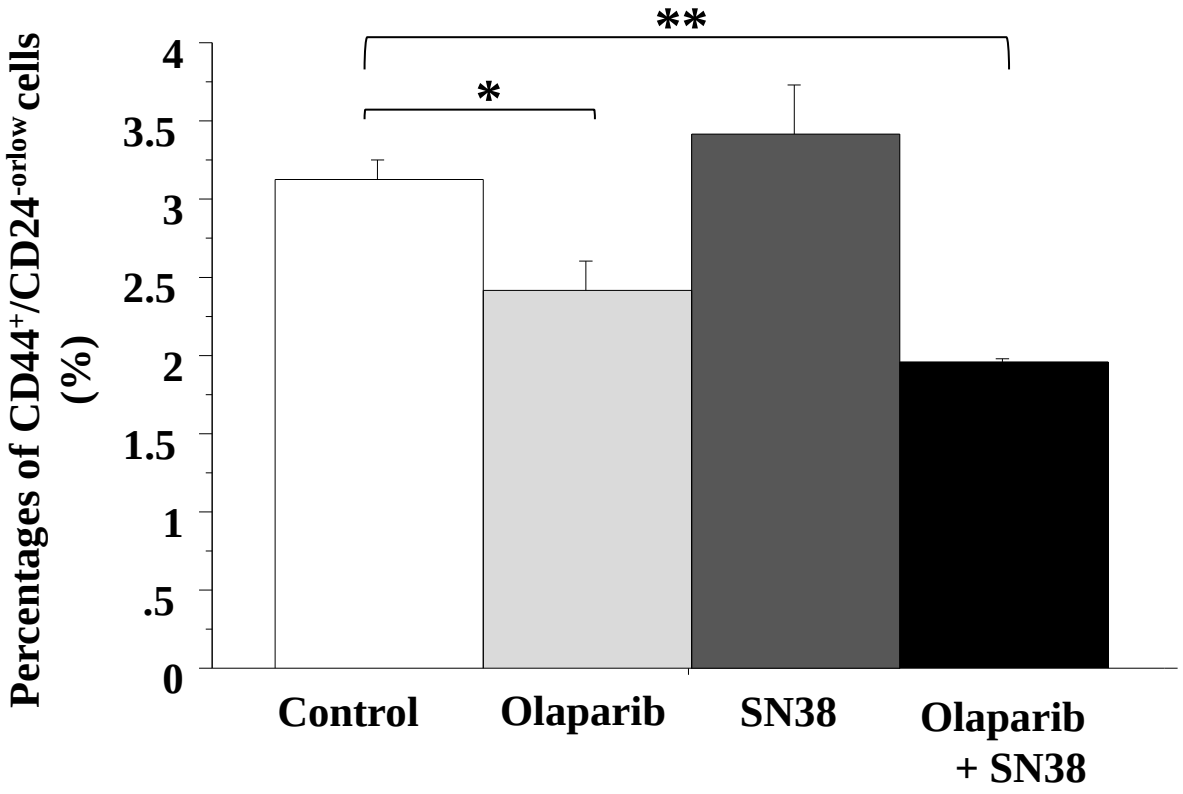
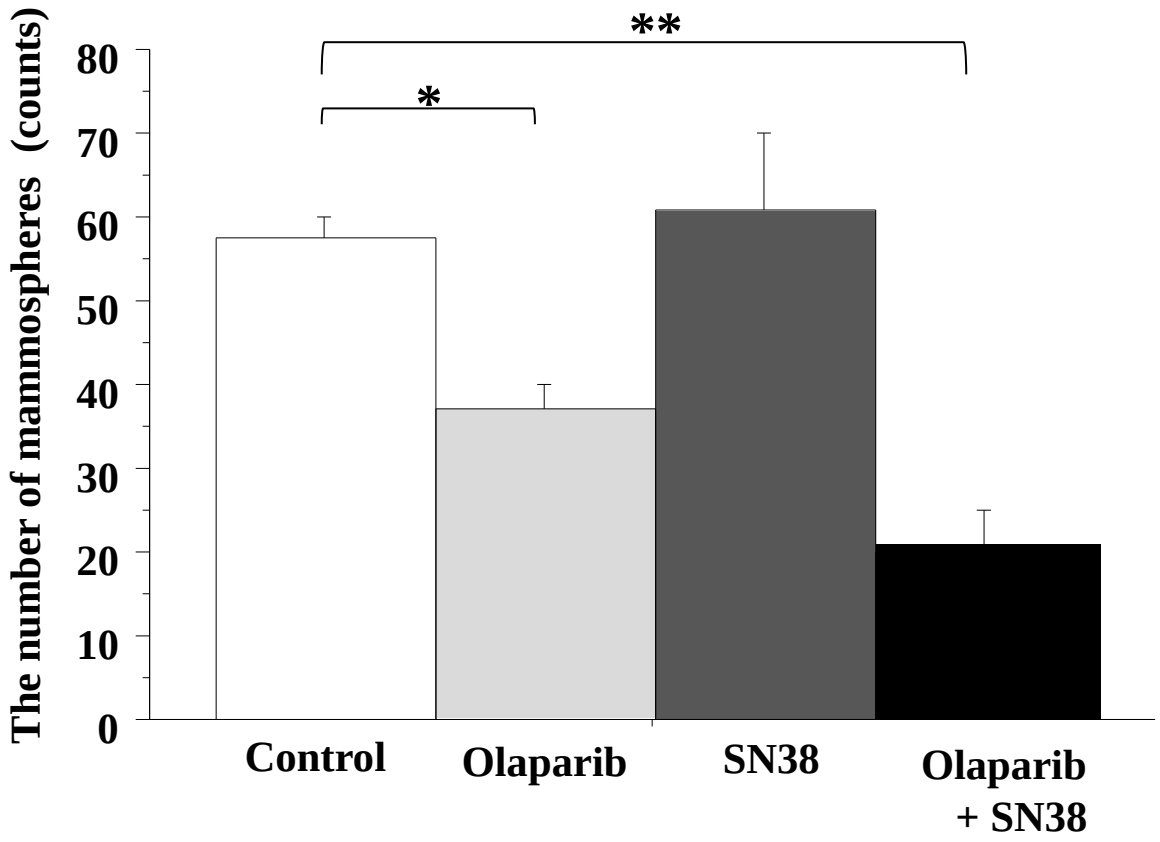

**
*
Percentages of CD44+/CD24-orlow cells (%)
4
3.5
3
2.5
2
1.5
1
.5
0
Control
Olaparib
SN38
Olaparib + SN38

**Figure 10B**


**
*
The number of mammospheres (counts)
80
70
60
50
40
30
20
10
0
Control
Olaparib
SN38
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