Sigma-2 ligands and PARP inhibitors synergistically trigger cell death in breast cancer cells

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Abstract

The sigma-2 receptor is overexpressed in proliferating cells compared to quiescent cells and has been used as a target for imaging solid tumors by positron emission tomography. Recent work has suggested that the sigma-2 receptor may also be an effective therapeutic target for cancer therapy. Poly (ADP-ribose) polymerase (PARP) is a family of enzymes involved in DNA damage response. In this study, we looked for potential synergy of cytotoxicity between PARP inhibitors and sigma-2 receptor ligands in breast cancer cell lines. We showed that the PARP inhibitor, **YUN3-6**, sensitized mouse breast cancer cell line, EMT6, to sigma-2 receptor ligand (**SV119**, **WC-26**, and **RHM-138**) induced cell death determined by cell viability assay and colony forming assay. The PARP inhibitor, olaparib, sensitized tumor cells to a different sigma-2 receptor ligand **SW43**-induced apoptosis and cell death in human triple negative cell line, MDA-MB-231. Olaparib inhibited PARP activity and cell proliferation, and arrested cells in G2/M phase of the cell cycle in MDA-MB-231 cells. Subsequently cells became sensitized to **SW43** induced cell death. In conclusion, the combination of sigma-2 receptor ligands and PARP inhibitors appears to hold promise for synergistically triggering cell death in certain types of breast cancer cells and merits further investigation.

1. Introduction

Members of the Poly (adenosine diphosphate [ADP]) ribose polymerase (PARP) enzyme family catalyze (ADP-ribosyl)ation, a post-translational modification that is important in many physiologic processes including DNA repair, replication, transcription, mitochondrial function, and cell division [1]. PARP1-catalyzed poly(ADP-ribosyl)ation is one of the earliest responses to DNA damage. Studies over the past decade also revealed that PARP1 plays additional roles in the repair of double strand breaks by the homologous recombination (HR) and alternative end joining (Alt-EJ) mechanisms. PARP may be an effective target for breast cancer, especially for cancers with inherent defects of homologous DNA repair such as BRCA-related cancers and likely some triple negative breast cancers (TNBC) [2]. It has been reported that dysfunctional PARP-1 and BRCA display the property of “synthetic lethality”, where inhibition or depletion of PARP-1 effectively results in cell death in BRCA-defective tumor cells [3]. This process of synthetic lethality has led to the development of small molecule PARP inhibitors as potential chemotherapeutic agents. PARP inhibitors are believed to work by inhibiting PARP enzyme activity and by trapping PARP1 and PARP2 enzymes at damaged DNA. Trapping of PARP1 and PARP2 bound to PARP inhibitors on DNA is thought to result in replication fork collapse and is more cytotoxic than catalytic inhibition alone [4]. Traditionally, PARP inhibitors have worked synergistically with DNA-damaging agents (DNA-damaging chemotherapy or radiotherapy); however, there has been inconsistent efficacy and notable toxicity [3], especially in breast cancer treatment [5, 6]. Thus, an alternative choice to traditional targeted therapy that has synergy with PARP inhibitors would be clinically meaningful.

Sigma-2 receptors are expressed in high density in nearly all human and rodent tumor cell lines [7]. Sigma-2 receptor densities have been correlated with the grade of some human and

bovine tumors [8-10]. The density of sigma-2 receptors in proliferating mouse mammary adenocarcinoma cells was found to be 10-fold higher than that in the corresponding quiescent tumor cells [11, 12]. These observations have led to the development of the sigma-2 receptor ligands not only as molecular probes for diagnostic imaging of solid tumors [11-13], but also as potential drugs for treatment of cancer [14, 15].

Data indicate that sigma-2 receptor ligands including **SV119** and **SW43** are potential therapeutic drugs as a single agent, a sensitizer for other chemotherapeutic drugs, or a drug delivery agent for treating breast, ovarian and pancreatic cancer [16-21]. Mechanistically, **SV119** and **SW43**, two structural congeners, are believed to kill cancer cells by multiple signaling pathways including induction of reactive oxygen species (ROS), lysosomal leakage, caspase-3 dependent and independent apoptosis. We hypothesized that sigma-2 ligand mediated apoptosis, which preferentially targets proliferating cells, would act synergistically with PARP inhibitors, which induce DNA damage through replication fork collapse. Furthermore, preclinical studies [11] and a human imaging study [22] showed that sigma-2 receptors are overexpressed in proliferating cancer cells and that sigma-2 radioligand is highly uptaken in proliferating tumors but not normal marrow. Thus the combination of sigma-2 ligands and PARP inhibitors could result in better selectivity to tumors, with lower toxicity to normal proliferating tissue such as marrow. The purpose of this study was to evaluate for synergy between PARP inhibitors and sigma-2 receptor ligands in mouse and human breast cancer cell lines to facilitate the design of future animal studies.

2. Materials and methods

2.1. Cell culture

EMT6 mouse breast cancer cells from American Type Culture Collection (ATCC, Manassas, VA) were grown in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. MDA-MB-231 cells from American Type Culture Collection (ATCC, Manassas, VA) were grown in MEM containing 5% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids (Mediatech Inc, Manassas, VA), 2% MEM vitamins (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 µg/ml streptomycin. Both cell lines were maintained at 37°C in a humidified incubator with a 5% CO₂/95% air atmosphere.

2.2. MTS cell viability assay and Trypan blue viability assay

The cytotoxicity of the compounds was determined using the CellTiter96 Aqueous One Solution (Promega, Madison, WI) as described previously [23]. The cytotoxicity and anti-proliferation effect of the compounds were assessed by Trypan blue viability assay. Briefly, 100 µl cell suspension was mixed with 100 µl Trypan blue solution (Sigma-Aldrich, St. Louis, MO), and then the live and dead cell numbers were counted using an automated cell counter (ThermoFisher Scientific, Grand Island, NY).

2.3. Caspase 3 activity assay

MDA-MB-231 cells were plated in black 96-well plate at a cell density of 5000 cells/well for 24h. The cells were then treated with **SW43** and olaparib at the indicated concentrations for

24h. Caspase 3/7 activity was measured by using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI).

2.4. PARP activity assay

PARP activity in MDA-MB-231 cells was determined using the HT Chemiluminescent PARP/Apoptosis Assay (Trevigen, Gaithersburg, MD), according to the manufacturer's protocol. Cells were plated in 96-well plates at a density of 1×10^4 cells/well for 24h. The cells were treated with 0, 50 or 100 μ M olaparib for 24h. The cells were lysed with 50 μ L/well cell extraction buffer. The cell lysate (25 μ L/well) was mixed with PARP substrate cocktail (25 μ L/well) in a histone-coated 96-well plate for 30 min. PAR deposited onto immobilized histone proteins were then detected with an anti-PAR monoclonal antibody, goat anti-mouse IgG-HRP conjugate, and HRP substrate to generate a chemiluminescent signal that were quantified in an Enspire plate reader (PerkinElmer Life and Analytical Sciences, Shelton, CT).

2.5. Clonogenic assay

The clonogenic assay enables an assessment of the differences in reproductive viability (capacity of cells to produce progeny; i.e. a single cell to form a colony of 50 or more cells) between untreated and treated cells. Cells were plated in 6-well plates at a density of 1000 cells/well. After 24h of drug treatment, cells were washed with PBS twice and allowed to grow 1-3 weeks in drug free media. The colonies of 50 or more cells were visualized by crystal violet staining and counted.

2.6. Annexin V assay

Apoptotic cells were detected using Muse Annexin V and Dead Cell Assay Kit (EMD Millipore, MA), according to the manufacture's protocol. Briefly, cells were treated with drugs for 24h. The cells, which included attached and detached cells, were collected, washed twice with cold PBS and then resuspended in the culture media at a concentration of 2×10^5 cells/ml. 100 μ l of the solution (2×10^4 cells) was incubated with 100 μ l Muse Annexin V and Dead Cell Reagent for 20 min at room temperature and analyzed by flow cytometry within 1h using Muse[®] Cell Analyzer (EMD Millipore, MA).

2.7. Cell cycle analysis

Cell cycle analysis was performed using Muse Cell Cycle Assay Kit (EMD Millipore, MA), according to the manufacture's protocol. Briefly, cells were treated with drugs for 24h. The cells, which included attached and detached cells, were collected, washed twice with cold PBS. Cells were then fixed in cold 70% ethanol for at least 3 hours at -20°C. Cells were then washed once with PBS. Cell pellets (2×10^5 cells) were resuspended in 200 μ l of Muse Cell Cycle Assay Reagent for 30 min at room temperature. Cells were analyzed by Muse[®] Cell Analyzer (EMD Millipore, MA).

2.8. Statistical analysis

The results are expressed as the mean $\pm$ s.d. based on three independent experiments performed in triplicate. Differences among groups were analyzed by one-way or two-way ANOVA with a Tukey's post-hoc test. A p-value of <0.05 was considered significant.

3. Results

3.1. The PARP inhibitor, YUN3-6, sensitized mouse breast cancer cells EMT6 to sigma-2 ligand-induced cell death

Sigma-2 receptor ligands, **SV119**, **WC-26**, **RHM-138** and PARP inhibitor, **YUN3-6**, were synthesized previously [20, 24]. The structures of the compounds are shown in the Supplementary Data. Mouse breast cancer cells EMT6 were pretreated with vehicle or the PARP inhibitor, 5 μ M **YUN3-6**, for 1 h and then treated with vehicle or increasing concentrations of sigma-2 ligands (**SV119**, **WC26** and **RHM-138**) for 24h, 48 h or 72 h. Cell viability was determined using MTS assay. The data showed that 5 μ M **YUN3-6** leftward shifted the dose response curve for all the three sigma-2 ligands (Fig. 1), suggesting that the PARP inhibitor, **YUN3-6**, sensitized EMT6 cells to sigma-2 ligand-induced cell death. The EC₅₀ values of sigma-2 receptor ligands in the absence and presence of **YUN3-6** are listed in Table 1.

The clonogenic assay was also used to assess the effect of the PARP inhibitor, **YUN3-6**, on sigma-2 receptor ligand-induced cell death in EMT6 cells. EMT6 cells were treated with the sigma-2 ligand, **SV119**, in the absence or presence of **YUN3-6** for 24 or 48h and then were cultured in drug free media for two weeks. Cell colonies were visualized and counted. The data show that **YUN3-6** potentiated **SV119**-induced decrease in cell viability (Fig. 2A and Fig. 2B), suggesting synergistic effects of the two agents together. EMT6 cells were treated with **SV119** (0, 20 or 30 μ M) and **YUN3-6** (0 or 5 μ M) for 72h. There were morphologic changes in the cells treated with both drugs, which also suggests that **YUN3-6** potentiates toxicity induced by **SV119** (Fig. 2C).

3.2. The PARP inhibitor, olaparib, sensitized human triple negative breast cancer cell line MDA-MB-231 to sigma-2 ligand induced cell death and apoptosis

Olaparib is a PARP inhibitor currently used for targeted therapy in a variety of cancers including breast and ovarian cancers. Therefore, we tested the effect of olaparib on sigma-2 receptor ligand-induced cell death in the human triple negative breast cancer cell line, MDA-MB-231. The cells were treated with 50 μ M or 100 μ M olaparib for 24 h. The cells were then lysed and the PARP activity was measured. We confirmed that olaparib inhibited PARP activity in MDA-MB-231 cells (Fig. 3A). We then tested whether olaparib would work synergistically with the sigma-2 receptor ligand, **SW43**, to induce cell death in MDA-MB-231 cells. The cells were treated with olaparib and increasing concentrations of **SW43** for 48 h and assessed for cell viability by MTS assay. There was significantly more killing with the combination olaparib and **SW43** than either ligand alone, demonstrating an additive effect of the two drugs (Fig. 3B and C).

We then studied whether **SW43** and/or olaparib induced cell death was through a pathway involving cellular apoptosis. **SW43** significantly increased Annexin V positive cells (Fig. 4A). Olaparib alone did not induce Annexin V positive cells and did not significantly change **SW43** induced Annexin V positive cell numbers. The data suggest that olaparib had little effect on **SW43**-induced apoptosis. On the other hand, the combination of 25 μ M **SW43** and 50 μ M olaparib significantly increased caspase-3 activation compared to the single agent (**SW43** or olaparib) treatment (Fig. 3D). Thus, olaparib potentiated caspase-3 activation may be through non-apoptotic mechanisms. Olaparib did not cause cell death by itself but significantly increased the number of dead cells induced by **SW43** (Fig. 4B). In addition, flow cytometry analysis data

showed that the combination of both **SW43** and olaparib significantly increased the subG1 cell population compared to the single agent (**SW43** or olaparib) alone (Fig. 4C). Sub-G1 cells have a lower DNA content than normal cells and are generally thought to represent the proportion of cells in late stage apoptosis [25]. Collectively, these data suggest that olaparib sensitized cells to **SW43** induced apoptosis and cell death.

3.3. Olaparib inhibited proliferation and arrested cells in G2/M phase in MDA-MB-231 cells

To analyze the mechanism of the synergistic effect of PARP inhibitor and sigma-2 receptor ligand in inducing cell death in tumor cells, we performed cell cycle analysis. MDA-MB-231 cells were treated with **SW43**, olaparib or combination of the both. All cells, which included attached and detached cells, were harvested for cell cycle analysis. The result showed that olaparib treatment increased the G2/M cell population from 23% to 47% (Fig. 4C), suggesting that olaparib arrested cells in G2/M phase. Furthermore, olaparib alone decreased the total cell number by 41% compared to the control, but did not induce cell death after 24h treatment (Fig. 4A and B). These data suggest that olaparib inhibited cell proliferation, at least partially by arresting cells in G2/M phase. G2/M phase arrest or/and inhibition of proliferation may sensitize cells to **SW43**-induced cytotoxicity.

4. Discussion

This study demonstrated that a PARP inhibitor, **YUN3-6**, and sigma-2 receptor ligand, **SV119**, have moderate synergistic cytotoxicity in a mouse breast cancer cell line by cell viability assay and clonogenic assay (Fig. 1 and 2). The combination of the sigma-2 receptor ligand **SW43** and PARP inhibitor, olaparib, resulted in additive cytotoxic effect on MDA-MB-231 cells by cell viability assay and caspase-3 assay (Fig. 3B-D and Fig. 4B and C) and synergistic effects

on dead cell number and sub-G1 populations. In addition, olaparib arrested MDA-MB-231 cells in G2/M phase.

Prior work has suggested that **SW43** and olaparib induce cell death by separate signaling pathways. It was reported previously that **SW43** induced oxidative stress [18]. It was reported that PARP inhibitors including olaparib trap PARP1 and 2 at damaged DNA [4]. Trapping PARP1 and 2 in the DNA in the presence of olaparib was shown to be cytotoxic, possibly by posing an obstacle to DNA replication. The combination of oxidative stress induced by **SW43**, together with PARP inhibition by olaparib, may lead to synthetic lethality. Moreover, our data showed that olaparib arrested MDA-MB-231 cells at G2/M phase. Other reports showed that olaparib can cause Chk1 phosphorylation, and that Chk1 activation is required for the G2/M arrest in response to DNA-damage agents [26, 27]. Therefore, it is possible that trapping PARP-1 in DNA by olaparib may stall replication forks, thereby causing replication stress [28], activating G2 checkpoint (e.g., Chk1) signaling cascade, leading to G2/M phase arrest. Trapping PARP-1 to DNA and arresting cells in G2/M may increase cyclin A and B expression level [27], which expressed in G2 and M phase, respectively. **SV119** has been shown to completely degrade cyclin A and B [20]. Thus, olaparib induced G2/M arrest make cells more vulnerable to sigma-2 ligands.

Combination of sigma-2 ligands and PARP inhibitors may allow lower therapeutic concentrations for each drug and less toxicities to areas with little sigma receptors such as the bone marrow. Many therapeutic advances have been made in breast cancer treatment using animal models based on preliminary culture results, and we will use this data as a foundation for future studies to move towards clinical translation. Future studies will clarify the mechanism of

action of combined sigma-2 ligands and PARP inhibitors using *in vitro* cell culture models.
They will also combine sigma-2 ligands and PARP inhibitors *in vivo* to assess toxicity reduction
and treatment response in animal models of disease. In conclusion, a carefully chosen
combination of PARP inhibitors, sigma-2 receptor ligands and tumor cell types may result in
better anti-tumor effects than PARP inhibitors or sigma-2 receptor ligands alone.

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Table 1, EC₅₀ values of sigma-2 receptor ligands in the absence and presence of 5 µM **YUN3-6** in EMT6 cells

	SV119		WC-26		RHM-138	
	No YUN3-6	5 µM YUN 3-6	No YUN3-6	5 µM YUN 3-6	No YUN3-6	5 µM YUN 3-6
EC _{50, 24h} (µM)	39.2±1.6	34.7±0.5	55.1±1.5	56.4±2.0	35.6±2.9	37.8±4.5
EC _{50, 48h} (µM)	34.7±3.2	19.4±0.5	26.8±0.6	20.6±1.0	25.5±4.2	21.2±0.5
EC _{50, 72h} (µM)	32.8±1.1	13.2±1.4	21.8±0.2	16.1±0.2	24.6±1.4	17.8±0.4

Figure legends:

Figure 1. The PARP inhibitor, **YUN3-6**, sensitized sigma 2 ligand-induced cell death in EMT6 cells by MTS assay. Mouse breast cancer cells EMT6 were pretreated with vehicle or 5 μ M **YUN3-6**, for 1 h and then treated with increasing concentrations of sigma-2 ligands (**SV119**, **WC-26** and **RHM-138**) for 48 h or 72 h. Cell viability was determined using MTS assay. The bars represent mean $\pm$ SD in a representative experiment performed in triplicates.

Figure 2. **SV119** and **YUN3-6** synergistically induced cell death by clonogenic assay in EMT6 cells. (A) EMT6 cells were treated with **SV119** (0 or 30 μ M) and **YUN3-6** (0 or 5 μ M) for 24h and then were cultured in drug free media for two weeks. Cell colonies were visualized and counted. The bars represent mean $\pm$ SD in a representative experiment performed in triplicates. *p = 0.0125 (control vs 5 μ M **YUN3-6**), *p < 0.0001 (control vs 30 μ M **SV119**), **p < 0.0001 (5 μ M **YUN3-6** vs 5 μ M **YUN3-6** + 30 μ M **SV119**). One-way ANOVA with a Tukey's post-hoc test. (B) EMT6 cells were treated with **SV119** (0, 10, or 30 μ M) and **YUN3-6** (0 or 5 μ M) for 48h and then were cultured in drug free media for two weeks. Cell colonies were visualized. Representative images of EMT6 colonies were taken using a digital camera. (c) The morphology

of EMT6 cells treated with **SV119** (0, 20 or 30 μ M) and **YUN3-6** (0 or 5 μ M) for 72h. The images of cells were taken under a phase contrast microscope. The cells were shrunk and damaged by the treatment of **SV119** alone or the combination of **SV119** and **YUN3-6**.

Figure 3. Olaparib sensitized MDA-MB-231 cells to **SW43** induced cytotoxicity. (A) The cells were treated with 50 or 100 μ M olaparib for 24h and assessed for PARP activity. (B) The cells were treated with increasing concentrations of **SW43** in the absence or presence of 50 μ M olaparib for 48h and then assessed for cell viability by MTS assay. (C) The combination of 25 μ M **SW43** and 50 μ M olaparib induced greater cytotoxicity than single agent, **SW43** or olaparib. (D) The combination of **SW43** (25 μ M) and olaparib (10 or 50 μ M) enhanced caspase 3/7 activation compared to either **SW43** or olaparib treatment alone. *p<0.0001 (0 μ M **SW43** + 0 μ M olaparib vs 25 μ M **SW43** + 0 μ M olaparib), **p < 0.0001 (0 μ M **SW43** + 0 μ M olaparib vs 25 μ M **SW43** + 10 μ M olaparib), **p = 0.0254 (25 μ M **SW43** + 0 μ M olaparib vs 25 μ M **SW43** + 10 μ M olaparib), ***p < 0.0001 (0 μ M **SW43** + 50 μ M olaparib vs 25 μ M **SW43** + 50 μ M olaparib), ***p < 0.0001 (25 μ M **SW43** + 0 μ M olaparib vs 25 μ M **SW43** + 50 μ M olaparib).
Two-way ANOVA with a Tukey's post-hoc test.

Figure 4. Olaparib sensitized MDA-MB-231 cells to **SW43** induced cell death and arrested cells at G2/M phase. (A) The cells were treated with 25 μ M **SW43** or/and 50 μ M olaparib for 24h and then assessed by Annexin V assay. (B) The cells were treated with 25 μ M **SW43** or/and 50 μ M olaparib for 24h. Live and dead cell numbers were accounted using Trypan blue viability assay. *p<0.0001 (for live cells, control vs 25 μ M **SW43** or 50 μ M olaparib), **p=0.0042 (for live cells, 25 μ M **SW43** vs 25 μ M **SW43** + 50 μ M olaparib) or **p<0.0001 (for live cells, 50 μ M olaparib vs 25 μ M **SW43** + 50 μ M olaparib), ***p=0.039 (for dead cells, control vs 25 μ M

SW43), **** $p < 0.0001$ (for dead cells, 50 μM olaparib vs 25 μM **SW43** + 50 μM olaparib).

Two-way ANOVA with a Tukey's post-hoc test. (C) Cell cycle analysis was performed by flow cytometry. Olaparib arrested cells at G2/M phase and sensitized cells to **SW43** induced subG1 formation.

Figure

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