Small Molecule Therapeutics

The Use of Olaparib (AZD2281) Potentiates SN-38 Cytotoxicity in Colon Cancer Cells by Indirect Inhibition of Rad51-Mediated Repair of DNA Double-Strand Breaks

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Abstract

Potent application of topoisomerase I inhibitor plus PARP inhibitor has been suggested to be an effective strategy for cancer therapy. Reportedly, mismatch repair (MMR)–deficient colon cancer cells are sensitive to topoisomerase I inhibitor, presumably due to microsatellite instability (MSI) of the MRE11 locus. We examined the synergy of SN-38, an active metabolite of irinotecan, in combination with the PARP inhibitor olaparib in colon cancer cells showing different MMR status, such as MSI or microsatellite stable (MSS) phenotype. Treatment with SN-38 and olaparib in combination almost halved the IC50 of SN-38 for a broad spectrum of colon cancer cells independent of the MMR status. Furthermore, olaparib potentiated S-phase–specific double-strand DNA breaks (DSB) induced by SN-38, which is followed by Rad51 recruitment. siRNA-mediated knockdown of Rad51, but not Mre11 or Rad50, increased the sensitivity to olaparib and/or SN-38 treatment in colon cancer cells. In vivo study using mouse xenograft demonstrated that olaparib was effective to potentiate the antitumor effect of irinotecan. In conclusion, olaparib shows a synergistic effect in colon cancer cells in combination with SN-38 or irinotecan, potentiated by the Rad51-mediated HR pathway, irrespective of the Mre11-mediated failure of the MRN complex. These results may contribute to future clinical trials using PARP inhibitor plus topoisomerase I inhibitor in combination. Furthermore, the synergistic effect comprising topoisomerase I-mediated DNA breakage–reunion reaction, PARP and Rad51-mediated HR pathway suggests the triple synthetic lethal pathways contribute to this event and are applicable as a potential target for future chemotherapy. Mol Cancer Ther; 13(5); 1170–80. ©2014 AACR.

Introduction

Colorectal cancer is the third most common cancer in men and the second in women worldwide, while almost 60% of the cases occur in developed countries. Colorectal cancer is estimated to be the cause of about 8% of all cancer-related deaths, making it the fourth most common cause (1).

The incidence of distant metastasis (stage IV) is reported to be 18% to 25%, with differences depending on ethnicity, and the 5-year relative survival rate for stage IV disease is as low as 12% (2), which shows the need for developing novel systemic chemotherapy. Conventionally, 5-fluorouracil (5-FU) and its analogs were employed as the first-line or adjuvant chemotherapy for colon cancer, while cases relapsing after 5-FU–based adjuvant chemotherapy were mostly resistant to 5-FU–based systemic chemotherapy. Irinotecan (CPT-11) was introduced as a drug effective for 5-FU–resistant colorectal cancer, the molecular target of which is type I topoisomerase. At present, either FOLFOX (5-FU + leucovorin + oxaliplatin) or FOLFIRI (5-FU + leucovorin + irinotecan) is recommended as the first-line regimen for stage IV colorectal cancer.

DNA repair systems have drawn attention as molecular targets of cancer therapy. Cancers with DNA repair deficiency could be more sensitive to certain therapeutic agents. One of the well-known syndromes is hereditary breast/ovarian cancer (HBOC) harboring a mutated BRCA1 or BRCA2 gene, which is involved in double-strand DNA break (DSB) repair. PARP inhibitors showed synthetic lethality to HBOC-related tumors (3, 4) and clinical trials on cases with HBOC have been ongoing worldwide. PARP inhibitors mainly target PARP-1 and PARP-2, which synthesize poly(ADP-ribose) (PAR) and are involved in DNA repair, cell differentiation, regulation of chromatin structure, and gene regulation (5–7).
PARP-1 has an important role in single-strand DNA break (SSB) repair and base excision repair (8). Recently, PARP has been reported to participate in the DSB repair pathway by nonhomologous end joining (NHEJ; refs. 9, 10) or homologous recombination (HR; ref. 11). The mechanism of PARP in DSB repair has been elucidated.

On the other hand, mismatch repair (MMR) deficiency showing microsatellite instability (MSI) due to aberration of mismatch repair genes such as MSH2, MLH1, MSH6, or PMS2 could be a novel target for cancer treatment because such cases often show frameshift mutation in MRE11 and/or RAD50 genes, of which the gene products form a heterotrimer with Nbs1 and are associated with DSB repair (12–15). Reportedly, MMR deficiencies in colorectal cancer could be available as a surrogate marker for various chemotherapeutic agents. MMR-deficient cells were sensitive to topoisomerase I inhibitors (16–19), such as irinotecan and camptothecin, which form a covalent bond with topoisomerase I and inhibit the breakage–reunion reaction in DNA replication or translation; they are now available as the major chemotherapeutic drugs for colorectal cancer (20, 21). In vivo, irinotecan is converted to its active metabolite, SN-38, in the presence of carboxylesterase (22, 23).

The combinational treatment of PARP inhibitor and topoisomerase I inhibitor has been considered as a potent strategy for cancer therapeutics, especially for colorectal cancer cells (24–30). However, the mechanisms of inducing topoisomerase I inhibitor hypersensitivity using PARP inhibitor remain unclear and the correlation of the MMR status with the sensitivity to topoisomerase I inhibitor treatment in combination with PARP inhibitor has not been elucidated yet.

Olaparib (AZD2281) is a PARP inhibitor that has been used in clinical trials targeting breast or ovarian cancers. In this study, first, we investigated the sensitivity to SN-38 plus olaparib combinational treatment in colon cancer cell lines characterized by their MMR status, using the 3H-thymidine incorporation assay, the cell counting assay and the clonogenic assay. Second, we investigated the intracellular response of treated cells by using cell diameter measurements as a surrogate marker of G2-M arrest and apoptosis. Third, we examined the expression of DSB repair-related proteins and the incorporation of 5-bromo-2′-deoxyuridine (BrdUrd), that is, a S-phase–specific cell-cycle marker, using immunofluorescence microscopy to elucidate the mechanisms of enhanced chemosensitivity evoked by olaparib. Finally, we examined the antitumor effect of irinotecan plus olaparib in mouse xenograft models to confirm the results of in vitro study as the final stage of the preclinical setting.

**Materials and Methods**

**Reagents**

SN-38 and irinotecan (CPT-11) were purchased from Sigma-Aldrich and SANDOZ, respectively. Olaparib (AZD2281) was kindly provided by AstraZeneca K.K. SN-38 and olaparib were dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L and stored at −20°C until the in vitro experiment. Irinotecan (20 mg/mL) was stored at room temperature for the in vitro experiment.

**Cell lines and culturing**

SW48, RKO, HCT116, and HT29 were purchased from American Type Culture Collection (ATCC; Summit Pharmaceutical International) from January 2010 through March 2011, which had been characterized using short tandem repeat polymorphism analysis by ATCC. LoVo was purchased from Health Science Research Resources Bank (HSRRB) in January 2011, which had been verified using isoenzyme analysis by HSRRB. These cell lines were passaged for less than 6 months after receipt. HCT-15 was kindly provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) in January 2011. SW1116, C-1, and colo320-HIS were provided by the National Cancer Center (Tokyo, Japan) in 1999. These four cell lines had not been authenticated in our laboratory.

All of these human colon cancer cell lines were cultured in RPMI-1640 medium (Life Technologies Japan) supplemented with 10% FBS (Thermo Fisher Scientific Japan) and 100 μg/mL kanamycin (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO2.

SW48 and RKO lack MLH1 expression due to promoter hypermethylation, causing MSI (31). H1116, LoVo, and HCT-15 have mutated MMR genes, MLH1 (p.S252X), MSH2 (p.R359fsX8), and MSH6 (p.L290fsX1 and p.D1171fsX4), respectively (Supplementary Table S1).

**Cell proliferation assay**

The procedures for the 3H-thymidine incorporation assay and the cell counting assay using the Scepter 2.0 cell counter were as described previously in detail (32) and are described in the Supplementary Methods.

**Western blot analysis**

The procedures for Western blot analysis were described in the Supplementary Methods. The primary antibodies used were as follows: anti-PAR (10H) mouse monoclonal antibody (1:500; GeneTex), anti–PARP-1 mouse monoclonal antibody (1:2,000, Santa Cruz Biotechnology), anti–PARP-2 mouse monoclonal antibody (1:1,000, Merck Millipore), anti-Mre11 rabbit polyclonal antibody (1:2,000, Cell Signaling Technology), anti-Rad50 mouse monoclonal antibody (1:1,000, GeneTex), anti-Nbs1 rabbit polyclonal antibody (1:1,000, Novus Biologicals), and anti-Rad51 rabbit polyclonal antibody (1:2,000, Santa Cruz). Anti-β-actin mouse monoclonal antibody (1:2,000, Santa Cruz Biotechnology) was used as an internal control.

**Immunofluorescence microscopy**

The detail of the procedures for the immunofluorescence staining is described in the Supplementary Methods. The primary antibodies were as follows: anti-53BP1 rabbit polyclonal antibody (1:500, Abcam), anti-Rad51 rabbit polyclonal antibody (1:50, Santa Cruz Biologicals) (33), and anti-TRF2 rabbit polyclonal antibody (1:200, Cell Signaling Technology) (34).
Biotechnology), anti-γ-H2AX mouse monoclonal antibody (1:500, Merck Millipore) and anti-BrdUrd mouse monoclonal antibody (1:500, Sigma-Aldrich). Fluorescence images were recorded with a BZ-9000 fluorescence microscope (KEYENCE) and analyzed using BZ-II Analyzer (KEYENCE). For quantification of 53BP1, Rad51, and γ-H2AX foci, haze-reduced images of about 100 to 200 nuclei from at least 4 fields were observed and the number of foci per nucleus was counted. For counting nuclei with BrdUrd incorporation, at least 6 fields (about 200–1,300 nuclei/field) were observed.

RNA interference

siRNAs directed to Mre11, Rad50, and Rad51 as well as a negative control were purchased from Life Technologies Japan. As commercially available negative control siRNA was cytotoxic for transfection with HCT116, we synthesized nontargeted siRNA using the Silencer siRNA Construction Kit (Life Technologies Japan). The nontarget siRNA sequences were designed as the scrambled sequences of the siRNA directed to RADS1: ACCTAATGGGATCTAAGATGGtt (sense) and CCACCTCGATCCATCAGTatt (antisense).

A total of 1–3 × 10^5 cells were plated in 24-well culture plates (300 µL/well). After 24 hours, the cells were transfected with 10 nmol/L siRNA using Lipofectamine RNAiMAX transfection reagent (Life Technologies Japan) in accordance with the manufacturer’s recommended protocol. After 24 hours, the medium was replaced with fresh growth medium and exposed to SN-38 and/or olaparib for 48 hours, and then cells were harvested for the cell counting assay. For Western blot analysis, cells were plated in 100 mm dishes (10 mL/dish) and harvested 24 hours after transfection.

In vivo growth inhibitory assay

Five-week-old SCID hairless outbred (SHO) mice (Crl:SHO-Prdk◔[H2][H3]) were purchased from Charles River Japan, Inc. Xenograft models were established by injecting HT29 or SW1116 cells (5 × 10^6) subcutaneously into the left flank of mice. When the tumors reached a size of 100 mm^3, diameters were measured with calipers and the tumor volumes were calculated using the following formula: V = (L × W^2)/2, where V is tumor volume (mm^3), L is the longest tumor diameter (mm), and W is the shortest tumor diameter (mm). The data of the relative tumor volume are shown as means ± SEMs.

Statistical analysis

The data of the in vitro study are presented as means ± SDs and that of the in vivo study are presented as means ± SEMs. Statistical significance of in vitro study was either analyzed by the Student t test (between 2 groups) or one-way ANOVA with the Tukey test or Tukey–Kramer method (between 4 groups). In in vivo study, a linear trend test was performed as a utility of one-way ANOVA in each group, and statistical significance between four groups was analyzed by one-way ANOVA with the Tukey–Kramer method. All statistics were calculated using the GraphPad Prism V5.0 software (GraphPad Software, Inc.).

Results

Molecular profiles of the colon cancer cell lines

The molecular profiles of the cell lines used for the assay were examined and summarized as regards MSI status, genetic or epigenetic alterations of the MMR genes, and mutational status of TP53, MRE11, and RAD50 (Supplementary Table S1). Of the 9 cell lines examined, 5 cell lines (SW48, RKO, HCT116, LoVo, and HTC-15) showed MSI, whereas 4 cell lines (C-1, colo320HR, HT29, and SW116) had a MSS phenotype. MMR-deficient cell lines also had MSI in the genes responsible for HR, such as MRE11 and RAD50. SW48, RKO, HCT116, and LoVo had biallelic MSI in the poly(T) tract of MRE11, whereas HCT-15 had monoallelic MSI. As for RAD50, poly(A) mutations, RKO, HCT116, and LoVo were heterozygous whereas SW48 and HCT-15 showed the wild-type phenotype. TP53 gene mutations were detected in 1 MMR-deficient cell line (HCT-15) and 3 MMR-proficient cell lines (colo320HR, HT29, and SW1116).

Olaparib potentiates SN-38 sensitivity by inhibiting the synthesis of PAR polymer

To assess the effect of olaparib, we first evaluated the cell growth inhibitory effects of olaparib alone by using the ^3H-thymidine incorporation assay (Fig. 1A). Of all the cell lines examined, HCT116 was the most sensitive to olaparib, the IC_50 of which was 2.5 µmol/L and SW116 was the most resistant, with the IC_50 of more than 100 µmol/L. We also evaluated the expression of PAR, PARP-1, and PARP-2 in HCT116 cells treated with various concentrations of olaparib. Poly (ADP-ribose)ylation (PARylation) induced by 1 nmol/L SN-38 in HCT116 was inhibited by olaparib in a dose-dependent manner without affecting the expression levels of PARP-1 and PARP-2 (Fig. 1B).

Next, we evaluated the cell growth inhibitory effects of SN-38 with various concentrations of olaparib in HCT116 and HT29, which have different MMR status and sensitivities to SN-38 (17, 32, 34). In the presence of 0.5 nmol/L SN-38, cell growth was inhibited with 10 nmol/L (P < 0.01) or...
100 nmol/L (P < 0.001) olaparib, and these effects were enhanced when cells were treated with 1 nmol/L SN-38 (P < 0.0001 for 10 nmol/L and P < 0.0001 for 100 nmol/L olaparib) in HCT116. Concentrations of olaparib less than 1 nmol/L had no inhibitory effect on cell growth in HCT116 treated with 0.5 nmol/L or 1 nmol/L SN-38 (Fig. 1C). Likewise, olaparib also potentiated SN-38 sensitivity at concentrations above 10 nmol/L in HT29 (Fig. 1D). Olaparib concentrations less than 1 nmol/L did not show cell cytotoxicity (Fig. 1A). Considering these results, we determined the concentration of olaparib as 10 nmol/L in the subsequent in vitro experiments.

Olaparib potentiates SN-38 cytotoxicity irrespective of MMR status

To assess whether the potentiating effects of olaparib on SN-38 sensitivity were correlated with MMR status or not, we evaluated the SN-38 sensitivity in the presence or absence of olaparib by a variety of assays using colon cancer cell lines showing either MMR-deficient or proficient phenotypes.

First, we evaluated the IC$_{50}$ of SN-38 in the presence or absence of olaparib by the $^3$H-thymidine incorporation assay (Fig. 2). Each cell line was treated with SN-38 and/or olaparib for 48 hours. Olaparib significantly potentiated the sensitivity to SN-38 for all cell lines except HCT-15 (Fig. 2 and Supplementary Table S2). SN-38 in combination with olaparib almost halved the IC$_{50}$ of SN-38 in each cell line.

In four cell lines (HCT116, RKO, HT29, and SW1116) showing different MMR status, the results of the $^3$H-thymidine incorporation assay were reproducible with those measured by the Scepter 2.0 cell counter (Supplementary Fig. S1). We previously reported that cell diameter changes measured by the Scepter 2.0 cell counter could be used as a marker of G2–M arrest and apoptosis evoked by SN-38 (32). The increase of the mean cell diameter evoked by SN-
SN-38 plus olaparib was greater than that of SN-38 alone in each cell line (Supplementary Table S3). These results indicate that olaparib enhanced G2–M arrest induced by SN-38, together with the increase of the apoptotic cell fraction shown by the appearance of small-sized particles. These results show that olaparib potentiates SN-38 sensitivity in various colon cancer cell lines by inducing G2–M arrest and apoptosis, irrespective of MMR status.

To assess the efficacy of the treatments with shorter duration of treatment, we performed robust colony assays. Two cell lines (HCT116 and HT29) were exposed to SN-38 and/or olaparib for 6, 12, 24, and 48 hours and subjected to colony formation. Olaparib potentiated the inhibitory effect of colony formation by SN-38, while these effects were not observed in the treatment using SN-38 alone after 12 hours of exposure in both cell lines (Supplementary Fig. S2). Colony formation was suppressed significantly after 24 hours in both cell lines treated with SN-38 alone or SN-38 plus olaparib. These results were well correlated with the data obtained by the 3H-thymidine incorporation assay shown in Fig. 1C. When exposed to SN-38 plus olaparib for 6 hours, 53BP1 foci seemed to increase in the nuclei with BrdUrd incorporation (Supplementary Fig. S3B). This finding lasted until 12 hours of treatment, but BrdUrd-stained cell decreased markedly when they were exposed over 24 hours, leaving 53BP1 foci in the nuclei without BrdUrd incorporation (Supplementary Fig. S3B).

Olaparib increases Rad51 recruitment to the DSB sites induced by SN-38

As the major DSB repair pathway is mediated by HR (36–38), we examined formation of the Rad51 and γ-H2AX foci and their colocalization in HT29 cells. As antibodies directed against 53BP1 and Rad51 were both rabbit polyclonal antibodies and unsuitable for double staining, we used mouse anti-γ-H2AX monoclonal antibody as a DSB marker and examined its colocalization with Rad51 foci using double staining. Numbers of γ-H2AX foci and Rad51 foci showed a significant increase between SN-38 versus SN-38 plus olaparib and a part of γ-H2AX foci colocalized with Rad51 foci (Fig. 4). These data indicate that olaparib potentiates the recruitment of Rad51 to the DSB site in SN-38-treated cells.

Baseline levels of the DNA repair–related protein expression in cell lines were examined by Western blot analysis (Supplementary Fig. S4). It was found that the potentiating effects of olaparib on SN-38 sensitivity might not be correlated with the baseline levels of PARP-1, PARP-2, Mre11, Rad50, Nbs1, and Rad51 expressions. Furthermore, Rad51 expression by Western blot analysis did not differ between cells treated with SN-38 alone and SN-38 plus olaparib in RKO, HCT116, HT29, and SW1116 (data not shown).

Rad51 knockdown increases sensitivity to SN-38 and/or olaparib

To elucidate an effector molecule concerned with the potentiating effects of olaparib on SN-38 sensitivity, we performed a siRNA-mediated knockdown experiment of HR-related molecules, including Mre11, Rad50, and Rad51. SW1116 cells harboring wild-type MRE11 and RAD50 were used for the assay in that it is suitable for siRNA transfection. Drug sensitivities were evaluated by the cell counting assay. SW1116 cells transfected with
Rad51 siRNA became significantly sensitive in all conditions, that is, with SN-38, SN-38 plus olaparib, and even with olaparib alone, as compared with the cells transfected with control siRNA (Fig. 5A, Supplementary Fig. S5). In cells transfected with Mre11 or Rad50 siRNA, there were no changes in the sensitivities to the olaparib or SN-38 treatments. Cell diameters of the transfectant with Rad51 siRNA increased according to the order of the treatments, namely, control < olaparib < SN-38 < SN-38 plus olaparib, indicating that G2–M arrest occurred preferentially for SN-38 or SN-38 plus olaparib compared with olaparib alone (Fig. 5B and Supplementary Table S4). Furthermore, the formation of small particles indicated the increase of cell apoptosis in each treatment (Fig. 5B and Supplementary Table S4). Knockdown effects of these cells were confirmed by Western blot analysis, in which the protein expressions of Mre11, Rad50, and Rad51 were diminished 24 hours after transfection with each targeted siRNA (Fig. 5C–E). In addition, we observed the formation of Rad51 foci in cells transfected with Rad51 or control siRNA after SN-38 treatment by immunofluorescence microscopy and confirmed that the formation of Rad51 foci was inhibited in cells transfected with Rad51 siRNA compared with control siRNA (Fig. 5F).

We also evaluated the effects of Rad51 knockdown in HCT116 cells, which have biallelic MRE11 mutations. HCT116 cells have endogenously weak expression of Mre11 and Rad50 (Supplementary Fig. S4). In cells transfected with Rad51 siRNA, the sensitivities to olaparib, SN-38, and combinational treatment increased significantly more than in cells transfected with control siRNA (Supplementary Fig. S6A). Moreover, the cell diameter changes indicated the increase of G2–M arrest and apoptosis in cells transfected with Rad51 siRNA and subsequently treated with SN-38 and/or olaparib (Supplementary Fig. S6B and Supplementary Table S5). The expression of Rad51 protein was markedly diminished 24 hours after transfection with Rad51-targeted siRNA (Supplementary Fig. S6C). These results suggest that Rad51 may play an important role in DSB repair upon SN-38 and/or

Figure 3. Olaparib potentiates DNA DSBs induced by SN-38. The formation of 53BP1 foci was examined by immunofluorescence microscopy upon counterstaining with 4’, 6-diamidino-2-phenylindole (DAPI) in HCT116 (A) and HT29 (B) exposed to SN-38 (1 nmol/L for HCT116 and 2 nmol/L for HT29) in the presence or absence of 10 nmol/L olaparib for 12 hours. Counts of 53BP1 foci per nucleus were performed for HCT116 (C) and HT29 (D). Error bars, mean ± SD. The significance of differences between the four groups was analyzed using one-way ANOVA with the Tukey–Kramer method (NS, not significant).
olaparib treatment, irrespective of the Mre11 or Rad50 pathway.

**Olaparib potentiates the antitumor effect of irinotecan in mouse xenograft models**

To confirm whether the treatment with irinotecan plus olaparib in combination has an antitumor effect compared with irinotecan alone in an *in vivo* experiment, we established two mouse xenograft models using HT29 and SW1116, of which the *in vitro* sensitivities to SN-38 were different. In HT29 xenografts, tumor growth in the irinotecan-treated group was not significantly suppressed compared with that in the control group. However, the group treated with irinotecan plus olaparib showed a significant decrease of the tumor volumes compared with the control group and the irinotecan-treated group (*P* < 0.01 for control vs. combination and olaparib vs. combination, *P* < 0.05 for irinotecan vs. combination; Fig. 6A). In SW1116 xenografts, tumor growth in the irinotecan-treated group was significantly suppressed compared with that in the control group. In the combinational treatment group, antitumor effects also increased significantly compared with those in the control group and the olaparib-treated group (*P* < 0.001 for control vs. irinotecan or combination and olaparib vs. irinotecan or combination; Fig. 6B). There was no significant change between irinotecan- and combinational-treated group, due to high sensitivity of SW1116 to irinotecan; however, the result of the trend test showed decrease of the slope in the irinotecan plus olaparib-treated group compared with the irinotecan alone (0.008 vs. 0.04; *P* < 0.0001; Fig. 6B). No significant body weight loss was observed in the treated groups compared with the control group (data not shown). In the experiment using SW1116 mouse xenograft, clinical blood chemistry was examined to analyze hematologic toxicity and liver dysfunction. In each treated group, neither...
hematocytopenia nor liver dysfunction was observed at the doses of irinotecan and olaparib used in this study, and the regimen was well tolerable in the mouse experiments (Supplementary Table S6).

Discussion

In this study, we assessed the effect of olaparib on potentiating the tumor cytotoxicity of SN-38 in colon cancer cell lines and whether the administration of olaparib in combination with irinotecan had suppressed the tumor growth in mouse xenograft models. As reported previously, the IC50 of SN-38 were lower in MMR-deficient cells harboring homozygous mutations in MRE11 and genetic or epigenetic alterations of MLH1 as compared with the MMR-proficient cells, suggesting that Mre11 deficiency brought about the sensitization of the MMR-deficient cells to the topoisomerase I inhibitor (19, 34, 39). In analysis using 3H-thymidine incorporation assay, olaparib almost halved the IC50 of SN-38 in each cell lines, irrespective of MMR status (Fig. 2 and Supplementary Table S2).

We counted the number of the 53BP1 foci formation in 2 cell lines, HCT116 and HT29 exposed to SN-38 or SN-38 plus olaparib for 12 hours: the former as a SN-38–sensitive cell line and the latter as a SN-38–resistant one. There was a significant increase in the number of 53BP1 foci in cells treated with SN-38 plus olaparib as compared with those treated with SN-38 alone, irrespective of the original sensitivity in each cell line (Fig. 3).

Figure 5. The siRNA-mediated knockdown targeting Rad51 potentiates the sensitivity to SN-38 and/or olaparib in SW1116. A, cell counting assay of SW1116 exposed to 1 nmol/L SN-38 and/or 10 nmol/L olaparib for 48 hours after transfection with control siRNA, Mre11 siRNA, Rad50 siRNA, or Rad51 siRNA. Data, mean ± SD of triplicate experiments. Control means nontreated cells. The Student t test was performed between cells transfected with control siRNA and those with the other siRNA (*, P < 0.05; **, P < 0.01; ***, P < 0.0001). B, measurement of the cell diameter and cell count was performed using the Scepter 2.0 cell counter. The x-axis of each graph represents the cell diameter (µm) and the y-axis represents the cell count (demonstrated in the inset of Supplementary Fig. S5). Experiments were performed in triplicate in each condition and each graph of cells transfected with control siRNA and Rad51 siRNA represents the result of a single run in triplicate experiments. The mean ± SD of the cell diameters and the proportion of the cells with small diameter in each condition are shown in Supplementary Table S4. Graphs of cells transfected with Mre11 siRNA and RAD50 siRNA are shown in Supplementary Fig. S5. Effects of knockdown were confirmed by Western blot analysis 24 hours after siRNA-mediated transfection targeting Mre11 (C), Rad50 (D), or Rad51 (E). F, formation of the Rad51 foci 24 hours after transfection was examined by immunofluorescence microscopy. Cells were exposed to 10 nmol/L SN-38 for 2 hours before PFA fixation to induce double-strand DNA breaks.
Results of the 3H-thymidine incorporation assay and the correlation with the formation of 53BP1 foci were further analyzed by immunofluorescence double staining using antibodies directed to BrdUrd and 53BP1. Accumulation of nuclear BrdUrd incorporation persisted at 12 hours and decreased after 24 hours of exposure to SN-38 with or without olaparib (Supplementary Fig. S3A), while the formation of 53BP1 foci was consistent with positive BrdUrd nuclear staining in cells exposed to SN-38 or SN-38 plus olaparib for 12 hours, while in cells exposed for over 24 hours, 53BP1 foci was observed in the nuclei without BrdUrd incorporation (Supplementary Fig. S3B). Furthermore, results of the clonogenic assay showed that colony formation was suppressed significantly when cells were exposed to SN-38 or SN-38 plus olaparib over 24 hours as compared with the control or olaparib alone, whereas those exposed to SN-38 or SN-38 plus olaparib for less than 12 hours did not show reduced colony formation (Supplementary Fig. S2). Wu and colleagues reported that DSBs induced by SN-38 were biphasic events: an immediate phase was S-phase specific and inhibited by aphidicolin, a DNA polymerase inhibitor and a lagging phase, associated with apoptotic cell death (35). The data of BrdUrd and 53BP1 double staining indicated that DSBs at 12 hours after exposure to SN-38 or SN-38 plus olaparib seemed to be S-phase–specific immediate phase, and the cytotoxic effects observed at 48 hours exposure correspond to the lagging phase. The data of 3H-thymidine incorporation assay were well compatible with those of the clonogenic assay (Fig. 2 and Supplementary Fig. S2).

\[ IC_{50} \text{so for olaparib were more than } 2.5 \mu \text{mol/L in all cell lines and } 10 \text{ nmol/L olaparib used in in vitro experiments seemed to have the least cytotoxic effect (Fig. 1). At this concentration, olaparib alone did not increase DSBs and there were no differences in cell counts, cell cycle, and apoptotic change between control and olaparib-treated cells. } \]
Olaparib Potentiates SN-38 Sensitivity in Colon Cancer Cells

Nonetheless, olaparib in combination with SN-38 increased DSBs and potentiated SN-38 sensitivity, leading to G2–M arrest and apoptosis. SN-38 plus olaparib treatment in HT29 cells increased the number of Rad51 foci colocalized with γ-H2AX, another marker of DSB, implying that DSBs induced by SN-38 would be repaired by the Rad51-mediated DNA repair pathway (Fig. 4). On the other hand, Mre11-Rad50-Nbs1 complex forms a MRN trimmer, which has 3′ to 5′ exonuclease activity and acts as a sensor molecule in the initiating process of DSB repair with the activation of the checkpoint kinase ATM (40–45). If the hypersensitivity to SN-38 is caused by MRE11 mutations resulting in disruption of the MRN complex, does it arise independent of the Rad51-mediated HR or not? To address this, we performed a knockout experiment using Mre11, Rad50, and Rad51 siRNAs for SW1116 cells, a cell line with MMR-proficient phenotype without MSI in the MRE11 locus. As a result, cell proliferation was suppressed significantly in the absence of Rad51, but not MRE11 nor RAD50 (Fig. 5).

A series of experiments denoted that single-strand DNA breaks caused by treating cells with SN-38 proceeds to S-phase-specific DSBs, which is repaired through HR-mediated DNA repair. The addition of olaparib in combination with SN-38 increased the number of Rad51 foci, which is colocalized with γ-H2AX foci, indicating the DSBs repaired by the HR-mediated DNA repair pathway (Figs. 3 and 4). Suppression of PARylation enhanced the sensitivity to SN-38, leading to G2–M arrest and apoptosis in most of the colon cancer cell lines used in this study (Fig. 1 and Supplementary Fig. S1). The use of olaparib in combination with SN-38 may indirectly inhibit HR-mediated DNA repair through suppressing PARylation. This effect was also reproducible in the experiment using HCT116, an MRN-deficient cell line harboring biallelic MRE11 mutations (Supplementary Fig. S6). SiRNA-mediated Rad51 knockdown increases the sensitivity of HCT116 cells to SN-38 and olaparib, indicating that PARP plays an indirect role for this synthetic lethality mediated by Rad51, irrespective of neither MMR deficiency nor MRE11 inactivation.

In combination using the mouse xenograft models, combination of irinotecan and olaparib suppressed tumor growth significantly, without critical systemic or hematologic toxicity (Fig. 6 and Supplementary Table S6). The results of the in vivo study well confirmed the synergistic effect observed in the in vitro study. Dose of irinotecan employed in this study seems to be lower than LD50 reported in the nude mouse (177 mg/kg, i.p.), although LD50 of irinotecan in SHO mouse was not reported elsewhere (46). Tentori and colleagues reported that PARP inhibitor prevents irinotecan-induced intestinal damage in in vivo study using rat models (29). In the clinical settings, topoisomerase I inhibitor has adverse effects such as gastrointestinal symptoms, hematologic toxicity, and liver dysfunction, while olaparib was reported to be less toxic than irinotecan and its combined use may improve chemotherapeutic compliance by reducing the dose or frequency of irinotecan administration.

In summary, we found that SN-38 or irinotecan plus olaparib in combination could be applicable as a useful approach in a broad spectrum of colon cancer cells, regardless of the MMR status. In vivo study demonstrated that olaparib would be effective to potentiate the antitumor effect of irinotecan without serious adverse effects. Results of this preclinical study endorse further approaches to be pursued in clinical settings. Furthermore, triple synthetic lethality comprising topoisomerase I-mediated DNA breakage–reunion reaction, PARylation, and Rad51-mediated HR pathway may contribute as a potential target for future chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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