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

Makiko Tahara1,3, Takeshi Inoue1, Futoshi Sato1, Yasuyuki Miyakura3, Hisanaga Horie3, Yoshikazu Yasuda3, Hirofumi Fuji4, Kenjiro Kotake2, and Kokichi Sugano1

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).

Authors’ Affiliations: 1Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, 2Department of Surgery, Tochigi Cancer Center, Utsunomiya; and 3Department of Gastrointestinal Surgery and 4Division of Clinical Oncology, Jichi Medical University, Shimotsuke, Tochigi, Japan

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Kokichi Sugano, Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, 4-9-13 Yohnan, Utsunomiya, Tochigi 320-0834, Japan. Phone: 812-8658-5151; Fax: 812-8684-5756; E-mail: ksugano@tcc.pref.tochigi.lg.jp
doi: 10.1158/1535-7163.MCT-13-0683
©2014 American Association for Cancer Research.
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 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 Bio-medical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) in January 2011. SW1116, C-1, and colo320HISR 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). HCT116, 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 (1:200, 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).
Biotekr Technology, 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 RAD51: ACGTATGTGATCTAAGATGGtt (sense) and CCATCTCGATCCATTACTGtat (antisense).

A total of 1–3 × 10^5 cells were plated in 24-well culture plates (500 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-Prkdc<sup>scid</sup>+/+H<sup>+3</sup>) were purchased from Charles River Japan, Inc.. Xenograft models were established by injecting HT29 or SW1116 cells (5 × 10<sup>6</sup>) subcutaneously into the left flank of mice. When the tumors reached a size of 100 mm<sup>3</sup>, the study of SW1116 xenografted mice, blood sampling and body weights were measured three times weekly. In day 18 for HT29 and on day 30 for SW1116. The tumor sizes in vivo 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 HCT-15) showed MSI, whereas 4 cell lines (C-1, colo320HBR, HT29, and SW1116) 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(D1) tract of MRE11, whereas HCT-15 had monoallelic MSI. As for RAD50, poly(A)8 mutations, RKO, HCT116, and LoVo were heterozygous while 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 <sup>3</sup>H-thymidine incorporation assay (Fig. 1A). Of all the cell lines examined, HCT116 was the most sensitive to olaparib, the IC<sub>50</sub> of which was 2.5 μmol/L and SW1116 was the most resistant, with the IC<sub>50</sub> of more than 10 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-ribosyl)ation (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 IC₅₀ of SN-38 in the presence or absence of olaparib by the ³H-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 IC₅₀ of SN-38 in each cell line.

In four cell lines (HCT116, RKO, HT29, and SW1116) showing different MMR status, the results of the ³H-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-
Olaparib increases DNA double-strand breaks induced by SN-38

Reportedly, SN-38 induces DSBs and PARP inhibitor is involved in the inhibition of SSB repair and PARylation of the proteins involved in DNA repair (5–8, 35). To elucidate whether olaparib increases DSBs induced by SN-38, we examined the formation of the 53BP1 nuclear foci, for example, a DSB marker in HCT116 and HT29 exposed to SN-38 and/or olaparib for 12 hours (Fig. 3).

The formation of 53BP1 nuclear foci did not increase by the treatment with olaparib alone, while they increased significantly in the groups treated with SN-38 or SN-38 plus olaparib (Fig. 3A and B). In analysis using one-way ANOVA, numbers of foci were significantly increased in the group treated with SN-38 plus olaparib as compared with SN-38 alone in both cell lines (P < 0.001; Fig. 3C and D).

We compared the results of the 53BP1 foci formation with those of 3H-thymidine incorporation assay, using immunofluorescence double staining for BrdUrd and 53BP1 in HCT116 cells exposed to SN-38 and/or olaparib for different periods ranging from 6, 12, 24, and 48 hours (Supplementary Fig. S3). Exposed to SN-38 plus olaparib for 48 hours, BrdUrd incorporation decreased significantly as compared with SN-38 alone (Supplementary Fig. S3A). As BrdUrd incorporation is a surrogate marker of the S-phase, this was compatible with the data of 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 transected 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 increase 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 olaparib treatment.
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 *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

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Olaparib potentiates Rad51 recruitment for double-strand break sites induced by SN-38. A, the formation of Rad51 and γ-H2AX foci was examined by immunofluorescence microscopy in HT29 exposed to 2 nmol/L SN-38 and/or 10 nmol/L olaparib for 12 hours. For analysis of the colocalization of Rad51 and γ-H2AX, fluorescence intensity of Rad51 (green) and γ-H2AX (red) above the white line in the merged image was measured and we adopted two-color line plots to overlay the two-color histograms as shown in graphs B (SN-38-treated cell) and C (SN-38 plus olaparib-treated cell). Counts of Rad51 foci (D) and γ-H2AX foci (E) per nucleus in HT29 were carried out. 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).
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 IC_{50} 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 IC_{50} 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. S3). 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 \(^3\)H-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 \(^3\)H-thymidine incorporation assay were well compatible with those of the clonogenic assay (Fig. 2 and Supplementary Fig. S2).

\(\text{IC}_{50}\) of olaparib were more than 2.5 \(\mu\)mol/L in all cell lines and 10 \(\text{nmol/L} \) olaparib used 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.

**Figure 6.** Olaparib increases the antitumor effects of irinotecan in mouse xenograft models. Tumor xenografts of HT29 and SW1116 were treated with olaparib (50 mg/kg, per os, every day consecutively for 5 days with intermission for 2 days per week) and/or irinotecan (10 mg/kg i.p., twice a week). A, relative tumor volumes (RTV) were measured in HT29 xenograft models (control, \(n = 6\); olaparib, \(n = 7\); irinotecan, \(n = 5\); irinotecan plus olaparib, \(n = 5\)). B, relative tumor volumes were measured in SW1116 xenograft models (control, \(n = 7\); olaparib, \(n = 7\); irinotecan, \(n = 6\); irinotecan plus olaparib, \(n = 7\)). Data, mean ± SEM. A linear trend test was performed in each group, and statistical significance between four groups was analyzed by one-way ANOVA with the Tukey-Kramer method (NS, not significant).
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

Conception and design: M. Tahara, T. Inoue, K. Sugano
Development of methodology: M. Tahara, T. Inoue
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Tahara, F. Sato, K. Kotake
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Tahara, K. Sugano
Writing, review, and/or revision of the manuscript (i.e., drafting discussions of experimental results or revise drafts): M. Tahara, Y. Miyakura, Y. Yasuda, K. Sugano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Tahara, H. Horie, Y. Yasuda, H. Fuji, K. Sugano
Study supervision: Y. Miyakura, Y. Yasuda, K. Kotake, K. Sugano

Grant Support

This study was supported, in part, by Grants-in-Aid for Cancer Research and for the Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare, Japan, as well as by the National Cancer Center Research and Development Fund (21bunshi-3, 23-A-2, and 23-A-7 to K. Sugano).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 30, 2013; revised January 26, 2014; accepted February 17, 2014; published OnlineFirst February 27, 2014.

References


Molecular Cancer 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

Makiko Tahara, Takeshi Inoue, Futoshi Sato, et al.

Mol Cancer Ther 2014;13:1170-1180. Published OnlineFirst February 27, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0683

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/02/27/1535-7163.MCT-13-0683.DC1

Cited articles
This article cites 44 articles, 21 of which you can access for free at:
http://mct.aacrjournals.org/content/13/5/1170.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/13/5/1170.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.