Combining inotuzumab ozogamicin with PARP inhibitors olaparib and talazoparib exerts synergistic cytotoxicity in acute lymphoblastic leukemia by inhibiting DNA strand break repair

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Abstract. Inotuzumab ozogamicin (IO), a novel therapeutic drug for relapsed or refractory acute lymphoblastic leukemia (RR)-(ALL), is a humanized anti-cluster of differentiation (CD) 22 monoclonal antibody conjugated with calicheamicin that causes DNA single- and double-strand breaks. Although the efficacy of IO is significantly improved compared with that of conventional chemotherapies, the prognosis for RR-ALL remains poor, highlighting the need for more effective treatment strategies. The present study examined the role of DNA damage repair inhibition using the poly (ADP-ribose) polymerase (PARP) inhibitors olaparib or talazoparib on the enhancement of the antitumor effects of IO on B-ALL cells *in vitro*. The Reh, Philadelphia (Ph)$^-$B-ALL and the SUP-B15 Ph$^+$ B-ALL cell lines were used for experiments. Both cell lines were ~90% CD22$^+$. The half-maximal inhibitory concentration (IC_{50}) values of IO were 5.3 and 49.7 ng/ml for Reh and SUP-B15 cells, respectively. The IC_{50} values of IO combined with minimally toxic concentrations of olaparib or talazoparib were 0.8 and 2.9 ng/ml for Reh cells, respectively, and 36.1 and 39.6 ng/ml for SUP-B15 cells, respectively. The combination index of IO with olaparib and talazoparib were 0.19 and 0.56 for Reh cells and 0.76 and 0.89 for SUP-B15 cells, demonstrating synergistic effects in all combinations. Moreover, the addition of minimally toxic concentrations of PARP inhibitors augmented IO-induced apoptosis. The alkaline comet assay, which quantitates the amount of DNA strand breaks, was used to investigate the degree to which DNA damage observed 1 h after IO administration was repaired 6 h later, reflecting successful repair of DNA strand breaks. However, DNA strand breaks persisted 6 h after IO administration combined with olaparib or talazoparib, suggesting inhibition of the repair processes by PARP inhibitors. Adding olaparib or talazoparib thus synergized the antitumor effects of IO by inhibiting DNA strand break repair via the inhibition of PARP.

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Abbreviations: IO, inotuzumab ozogamicin; RR-ALL, relapsed or refractory acute lymphoblastic leukemia; CD, cluster of differentiation; B-ALL, B-acute lymphoblastic leukemia; PARP, poly (ADP-ribose) polymerase; Ph, Philadelphia; IC_{50}, half-maximal inhibitory concentration; OS, overall survival; HSCT, hematopoietic stem cell transplantation; CR, complete remission; SSBs, single strand breaks; DSBs, double strand breaks; BRCA, breast cancer susceptibility gene; ATCC, American Type culture collection; FBS, fatal bovine serum; FITC, fluorescein isothiocyanate; CCK-8, Cell Counting Kit-8; CI, combination index; BER, base excision repair; Chk1, checkpoint kinase 1; P-gp, P-glycoprotein; CVD, cyclophosphamide, vincristine and dexamethasone; CVAD, cyclophosphamide, vincristine, doxorubicin and dexamethasone

Key words: IO, PARP, olaparib, talazoparib, ALL, DNA repair

Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy characterized by malignant transformation and proliferation of lymphoid progenitor cells in bone marrow. The prognosis of relapsed or refractory ALL (RR-ALL) remains poor, with a reported median overall survival (OS) of 6 months and a 5-year survival rate of 7% (1,2). Hematopoietic stem cell transplantation (HSCT) is the only treatment option for achieving long-term survival in RR-ALL. Although achieving complete remission (CR) before the initiation of HSCT is preferable, conventional salvage chemotherapy regimens are associated with CR rates of only 30-40% (3).

Inotuzumab ozogamicin (IO), a novel therapeutic drug for RR-ALL, is a humanized anti-cluster of differentiation (CD) 22 monoclonal antibody conjugated with calicheamicin. IO was approved in August 2017 in the United States for the treatment of CD22$^+$ RR-ALL. The pivotal INO-VATE study compared clinical efficacy of IO with that of conventional chemotherapies used as the control; it revealed that CR and CR with incomplete hematologic recovery were markedly higher in the IO group (80.7%) compared with those in the control

group (29.4%). On the other hand, the duration of remission and median OS in the IO group were only 4.6 months and 7.7 months, respectively, which is only 1.5 months longer than in the control group (4). The final report from the INO-VATE study revealed 2-year OS rates of 22.8 and 10.8% in the IO group and in the control group, respectively (5,6). Thus, while IO is far more effective for treating RR-ALL than conventional regimens, the duration of remission remains short. As a result, effective combination strategies are needed to enhance the anti-leukemic effects of IO.

IO comprises the cytotoxic antibiotic N-acetyl-gamma-calicheamicin dimethylhydrazine, a calicheamicin derivative, attached to a humanized monoclonal IgG4 antibody via the 4-(4 acetylphenoxy) butanoic acid (acetyl butyrate) linker. Once IO is administered to patients, the drug binds to the CD22 leukemic cell surface antigen. The complex is then internalized and fuses with lysosomes inside the cell. Calicheamicin detaches from the antibody site following the breakdown of the linker, inducing DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) in the nucleus and subsequent apoptosis (7).

PARPs are a family of 17 proteins involved in the repair of DNA strand breaks. The most well-studied members of the PARP family are PARP-1 and PARP-2, which play crucial roles in the repair of SSBs. PARP inhibitors are novel anticancer drugs targeting the DNA damage response (8). Olaparib, a potent inhibitor of PARP-1/2, was first approved for the treatment of breast cancer susceptibility gene (*BRCA*)-mutant ovarian cancer in 2014. Since then, several other PARP inhibitors have been developed and approved, and their use has been expanded to the treatment of various types of cancer (9,10). Talazoparib is the latest approved PARP-1/2 inhibitor with the most potent PARP1 inhibitory activity for the treatment of *BRCA*-mutant breast cancer. Loss of *BRCA* function causes failure of the DSB repair pathway. In *BRCA*-defective tumor cells, PARP inhibitors prevent the repair of SSBs, which then convert to irreparable and toxic DSBs, effectively exerting cytotoxicity through synthetic lethality (11).

One strategy to increase the cytotoxicity of IO is inhibition of DSB repair. Leukemic cells evoke a DSB repair response following DNA damage triggered by IO. Inhibition of the repair response is hypothesized to accumulate unrepaired DNA strand breaks, thereby enhancing the cytotoxicity of IO. The present study aimed to compare synergistic anti-leukemic effects between IO and PARP inhibitors in B-ALL cells. For this purpose, the cytotoxicity of IO was first evaluated in detail using Philadelphia $(Ph)^-$ and Ph^+ B-ALL cell lines *in vitro*. Next, the alkaline comet assay was used to determine DNA damage repair kinetics in cells treated with IO. Finally, attempts were made to increase the cytotoxic effects of IO by adding either olaparib or talazoparib, which are PARP inhibitors, under a hypothesized interaction between these drugs.

Materials and methods

Cell cultures. The Reh Ph^- B-ALL cell line (cat. no. CRL-8286), the SUP-B15 Ph^+ B-ALL cell line (cat. no. CRL-1929) and the HL-60 acute promyelocytic leukemia cell line (cat. no. CLL-240) were purchased from American Type Culture Collection (ATCC). Reh and HL-60 cells were cultured in RPMI-1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA), at 37˚C under 5% CO_2 in a humidified atmosphere. SUP-B15 cells were cultured in Dulbecco's medium (FUJIFILM Wako Pure Chemical Corporation) with 20% FBS in the same atmosphere. Reverse transcription PCR testing was used to ensure the absence of mycoplasma contamination (Biotherapy Institute of Japan, Inc.). Reh and SUP-B15 cells were confirmed to be free of mycoplasma.

Chemicals and reagents. IO was kindly provided by Pfizer, Inc. and dissolved in water for injection (FUSO Pharmaceutical Industries, Ltd.) to a stock concentration of 250 μg/ml. Olaparib and talazoparib were obtained from Selleck Chemicals and dissolved in dimethylsulfoxide (Wako Yakuhin Co., Ltd.) to a stock concentration of 10 mM.

Determination of CD22 positivity. Flow cytometry was performed to determine CD22 expression on the cell surface of Reh and SUP-B15 cells using antibodies against CD22. The analysis of CD22 expression was performed by LSI Medience Corporation. CD22 expression was measured using a flow cytometer (FACSCanto II; Becton, Dickinson and Company) using undiluted IOTest CD22-Fluorescein isothiocyanate (FITC; cat. no. IM0779U; Beckman Coulter, Inc.) and undiluted IgG2a mouse-FITC (cat. no. A12689; Beckman Coulter, Inc.). Twice-diluted IgG1 mouse-RD1 (cat. no. 6602884; Beckman Coulter, Inc.) was used as a control. The acute promyelocytic leukemia cell line HL-60 was used as a $CD22^-$ control.

Cell proliferation inhibition assay. Cells ($2.0x10^5$/ml Reh cells and $5.0x10^5$/ml SUP-B15 cells) were continuously exposed to various concentrations of IO ($1x10^{-3}$-$1x10^2$ ng/ml), olaparib ($1x10^{-3}$-$1x10^2$ μM) and talazoparib ($1x10^{-1}$-$1x10^4$ nM) to evaluate proliferation inhibition effects for 48 h. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay (cat. no. CK04; Dojindo Laboratories, Inc.) in accordance with the manufacturer's instructions. A total of 10 μl of the CCK-8 reagent was added to 100 μl of cell suspension and incubated at 37˚C for 3 h. Absorbance was measured at 450 nm using a plate reader (SPECTRA MAX iD3; Molecular Devices, LLC).

Annexin-V binding assay. To detect the induction of apoptosis in Reh and SUP-B15 cells, a total of $2.0x10^5$/ml cells were treated with various concentrations of IO, olaparib and talazoparib for 24 or 48 h. After drug administration, cells were stained with annexin-V and propidium iodide using Annexin V FLUOS Staining Kit (cat. no. 49734400; Sigma-Aldrich; Merck KGaA) and analyzed by flow cytometry (FACSDiva Software version 6.1.3; Becton, Dickinson and Company) according to the manufacturer's instructions. Untreated cells were used as negative controls and cells treated with drug concentrations sufficient to induce apoptosis were used as positive controls.

Calculation of combination index (CI). The effects of combining IO and PARP inhibitors were calculated using the CI method (12), with values determined using CompuSyn (version 2.11; Informert Technologies, Inc.). CI values were classified as follows: i) CI >1.1, antagonism; ii) CI, 0.9-1.1,

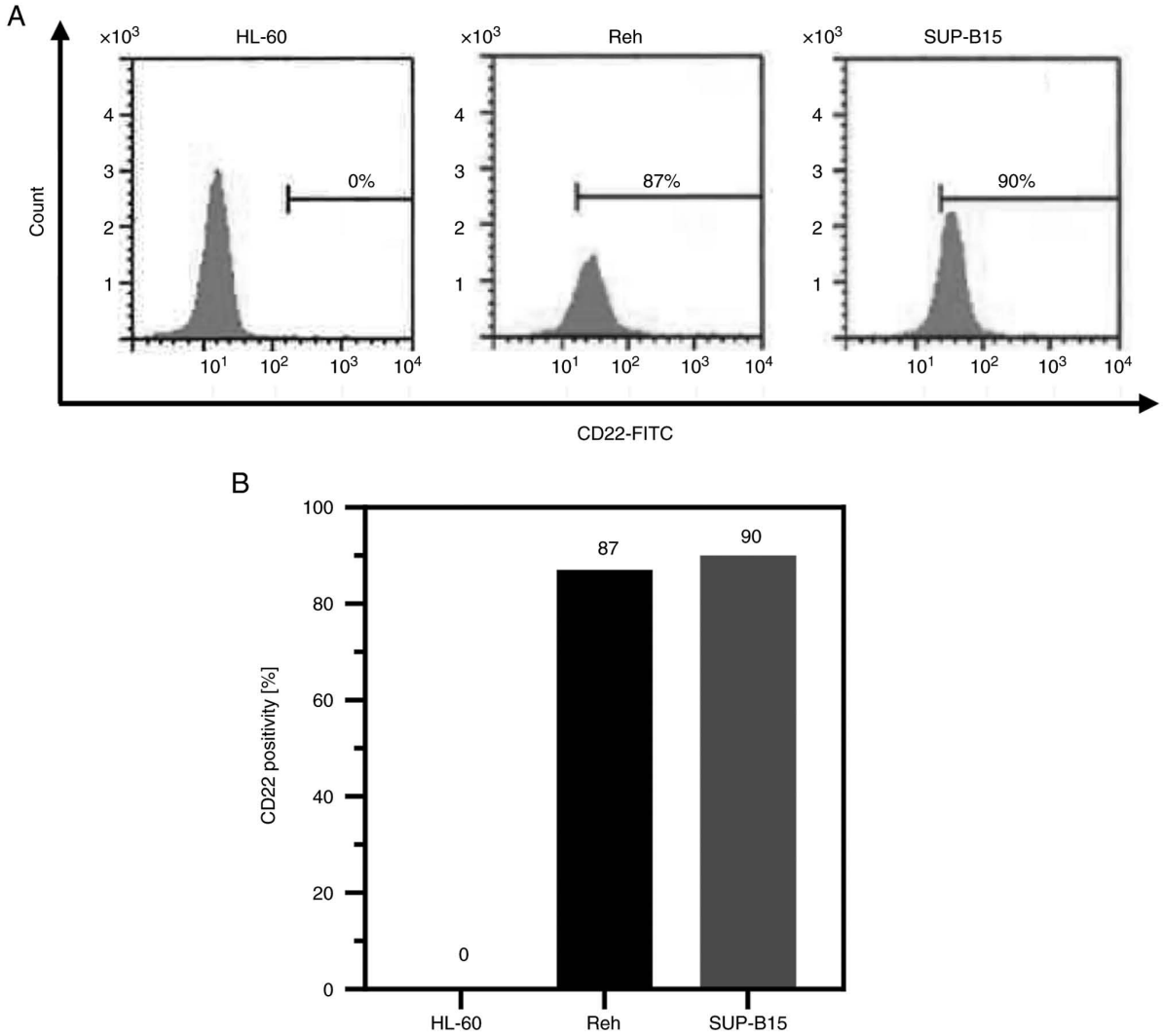

Figure 1. Cell surface CD22 expression. (A) CD22 positivity was determined by flow cytometry in HL-60, Reh and SUP-B15 cells. (B) Histogram showing the ratio of CD22 expression. CD, cluster of differentiation.

additive; iii) CI, 0.85-0.9, slight synergism; iv) CI, 0.7-0.85, moderate synergism; v) CI, 0.3-0.7, synergism; vi) CI, 0.1-0.3, strong synergism; and vii) CI <0.1, very strong synergism (13).

Alkaline comet assay. Alkaline comet assay was conducted to quantify the amount of DNA strand breaks using an OxiSelect Comet Assay Kit (cat. no. STA-351; Cell Biolabs, Inc.) according to the manufacturer's instructions. A total of $1x10^5$ Reh cells/ml were incubated with 2 ng/ml of IO in the presence or absence of 1 μM olaparib or 100 nM talazoparib for 1 or 6 h at 37˚C. A total of $1x10^5$ SUP-B15 cells/ml were incubated with 20 ng/ml IO in the presence or absence of 10 μM olaparib or 1 nM talazoparib under the same conditions used for Reh cells. Resuspended cells were then mixed with melted 90% agarose gel and transferred onto the base layer. These embedded cells were treated with lysis buffer (included in OxiSelect Comet Assay Kit) and alkaline solution. After electrophoresis, samples were washed and fixed in ice-cold 70% ethanol at room temperature for 5 min. Slides were dried, stained with Vista Green DNA dye (OxiSelect Comet Assay Kit), and visualized using an epifluorescence microscope (BX50F; Olympus corporation). The Olive tail moment was calculated using Comet (version 4.0; Oxford Instruments plc). For each sample, 50 cells were selected and analyzed.

Statistical analysis. Data analysis and graph creation were conducted using GraphPad Prism (version 10.0.3; Dotmatics). The results are shown as the mean ± standard division, and two-way ANOVA with Bonferroni corrections for multiple comparisons on the interaction between IO and PARP inhibitors. All of the results were derived from at least triplicate independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell surface CD22 expression. Cell surface CD22 expression, which is indispensable to the internalization of IO in B-ALL cells, was determined by flow cytometry (14). The expression levels of CD22 in Reh and SUP-B15 cells were 87 and 90%, respectively (Fig. 1). These two cell lines expressed sufficient levels of CD22 for IO to show cytotoxicity (7). CD22 was not expressed in the negative control HL-60 cells.

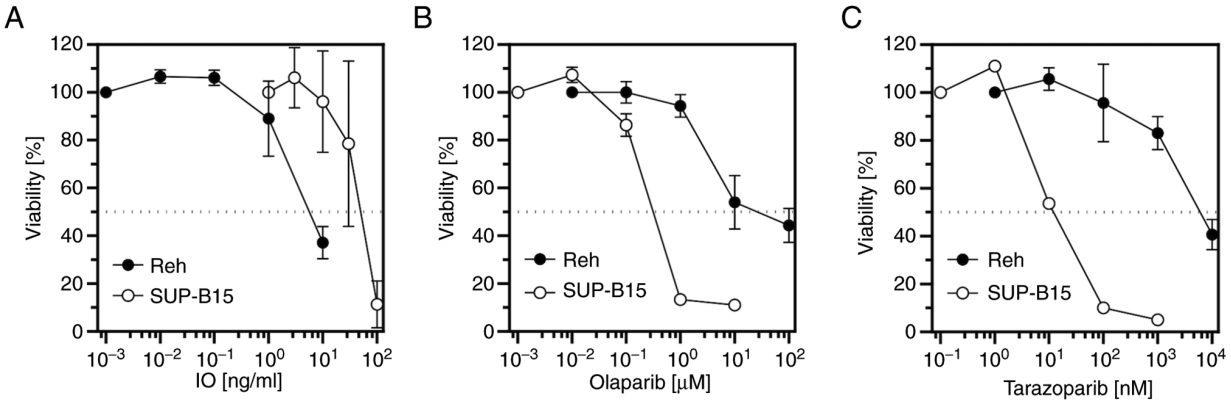

Figure 2. Cell proliferation inhibition by IO, olaparib or talazoparib. Reh and SUP-B15 cells were incubated with various concentrations of (A) IO and (B) olaparib or (C) talazoparib for 48 h. Cell viability was assessed using the water-soluble tetrazolium assay. Error bars represent standard deviation from at least triplicate experiments. IO, inotuzumab ozogamicin.

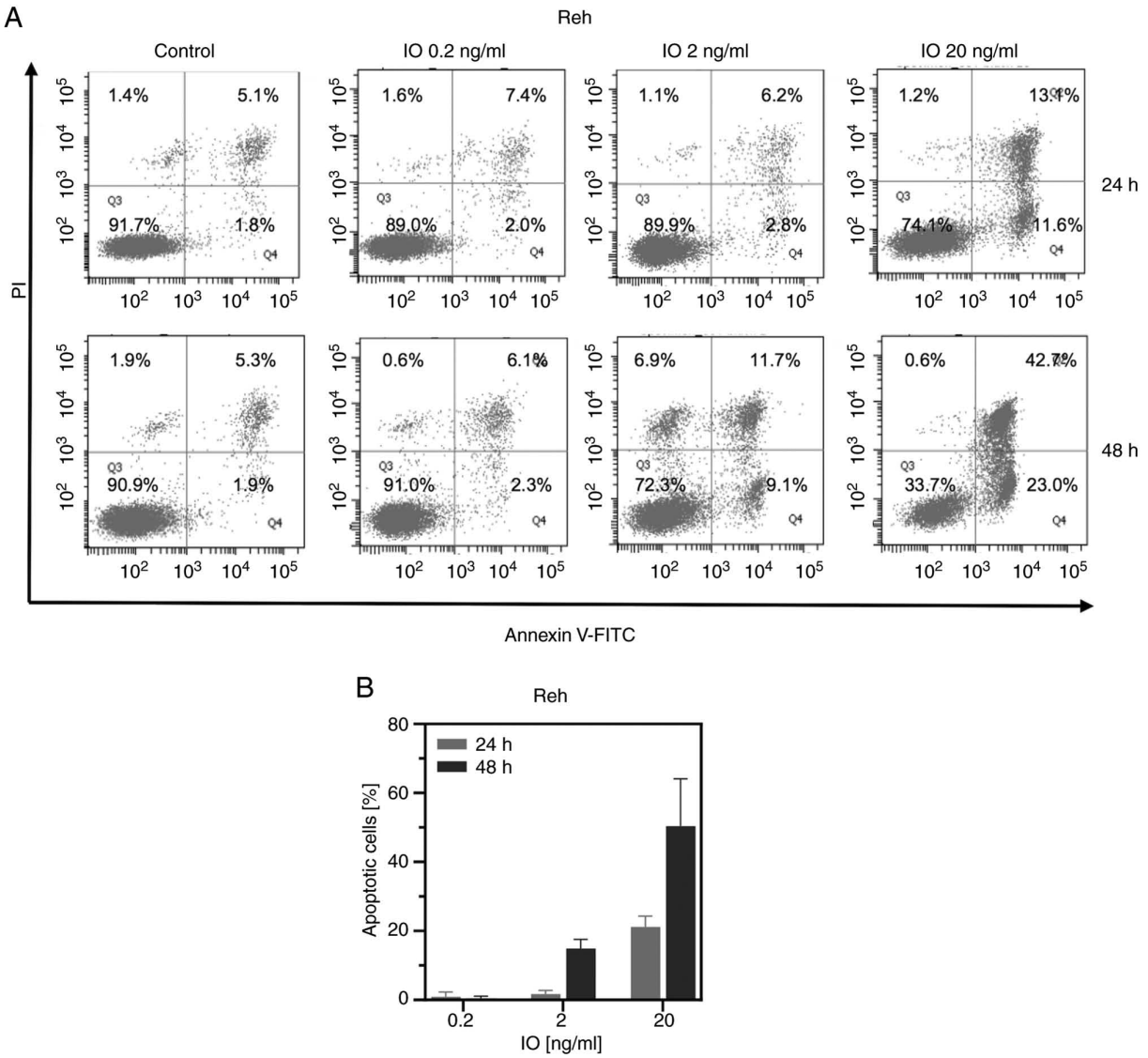

Figure 3. Continued.

Cell proliferation inhibition and induction of apoptosis by IO or PARP inhibitors. The anti-proliferative activity of IO in Reh and SUP-B15 cells was examined. Cells were incubated with increasing concentrations of IO for 48 h, then cell viability was measured by water soluble tetrazolium assay using CCK-8. IO inhibited the proliferation of these cells in a

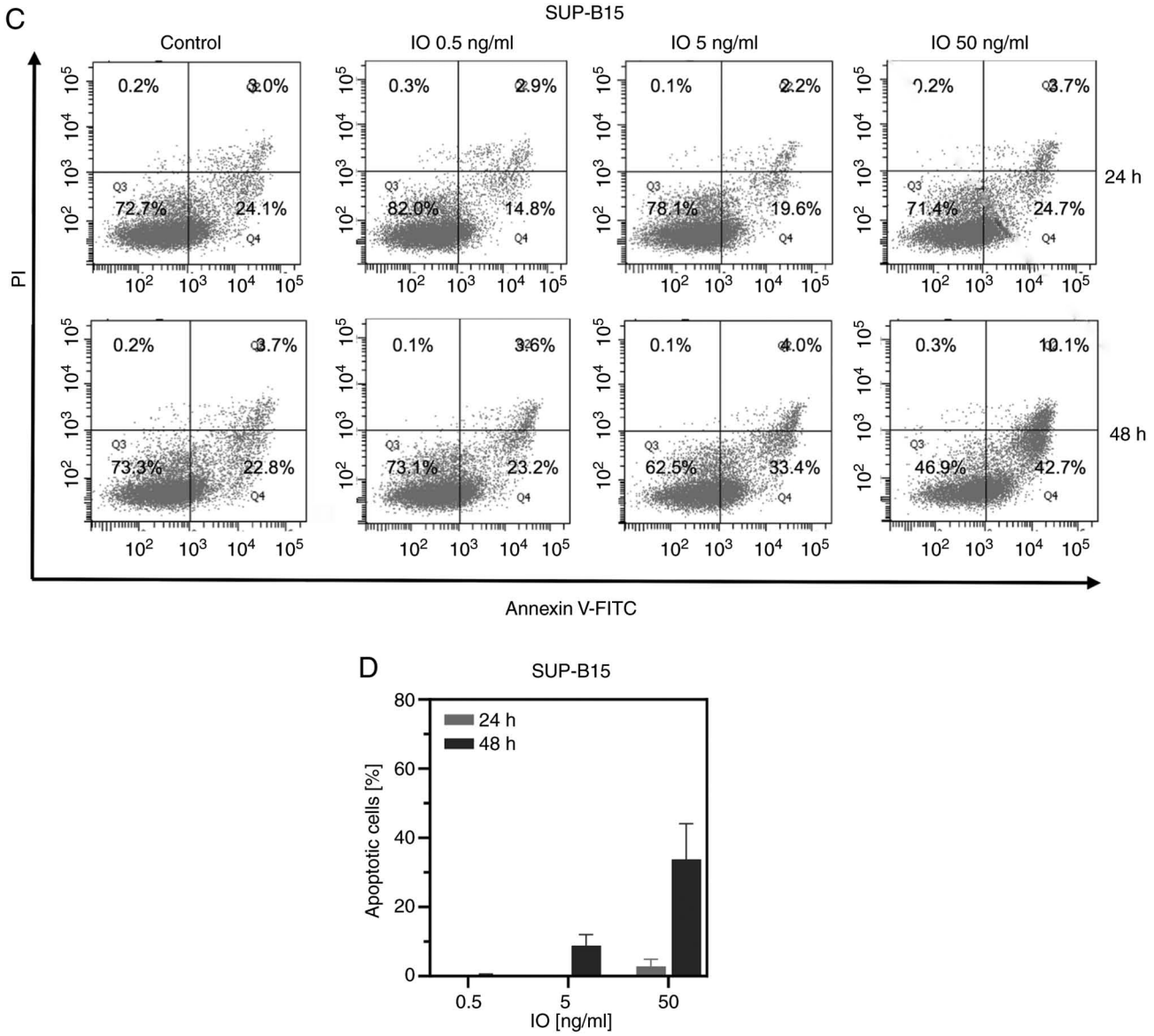

Figure 3. Induction of apoptosis by IO. Cells were incubated with (A and B) 0.2, 2, or 20 ng/ml IO for Reh cells; (C and D) 0.5, 5, or 50 ng/ml IO for SUP-B15 cells for 24 or 48 h, followed by (A and C) Annexin-V and propidium iodide co-staining and flow cytometry. The left lower quadrant shows live cells, and the left upper quadrant shows necrotic cells. The right lower and upper quadrants, as annexin-V+ quadrants, show early and late apoptotic cells, respectively. The percentage of cells in every quadrant is indicated. (B and D) Histograms represent the percentage of apoptotic cells (drug-treated apoptotic cells minus control apoptotic cells). Error bars represent standard deviation from triplicate experiments. IO, inotuzumab ozogamicin.

dose-dependent manner (Fig. 2A). The half-maximal inhibitory concentration (IC_{50}) values for IO are shown in Table I. Similarly, cell proliferation inhibition by the PARP inhibitors olaparib and talazoparib in Reh and SUP-B15 cells was examined. Olaparib and talazoparib inhibited cell proliferation in a dose-dependent manner in both cell lines (Fig. 2B and C). The IC_{50} values of olaparib and talazoparib are shown in Table II, revealing that talazoparib was more potent than olaparib. An Annexin-V binding assay was conducted to determine IO-induced apoptosis. When Reh and SUP-B15 cells were treated with various concentrations of IO for 24 or 48 h, IO increased early and late cell apoptosis in a dose-dependent manner. Moreover, exposure to IO induced more apoptosis at 48 h than at 24 h for every IO concentration tested (Fig. 3). In Fig. 3C, untreated SUP-B15 cells underwent >20% apoptosis. SUP-B15 cells are prone to elimination by apoptosis very easily and spontaneously. Previous studies using SUP-B15 cell line also revealed some extent of cell death of untreated SUP-B15 cells (15,16). The live cell percentage of untreated SUP-B15 was ~83-88% in these studies. These present results indicated that IO was cytotoxic to both Ph^- and Ph^+ B-ALL cells, and talazoparib was more cytotoxic to both Ph^- and Ph^+ B-ALL cells than olaparib.

Cell proliferation-inhibiting effects of combining IO with PARP inhibitors. Both Reh and SUP-B15 cells were incubated with IO for 48 h with minimally toxic concentrations of either olaparib (1 μM in Reh cells; 10 nM in SUP-B15 cells) or talazoparib (100 nM in Reh cells; 1 nM in SUP-B15 cells) to investigate the cell proliferation inhibition effects of IO combined with olaparib or talazoparib. Combined with olaparib or talazoparib, IC_{50} values of IO were apparently

Table I. IC_{50} values for IO, Ola and Tala.

	Drugs		
	IO	Ola	Tala
IC_{50}			
Reh	5.3 ng/ml	24.0 μM	4.9 μM
SUP-B15	49.7 ng/ml	0.3 μM	0.01 μM

Cells were treated with IO, Ola, or Tala for 48 h, followed by water soluble tetrazolium assay. IC_{50}, half-maximal inhibitory concentration; IO, inotuzumab ozogamicin; Ola, olaparib; Tala, talazoparib.

Table II. IO sensitivity in combination with Ola or Tala.

	Drugs	
	IO + Ola	IO + Tala
IC_{50}		
Reh	0.8 ng/ml	2.9 ng/ml
SUP-B15	36.1 ng/ml	39.6 ng/ml

Cells were treated with IO alone or in combination with Ola or Tala for 48 h, followed by water soluble tetrazolium assay. IO, inotuzumab ozogamicin; Ola, olaparib; Tala, talazoparib; IC_{50}, half-maximal inhibitory concentration.

decreased (Table II). These cells became more sensitive to IO administered in combination with PARP inhibitors.

Determination of synergism between IO and PARP inhibitors. To further investigate the combination effects between IO and olaparib or talazoparib, the CI was calculated. Reh and SUP-B15 cells were incubated with various concentrations of IO in the presence of minimally toxic concentrations of olaparib or talazoparib. When combined with olaparib or talazoparib, the CI values were 0.19 and 0.56 for Reh cells, and 0.76 and 0.89 for SUP-B15 cells, respectively (Fig. 4). These results revealed the synergism between IO and both olaparib and talazoparib for Reh and SUP-B15 cells.

Induction of cell apoptosis by IO combined with PARP inhibitors. Apoptosis was measured after cells were treated with IO (0.2 ng/ml in Reh cells; 3.0 ng/ml in SUP-B15 cells) in the presence or absence of minimally toxic concentrations of either olaparib or talazoparib. Combining IO with olaparib or talazoparib induced higher apoptosis than IO alone (Fig. 5). In both Reh and SUP-B15 cells, the interaction between IO and olaparib was significant. In Reh cells, the interaction between IO and talazoparib was also significant ($P<0.05$; two-way ANOVA and Bonferroni multiple comparisons test). The data indicated that combining IO with PARP inhibitors augmented IO-induced apoptosis.

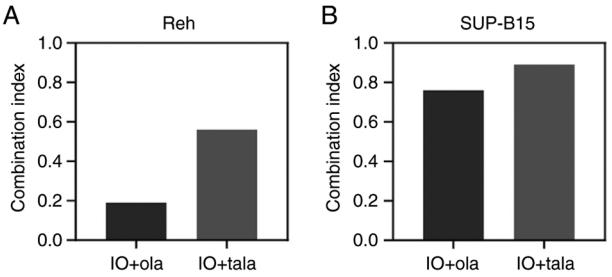

Figure 4. Combination index. (A) Reh and (B) SUP-B15 cells were incubated for 48 h with minimally toxic concentrations of Ola or Tala. CI<1 indicated synergism. Ola, olaparib; Tala, talazoparib; IO, inotuzumab ozogamicin.

Inhibition of IO-induced DNA damage repair by PARP inhibitors. The comet assay was conducted to verify DNA damage and repair. The tail moment is an appropriate indicator of the amount of DNA damage induced since this value takes under consideration both the amount of DNA migration and the relative amount of DNA in the tail (17). Reh and SUP-B15 cells were treated with IO (2 ng/ml in Reh cells; 20 ng/ml in SUP-B15 cells) in the presence or absence of minimally toxic concentrations of olaparib or talazoparib for 1 or 6 h. Representative comet images are shown in Fig. 6A and C. A total of 1 h treatment with IO induced the comet tail, indicating the induction of DNA strand breaks. However, the image at 6 h did not reveal any tails, suggesting that breaks were repaired after that time. Conversely, in the presence of olaparib or talazoparib, comet tails remained at 6 h, indicating that breaks remained unrepaired by the inhibition of PARP function. Line graphs revealed that the line for the Olive tail moment of combination went upwards from 1 to 6 h after drug administration; by contrast, that of IO alone went down (Fig. 6B and D). Olaparib and talazoparib were thought to inhibit PARP-dependent SSB repair. These results suggested that the enhancement of IO cytotoxicity by PARP inhibitors was attributable to the inhibition of IO-induced DNA damage repair.

Discussion

The present study demonstrated that the combination of IO with olaparib or talazoparib demonstrated synergistic anti-leukemic effects via the inhibition of DNA damage repair mechanisms. As single agents, IO, olaparib and talazoparib all inhibited cell proliferation in B-ALL cell lines. Combining IO with either olaparib or talazoparib resulted in greater inhibition of cell proliferation, showing synergistic effects. Furthermore, the addition of minimally toxic concentrations of olaparib or talazoparib combined with IO increased apoptosis. The comet assay demonstrated that DNA strand breaks induced by IO alone had almost disappeared by 6 h, suggesting successful completion of DNA repair. However, in the presence of olaparib or talazoparib, DNA strand breaks persisted even at 6 h, suggesting the inhibition of the DNA repair function. These results indicated that the cytotoxicity of IO was enhanced by olaparib or talazoparib via inhibition of DNA damage repair.

SSBs are repaired by base excision repair (BER) and nucleotide excision repair (18), while DSBs are repaired by homologous recombination and non-homologous end rejoining (19-21). Calicheamicin, as the payload of IO, exhibits cytotoxicity by inducing both SSBs and DSBs. The ratio of

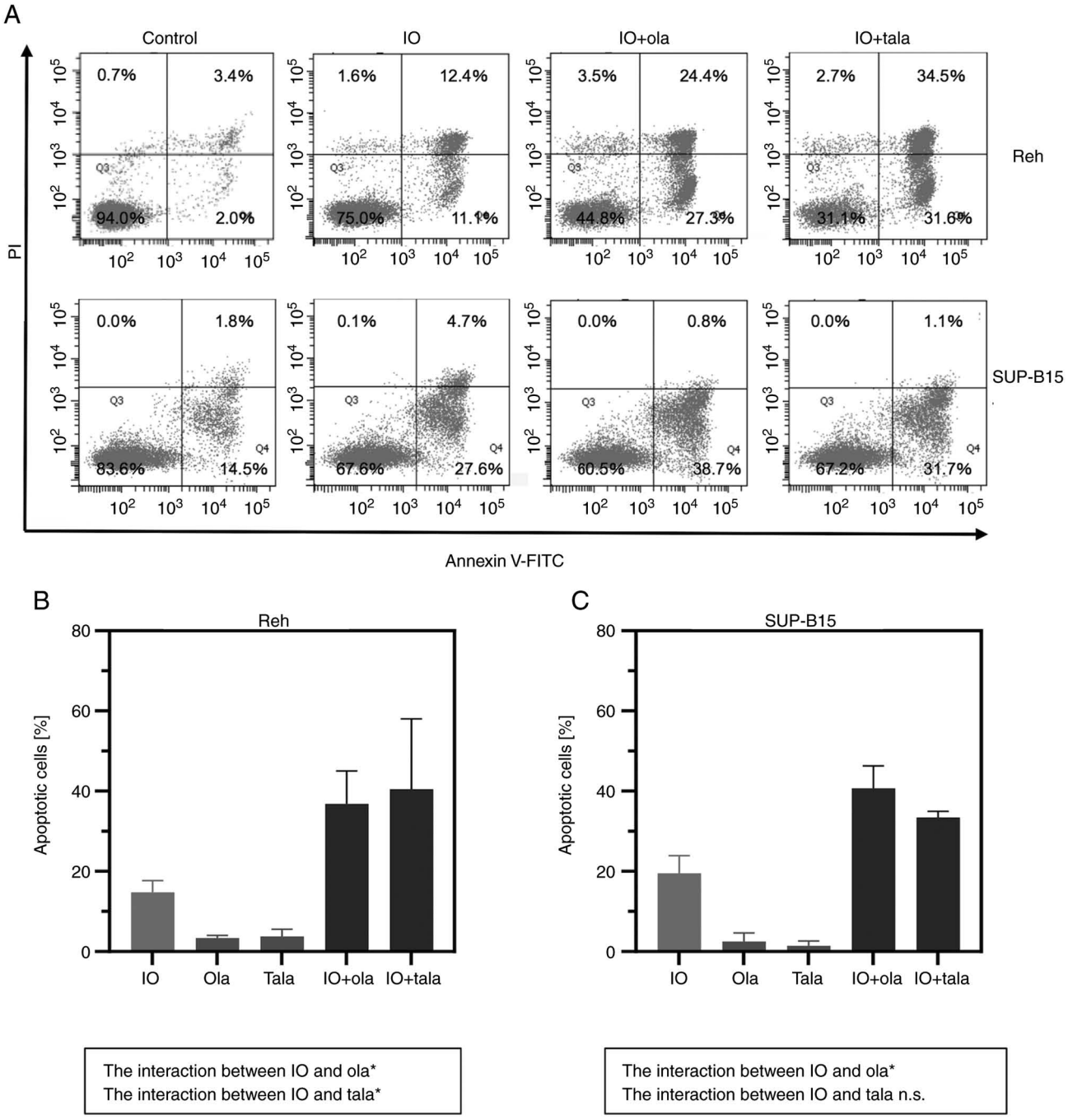

Figure 5. Induction of apoptosis by IO combined with Ola or Tala. (A) Reh and SUP-B15 cells were incubated for 48 h with IO (0.2 ng/ml IO in Reh cells; 3 ng/ml IO in SUP-B15 cells) in the presence or absence of minimally toxic Ola or Tala followed by an annexin-V binding assay. The left lower quadrant shows live cells, and the left upper quadrant shows necrotic cells. The right lower and upper quadrants, as annexin-V$^+$ quadrants, demonstrate early and late apoptotic cells, respectively. The percentage of cells in each quadrant is indicated. (B and C) Histograms represent the percentage of apoptotic cells (drug-treated apoptotic cells-vehicle-treated apoptotic cells). Error bars represent standard deviation from at least triplicate experiments. *P<0.05; two-way ANOVA and Bonferroni multiple comparisons test on the interaction between the apoptotic rate of IO and PARP inhibitors. IO, inotuzumab ozogamicin; Ola, olaparib; Tala, talazoparib; PARP, poly (ADP-ribose) polymerase.

DSBs to SSBs among cellular DNA is 1:3, close to the 1:2 ratio observed when calicheamicin g1 cleaves purified plasmid DNA (22). PARP senses and binds to SSBs, then forms long chains of poly(ADP-ribose) to recruit DNA repair proteins involved in BER (23). PARP inhibitors achieve cytotoxicity by inhibiting SSB repair. In the presence of PARP inhibitors, calicheamicin-induced SSBs are not repaired, and unrepaired SSBs convert to accumulating DSBs during DNA replication, resulting in cell death. The strategy of combining IO with PARP inhibitors thus achieves synergistic effects based on a different concept from synthetic lethality. Previous studies have reported that doxorubicin, which causes both SSBs and DSBs, similar to calicheamicin, in combination with olaparib also demonstrated synergistic effects in breast cancer and leukemia cells (24,25). Gemtuzumab ozogamicin, an antibody-drug conjugate similar to IO, reportedly demonstrated synergistic effects on CD33$^+$ myeloid leukemia cells in combination with olaparib (26). Moreover, PARP1 was previously revealed to

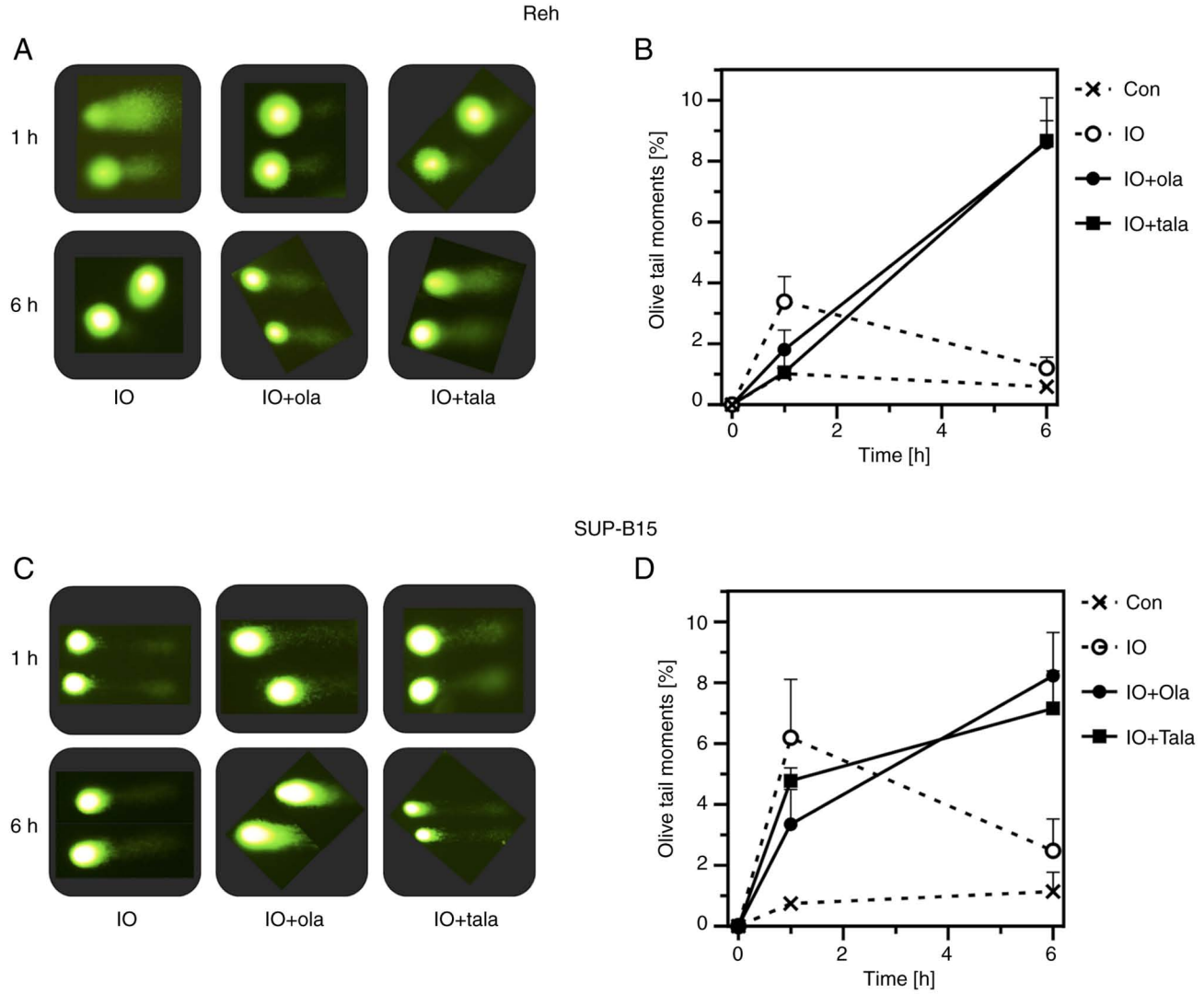

Figure 6. DNA strand breaks and repair. Cells were incubated with (A and B) 2 ng/ml IO for Reh cells and (C and D) 20 ng/ml IO for SUP-B15 cells in the presence or absence of Ola (1 μM for Reh cells and 10 nM for SUP-B15 cells) or Tala (100 nM for Reh cells and 1 nM for SUP-B15 cells) for 1 or 6 h, followed by an alkaline comet assay. (A and C) Representative comet figures. Olive tail moment is calculated as tail DNA % x tail moment length using the Comet software. (B and D) A total of 50 cells were analyzed for each sample. Percentages are indicated as the mean ± standard deviation from three independent experiments. IO, inotuzumab ozogamicin; Ola, olaparib; Tala, talazoparib.

be involved in DSB repair (27-30). Combining IO with PARP inhibitors thus appears to represent a reasonable strategy to enhance the cytotoxicity of IO.

Tirrò *et al* (31) investigated the cytotoxicity of IO in $CD22^+$ cells from the perspective of checkpoint kinase1 (Chk1) inhibition. When $CD22^+$ cells were treated with IO, the surviving cells were arrested in the G2/M phase during which DNA repair was performed. The Chk1 inhibitor UCNO-1 abrogated this IO-induced G2/M arrest. Such treatment increased cell death rates among cells showing mutant p53. That study suggested the cellular damage response as a therapeutic target for patients with ALL receiving IO therapy. Takeshita *et al* (32) reported that cancer cells expressing the multidrug resistance protein P-glycoprotein (P-gp) were resistant to IO treatment *in vitro*. The cell lines used in the present study did not exhibit P-gp upregulation, therefore the results of the cytotoxicity experiments performed were not biased in that regard. Nevertheless, the expression of P-gp should be evaluated to further clarify the cytotoxicity of IO for RR-ALL in the light of P-gp.

Jabbour *et al* (33) investigated the clinical efficacy of IO combined with mini-hyper-CVD (cyclophosphamide, vincristine and dexamethasone) chemotherapy with or without blinatumomab in newly diagnosed elderly patients with Ph^-ALL. A total of 135 patients were treated prospectively with standard hyper-CVAD (cyclophosphamide, vincristine, doxorubicin and dexamethasone) (n=77) or with the combination of IO plus mini-hyper-CVD with or without blinatumomab (n=58). The identified 38 patients of each cohort were compared with standard hyper-CVAD and mini hyper-CVD plus IO using a propensity score matching. The 3-year event-free survival rates were 34 and 64%, respectively (P=0.003). The 3-year OS rates were 34 and 63%, respectively (P=0.004). Moreover, the authors also investigated the clinical efficacy of IO combined with mini-hyper-CVD chemotherapy. Mini-hyper CVD plus IO were performed in combination with or without blinatumomab. Of 110 patients (median age 37 years), 91 patients (83%) responded with 69 CR (63%). Median OS was 17 months (34). Thus, more effective and less

toxic combination regimens are needed to improve the clinical efficacy of ALL treatment.

A limitation of the present study includes the use of cell lines only for experimentation. Although cancer cell lines are widely used in basic research, cell lines do not completely represent the molecular features of primary tumor cells (35). Cell lines likely represent a subpopulation of the original tumor because of cell culture without the original micro-environment, resulting in genetic or epigenetic differences between cell lines and primary tumor cells (36). Therefore, experiments using primary ALL cells for evaluation the efficacy of the combination therapy of IO with PARP inhibitors are desired. The present study has another limitation with regards to safety evaluation. The maximum drug concentration of IO administration reached 200 ng/ml with the half-life of 130 h in clinic (4,37). Therefore, the IO concentrations used in the present study (2-100 ng/ml) were markedly lower, which can assure the safety. Moreover, PARP inhibitors were used at minimally toxic concentrations in the combination with IO [olaparib (1 μM in Reh cells and 10 nM in SUP-B15 cells] or talazoparib (100 nM in Reh cells and 1 nM in SUP-B15 cells)) here. These concentrations also were markedly lower than the maximum drug concentration in clinic (olaparib 8.43 μg/ml, talazoparib 13.78 ng/ml) (38,39). Several clinical trials for combination therapy of chemotherapeutic drugs with PARP inhibitors have been reported (40-42). There was a major issue with a narrow therapeutic window. Both chemotherapeutic drugs and PARP inhibitors are not selective for tumor cells, therefore PARP inhibition of normal cells enhances the toxicity of chemotherapeutic drugs, including myelosuppression. On the other hand, IO targets $CD22^+$ leukemic cells, not other normal cells including neutrophils. The interaction between IO and PARP inhibitors theoretically occurs in $CD22^+$ leukemic cells, which will not increase adverse reactions in the patient's body. To confirm the safety of this combination therapy, experiments using patient-derived tumor xenografts as pre-clinical models are also necessary (43).

In conclusion, the present study demonstrated synergistic anti-leukemic effects from the combination of IO with olaparib or talazoparib. There were no previous studies of clinical trials on the combination of IO with PARP inhibitors. Therefore, the present study is considered to be highly novel. The impact of such combination is dependent on the inhibition of DNA damage repair. In ALL cells, IO-induced DNA strand breaks were inhibited by PARP inhibitors. Such combination may not increase damage to normal cells, since IO specifically targets $CD22^+$ cancer cells. Targeting the inhibition of DNA damage repair may thus represent a potent therapeutic strategy for ALL.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

NI, MO, ST, NH and TY have participated sufficiently in the conception and design of the study and were involved in the acquisition, analysis and interpretation of the data, as well as drafting the manuscript. NI acquired and analyzed the data, produced the figures, and wrote the manuscript as principal investigator. ST was involved in acquisition and interpretation of data. MO and TY confirm the authenticity of all the raw data. NH supervised the study design and critically revised the work for important intellectual content. TY developed the study concept and designed the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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