

# Gemtuzumab Ozogamicin and Olaparib Exert Synergistic Cytotoxicity in CD33-positive HL-60 Myeloid Leukemia Cells

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**Abstract.** *Background/Aim:* Gemtuzumab ozogamicin (GO) consists of the cluster of differentiation 33 (CD33) antibody linked to calicheamicin. The binding of GO to the CD33 antigen on leukemic cells results in internalization and subsequent release of calicheamicin, thereby inducing DNA strand breaks. We hypothesized that the poly (ADP-ribose) polymerase inhibitor olaparib might inhibit DNA repair initiated by GO-induced DNA strand breaks, thereby increasing cytotoxicity. *Materials and Methods:* The human myeloid leukemia cell line HL-60 and a GO-resistant variant (HL/GO20) were used. *Results:* The 50% growth-inhibitory concentrations ( $IC_{50}$ ) were 24 ng/ml for HL-60 cells and 550 ng/ml for GO-resistant variant HL/GO20 cells. HL/GO20 cells were also refractory to GO-induced apoptosis. CD33 positivity was reduced in HL/GO20 cells. Olaparib-alone did not inhibit the cell growth and did not induce apoptosis in either HL-60 cells or HL/GO20 cells at concentrations of up to 10  $\mu$ M. When cells were treated with different concentrations of GO in the presence of 10  $\mu$ M olaparib, the  $IC_{50}$  of GO for HL-60 cells was 13 ng/ml. The combination index was 0.86, indicating synergistic cytotoxicity of GO and olaparib in combination. Such a combination was ineffective for HL/GO20 cells. *Conclusion:* GO and olaparib exerted synergistic cytotoxicity in CD33-positive myeloid leukemia cells in vitro.

*Abbreviations:* AML, Acute myeloid leukemia; GO, Gemtuzumab ozogamicin; CD33, cluster of differentiation 33; PARP, poly (ADP-ribose) polymerase;  $IC_{50}$ , 50%- growth inhibitory concentration; P-gp, P-glycoprotein; XTT, sodium 3'-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; PARP1, Poly(ADP-ribose) polymerase-1; CI, combination index; real-time reverse transcriptase - polymerase chain reaction; RT-PCR.

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*Key Words:* Gemtuzumab ozogamicin, olaparib, DNA repair, acute myeloid leukemia, HL-60 cells.

Gemtuzumab ozogamicin (GO, Mylotarg™, Pfizer, New York, NY, USA) is a humanized monoclonal antibody directed against the cluster of differentiation 33 (CD33) surface antigen that is conjugated to a derivative of the cytotoxic antibiotic calicheamicin (1-3). CD33 is expressed on leukemic blasts in 90% of cases of newly-diagnosed acute myeloid leukemia (AML) but not on normal stem cells (1-3). Because CD33 is specific to leukemia cells, GO is thought to be an attractive targeted-agent for AML chemotherapy with limited toxicity.

After intravenous administration, GO binds to the CD33 antigen on the surface of leukemic cells, and the complex is incorporated into the cells. ATP-binding cassette transporters, such as P-glycoprotein (P-gp, ABCB1) or multidrug resistance protein-1 (ABCC1), export GO from cells (4-6). Calicheamicin is released from the complex after internalization, and reduced to 1,4-dehydrobenzene, which then enters the cell nucleus, intercalates within the minor groove of the DNA helix, and consequently induces site-specific DNA strand breaks (7-9). The induction of DNA strand breaks by GO is considered to be the cumulative effect of CD33-mediated internalization of the drug, efflux by transporters, and equilibrium between GO-induced DNA damage and DNA repair response (10).

When calicheamicin cleaves purified DNA, it produces both double-strand and single-strand breaks. The double-strand break/single-strand break ratio in DNA is 1:1 to 1:3 (9, 11). In eukaryotic cells, DNA double-strand breaks are repaired through non-homologous end-joining and homologous recombination (12-14), whereas single-strand breaks are repaired by using several excision repair methods, including base excision repair (15, 16). Poly(ADP-ribose) polymerase-1 (PARP1) interacts with several proteins involved in single-strand break repair, base excision repair, and double strand break repair and plays important roles in multiple DNA damage response pathways (16-20). Therefore, it was hypothesized that the inhibition of PARP1 would sustain GO-induced DNA strand breaks, thereby enhancing GO cytotoxicity.

In the present study, the cytotoxicities of GO and the PARP inhibitor olaparib were evaluated in cultured myeloid leukemia cell line HL-60 expressing CD33 antigen. The

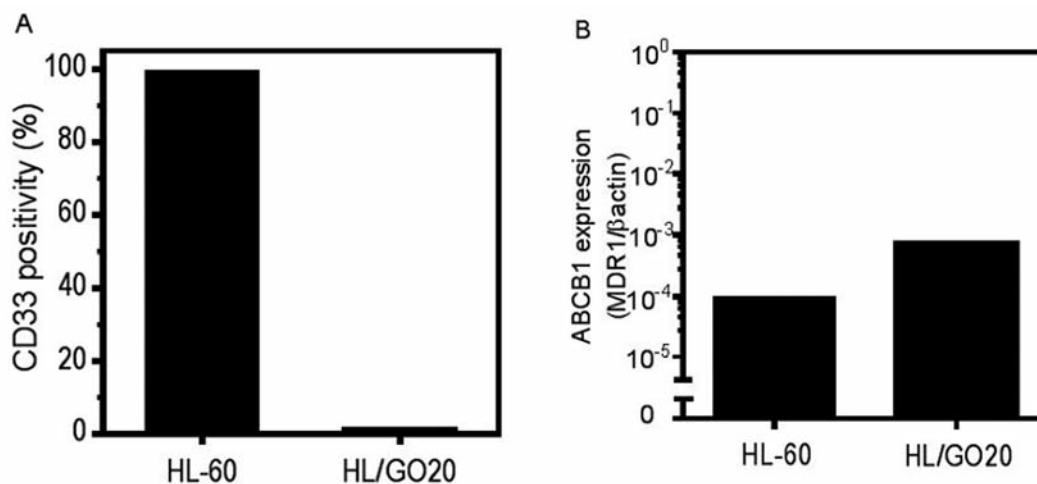


Figure 1. Characterization of GO-resistant HL-60 variant cell line HL/GO20 in comparison with the parental HL-60 cell line. (A) The CD33 positivity was determined by flow cytometry. (B) P-gp (ABCB1) mRNA levels determined by using real-time RT-PCR. GO, Gemtuzumab ozogamicin; CD33, cluster of differentiation 33; RT-PCR, reverse transcriptase – polymerase chain reaction.

cytotoxicity of olaparib was also evaluated in the context of GO-resistant cells and CD33-negative cells.

## Materials and Methods

**Chemicals and reagents.** GO was kindly supplied by Pfizer Japan Inc. (Tokyo, Japan) and dissolved in phosphate-buffered saline (PBS) to a stock concentration of 10 mg/ml. Olaparib was purchased from Sigma-Aldrich (St Louis, MO, USA).

**Cell culture.** The human myeloid leukemia cell lines HL-60 (American Type Culture Collection, ATCC, Manassas, VA, USA) and HL/GO20, a GO-resistant HL-60 subclones, were cultured in RPMI-1640 media in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C. The HL/GO20 cell line was developed along with two other GO-resistant subclones that have been characterized and reported in our previous study (10). HL/GO20 cell line had not yet been characterized. The CD33-negative T-lymphoblastic leukemia cell line CCRF-CEM (ATCC) was used for comparison.

**Proliferation assay.** To evaluate the growth inhibition effects, the sodium 3'-[1-[(phenylamino)-carbonyl-3,4-tetrazolium]]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN, USA) with slight modifications. (10)

**Cell-cycle analysis and quantitation of apoptotic cell death.** Flow cytometric analysis was performed to evaluate the cell-cycle distribution. Samples treated with GO or olaparib or both were fixed in 80% ethanol, stained with 20 µg/ml propidium iodide (Beckman Coulter, Fullerton, CA, USA), and analyzed using FACSCanto II (BD Bioscience, Franklin Lakes, NJ, USA). The population of cells in the sub-G<sub>1</sub> phase was measured to assess apoptotic cell death (21).

**Determination of CD33 positivity.** Flow cytometric analysis was performed as described previously to detect the expression of

CD33 using antibody against CD33 (LSI Medience, Corp., Tokyo, Japan) (10).

**Determination of P-gp (ABCB1).** To evaluate the expression levels of P-gp (accession: P08183), real-time reverse transcriptase - polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). The primers, the sequences of which were not open to the public, were prepared by LSI Medience, Corp.

**Calculation of the combination index (CI).** CI analysis provides quantitative information on the nature of drug interactions, especially the combination effects between 2 different agents. The CI method was based on that described by Chou and Talalay (22), and the values were determined by using the CalcuSyn (version 2.0) computer software (Biosoft, Great Shelford, Cambridge, UK). The CI values less than, equal to, and greater than 1 indicate synergy, additivity, and antagonism, respectively (22).

**Statistical analyses.** All graphs were generated using GraphPad Prism software (version 6.0) (GraphPad Software, Inc. San Diego, CA, USA). Values of  $p < 0.05$  were considered statistically significant.

## Results

**Characterization of HL/GO20 cells.** Surface CD33 expression is indispensable to the internalization of GO in leukemia cells, and via ATP-binding cassette transporters including P-gp, GO is effluxed out of the cells (4-6). Thus, the expression of cell surface CD33 antigen and P-gp were determined. The positivity of CD33 was severely reduced in HL/GO20 cells compared to that in HL-60 cells (Figure 1A). However, P-gp was not overexpressed in HL/GO20 cells (Figure 1B). Thus, the reduction in CD33 antigen on the cell surface was suggested to be the major mechanism of drug resistance.

Table I. Drug sensitivity of HL-60 and HL/GO20.

| Drugs                                | IC <sub>50</sub> |         |
|--------------------------------------|------------------|---------|
|                                      | HL-60            | HL/GO20 |
| GO (ng/ml)                           | 24               | 550     |
| Olaparib (μM)                        | >10*             | >10*    |
| GO +1 μM olaparib (ng/ml)            | 10               | 750     |
| GO + 10 μM olaparib (ng/ml)          | 13               | 330     |
| GO + olaparib (equivalently) (ng/ml) | 10               | 800     |

Cells were incubated with various concentrations of GO, olaparib or GO + 1 μM olaparib or 10 μM olaparib for 72 h. The IC<sub>50</sub> was then determined using the XTT assay. \*The IC<sub>50</sub> was beyond the upper detection limit. GO, Gemtuzumab ozogamicin; IC<sub>50</sub>, 50%-growth inhibitory concentration.

Table II. Drug sensitivity of CEM.

| Drugs                                | CEM (IC <sub>50</sub> ) |
|--------------------------------------|-------------------------|
| GO (ng/ml)                           | 420                     |
| Olaparib (μM)                        | >10*                    |
| GO + olaparib (equivalently) (ng/ml) | 415                     |

Cells were incubated with various concentrations of GO, olaparib or GO + 10 μM olaparib for 72 h. The IC<sub>50</sub> was then determined using the XTT assay. \*The IC<sub>50</sub> was beyond the upper detection limit. GO, Gemtuzumab ozogamicin; IC<sub>50</sub>, 50%-growth inhibitory concentration.

**Drug sensitivity of HL-60 cells and HL/GO20 cells.** The XTT growth inhibition assay revealed that GO-resistant variant HL/GO20 cells were 20-fold more GO-resistant than HL-60 cells (Table I) (Figure 2A). Both HL-60 cells and HL/GO20 cells were insensitive to olaparib as a single agent (Figure 2B). In the presence of non-toxic concentrations of olaparib (1 μM, 10 μM), HL-60 cells were sensitized to GO with a 50% reduction in the 50%-growth inhibitory concentration (IC<sub>50</sub>) value (Table I) (Figures 2C and D). Nevertheless, olaparib did not alter the sensitivity of HL/GO20 cells to GO (Table I) (Figures 2C and D).

**Determination of synergism between GO and olaparib.** To further investigate the combination effect between GO and olaparib, CI was calculated by incubating the cells for 72 h with both GO and olaparib at equivalent concentrations, followed by the XTT assay (Table I) (Figure 3). The IC<sub>50</sub> value was reduced for HL-60 cells, but the value was unchanged for HL/GO20 cells (Figures 3A and B). The CI values were 0.86 for HL-60 cells and 1.40 for HL/GO20 cells (Figure 3C). These results thus suggest that the synergism between GO and olaparib was obtained for HL-60 cells, but not for HL/GO20 cells.

**The induction of apoptosis.** Cytotoxic effects were determined by measuring apoptotic cell death after treatments with GO or olaparib or both in combination. When HL-60 cells were treated with a minimally toxic concentration of GO in the presence of olaparib, the induction of apoptosis was significantly enhanced compared to the treatment with GO-alone ( $p < 0.05$ , Mann-Whitney test) (Figure 4A). However, HL/GO20 cells were refractory to GO-induced apoptosis regardless of the addition of olaparib (Figure 4B).

**Cell-cycle analysis.** The cell-cycle distribution was evaluated at 72 h after HL-60 cells had been treated with GO or olaparib or both in combination using flow cytometry. Olaparib alone did not alter the cell-cycle distribution (Figures 5A and B). By contrast, the GO-treated cells exhibited an increased G<sub>2</sub>M population (Figure 5C) compared to the untreated control (Figure 5A), suggesting that GO-induced DNA damage might trigger cell-cycle arrest for checkpoint (23). The addition of olaparib resulted in the further accumulation of the G<sub>2</sub>M population (Figure 5D), suggesting that the inhibition of PARP might augment DNA damage, thereby increasing the time for DNA repair.

**The effect of the combination of GO and olaparib in CD33-negative cells.** The anti leukemic effect of the combination of GO and olaparib was also evaluated using the CD33-negative T-lymphoblastic leukemia cell line CCRF-CEM. The IC<sub>50</sub> value for GO in CEM cells was 20-fold higher than that of HL-60 cells (Table II) (Figure 6A). Although CEM cells are lacking in CD33, the high concentration of GO in the medium might allow-passive transport *via* the concentration gradient between intracellular and extracellular fluids. Olaparib-alone was not cytotoxic to CEM cells (Figure 6B). The addition of olaparib did not augment the growth-inhibitory effect of GO (Figure 6C).

## Discussion

Larson *et al.* reported the clinical efficacy of GO (9 mg/m<sup>2</sup>) as a single agent in two doses separated by two weeks for patients with CD33-positive AML in their first recurrence in three phase II studies, and found that 71 out of 277 patients achieved complete remission (26%) (24). The Southwest Oncology Group compared GO plus standard induction chemotherapy *versus* standard induction chemotherapy alone (25). In this trial, the addition of GO to induction or post-consolidation therapy failed to indicate improvement in complete remission rates, disease-free survival, or overall survival (25). The investigation conducted in the United Kingdom Medical Research Council AML15 revealed that there was a significant survival benefit for patients with AML with favorable cytogenetics (26). Castaigne *et al.*

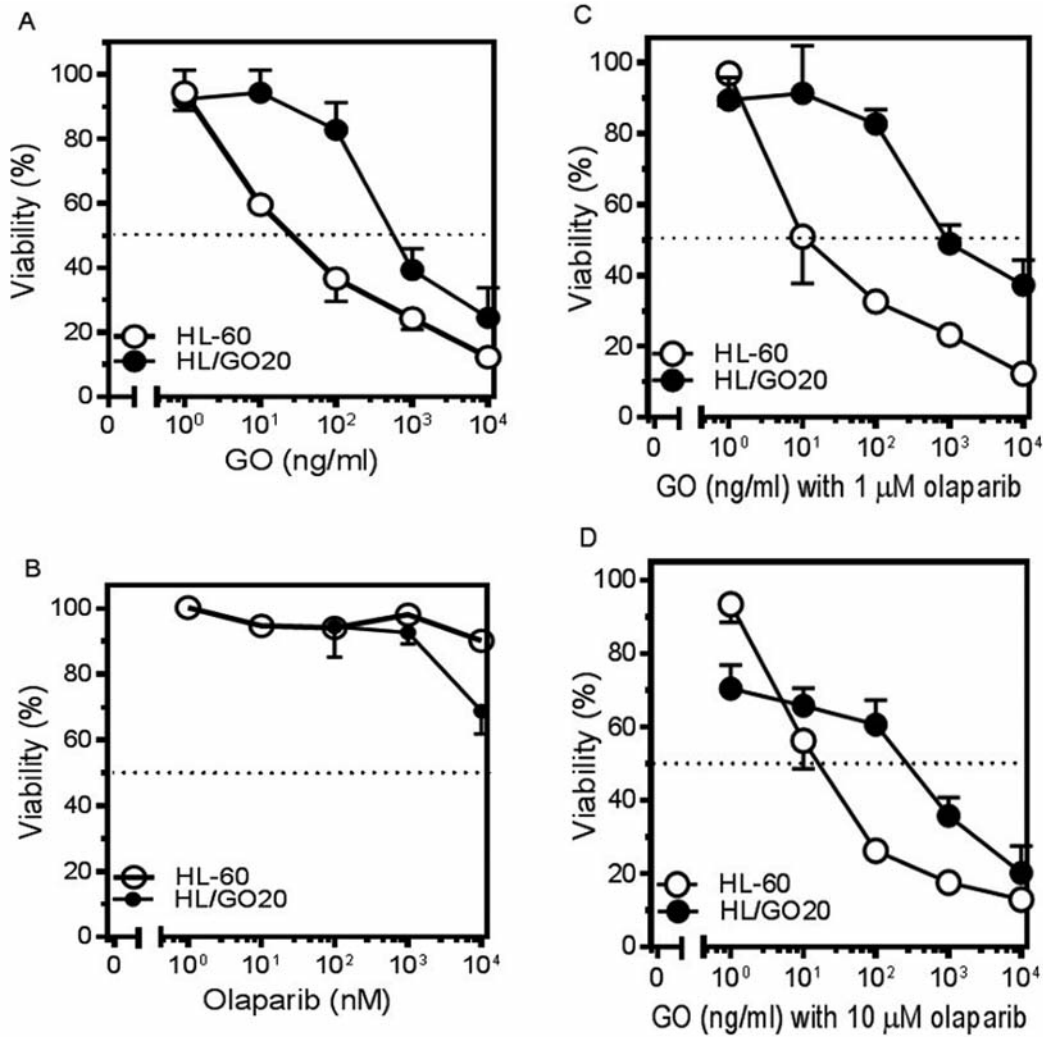


Figure 2. Growth inhibitory effects of GO and olaparib. HL-60 cells and HL/GO20 cells were incubated for 72 h with different concentrations of GO (A) and olaparib (B), followed by the XTT assay. These cells were also incubated for 72 h with different concentrations of GO in the presence of olaparib at 1  $\mu$ M (C) or 10  $\mu$ M (D), followed by the XTT assay. The values are the means $\pm$ SD of at least three independent experiments. GO, Gemtuzumab ozogamicin.

reported a phase III randomized trial in patients aged 50-70 years with previously untreated *de novo* AML to compare standard treatments with and without GO (27). The complete response rate with and without incomplete platelet recovery was 75% in the control group and 81% in the GO group. At 2 years, event-free survival and overall survival were estimated as 17.1% and 41.9% in the control group *versus* 40.8% and 53.2% in the GO-treated group, respectively (27). Despite spontaneous withdrawal of GO from the market in the US, certain subsets of patients with leukemia are suggested to benefit from the use of GO. It may be possible to optimize GO administration. The present study thus utilized olaparib to establish a strategy for enhancing the cytotoxicity of GO.

The present study clearly demonstrated that the combination of GO and olaparib exerts synergistic cytotoxicity against leukemia cells *in vitro* (Figures 2-4). The mechanistic interaction between GO and olaparib might be demonstrated by the alterations of the cell-cycle distribution. GO induced G<sub>2</sub>/M arrest (Figure 5), suggesting an initiation of cell-cycle checkpoints and DNA repair after GO-induced DNA damage. Olaparib-alone did not affect the cell cycle. Treatment with GO and olaparib in combination further increased the G<sub>2</sub>/M population, suggesting the presence of unrepaired DNA strand breaks that might prolong the time for repair (28). The GO-resistant subclone HL/GO20 cells that lost CD33 positivity were 20-fold more GO-resistant than were HL-60 cells. The HL/GO20 cell line was



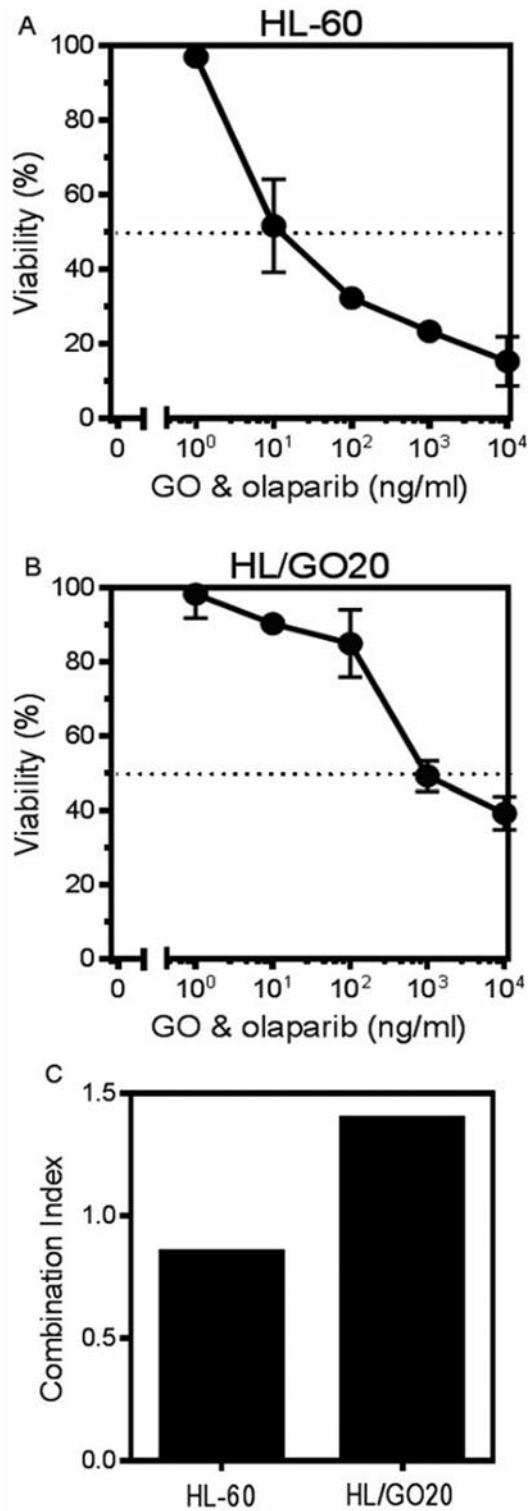


Figure 3. Combination index. HL-60 cells (A) and HL/GO20 cells (B) were incubated for 72 h with equivalent concentrations of GO and olaparib in combination, followed by the XTT assay. The values are the means±SD of at least three independent experiments. The combination index for GO and olaparib was determined in HL-60 cells and HL/GO20 cells (C). Values less than 1 indicate synergism. GO, Gemtuzumab ozogamicin.

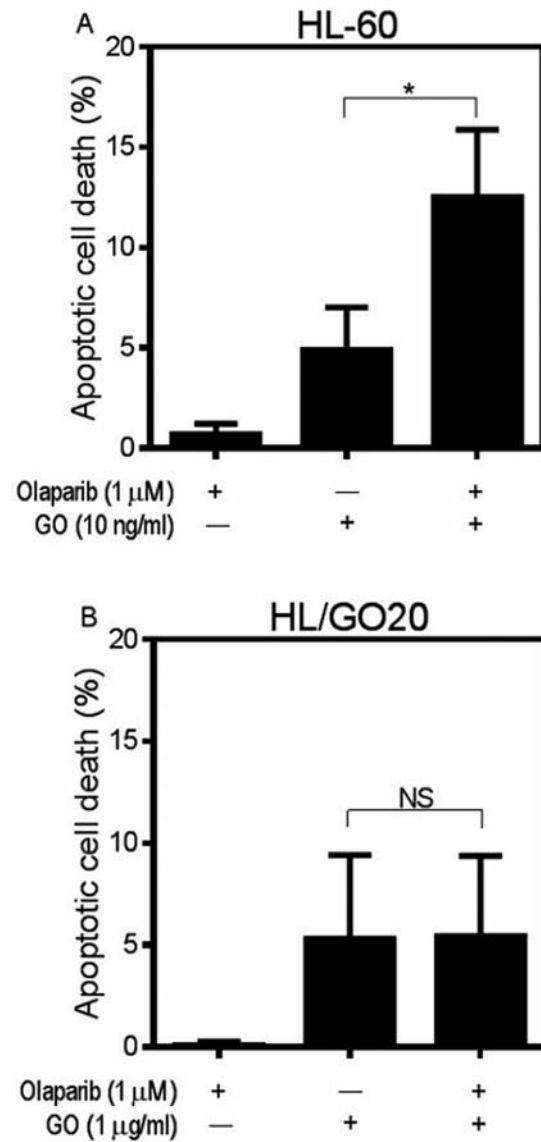


Figure 4. The induction of apoptosis. HL-60 cells (A) and HL/GO20 cells (B) were incubated for 72 h with GO, olaparib or both in combination, followed by the determination of apoptotic cell death of the sub-G<sub>1</sub> cell-cycle population. The values are the means±SD of at least three independent experiments. \**p*<0.05. NS, not significant. GO, gemtuzumab ozogamicin.

insensitive to olaparib both as a single agent and in combination with GO (Figures 2-4). Because HL/GO20 cells lack CD33 expression (Figure 1), GO is unlikely to be incorporated into the cells, a situation which could not be overcome mechanistically by the addition of olaparib.

The induction of DNA strand breaks by GO is considered the cumulative effect of CD33-mediated internalization of the drug, efflux by transporters, and equilibrium between

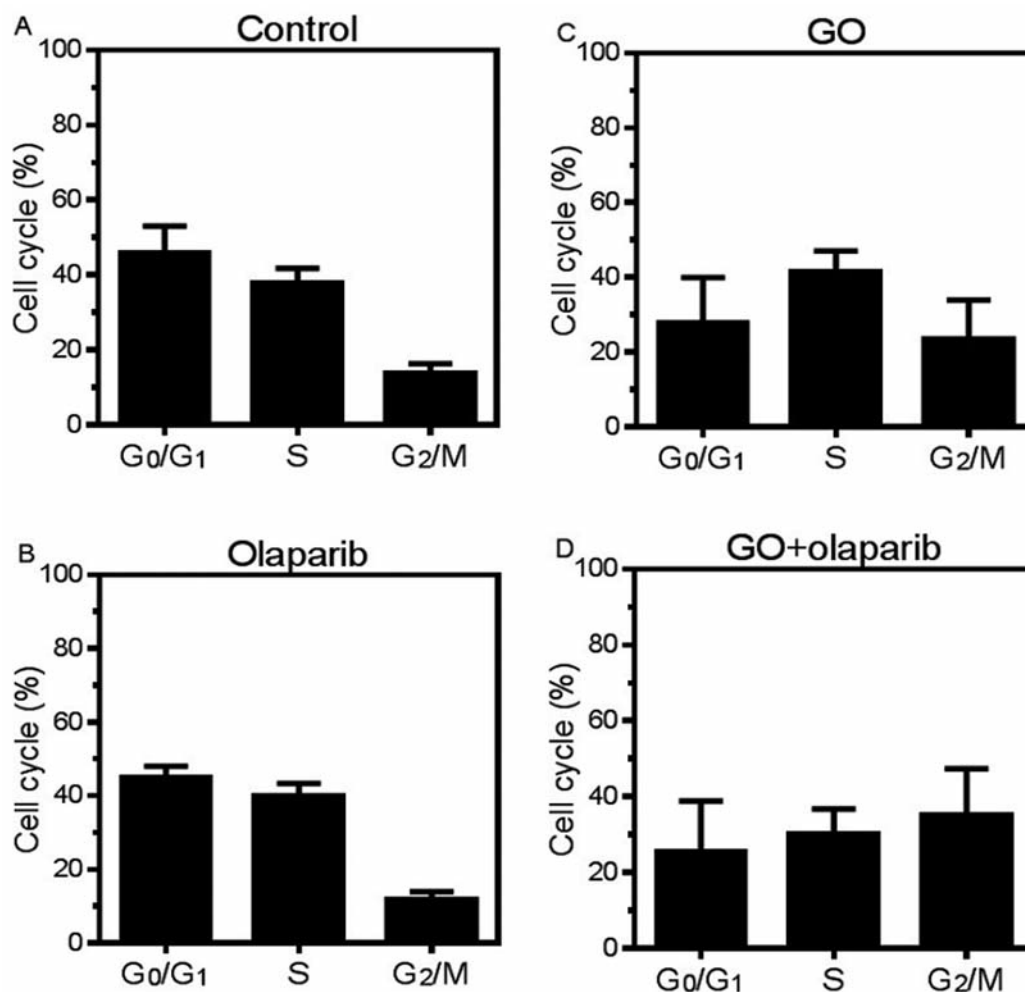


Figure 5. Cell-cycle distribution. HL-60 cells were incubated for 72 h with GO, olaparib or both in combination, followed by the determination of cell-cycle distribution by flow cytometry. A: Control, B: 1  $\mu$ M olaparib-alone, C: 10 ng/ml GO-alone, D: 1  $\mu$ M olaparib and 10 ng/ml GO in combination. The values are the means $\pm$ SD of at least three independent experiments. GO, Gemtuzumab ozogamicin.

GO-induced DNA damage and DNA repair responses (10). DNA single-strand breaks are normally rapidly repaired by a process known as base excision repair and single-strand break repair (20, 29). If the activity of PARP1 is inhibited, these repairs are not completed and are then converted into more cytotoxic DNA double-strand breaks during DNA replication. Moreover, PARP1 was recently found to interact with proteins of the DNA double-strand break response (30-33). These results prompted our attempts to combine the PARP inhibitor olaparib with GO in this study.

In conclusion, the present study demonstrated synergistic cytotoxicity between GO and olaparib against CD33-positive HL-60 leukemia cells. The administration of GO combined with olaparib may be a promising treatment for patients with refractory CD33-positive AML in the clinic. Moreover, if leukemia cells are deficient in breast cancer

susceptibility genes for DNA double-strand break repair, GO-induced DNA strand breaks and the inhibition of PARP by olaparib may induce the mechanism of synthetic lethality (34).

#### Disclosure Statement

The Authors have nothing to disclose concerning any of the drugs or agents considered in the present study.

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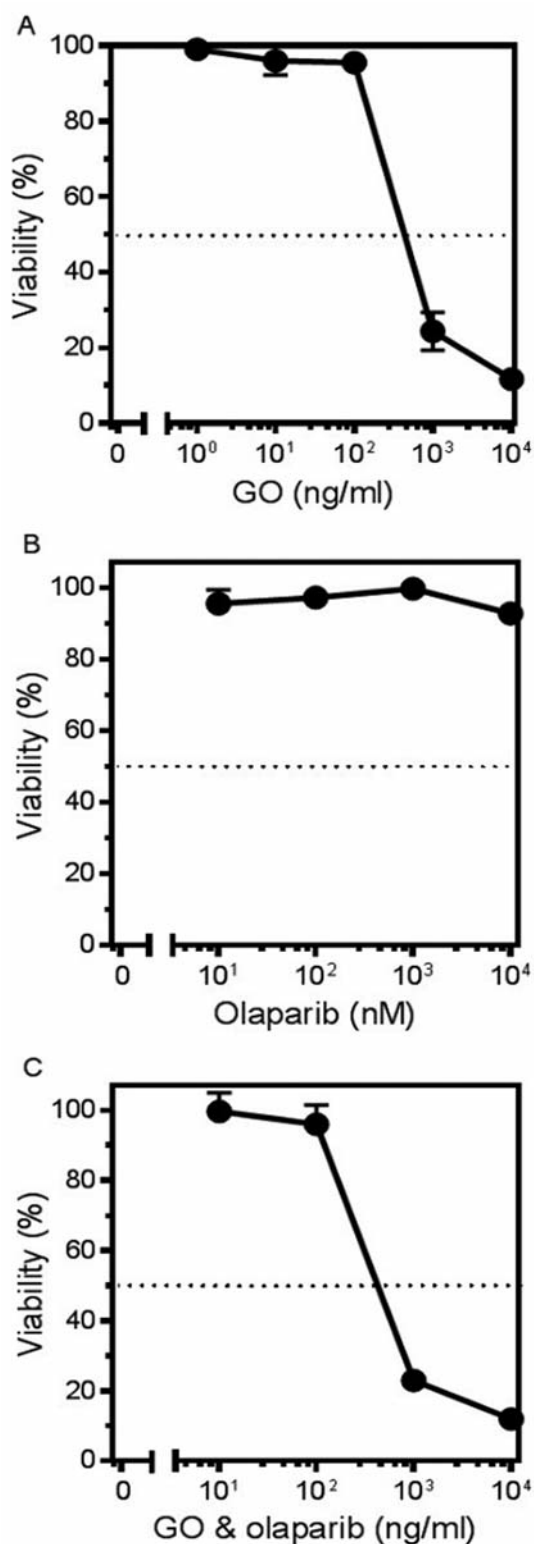


Figure 6. Growth inhibitory effects of GO and olaparib. CEM cells were incubated for 72 h with different concentrations of GO (A), olaparib (B), or both at equivalent concentrations (C), followed by the XTT assay. The values are the means $\pm$ SD of at least three independent experiments. GO, Gemtuzumab ozogamicin.

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