Obinutuzumab in Combination with Chemotherapy Enhances Direct Cell Death in CD20-Positive Obinutuzumab-resistant Non-Hodgkin Lymphoma Cells

Takaaki Fujimura, Yoriko Yamashita-Kashima, Natsumi Kawasaki, Shigeki Yoshiura, Naoki Harada, and Yasushi Yoshimura

ABSTRACT

Follicular lymphoma commonly recurs and is difficult to cure. Obinutuzumab is a humanized glycoengineered type II anti-CD20 antibody with a mode of action that includes induction of antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and direct cell death. There is no evidence on the effectiveness of retreatment with obinutuzumab in patients with prior obinutuzumab treatment. Using obinutuzumab-induced direct-cell-death–resistant cells, we investigated the efficacy of obinutuzumab retreatment in combination with chemotherapeutic agents used in follicular lymphoma treatment. Human non-Hodgkin lymphoma SU-DHL-4 cells were sustainably exposed to obinutuzumab in vitro, and 17 resistant clones expressing CD20 and showing 100-fold higher IC50 of obinutuzumab than parental cells were established. The growth inhibition effect of obinutuzumab in combination with bendamustine, 4-hydroperoxy-cyclophosphamide, doxorubicin, vincristine, or prednisolone was estimated using an interaction index based on the Bliss independence model. For each clone, there were various combinations of obinutuzumab and chemotherapeutic agents that showed supra-additive effects. Obinutuzumab combined with doxorubicin enhanced caspase-dependent apoptosis and growth inhibition effect. Obinutuzumab combined with prednisolone enhanced DNA fragmentation and G0–G1 arrest. These combinations also had an antitumor effect in mouse xenograft models. Our results indicate that retreatment with obinutuzumab, when it is combined with chemotherapeutic agents, is effective in the CD20-positive obinutuzumab-induced direct-cell-death–resistant cells.

Introduction

Follicular lymphoma is the second most common type of non-Hodgkin lymphoma (NHL), accounting for approximately 35% of NHLs and 70% of indolent lymphomas (1). Follicular lymphoma is usually slow growing and responds well to treatment; however, it commonly recurs and is difficult to cure. For patients with stage III/IV follicular lymphoma, regimens containing rituximab, a chimeric mouse–human type I anti-CD20 antibody, have become the standard of care (2, 3). Although the therapeutic outcomes of follicular lymphoma have greatly improved since rituximab was approved, it has become more important to determine which therapeutic agents are the best to follow obinutuzumab-containing regimens. In clinical practice, rituximab is widely used to retreat patients with follicular lymphoma who previously received it, and the efficacy of rituximab retreatment has also been reported with relapsed/refractory NHLs (10). However, there is no evidence on the efficacy of retreatment with obinutuzumab in patients who have already received obinutuzumab, and new scientific data are needed to address this. The purpose of our study was to explore the possibility of obinutuzumab retreatment for relapsed/refractory follicular lymphoma. Because direct cell death is a characteristic mode of action for type-II CD20 antibodies like obinutuzumab, in the current study we established human NHL cell clones that were resistant to obinutuzumab-induced direct cell death. Then we investigated the effectiveness of obinutuzumab in combination with other agents against these obinutuzumab-resistant cells.
Materials and Methods

Compounds and cells
Obinutuzumab was provided by F. Hoffmann-La Roche Ltd. Doxorubicin, vincristine, bendamustine (Selleck Chemicals LLC), 4-hydroperoxycyclophosphamide (4-OH-CY), an active metabolite of cyclophosphamide (Cayman Chemical), and prednisolone (Fuji) dimethyl sulfoxide (Sigma-Aldrich). N-ethyl-N-nitrosourea (ENU) and Z-VAD-FMK were purchased from Sigma-Aldrich and Promega Corporation, respectively.

SU-DHL-4 cells, a human germinal center B cell–like diffuse large B-cell lymphoma cell line, were obtained from the ATCC at 2017, and were maintained in RPMI1640 (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 10 mmol/L HEPES (Sigma-Aldrich), 0.45% D-glucose (Sigma-Aldrich), and 1 mmol/L sodium pyruvate (Thermo Fisher Scientific). All cells were cultured at 37 °C under 5% CO2, tested Mycoplasma contamination, and passed less than 20 times.

Animals
Animal procedures were approved by the Institutional Animal Care and Use Committee and the Biosafety Committee at Chugai Pharmaceutical Co., Ltd. Six-week-old female NOG mice (NOD/Shi-scid, IL-2Rγ−/−) were purchased from CLEA Japan, Inc. All animals were allowed to acclimatize and recover from shipping-related stress for more than 5 days prior to the study. Chlorinated water and irradiated food were provided ad libitum, and the animals were kept under a controlled 12-hour light/12-hour dark cycle.

Establishment of obinutuzumab-induced direct-cell-death–resistant clones
SU-DHL-4 cells were pretreated with 100 μg/mL of ENU for 1 day to establish resistant clones more efficiently by inducing random mutations (11) and were then treated with 200 μg/mL of obinutuzumab for 3 weeks. The mean serum trough concentration of obinutuzumab was used as a reference for the concentration (12). Regrown cells were single-cell cloned and cultured in medium without obinutuzumab for 12 days followed by the exposure of 10 μg/mL of obinutuzumab to eliminate clones that had only temporarily obtained insensitivity to obinutuzumab. The CD20 expression level of regrown cells was assessed by flow cytometry and the sensitivity to obinutuzumab-induced direct cell death was assessed by in vitro cell growth inhibition assay.

Flow cytometry
Cells were labeled with mouse phycoerythrin (PE)-conjugated anti-human CD20 antibody or isotype-matched mouse PE-conjugated isotype control antibody (BD Biosciences). Fluorescence was measured with an LSRFortessa X-20 cell analyzer (BD Biosciences), and analyzed using FlowJo v10 software (Tree Star Inc.).

In vitro cell growth inhibition assay
SU-DHL-4 cells and resistant clones were seeded on 96-well plates at 1–2 × 104 cells/well or 25 cm2 flask at 3 × 105 cells/flask, and treated with obinutuzumab and/or doxorubicin or prednisolone at indicated concentrations. Cells were washed with cell lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Nacalai Tesque, Inc.). Cells lysates were separated by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes by using an iBlot Gel Transfer System (Invitrogen). Immunoblotting was performed using the following primary antibodies: anti-IRE1, anti-phospho-JNK (pJNK), anti-phospho-Bcl2 (pBcl2), anti-Bcl2, anti-phospho-Rb at Ser807/Ser811 (pRb (Ser807/811]), anti-phospho-Rb at Ser795 (pRb (Ser795)), anti-phospho-Rb at Ser780 (pRb (Ser780)), anti-p27, anti-Skp2, and anti-ß-actin (Cell Signaling Technology), and anti-phospho-IRE1 (pIRE1), anti-JNK, and anti-Rb (Abcam). Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology), followed by chemiluminescence detection.

Immunoblot analysis
Cells were seeded on 6-well plates at 1 × 104 cells/well or 25 cm2 flask at 3 × 105 cells/flask, and treated with obinutuzumab and/or doxorubicin or prednisolone at indicated concentrations. Cells were lysed with cell lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Nacalai Tesque, Inc.). Cell lysates were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes by using an iBlot Gel Transfer System (Invitrogen). Immunoblotting was performed using the following primary antibodies: anti-IRE1, anti-phospho-JNK (pJNK), anti-phospho-Bcl2 (pBcl2), anti-Bcl2, anti-phospho-Rb at Ser807/Ser811 [pRb (Ser807/811)], anti-phospho-Rb at Ser795 [pRb (Ser795)], anti-phospho-Rb at Ser780 [pRb (Ser780)], anti-p27, anti-Skp2, and anti-ß-actin (Cell Signaling Technology), and anti-phospho-IRE1 (pIRE1), anti-JNK, and anti-Rb (Abcam). Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology), followed by chemiluminescence detection.

Measurement of caspase-3/7 activity
Resistant clones were seeded on 96-well plates at 1 × 104 or 5 × 104 cells/well, respectively, and were treated with obinutuzumab and/or doxorubicin at the indicated concentrations for 48 hours. Caspase-3/7 activity was measured using a Caspase-Glo 3/7 Assay Kit (Promega Corporation) in accordance with the manufacturer’s protocol. The ratio of caspase-3/7 activity was calculated as follows: (luminescence of treatment well – luminescence of blank well)/luminescence of non-treatment well – luminescence of blank well)

Measurement of caspase-3/7 activity
Resistant clones were seeded on 96-well plates at 1 × 104 or 5 × 104 cells/well, respectively, and were treated with obinutuzumab and/or doxorubicin at the indicated concentrations for 48 hours. Caspase-3/7 activity was measured using a Caspase-Glo 3/7 Assay Kit (Promega Corporation) in accordance with the manufacturer’s protocol. The ratio of caspase-3/7 activity was calculated as follows: (luminescence of treatment well – luminescence of blank well)/luminescence of non-treatment well – luminescence of blank well).
Obinutuzumab with Chemotherapy in OBI-resistant Cells

Cell-cycle analysis
Cells were seeded at 2 × 10^5 cells/well and were treated with obinutuzumab and/or prednisolone at the indicated concentrations for 48 hours. Cells were labeled with DAPI, and the fluorescence was measured with an Advanced Image Cytometer NucleoCounter NC-3000 (Chemometec) in accordance with the manufacturer’s protocol. Data were analyzed using FlowJo v7.6.5 software.

**In vivo anticancer assay**
Mice were inoculated subcutaneously in the right flank with 5 × 10^6 cells/mouse with Matrigel Matrix (Corning Inc.). Tumor-bearing mice were randomly allocated to the control group or treatment groups. Obinutuzumab (30 mg/kg), control human IgG (HulgG, 30 mg/kg), doxorubicin (2.5 mg/kg), or its vehicle was administered intravenously. Prednisolone (2 mg/kg) or its vehicle was administered orally. All drugs were treated on the same schedule as in clinical practice. Tumor volume (TV) and body weight were measured twice a week. TV was estimated as follows: TV = ab^2/2, where a and b are tumor length and width, respectively.

**Statistical analysis**
The statistical significance in in vitro experiments was analyzed with the Tukey honestly significant difference (HSD) test. Two-way ANOVA was used prior to Tukey HSD test for comparison of dose–response curve assay. P < 0.05 indicates a significant difference. In in vivo experiments, statistical significance was analyzed by Wilcoxon test. The significant P values were adjusted for multiple comparisons by the Holm method. All statistical analyses were performed with JMP software (SAS Institute).

**Results**

**Establishment of obinutuzumab-induced direct-cell-death–resistant clones**
Cells resistant to obinutuzumab-induced direct cell death were established from SU-DHL-4 cells as described in Materials and Methods. Among the obtained clones, 17 clones expressing CD20 had a 100-fold higher IC_{50} for obinutuzumab—with respect to obinutuzumab-induced direct cell death—than the parental cells. These were established as resistant and named SU-DHL-4-OR clones (Fig. 1A; Supplementary Fig. S1).

**Combined effect of obinutuzumab plus chemotherapeutic agent in obinutuzumab-induced direct-cell-death–resistant clones**
Next, to investigate the possibility of retreatment after acquisition of resistance to obinutuzumab, we assessed whether the sensitivity to obinutuzumab in these resistant clones changed when it was combined with chemotherapeutic agents used in the treatment of patients with follicular lymphoma. The combination effect was estimated using an interaction index based on the Bliss independence model (14). This index identifies combinations of agents with a supra-additive effect, whose efficacies when combined were higher than those estimated from the efficacies of the individual agents. The dose of each

![Figure 1](image-url)

**Figure 1.** In vitro combination effect of obinutuzumab plus each chemotherapeutic agent on resistant clones. A, Each of the cells was treated with various concentrations of obinutuzumab for 4 days, and the obinutuzumab IC_{50} values for SU-DHL-4 cells and each resistant clone were calculated. # indicates clones for which the IC_{50} was >200 μg/mL. B, Each clone was treated with obinutuzumab (1 μg/mL) and/or doxorubicin (10 nmol/L), prednisolone (1 μmol/L), 4-OOH-CY (100 nmol/L), vincristine (1 nmol/L), or bendamustine (20 μmol/L) for 4 days. The combination effect was estimated by an interaction index based on the Bliss independence model. An index value of <0, 0, or >0 indicates a supra-additive, additive, or subadditive effect, respectively. *, P < 0.05 versus the additive assumption calculated from each single agent by Student t test.
chemotherapeutic agent was set at the concentration that induced an approximately 30% growth inhibition effect in SU-DHL-4 cells (Supplementary Fig. S2), except for prednisolone which was set at a dose of 1 μmol/L with reference to its clinical mean Cmax (~1.79 μmol/L) because it required a higher concentration to show a 30% growth inhibition effect in SU-DHL-4 cells (16–20). As a result, for each clone, there were several combinations of obinutuzumab plus chemotherapeutic agent that showed supra-additive effects, and the agent producing the highest combination effect varied among clones (Fig. 1B). Remarkably, obinutuzumab plus doxorubicin exerted an additive effect or more in all of the resistant clones.

**Caspase-dependent mechanism enhanced the growth inhibition effect of obinutuzumab in combination with doxorubicin**

The growth inhibition effect of obinutuzumab combined with doxorubicin was verified at various concentrations using clones SU-DHL-4-OR-1A2 (1A2) and SU-DHL-4-OR-1C4 (1C4), in which the combination effects were stronger than in other clones. In these clones, cotreatment with doxorubicin enhanced the growth inhibition effect of obinutuzumab (Fig. 2A). In contrast, the combination only slightly enhanced the inhibitory effect in SU-DHL-4 cells (Supplementary Fig. S3A).

A recent report suggested that a Ca^{2+} signaling-mediated endoplasmic reticulum (ER) stress pathway is involved in obinutuzumab-induced direct cell death in SU-DHL-4 cells (21). Therefore, we first examined the effect of obinutuzumab plus doxorubicin on the induction of ER stress in clone 1A2. Treatment with either as a single agent increased the level of phospho-IKE1, an ER stress sensor protein, and the combination further increased this level (Fig. 2B). Furthermore, the combination increased levels of phospho-JNK and phospho-Bcl2 (Fig. 2C), which are downstream molecules of IRE1 (22).

Phosphorylation of Bcl2 by JNK is known to inhibit its antiapoptotic function and to induce apoptosis (22). Therefore, we next assessed the effect of this combination on the induction of apoptosis by detecting DNA fragmentation using TUNEL assay and caspase-3/7 activity. This combination increased DNA fragmentation more than each single agent, while obinutuzumab and doxorubicin alone each increased caspase-3/7 activity, their combination increased it significantly more (Fig. 2E). Furthermore, the combination enhanced DNA fragmentation and growth inhibition effect were suppressed by Z-VAD-FMK, a pan-caspase inhibitor (Fig. 2D and F). On the other hand, the growth inhibition effect of obinutuzumab alone was not suppressed by Z-VAD-FMK, either in resistant clones (Fig. 2F) or in parental cells (Supplementary Fig. S3C), although caspase-3/7 activity increased, and was suppressed by Z-VAD-FMK (Supplementary Fig. S3D).

**Enhanced induction of apoptosis and G1 arrest with the combination of obinutuzumab plus prednisolone**

Next, we investigated the effect of combining obinutuzumab with prednisolone, which was the second most common agent to show a strongest combination effect among clones (Fig. 1B). In clones SU-DHL-4-OR-2B3 (2B3) and SU-DHL-4-OR-6B3 (6B3), in which obinutuzumab plus doxorubicin showed the weakest combination effect, obinutuzumab plus prednisolone resulted in a stronger combination effect than in all other resistant clones except clone 1A2 (Fig. 1B). We confirmed that prednisolone increased obinutuzumab's growth inhibition effect at various concentrations in both clones (Fig. 3A), and also in the parental SU-DHL-4 cells (Supplementary Fig. S3B). Next, we examined the underlying processes behind this combination's enhancement of the growth inhibition effect. First, we evaluated the effect of cotreatment with prednisolone on cell death induced by obinutuzumab in clones 2B3 and 6B3. While prednisolone alone did not induce DNA fragmentation, the combination increased it more than obinutuzumab alone (Fig. 3B). However, unlike with doxorubicin, cotreatment with Z-VAD-FMK did not suppress the enhanced growth inhibition effect of obinutuzumab plus prednisolone (Supplementary Fig. S4).

Glucocorticoids such as prednisolone induce growth inhibition through G1 arrest (23). The percentage of cells in the G0–G1 phase was increased by the prednisolone treatment and was significantly more increased by the addition of obinutuzumab, whereas obinutuzumab alone failed to induce G1 arrest in these resistant clones (Fig. 3C). We also examined the protein levels of cell-cycle regulators. This combination decreased the protein level of Skp2, which is a substrate recruiting component of the ubiquitin–proteasome system that targets cell-cycle control elements (Fig. 3D). Furthermore, p27, a cyclin-dependent kinase inhibitor and a substrate of Skp2, was increased and the level of phosphorylated Rb was decreased by this combination (Fig. 3D).

In clone SU-DHL-4-OR-7C2 (7C2), in which the combination of obinutuzumab plus prednisolone showed a supra-additive effect but to a lesser extent than that in clones 2B3 and 6B3 (Fig. 1B), this combination increased DNA fragmentation more than each single agent, without enhancing G0–G1 arrest (Supplementary Fig. S5). Furthermore, in clone SU-DHL-4-OR-1D6 (1D6), in which this combination showed a subadditive effect (Fig. 1B), although the combination slightly decreased the protein level of Skp2, it did not modulate downstream molecules or enhance G0–G1 arrest more than prednisolone, and did not enhance DNA fragmentation more than obinutuzumab (Supplementary Fig. S5).

**In vivo antitumor effect of the combination of obinutuzumab and chemotherapeutic agents in mouse xenograft model**

We evaluated the antitumor efficacy of the combination of obinutuzumab plus doxorubicin or prednisolone in mouse xenograft models. To evaluate the effect of obinutuzumab-induced direct cell death, as opposed to ADCC, ADCP, or CDC, we used NOG mice which lack natural killer (NK) cells, have dysfunctional macrophages, and display reduced complement activity. Obinutuzumab alone significantly decreased the tumor volume compared with the control group in SU-DHL-4 xenograft model (Supplementary Fig. S6), but not in the models with resistant clones (Fig. 4A and B). In the A2 xenograft model, doxorubicin significantly decreased the tumor volume compared with control group, and the combination of obinutuzumab plus doxorubicin significantly enhanced the antitumor effect compared with each single agent on day 22 (Fig. 4A). In the 2B3 xenograft model, although neither obinutuzumab nor prednisolone decreased the tumor volume, the combination did show a significant antitumor effect compared with each single agent on day 22 (Fig. 4B). Furthermore, in both models, although tumors in combination groups regrew 11–15 days after initial treatment, retreatment using the combination still showed antitumor effects (Fig. 4).

**Discussion**

Our data demonstrate the efficacy of obinutuzumab against CD20-positive NHL cells resistant to obinutuzumab-induced direct cell death. Remarkably, our data showed that for all resistant cells, there
were several combinations of obinutuzumab plus chemotherapeutic agents used in the treatment of patients with follicular lymphoma that exerted a supra-additive effect. Our results indicated that doxorubicin enhances the growth inhibition effect of obinutuzumab through a caspase-dependent mechanism in the resistant cells. Our results also suggest that the combination of obinutuzumab plus prednisolone enhances the growth inhibition effect by increasing cell death and further enhances it by inducing G0–G1 arrest in the resistant cells.

Figure 2.
Enhanced growth inhibition effect of obinutuzumab in combination with doxorubicin through caspase-dependent apoptosis induction in resistant clones. A, Cell growth inhibition by obinutuzumab (OBI) alone or the combination of OBI plus doxorubicin (DXR) was examined after 4 days of treatment in clone 1A2 and clone 1C4. Data points represent mean + SD (n = 3). a, P < 0.05 versus DXR 0 nmol/L; b, P < 0.05 versus DXR 2.5 nmol/L; c, P < 0.05 versus DXR 5 nmol/L by Tukey HSD test with two-way ANOVA. Immunoblots evaluating the phosphorylation levels of IRE1 (B), and JNK and Bcl2 (C) of clone 1A2. Cells were treated with OBI (1 μg/mL) and/or DXR (10 nmol/L) for the indicated times. β-Actin was used as a loading control. D, DNA fragmentation was evaluated by TUNEL assay. Clone 1A2 and clone 1C4 were treated with OBI (1 μg/mL), DXR (10 nmol/L), and/or Z-VAD-FMK (40 μmol/L) for 3 days. E, Caspase 3/7 activity was measured after 48 hours treatment with OBI (1 μg/mL) and/or DXR (10 nmol/L). Each bar represents mean ± SD (n = 3). a, P < 0.05 versus nontreatment; b, P < 0.05 versus treatment with OBI alone; c, P < 0.05 versus treatment with DXR alone by Tukey HSD test. F, Cell growth inhibition by OBI, DXR, and/or 40 μmol/L of Z-VAD-FMK (Z-VAD) was examined 4 days after treatment in clone 1A2 and clone 1C4. Data points represent mean ± SD (n = 3).
Furthermore, resistant cells retained their response to obinutuzumab-induced direct cell death in vivo, and the combination of obinutuzumab plus doxorubicin or prednisolone also significantly enhanced antitumor effect in the resistant clone xenograft models. Moreover, these combination effects were also confirmed in obinutuzumab-refractory cells (Supplementary Fig. S7).

Our findings suggest that obinutuzumab induces direct cell death independent of caspase in SU-DHL-4 cells (Supplementary Fig. S3), and according to a study by Latour and colleagues (21), obinutuzumab-induced direct cell death occurs via the Ca^{2+} signaling-mediated ER stress pathway in SU-DHL-4 cells. Furthermore, our data indicate that the combination effect of obinutuzumab plus doxorubicin in resistant clones involves caspase-dependent mechanism, likely through the activation of IRE1–JNK–Bcl2 signaling, which is known to be one of the main contributors to ER stress–induced apoptosis in resistant clones (Fig. 2; refs. 22, 24). ER stress kills cancer cells by inducing either caspase-dependent apoptosis or caspase-independent cell death, such as necroptosis, and either of these cell death mechanisms can switch to the other (25–27). Together, our results suggest that, in resistant clones, the mechanism of obinutuzumab-induced cell death via ER stress changes based on the treatment: obinutuzumab alone causes caspase-independent cell death, whereas the combination with doxorubicin causes caspase-dependent apoptosis.

While doxorubicin decreases the activity of sarcoplasmic reticulum Ca^{2+}-ATPase, an ATP pump that restores luminal ER calcium levels, and activates ER stress (28, 29), the combination effect of obinutuzumab plus doxorubicin was slight in SU-DHL-4 cells (Supplementary Fig. S3A). This could be because each compound works in different phase of cell cycle. Doxorubicin exerts its cytotoxic effect in the G2–M phase, but obinutuzumab induces G0–G1 arrest in SU-DHL-4 cells (Supplementary Fig. S3E). We investigated the combination of obinutuzumab and doxorubicin against obinutuzumab-sensitive cells

Figure 3.
Enhanced combination effect of obinutuzumab plus prednisolone on cell growth inhibition, cell death induction, and G0–G1 arrest in resistant clones. A, Cell growth inhibition by obinutuzumab (OBI) alone or the combination of OBI plus prednisolone (PSL) was examined after 4 days of treatment in each clone. Data points represent mean ± SD (n = 3). *, P < 0.05 versus PSL 0 μmol/L; #, P < 0.05 versus PSL 0.04 μmol/L; $, P < 0.05 versus PSL 0.04 μmol/L by Tukey HSD test with two-way ANOVA. B, DNA fragmentation was evaluated by TUNEL assay. Clone 2B3 and clone 6B3 were treated with OBI (1 μg/mL) and/or PSL (1 μmol/L) for 3 days. C, Cell cycle was analyzed after 48 hours of treatment with OBI (1 μg/mL) and/or PSL (1 μmol/L) in each clone. Each bar represents mean ± SD (n = 3, independent assay). Statistical analysis was performed on the percentage of cells in the G0–G1 phase. *, P < 0.05 versus non-treatment control; #, P < 0.05 versus OBI alone; $, P < 0.05 versus PSL alone by Tukey HSD test. D, Immunoblots evaluating the protein levels of cell-cycle regulators. Each clone was treated with OBI (1 μg/mL) and/or PSL (1 μmol/L) for 48 hours. β-Actin was used as a loading control.
Obinutuzumab with Chemotherapy in OBI-resistant Cells

Figure 4.
The in vivo antitumor effect of obinutuzumab plus chemotherapeutic agents in obinutuzumab-induced direct-cell-death-resistant cells transplanted mouse xenograft model. A, In vivo antitumor effect of combination of obinutuzumab 30 mg/kg (OBI) plus doxorubicin 2.5 mg/kg in NOG mice bearing clone 1A2 (n = 6/group). Control HuIgG and OBI were intravenously treated on day 1, 8, 15, and 22. Vehicle and DXR were intravenously treated on day 1 and 22. a, significant versus control group; b, significant versus obinutuzumab group; c, significant versus doxorubicin group by Wilcoxon test and the Holm method on day 22. B, In vivo antitumor effect of combination of OBI 30 mg/kg plus prednisolone (PSL) 2 mg/kg in NOG mice bearing clone 2B3 (n = 6/group). Control HuIgG and OBI were intravenously treated on day 1, 8, 15, and 22. Vehicle and PSL were orally treated on day 1–5, and day 22–26. a, significant versus control group; b, significant versus obinutuzumab group; c, significant versus prednisolone group by Wilcoxon test and the Holm method on day 22.

Using only one in vitro model, and further study is needed to fully clarify its effect.

Unlike in the doxorubicin combination, with prednisolone the caspase inhibitor failed to suppress the enhanced growth inhibition effect in resistant clones (Supplementary Fig. S4) despite the promoted cell death (Fig. 3B); this suggests that prednisolone strengthens obinutuzumab-induced caspase-independent cell death. The resistant clones subject to stronger supra-additive effects exhibited both enhanced cell death and G0–G1 arrest, while clones subject to weaker supra-additive effects only exhibited enhanced cell death (Fig. 3; Supplementary Fig. S5). This suggests that obinutuzumab plus prednisolone enhances growth inhibition primarily by inducing caspase-independent cell death, and that additional enhancement of G0–G1 arrest further increases it.

Because NOG mice lack NK cells, have a less efficient complement system, and have dysfunctional macrophages (30), obinutuzumab’s antitumor effect in these mice transplanted with parental SU-DHL-4 cells seems to indicate the efficacy of direct cell death (Supplementary Fig. S6). This effect was diminished in mice transplanted with resistant clones (Fig. 4). These results indicate that obinutuzumab-induced direct cell death at least partially involves an in vivo antitumor effect and in vivo resistance, which suggests that direct cell death is integral to obinutuzumab’s in vivo mechanism of action. Furthermore, because the combination of obinutuzumab plus doxorubicin or prednisolone showed a significant antitumor effect not only as an initial treatment but also as post-regression treatment, this result contributes to the possibility of an effective clinical treatment option.

Rituximab is another anti-CD20 antibody commonly used in the treatment of follicular lymphoma. We investigated the combination effect of rituximab plus chemotherapeutic agents in obinutuzumab-resistant clones. The combination of rituximab plus doxorubicin or prednisolone also significantly inhibited growth compared with each single agent in clones 1A2 and 2B3, in which the combination of obinutuzumab plus doxorubicin or prednisolone showed a supra-additive effect, respectively (Supplementary Fig. S8). These results suggest the possibility that not only the combination of obinutuzumab plus chemotherapeutic agent but also that of rituximab plus chemotherapeutic agent is useful after acquisition of obinutuzumab resistance. However, we think the important takeaway from this non-clinical study is the possibility that obinutuzumab can still be an effective treatment option when combined with a chemotherapeutic agent, even after obinutuzumab treatment.

In this study, we focused on the efficacy of obinutuzumab against CD20-positive NHL cells resistant or refractory to obinutuzumab-induced direct cell death. The loss or reduction of CD20 expression is known to be one of the mechanisms of resistance to the anti-CD20 mAb rituximab (31, 32). Obinutuzumab is more effective than rituximab at depleting the CD20-low B-cell population, and its inability to interact with FcRRIb is thought to contribute to the lower CD20 internalization (33, 34); however, the loss or reduction of CD20 expression could still be an obinutuzumab-resistant mechanism. At the same time, ADCC and ADCP are also important mechanisms of action for obinutuzumab. Further study is required to investigate how to overcome resistance to obinutuzumab caused by either the reduction of CD20 expression or by resistance to ADCC/ADCP. Furthermore, we could not identify any genetic resistant mechanism in this study. The mechanism of action (MoA) of obinutuzumab-induced direct cell death in SU-DHL-4 is still unclear and is just beginning to understand that it is different from the more well-known mechanism (21, 35, 36), which makes the analysis of the resistance mechanisms more difficult. In the future, the elucidation of the MoA of obinutuzumab-induced direct cell death along with technological breakthroughs can eventually unravel this resistant mechanism, and it is believed that this will lead to the identification of biomarkers in patients who would benefit from obinutuzumab retreatment.

The results of this preclinical study suggests that, combined with chemotherapy, obinutuzumab retreatment may be a possible option in the clinical treatment of CD20-positive obinutuzumab-resistant lymphoma. Further investigation is needed to confirm the clinical efficacy of obinutuzumab retreatment. Furthermore, because the chemotherapeutic agent showing the highest combination effect with obinutuzumab differed for each resistant clone, biomarkers to identify the best combination for each patient need to be elucidated.

Authors’ Disclosures
T. Fujimura reports grants from Nippon Shinyaku Co., Ltd. and personal fees from Chugai Pharmaceutical Co., Ltd. during the conduct of the study; in addition, T. Fujimura has a patent for JP2019-184149 pending. Y. Yamashita-Kashima reports...
grants from Nippon Shinyaku Co., Ltd. and personal fees from Chugai Pharmaceutical Co., Ltd. during the conduct of the study; in addition, Y. Yoshimura-Kashima has a patent for JP2019-184149 pending. N. Kawasaki reports grants from Nippon Shinyaku Co., Ltd. and personal fees from Chugai Pharmaceutical Co., Ltd. during the conduct of the study; in addition, Y. Yoshimura has a patent for JP2019-184149 pending.

Acknowledgments

The authors thank Yoshiaki Ishiki, Kazushige Mori, Kaori Fujimoto-Ouchi, and Osama Kondoh (Chugai Pharmaceutical Co., Ltd.) for their helpful advice.

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 October 8, 2020; revised March 3, 2021; accepted March 31, 2021; published first April 13, 2021.

References


Molecular Cancer Therapeutics

Obinutuzumab in Combination with Chemotherapy Enhances Direct Cell Death in CD20-Positive Obinutuzumab-resistant Non-Hodgkin Lymphoma Cells


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-20-0864

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2021/04/02/1535-7163.MCT-20-0864.DC1

Cited articles
This article cites 36 articles, 10 of which you can access for free at:
http://mct.aacrjournals.org/content/20/6/1133.full#ref-list-1

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, use this link http://mct.aacrjournals.org/content/20/6/1133.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.