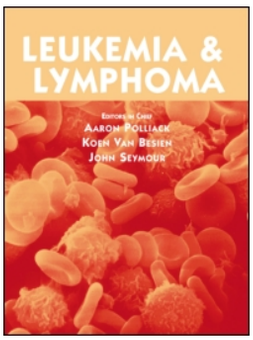

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Anti-tumor efficacy study of the Bruton's tyrosine kinase (BTK) inhibitor, ONO/GS-4059, in combination with the glycoengineered type II anti-CD20 monoclonal antibody obinutuzumab (GA101) demonstrates superior in vivo efficacy compared to ONO/GS-4059 in combination with rituximab

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Anti-tumor efficacy study of the Bruton's tyrosine kinase (BTK) inhibitor, ONO/GS-4059, in combination with the glycoengineered type II anti-CD20 monoclonal antibody obinutuzumab (GA101) demonstrates superior *in vivo* efficacy compared to ONO/GS-4059 in combination with rituximab

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ABSTRACT

The activated B-cell diffuse large B-cell-like lymphoma (ABC-DLBCL) correlates with poor prognosis. The B-cell receptor signaling pathway is known to be dysregulated in NHL/CLL and given BTK is a downstream mediator of BCR signaling, BTK constitutes an interesting and obvious therapeutic target. Given the high potency and selectivity of the BTK inhibitor, ONO/GS-4059, it was hypothesized that, the anti-tumor activity of ONO/GS-4059 could be further enhanced by combining it with the anti-CD20 Abs, rituximab (RTX) or obinutuzumab (GA101). ONO/GS-4059 combined with GA101 or RTX was significantly better than the respective monotherapy with tumor growth inhibition (TGI) of 90% for the GA101 combination and 86% for the RTX combination. In contrast, ibrutinib (PCI-32765) combined with RTX did not result in improved efficacy compared with respective monotherapy. Taken together these data indicate that the combination of ONO/GS-4059 with rituximab and particularly obinutuzumab may be an effective treatment for ABC-DLBCL.

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ABC-DLBCL; Bruton's tyrosine kinase; obinutuzumab; rituximab

Introduction

Bruton's tyrosine kinase (BTK) is a member of TEC family kinase [1] that is broadly expressed in cells of several hematopoietic lineages, but not in T-cells, NK cells and plasma cells.[2] Genetic evidence supports a critical role for BTK in multiple hematopoietic signaling pathways including the B-cell receptor (BCR), several cytokine receptors and a potential novel role in heterotrimeric G-protein-associated receptors related to B-cell migration and adhesion, such as the CXCR4 and CXCR5 chemokine receptors and adhesion molecules, integrins.[3,4] Mutations within BTK result in arrested B-cell development leading to severe X-linked agammaglobulinemia (XLA).[5] These observations have indicated that BTK is a functional as opposed to a genetically-defined therapeutic target and has led to the development of many small molecule BTK inhibitors for the treatment of B-cell malignancies [6] and autoimmune disorders.[7]

BTK has an interesting cysteine residue at the position as Cys481 in ATP-binding pocket, which has been explored in the design of irreversible inhibitors.[8] Only nine other kinases in human genome have similarly placed cysteine residue. These nine kinases include three EGFR family kinases (EGFR, ErbB2 and ErbB4), four Tec family kinases (BMX, ITK, TEC and TXK), one Src family member (BLK) and JAK3.[9] The first-in-class BTK inhibitor, ibrutinib (Imbruvica, Pharmacyclics, Sunnyvale, CA) has shown remarkable efficacy in some B-cell malignancies and has now been approved for the treatment of CLL, MCL and Waldenström's macroglobulinemia (WM).[10–12] The kinome of ibrutinib is broad and includes low nanomolar inhibition of several other key kinases such as EGFR, JAK3, ErbB2 and ITK.[13] Especially inhibition of ITK may mitigate the antibody-dependent cellular cytotoxicity (ADCC) of NK cells when combined with anti-CD20 monoclonal antibodies.[14,15] ITK expressed in NK cells may have an important regulatory role in NK cell-mediated cytotoxicity via activation of PLCγ and MAPKs.[16]

ONO-4059 (now known as ONO/GS-4059) is a covalent type inhibitor and has greater selectivity for BTK (*In vitro* IC50, 2 nmol/L) than Lck, Fyn, LynA and ITK.

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ONO/GS-4059 only inhibits anti-IgM-induced B-cell activation in a concentration-dependent manner but not inhibit anti-CD3/CD28-induced activation of T-lymphocytes from human PBMCs.[6,17] ONO/GS-4059 inhibits cell proliferation in some malignant B-cell lines but also induces classical apoptosis at nanomolar concentrations in the activated-B cell (ABC) DLBCL cell line, TMD8.[18] Despite the efficacy of ONO/GS-4059 in DLBCL, the low response rates have been observed in relapsed/refractory non-GCB DLBCL.[19] Similarly in a phase 1/2 study of ibrutinib, responses were seen in 37% of non-GCB DLBCL.[20] To improve outcomes with targeted therapies for ABC-DLBCL, rational mechanistic approaches are important. Preferable combination responses with ibrutinib were observed with chemotherapeutic agents in ABC-DLBCL cell line.[21] Given the need to treat and overcome disease resistance, selective BTK inhibitor, ONO/GS-4059, combined with anti-CD20 antibodies is a novel mechanistic approach to the treatment of ABC-DLBCL. This study evaluates the effective of ONO/GS-4059 and ITK inhibitor on NK cell-mediated ADCC and indicates that the combination of ONO/GS-4059 with RTX, and particularly the glycoengineered Type II CD20 antibody GA101 (obinutuzumab) [22] may be an effective treatment for ABC-DLBCL.

Materials and methods

Reagents and cell lines

GA101 and RTX were supplied by Roche Glycart AG (Schlieren, Switzerland). ONO/GS-4059, ONO-7790500 and PCI-32765 were obtained from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). CGI1746 was from Selleckchem (Houston, TX). TMD8 cell line [23] was provided by Dr. Shuji Tohda, Tokyo Medical and Dental University. SU-DHL-4 cell line was purchased from American Type Culture Collection. These cells were kept in RPMI1640 (Life Technologies, Japan) supplemented with 10% (v/v) heat-inactivated FBS and penicillin-streptomycin.

Preparation of NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare, Japan) from adult healthy donors. The use of the donated blood for the specific experiments was approved by the Ethical guideline "Guidance for Human Tissue Experiments" established by Ono Pharmaceutical Co., Ltd. NK cells were obtained from PBMCs using Human NK Cell Isolation Kit (Miltenyi Biotec, Japan) in accordance with the manufacture's protocol.

Immunoprecipitation

NK cells were stimulated with cross-linking anti-CD16 and goat anti-mouse IgG.[16] Lysis buffer was added to the cells (Cell Signaling Technology, CST, Japan) including Protease Inhibitor Cocktail (Sigma-Aldrich, Japan). The cell lysate was immunoprecipitated with mouse anti-Itk antibody (2F12, CST) overnight at 4 °C on a rotator and Dynabeads Protein G system (Life Technologies). Western blot analysis was performed by using primary antibodies, anti-Btk antibody (C82B8, CST), anti-phospho-Btk (Tyr223) antibody (Novus Biologicals, Littleton, CO), anti-Itk antibody (Y401) (Abcam, Japan), and anti-phospho-Tyr antibody (4G10) (Merck Millipore, Japan).

Cytotoxicity assay

SU-DHL-4 cells were used as target cells and NK cells were used as effector cells for ADCC assay.[14,15] SU-DHL-4 were incubated with 0.2 μg/mL of RTX or Human IgG1 (Sigma-Aldrich) for 30 min and subsequently added BTK or ITK inhibitor. NK cells were incubated with or without BTK or ITK inhibitor for 30 min, followed by addition of the pretreated SU-DHL-4 at the E:T ratio of 2.5:1, 5:1 or 10:1 and cultured for 4 h. The supernatant was collected and the cytotoxicity was evaluated by a CytoTox96 nonradioactive cytotoxicity assay (Promega, Madison, WI).[24] The % lysis value was calculated from the measured absorbance obtained for each sample using the formula shown below and was used as an index of cytotoxicity.

$$\%\ \text{lysis} = (\text{Abs. [experimental]} - \text{Abs. [BG : No cells]}) \times 100 / (\text{Abs. [Max : target cells]} - \text{Abs. [Max : BG]})$$

The inhibition rate was calculated from the % lysis value using the following formula:

$$\%\ \text{inhibition} = 100 - (\%\ \text{lysis [experimental]} \times 100 / \%\ \text{lysis [without inhibitor]})$$

Animals and TMD8 xenograft model

Six-week-old female C.B17/Icr-scid/scid Jcl mice (CLEA Japan, Inc.) were used. The mice were allowed free access to pelleted CRF-1 diet (Oriental Yeast Co., Ltd., Japan) and tap water. The present study was conducted in compliance with the "Guidance for Animal

Experiments," the "Ethical Standards for Experiments using Human Tissues," and the "Standards for Safety Management of Pathogens" established by Ono Pharmaceutical Co., Ltd.

TMD8 cells were collected in a centrifugal tube and re-suspended in growth medium on ice to obtain a 2×10^8 cells/mL suspension. The equal volume of BD Matrigel GFR (BD Biosciences, Japan) was combined to the cell suspension. TMD8 (1×10^7 cells per animal, 0.1 mL volume of the suspension) were subcutaneously injected in the right flank of the mice under pentobarbital anesthesia, using a 25-guage needle (Terumo Corp, Japan). Tumor volume was assessed by caliper. TGI rate was calculated as previously described.[22]

Statistics

Dunnett tests were performed between the vehicle group and each of test substance groups. T-tests were performed between the RTX monotherapy and combination groups, or between the GA101 monotherapy and combination groups. All statistical tests were two-sided with a significance level of 5%.

Results

Active BTK but inactive ITK was observed in NK cells

We evaluated whether BTK and ITK are expressed in human NK cells. Figure 1(A) shows the Western blot analysis of NK cells in peripheral blood from normal healthy volunteers. Bands with mobility equivalent to the molecular weights of BTK (77kDa) and ITK (72kDa) were observed.[2] Despite the presence of protein levels of BTK and ITK in NK cells, the protein expression level of ITK was quite low in NK cells compared with BTK. However, immunoprecipitation using anti-ITK antibody (2F12) clearly demonstrated the presence of ITK in NK cells. Figure 1(B) shows the results of Western blotting for BTK and ITK phosphorylation in NK cells, stimulated with cross-linking anti-CD16 and secondary antibodies. The phosphorylation signal at the site of BTK autophosphorylation (Y223) was observed in both unstimulated and stimulated NK cells, and stimulation did not cause any changes (lanes 1 and 2). The phosphorylation signals were completely inhibited after treatment with ONO/GS-4059 or PCI-32765 (lanes 4 and 5). In contrast, an ITK phosphorylation signal was not observed in both unstimulated and stimulated NK cells (lanes 1 to 5).

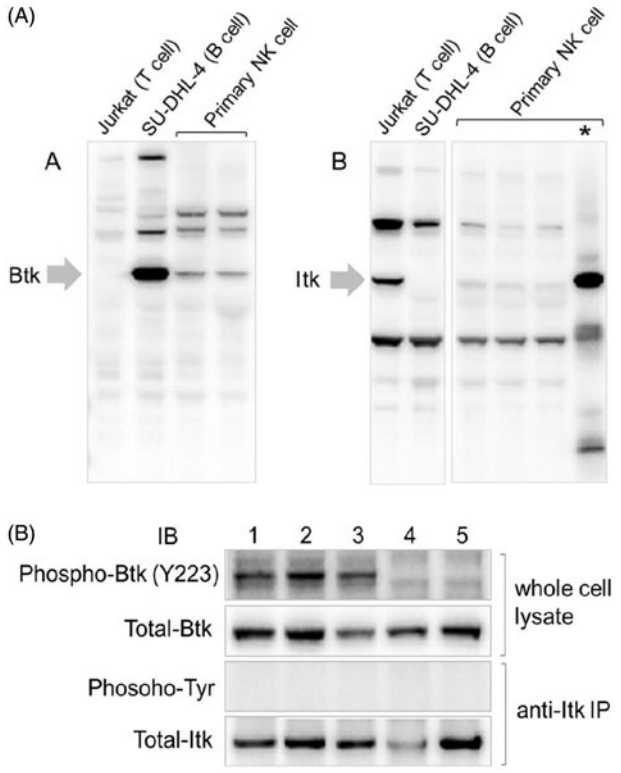

Figure 1. Active BTK but inactive ITK was observed in NK cells. (A) BTK and ITK expression in NK cells in peripheral blood from normal healthy volunteers: Western blotting was performed on lysates of NK cells in peripheral blood from normal healthy volunteers using an anti-Btk antibody (A) or anti-Itk antibody (B). The figure shows the expression of Btk from 2 donors and Itk from 3 donors. For Itk, Western blotting was also performed on a sample of NK cell lysate immunoprecipitated with an anti-Itk antibody (marked as asterisk). Western blotting was performed on lysates of Jurkat T cell and SU-DHL-4 B cell lines as a positive control for Itk and Btk, respectively. The bands with mobility equivalent to the molecular weights of Btk (77kDa) and Itk (72kDa) are marked with arrows. (B) BTK and ITK phosphorylation in NK cells in peripheral blood from normal healthy volunteers: Vehicle (lanes 1 and 2), 5 μmol/L of ONO-7790500, specific ITK inhibitor (lane 3), 1 μmol/L of ONO/GS-4059 (lane 4), and 1 μmol/L of PCI-32765 (lane 5) were added to NK cells in peripheral blood from normal healthy volunteers, and the mixtures were incubated for 20 min at 37 °C. Medium (lane 1) or an anti-CD16 antibody (lanes 2 to 5) was added, and the mixtures were incubated for another 10 minutes. Medium (lane 1) or an anti-mouse Ig antibody (lanes 2 to 5) was added, mixed, and incubated for 1 min. Cell lysates were prepared, and Western blotting was performed to detect phosphorylated Btk (Phospho-Btk (Y223)) and total Btk protein (Total-Btk). Cell lysates were immunoprecipitated using an anti-Itk antibody, and Western blotting was performed to detect phosphorylated tyrosine (Phospho-Tyr) and total Itk protein (Total-Itk).

Effect of BTK and ITK inhibitors on antibody-dependent NK cell-mediated cytotoxicity

Figure 2(A) shows the NK cell-mediated cytotoxicity in RTX-treated SU-DHL-4 cells that was measured by colorimetrically quantifying LDH in culture supernatant.

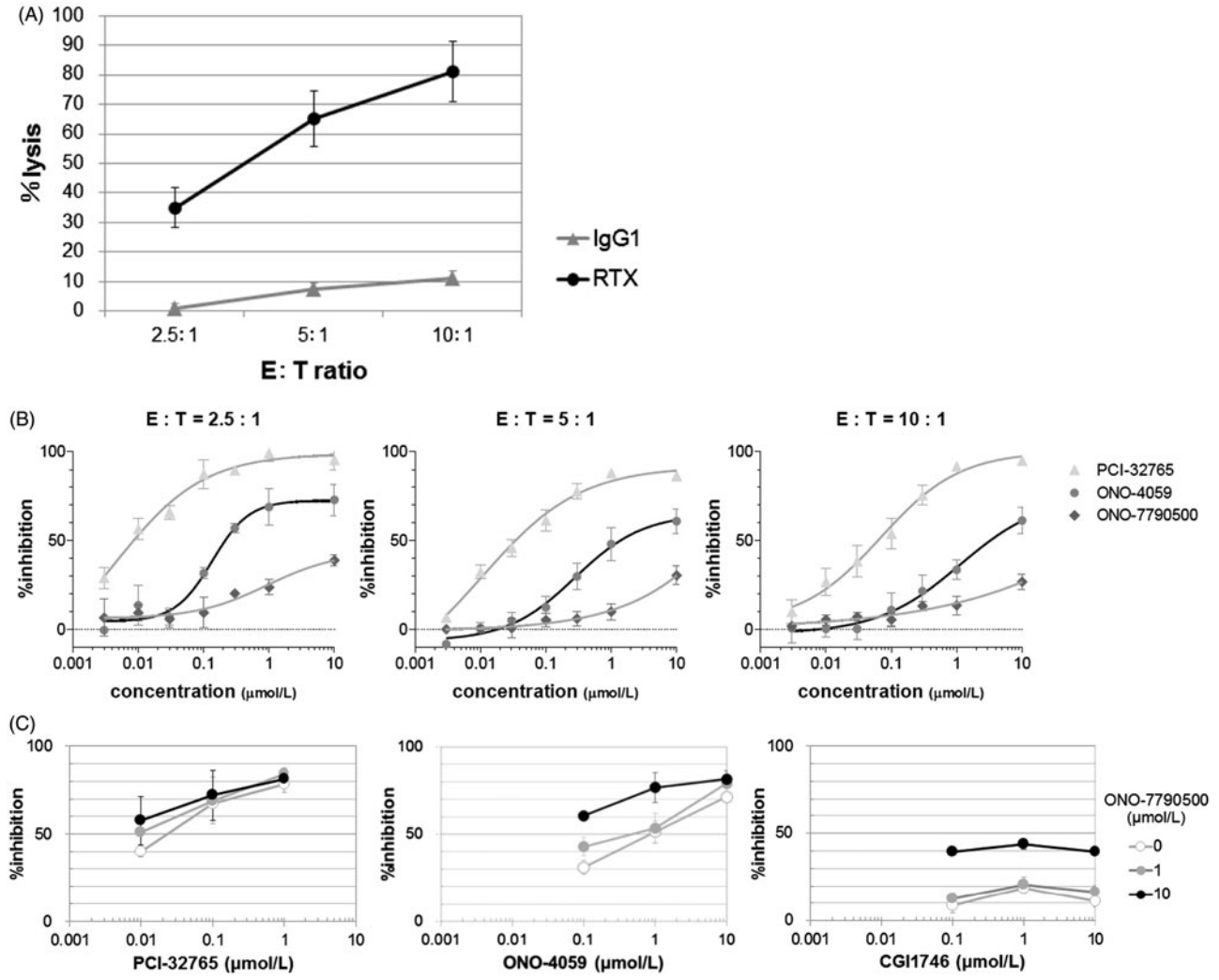

Figure 2. Antibody-dependent cytotoxicity of NK cells in peripheral blood from normal healthy volunteers. (A) NK cells in peripheral blood from normal healthy volunteers (E) were mixed with RTX- or IgG1-treated SU-DHL-4 cells (T) at ratios of 2.5:1, 5:1, and 10:1, and cultured for 4 h. LDH in the supernatant was quantified colorimetrically, and the %lysis value was calculated. The figure shows the mean value and standard error in 2 NK cell donors when the E:T ratio was 2.5:1, and those in 3 donors when E:T ratios were 5:1 and 10:1. (B) Effects of BTK and ITK inhibitors on the antibody-dependent cytotoxicity of NK cells in peripheral blood from normal healthy volunteers: Vehicle, PCI-32765, ONO/GS-4059, or ONO-7790500 was added to NK cells in peripheral blood from normal healthy volunteers (E), and cultured for 30 min. As with the NK cells, vehicle, PCI-32765, ONO/GS-4059, or ONO-7790500 was added to RTX-treated SU-DHL-4 cells (T). (E) and (T) cells were mixed at ratios of 2.5:1, 5:1, and 10:1, and cultured for 4 h. The inhibition rate in samples containing each test substance relative to that in samples containing vehicle (%inhibition) was calculated from the % lysis value (as indicated above). These figures show the mean value and standard error in 2 NK cell donors when the E:T ratio was 2.5:1 and those in 3 donors when E:T ratios were 5:1 and 10:1. (C) Effects of combinations of BTK and ITK inhibitors on the antibody-dependent cytotoxicity of NK cells in peripheral blood from normal healthy volunteers: Vehicle, PCI-32765, ONO/GS-4059, or CGI1746 was added to NK cells in peripheral blood from normal healthy volunteers (E) in combination with vehicle or ONO-7790500, and cultured for 30 min. As with NK cells, vehicle, PCI-32765, ONO/GS-4059, or CGI1746 was added to RTX-treated SU-DHL-4 cells (T) in combination with vehicle or ONO-7790500. (E) and (T) cells were mixed at a ratio of 5:1, and cultured for 4 hours. These figures show the mean value and standard error in 3 NK cell donors.

SU-DHL-4, a GCB-DLBCL cell line, are known to carry the t(14;18) chromosomal translocation and BCL2 gene rearrangement.[25,26] TMD8 are very sensitive to BTK inhibitors, which makes difficult to recognize the antibody driven-ADCC activity. We therefore attempted to evaluate the ADCC activity by using a less sensitive cell line, SU-DHL-4. The cytotoxicity increased as the ratio of NK cells to RTX-treated SU-DHL-4 increased. Figure 2(B) shows the effects of PCI-32765, ONO/GS-4059, or ONO-7790500, specific ITK inhibitor, on cytotoxicity at each mix ratio. PCI-32765 inhibited cytotoxicity even at low concentrations at any mix ratio, with an inhibition

rate of ≥50% at a concentration of 0.1 μmol/L. In contrast, ONO/GS-4059 inhibited cytotoxicity by approximately 10% to 30%, and ONO-7790500 had almost no inhibitory effect at the same concentration (0.1 μmol/L). Figure 2(C) shows the effects of PCI-32765, ONO/GS-4059 or another potent Btk inhibitor, CGI1746 [6,27] in combination with ONO-7790500 under the mix ratio of NK cells to RTX-treated SU-DHL-4 cells was 5:1. PCI-32765 inhibited NK cell-mediated cytotoxicity by approximately 40% at a concentration of 0.01 μmol/L, and by approximately 60% when combined with ONO-7790500 (10 μmol/L). On the other hand, the inhibition rates of PCI-32765 at concentrations of 0.1 μmol/L and 1 μmol/L were approximately 70% and 80%, respectively, showing no combined effects with ONO-7790500. The inhibition rates of ONO/GS-4059 at concentrations of 0.1 μmol/L and 1 μmol/L were approximately 30% and 50%, respectively, and those of ONO/GS-4059 at concentrations of 0.1 μmol/L and 1 μmol/L when combined with ONO-7790500 (1 μmol/L) were approximately 40% and 50%, respectively, showing that the inhibition rate did not increase significantly. When ONO/GS-4059 at concentrations of 0.1 μmol/L and 1 μmol/L were combined with ONO-7790500 (10 μmol/L), the inhibition rates increased to approximately 60% and 75%, respectively. The inhibition rates of CGI1746 at concentrations of 0.1 μmol/L and 1 μmol/L were approximately 10% and 20%, respectively, suggesting that the selectivity profile of CGI1746 is superior to ONO/GS-4059. When CGI1746 at concentrations of 0.1 μmol/L and 1 μmol/L were combined with ONO-7790500 (1 or 10 μmol/L), the inhibition rates increased to approximately 10–40%.

Anti-tumor activity of BTK inhibitors and anti-CD20 antibodies in advanced tumor model

A TMD8 xenograft model was examined and its temporal changes in tumor volume are shown in Figure 3(A). The treatment with ONO/GS-4059 or PCI-32765 was commenced when mean tumor volume reached approximately 300 mm^3. Evaluation of tumor volumes in both the ONO/GS-4059 and PCI-32765 groups showed lower growth rate than in the vehicle group, with significant decreases observed from Day 23 in the ONO/GS-4059 group and from Day 21 in the PCI-32765 group. In terms of TGI on Day 38 (final assessment day), the ONO/GS-4059 and PCI-32765 groups had comparable efficacy (60.2 and 66.0%, respectively).

Treatment with ONO/GS-4059 or PCI-32765 was commenced when mean tumor volume reached approximately 500 mm^3, shown in Figure 3(B). Each treatment demonstrated a statistical significant TGI effect on Day 42, but less efficacious when compared to the treatment initiated at 300 mm^3 (A). In terms of TGI on Day 42 (final assessment day), the ONO/GS-4059 and PCI-32765 groups had comparable efficacy (27.5 and 35.6%, respectively). No decreases in body weight were observed in any of the treatment groups (data not shown). To confirm that both single agent GA101 and RTX have activity in a TMD8 xenograft model, the treatment was commenced when mean tumor volume reached approximately 300 mm^3, shown in Figure 3(C). Significant decreases in tumor volume were observed from Day 17 in the single agent GA101 10 mg/kg group and from Day 21 in the single agent RTX 10 mg/kg group. In the group of GA101, tumor volume on the last evaluation day, the primary endpoint, was lower compared to the group of RTX. Of the three mice (one mouse in the group of GA101 and two mice in the group of RTX) for which tumor volume on the start day of the treatment (Day 13) exceeded 400 mm^3, the two in the group of RTX showed marked increases in tumor volume over time and were resistant to the treatment (Data not shown). No decreases in body weight were observed in both GA101 and RTX treatment groups (data not shown).

Anti-tumor activity of BTK inhibitors in combination with RTX in advanced tumor model

Next, we investigated whether combination treatment with RTX was able to increase the responses to BTK inhibitors. Due to the lower PK of PCI-32765 in mice, we initially explored an appropriate dose for PCI-32765 in the advanced tumor model. 0.012% PCI-32765 treatment showed significant anti-tumor effects, and had comparable efficacy to the 0.0037% ONO/GS-4059 treatment (47.1% of TGI for PCI-32765 and 56.1% for ONO/GS-4059, respectively) shown in Figure 4. In the RTX and 0.0037% ONO/GS-4059 combination group, tumor volumes remained low compared with RTX monotherapy group, and the observed inter-group differences in tumor volume were significant on Day 21 and all subsequent evaluation time points. In particular, 7 out of the 9 mice in RTX and 0.0037% ONO/GS-4059 combination group showed decreases from baseline in tumor volume on the last evaluation day and a complete remission was observed in 3 animals, whereas only 1 out of the 9 mice in the RTX monotherapy group showed decreases from baseline in tumor volume on the last evaluation day. In RTX and 0.012% PCI-32765 combination group, tumor volumes remained low compared with RTX monotherapy group; however, the observed inter-group differences in tumor volume were not significant at any evaluation time points. In addition, only

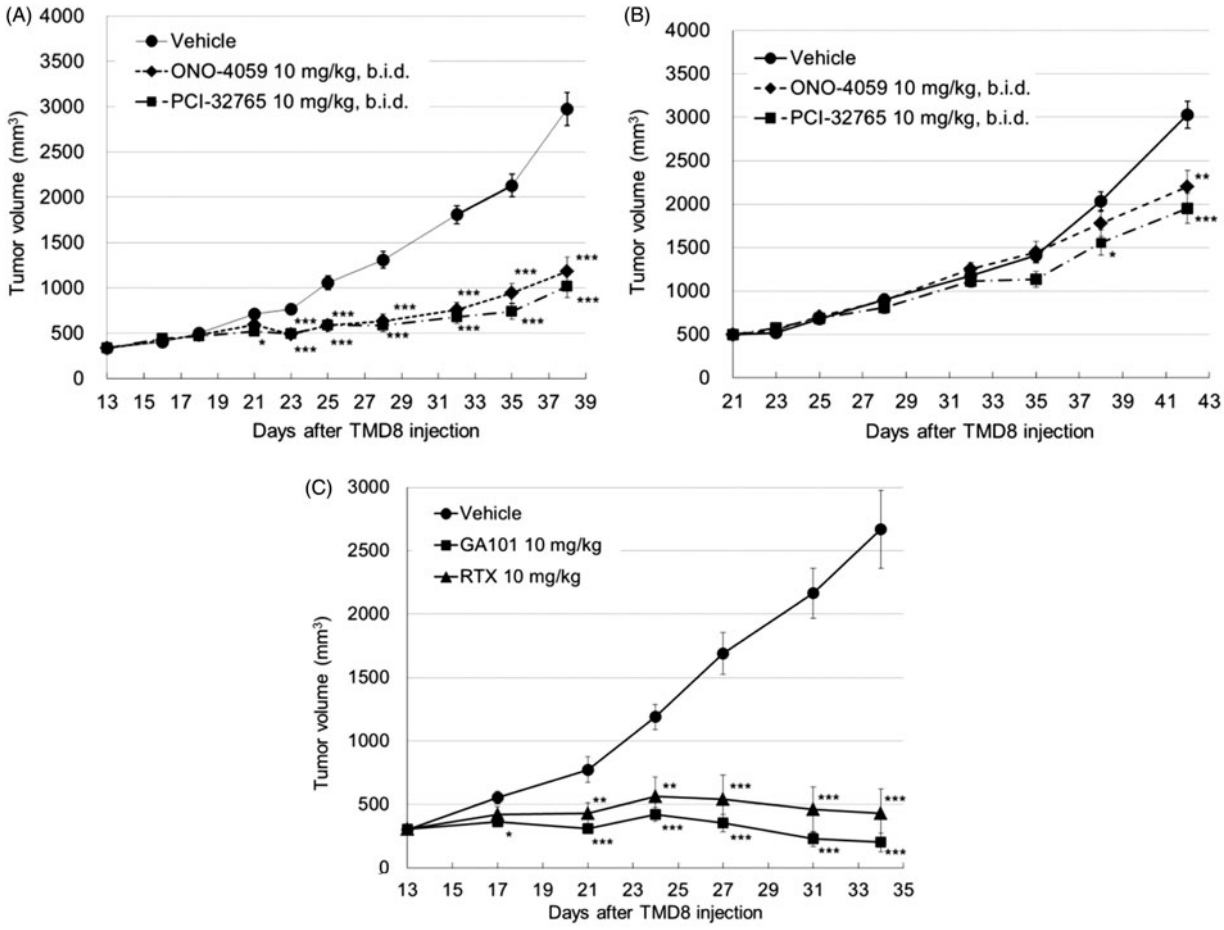

Figure 3. Temporal changes in tumor volume in mouse advanced TMD8 subcutaneous xenograft models: anti-tumor effects of Btk inhibitors and anti-CD20 antibodies. (A) The day of injection was defined as Day 0. Mice were randomized into groups, based on tumor volume calculated from measurement of tumor diameter on Day 13 when tumor volume was approximately 300 mm^3 (13 days after injection). ONO/GS-4059, PCI-32765, or 0.5% MC was administered orally twice daily for 25 days, starting on Day 13. Tumor diameters were measured and tumor volume was calculated every 2 to 4 days after group assignment. The tumor volumes measured for 9 mice in each group at each measurement time point are presented as the mean ± standard error. The Dunnett-test was used to compare data on tumor volume in the ONO/GS-4059 and PCI-32765 groups, versus the vehicle. A p value of less than 5% was considered statistically significant. *, $p < 0.05$. ***, $p < 0.001$. (B) Mice were randomized into groups, based on tumor volume calculated from measurement of tumor diameter on Day 21 when tumor volume was approximately 500 mm^3 (21 days after injection). ONO/GS-4059, PCI-32765, or 0.5% MC was administered orally twice daily for 21 days, starting on Day 21. Tumor volumes were calculated in the same manner as in (A). The tumor volumes measured for 9 mice in each group at each measurement time point are presented as the mean ± standard error. The Dunnett-test was also used. A p value of less than 5% was considered statistically significant. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. (C) Mice were randomized into groups, based on tumor volume calculated from measurement of tumor diameter on Day 13 when tumor volume was approximately 300 mm^3 (13 days after injection). On Days 13, 20 and 27, each mouse received either GA101 or RTX intravenously. Tumor volumes were calculated in the same manner as in (A). The tumor volumes measured for 8 mice in each group at each measurement time point are presented as the mean ± standard error. The Dunnett-test was also used. A p value of less than 5% was considered statistically significant. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

2 out of the 9 mice in RTX and 0.012% PCI-32765 combination group showed decreases from baseline in tumor volume on the last evaluation day. When ONO/GS-4059 or PCI-32765 was administered via mixed diet at an indicated relative content of 0.0037 or 0.012%, the daily dosages for both ONO/GS-4059 and PCI-32765 were found to be comparable to the target doses, 6 and 20 mg/kg/day, respectively. No decreases in body weight were observed in any of the treatment groups (data not shown).

Anti-tumor effects of ONO/GS-4059 used in combination with GA101 or RTX

We then evaluated the combination with GA101 in advanced tumor model. Because GA101 induced a

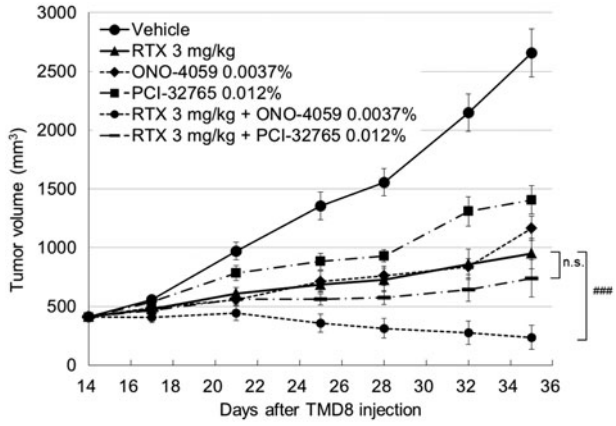

Figure 4. Temporal changes in tumor volume in a mouse TMD8 subcutaneous xenograft model: Anti-tumor effects of ONO/GS-4059 or PCI-32765 used in combination with RTX. The day of injection of TMD8 cells was defined as Day 0. Mice were randomized into groups, based on tumor volume calculated from measurement of tumor diameter on the day when tumor volume was approximately 400 mm^3 (14 days after injection). On Days 14, 21 and 28, each mouse received RTX or physiological saline intravenously, in addition to 21 days of free access (from Day 14) to diet containing either ONO/GS-4059 or PCI-32765. After the group assignment, tumor diameters were measured every 3–4 days to calculate tumor volume. The tumor volumes measured for 9 mice in each group at each measurement time point are presented as the mean ± standard error. The student's *t*-test at 5% was performed for comparison. ###, $p < 0.001$, n.s: non significant.

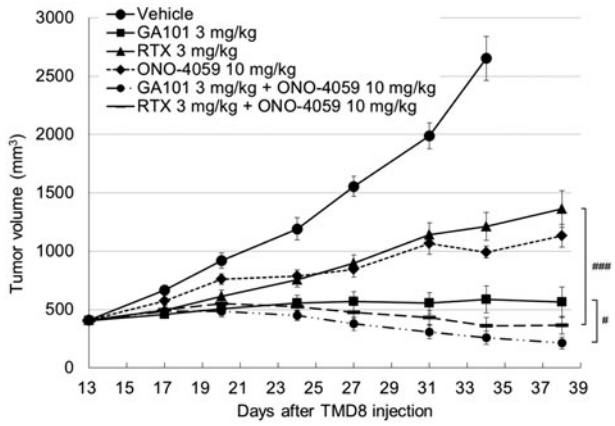

Figure 5. Temporal changes in tumor volume in a mouse TMD8 subcutaneous xenograft model: anti-tumor effects of ONO/GS-4059 used in combination with GA101 or RTX. The day of injection of TMD8 cells was defined as Day 0. Mice were randomized into groups, based on tumor volume calculated from measurement of tumor diameter on the day when tumor volume was approximately 400 mm^3 (13 days after injection). On Days 13, 20, 27, and 34, each mouse received either an anti-CD20 monoclonal antibody (GA101 or RTX) or physiological saline intravenously, in addition to twice-daily repeated oral administration of either ONO/GS-4059 or 0.5% MC for 25 days from Day 13. After the group assignment, tumor diameters were measured every 3–4 days to calculate tumor volume. The tumor volumes measured for 10 mice in each group at each measurement time point are presented as the mean ± standard error. The student's *t*-test at 5% was performed for comparison. #, $p < 0.05$, ###, $p < 0.001$.

higher level of ADCC than RTX does, ibrutinib had minimal effects on GA101-mediated ADCC.[28] Therefore, we considered the hypothesis that the anti-tumor activity of ONO/GS-4059 could be further enhanced by combining it with GA101, shown in Figure 5. On the last evaluation day, the obtained TGI were 77.9, 54.3 and 62.6% in GA101, RTX and ONO/GS-4059 groups, respectively. In the GA101 and ONO/GS-4059 combination and RTX and ONO/GS-4059 combination groups, tumor volumes remained lower compared with GA101 monotherapy and RTX monotherapy groups. The observed differences in tumor volume between the GA101 and ONO/GS-4059 combination and GA101 monotherapy groups were significant on Day 31 and all subsequent evaluation time points. Similarly, the observed differences in tumor volume between the RTX and ONO/GS-4059 combination and RTX monotherapy groups were significant on Day 24 and all subsequent evaluation time points. In particular, all 10 mice in the GA101 and ONO/GS-4059 combination group showed decreases from baseline in tumor volume on the last evaluation day and a complete remission was observed on the last evaluation day for 3 of the animals, whereas only 4 out of the 10 mice in the GA101 monotherapy group showed decreases from baseline in tumor volume on the last evaluation day and a complete remission was only observed in 1 animal. In contrast, 6 out of the 10 mice in the RTX and ONO/GS-4059 combination group showed decreases from baseline in tumor volume and a complete remission was observed in 1 animal, despite all 10 mice in the RTX monotherapy group showing increases from baseline in tumor volume. No differences in body weight were observed in any of the treatment groups (data not shown).

Discussion

The mechanism of action of PCI-32765 (ibrutinib) on the antibody-dependent cellular cytotoxicity (ADCC) of NK cells has been considered to be based on ITK inhibition, because selective BTK inhibitors demonstrate weak inhibitory activity. However, no reports have been published on the same mechanism of action with selective ITK inhibitors. In the present study, ibrutinib inhibited the NK cell-mediated ADCC, and ONO/GS-4059 and CGI1746, which are more selective for BTK than ibrutinib,[6,17,18,27] had much less inhibitory effects as previously reported. ONO-7790500, which

has greater enzyme inhibitory activity against ITK and is more selective for ITK than ibrutinib (IC_{50} for ITK enzyme: 0.011 μmol/L [13] (ibrutinib) and <0.004 μmol/L (ONO-7790500)), also had much less inhibitory effect, and a combination of a selective BTK inhibitor and 1 μmol/L of ONO-7790500 was also less effective than ibrutinib monotherapy. Moreover, ITK expression in NK cells was confirmed; however, stimulated with cross-linking antibodies-induced phosphorylation was not observed, suggesting that ITK may not play a role in the cytotoxicity of NK cells. Most of the activity of ITK is inhibited by treatment with 1 μmol/L of ONO-7790500 even if ITK activation may play a role in NK cells, because ONO-7790500 inhibits IL-2 production by T cells in peripheral blood from normal healthy volunteers and PLCγ1 (substrate) phosphorylation in Jurkat cells by greater than 80% at a concentration of 1 μmol/L. On the other hand, ONO-7790500 inhibited the NK cell-mediated ADCC by approximately 30% to 40% at a concentration of 10 μmol/L, and this effect is likely to be due to other targets of ONO-7790500. Given the results of selective BTK and ITK inhibitors on the NK cell-mediated ADCC are weaker than that of ibrutinib, suggesting that the inhibitory effect of ibrutinib may be due at least in part to other targets of ibrutinib.

The mouse xenograft model is widely used in order to confirm the anti-tumor effect of novel anti-cancer agents *in vivo*. As well as early-stage models in which administration of the test substance is commenced when the xenografted tumor reaches 50 to 200 mm^3, recently an advanced-stage model in which a treatment is initiated when the tumor reaches to advanced volumes has been reported.[29,30] This model has been used for extrapolating the anti-tumor effect to patients with advanced cancer. In the present study, the anti-tumor effect in the TMD8 advanced-stage model was examined by commencing administration when tumor volume reached 300 and 500 mm^3, respectively, in order to extrapolate the effectiveness of BTK inhibitors to patients with more advanced B-cell malignancies. Since both ONO/GS-4059 and PCI-32765 showed an anti-tumor effect even in this advanced-stage model, it was suggested that BTK inhibitors may also be effective in patients with advanced B-cell malignancies. B-cell lymphoma is a disease that can exist as various clinical types.[31] Among all types of B-cell lymphoma, ABC-DLBCL has approximately 40% cure rate with existing R-CHOP regimen (combination of Rituxan with CHOP) [32] and highlights the need for ABC subtype-specific treatment strategies.

The present study demonstrated that, when used alone, both anti-CD20 monoclonal antibodies, RTX and GA101, showed strong anti-tumor effects on tumors with volumes around 100–250 mm^3, without affecting changes in body weight. For tumors with volumes around 300–550 mm^3, however, the anti-tumor effects of RTX alone and GA101 alone diminished, in particular, tumors with volumes ≥400 mm^3 were mostly resistant to these two anti-CD20 monoclonal antibodies. The observed treatment resistance was probably because the ADCC activity of these drugs could not exerted in the interior of the tumor mass.[33] With this background, the present study was designed to investigate the effect on tumors that had grown to mean volumes ≥400 mm^3 as a model representing tumors which are refractory to RTX in a clinical setting. When used in combination with an anti-CD20 monoclonal antibody, ONO/GS-4059 showed higher anti-tumor effects and induced complete remission in greater number of mice compared with ONO/GS-4059 alone, RTX alone, and GA101 alone, without affecting changes in body weight. The use of ONO/GS-4059 in combination with either anti-CD20 monoclonal antibody was therefore suggested as possibly serving as a novel therapy for ABC-DLBCL that could be superior to monotherapy with each constituent drug. Unlike combinations of ONO/GS-4059, PCI-32765 used in combination with RTX did not result in a significantly better efficacy than the RTX alone. This result supported findings from preceding reports that PCI-32765 inhibited the ADCC activity of RTX because of the poor kinase selectivity.[14] Furthermore, GA101 showed a stronger effect than the other anti-CD20 monoclonal antibody, RTX, when used in combination with ONO/GS-4059.

In conclusion, this study demonstrates that ONO/GS-4059 would not inhibit the ADCC-based anti-tumor effects and direct cell death-inducing effects elicited with GA101.

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