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Development of a Bruton's Tyrosine Kinase (Btk) inhibitor - ONO-WG-307, a potential treatment for B-cell malignancies

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ABSTRACT

Purpose: Signals from B cell receptors (BCR) play a central role in signal transduction pathways regulating survival, activation, proliferation, and differentiation of B-lineage lymphoid cells. BCR signaling is implicated in the survival of malignant B cells and recent studies indicate that targeting Bruton's tyrosine kinase (Btk), an essential component of the BCR pathway, may be effective in the treatment of B-cell lymphoma. ONO-WG-307 is a highly potent and selective Btk inhibitor with an IC50 in the sub-nmol/L range. Diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) account for approximately more than 50% of all types of Non-Hodgkin lymphoma (NHL). Combination of the anti-CD20 monoclonal antibody (Rituximab) with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisolone) is first-line treatment of FL and DLBCL. Therefore, the inhibitory effect of ONO-WG-307 in combination with rituximab was evaluated in *in vitro* tumour growth assays.

Results: Treatment with ONO-WG-307 resulted in a dose-dependent inhibition of tumour growth in the TMD-8 xenograft model (TMD-8 cells represent an activated B-cell-like (ABC) sub-type of DLBCL). Results from an *in vitro* cytotoxic cell assay using TMD-8 cells, show that the TMD-8 cells were much more sensitive to ONO-WG-307 when administered as monotherapy compared to DOHH-2 cells (follicular lymphoma cell line). However, it was noted that rituximab was more efficacious than ONO-WG-307 in DOHH-2 cells. Results from *in vitro* combination studies (using DOHH-2 and TMD-8 cell lines) combining ONO-WG-307 with rituximab, show that a moderate antagonism was observed in DOHH-2 cells, whereas a good synergy was observed inTMD-8 cells.

Conclusion: ONO-WG-307 is a highly potent and selective oral Btk inhibitor with evidence of efficacy in the *in vitro* / *in vivo* ABC-DLBCL model. These results indicate that ONO-WG-307 is a promising new candidate targeted agent for ABC-DLBCL and support the potential clinical utility of ONO-WG-307 in the treatment of B-cell malignancies.

INTRODUCTION

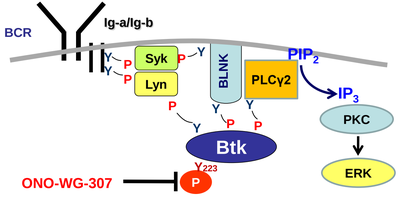
•Signals from B-cell receptors (BCR) play a central role in signal transduction pathways, by regulating survival, activation, proliferation, and differentiation of B-lineage lymphoid cells.
•Bruton's tyrosine kinase (Btk) is an essential component of the BCR signaling pathway and therefore is critical for normal B-lymphocyte function.
•The B-cell antigen receptor (BCR) is expressed on most B-cell lymphomas and is necessary for tumour expansion and proliferation, via activation of several downstream protein kinases, including Btk.
•Rituximab (RTX) inhibits BCR signaling by targeting proximal components of the BCR cascade. RTX has significant impact on the cells, which are subject to BCR stimulation.
•Diffuse large B-cell lymphoma (DLBCL) remains incurable in >50% of patients and remains an unmet medical need.
•DLBCL is the most common malignant lymphoma with studies indicating that chronic, active BCR signaling plays an important role in the pathogenesis of ABC-DLBCL.
•Inhibiting Btk-mediated signaling is an attractive treatment approach for DLBCL and other B-cell lymphomas with abberant BCR signaling.
•ONO-WG-307 is a highly potent and selective, oral Btk inhibitor in development for the treatment of B-cell lymphoproliferative diseases.

MATERIAL and METHODS

●**TMD-8 xenograft model :** TMD-8 tumour cells were implanted subcutaneously into female SCID mice and ONO-WG-307 was administered orally, twice a day (BID) at doses of 1 mg/kg, 3 mg/kg and 10 mg/kg. Treatment with ONO-WG-307 was initiated when the mean tumour volumes reached 100-200mm^3. All animal studies were conducted under approved animal care protocols by the Animal Care and Use Committee of ONO phamaceutical Co., Ltd.
● **Selectivity and inhibitory profile :** Human peripheral blood mononuclear cells (PBMC) were treated with ONO-WG-307 at concentrations from 0.3 to 10,000 nmol/L for 10 min. For continuous exposure of ONO-WG-307, the stimulation with anti-IgM for 22 h was performed. As for washout, the ONO-WG-307 was removed by replacing with the fresh media, then stimulation was performed as described above. The lymphocyte activation marker CD69 was measured by flow cytometry.
● **In vitro cytotoxic activity :** The IC50 of the *in vitro* cytotoxic activity was determined using a MTS assay 72 and 96 hours after incubation. Anti-tumor activity was defined as the ratio of the median tumor volume of treatment groups versus control group. The determination of combination index (CI) was calculated by the median-effect method. The CI was used to express synergism (≤ 0.9), additivity (0.9 - 1.1) or antagonism (≥1.1).

RESULTS

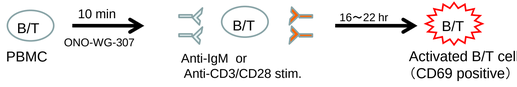
Figure 1. ONO-WG-307 blocks Btk phosphorylation (Y-223) in B-Cell Receptor signaling



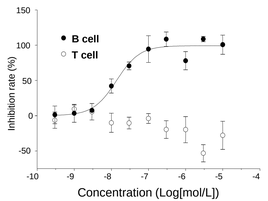
Kinase	IC_{50} (μmol/L)
Btk	0.002
Fyn	2.2
Lck	0.789
Lyn A	3.5

The effect of ONO-WG-307 on recombinant human Btk, Fyn, Lck and Lyna, the IC_{50} values were determined after the measurement of kinase activity with optimized Mobility Shift Assay (MSA). The ATP concentration in the assay was set at the concentration of K_m value of each kinase for ATP.

Figure 2. Selectivity and inhibitory profile of ONO-WG-307



Effects of ONO-WG-307 on PBMC activation



Effects of ONO-WG-307 on B-cells activation

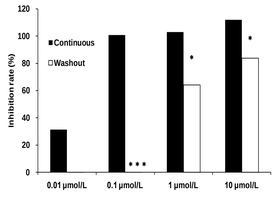
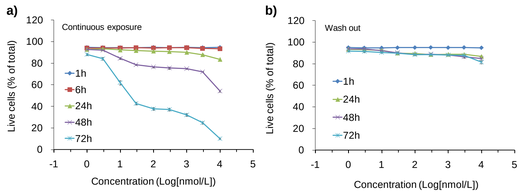
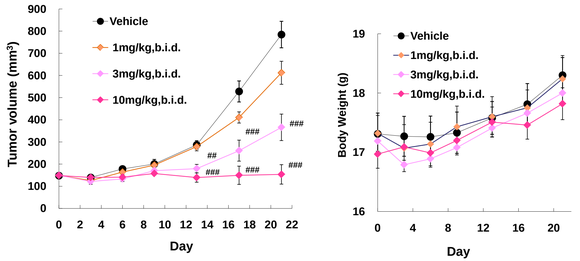


Figure 3. Continuous exposure of ONO-WG-307 is required to kill TMD-8 cells



a) Continuous treatment of ONO-WG-307 resulted in cell death. After completion of culturing for 4, 18, 28 and 52hr, live cells were determined by 7-AAD staining.
b) Wash out after 4h treatment of ONO-WG-307 resulted in no significant cell death. After washing out of ONO-WG-307, cells were cultured with media for 0, 14, 24, 48 and 72hr.

Figure 4. Anti-tumor activity of ONO-WG-307 in TMD-8 xenograft model



Twice-daily treatment with ONO-WG-307 resulted in a dose-dependent inhibition of tumor growth. Tumor volumes are described as the mean ± standard error from 10 mice.
Dunnett test was performed for comparison between vehicle and ONO-WG-307.
##: p<0.01, ###: p<0.001. There were no significant reductions of body weight in the treatment of ONO-WG-307 through the experiment.

Table 1. ONO-WG-307 inhibits proliferation in a range of NHL/CLL cell lines

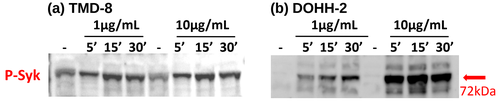
Cell lines	Cancer Type	IC_{50} (μmol/L)	% of inhibition (at 100 μmol/L)
TMD-8	ABC-DLBCL	0.004	-
DOHH-2	FL	1.28	100
DHL-4	GCB-DLBCL	7.30	99.0
DHL-10	GCB-DLBCL	5.23	99.7 (@30 μmol/L)
Jeko-1	MCL	7.74	99.8
Mino	MCL	2.92	100
MEC-1	CLL	31.2	92.1
TMD-2	CLL (Acute Phase)	5.69	99.3

Cells were treated with ONO-WG-307 for 72hr. Relative light units (RLU) were measured with the Cell Titer-Glo Luminescent Cell Viability Assay

Table 2. Rituximab inhibits proliferation of TMD-8 and DOHH-2 cells

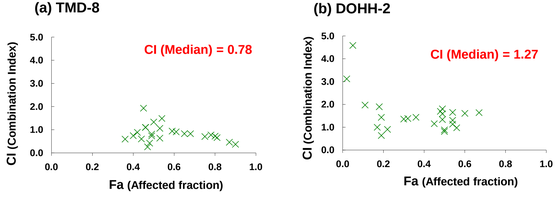
Cell lines	Cancer Type	IC_{50} (μg/mL)
TMD-8	ABC-DLBCL	6.0
DOHH-2	FL	0.2

Figure 5. Effect of anti-IgM/IgG on BCR activation in TMD-8 and DOHH-2 cell lines



TMD-8 (a) or DOHH-2 (b) cells were stimulated with anti-IgM (a) or anti-IgG (b) antibodies (1 or 10 μg/mL) for 5, 15 and 30 min. Non-stimulated (-) and stimulated cells were evaluated by Western blot analysis. BCR activation was determined by the autophosphorylation of Syk (Y525). BCR activation was observed in non-stimulated lane (=basal) of TMD-8 cells. In DOHH-2 cells, BCR stimulation by anti-IgG antibody had drastically effect on Syk phosphorylation.

Figure 6. ONO-WG-307 in Combination with Rituximab against TMD-8 or DOHH-2 cells *in vitro*



TMD-8 (a) or DOHH-2 (b) cells were treated with various concentrations of ONO-WG-307 or RTX and the combinations for 72 and 96hr, respectively. Each dot represents a CI value of combined treatment of an each dose of ONO-WG-307 with RTX. Combination index (CI) values of ≤ 0.9 indicate synergy, a CI value of 0.9 - 1.1 indicates additive effects and a CI value of ≥1.1 indicates antagonism. The treatment of ONO-WG-307 combined with RTX shows that a moderate antagonism was observed in DOHH-2 cells, whereas a good synergy was observed inTMD-8 cells.

CONCLUSIONS

● ONO-WG-307 is a highly potent and selective oral Btk inhibitor with preliminary evidence of efficacy in an ABC-DLBCL xenograft model (TMD-8) along with an anti-proliferative effects in a range of NHL and CLL cell lines.
● Our data also indicate that ONO-WG-307 binding may be reversible, potentially resulting in fewer off target effects in the clinical setting, when compared to the irreversible type of inhibition of other Btk inhibitors.
● Our data support that BCR signaling is constitutively active in TMD-8 cells but not in DOHH-2 cells, suggesting that RTX has less impact on TMD-8 cells.
● The treatment of ONO-WG-307 combined with RTX showed synergistic effect on TMD-8 cells *in vitro*.
●ONO-WG-307 is a promising new candidate targeted agent that is being developed for the treatment of B-cell lymphoproliferative diseases and our results support the potential clinical utility of ONO-WG-307 in the treatment of B-cell malignancies.