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ORIGINAL PAPER

The molecular rationale for the combination of polatuzumab vedotin plus rituximab in diffuse large B-cell lymphoma

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Summary

Polatuzumab vedotin (Pola) is an antibody–drug conjugate that targets the B-cell antigen CD79b and delivers monomethyl auristatin E (MMAE). It is approved in combination with bendamustine and rituximab (Rit) for relapsed/refractory diffuse large B-cell lymphoma (r/r DLBCL). Understanding the molecular basis of Pola combination therapy with Rit, the key component for the treatment of DLBCL, is important to establish the effective treatment strategies against r/r DLBCL. Here, we examined the rationale for the combination of Pola with Rit using Pola-refractory cells. We found that treatment with Pola increased CD20 expression and sensitivity to Rit-induced complement-dependent cytotoxicity (CDC) in several Pola-refractory cells. Pola treatment increased phosphorylation of AKT and ERK and both AKT- and MEK-specific inhibitors attenuated the Pola-induced increase of CD20 and CDC sensitivity, suggesting that these phosphorylation events were required for this combination efficacy. It was revealed that anti-CD79b antibody increased the phosphorylation of AKT but inhibited the phosphorylation of ERK. In contrast, MMAE potentiated phosphorylation of ERK but slightly attenuated the phosphorylation of AKT. Pola also increased CD20 expression on Pola-refractory xenografted tumours and significantly enhanced antitumour activity in combination with Rit. In conclusion, these results could provide a novel rationale for the combination of Pola plus Rit.

KEYWORDS
CD20, DLBCL, non-Hodgkin lymphoma, polatuzumab vedotin, rituximab

INTRODUCTION

Polatuzumab vedotin (Pola) is a first-in-class antibody–drug conjugate (ADC) targeting the B-cell antigen CD79b, a signalling component of the B-cell receptor (BCR).[1–3] On binding to CD79b, Pola is internalized and release monomethyl auristatin E (MMAE). The MMAE then binds to microtubules and kills dividing cells by inhibiting cell division and inducing apoptosis. In the randomized phase 1b/2 study (GO29365), treatment with Pola in combination with bendamustine and rituximab (Pola+BR) was compared with BR in patients with relapsed or refractory diffuse large B-cell lymphoma (r/r DLBCL).[4–6] In that study, Pola+BR demonstrated clinical improvements in complete response rate. Based on those results, Pola+BR has been approved for DLBCL patients in many countries.[7]

Moreover, in the POLARIX study, the combination of Pola with rituximab, cyclophosphamide, doxorubicin and prednisone (Pola-R-CHP) was compared with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) in patients with previously untreated DLBCL. Among these patients, Pola-R-CHP achieved a significantly improved progression-free survival compared to R-CHOP.[8]

Despite many years of research, advances in establishing more effective therapies for patients with r/r DLBCL have been limited. However, the introduction of CD19-directed chimeric antigen receptor (CAR) T-cell therapy, the anti-CD19 monoclonal antibody tafasitamab and Pola has been changing the treatment vision of these patients.[4,5,9–11] Because approaches to the treatment of DLBCL are changing dynamically, understanding the molecular basis of Pola combination therapy, especially with the key component for the treatment of DLBCL rituximab (Rit), which targets the surface antigen CD20, is important to determine the effective treatment strategies to r/r DLBCL.

Here, we examined the Pola-combination efficacy using cells showing low sensitivity to Pola (referred to hereafter as Pola-refractory cells) as model systems which enable the analysis of the novel effect of Pola as well as its cytotoxic effect. We assessed and identified Pola-refractory DLBCL cells. In these cells, pretreatment with Pola enhanced the CD20 expression and sensitivity to Rit-mediated complement-dependent cytotoxicity (CDC) by activating both AKT and ERK signalling. Combination of Pola plus Rit also significantly enhanced antitumour activity in the Pola-refractory xenograft mouse model. Based on these findings, we propose a novel mechanism underlying the combination therapy of Pola with Rit.

METHODS

Reagents

Polatuzumab vedotin and rituximab were obtained from Chugai Pharmaceutical Co., Ltd. A-1331852, MK-2206 2HCl and MMAE were purchased from Selleck Chemicals. Human IgG (HuIgG) was purchased from MP Biomedicals. Verapamil hydrochloride was obtained from Sigma-Aldrich. U0126 was purchased from Promega. Anti-human CD79b antibody (SN8) was purchased from BD Biosciences.

Cell lines and cell culture

DB, SU-DHL-5, SU-DHL-10, NU-DUL-1, SU-DHL-8 and HT cells were obtained from the American Type Culture Collection (ATCC) and STR-428 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank). These cells were maintained in RPMI-1640

ATCC modification (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Nichirei Biosciences). SU-DHL-4 and SU-DHL-2 cells obtained from ATCC were maintained in RPMI-1640 (Sigma-Aldrich) with 10% FBS, 10 mM HEPES (Sigma-Aldrich), 0.45% D-glucose (Sigma-Aldrich) and 1 mM sodium pyruvate (Thermo Fisher Scientific). U-2932 cells obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures were cultured in RPMI-1640 (Sigma-Aldrich) with 10% FBS. RC-K8 cells obtained from JCRB Cell Bank were cultured in RPMI-1640 ATCC modification containing 20% FBS. CD16(158 V)/NK92 (NK92) cells were established as previously described,[12] and grown in MEMα (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FBS, 10% horse serum (Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific), 0.02 mM folic acid (Sigma-Aldrich), 0.2 mM myo-inositol (Sigma-Aldrich) and 0.02 μg/ml recombinant human IL-2 (PeproTech). All cells were cultured at 37°C under 5% CO_2.

Animals

Female 5-week-old C.B-17/Icr-scid/scidJcl mice were obtained from CLEA Japan. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd., which is an institute accredited by AAALAC International and conformed to the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research.

CDC assay

Target cells were prelabelled with calcein-AM (FUJIFILM Wako Pure Chemical Corporation) for 1 h. Specific lysis was assessed 4 h after treatment with normal human serum (Complement Technology, 15% v/v) and rituximab at various concentrations. Fluorescence intensity was measured with a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific). Percentage CDC was calculated as follows: (experimental release − background)/(maximum lysis − background) × 100. Here, 'background' means labelled target cells only and 'maximum lysis' means labelled target cells lysed with 1% Triton X-100 (Sigma-Aldrich).

ADCC assay

Target cells were prelabelled with calcein-AM for 1 h. Specific lysis was assessed at 4 h after exposure to effector NK92 cells at an effector/target ratio of 1:1 with rituximab. Fluorescence intensity was measured with a Varioskan LUX Multimode Microplate Reader. Percentage antibody-dependent cellular cytotoxicity (ADCC) was calculated as follows: (experimental release − background)/(maximum

lysis − background)×100. Here, 'background' means labelled target cells only and 'maximum lysis' means labelled target cells lysed with 1% Triton X-100.

Flow cytometry

Tumour samples were digested using Tumour Dissociation Kit human (Miltenyi Biotec). Labelled cells were analysed by BD LSRFortessa X-20 cell analyser (BD Biosciences) and FlowJo v10 software (Tree Star). Anti-CD20 antibodies and IgG2bκ isotype control were purchased from BD Biosciences or BioLegend. Anti-CD19 antibodies, IgG2aκ isotype control and IgG1κ isotype control were obtained from BD Biosciences. Anti-CD79b antibodies were purchased from Thermo Fisher Scientific or Miltenyi Biotec. Anti-CD243 (anti-MDR1) antibodies were obtained from BioLegend. IgG1 isotype control antibodies were purchased from Miltenyi Biotec. Mean fluorescence intensity (MFI) ratio was calculated by dividing the MFI value of stained cells by the MFI value of the respective isotype control-stained cells.

Cell viability assay

Cells were incubated with Pola in varying concentrations. After three days, WST-8 solution (Dojindo) was added to each well and absorbance was measured using a Varioskan LUX Multimode Microplate Reader.

RT-PCR

The levels of mRNA expression were determined with a LightCycler 480 System II (Roche Diagnostics) using probe/primer sets (*MS4A1*, 4331182; *GAPDH*, 4352665; Thermo Fisher Scientific).

In vivo experiments

Each mouse was inoculated subcutaneously with 5×10^6 tumour cells. Several weeks after tumour inoculation, mice were randomly allocated to study groups. To evaluate the antitumour activity of the test agents, tumour volume was evaluated as described previously.[13] Control HuIgG, Pola, Rit, or Pola plus Rit was administered intravenously on Day 1.

RNA sequencing

Whole-transcriptome RNA sequencing was conducted by Takara Bio using a HiSeq 2500 (Illumina). Reads were mapped to human genome (GRCh38) with RefSeq transcript annotation using bowtie version 1.1.2 (http://bowtie-bio.sourceforge.net/index.shtml) and FPKM values were calculated using RSEM v1.2.31 (https://github.com/deweylab/RSEM).

Western blotting

Total lysates were subjected to SDS polyacrylamide gel electrophoresis. Immunoblotting was carried out using anti-pERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-pAKT (Ser473), anti-AKT and anti-β-actin (Cell Signaling Technology) antibodies and secondary antibodies (Cell Signaling Technology). Imaging was performed with a ChemiDoc Touch imaging PC system (Bio-Rad Laboratories).

FISH analysis

Probes for fluorescence *in situ* hybridization (FISH) were purchased from Leica Biosystems; BCL2 (KBI-XL008), BCL6 (KBI-XL009) and MYC (KBI-XL006). Cells were fixed with 10% neutral-buffered formalin for 24 h. FISH was performed and analysed by New Histo. Science Laboratory Co., Ltd. Cells were considered to have a translocation if more than 30% of cells were positive; cells were considered to not have a translocation if at most 30% of cells were negative.

Statistical analysis

Data were analysed with Student's *t*-test between two groups. Dunnett's test was used for multiple comparisons with the control group. Tukey's honestly significant difference (HSD) test was used for multiple comparisons within groups. For tumour growth experiments, *p*-values were adjusted for the Wilcoxon rank sum test by the Holm–Bonferroni method. All statistical analyses were performed in JMP 15 software (SAS Institute).

Data availability

The data generated in this study are available upon request from the corresponding author.

RESULTS

Identification and characterization of Pola-refractory DLBCL cells

To search for Pola-refractory cells, we tested sensitivity to Pola and calculated the IC_{50} value in a panel of 11 DLBCL cells, comprising eight germinal centre B-cell-like (GCB)-DLBCL (DB, STR-428, SU-DHL-5, SU-DHL-10, SU-DHL-4, NU-DUL-1, SU-DHL-8 and HT) and three activated B-cell-like (ABC)-DLBCL (U-2932, SU-DHL-2 and RC-K8) (Figure 1A; Figure S1A). We used an $IC_{50} = 1\,\mu g/ml$ as the
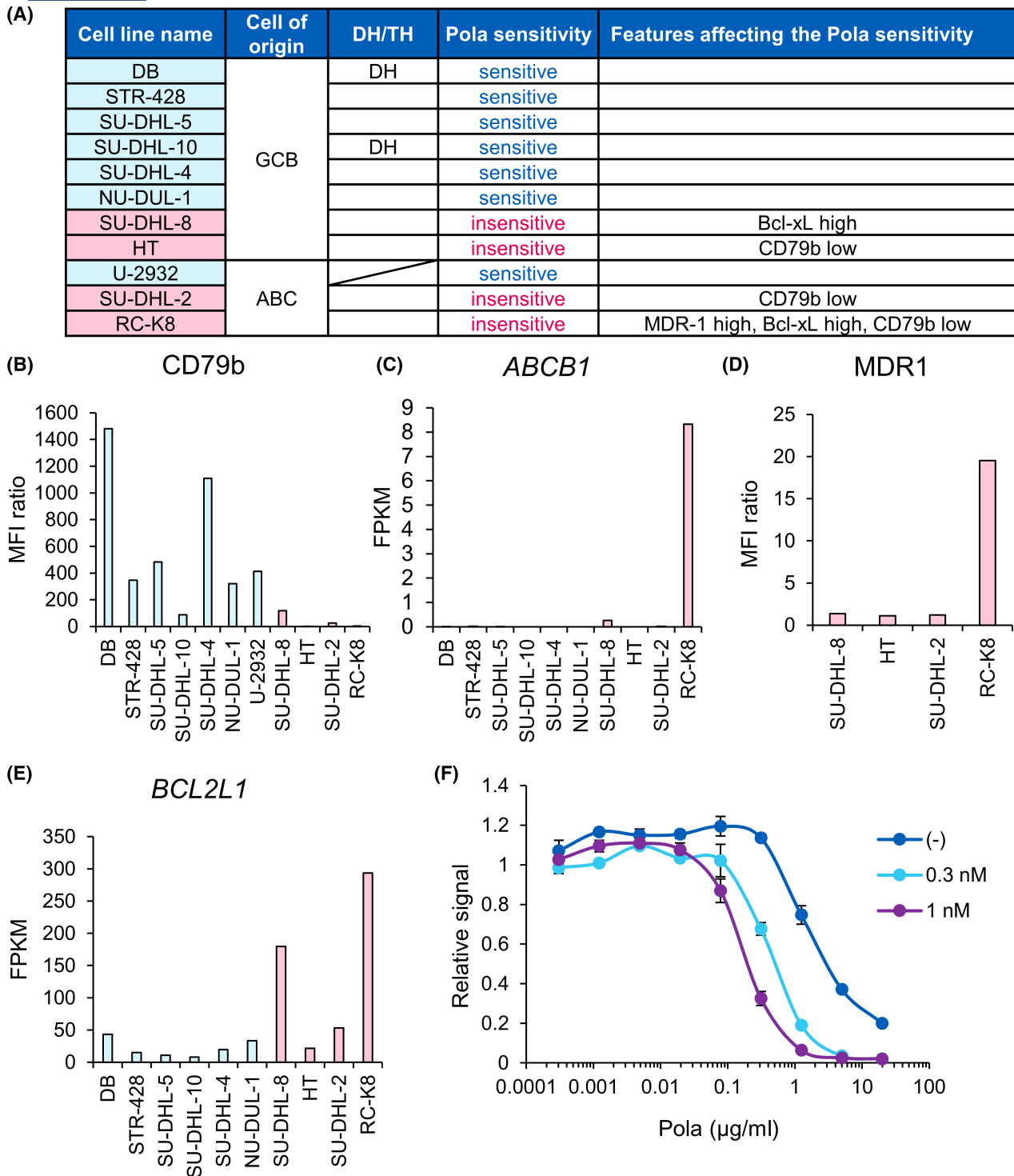
(A)

Cell line name	Cell of origin	DH/TH	Pola sensitivity	Features affecting the Pola sensitivity
DB	GCB	DH	sensitive	
STR-428			sensitive	
SU-DHL-5			sensitive	
SU-DHL-10		DH	sensitive	
SU-DHL-4			sensitive	
NU-DUL-1			sensitive	
SU-DHL-8			insensitive	Bcl-xL high
HT			insensitive	CD79b low
U-2932	ABC		sensitive	
SU-DHL-2			insensitive	CD79b low
RC-K8			insensitive	MDR-1 high, Bcl-xL high, CD79b low

FIGURE 1 Identification of four polatuzumab vedotin (Pola)-refractory DLBCL cells. (A) Results of molecular characterization and sensitivity of 11 DLBCL cells with respect to Pola. The cells that showed high sensitivity to Pola are shown in blue and Pola-refractory cells are shown in red. FISH analysis was not performed on U-2932 cells owing to a contractual issue. (B) Surface expression of CD79b on DLBCL cells was measured by flow cytometry. (C) Gene expression of *ABCB1* was obtained by RNA sequencing. The analysis was not performed on U-2932 cells owing to a contractual issue. (D) Surface expression of MDR1 on Pola-refractory cells was detected by flow cytometry. (E) Gene expression of *BCL2L1* was obtained by RNA sequencing. The analysis was not performed on U-2932 cells owing to a contractual issue. (F) Sensitivity to Pola was assessed with a Bcl-xL inhibitor (A-1331852) at the indicated concentrations in SU-DHL-8 cells. The results are presented as mean values ± SD. *n* = 3. ABC: activated B-cell-like; DH/TH: double-hit or triple-hit lymphoma; DLBCL: diffuse large B-cell lymphoma; FISH: fluorescence *in situ* hybridization; FPKM: fragments per kilobase of exon per million mapped reads; GCB: germinal centre B-cell-like; MFI: mean fluorescence intensity. [Colour figure can be viewed at wileyonlinelibrary.com]

cut-off value, based on the clinical trough drug concentration levels in the GO29365 study,[14] and identified four cells showing refractoriness to Pola.

Patients harbouring chromosomal rearrangements of MYC and BCL2 and/or BCL6, which are known as double-hit (DH) or triple-hit (TH) lymphoma,[15] show very poor clinical outcome; therefore, to determine if Pola-refractory cells have these features, gene rearrangements were determined by FISH analysis (see the DH/TH column in Figure 1A). Since neither of the two DH cells identified were Pola-refractory, these genotypic features may not affect sensitivity to Pola.

To elucidate the mechanisms of refractoriness to Pola, we assessed some factors that could affect the sensitivity to Pola. ADCs are highly dependent upon antigen expression.[16] We thus compared the surface expression of CD79b in the cells showing high sensitivity to Pola (DB, STR-428, SU-DHL-5, SU-DHL-10, SU-DHL-4, NU-DUL-1 and U-2932) and the cells showing refractoriness to Pola (SU-DHL-8, HT, SU-DHL-2 and RC-K8) using flow cytometry (Figure 1B). The expression of CD79b tended to be lower in Pola-refractory cells, but in SU-DHL-8 cells it was at a level similar to that of Pola-sensitive SU-DHL-10 cells. Another possible factor would be the expression of ATP-binding C cassette transporters, which are known to reduce the effectiveness of structurally diverse small-molecule chemotherapeutic agents, including MMAE.[17] Previous results suggest that the expression of MDR1 (ABCB1) multidrug resistance pump, which mediates MMAE efflux, is elevated in non-Hodgkin lymphoma cells resistant to anti–CD79b ADC.[18] We found that high expression of MDR1 was observed in RC-K8 cells by RNA sequencing and flow cytometry (Figure 1C,D) and the MDR1 inhibitor verapamil restored sensitivity to Pola (Figure S1B), indicating that overexpression of the MDR1 would be one of the mechanisms of refractoriness to Pola in RC-K8 cells. Moreover, the expression levels of Bcl-xL (BCL2L1), a member of the BCL2 anti-apoptotic family proteins, has been reported to be correlated with reduced sensitivity to anti–CD79b ADC.[1] High expression of Bcl-xL (BCL2L1) was observed in SU-DHL-8 and RC-K8 cells (Figure 1E). Inhibition of Bcl-xL by specific inhibitor A-1331852 increased sensitivity to Pola (Figure 1F), suggesting that high expression of Bcl-xL affects sensitivity to Pola in SU-DHL-8 cells. Collectively, these cells showed several features that have been shown to affect sensitivity to Pola.

Significant combination effect of Pola with Rit in SU-DHL-8 cells *in vitro* and *in vivo*

Rit induces CDC or antibody-dependent cellular cytotoxicity (ADCC).[19,20] We first investigated the efficacy of combination therapy with Rit against Pola-refractory cells by cell-based CDC and ADCC assays. Cells were pretreated with Pola for three days before the assays. Although SU-DHL-8 cells showed little Rit-mediated CDC sensitivity, pretreatment with Pola significantly increased its sensitivity (Figure 2A). Similarly, pretreatment with Pola also

upregulated the sensitivity to Rit-induced ADCC (Figure 2B). Furthermore, in the xenograft model, treatment with Pola (2 mg/kg) plus Rit (30 mg/kg) significantly increased antitumour activity compared with each single agent on Day 14 (Figure 2C,D), when tumour volume reached euthanasia criteria in the control group. In the combination group, it was possible to continue the study throughout one clinical cycle of treatment (21 days) (Figure 2C).

Treatment with Pola enhances CD20 expression and CDC sensitivity

Previous studies reported that Rit-induced CDC depends on the CD20 antigen expression levels.[21,22] Among DLBCL cells, baseline expression of CD20 was low in SU-DHL-8 cells (Figure S2A). We found that treatment with Pola upregulated cell surface and mRNA expression of CD20 (MS4A1) in a time- and concentration-dependent manner in these cells (Figure 3A–C). Treatment with Pola was confirmed to have little effect on the expression of cell surface CD79b (Figure S2B). Additionally, surface CD20 expression was significantly upregulated in SU-DHL-8 xenograft tumour samples after Pola (2 mg/kg) treatment compared with control HuIgG (2 mg/kg) on Day 4 (Figure 3D) or even on Day 14 (Figure 3E).

Pretreatment with Pola also enhanced CD20 expression and Rit-mediated CDC sensitivity in HT cells with low baseline CD20 expression (Figure S3A,B) and even in SU-DHL-2 cells with relatively high baseline CD20 expression (Figure S3C,D). However, the effect of pretreatment with Pola was not observed in RC-K8 cells, indicating that pretreatment with Pola does not show a combination effect with Rit in all Pola-refractory cells (Figure S3E,F). Although there seem to be some limitations, it was suggested that Pola plus Rit may have a combination effect on several types of Pola-refractory lymphomas.

Pola enhances CD20 expression and CDC sensitivity through AKT and ERK phosphorylation

Both AKT and ERK signalling have been reported to regulate CD20 (MS4A1) expression.[23–25] Immunoblotting analysis identified that AKT and ERK phosphorylation was increased after Pola treatment in SU-DHL-8 cells (Figure 4A). In SU-DHL-8 xenograft tumour samples, treatment with Pola (2 mg/kg) strongly upregulated AKT phosphorylation compared with control HuIgG (2 mg/kg) (Figure 4B). The involvement of AKT and ERK signalling in the regulation of CD20 expression was explored using specific inhibitors. AKT inhibitor MK-2206 disrupted the Pola-induced increase of CD20 and CDC sensitivity (Figure 4C; Figure S4A,B). Similarly, the MEK inhibitor U0126 inhibited Pola-mediated enhancement of CD20 and CDC sensitivity (Figure 4D; Figure S4C,D). It was confirmed that MK-2206
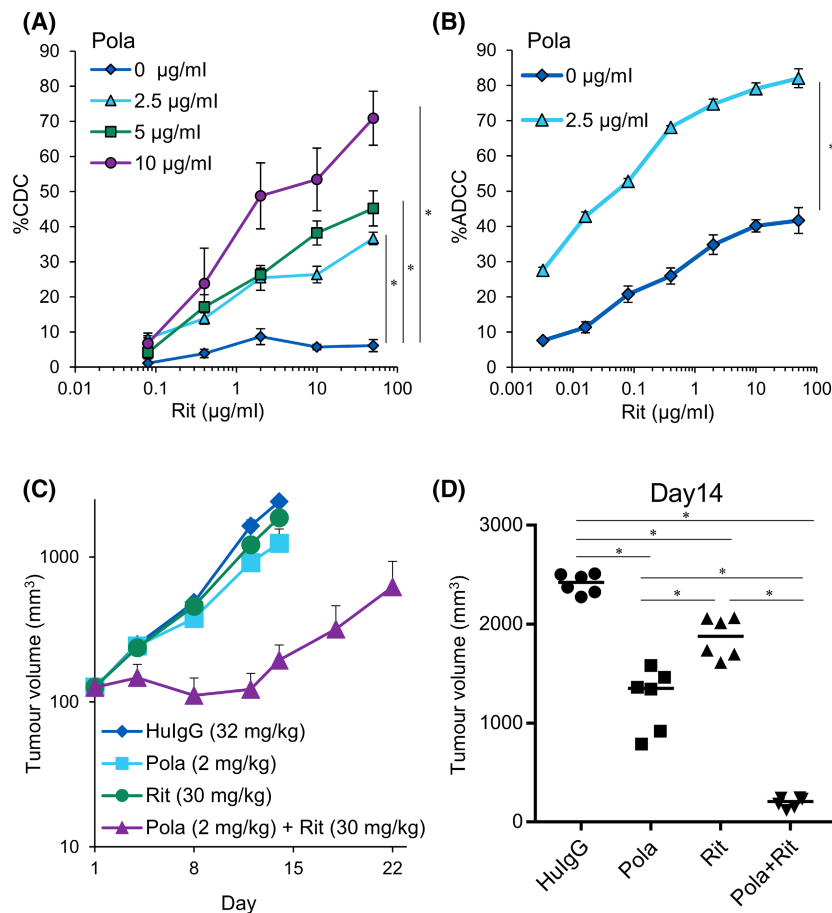
FIGURE 2 Pretreatment with polatuzumab vedotin (Pola) augments sensitivity to Rit. (A) SU-DHL-8 cells were pretreated with Pola at the indicated concentrations for three days. Cells were then collected and CDC assay was performed with Rit. The results of CDC (%) are presented as mean values ± SD. $n = 3$, *: $p < 0.05$ by Dunnett's test compared to Pola 0 µg/ml (Rit: 50 µg/ml). (B) SU-DHL-8 cells were treated as in (A). An ADCC assay was performed with Rit at the indicated concentrations. The results of ADCC (%) are presented as mean values ± SD. $n = 3$, *: $p < 0.05$ by Student's t-test (Rit: 50 µg/ml). (C) Mice bearing SU-DHL-8 cells were randomly divided into four groups ($n = 6$/group). Control HuIgG, Pola, Rit, or Pola + Rit was intravenously administered on day 1. The results of tumour volume (mm³) are presented as mean values + SD. (D) Tumour volumes measured on day 14, the day when control animals reached euthanasia criteria, are shown. Dots indicate individuals and bars represent median. p-values were adjusted for Wilcoxon's rank sum test by the Holm–Bonferroni method (*: $p < 0.05$). ADCC: antibody-dependent cellular cytotoxicity; CDC: complement-dependent cytotoxicity; HuIgG: human IgG; Rit: rituximab. [Colour figure can be viewed at wileyonlinelibrary.com]

did not affect the phosphorylation of ERK and U0126 did not inhibit the phosphorylation of AKT (Figure S4E). The combination of these inhibitors suppressed Pola-induced upregulation of CD20 and CDC sensitivity (Figure 4E,F). Therefore, activation of both AKT and ERK pathways is essential for demonstrating this combination efficacy in SU-DHL-8 cells.

Payload MMAE activates ERK and anti-human CD79b antibody activates AKT

Pola is comprised of a humanized IgG1 anti-CD79b monoclonal antibody conjugated with MMAE. To examine which part of Pola is responsible for activation of these signals, we used MMAE and the anti-human CD79b antibody SN8, the parental antibody for Pola. Pretreatment with MMAE or anti-CD79b antibody, as single agents, did not significantly upregulate CDC sensitivity (Figure 5A,B). Treatment with

Pola showed a more than fivefold increase in CD20 expression (Figure 3A), whereas treatment with MMAE and anti-CD79b antibody both showed a less than twofold increase (Figure 5C,D). Immunoblotting analysis revealed that MMAE potentiated phosphorylation of ERK but slightly attenuated the phosphorylation of AKT. In contrast, anti-CD79b antibody increased the phosphorylation of AKT but inhibited the phosphorylation of ERK (Figure 5E). Taken together, these results suggested the possibility that Pola, which consists of both anti-CD79b antibody and MMAE, activates both AKT and ERK signalling and shows a significant effect on the upregulation of CD20 and CDC sensitivity (Figure 6).

DISCUSSION

In this study, we found that treatment with Pola upregulated CD20 expression and sensitivity to Rit-mediated CDC in
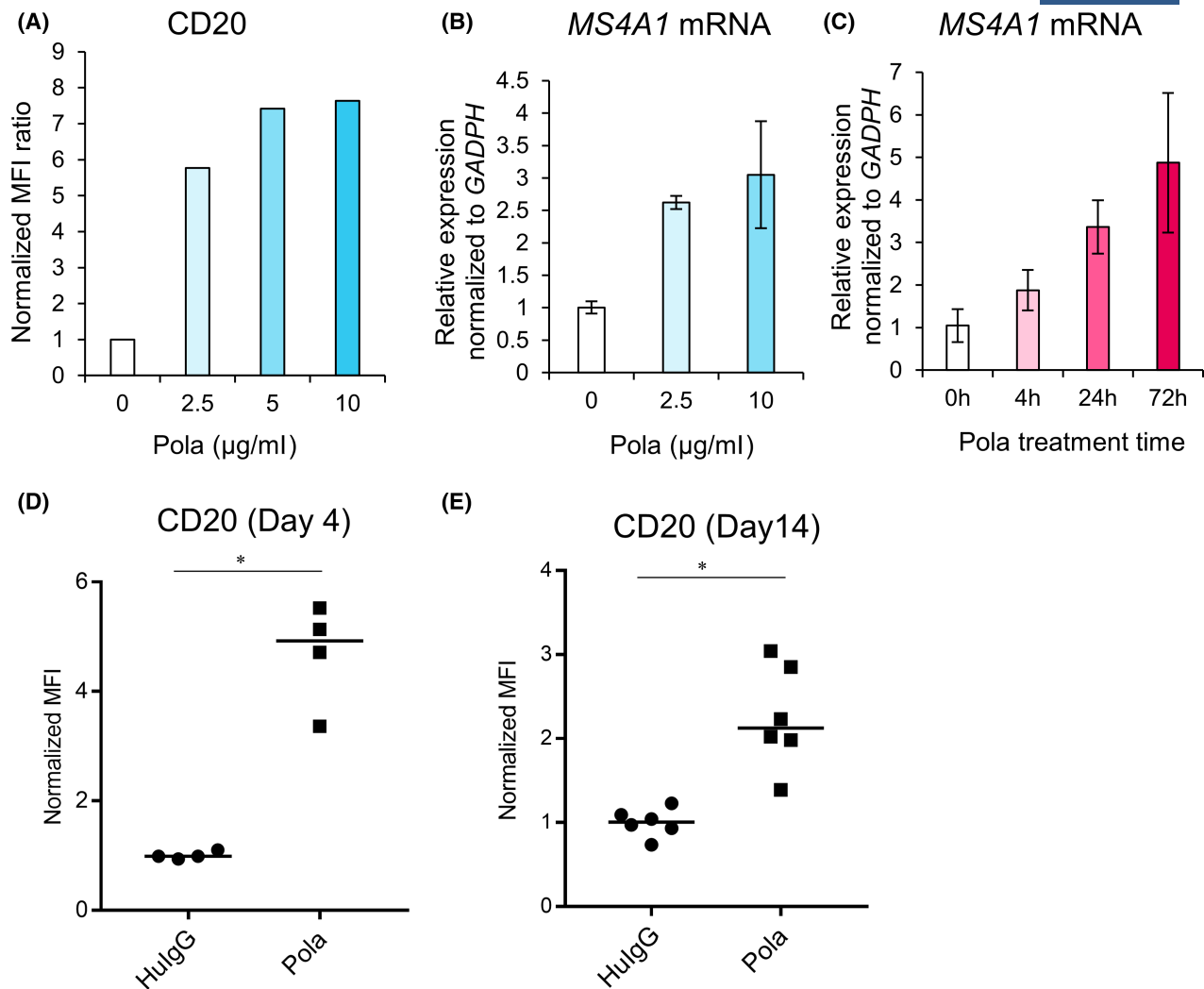
FIGURE 3 Treatment with polatuzumab vedotin (Pola) upregulates CD20 expression. (A) Surface expression of CD20 on SU-DHL-8 cells pretreated with Pola for three days was measured by flow cytometry. Normalized mean fluorescence intensity (MFI) ratio was calculated by dividing by the CD20 MFI ratio of Pola-untreated sample. (B) mRNA expression of *MS4A1* in SU-DHL-8 cells pretreated with Pola for three days was analysed using qPCR. The results are presented as mean values ± SD. $n = 3$. (C) mRNA expression of *MS4A1* in SU-DHL-8 cells pretreated with Pola (2.5 µg/ml) for the indicated number of hours was analysed by qPCR. The results are presented as mean values ± SD. $n = 3$. (D) Mice bearing SU-DHL-8 cells were intravenously administered control HuIgG or Pola (2 mg/kg) on day 1. Tumour samples were collected on day 4 and the surface expression of CD20 on CD19-positive cells was measured by flow cytometry. Normalized MFI was calculated by dividing by the average CD20 MFI of HuIgG-treated samples. Bar indicates median. $n = 4$, *: $p < 0.05$ by Student's *t*-test. (E) Mice bearing SU-DHL-8 cells were treated as in (D). Tumour samples were collected on day 14 and the surface expression of CD20 on CD19-positive cells was measured by flow cytometry. Normalized MFI was calculated by dividing by the average CD20 MFI of HuIgG-treated samples. Bar indicates median. $n = 6$, *: $p < 0.05$ by Student's *t*-test. HuIgG: human IgG; MFI: Mean fluorescence intensity. [Colour figure can be viewed at wileyonlinelibrary.com]

Pola-refractory SU-DHL-8 cells. Since previous studies have reported that the expression level of CD20 correlates with Rit-mediated CDC sensitivity,[21,22] this combination effect of Pola with Rit can be explained by this increased CD20 expression. Additionally, treatment with Pola showed a similar effect of increasing CD20 expression and CDC sensitivity in other Pola-refractory cells, HT and SU-DHL-2 with different expression of CD20, indicating that Pola could be effective regardless of baseline CD20 expression. Moreover, treatment with Pola plus Rit significantly enhanced antitumour activity in SU-DHL-8 xenografted tumours. Pola-mediated CD20 upregulation was also observed on Day 4 and even Day 14,

after Pola administration, indicating the combination effect could be expected to persist over the long term. Further investigations are required to examine whether these findings could be applied to Pola-sensitive cells.

Loss or reduction of CD20 expression is known to be one of the mechanisms of resistance to anti-CD20 monoclonal antibodies. Further studies are therefore warranted to examine whether the Pola–Rit combination is effective even in CD20-low/negative DLBCL or whether Pola is effective to prevent the CD20-negative conversion in clinical settings. Moreover, novel drugs targeting CD20 are under development in B-cell lymphoma, such as a T-cell-dependent
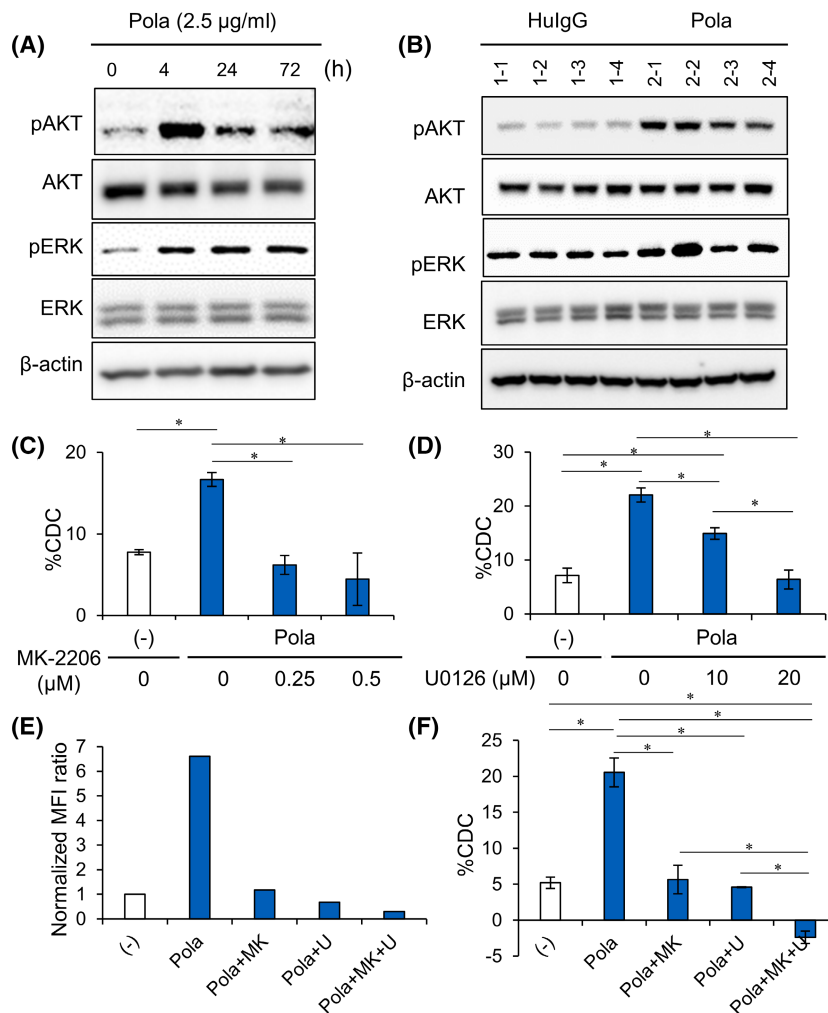
FIGURE 4 AKT and ERK pathway has an essential role in polatuzumab vedotin (Pola)-mediated enhancement of CD20 and CDC sensitivity. (A) SU-DHL-8 cells were treated with Pola for the indicated hours and cell lysates were then collected and analysed by Western blotting. (B) Mice bearing SU-DHL-8 cells were intravenously administered with control human IgG (HuIgG) or Pola (2 mg/kg) on day 1. Tumour samples were collected on day 4 and lysates were analysed by Western blotting. (C) SU-DHL-8 cells were treated with Pola (2.5 μg/ml) and MK-2206 at the indicated concentrations for three days. Cells were then collected and CDC assay was performed with rituximab (Rit) (0.4 μg/ml). The results of CDC (%) are presented as mean values ± SD. $n = 3$, *: $p < 0.05$ by Tukey's HSD test. (D) SU-DHL-8 cells were treated with Pola (2.5 μg/ml) and U0126 at the indicated concentrations for three days. Cells were then collected and CDC assay was performed with Rit (2 μg/ml). The results of CDC (%) are presented as mean values ± SD. $n = 3$, *: $p < 0.05$ by Tukey's HSD test. (E) SU-DHL-8 cells were treated with Pola (2.5 μg/ml), MK-2206 (MK, 0.5 μM) and U0126 (U, 10 μM) for three days. Cells were then collected and CD20 expression was measured by flow cytometry. Normalized MFI ratio was calculated by dividing by the CD20 MFI ratio of untreated sample. (F) SU-DHL-8 cells were treated as in (E). Cells were then collected and CDC assay was performed with Rit (10 μg/ml). The results of CDC (%) are presented as mean values ± SD. $n = 3$, *: $p < 0.05$ by Tukey's HSD test. CDC: complement-dependent cytotoxicity; HSD: honestly significant difference. [Colour figure can be viewed at wileyonlinelibrary.com]

bispecific antibody that binds to CD20 and CD3.[26] Future investigations are expected to determine if Pola-induced up-regulation of CD20 also increases the efficacy of these drugs.

Our data suggested that both AKT and ERK signalling is activated by treatment with Pola. AKT- and ERK-specific inhibitors each prevented the Pola-induced increase of CD20 and CDC sensitivity, indicating that the phosphorylation of both AKT and ERK after Pola treatment was essential for this combination efficacy. In the SU-DHL-8 xenografted tumour samples, the levels of ERK phosphorylation were not as enhanced after administration of Pola as those in *in vitro* samples. There is a possibility that ERK signalling is already fully activated in the *in vivo* tumour environment by

surrounding factors, creating a state in which the combination effect with Pola depends on Pola-induced AKT signalling. Thus, the relative impacts of AKT and ERK signalling on Pola-mediated enhancement of CD20 expression and CDC sensitivity may vary depending on the tumour microenvironment or the characteristics of each tumour.

The data shown here revealed that the activation of AKT is mediated by the anti-CD79b antibody, SN8. CD79b plays an essential role in BCR signalling.[1,2,4] Since AKT is one of the downstream targets of BCR signalling,[27] it could be activated by anti-CD79b antibody through the BCR pathway. Our data also showed that the activation of ERK is mediated by payload MMAE. Previous reports showed that the
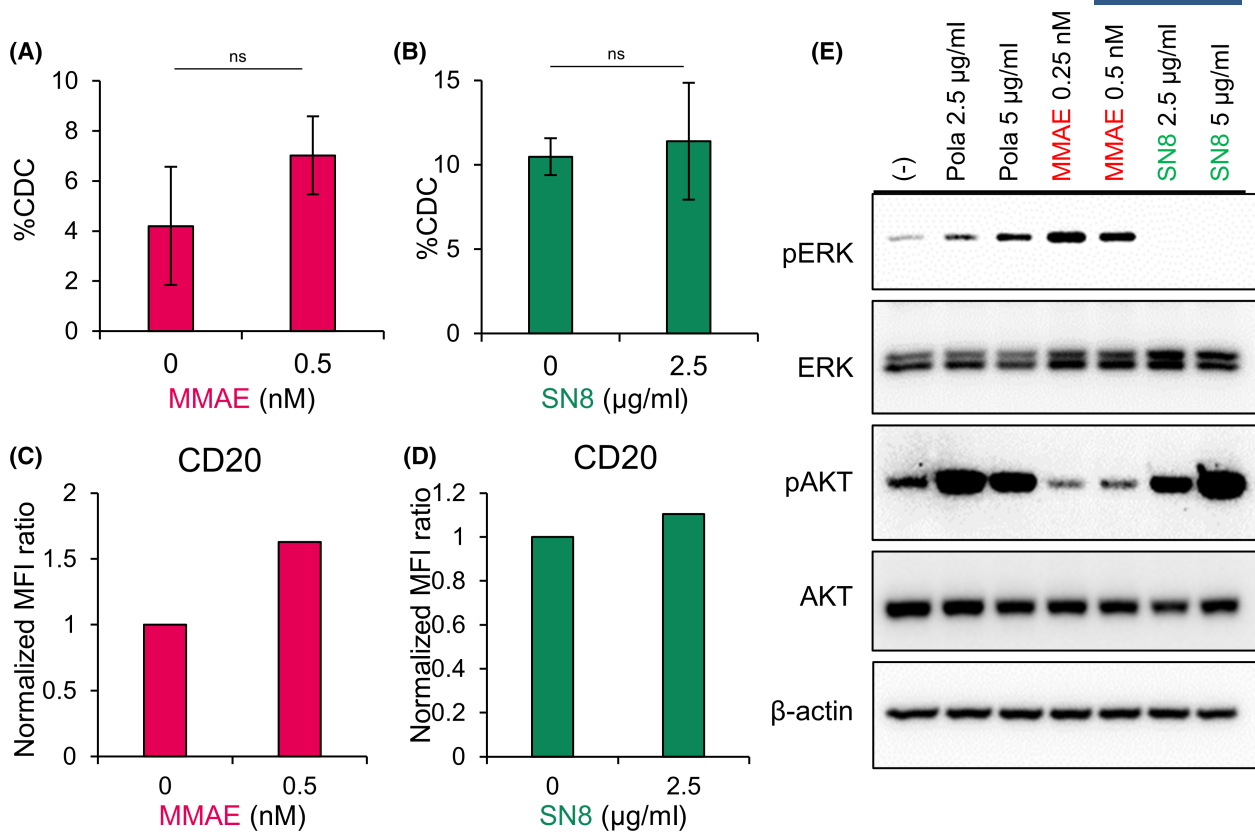
FIGURE 5 Both payload MMAE and anti-human CD79b antibody are essential for the polatuzumab vedotin (Pola) plus Rit combination efficacy. (A) SU-DHL-8 cells were pretreated with MMAE (0.5 nM) for three days. Cells were then collected and CDC assay was performed with Rit (2 µg/ml). The results of CDC (%) are presented as mean values ± SD. $n = 3$, ns indicates not significant ($p \geq 0.05$) by Student's t-test. (B) SU-DHL-8 cells were pretreated with anti-CD79b antibody, SN8 (2.5 µg/ml) for three days. Cells were then collected and CDC assay was performed with Rit (2 µg/ml). The results of CDC (%) are presented as mean values ± SD. $n = 3$, ns indicates not significant ($p \geq 0.05$) by Student's t-test. (C) SU-DHL-8 cells were treated as in (A). Cells were then collected and CD20 expression was measured by flow cytometry. Normalized MFI ratio was calculated by dividing by the CD20 MFI ratio of MMAE-untreated sample. (D) SU-DHL-8 cells were treated as in (B). Cells were then collected and CD20 expression was measured by flow cytometry. Normalized MFI ratio was calculated by dividing by the CD20 MFI ratio of SN8-untreated sample. (E) SU-DHL-8 cells were treated with Pola, MMAE, or SN8 at indicated concentrations for three days. Cell lysates were then collected and analysed by Western blotting. CDC: complement-dependent cytotoxicity; MFI:mean fluorescence intensity; MMAE: monomethyl auristatin E; Rit: rituximab. [Colour figure can be viewed at wileyonlinelibrary.com]
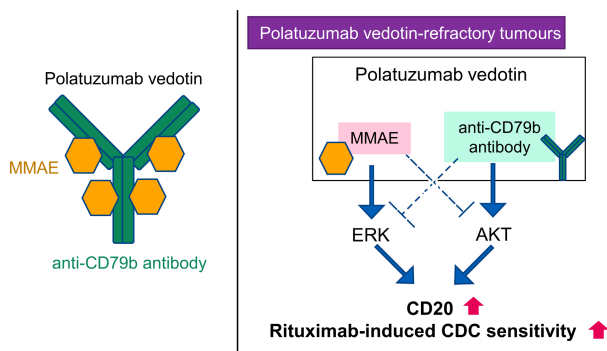

FIGURE 6 Schematic diagram of combination mechanism of polatuzumab vedotin (Pola) with Rit. Pola, which consists of both anti-CD79b antibody and MMAE, activates both AKT and ERK signalling and has a significant effect on CD20 upregulation and increased sensitivity to Rit-mediated CDC. CDC: complement-dependent cytotoxicity; MMAE: monomethyl auristatin E; Rit: rituximab. [Colour figure can be viewed at wileyonlinelibrary.com]

treatment of cells with taxane, a different type of microtubule inhibitor than MMAE, leads to the activation of ERK by causing nuclear localization of the transcription factor,

FOXO1[28,29]; a similar mechanism, therefore, might be responsible for the activation of ERK by MMAE. On the other hand, it was also observed that MMAE and anti-CD79b antibody show inhibitory effects on AKT and ERK, respectively. Consequently, these results indicated that Pola, which consists of both the anti-CD79b antibody and MMAE, could be a promising agent for enhancing CD20 expression and CDC sensitivity. Besides, it has been shown that taxane-mediated ERK activation is one of the resistance mechanisms that limits the efficacy of taxanes.[30] Our results indicated that, by the action of its anti-CD79b antibody, Pola inhibits the phosphorylation of ERK to some degree, which may counter this negative aspect of MMAE.

Here we provided a novel rationale for the combination treatment of Pola with Rit using Pola-refractory cells as a model and suggested that the Pola combination regimen could be an attractive treatment option for these Pola-refractory tumours. Further studies are warranted to examine whether these findings could be applied to Pola-sensitive cells and cells that are refractory or resistant to Pola due to other possible factors.

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