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Inhibition of MDR1 Overcomes Resistance to Brentuximab Vedotin in Hodgkin Lymphoma

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ABSTRACT

Purpose: In classical Hodgkin lymphoma, the malignant Reed–Sternberg cells express the cell surface marker CD30. Brentuximab vedotin is an antibody–drug conjugate (ADC) that selectively delivers a potent cytotoxic agent, monomethyl auristatin E (MMAE), to CD30-positive cells. Although brentuximab vedotin elicits a high response rate (75%) in relapsed/refractory Hodgkin lymphoma, most patients who respond to brentuximab vedotin eventually develop resistance.

Patients and Methods: We developed two brentuximab vedotin–resistant Hodgkin lymphoma cell line models using a pulsatile approach and observed that resistance to brentuximab vedotin is associated with an upregulation of multidrug resistance-1 (MDR1). We then conducted a phase I trial combining brentuximab vedotin and cyclosporine A (CsA) in patients with relapsed/refractory Hodgkin lymphoma.

Results: Here, we show that competitive inhibition of MDR1 restored sensitivity to brentuximab vedotin in our brentuximab vedotin–resistant cell lines by increasing intracellular MMAE levels, and potentiated brentuximab vedotin activity in brentuximab vedotin–resistant Hodgkin lymphoma tumors in a human xenograft mouse model. In our phase I trial, the combination of brentuximab vedotin and CsA was tolerable and produced an overall and complete response rate of 75% and 42% in a population of patients who were nearly all refractory to brentuximab vedotin.

Conclusions: This study may provide a new therapeutic strategy to combat brentuximab vedotin resistance in Hodgkin lymphoma. This is the first study reporting an effect of multidrug resistance modulation on the therapeutic activity of an ADC in humans. The expansion phase of the trial is ongoing and enrolling patients who are refractory to brentuximab vedotin to confirm clinical activity in this population with unmet need.

Introduction

Hodgkin lymphoma is a lymphoid malignancy characterized by the presence of Reed–Sternberg cells within an inflammatory milieu (1). It is most commonly diagnosed in young adults and after age 55. In 2019, it is estimated that 8,110 cases of Hodgkin lymphoma will be diagnosed in the United States and 1,000 will die from their disease (2). Brentuximab vedotin is a novel therapeutic targeting the CD30 surface antigen, a defining marker of Hodgkin lymphoma (3). This antibody–drug conjugate (ADC) is composed of (1) the chimeric mAb (cAC10) specific for human CD30 (2), the potent antimitotic agent monomethyl auristatin E (MMAE), which inhibits cell division by blocking tubulin polymerization, and (3) a protease-cleavable linker that covalently attaches MMAE to cAC10. Upon binding to CD30, the complex is internalized, MMAE is released by proteolysis in the lysosomes, and triggers cell-cycle arrest and apoptosis (4). In a pivotal phase II clinical trial in patients with relapsed or refractory (R/R) Hodgkin lymphoma post-autologous hematopoietic cell transplantation, brentuximab vedotin demonstrated an overall response rate (ORR) of 75% and a complete response (CR) rate of 34% (5), leading to its accelerated FDA approval in the relapsed setting (6). The final end-of-study results from this trial showed that patients who reach a CR after brentuximab vedotin therapy can achieve durable remissions (7). However, a majority of patients with R/R Hodgkin lymphoma who achieve CR with brentuximab vedotin will ultimately relapse and patients who achieve only partial responses (PR) eventually develop progressive disease despite ongoing treatment with brentuximab vedotin. Brentuximab vedotin is also approved as maintenance therapy after autologous hematopoietic cell transplantation in patients with Hodgkin lymphoma at high risk of relapse or progression (8). More recently, brentuximab vedotin was approved as part of initial therapy for advanced stage Hodgkin lymphoma in combination with chemotherapy (9).

Several mechanisms can lead to the development of resistance to ADCs, such as (i) downregulation of antigen surface expression, (ii) altered antigen-ADC internalization, intracellular trafficking, or drug release mechanism, (iii) resistance to the cytotoxic payload, or (iv) overexpression of ATP-binding cassette (ABC) efflux pumps, which transport a wide variety of chemotherapeutic agents across the cell

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Translational Relevance

Multidrug resistance-1 upregulation is a mechanism of resistance to brentuximab vedotin in Hodgkin lymphoma cell lines and human xenograft mouse models, which we overcame via competitive inhibition of the export pump with cyclosporine and verapamil. We then demonstrated that the combination of brentuximab vedotin + cyclosporine is feasible and tolerable in a phase I clinical trial. Brentuximab vedotin + cyclosporine was associated with a high response rate of 75% and complete response rate of 42% in a heavily pretreated population of patients with relapsed/refractory (R/R) Hodgkin lymphoma, nearly all of whom were previously refractory to both brentuximab vedotin and PD-1 blockade. Our findings have the potential to significantly impact the treatment of R/R Hodgkin lymphoma. Many patients with R/R Hodgkin lymphoma develop resistance to brentuximab vedotin and ultimately fail PD-1 blockade; the ability to reinduce sensitivity to brentuximab vedotin in these patients fulfills an important unmet need and may allow for bridging to subsequent stem cell transplantation or other novel therapies in development.

membrane, thereby reducing their effectiveness (10). We previously showed that resistance to brentuximab vedotin was associated with upregulation of the multidrug resistance gene *MDR1* (*ABCB1*) and its protein product PgP/MDR1 (ATP-dependent translocase ABCB1), rather than to a downregulation of CD30 expression, in a brentuximab vedotin–resistant Hodgkin lymphoma cell line model (L428-R; ref. 11). We also showed that, although L428-R cells displayed intrinsic MMAE resistance, intracellular MMAE accumulation was significantly decreased in L428-R cells compared with parental cells, and MDR1 activity and efflux of MMAE out of cells appeared to play an important role in this process (11). We also reported that tumor samples from patients with brentuximab vedotin–resistant Hodgkin lymphoma remained CD30 positive by IHC, with a subset (4/10) that stained positive for multiresistance drug transporters (11). To confirm our prior findings and generate a stronger rationale for further study, we used the same approach of pulsatile exposure to brentuximab vedotin employed to generate L428-R cells to generate a second brentuximab vedotin–resistant Hodgkin lymphoma cell line model, KMH2-R, and demonstrated that these cells also overexpress MDR1. We hypothesized that sensitivity to brentuximab vedotin could be restored by competitively inhibiting MDR1 in our cell line models *in vitro* and in mouse xenografts. To test this hypothesis, we used two broad multidrug-resistant modifiers, cyclosporine A (CsA) and verapamil (VRP), which are both clinically available and have been extensively tested in humans as an immunosuppressant and an anti-hypertensive agent, respectively. We then conducted a phase I trial combining CsA with brentuximab vedotin in patients with R/R Hodgkin lymphoma. Although MDR1 inhibitors have already been tested in combination with chemotherapies, this is the first clinical study reporting an effect of multidrug-resistant inhibition on the therapeutic activity of an ADC, which is capable of targeted drug delivery.

Patients and Methods

Preclinical experiments

Reagents and cells

VRP and CsA were purchased from Sigma and Selleckchem, respectively. The L428 and KMH2 Hodgkin lymphoma cell lines were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, which authenticates cell lines using short tandem repeat DNA typing. Cells were passaged in the laboratory for fewer than 6 months following purchase and original authentication. Brentuximab vedotin was obtained from the City of Hope Pharmacy. The development of the brentuximab vedotin–resistant cell line KMH2-R used a pulsatile approach, as previously described for the generation of L428-R cells (11). Selection was considered successful when consistent proliferation was observed at 20 μg/mL of brentuximab vedotin.

RNA extraction, qPCR, and RNA sequencing

Total RNA was extracted using TRIzol (Invitrogen). cDNA was obtained by reverse transcription of 10 μg of RNA using the SuperScript III reverse transcriptase (Invitrogen) and random primers. Expression of human *MDR1* transcripts was determined by qPCR using iQ SYBR Green Supermix and a CFX96 Detection System (Bio-Rad). *GAPDH* gene expression was used as an internal control. Primers (sequences provided in Supplementary Materials and Methods) were purchased from Integrated DNA Technologies. RNA sequencing of the parent and brentuximab vedotin–resistant cell lines was also performed. RNAs were converted to cDNA libraries and libraries were sequenced on the Illumina Hiseq 2500 (data processing described in Supplementary Materials and Methods).

MTS cell proliferation assay

Cells were seeded in 96-well plates at 10,000 cells per well, and then incubated with increasing amounts of brentuximab vedotin in triplicate. Cell viability was measured after 72 hours using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. IC_{50} value is the concentration of drug which produced a 50% reduction in viability compared with no drug (t_0) control and was calculated from the dose–response curves. For cell inhibition dose–response experiments, a four-parameter log-logistic model was fitted to the curves. Absolute IC_{50} values and the corresponding SEs were estimated from the fitted dose–response curves.

Flow cytometry

Flow cytometry was performed as described previously (11). Briefly, cells were incubated at room temperature for 15 minutes with either PE-conjugated anti-human CD30 antibodies (BD Biosciences), APC-conjugated anti-human MDR1 (BioLegend), or an isotype control. Flow cytometry was performed on a BD Fortessa (BD Biosciences) flow cytometer and data were analyzed using FlowJo (TreeStar).

Intracellular MMAE accumulation

KMH2-P (parental, brentuximab vedotin naïve) and KMH2-R cells (brentuximab vedotin resistant) were incubated with 15 μg/mL of brentuximab vedotin for up to 48 hours. L428-R and KMH2-R cells were incubated with brentuximab vedotin (20 or 15 μg/mL, respectively) for 24 and 48 hours in presence or absence of VRP or CsA. MMAE concentration in cells was measured by LC/MS-MS according to a modification of a previously published method (11, 12). Under optimized assay conditions, the lower limit of quantitation was 0.01 ng/10^6 cells or 0.17 pg on column. Inter- and intraday precision and accuracy of the method was within ±10% of target values.

MDR1 protein extraction and immunoblotting

Cell lysates were collected in RIPA buffer with 1 mmol/L PMSF. Protein concentrations were determined with the BCA Protein Assay

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(Pierce). Protein (20 μg) was loaded onto a 7.5% SDS-PAGE gel and proteins were transferred to Hybond-LFP membranes (Amersham), followed by primary and secondary antibody incubation. The following primary antibodies were used: mouse monoclonal anti-P Glycoprotein (GeneTex), rabbit monoclonal anti-PgP (MDR1/ABCB1) (Cell Signaling Technology), rabbit monoclonal anti-beta Tubulin (Thermo Fisher Scientific), and polyclonal anti-β-Actin (Cell Signaling Technology). Horseradish peroxidase–conjugated secondary antibodies (KPL) were detected using an ECL SuperSignal West Femto Kit (Thermo Fisher Scientific). Signals were detected using BIO-RAD ChemiDoc TM MP Imaging System.

MDR1 overexpression

MDR1 was exogenously overexpressed in the brentuximab vedotin–sensitive L428-P cell line by lentiviral transduction. Detailed methods are provided in Supplementary Materials and Methods.

Human xenograft mouse model

Animal models were established by subcutaneous injection of 1×10^7 Hodgkin lymphoma KMH2-R (brentuximab vedotin resistant) cells into the right flank of female NSG mice from The Jackson Laboratory. Technical details of the experiments, including doses and schedules are provided in Supplementary Materials and Methods. All animal experiments were approved by the City of Hope Institutional Animal Care and Use Committee.

Phase I clinical trial

Patients

This prospective, phase I trial in patients with biopsy-proven relapsed/refractory (R/R) Hodgkin lymphoma was approved by the City of Hope Institutional Review Board and all patients were consented in accordance with the Declaration of Helsinki (NCT03013933). Subjects were ≥15 years of age, and were allowed to be refractory to prior brentuximab vedotin defined as having achieved a best response of stable disease (SD) or progressive disease, or having achieved a best response of CR or PR but progressed while on active brentuximab vedotin treatment. Patients who had undergone prior autologous or allogeneic hematopoietic cell transplantation (HCT) were eligible as long as there was no evidence of graft-versus-host disease and the patient was not taking immunosuppressive agents. Patients had to have measurable disease of at least 1.5 cm, Eastern Cooperative Oncology Group performance status of 0–2, adequate hematologic function (absolute neutrophil count > 1,000/mm^3, platelets ≥ 50,000/mm^3, and hemoglobin ≥ 8.5 g/dL), and organ function [creatinine clearance ≥ 50 mL/minute, alanine and aspartate aminotransferases ≤ 3 times upper limit of normal (ULN), total bilirubin ≤ 1.5 times ULN]. Patients who had grade 2 or higher peripheral neuropathy were excluded.

Study design and assessments

Four dose levels were planned: (i) 1.2 mg/kg brentuximab vedotin on day 1 and 5 mg/kg CsA orally twice a day on days 1–5, (ii) 1.8 mg/kg brentuximab vedotin on day 1 and 5 mg/kg CsA orally twice a day on days 1–5, (iii) 1.8 mg/kg brentuximab vedotin on day 1 and 7.5 mg/kg CsA orally twice a day on days 1–5, and (iv) 1.8 mg/kg brentuximab vedotin on day 1, 7.5 mg/kg CsA orally twice a day on days 1–5, and 120 mg VRP orally four times a day on days 1–5. The first dose of MDR1 inhibitor(s) for each cycle was scheduled to be given 4 hours prior to brentuximab vedotin infusion. Cycle length was 21 days. Dose finding followed a "3+3" design, with dose escalation occurring after confirmation that 0 of 3 or ≤1 of 6 patients experienced dose-limiting toxicity (DLT) and terminating any time ≥2 DLTs occurred on the same dose. The highest dose with ≤1 of 6 DLT was considered the MTD. DLT was defined as any nonhematologic grade ≥3 toxicity or any grade ≥3 hematologic toxicity that did not resolve to grade 1 or 2 within 7 days, which was considered at least possibly related to CsA or brentuximab vedotin, or any other regimen-related cause of death. Grade 3 or 4 laboratory abnormalities (both hematologic and nonhematologic) that resolved to grade 1 or 2 within 7 days were not considered a DLT. The DLT period was 21 days and evaluation occurred after cycle 1. Patients who did not receive either brentuximab vedotin or at least 80% of the planned CsA dose for cycle 1 and did not experience DLT during the DLT period were considered inevaluable for DLT and replaced. Treatment continued until disease progression, DLT or other unacceptable toxicity, patient refusal, or at the discretion of the investigator (e.g., to proceed to HCT). Adverse events (AEs) were assessed according to the NCI CTCAE v4.3 scale. Patients were considered evaluable for response if they received brentuximab vedotin and at least 80% of the dose of CsA during the first two cycles and had at least one disease evaluation. Response was assessed by CT scan after cycle 2, by PET-CT after cycle 4, and then subsequently by alternating CT and PET-CT scans every three cycles. Responses were assessed by investigators according to the 2014 Lugano classification (13).

Statistical analysis

For preclinical data, statistical analysis was carried out using SAS (version 9.4), R (version 3.4.1), and R package "drc." Plots were generated using Excel and R package "easyGgplot2." All P values cited were two-sided and $P < 0.05$ was considered statistically significant.

For clinical trial data, patient demographics and baseline disease/prior treatment characteristics were summarized using descriptive statistics. Median and range were provided for continuous variables; counts and percentages were provided for categorical variables. The primary endpoint of the study was DLT and toxicities. Secondary endpoints were overall response, CR, duration of response (DOR), progression-free survival (PFS), and overall survival (OS). DOR was defined as time from first achieving at least PR until disease progression or death. PFS was defined as time from start of treatment until disease progression or death. OS was defined as time from start of treatment until death due to any cause. DOR and PFS were censored at last contact or at start of other anticancer treatment. OS was censored at last contact. DOR, PFS, and OS were estimated using the Kaplan–Meier method.

Results

Preclinical data

Brentuximab vedotin–resistant Hodgkin lymphoma KMH2 cell line model overexpresses MDR1

Using the same pulsatile approach we previously described to generate the brentuximab vedotin–resistant Hodgkin lymphoma cell line L428-R (11), we generated another brentuximab vedotin–resistant Hodgkin lymphoma cell model KMH2-R (**Fig. 1A**). KMH2-R IC_{50} was approximately 17-fold higher (172 ± 17 μg/mL) than that of parental cells (10 ± 2.4 μg/mL; **Fig. 1B**). By flow cytometry, we observed that CD30 expression was unchanged in KMH2-R compared with KMH2-P (**Fig. 1C**). We confirmed that the brentuximab vedotin–resistant KMH2 cells, like brentuximab vedotin–resistant L428 cells, overexpress the multidrug-resistant gene *MDR1* by qPCR; *MDR1* transcripts were threefold higher in KMH2-R cells compared with

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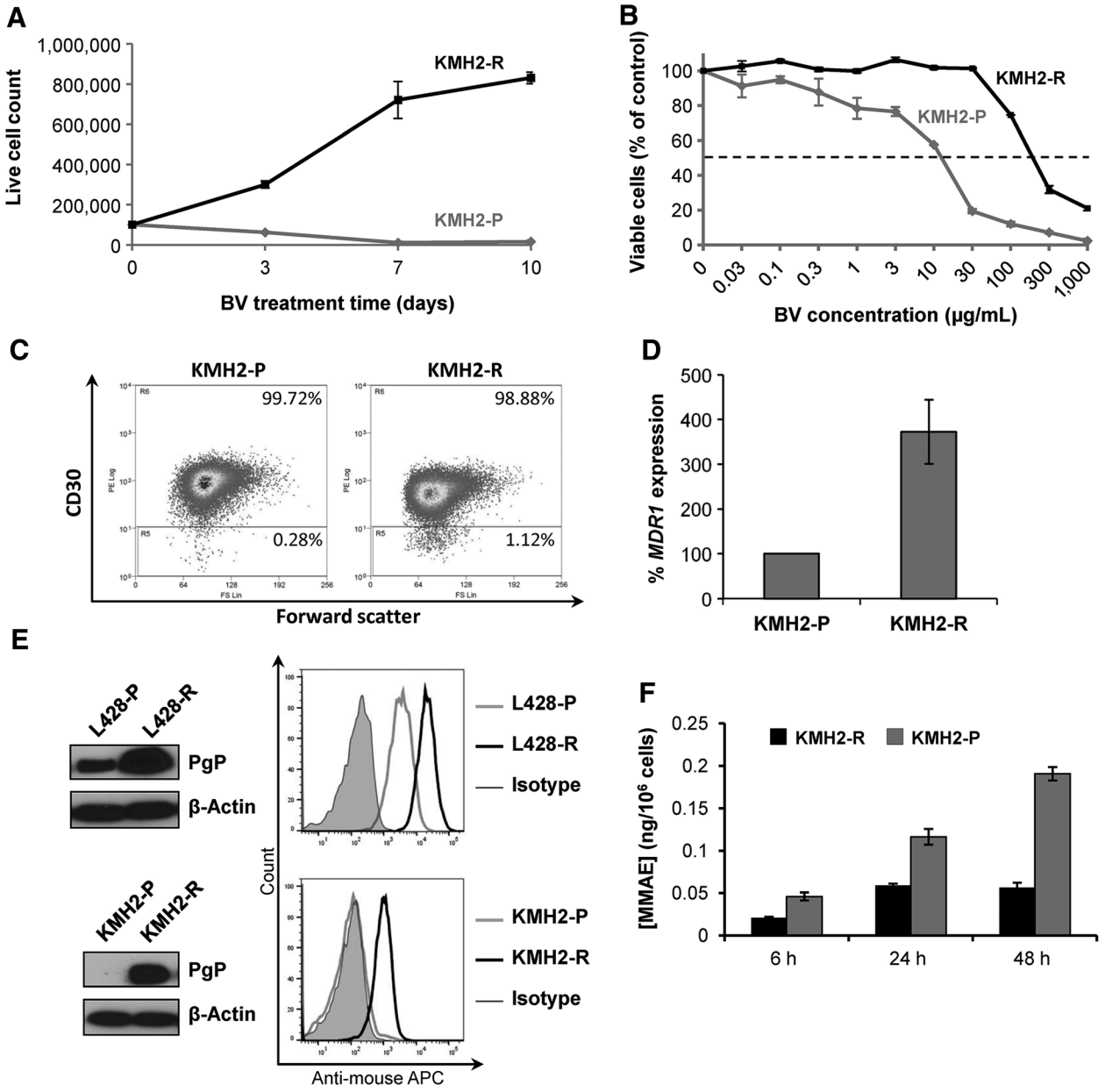

Figure 1.

Brentuximab vedotin (BV)-resistant Hodgkin lymphoma KMH2 cell line model and MDR1 upregulation. **A,** Viable cell counts of KMH2-P and KMH2-R seeded at 10×10^4 cells/well and incubated with brentuximab vedotin at 20 μg/mL. Error bars represent SD from duplicates. **B,** Dose–response curves of KMH2-P and KMH2-R cells incubated at the indicated brentuximab vedotin concentrations. Error bars represent SD from two experiments performed in triplicate. A four-parameter log-logistic model was fitted to assess the inhibitory effect of brentuximab vedotin on KMH2-P and KMH2-R cells. **C,** Flow cytometry showing surface CD30 expression in KMH2-P and KMH2-R cells after sorting. **D,** *MDR1* transcript expression by qPCR in KMH2-P and KMH2-R cells using *GAPDH* gene expression as internal control. Error bars represent SD from two experiments performed in triplicate. Results shown relative to *MDR1* expression in KMH2-P cells. **E,** MDR1 protein (PgP) expression (Western blot, β-actin used as control; left) and cell surface expression (flow cytometry, isotype antibody used as control; right) in parental and brentuximab vedotin–resistant L428 and KMH2 cell lines. **F,** Intracellular MMAE concentrations in KMH2-P and KMH2-R cells treated with brentuximab vedotin (15 μg/mL) over 48 hours. Error bars represent SD from triplicates.

KMH2-P cells (**Fig. 1D**). We confirmed this higher *MDR1* expression in resistant cell lines by RNA sequencing on L428-P, L428-R, KMH2-P, and KMH2-R cells; *MDR1* RNA expression was threefold higher in KMH2-R cells (10.37 vs. 2.88 using RPKM values); and sevenfold higher in L428-R cells (55.86 vs. 7.27). In fact, among all ABC transporter family genes, *MDR1* was the only one found to be over-expressed in resistant versus parental cells across both cell lines. We then looked at MDR1 protein (PgP) expression in both cell lines (**Fig. 1E**). L428 parental cells had a baseline level of PgP expression, yet expression was greatly increased in resistant cells (left). We did not detect PgP expression in KMH2-P cells, but observed a high expression in KMH2-R cells. Results were confirmed by flow cytometry (right).

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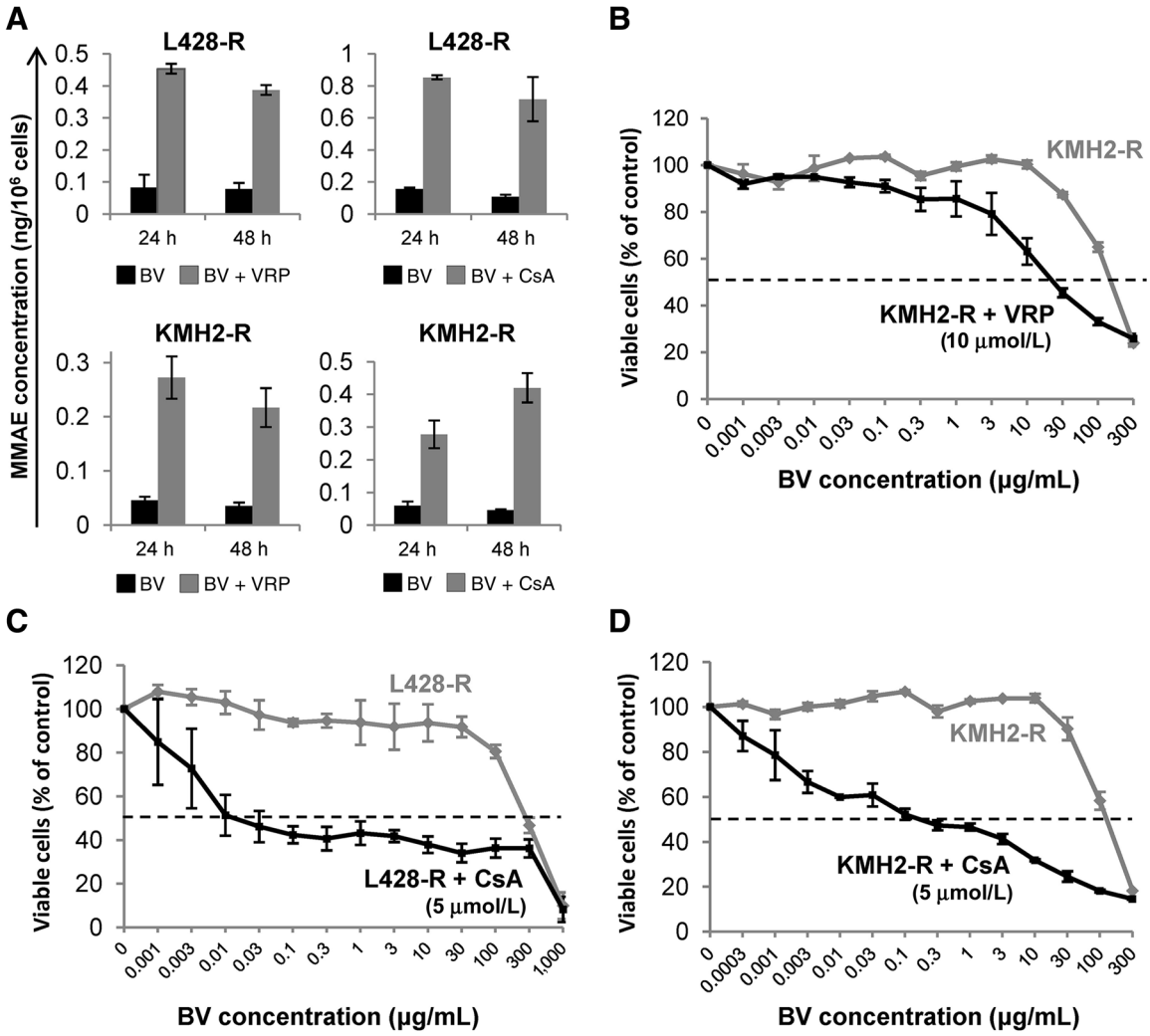

Figure 2.

MDR1 inhibition in brentuximab vedotin (BV)-resistant cell line models. **A,** Intracellular MMAE concentrations in L428-R (top) treated with brentuximab vedotin (20 μg/mL) and KMH2-R (bottom) cells treated with brentuximab vedotin (15 μg/mL) for 24 and 48 hours in the presence or absence of VRP (10 μmol/L, left) or CsA (5 μmol/L, right). Error bars represent SD from triplicates. **B–D,** Viability curves of the model cell lines incubated with the indicated concentrations of brentuximab vedotin in the presence or absence of VRP (10 μmol/L) for KMH2-R (**B**), CsA (5 μmol/L) for L428-R (**C**), or CsA (5 μmol/L) for KMH2-R (**D**). Error bars represent SD from two or three experiments performed in triplicate. A four-parameter log-logistic model was fitted to assess the effect of brentuximab vedotin.

Similar to the L428 model, intracellular MMAE accumulation was significantly reduced in KMH2-R cells compared with the parental line (**Fig. 1F**). We have thus developed a new brentuximab vedotin–resistant Hodgkin lymphoma cell model by pulsatile exposure to brentuximab vedotin and, as we previously reported for the L428 cell line model (11), resistance to brentuximab vedotin in this new model is associated with an upregulation of MDR1, while CD30 surface expression is maintained.

MDR1 inhibition restores sensitivity to brentuximab vedotin in brentuximab vedotin–resistant Hodgkin lymphoma cell line models and improves brentuximab vedotin therapeutic activity in a human xenograft mouse model

To determine whether competitive inhibition of MDR1 could restore sensitivity to brentuximab vedotin in the brentuximab vedotin–resistant L428-R and KMH2-R cell lines, we used the clinically available CsA and VRP, which are both substrates for and thus competitive inhibitors of the MDR1 protein. Building on our previous finding that L428-R cells consistently have decreased intracellular MMAE compared with L428-P cells (11), we measured the intracellular MMAE levels at 24 and 48 hours in L428-R and KMH2-R cells that were incubated with brentuximab vedotin in the presence or absence of VRP (10 μmol/L) or CsA (5 μmol/L). Both MDR1 inhibitors led to a greater than threefold increase in intracellular MMAE levels in both cell lines (**Fig. 2A**).

We then examined the effect of CsA and VRP on the IC_{50} of brentuximab vedotin in L428-R and KMH2-R cells. We previously reported that the addition of VRP to L428-R cells led to a 3.9-fold reduction in brentuximab vedotin IC_{50} (297 ± 12 to 76 ± 23 μg/mL; ref. 11). Similarly, treating KMH2-R cells with VRP (10 μmol/L) decreased the IC_{50} from 144 ± 49 to 25 ± 10 μg/mL (sixfold; **Fig. 2B**). CsA (5 μmol/L) was even more efficient in restoring

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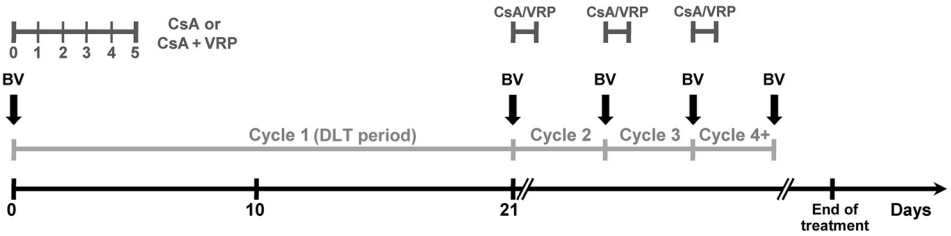

Figure 3.
Phase I trial treatment schema. Experimental design schema of the phase I trial. Four dose levels were planned as described in Patients and Methods. Cycle 1 was the DLT period. BV, brentuximab vedotin.

sensitivity to brentuximab vedotin, reducing the IC_{50} from 265 ± 66 to 0.025 ± 0.036 μg/mL (~10,000 fold) in L428-R cells, and from 121 ± 27 to 0.21 ± 0.28 μg/mL (~600 fold) in KMH2-R cells (**Fig. 2C** and **D**, respectively). VRP and CsA did not affect the viability of parental or brentuximab vedotin–resistant L428 and KMH2 cells in the absence of brentuximab vedotin, and *MDR1* mRNA expression and PgP protein expression did not change upon treatment with CsA (Supplementary Fig. S1). Competitive MDR1 inhibition thus increased intracellular MMAE levels, and resensitized the two brentuximab vedotin–resistant Hodgkin lymphoma cell lines to brentuximab vedotin. In addition, we showed that resistance to brentuximab vedotin could be induced by stably overexpressing exogenous MDR1 in L428-P cells, and the addition of CsA to these resistant cells could restore brentuximab vedotin sensitivity (Supplementary Fig. S2).

In a subcutaneous tumor mouse model (generated using KMH2-R cells as described in Patients and Methods), treatment with CsA and brentuximab vedotin led to a significant decrease in tumor growth, compared with brentuximab vedotin alone ($P = 0.017$) and with control mice ($P < 0.001$), while CsA alone had no effect on tumor growth compared with control (Supplementary Fig. S3). Addition of CsA to brentuximab vedotin treatment led to a significantly higher accumulation of MMAE in the tumors, as compared with brentuximab vedotin alone, while not significantly affecting MMAE levels in the liver and kidney (Supplementary Table S1). This suggests that brentuximab vedotin activity can be enhanced by combining brentuximab vedotin treatment with CsA without increased toxicity to other organs, due to the specific CD30 targeting of brentuximab vedotin. Altogether, our results show that competitive inhibition of MDR1 with CsA is a strategy that deserves to be tested clinically.

Table 1. Dose finding cohort baseline characteristics ($N = 14$).

Characteristics	*n* (%) or median (range)
Gender	
Female	7 (50%)
Male	7 (50%)
Race	
White	12 (86%)
Other	2 (14%)
Hispanic	9 (64%)
Age	36 (23–69)
Stage at treatment	
I—II	4 (29%)
III—IV	10 (71%)
Extra-nodal disease	6 (43%)
B symptoms	3 (21%)
Prior HCT	7 (50%)
Prior autologous	5 (36%)
Prior allogenic	3 (21%)
Prior BV	14 (100%)
Refractory to prior BV	12 (86%)
Prior PD-1/PD-L1 therapy	13 (93%)
Refractory to prior PD-1/PD-L1 therapy	12 (86%)

Abbreviation: BV, brentuximab vedotin.

Phase I clinical trial of brentuximab vedotin + cyclosporine in patients with R/R Hodgkin lymphoma

Patients

On the basis of our preclinical studies, we designed a phase I clinical trial in patients with R/R Hodgkin lymphoma combining brentuximab vedotin with MDR1 inhibitors (NCT03013933; **Fig. 3**). Treatment plan/dose levels are described in Patients and Methods. The trial was initially designed to administer CsA on days 1–10; however, the first patient on dose level 1 who received a 10-day CsA dosing experienced DLTs (grade 3 hyperbilirubinemia, abdominal pain, and hypertension). The protocol was subsequently amended to administer CsA on days 1–5 in all CsA-containing dose levels, and accrued an additional 13 patients to complete the dose-finding portion. Three patients were treated at dose level 1, six were treated at dose level 2, and three were at dose level 3. There was one additional patient treated at dose level 2 who was inevaluable for DLT due to early withdrawal from the study during cycle 1. The baseline characteristics for these 14 patients of the dose-finding portion are shown in **Table 1**. The median age was 36 years old (range, 23–69), 50% of the patients were male, 71% had advanced stage disease at baseline, and 50% had prior HCT including five (36%) autologous and three (21%) allogeneic. All patients had prior treatment with brentuximab vedotin (100%), and 86% were refractory to prior brentuximab vedotin. In addition, 93% of patients had prior treatment with an anti-PD-1/PD-L1 antibody, 86% had PD-1/PD-L1 therapy as their most recent therapy, and 86% were refractory to PD-1/PD-L1 therapy.

Safety

Among the 12 DLT-evaluable patients, the median number of cycles administered was four (range, 1–16). There were no DLTs observed among the three patients treated at dose level 1. There were two DLTs of grade 3 abdominal pain and grade 3 neutropenia in the same patient among the six patients treated at dose level 2 (one of six patients). At dose level 3, there were four DLTs in two of three patients, including grade 4 hyperglycemia in one patient and grade 3 bone pain, grade 3 constipation, and grade 4 lymphopenia in another patient. Therefore, dose level 2 was determined to be the MTD and recommended phase 2 dose (RP2D). All (100%) patients experienced treatment-related AEs. The most common AEs considered at least possibly due to protocol treatment for the 12 DLT-evaluable patients treated with 5-day CsA dosing are documented in **Table 2**. The most common AEs were anemia (92%), hypertension (92%), nausea (83%), fatigue (83%), weight loss (83%), leukopenia (83%), neutropenia (75%), anorexia (75%), hypomagnesemia (75%), and peripheral sensory neuropathy (75%). The most common grade 3–4 AEs (**Table 3**) were neutropenia (67%), leukopenia (50%), anemia (33%), lymphopenia (33%), hypophosphatemia (25%), and hyponatremia (25%). Reasons for treatment discontinuation among DLT-evaluable patients include: disease

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Table 2. AEs at least possibly related to any study agent occurring in 50%+ of patients.

Treatment	Dose level 1 ($n = 3$)			Dose level 2 ($n = 6$)			Dose level 3 ($n = 3$)			Total
Grade, *N* (%)	1–2	3+	All	1–2	3+	All	1–2	3+	All	Any
Anemia	1	1	2 (67)	4	2	6 (100)	2	1	3 (100)	11 (92)
Hypertension	2	1	3 (100)	5	0	5 (83)	3	0	3 (100)	11 (92)
Nausea	3	0	3 (100)	4	0	4 (67)	2	1	3 (100)	10 (83)
Fatigue	2	0	2 (67)	5	0	5 (83)	2	1	3 (100)	10 (83)
Weight loss	2	0	2 (67)	4	1	5 (83)	3	0	3 (100)	10 (83)
White blood cell decreased	1	1	2 (67)	2	4	6 (100)	1	1	2 (67)	10 (83)
Neutrophil count decreased	1	1	2 (67)	0	6	6 (100)	0	1	1 (33)	9 (75)
Anorexia	1	0	1 (33)	5	0	5 (83)	3	0	3 (100)	9 (75)
Hypomagnesemia	2	0	2 (67)	5	0	5 (83)	2	0	2 (67)	9 (75)
Peripheral sensory neuropathy	3	0	3 (100)	4	0	4 (67)	2	0	2 (67)	9 (75)
Abdominal pain	2	0	2 (67)	2	1	3 (50)	3	0	3 (100)	8 (67)
Vomiting	1	0	1 (33)	4	0	4 (67)	2	1	3 (100)	8 (67)
Hyponatremia	2	0	2 (67)	3	1	4 (67)	0	2	2 (67)	8 (67)
Sinus tachycardia	1	0	1 (33)	3	0	3 (50)	3	0	3 (100)	7 (58)
Constipation	1	0	1 (33)	4	0	4 (67)	1	1	2 (67)	7 (58)
Aspartate aminotransferase increased	0	0	0 (0)	4	1	5 (83)	2	0	2 (67)	7 (58)
Hypoalbuminemia	0	0	1 (33)	4	0	4 (67)	2	0	2 (67)	7 (58)
Myalgia	1	0	1 (33)	4	0	4 (67)	2	0	2 (67)	7 (58)
Hypophosphatemia	2	0	2 (67)	2	2	4 (67)	0	1	1 (33)	7 (58)
Alanine aminotransferase increased	0	0	0 (0)	4	0	4 (67)	3	0	3 (100)	7 (58)
Alkaline phosphatase increased	0	0	0 (0)	3	0	3 (50)	3	0	3 (100)	6 (50)
Hypokalemia	1	0	1 (33)	3	1	4 (67)	1	0	1 (33)	6 (50)
Lymphocyte count decreased	0	1	1 (33)	2	1	3 (50)	0	2	2 (67)	6 (50)
Proteinuria	1	0	1 (33)	3	0	3 (50)	2	0	2 (67)	6 (50)
Chills	0	0	0 (0)	4	0	4 (67)	2	0	2 (67)	6 (50)

Note: Dose level 1, 1.2 mg/kg brentuximab vedotin on day 1 and 5 mg/kg CsA twice a day on days 1–5; dose level 2, 1.8 mg/kg brentuximab vedotin on day 1 and 5 mg/kg CsA twice a day on days 1–5; and dose level 3, 1.8 mg/kg brentuximab vedotin on day 1 and 7.5 mg/kg CsA twice a day on days 1–5.

progression ($n = 6$, 50%), patient withdrawal ($n = 4$, 33%), death on treatment ($n = 1$, 8%), and lost to follow-up ($n = 1$, 8%). The DLT-inevaluable patient voluntarily withdrew from the study early during cycle 1 of therapy, and the patient treated on the 10-day CsA schedule resumed treatment after resolution of toxicity and completed the study after 10 cycles, remaining in CR since cycle 4.

Table 3. Grade ≥3 AEs at least possibly related to any study agent.

Treatment	Dose level 1 ($n = 3$)				Dose level 2 ($n = 6$)			Dose level 3 ($n = 3$)			Total
Grade, *N* (%)	3	4	5	3+	3	4	3+	3	4	3+	3+
Neutrophil count decreased	0	1	0	1 (33)	1	5	6 (100)	0	1	1 (33)	8 (67)
White blood cell decreased	1	0	0	1 (33)	4	0	4 (67)	1	0	1 (33)	6 (50)
Anemia	1	0	0	1 (33)	2	0	2 (33)	1	0	1 (33)	4 (33)
Lymphocyte count decreased	1	0	0	1 (33)	1	0	1 (17)	1	1	2 (67)	4 (33)
Hypophosphatemia	0	0	0	0 (0)	2	0	2 (33)	1	0	1 (33)	3 (25)
Hyponatremia	0	0	0	0 (0)	1	0	1 (17)	2	0	2 (67)	3 (25)
Abdominal pain	0	0	0	0 (0)	1	0	1 (17)	0	0	0 (0)	1 (8)
Acidosis	1	0	0	1 (33)	0	0	0 (0)	0	0	0 (0)	1 (8)
Pneumonitis	0	0	1	1 (33)	0	0	0 (0)	0	0	0 (0)	1 (8)
Aspartate aminotransferase increased	0	0	0	0 (0)	1	0	1 (17)	0	0	0 (0)	1 (8)
Hypokalemia	0	0	0	0 (0)	1	0	1 (17)	0	0	0 (0)	1 (8)
Nausea	0	0	0	0 (0)	0	0	0 (0)	1	0	1 (33)	1 (8)
Vomiting	0	0	0	0 (0)	0	0	0 (0)	1	0	1 (33)	1 (8)
Fatigue	0	0	0	0 (0)	0	0	0 (0)	1	0	1 (33)	1 (8)
Hyperglycemia	0	0	0	0 (0)	0	0	0 (0)	0	1	1 (33)	1 (8)
Hypertension	1	0	0	1 (33)	0	0	0 (0)	0	0	0 (0)	1 (8)
Colitis	0	0	0	0 (0)	1	0	1 (17)	0	0	0 (0)	1 (8)
Weight loss	0	0	0	0 (0)	1	0	1 (17)	0	0	0 (0)	1 (8)
Constipation	0	0	0	0 (0)	0	0	0 (0)	1	0	1 (33)	1 (8)
Bone pain	0	0	0	0 (0)	0	0	0 (0)	1	0	1 (33)	1 (8)

Note: Dose level 1, 1.2 mg/kg brentuximab vedotin on day 1 and 5 mg/kg CsA twice a day on days 1–5; dose level 2, 1.8 mg/kg brentuximab vedotin on day 1 and 5 mg/kg CsA twice a day on days 1–5; dose level 3, 1.8 mg/kg brentuximab vedotin on day 1 and 7.5 mg/kg CsA twice a day on days 1–5.

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The death on study treatment was due to an episode of respiratory failure with alveolar hemorrhage in a patient with a known bleeding diathesis during cycle 4 of study treatment. There was no evidence of infection on bronchoscopy but pneumonitis could not be ruled out and thus the event was considered possibly related to treatment.

Efficacy

Among the 14 patients treated, two patients were not evaluable for response, including the 1 patient also inevaluable for DLT (due to early withdrawal and therefore too few CsA doses during cycle 1 for DLT or response assessment) and 1 of the 12 DLT-evaluable patients treated at dose level 2 (who terminated after one cycle without disease assessment due to patient withdrawal). The best overall response rate achieved in all treated patients who were evaluable for response (including the one patient treated at the 10-day CsA dosing) was 75% (9/12) with a CR rate of 42% (5/12). In the 11 DLT- and response-evaluable patients treated on the 5-day CsA dosing, the best overall response rate was 72% (8/11), with a best response of four CR (36%), four PR (36%), and three SD (27%). The best overall response rate for the 8 response-evaluable patients treated at dose levels 1 and 2 (dose level 3 was too toxic and not expanded) was 63% (5/8), with three CR (38%), two PR (25%), and three SD (38%). After protocol treatment, four of 14 patients proceeded to allogeneic HCT, and three patients received other chemotherapy. The median follow-up in survivors was 18.6 months (range, 7.1–27.6 months). In all treated patients, the median PFS was 4.8 months [95% confidence interval (CI), 2.5–13.6 months] and the 1-year PFS was 19% (95% CI, 1%–53%). The median OS was not reached, and the 1-year OS was 86% (95% CI, 54%–96%). In nine patients with evidence of objective response, the median DOR was 7.2 months (95% CI, 1.5–12.4 months).

Discussion

In this study we generated a brentuximab vedotin–resistant cell line from KMH2 Hodgkin lymphoma cells, in which MDR1 is upregulated, similar to our previous results with brentuximab vedotin–resistant Hodgkin lymphoma cell line L428-R (11). We then demonstrated that both CsA and VRP could restore sensitivity to brentuximab vedotin in these two cell lines. CsA was also effective *in vivo*, leading to reduced tumor growth in brentuximab vedotin–resistant KMH2 human xenograft mice when used in conjunction with brentuximab vedotin treatment. We showed that this was not due to downregulation of MDR1 expression as proven by unchanged levels of *MDR1* mRNA and protein in the presence of CsA. We also showed that CsA or VRP alone had no direct cytotoxic effect on Hodgkin lymphoma cell lines, and single agent CsA without brentuximab vedotin had no direct effect on tumors in our human xenograft mouse models. In addition, MMAE levels were elevated in the KMH2-R tumors of mice treated with the combination, but not in control tissues, which is consistent with specific targeting of CD30-positive cells by brentuximab vedotin. To further confirm our hypothesis that MDR1 upregulation is associated with resistance to brentuximab vedotin, we overexpressed exogenous MDR1 in L428-P cells, which conferred brentuximab vedotin resistance to the cells. Treatment with CsA restored sensitivity to brentuximab vedotin in these resistant cells, similar to L428-R. These results strongly support our hypothesis that overexpression of the ABC drug transporter MDR1/PgP, which exports MMAE out of the Hodgkin lymphoma cell, is a mechanism of resistance to brentuximab vedotin in Hodgkin lymphoma.

On the basis of our preclinical findings, we performed a phase I trial using brentuximab vedotin + CsA in patients with R/R Hodgkin lymphoma (NCT03013933). We initially had chosen a 10-day administration of CsA but, due to unacceptable toxicity observed in the first patient treated, we ultimately selected 5 days of CsA administration in conjunction with brentuximab vedotin. Five days of CsA allows concurrent exposure through approximately 2 half-lives of brentuximab vedotin, thereby potentiating antitumor effects while minimizing toxicities of prolonged administration of CsA. We found that dose level 2 (brentuximab vedotin 1.8 mg/kg on day 1, CsA 5 mg/kg twice a day on days 1–5) was the MTD, and at this dose level, the combination was tolerable and feasible. The combination does appear to have increased toxicity compared with brentuximab vedotin alone, including myelosuppression and gastrointestinal toxicities (nausea, constipation, abdominal pain, and anorexia) with resulting electrolyte abnormalities. While this may be related to nontumor tissue–associated MMAE exposure, our animal model showed that CsA clearly increased the MMAE concentration in tumor tissues but not in other organs. Therefore, this may be from the high dose of CsA used rather than diffusion of MMAE to nontumor tissues. We were encouraged by the high ORR and CR rate in such a heavily pretreated population, especially as the great majority of patients were previously refractory to brentuximab vedotin. Retreatment with brentuximab vedotin monotherapy in patients who previously responded to brentuximab vedotin and discontinued while in response is associated with a response rate of 53% to 60% (14, 15). One study evaluated an intensified dosing schedule of brentuximab vedotin monotherapy in a small cohort of patients who were refractory to brentuximab vedotin using criteria similar to ours. The ORR was only 13% (one patient with a PR) suggesting that brentuximab vedotin monotherapy retreatment in brentuximab vedotin–refractory patients is unlikely to be effective (16). The median DOR in responders to brentuximab vedotin + CsA was 7.2 months, which is comparable with single-agent chemotherapy such as bendamustine in this setting (17). The 1-year PFS in our small cohort was only 19%, suggesting that brentuximab vedotin retreatment combined with CsA may not yield a durable response in most patients. With the increasing use of brentuximab vedotin as part of first-line and salvage therapy for Hodgkin lymphoma, more patients with relapsed or refractory disease have brentuximab vedotin–refractory Hodgkin lymphoma. Although we did observe increased toxicity relative to brentuximab vedotin monotherapy, brentuximab vedotin retreatment in combination with CsA could potentially serve as a bridge to allogeneic HCT or other novel therapies (e.g., CAR T cells) or provide a treatment option in patients who are chemorefractory and refractory to brentuximab vedotin and PD-1 blockade. Patients who have failed standard chemotherapy approaches and who have also failed brentuximab vedotin and PD-1 blockade similar to the patients treated on this trial have very limited treatment options and developing effective treatments for these patients is a major unmet need. The toxicities we observed at the RP2D were not dose limiting, allowed for outpatient administration, and the treatment resulted in objective responses.

Strategies to overcome multidrug resistance by drug transporter pump inhibition have been the subject of intense research. CsA and VRP were among the first MDR modulators to be described. Multiple preclinical studies demonstrated that VRP and CsA could reverse MDR activity, enhance intracellular accumulation of chemotherapeutic agents, and resensitize drug-resistant cell lines to chemotherapies (18–24). Both CsA and VRP have been extensively tested with the goal of modulating PgP function and increasing sensitivity to chemotherapy in clinical trials of patients with solid and hematologic cancers (25). However, because high doses of inhibitors were often needed in humans to reverse MDR activity and were

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associated with toxicities (26, 27), second- (e.g., dexverapamil and valspodar) and third-generation (e.g., zosuquidar and tariquidar) MDR modulators were developed in an attempt to improve efficacy and reduce side effects. Unfortunately, despite promising preclinical activity, clinical trials with second- and third-generation modulators led to discouraging results and these drugs are no longer clinically available (28–32). In this study, the rationale to revisit the use of MDR modulators was the context of the targeted delivery of chemotherapy by an ADC, which minimizes off-target effects on normal cells. For practical purposes, we chose to use the clinically available agents CsA and VRP in our preclinical studies and clinical trial rather than a second- or third-generation MDR modulator. With the encouraging results from our work, one next possible step could be to utilize a newer generation MDR modulator to reduce toxicity.

Although MDR1 expression and other drug transporters have been implicated in drug resistance in other tumor types (33, 34), to our knowledge, MDR1 expression in Hodgkin lymphoma has not been demonstrated prior to our work. Other studies have demonstrated genetic alteration or IHC overexpression of other ABC transporters in Hodgkin lymphoma cell lines or patient samples that was associated with resistance to standard chemotherapies, but MDR1 expression was not present (35, 36). This is consistent with our findings, where MDR1 was not significantly expressed until Hodgkin lymphoma cells became resistant to brentuximab vedotin. It appears that in Hodgkin lymphoma, MDR1 expression may be relevant for resistance to brentuximab vedotin and not to standard chemotherapies.

Modulating multidrug resistance transporters to enhance ADC activity may be applicable to other ADCs directed against other malignancies. For example, increased multidrug transporter ABCC1 (MRP1) expression and reduced target antigen expression (Her2) were reported as the primary mediators of resistance in breast cancer cell lines made resistant to trastuzumab emtansine (T-DM1; ref. 37). Although our strategy of modulating MDR1 would be best suited to ADC-resistant tumors exhibiting MDR1 overexpression, a similar approach could be studied in situations where the cytotoxic payload is a substrate for the multidrug transporter (e.g., an MRP1 substrate in T-DM1–resistant breast cancer).

It should be noted in that in our preclinical *in vivo* models, while the rate of KMH2-R tumor growth was significantly lower in mice treated with brentuximab vedotin + CsA as compared with brentuximab vedotin alone, the magnitude of the difference was less than that observed *in vitro*. This suggests that there are other mechanisms of resistance *in vivo* not accounted for solely by MDR1 upregulation. Nevertheless, the objective responses observed with brentuximab vedotin + CsA in humans with brentuximab vedotin–resistant Hodgkin lymphoma who would not be expected to respond to brentuximab vedotin alone suggest that while enhanced MDR1 activity is not the only mechanism of resistance to brentuximab vedotin, it is an important one that can be therapeutically targeted. Patients still do progress after brentuximab vedotin plus cyclosporine, again confirming that other important mechanisms of resistance to brentuximab vedotin remain to be elucidated. The majority of patients in our study received PD-1 blockade as the most recent prior therapy to brentuximab vedotin + CsA. It has been postulated that PD-1 blockade may sensitize patients to subsequent therapy including traditional chemotherapy (38, 39). We cannot exclude such an effect in our patients, however, this is the reality of treating patients with Hodgkin lymphoma in the post-checkpoint blockade era and any novel therapy tested in R/R Hodgkin lymphoma will have to be considered through this lens. Of note, the best response in the 2 patients who had not received PD-1/PD-L1 therapy as the most recent therapy was PR and SD. A potential limitation to applying our brentuximab vedotin + CsA combination is the use of an immunosuppressant like CsA in Hodgkin lymphoma, which is known to have an immunosuppressive tumor microenvironment that plays an important role in the pathogenesis of the disease (40). On the basis of the clinical responses observed thus far in the clinical trial, the MDR1/PgP-modulating and thus brentuximab vedotin–potentiating effects of CsA may outweigh the effects of additional immunosuppression in the Hodgkin lymphoma microenvironment. Finally, while we have pursued modulation of cellular drug transport as an approach to combat ADC resistance, there are alternate approaches that may be applicable to brentuximab vedotin resistance in Hodgkin lymphoma, because downregulation of the CD30 target antigen does not appear to be a major resistance mechanism. One possible approach to overcome brentuximab vedotin resistance could be to simply change the cytotoxic payload, which has been shown to overcome auristatin-based ADC resistance in a B-cell lymphoma model (41).

In conclusion, we induced resistance to brentuximab vedotin in two Hodgkin lymphoma cell lines, demonstrated that brentuximab vedotin resistance is associated with upregulation of MDR1, and performed *in vitro* and *in vivo* studies that showed competitive MDR1/PgP inhibition could potentiate the antitumor activity of brentuximab vedotin in brentuximab vedotin–resistant Hodgkin lymphoma models. We then performed a phase I trial and determined that brentuximab vedotin in combination with MDR modulation using CsA was tolerable and produced antitumor responses in patients with brentuximab vedotin–refractory Hodgkin lymphoma. Our preclinical investigations in conjunction with our clinical trial data using brentuximab vedotin combined with cyclosporine to competitively inhibit MDR1 demonstrate proof of the concept that resensitization to brentuximab vedotin is a viable and effective strategy in Hodgkin lymphoma. The expansion phase of this phase I trial is currently enrolling patients who have brentuximab vedotin–refractory Hodgkin lymphoma to confirm the efficacy of this therapeutic approach.

Disclosure of Potential Conflicts of Interest

R. Chen is an employee/paid consultant for and reports receiving speakers bureau honoraria from Seattle Genetics. A.F. Herrera is an advisory board member/unpaid consultant for Bristol-Myers Squibb, Genentech, Merck, Adaptive Biotechnologies, Kite Pharma, and Gilead. No potential conflicts of interest were disclosed by the other authors.

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