

Development of a clinically relevant chemoresistant mantle cell lymphoma cell culture model

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Impact statement

Mantle cell lymphoma remains one of the deadliest subtypes of non-Hodgkin's lymphoma, in large part because patients become resistant to frontline chemotherapy. The development of strategies to treat advanced disease will be contingent upon testing in appropriate models. Most *in vitro* models of resistant mantle cell lymphoma are laboratory grade models that do not recapitulate the low level of chemoresistance typically observed in patients, limiting their utility. This study develops a clinically relevant *in vitro* model that can be used to establish the mechanisms of resistance and test new therapeutics intended to treat recurrent disease.

Abstract

Mantle cell lymphoma is an aggressive subtype of non-Hodgkin's lymphoma that claims the lives of tens of thousands of people every year. Although combination chemotherapy treatments such as CHOP have yielded promising outcomes in the clinic, the development of chemoresistance in patients has limited their long-term success. The lack of *in vitro* chemoresistance models has limited our ability to understand the mechanisms by which cells develop resistance, and thus our ability to develop novel therapeutics to overcome this issue. Here, we describe the development of a clinically relevant chemoresistant mantle cell lymphoma model using the JeKo-1 cell line. This was achieved through a stepwise treatment selection strategy using gradually increasing concentrations of CHOP. We show that resistant JeKo-1 cells display strong recovery and fast proliferation after treatment with an IC₅₀ dose of CHOP. We also found that resistant JeKo-1 cells overexpress three oncogenes implicated in the development of mantle cell lymphoma—Cyclin D1, Mcl-1, and Bcl-2—compared to normal JeKo-1 cells. We anticipate that *in vitro* models such as this one will enable the discovery of new therapeutic strategies for overcoming chemoresistance and improve clinical outcomes in mantle cell lymphoma patients.

Keywords: Chemoresistance, lymphoma, *in vitro* models, CHOP

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Introduction

Non-Hodgkin lymphoma is the seventh most common cancer in the United States, accounting for approximately 4% of all cancer diagnoses.¹ According to the American Cancer Society's estimates for 2018, about 75,000 people will be diagnosed with non-Hodgkin lymphoma, and ~19,000 people will succumb to the disease.² One subtype of non-Hodgkin lymphoma that is in particular need of new therapeutic approaches is mantle cell lymphoma.

Mantle cell lymphoma comprises 6% of all non-Hodgkin lymphoma cases in the United States. With a median age of diagnosis of over 60 years, many mantle cell lymphoma patients are unable to withstand intensive chemotherapy regimens.³ Furthermore, the disease is typically discovered in its later stages after metastasis to secondary sites such

as the gastrointestinal tract and the bone marrow.⁴ The defining characteristic of mantle cell lymphoma is the translocation of the gene cyclin D1 from chromosome 11 to chromosome 14, where it is placed in front of an immunoglobulin heavy chain enhancer. This leads to significant overexpression of cyclin D1 in over 90% of patients.^{4,5} As a cell cycle regulator, its overexpression causes rapid cell proliferation and oncogenesis.

The best treatment option for an individual mantle cell lymphoma patient often depends on the stage of the disease, age, and the overall health of the patient.^{4,6} Combination chemotherapy remains the standard treatment strategy, given the aggressive nature of mantle cell lymphoma. CHOP (Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednisone) is the most commonly used combination therapy, consisting of the

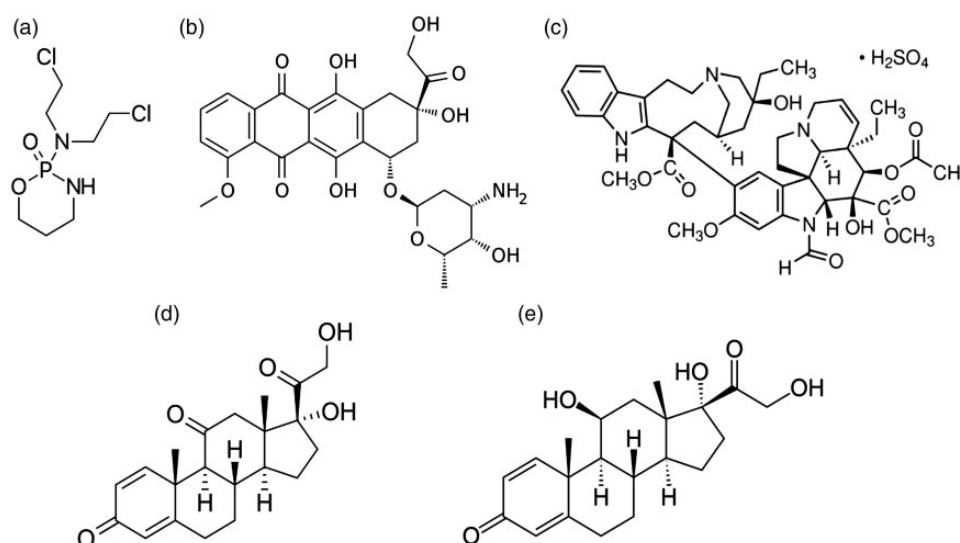


Figure 1. Structures of the drugs used in CHOP therapy. (a) Cyclophosphamide, (b) Doxorubicin, (c) Vincristine, (d) Prednisone, and (e) Prednisolone, the biologically active form of prednisone.

Table 1. Comparison of high-level laboratory and clinically relevant cell resistance models.

	High-level laboratory models	Clinically relevant models
Resistance concentration	IC ₇₀ –IC ₈₀	IC ₁₀ –IC ₄₀
Exposure time	Constantly in the presence of drug or on-off cycles	2–24 h with recovery in drug-free media
Resistance level	20–2000 fold	2–8 fold
Stability	Very stable	Unstable, needs to be exposed to several drug cycles
Disadvantage	Molecular changes occur that are not clinically relevant	Unstable

chemotherapeutics cyclophosphamide, doxorubicin (also known as hydroxydaunorubicin), vincristine (also known as oncovin), and prednisone (Figure 1).⁷ This combination treatment is often used in conjunction with the monoclonal antibody Rituximab (R-CHOP) for combined chemotherapy and immunotherapy.⁸

Although new treatment strategies have yielded encouraging results in the clinic, mantle cell lymphoma is still considered incurable. Common treatments such as R-CHOP have shown high response rates in patients, but the duration of response is often less than three years.^{4,9} Recurrence can be primarily attributed to the development of minimum residual disease and resistance to chemotherapy.¹⁰

Unfortunately, conventional cell culture and preclinical models of mantle cell lymphoma do not recapitulate the chemoresistance that develops in a typical patient. This limits the applicability of such studies in the treatment of advanced disease. To enable *in vitro* and *in vivo* testing that addresses the challenges of recurrent disease, the development of a clinically relevant chemoresistant mantle cell lymphoma cell line models is needed.

Currently, there are two categories of *in vitro* drug resistance models: high-level laboratory models and clinically relevant models. The characteristics of each model are summarized in Table 1.¹¹ In high-level laboratory models, cells are treated with a high concentration of chemotherapy

and are cultured in the continual presence of the drug(s). Cells developed with this method can display up to 2500-fold higher resistance compared to non-resistant (parental) cells.¹² Unfortunately, this extreme degree of resistance, together with the high drug doses involved, are not representative of the resistance observed in a clinical setting. Clinically relevant models, on the other hand, are developed by continuous on-off cycling of low drug concentrations. Cells developed in this way typically show between 2 and 8-fold higher resistance compared to parental cells.¹¹ However, resistance in these cells is typically unstable, meaning that consistent drug cycling is required to maintain resistance.

Here, we describe the development of a mantle cell lymphoma cell line that mimics the low-level resistance to frontline chemotherapeutics observed in clinical settings. Resistance was developed by treating JeKo-1 cells with increasing concentrations of the chemotherapy cocktail, CHOP. Using these resistant cells, we show increased tolerance to CHOP as well as strong recovery and rapid proliferation after exposure to an IC₅₀ dose of CHOP. Furthermore, we show that resistant cells overexpressed three genes—Cyclin D1, Bcl-2, and Mcl-1—implicated in the development of mantle cell lymphoma. Together these results demonstrate the development of a CHOP-resistant mantle cell lymphoma cell line that can be used to elucidate drug resistance mechanisms or to discover agents that combat drug resistance.

Materials and methods

Materials

JeKo-1 human mantle cell lymphoma cells were purchased from ATCC (Manassas, VA). Doxorubicin hydrochloride and vincristine sulfate were obtained from Alfa Aesar (Ward Hill, MA). Cyclophosphamide monohydrate was purchased from VWR (Radnor, PA). Prednisolone was purchased from Sigma Aldrich (St. Louis, MO). Ames rat liver S9 fraction (lyophilized) and S9 cofactor kit were purchased from Aniera (West Chester, OH). Roswell Park Memorial Institute (RPMI) Medium 1640, phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin/streptomycin, trypan blue reagent, siRNAs, and quantitative polymerase chain reaction (qPCR) primers were purchased from Thermo Fisher Scientific (Waltham, MA).

Cell culture

JeKo-1 cells were cultured in RPMI 1640 medium supplemented with 20% FBS, 20 U/mL penicillin, and 20 U/mL streptomycin at 37°C and with 5% CO₂ in a Heracell 240i CO₂ incubator (Thermo Fisher Scientific, Grand Island, NY).

Activation of cyclophosphamide using S9 mix

Cells were seeded at a concentration of 2.5×10^5 cells/mL and treated with cyclophosphamide at a concentration of 27.92 µg/mL for 22 h. Cyclophosphamide was activated using the S9 Cofactor Kit from Xenometrix (Allschwil, Switzerland) as per the manufacturer's instructions. Two strengths of "S9 mix" were prepared with either 15% or 30% S9 fraction. Twenty-two hours after cells were seeded, S9 mix was added to the cells to make up 10% of the final volume for a 2 h co-incubation in the presence or absence of cyclophosphamide. After this 2 h co-incubation, media was removed and cells were washed with PBS and cultured in drug-free media. Cell numbers were determined using the trypan blue reagent.

Growth curves in response to individual drugs

Cell growth was evaluated using the MTT assay. Cyclophosphamide, doxorubicin, and vincristine were dissolved in PBS. Prednisolone was dissolved in 100% DMSO and then diluted in PBS such that the final drug solution contained no more than 0.5% DMSO. Cells were seeded in 96-well plates at a concentration of 7.5×10^5 cells/mL and incubated for 24 h with various concentrations of either doxorubicin (0.28–72.6 µg/mL), vincristine (0.01–45.37 µg/mL), or prednisolone (0.07–71.97 µg/mL). At 24 h, some cells were assessed for viability, while the remainder were washed and incubated in fresh, drug-free media for another 24–48 h. Viability was determined again at total time points of 48 and 72 h. Cell viability was determined by adding 10 µL of MTT reagent to the cells for a 3-h incubation at 5% CO₂ and 37°C, followed by the addition of 100 µL detergent reagents overnight at room temperature. Data were collected by measuring the absorbance on a plate reader, and cell viability was determined by normalizing

the absorbance of treated samples to the untreated control. The cell growth curve for cyclophosphamide (6.98 µg/mL–55.84 µg/mL) was determined by cell counting using the trypan blue reagent. Cell viability was calculated by normalizing the cell count of treated samples to the untreated control. IC₅₀ values were identified as the drug concentration that corresponded to 50% cell viability.

Growth curves in response to combination CHOP therapy

For the combination CHOP therapy, the ratio of cyclophosphamide, doxorubicin, vincristine, and prednisolone was 80:5.5:0.16:11.1, respectively. Cells were seeded at a concentration of 2.5×10^5 cells/mL and incubated for 24 h with six concentrations of CHOP (0.4, 1.6, 6.4, 25.6, 102.4, and 409.6 µg/mL). At 22 h, 15% S9 mix was added to the cells such that it made up 10% of the final volume for a 2 h co-incubation. After the 2 h co-incubation, drug containing medium was removed and cells were washed with PBS and cultured in drug-free media. Cell numbers were determined by counting using the trypan blue reagent immediately following washing and every 24 h thereafter. IC₅₀ values were determined by assessing the drug concentration that corresponded to 50% cell viability.

Development of CHOP-resistant JeKo-1 cell lines

To develop CHOP-resistant cell lines, JeKo-1 cells were initially incubated with CHOP at a concentration of 0.4 µg/mL for a total of 24 h. At 22 h, 15% S9 mix was added to the cells such that it made up 10% of the final volume for a 2 h co-incubation. At 24 h, drug-containing medium was removed, and cells were washed with PBS and cultured in drug-free media. Cell numbers were determined using trypan blue reagent immediately following washing and every 24 h thereafter until the cells showed complete recovery to 100% viability. Cells were then treated with this drug concentration for several cycles of on-off CHOP regimen until cell viability remained between 85 and 90% after treatment. This established a first generation of cells resistant to the 0.4 µg/mL concentration. This process was then repeated using the same cells with CHOP concentrations of 4.8 µg/mL, 12 µg/mL, and 25.6 µg/mL to generate the second, third, and fourth generations of resistant cells.

Quantification of gene expression by quantitative PCR

JeKo-1 cells were seeded at a density of 2.5×10^5 cells/mL. Total RNA was isolated using the Qiagen RNeasy kit according to the manufacturer's instructions. Total RNA concentration was determined using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized by reverse transcriptase PCR using the high capacity cDNA reverse transcription kit (Thermo Fisher Scientific).

Quantitative PCR (qPCR) was performed on cDNA samples using the Taqman universal PCR master mix (Thermo Fisher Scientific). Each qPCR reaction was performed using a total of 100 µg of cDNA with 10 µL Taqman mastermix, 1 µL endogenous control primer, and 1 µL gene expression

primer (in a total volume of 20 μ L). All runs were performed using the ViiA 7 Real-Time PCR system in the comparative Ct mode using the following program: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The expression of Mcl-1, Bcl-2, and CCND1 was normalized to an endogenous control (GAPDH) and expression is shown relative to the expression in control samples.

Results

Development of treatment protocol for S9-activated cyclophosphamide in JeKo-1 cells

Three of the components of the CHOP regimen—doxorubicin, vincristine, and prednisone—are soluble in cell culture media and readily induce cytotoxicity in lymphoma cells. Cyclophosphamide, however, is a prodrug that requires the presence of liver enzymes for conversion into cytotoxic components. Specifically, the enzyme family cytochrome P450 converts cyclophosphamide into phosphoramidate mustard and alkylating metabolites such as 4-hydroxycyclophosphamide.¹³ Because hepatic activity is absent in an *in vitro* model, an alternative strategy is required for prodrug activation. This was accomplished using S9 fractions, which are tissue homogenates that contain biologically active cytochrome P450 enzymes. The mechanism for this is shown in Figure 2(a). In the presence of co-factors such as NADPH, glucose-6-phosphate, and a buffer salt solution, S9 fractions simulate hepatic activity and convert cyclophosphamide into its cytotoxic components.

As shown in Figure 2(b), cyclophosphamide alone did not induce toxicity in JeKo-1 cells. As such, S9 fractions were used for drug activation in this *in vitro* model. When co-incubated with cyclophosphamide for the final 2 h of a 24-h treatment, both the 15% and 30% S9 mixes induced potent cytotoxicity in JeKo-1 cells. There was no statistically significant difference in cell death using the two concentrations of S9 fractions. As a control, 15% and 30% S9 mixes were applied to cells alone in the absence of cyclophosphamide to determine their toxicity. Although the 30% S9 mix alone induced significant toxicity, the 15% S9 mix did not. As such, the 15% S9 mix was used in all subsequent experiments.

JeKo-1 dose response to CHOP and its components

Development of a chemoresistant cell line requires an understanding of how unexposed cells respond to treatment. This facilitates calculation of the inhibitory concentrations (e.g. IC₂₀, IC₅₀) needed to induce resistance. Towards this goal, we first assessed the cytotoxicity of the four CHOP components, individually, on JeKo-1 cells. Cells were treated with increasing concentrations of each drug for 24 h, after which the cells were washed and cultured in drug-free media. Cell viability was determined immediately after removing treatment and every 24 h thereafter to assess the response of JeKo-1 cells to these treatments.

Cells treated with doxorubicin and vincristine showed significant concentration-dependent growth suppression at

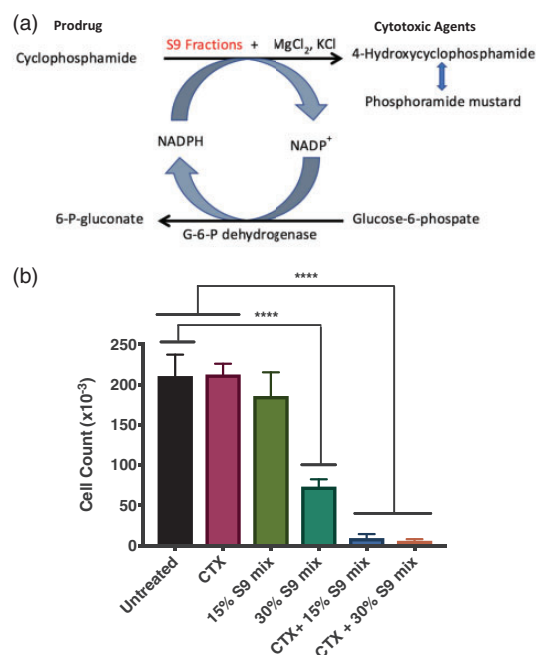


Figure 2. The cytotoxicity of cyclophosphamide was induced only in the presence of S9 mix. (a) Cyclophosphamide, a prodrug, is converted into 4-hydroxycyclophosphamide and phosphoramidate mustard, which are cytotoxic. In the presence of co-factors such as NADPH, S9 fractions simulate hepatic metabolism. (b) Cyclophosphamide (CTX) required S9 activation to induce JeKo-1 mantle cell lymphoma cell death. Cells were incubated with either cyclophosphamide (27.92 μ g/mL) or media for 24 h. Cells exposed to S9 mix were incubated with either 15% or 30% S9 mix for the last 2 h of CTX treatment. Cells were counted 24 h later. Cyclophosphamide alone did not result in cell death, but was extremely cytotoxic in the presence of 15% or 30% S9 mix. 30% S9 mix alone was also cytotoxic. Error bars represent s.d. ($n = 3$). **** $P < 0.0001$. (A color version of this figure is available in the online journal.)

24, 48, and 72 h post-treatment (Figure 3(a) and (b)). For both drugs, the growth curve shifted substantially to the left between the 24 and 48 h time points. IC₅₀ values for doxorubicin and vincristine, as determined by the 48-h curve, were 1.55 μ g/mL and 0.03 μ g/mL, respectively (Table 2). Prednisolone, the active form of the steroidal prodrug prednisone, did not induce dose-responsive cytotoxicity in JeKo-1 mantle cell lymphoma cells (Figure 3(c)). This is not unexpected, however, as steroids are used in chemotherapy cocktails to suppress the immune system and not to directly induce cytotoxicity.¹⁴

Cyclophosphamide inhibited cell growth at all of the tested doses at 48 h post-treatment (Figure 3(d)). At 48 h, the IC₅₀ value was calculated as 9.21 μ g/mL (Table 2). Five days after treatment, cells treated with the lowest 6.98 μ g/mL dose had recovered (data not shown). Although the colorimetric MTT assay was used to determine cell viability for doxorubicin, vincristine, and prednisolone, cell counting was used in the case of cyclophosphamide, as the S9 fractions influenced sample absorbance.

Finally, we explored the cytotoxicity of the combined CHOP therapy. In this experiment, CHOP was applied at the clinically used ratio of 80:5.5:0.16:11.1 (by weight) cyclophosphamide to doxorubicin to vincristine to prednisolone.¹⁵ We varied the CHOP concentration between 0.4 and 409.6 μ g/mL and examined cytotoxicity every 24 h for three days. As shown in Figure 4, we observed a

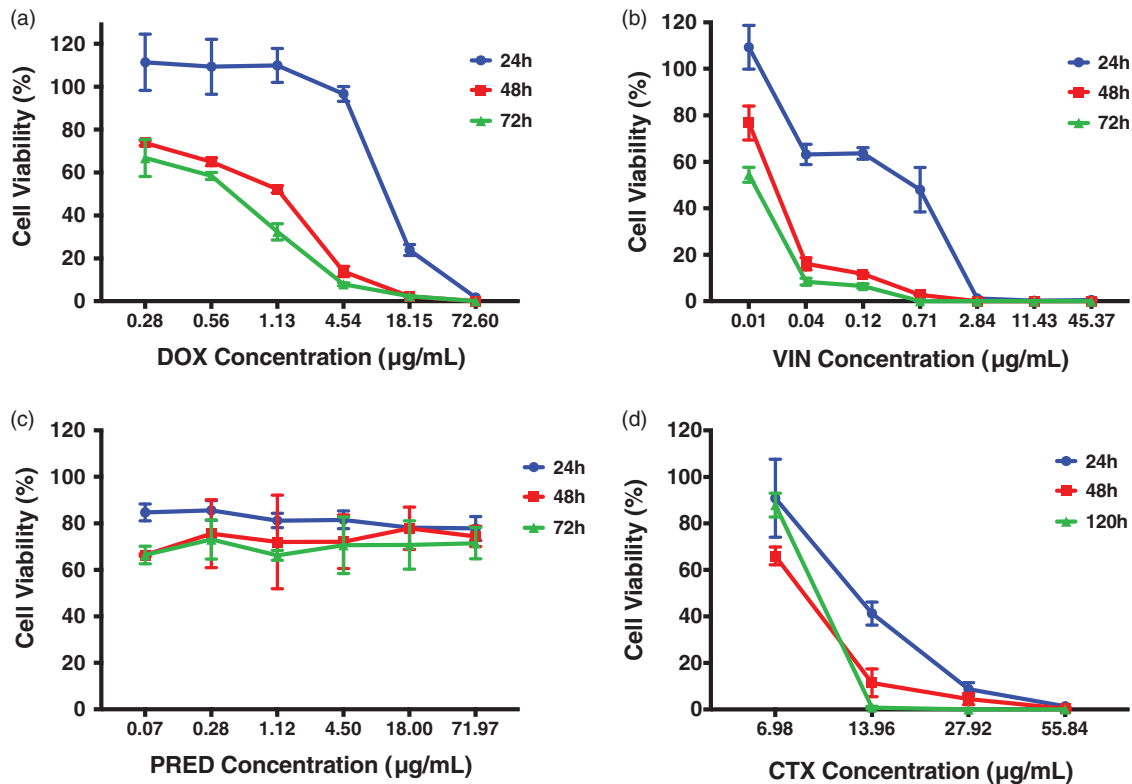


Figure 3. JeKo-1 cells were chemosensitive to all components of CHOP therapy except prednisolone. Cell viability for (a) doxorubicin, (b) vincristine, and (c) prednisolone treatments was determined by MTT assay. Cells were treated with increasing concentrations of drugs for 24 h, 48 h, or 72 h. Cell viability for (d) cyclophosphamide was determined by cell counting using the trypan blue reagent. Cells were treated with increasing concentrations of CTX for 24 h, with 15% S9 mix applied for the final two hours. At 24 h, drugs-containing medium were removed and replaced with fresh medium. Error bars represent s.d. ($n = 3$). (A color version of this figure is available in the online journal.)

Table 2. The 24 h IC_{50} values for doxorubicin, vincristine, cyclophosphamide, and the combination CHOP therapy were determined from the cell viability curves in Figures 3 and 4.

Doxorubicin ($\mu\text{g/mL}$)	Vincristine ($\mu\text{g/mL}$)	Cyclophosphamide ($\mu\text{g/mL}$)	CHOP ($\mu\text{g/mL}$)
13.42	0.65	13.02	4.8

dose-dependent suppression of cell growth at all three time points, with an IC_{50} value of 4.8 $\mu\text{g/mL}$ at 24 h (Table 2). Complete cell death was observed at the two highest doses of 102.4 and 409.6 $\mu\text{g/mL}$ at both 48 and 72 h post-treatment. No cell recovery was observed at 72 h using CHOP, even at the lowest dose, highlighting the synergistic effect of the combination therapy.

Development of CHOP-resistant JeKo-1 cells

To develop a clinically relevant resistance model, we subjected JeKo-1 cells to a stepwise treatment selection strategy, beginning with an IC_{20} CHOP dose (0.4 $\mu\text{g/mL}$) with component ratios as described above. This treatment process is depicted in the flow chart shown in Figure 5. After applying an initial IC_{20} dose to JeKo-1 cells for 24 h, cells were assessed for viability. If the cells were less than 85% viable, they were allowed to recover in drug-free media before being subject to a second IC_{20} dose. If the cells were more than 85% viable, they were deemed resistant

to the IC_{20} dose ("first generation" resistant cells). They were incubated in drug-free media before being advanced to an IC_{50} dose (4.8 $\mu\text{g/mL}$), when this process was repeated. This strategy ensured that after each CHOP treatment, resistant cells would compose the bulk of the population advancing to the next dose.

Growth behavior and recovery of resistant JeKo-1 cells

To study the behavior of resistant JeKo-1 cells, we looked at the proliferation and viability of second generation cells (resistant to a 4.8 $\mu\text{g/mL}$ dose) to CHOP. Cells were treated with CHOP at varying concentrations, and cell viability was analyzed 24 h post-treatment, immediately once the treatment was removed. As shown in Figure 6(a), the resistant cell growth curve shifted significantly to the right relative to parental JeKo-1 cells, indicating reduced cytotoxicity at all doses applied. The difference was most prominent at low CHOP doses, around the 4.8 $\mu\text{g/mL}$ concentration to which these cells were resistant. More importantly, the IC_{50} value increased from 4.8 $\mu\text{g/mL}$ in parental cells to 18.57 $\mu\text{g/mL}$ in resistant cells, representing nearly a 4-fold increase.

In addition to the increased IC_{50} value, the rate of recovery after CHOP treatment is another important consideration for resistant cells. Parental and resistant cells were treated with 4.8 $\mu\text{g/mL}$ CHOP (the IC_{50} for parental cells) for 24 h, after which cell numbers were determined by counting every day for five days. In parental cells, cell

numbers began decreasing after one day, and gradually continued to do so until day 5 where almost all cells were dead (Figure 6(b)). In contrast, resistant cells showed almost no decrease in numbers for the first three days

after treatment, after which strong recovery was observed. At day 4, cell counts had exceeded the pre-treatment numbers. By day 5, cells had rapidly proliferated, reaching almost double the initial cells count, highlighting the resistant behavior of these cells.

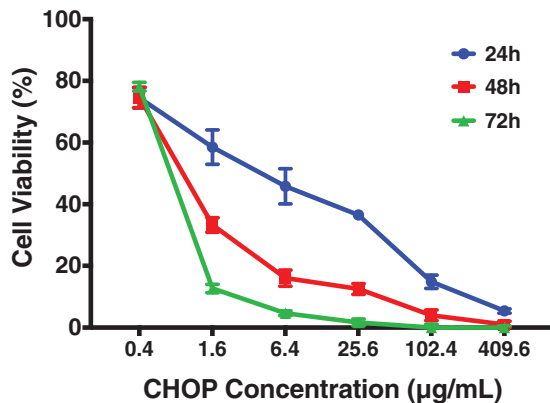


Figure 4. JeKo-1 cells showed dose responsive chemosensitivity to the combined CHOP therapy. Cells were treated with increasing concentrations of CHOP at the clinically relevant weight ratio of 80:5:5:0.16:11.1 (CTX:DOX:VIN:PRED) for 24 h; 15% S9 mix was added to the cells for the final 2 h of treatment (from 22 h to 24 h post-treatment). After 24 h, the drug-containing medium was removed and replaced with fresh medium. Cell growth was determined every 24 h by trypan blue reagent. Error bars represent s.d. ($n = 3$). (A color version of this figure is available in the online journal.)

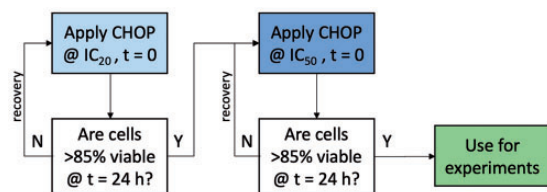


Figure 5. Resistant JeKo-1 cells were developed through a stepwise treatment selection strategy. Parental JeKo-1 cells were first treated with CHOP at the IC_{20} concentration of 0.4 µg/mL. After 24 h, cell counts were determined to see if viability remained between 85 and 90%. If cell viability was lower, cells were allowed to recover and the process was repeated with the same dose. If viability was between 85 and 90%, cells were allowed to recover before being treated with the IC_{50} concentration (4.8 µg/mL) when the cycling was repeated. (A color version of this figure is available in the online journal.)

Oncogene expression in resistant JeKo-1 cells

Having developed an *in vitro* mantle cell lymphoma model exhibiting clinically relevant CHOP resistance, we conducted gene expression analysis to better understand the molecular mechanisms underlying CHOP resistance. Specifically, we analyzed the expression of three genes implicated in the development of mantle cell lymphoma: Mcl-1, Bcl-2, and cyclin D1 (CCND1). As shown in Figure 7, we found that resistant cells (denoted R-JeKo-1, blue bars) significantly overexpressed all three of these oncogenes compared to parental JeKo-1 cells (red bars). Mcl-1 expression was 50% higher in resistant cells, while Bcl-2 and CyclinD1 expression increased by 38% and 11%, respectively.

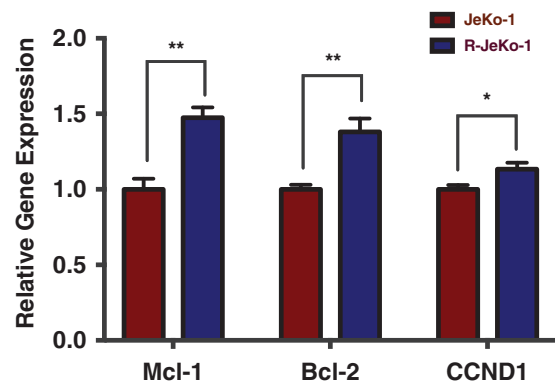


Figure 7. Resistant JeKo-1 cells overexpressed the three oncogenes Mcl-1, Bcl-2, and CCND1, which are implicated in mantle cell lymphoma. Relative gene expression was determined by extracting mRNA from parental and resistant JeKo-1 cells followed by qPCR analysis. Gene expression was quantified relative to the values for parental JeKo-1 cells. Error bars represent s.d. ($n = 5$). * $P < 0.05$ and ** $P < 0.01$. (A color version of this figure is available in the online journal.)

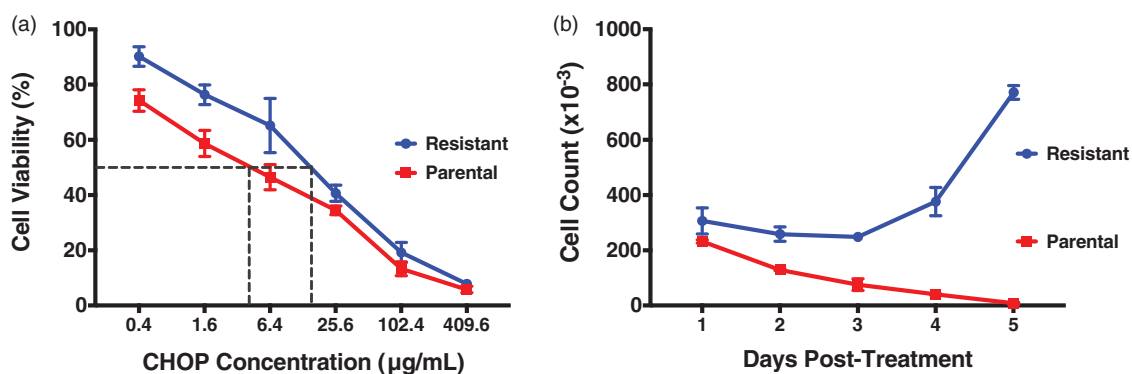


Figure 6. CHOP-resistant cells had a higher IC_{50} value and improved recovery after treatment. (a) Parental and CHOP-resistant JeKo-1 cells were treated with increasing concentrations of CHOP (between 0.4 and 409.6 µg/mL) for 24 h. Resistant cells had an IC_{50} of 18.57 µg/mL, whereas parental cells had an IC_{50} of 4.8 µg/mL. (b) Parental and resistant JeKo-1 cells were treated with 4.8 µg/mL CHOP. Cell numbers were determined every 24 h by trypan blue reagent. Resistant cells showed strong recovery after three days, while parental cell numbers continued decreasing. Error bars represent s.d. ($n = 3$). (A color version of this figure is available in the online journal.)

Discussion

Mantle cell lymphoma is an aggressive cancer that often affects the elderly, with a median age of diagnosis between 60 and 68 years.¹⁶ Over the last decade, new immunochemotherapy strategies such as R-CHOP have emerged that improve outcomes in older mantle cell lymphoma patients. Yet these treatment strategies often fail to produce complete remission due to the development of chemoresistance, which leads to rapid disease progression.¹⁷ As such, new therapeutic methods are urgently needed that address CHOP resistance. Here, we described the development of a clinically relevant *in vitro* CHOP-resistance model that mimics the low levels of resistance observed in a typical mantle cell lymphoma patient. This model stands in contrast to high-level laboratory models in which the cancer cells are stably resistant to extremely high doses of CHOP. Use of a clinically relevant resistance model will facilitate the identification cellular resistance mechanisms and novel therapeutic methods that address the unique challenges posed by low-level resistance to chemotherapy.

One of the first obstacles in studying CHOP *in vitro* was activating the prodrugs prednisone and cyclophosphamide, which require hepatic enzymes to be converted to cytotoxic metabolites. In the case of prednisone, we simply used its active form, prednisolone, in the CHOP mixture. In the case of cyclophosphamide, however, the direct use of its active forms 4-hydroxycyclophosphamide and phosphoramidate mustard are not viable options due to their low biological stability *in vitro*.^{13,18} As such, we took the approach of activating cyclophosphamide *in vitro* by mimicking hepatic activity using an S9 mix which contains the cytochrome P450 enzymes as well as the co-factors necessary for proper enzymatic activity. In this study, we applied the S9 mix for a 2-h co-incubation with cyclophosphamide, as previous studies have shown that longer exposure times to S9 mix may lead to deactivation of active metabolites.¹⁹ We used the 15% S9 mix in all of our studies because the 30% S9 mix displayed cytotoxicity in JeKo-1 cells (Figure 2(b)). Further, there was no difference in the efficacy of cyclophosphamide in the presence of 15% versus 30% S9 mix. No cytotoxicity was observed when cyclophosphamide was applied without S9 mix. These results are consistent with previous studies showing that the active form of cyclophosphamide is over 200-fold more effective than the prodrug.²⁰

While JeKo-1 cells displayed dose-dependent chemosensitivity to doxorubicin, vincristine, and cyclophosphamide, this was not the case for prednisolone (Figure 3). This, however, is not surprising given that prednisone is not a chemotherapeutic that induces cytotoxicity, but rather a corticosteroid that is used to suppress the immune system to improve the efficacy of chemotherapy and to reduce allergic reactions to CHOP.²¹ A study by Lesovaya *et al.*¹⁴ found that the use of the dexamethasone (a more potent steroid than prednisone) in JeKo-1 cells resulted in negligible inhibition of cell growth,¹⁴ which is consistent with our results (Figure 3(c)).

Our primary objective was to develop an *in vitro* mantle cell lymphoma model that displayed resistance to

conventional CHOP therapy. As previously mentioned, there are two main types of *in vitro* drug resistance models: clinically relevant models and high-level laboratory models. Although the high-level laboratory model is more stable, we chose to develop a clinically relevant model to mimic the conditions of resistance in patients as closely as possible. To develop this drug-resistant cell line, it was important to first analyze the response of parental cells to CHOP (Figure 4) to determine a suitable starting concentration. In doing so, we chose to begin the on-off selection strategy with a CHOP dose of 0.4 $\mu\text{g/mL}$, which is roughly the IC_{20} value at 24 h. The resistance level in a clinically relevant model is typically 2 to 8-fold higher than the parental cell. Using the second generation of resistant cells (resistant to 4.8 $\mu\text{g/mL}$ CHOP, which is the IC_{50}), we found that these cells were 4-fold more resistant than parental cells (Figure 6(a)). Furthermore, resistant cells displayed strong recovery three days after treatment with a 4.8 $\mu\text{g/mL}$ dose, with very fast proliferation observed between days four and five post-treatment. Although these cells exhibited a strongly resistant phenotype, the main disadvantage of a clinically relevant model is their instability. This means that they require regular dosing of CHOP in order to maintain their resistance.

We also wanted to utilize this model for elucidating a potential mechanism of CHOP resistance linked to the overexpression of oncogenes implicated in mantle cell lymphoma. While the overexpression of cyclin D1 is a hallmark of mantle cell lymphoma, increased expression of the anti-apoptotic proteins Mcl-1 and Bcl-2 is also involved in resistance to cell death.²² Here we showed that all three of these genes are overexpressed in resistant cells, suggesting a potential mechanistic factor in drug resistance.

In conclusion, we show here that combination CHOP therapy effectively inhibited cell growth in JeKo-1 cells. Using a stepwise treatment selection strategy beginning with the IC_{20} concentration, we developed several generations of JeKo-1 cells resistant to various CHOP concentrations. Second generation resistant cells had an IC_{50} value 4-fold higher than parental cells. These cells also displayed robust recovery and strong proliferation after treatment with CHOP. Using this cell line, we showed that resistant cells overexpressed the oncogenes Mcl-1, Bcl-2, and CCND1. We anticipate that this clinically relevant CHOP-resistant cell line can be used to further investigate resistance mechanisms and to facilitate the discovery of novel therapeutics for overcoming drug resistance to improve the treatment of patients with mantle cell lymphoma.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; JH, and KAH conducted experiments, KAH, and KAW wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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