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Over-expression of Mcl-1 confers multi-drug resistance while topo IIβ down-regulation introduces mitoxantrone-specific drug resistance in acute myeloid leukemia*

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Abbreviations

AML, acute myeloid leukemia; MOI: multiplicity of infection; SEM: standard error of the mean; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; topo, topoisomerase; ABC, ATP-binding cassette; Bcl-2; B-cell lymphoma 2; MEK, mitogen-activated protein kinase; shRNA, small hairpin RNA.

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ABSTRACT

Drug resistance is a serious challenge in cancer treatment and can be acquired through multiple mechanisms. These molecular changes may introduce varied extents of resistance to different therapies and need to be characterized for optimal therapy choice. A recently discovered small molecule, CXL017, reveals selective cytotoxicity towards drug resistant leukemia. A drug resistant AML cell line, HL60/MX2, also failed to acquire resistance to CXL017 upon chronic exposure and regained sensitivity towards standard therapies. In this study, we investigated the mechanisms responsible for HL60/MX2 cells' drug resistance and the molecular basis for its re-sensitization. Results show that the HL60/MX2 cell line has an elevated level of Mcl-1 protein relative to the parental cell line, HL60, and its re-sensitized cell line, HL60/MX2/CXL017, while it has a reduced level of topoisomerase IIβ. Mcl-1 over-expression in HL60/MX2 cells is mainly regulated through phospho-ERK1/2 mediated Mcl-1 stabilization while the reduction of topoisomerase IIβ in HL60/MX2 cells is controlled through genetic down-regulation. Up-regulating Mcl-1 introduces multi-drug resistance to standard therapies while its down-regulation results in significant cell death. Down-regulating topoisomerase IIβ confers resistance specifically to mitoxantrone, not to other topoisomerase II inhibitors. Overall, these data suggest that Mcl-1 over-expression is a critical determinant for cross-resistance to standard therapies while topoisomerase IIβ down-regulation is specific to mitoxantrone resistance.

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Introduction

Drug resistance is a serious problem in cancer therapy because it is an inevitable phenomenon among all malignancies during therapy treatment with no effective solution. For example, in acute myeloid leukemia (AML) although 60–80% of patients show an initial positive response to cancer therapies, only ~20% obtain long-term remission. The remaining patients relapse from residual disease that is typically drug resistant (Shipley and Butera, 2009). Therefore, there is an unmet clinical need for new therapies to treat drug-resistant malignancies.

One such mechanism is modulating a therapy-specific target/pathway, leading to reduction in damages induced by the therapy. For instance, cancer cells can mutate or down-regulate topoisomerase upon treatment with topoisomerase inhibitor to gain resistance (Harker *et al.*, 1991; Chen and Beck, 1995). Such resistance is unlikely cross-resistant to therapies with a different mechanism of action. Other mechanisms are more general that render cancer cells resistant to therapies of varied mechanisms, such as the over-expression of the anti-apoptotic Bcl-2 family proteins (Reed and Pellecchia, 2005; Kuroda and Taniwaki, 2009; Adams and Cory, 1998). Among the anti-apoptotic family members, Mcl-1 has been reported to be essential to drug resistance in AML (Glaser *et al.*, 2012; Breitenbuecher *et al.*, 2009; Kaufmann *et al.*, 1998). Another major mechanism for multi-drug resistance is the over-expression of ABC transporter proteins, such as p-glycoprotein. The over-expressed ABC proteins decrease the concentration of anticancer drugs in tumor cells via efflux, leading to multidrug resistance. Cancer cells can simultaneously utilize multiple mechanisms to acquire resistance (Wu and Singh, 2011; Fodale *et al.*, 2011; Deffie *et al.*, 1992). In order to design therapies that can effectively treat drug resistant malignancies, a detailed characterization of the molecular basis contributing to drug resistance is required.

We have recently developed an anticancer drug candidate – CXL017, derived from HA 14-1 (a putative anti-apoptotic Bcl-2 family protein inhibitor). CXL017 reveals selective cytotoxicity towards several multidrug resistant leukemia, including HL60/MX2 (Das *et al.*, 2009; Das *et al.*,

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2011; Aridoss *et al.*, 2012). HL60/MX2 was developed from HL60 cells through chronic exposure to mitoxantrone, a topo II inhibitor. As expected, drug resistant cancer cells over-express anti-apoptotic Bcl-2 family proteins (Das *et al*., 2009) and demonstrate cross-resistance to standard cancer therapies (Das *et al*., 2011; Aridoss *et al*., 2012). In the case of HL60/MX2, it over-expresses Mcl-1 protein (Das *et al*., 2009) and is cross-resistant to mitoxantrone, doxorubicin, etoposide, and ABT-737 – a classical Bcl-2 inhibitor (Aridoss *et al*., 2012). To explore CXL017's potential in preventing drug resistance development, HL60/MX2 cells were exposed to CXL017 for 6 months. The resulting cell line, HL60/MX2/CXL017, reveals no resistance towards CXL017 (Das *et al.*, 2013). In comparison, HL60/MX2 acquired >2000 fold stable resistance to cytarabine with a 6-month exposure and >20 fold stable resistance to ABT-737 with a 3-month exposure (Das *et al*., 2013). More strikingly, HL60/MX2/CXL017 was 10-100 fold more sensitive to standard therapies than HL60/MX2. These data suggest that CXL017 targets unique pathways to overcome drug resistance and to prevent its development. CXL017 exposure had no impact on ABC-transporter proteins or anti-apoptotic Bcl-2 family proteins except for Mcl-1, which was reduced in HL60/MX2/CXL017 cells (Das *et al*., 2013). These results suggest that Mcl-1 protein may be involved in the drug resistance of HL60 cells to standard therapies.

In this study, we have investigated the molecular mechanisms contributing to the drug resistance in HL60/MX2 cells and those responsible for the re-sensitization in HL60/MX2/CXL017 cells. Our data show that HL60/MX2 cells acquire part of their resistance to mitoxantrone via the down-regulation of topo IIβ. Interestingly, such resistance is mitoxantrone specific that it even introduces no resistance to other topo II inhibitors. Our data also show that Mcl-1 over-expression is the major contributor to the cross-resistance of HL60/MX2 cells and its reduction in HL60/MX2/CXL017 cells contributes to the re-sensitization. We further demonstrate that phospho-ERK1/2 mediated post-translational stabilization of Mcl-1 is one major mechanism that controls the level of Mcl-1 protein in these cells.

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Materials and Methods

Materials. CXL017 (ethyl-2-amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-*4H*-chromene-3-carboxylate) was synthesized as previously described (Das *et al*., 2009). ABT-737 was synthesized following published procedures (Oltersdorf *et al.*, 2005). Standard anticancer drugs (mitoxantrone, vincristine, etoposide, and doxorubicin), cycloheximide, MG-132, and U0126 were obtained from Sigma Aldrich (St. Louis, MO).

Cell lines and culturing conditions. HL60 and HL60/MX2 were obtained from ATCC. HL60/MX2/CXL017 cells were developed from HL60/MX2 (Das *et al*., 2013). HL60 cell line was grown in IMDM Glutamax media supplemented with 20% FBS. HL60/MX2 and HL60/MX2/CXL017 cell lines were grown in RPMI 1640 media supplemented with 10% FBS. All cells were maintained at 37ºC with 5% CO_2 in air atmosphere.

Cell viability assays. 48-hour cell viability was evaluated following an established procedure (Das *et al*., 2011). In brief, tumor cells were plated in a 96-well plate at a density of 1 x 10^4 cells/well. The cells were treated with a series of 3-fold dilutions of test compounds with final concentrations of 1% DMSO in the final volume. Cells treated with 1% DMSO served as controls. Cells were incubated for 48 hours at 37 °C, and the relative viability was measured using CellTiter-Blue from Promega (Madison, Wisconsin). IC_{50} values were determined by plotting the relative viability vs. the drug concentration and fitting to a sigmodial dose-response (variable slope) model in GraphPad Prism software (San Diego, CA). For Mcl-1 transient over-expression, drug treatment was 24 hours instead of 48 hours.

mRNA isolation and quantitative real-time PCR. Total RNA was isolated from cells using TRIzol Reagent from Invitrogen (Grand Island, NY) followed by PureLink RNA Mini kit from Life

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Technologies (Grand Island, NY) following manufactures protocols. The isolated RNA was quantified by measuring absorbance at 260 nm. Purity was assessed using A260/A280 nm. Q-RT PCR for genes of interested was performed by the Biomedical Genomics Center at the University of Minnesota (Minneapolis, MN) following established procedures. Data was processed using the $2^{-\Delta\Delta C}{}_T$ method as described (Livak and Schmittgen, 2001).

Topo IIβ and Mcl-1 shRNA transfection. 1 x 10^5 cells in 1 mL of complete media containing 5 µg/mL polybrene (Santa Cruz Biotechnology, Santa Cruz, CA) were plated in a 12-well tissue culture plate. Cells were transduced with lentivirus containing either scrambled shRNA or shRNA targeting topo IIβ at a multiplicity of infection (MOI) of 3. Lentivirus was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). After 8 hours, the cells were centrifuged and re-suspended in 1 mL of fresh media. 48 hours after transduction, cells were selected with 3 µg/mL puromycin for a period of 72 hours. 1-2 weeks after transduction, cells were analyzed via qRT-PCR to evaluate the extent of target down-regulation. Similar protocols were used for Mcl-1 as well.

Mcl-1 siRNA and open-reading frame transfection. Cells were transfected using the Neon electroporation system from Life Technologies (Grand Island, NY) following manufacture protocols. Briefly, 1 x 10^6 cells were suspended in 100 µL PBS buffer. Cells were electroporated with one pulse at 1350 V, pulse width of 35 ms, and then re-suspended in 2.5 mL media in a 6-well plate. Cells were either transfected with 12.5 nM siRNA (based on 2.5 mL re-suspension volume) from Life Technologies or 5 µg of plasmid containing the Mcl-1 open reading frame (SC315538) from OriGene (Rockville, MD). Down-regulation was assessed after 3 hours and up-regulation was assessed after 24 hours via Western Blotting.

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Western Blotting. Cells were lysed in RIPA buffer containing 1% protease inhibitor from Sigma Aldrich (St. Louis, MO) and the protein concentration was determined by BCA assay from Pierce (ThermoScientific, Rockford, IL). 40 µg of total protein was separated at 150 V on a NuPAGE 4-12% Bis-Tris Gel from Invitrogen (Carlsbad, CA). Proteins were then transferred at 40 V to a PVDF membrane from Millipore (Billerica, MA). Membranes were blocked in 5% milk in TBST for 1 hour at room temperature followed by incubation with primary antibody overnight at 4 °C. Membranes were washed 3 times in TBST and then incubated in the appropriate HRP conjugated secondary antibody (1:3000) for 3 hours at room temperature. Detection was performed using supersignal chemiluminescence system from Pierce (Rockford, IL). Mcl-1 S-19 antibody (1:400) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-ERK1/2 antibody and total ERK1/2 antibody (1:1000) were from Cell Signaling (Danvers, MA). β-actin antibody (1:40,000) and all secondary antibodies (1:2000 – 1:20,000) were from Sigma–Aldrich (St. Louis, MO). The relative level of a protein was quantified via densitometry using Image J software and corrected by β-actin level in the same sample.

The rate of Mcl-1 translation. HL60, HL60/MX and HL60/MX/CXL017 were incubated with cycloheximide (10 ug/ml) for 6 hours. Upon removal of cycloheximide, cells were treated with the proteasome inhibitor MG-132 (10 uM) for 1, 2, and 4 hours. Cell lysates were prepared as detailed above and Mcl-1 protein was analyzed by Western Blotting. In order to compare the absolute translation rate among these cell lines, different cell lysate samples were analyzed on the same SDS-PAGE and blotted under identical conditions.

The half-life of Mcl-1 protein. HL60, HL60/MX and HL60/MX/CXL017 cells were incubated with cycloheximide (10 ug/ml) for 1, 2, 4 and 6 hours. Cell lysates were prepared as detailed above and Mcl-1 protein was analyzed by Western Blotting. Densitometry was performed and GraphPad Prism 4 was used to fit the data and determine the half-life.

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The impact of ERK1/2 inhibition on Mcl-1 half-life. HL60/MX cells were incubated with U0126 (a MEK inhibitor, 3 uM) for 2 hours, followed by cycloheximide treatment (10 ug/ml) for 1, 2, 4 and 6 hours in the presence of U0126. Cell lysates were prepared as detailed above and Mcl-1 protein was analyzed by Western Blotting.

Statistic analysis. All biological experiments were performed at least twice with representative results shown in this report. Quantitative data are presented as means ± standard error of the mean (SEM), and comparisons were made using un-paired Student's *t* test in GraphPad Prism 4 (San Diego, CA). A p value of 0.05 or less was considered statistically significant.

Results

Topoisomerase IIβ is down-regulated in HL60/MX2 cells and up-regulated in HL60/MX2/CXL017 cells relative to HL60. Topoisomerase IIβ (topo IIβ) has been reported to be down-regulated in HL60/MX2 cells (Harker *et al*., 1991), which may contribute to HL60/MX2 cells' resistance to mitoxantrone and other topo II inhibitors. In order to validate the function of topo IIβ reduction in HL60/MX2 for its cross-resistance, as well as to explore its potential contribution to drug re-sensitization in HL60/MX2/CXL017 cells, quantitative real-time PCR (qRT-PCR) was performed to measure the mRNA levels of topo IIβ among these cell lines. HL60 cells were found to have a 12-fold increase in topo IIβ mRNA relative to HL60/MX2 cells (Fig. 1A). A 28-fold increase was observed in HL60/MX2/CXL017 cells (Fig. 1A).

Down-regulation of topoisomerase IIβ in HL60 and HL60/MX2/CXL017 leads to drug resistance specific to mitoxantrone. Next, small hairpin RNA (shRNA) was used to stably

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down-regulate topo IIβ in HL60 and HL60/MX2/CXL017 cells, respectively. Knockdown efficiency was measured by qRT-PCR. Levels of topo IIβ mRNA were reduced by 5-fold in HL60/TOP2B cells and 3-fold in HL60/MX2/CXL017/TOP2B when compared to their respective parental control cells with scrambled shRNA treatment (Fig. 1B). Nonetheless, HL60/TOP2B and HL60/MX2/CXL017/TOP2B still retained a 1.8- and 6-fold increase in the level of topo IIβ mRNA relative to HL60/MX2.

The transduced cell lines were then tested for their sensitivity to mitoxantrone. HL60/TOP2B and HL60/MX2/CXL017/TOP2B demonstrated a 3.0-fold and a 1.8-fold resistance to mitoxantrone, relative to their scrambled shRNA controls (Fig. 1C). To explore the potential contribution of topo IIβ reduction to cross-resistance in HL60/MX2 cells, the transduced cell lines were evaluated against a number of standard therapies as well as CXL017. Knock-down of topo IIβ had no effect on therapies with non-topoisomerase II mechanisms, including cytarabine, vincristine and CXL017. The topo IIβ knock-down cell lines also failed to exhibit resistance against two other topo II inhibitors – etoposide and doxorubicin (Table 1).

Mcl-1 protein is differentially regulated in HL60 and HL60/MX2/CXL017 for its reduced levels relative to HL60/MX2. Western blotting analyses revealed that the level of Mcl-1 protein in HL60/MX2 was ~10 fold higher relative to that in HL60 and was ~4 fold higher relative to HL60/MX2/CXL017 (Fig. 2A). qRT-PCR analyses were performed to determine whether Mcl-1 protein abundance was mediated through changes at the transcription level. Mcl-1 mRNA in HL60/MX2 cells was 2-fold higher relative to that in HL60 cells while Mcl-1 mRNA in HL60/MX2/CXL017 was ~6-fold higher (Fig. 2B). These data suggest that the low level of Mcl-1 protein in HL60/MX2/CXL017 cells is not transcriptionally regulated while Mcl-1 mRNA levels may partially account for the lower abundance of Mcl-1 protein in HL60 cells relative to HL60/MX2. Other mechanisms, such as the rate of translation or the stability of Mcl-1 protein,

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may be more important, particularly in HL60/MX2/CXL017 cells, where increased Mcl-1 transcription was observed but its protein level was reduced relative to HL60/MX2 cells.

Down-regulation of Mcl-1 in HL60/MX2 and HL60 cells results in cell death. In order to characterize the function of Mcl-1 protein in drug resistance, Mcl-1 protein was first attempted to be down-regulated in HL60/MX2 via the shRNA approach as demonstrated for topo IIβ. Massive cell death was observed during the transfection period when Mcl-1 shRNA was applied, suggesting Mcl-1 down-regulation leads to decreased cell survival in HL60/MX2 cells. Therefore, small interfering RNA (siRNA) was tried to transiently down-regulate Mcl-1 protein. As shown in Fig. 2C, Mcl-1 siRNA treatment successfully down-regulated Mcl-1 protein within 3 hours in both HL60 and HL60/MX2 cells. However, reduced cell viability was observed 2 hours after transfection in HL60/MX2 cells with nearly 80% reduction by 48 hours (Fig. 2D). The amount of viable cells in HL60 was also reduced upon Mcl-1 siRNA treatment although only a ~30% reduction was observed after 48 hours. These data suggest that HL60/MX2 cells are more dependent on its up-regulated Mcl-1 protein for survival and that Mcl-1 down-regulation is not a feasible approach to delineate its function in drug resistance in HL60/MX2 cells.

Transient over-expression of Mcl-1 in HL60 confers cross-resistance to standard therapies. Over-expression of Mcl-1 was then attempted in HL60 and HL60/MX2/CXL017 cells in order to evaluate its contribution to drug resistance. The over-expression of Mcl-1 in HL60/MX2/CXL017 cells was unsuccessful (data not shown), potentially because the reduction of Mcl-1 protein in HL60/MX2/CXL017 cells is not due to the shortage of Mcl-1 mRNA - HL60/MX2/CXL017 has the highest level of endogenous Mcl-1 mRNA (Fig. 2B). On the other hand, Mcl-1 protein was successfully over-expressed in HL60 cells (Fig. 3A). The transfected cells were evaluated for their sensitivity to a panel of therapies, including mitoxantrone, ABT-737 and CXL017. Mitoxantrone was selected for evaluation as a standard therapy while ABT-

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737 was selected because over-expression of Mcl-1 is known to induce resistance to ABT-737 (Oltersdorf *et al*., 2005; Mazumder *et al.*, 2012; Tromp *et al.*, 2012; Lucas *et al.*, 2012). CXL017 was evaluated to explore whether Mcl-1 may be less effective in conferring resistance to CXL017. As shown in Fig. 3B-D, HL60/Mcl-1 cells gained significant resistance to mitoxantrone (~8-fold) and ABT-737 (23-fold) while it conferred slight resistance to CXL017 (2-fold). Of note, drug treatment period was short (24 hours) because of the nature of transient Mcl-1 up-regulation, which resulted in incomplete dose-response curves.

HL60/MX2/CXL017 has the slowest rate while HL60 has the fast rate of Mcl-1 translation. Given that Mcl-1 protein abundance among these cells does not correlate with its transcriptional level, we next characterized the impact of Mcl-1 translation rate on protein abundance. These cells were first treated with cycloheximide, a eukaryotic translation inhibitor (Schneider-Poetsch *et al.*, 2010), for six hours to stop Mcl-1 synthesis and to allow for the existing Mcl-1 to degrade. Upon removing cycloheximide, newly synthesized Mcl-1 was allowed to accumulate in the presence of MG-132, a proteasome inhibitor, and quantified (Fig. 4A). Surprisingly, the translation rate for Mcl-1 was the highest in HL60 cells while that in HL60/MX2/CXL017 was slightly lower than HL60/MX2 cells, which can not account for their protein level differences. These data in combination with Mcl-1 mRNA information suggest that the abundance difference of Mcl-1 protein among these cell lines is likely controlled via post-translational regulation.

Mcl-1 has the shortest half-life in HL60/MX2/CXL017 cells. Among the anti-apoptotic Bcl-2 family proteins, Mcl-1 is unique that can be rapidly degraded via a proteasome-dependent mechanism (Inuzuka *et al.*, 2011). Its turnover rate is delicately regulated through various mechanisms, resulting in significantly different half-lives of Mcl-1 protein under different conditions (Thomas *et al.*, 2010). We therefore characterized the half-life of Mcl-1 protein

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among these cells. Cells were treated with cycloheximide for varying times and remaining Mcl-1 protein was quantified (Fig. 4B). The half-life of Mcl-1 in HL60/MX2 cells was the longest (2.1 hours) whereas HL60 cells had a slightly shorter Mcl-1 half-life (1.8 hours). The half-life of Mcl-1 in HL60/MX2/CXL017 cells (1.2 hours) was the shortest.

ERK1/2 activation contributes to the increased Mcl-1 stability in HL60/MX2 cells. Phospho-ERK1/2 has been reported to improve Mcl-1 stability (Liao *et al.*, 2011; Nishioka *et al.*, 2010). Therefore, the level of phospho-ERK1/2 in these cell lines was characterized (Fig. 5A). HL60/MX2 cells had an elevated level of phospho-ERK1/2 relative to HL60 cells while HL60/MX2/CXL017 cells completely lacked phospho-ERK1/2, despite similar levels of total ERK1/2 among these cell lines. Before investigating the role of phospho-ERK1/2 in stabilizing Mcl-1, we first explored whether phospho-ERK1/2 is essential to cell survival. HL60, HL60/MX2 and HL60/MX2/CXL017 were treated with U0126, a MEK inhibitor that leads to rapid inhibition of ERK1/2 phosphorylation (Fig. 5C). It was found that U0126 was equally cytotoxic to HL60 and HL60/MX2 cells but was non-toxic to HL60/MX2/CXL017 cells (Fig. 5B). These results suggest that phospho-ERK1/2 is essential to the survival of HL60 and HL60/MX2 cells and inhibiting ERK1/2 is equally cytotoxic in these cell lines and is independent of their endogenous Mcl-1 levels. HL60/MX2/CXL017, despite being more sensitive to standard therapies (Das *et al*., 2013), is uniquely resistant to ERK1/2 inhibition, likely because of its lack of phospho-ERK1/2-regulation of Mcl-1. These data suggest that phospho-ERK1/2 may be an up-stream regulator in controlling Mcl-1 abundance and function, possibly via influencing its stability. In order to characterize the impact of phospho-ERK1/2 on Mcl-1 stability, we pre-treated HL60/MX2 cells with U0126 for 30 min to achieve complete inhibition of ERK1/2 phosphorylation and then measured Mcl-1 stability, in the presence of U0126 to maintain complete ERK1/2 inhibition. As shown in Fig. 5C, the half-life of Mcl-1 in HL60/MX2 cells was reduced to ~ 1 hour upon ERK1/2 inhibition, similar to that in HL60/MX2/CXL017 cells. This

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suggests that the reduced half-life of Mcl-1 in HL60/MX2/CXL017 cells is primarily due to its lack of phospho-ERK1/2.

Discussion

CXL017 is a unique anticancer drug candidate, because of its selective cytotoxicity towards drug resistant leukemia and the inability of leukemia cells to acquire resistance. In addition, chronic CXL017 treatment re-sensitized leukemia cells towards standard therapies (Das *et al*., 2013). These data prompted investigation into the molecular mechanisms responsible for multidrug resistance and for CXL017 induced re-sensitization, using HL60 cells as the model system.

Previous work has characterized the reduction of topo IIβ in HL60/MX2 cells, which may contribute to drug resistance (Harker *et al*., 1991). Our results confirmed the reduction of topo IIβ in HL60/MX2 cells relative to HL60 and revealed a significant increase of its level in HL60/MX2/CXL017 (Fig. 1A). Upon down-regulation of topo IIβ, HL60/TOP2B cells gain a 3-fold resistance to mitoxantrone while HL60/MX2/CXL017/TOP2B cells gain a 1.8-fold resistance (Fig. 1C). The difference in the extent of resistance in these two cell lines likely results from the varied level of topo IIβ down-regulation in these cells (Fig. 1B). The limited level of resistance to mitoxantrone in both transduced cells may be partially due to the fact that topo IIβ level remained higher in HL60/MX2/CXL017/TOP2B cells and HL60/TOP2B cells relative to HL60/MX2 cells. Nevertheless, these data confirm that the down-regulation of topo IIβ in HL60/MX2 cells contributes to its resistance to mitoxantrone. The extent of resistance induced by topo IIβ knockdown to mitoxantrone is moderate compared to the 45-fold resistance observed in HL60/MX2 cells (Das *et al*., 2013), suggesting that alternative pathways contribute to mitoxantrone resistance in HL60/MX2 cells as well.

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The down-regulation of topo IIβ was also investigated for its role in cross-resistance. As expected, down-regulation of topo IIβ introduced no resistance to Ara-C, vincristine or CXL017 because of their topoisomerase-independent mechanisms (Table 1). Surprisingly, topo IIβ down-regulated cells revealed no resistance to two other topo II inhibitors, etoposide and doxorubicin, suggesting that topo IIβ is not essential for etoposide and doxorubicin. As these topo II inhibitors vary significantly in structure, they may have varied dependence on topo IIα and topo IIβ. Indeed, topo IIα seems to be more responsible for the anticancer activity of etoposide and doxorubicin than topo IIβ because several studies have demonstrated that topo IIα is down-regulated while topo IIβ remains unchanged in etoposide/doxorubicin resistant cancer cells (Matsumoto *et al.*, 2001; Andoh *et al.*, 1996; Mirski *et al.*, 1993; Jain *et al.*, 1996; Wessel *et al.*, 1997; Drake *et al.*, 1989; Meliksetian *et al.*, 1999; Eijdems *et al.*, 1995). Mirski et al. and Jain et al. also demonstrated that topo IIα-down-regulated etoposide-resistant cell lines reveal no cross-resistance to mitoxantrone (Mirski *et al*., 1993; Jain *et al*., 1996), indicating that topo IIα is not essential to the anticancer mechanism of mitoxantrone. Our data, together with these reports, suggest that mitoxantrone preferentially targets topo IIβ while etoposide and doxorubicin mainly target topo IIα. The independence of etoposide and doxorubicin on topo IIβ in HL60/MX2 cells is also consistent with the different extent of cross-resistance of HL60/MX2 to these topo II inhibitors (Das *et al*., 2013) and further supports topo IIβ-independent drug resistance mechanism.

Given the reported function of Mcl-1 in preventing apoptosis and in conferring drug resistance in AMLs (Glaser *et al.*, 2012; Kaufmann *et al*., 1998; Breitenbuecher *et al*., 2009), the over-expression of Mcl-1 in HL60/MX2 cells represented another possible resistance mechanism. Knockdown of Mcl-1 by siRNA resulted in 80% cell death in HL60/MX2 cells and 30% cell death in HL60 cells, revealing that Mcl-1 is essential to cell survival in HL60/MX2 and, to a lesser extent, in HL60 cells (Fig. 2D). The increased sensitivity in HL60/MX2 cells to Mcl-1

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siRNA treatment suggests that the elevated Mcl-1 protein is critical to the survival of HL60/MX2 cells and is potentially involved in multidrug resistance.

Transient over-expression of Mcl-1 in HL60 cells introduced cross-resistance to mitoxantrone and ABT-737 respectively (Fig. 3A – C). These data confirm the contribution of the over-expressed Mcl-1 protein to the cross-resistance observed in HL60/MX2 cells. On the other hand, HL60/Mcl-1 cells only showed a 2-fold resistance to CXL017 (Fig. 3D), further supporting the potential of CXL017 to overcome drug resistance induced by anti-apoptotic Bcl-2 family proteins.

Upon confirming the function of Mcl-1 in drug cross-resistance, we characterized the molecular basis for the different levels of Mcl-1 proteins among these leukemia cells. Despite the 10-fold increase in protein level, HL60/MX2 cells only had a 2-fold increase in Mcl-1 mRNA relative to HL60 cells (Figure 2 A&B). Furthermore, HL60/MX2/CXL017 cells expressed the highest level of Mcl-1 mRNA (~6 fold), but only had a 2.5-fold increase in protein levels relative to HL60 cells. As the translation rate of Mcl-1 protein was highest in HL60 cells than those in HL60/MX2 and HL60/MX2/CXL017 (Fig. 4A), Mcl-1 translation also does not explain the low abundance of Mcl-1 protein in HL60 and HL60/MX2/CXL017 relative to HL60/MX2 cells, indicating the contribution of post-translational regulation of Mcl-1 protein. We therefore determined the half-life of Mcl-1 protein among these cells (Fig. 4B) and found that HL60/MX2/CXL017 cells have a much shorter Mcl-1 half-life than HL60 and HL60/MX2 cells, likely contributing to its low static Mcl-1 level. The slightly shorter half-life of Mcl-1 in HL60 cells may contribute to its low abundance of Mcl-1 protein as well.

Consistent with previous report that phospho-ERK1/2 can increase Mcl-1 stability (Liao *et al*., 2011; Nishioka *et al*., 2010), the level of phospho-ERK1/2 was proportional to the half-life of Mcl-1 proteins among these HL60 cell lines (Fig. 5A and 4B). HL60/MX2 cells expressed the highest level of phospho-ERK1/2 and possessed the longest Mcl-1 half-life while HL60/MX2/CXL017 cells had the shortest Mcl-1 half-life with undetectable phospho-ERK1/2.

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We then confirmed that phospho-ERK1/2 is the key regulator for Mcl-1's stability since inhibiting ERK1/2 phosphorylation via U0126 treatment shortens the half-life of Mcl-1 in HL60/MX2 cells to the same level as that in HL60/MX2/CXL017 (Fig. 5C). Consistent with its impact on Mcl-1 stability and the protective function of Mcl-1 protein, U0126 also revealed selective cytotoxicity towards HL60 and HL60/MX2, but was completely non-toxic to HL60/MX2/CXL017 cells (Fig. 5B). These data overall support the mechanism by which Mcl-1 protein level is reduced in HL60/MX2/CXL017 cells and the role of phospho-ERK1/2 in stabilizing Mcl-1 in HL60/MX2 cells.

In summary, our study characterizes the mechanisms responsible for the resistance of HL60/MX2 to standard therapies and rationalizes the re-sensitization of CXL017 exposed HL60/MX2/CXL017 cells, which involves two pathways: (i) topo IIβ down-regulation, which confers resistance specific to mitoxantrone, and (ii) Mcl-1 over-expression, which introduces cross-resistance to various cancer therapies. Mcl-1 over-expression in HL60/MX2 cells mainly results from its increased stability. Re-sensitization in HL60/MX2/CXL017 cells is a result of increased topo IIβ gene expression and decreased Mcl-1 protein stability. The stability of Mcl-1 protein was mainly regulated by ERK1/2 in these cells, which is likely regulated by the MEK pathway (Huang *et al*., 2000). The topo II isoform knowledge that topo IIβ is the primary target for mitoxantrone while topo IIα is the primary target for doxorubicin and etoposide suggest that information about patients' topo II isoform should be considered for the selection of specific topo II inhibitors and that the combination of topo II inhibitors targeting both isoform may produce more pronounced anticancer effect. Research is ongoing to validate these discoveries in primary AML cell samples from patients.

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Authorship Contributions

Participated in research design: Hermanson and Xing

Conducted experiments: Hermanson, Li, and Das

Performed data analysis: Hermanson, Li, and Xing

Wrote or contributed to the writing of the manuscript: Hermanson, Li, Das, and Xing

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Footnotes:

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Figure Legend

Fig. 1. The levels of topo IIβ mRNA among HL60, HL60/MX2, and HL60/MX2/CXL017 cells and their impact to drug sensitivity. A. Q-RT-PCR analysis was performed on HL60, HL60/MX2 and HL60/MX2/CXL017 and normalized to the levels of HL60/MX2. Three independent experiments were performed. B. mRNA levels of topo IIβ in HL60/TOP2B, HL60/MX2/CXL017/TOP2B, and their scramble controls relative to that in HL60/MX2. Two independent experiments were performed. C. Drug sensitivity of HL60/TOP2B and HL60/MX2/CXL017/TOP2B to mitoxantrone relative to the scramble shRNA controls. Differences were analyzed using unpaired Students t-test. p-values: * <0.05 and ** <0.01.

Fig. 2. The levels of Mcl-1 among HL60, HL60/MX2, and HL60/MX2/CXL017 cells and the impact of Mcl-1 down-regulation on cell survival. A. Western Blot of protein levels of Mcl-1 in various cell lines. Densitometry was performed and normalized to β-actin levels as relative abundance to HL60 cells. Representative example of three independent experiments is shown. B. mRNA levels of Mcl-1 were analyzed by Q-RT-PCR of all three cell lines and are displayed relative to HL60. Three independent experiments were analyzed using students t-test with p-value shown. C. Western Blot of Mcl-1 down-regulation by siRNA in HL60 and HL60/MX2 cells as described in materials and methods. Control represents transfection with scrambled siRNA. Representative example of three experiments shown. D. Cell viability was determined by Cell Titer Blue of Mcl-1 siRNA transfected HL60 and HL60/MX2 cells. Data representative of three independent experiments.

Fig. 3. The impact of Mcl-1 up-regulation on drug sensitivity. A. Western Blot of Mcl-1 levels in HL60 cells 24 hours after transfection with Mcl-1 open reading frame. B – D. Drug sensitivity of HL60/Mcl-1 cells to mitoxantrone, ABT-737 and CXL017 relative to scrambled controls. Drugs were applied to cells 24 hours after transfection and drug treatment was 24 hours instead of typical 48 hours because Mcl-1 up-regulation was detectable 24 and 48 hours after transfection but not detectable 72 hours later. Data representative of three independent experiments.

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Fig. 4. The regulation of Mcl-1 protein among HL60, HL60/MX2, and HL60/MX2/CXL017 cells. A. Western Blot of Mcl-1 translation following addition of the proteasome inhibitor MG-132 for the indicated time points. Densitometry was performed and normalized to β-actin with relative abundance to time 0 reported. B. Cells were treated with the translation inhibitor, cycloheximide, for the indicated time points followed by Western Blotting for Mcl-1 levels. Densitometry was performed to on three independent experiments and half-life was determined using GraphPad Prism. Statistical analysis was performed using unpaired Students t-test. p-value: * <0.05.

Fig. 5. The regulation of Mcl-1 stability by ERK1/2 and the sensitivity of cells to U0126. A. The levels of phospho-ERK1/2 and total ERK1/2 as determined by Western Blot. Representative example of three blots shown. B. Drug sensitivity to U0126, a MEK inhibitor. C. The effect of U0126 treatment on the half-life of Mcl-1 in HL60/MX2 cells. Cells were treated with CHX alone or with U0126 pretreatment for 30 mins followed by CHX for varying time points. Densitometry was performed on Western Blots to determine Mcl-1 half-life in GraphPad Prism. Three independent experiments were performed and unpaired students t-test was applied. p-value: * < 0.05.

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Table 1. Drug sensitivity (IC_{50}) of HL60/TOP2B, HL60/MX2/CXL017/TOP2B and their scrambled shRNA controls to different therapies.

	HL60/Ctrl	HL60/TOP2B	HL60/MX2/ CXL017/Ctrl	HL60/MX2/ CXL017/TOP2B
Mitoxantrone (nM)	14 ± 3	44 ± 1	6.7 ± 0.5	10.7 ± 0.2
CXL017 (μM)	10 ± 1	10 ± 0.2	3.5 ± 0.1	3.5 ± 0.1
Etoposide (μM)	1.7 ± 0.4	2.5 ± 0.2	0.23 ± 0.04	0.27 ± 0.01
Doxorubicin (μM)	0.15 ± 0.05	0.18 ± 0.03	0.036 ± 0.003	0.042 ± 0.006
Cytarabine (μM)	5.6 ± 0.5	3.8 ± 0.1	0.034 ± 0.006	0.044 ± 0.006
Vincristine (nM)	2.8 ± 0.2	2.5 ± 0.5	0.60 ± 0.01	0.65 ± 0.05

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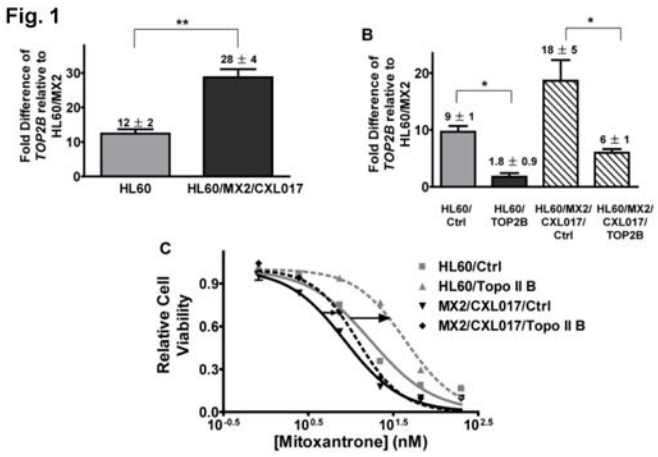

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Fig. 2

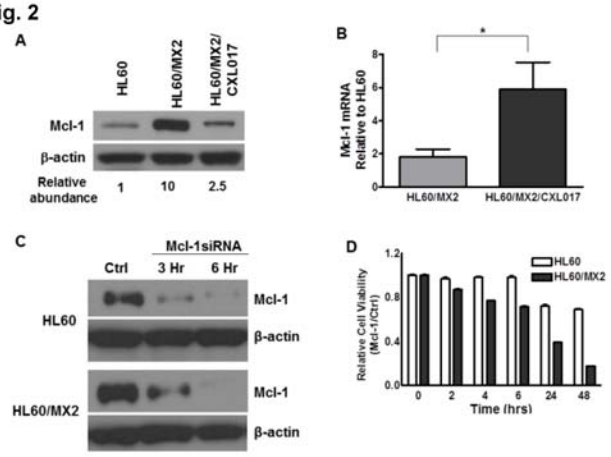

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Fig. 3

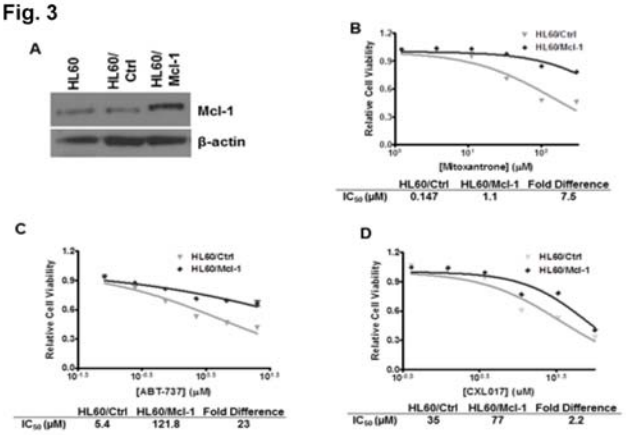

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Fig. 4

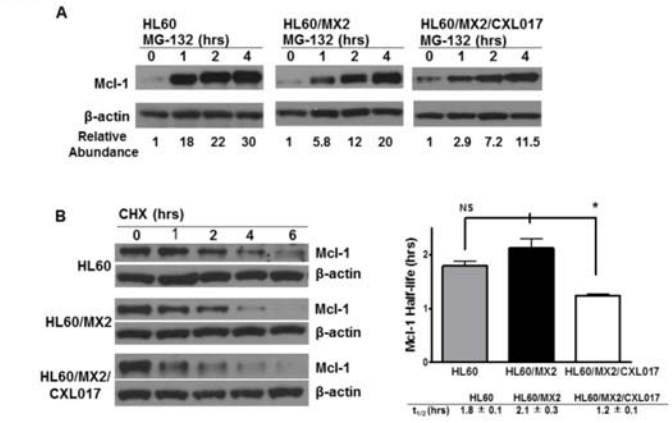

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Fig. 5