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Ethyl 2-Amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (CXL017): A Novel Scaffold That Resensitizes Multidrug Resistant Leukemia Cells to Chemotherapy

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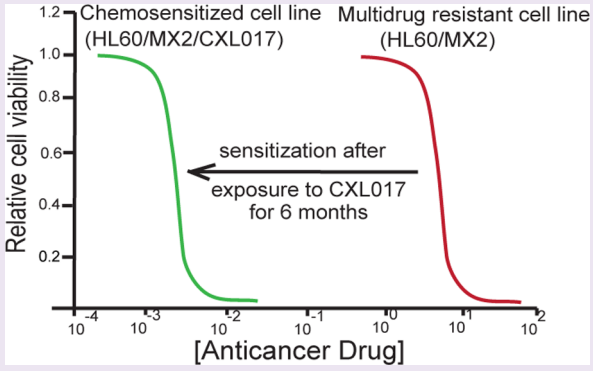
Supporting Information

ABSTRACT: Multidrug resistance (MDR) is a major hurdle in the treatment of cancer, and there is a pressing need for new therapies. We have recently developed ethyl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (CXL017), derived from a dual inhibitor of Bcl-2 and SERCA proteins, sHA 14-1, with selective cytotoxicity toward MDR cancer cell lines *in vitro*. In this study, we present new evidence for its therapeutic potential in treatment of MDR cancers and offer mechanistic insights toward its preferential targeting of drug-resistant cancer. CXL017 selectively suppressed the growth of tumors derived from the MDR cancer cell line, HL60/MX2, *in vivo*. In addition, even after chronic exposure to CXL017, HL60/MX2 failed to develop stable resistance to CXL017, whereas it acquired >2000-fold resistance to cytarabine (Ara-C), the major first-line chemotherapy for the treatment of acute myeloid leukemia (AML). Remarkably, instead of acquiring further cross-resistance, HL60/MX2 cells exposed to CXL017 were resensitized to standard therapies (10- to 100-fold). Western blotting analyses revealed that CXL017 exposure significantly down-regulated Mcl-1 and Bax and up-regulated Noxa, Bim, Bcl-X_L, SERCA2, and SERCA3 proteins, along with a reduction in endoplasmic reticulum (ER) calcium content. Given the well-established functions of Bcl-2 family proteins and ER calcium in drug resistance, our results suggest that the down-regulation of Mcl-1 and the up-regulation of Noxa and Bim along with the decrease in ER calcium content are likely responsible for CXL017-induced resensitization of MDR cancer cells. These data also demonstrate the unique potential of CXL017 to overcome MDR in cancer treatment.



The rapid development of drug resistance is a major obstacle in the treatment of cancer. Multidrug resistance (MDR) is a common form of acquired drug resistance in which cancer cells, upon exposure to a single chemotherapeutic drug, develop resistance to a variety of drugs that are structurally and mechanistically unrelated. Due to the rapid development of MDR, many patients do not respond well to chemotherapy and often relapse with a more aggressive form of cancer. Therefore, there is a need for new anticancer agents that can selectively target MDR cancers and ideally resensitize them to standard therapies.

Cancer cells can acquire MDR through various mechanisms. One major mechanism is the overexpression of ATP-binding cassette (ABC) transporter proteins, such as p-glycoprotein (P-gp).[1−7] Tumor cells with elevated ABC transporter proteins can decrease the intracellular concentration of anticancer drugs through ABC transporter-mediated enhanced efflux, leading to MDR. The other major mechanism is through altering the apoptotic pathway. Apoptosis is a programmed cell death process regulated by a variety of cellular signals. The two main apoptotic pathways are the intrinsic and extrinsic pathways.[8−10] The intrinsic pathway is of particular interests because a majority of chemotherapeutic drugs are believed to rely on this pathway to induce

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programmed cell death among cancer cells.[11−16] B-cell lymphoma-2 (Bcl-2) family proteins play an important role in the intrinsic apoptosis, consisting of anti-apoptotic members, such as Mcl-1, Bcl-2, and Bcl-X_L, and pro-apoptotic members, including Bax, Bad, Bak, Bid, Bim, and Noxa. The anti-apoptotic and pro-apoptotic members of the Bcl-2 family proteins antagonize each other to control cytochrome *c* release from mitochondria and to determine the fate of a cell − death or survival.[17] As key regulators that prevent programmed cell death, the anti-apoptotic Bcl-2 family proteins are overexpressed in 60−90% of all cancers,[18,19] being considered as one general mechanism by which cancer cells gain resistance against standard therapies.

Results of recent studies have revealed that the Bcl-2 family proteins also localize on other cellular compartments besides mitochondria, including the nuclear envelope and endoplasmic reticulum (ER).[20−22] Although the function of the Bcl-2 family proteins on the nuclear envelope is not fully understood, there have been some insights into their role on the ER membrane. Calcium release from the ER can act as a secondary messenger for cell death signaling, which involves multiple communication loops between ER and mitochondria that help regulate apoptosis.[23,24] The ER membrane-localized Bcl-2 family proteins regulate calcium homeostasis and subsequently, programmed cell death, by interacting with various ER calcium transporters. For instance, Bcl-X_L and Bax can interact with and regulate the inositol triphosphate receptors (IP_3Rs), which release ER Ca^{2+} into the cytosol.[25,26] Some reports also suggest that Bcl-2 interacts with and regulates the function of sarco-endoplasmic reticulum ATPase (SERCA) pumps, which transport cytosolic Ca^{2+} into the ER.[27−29] Given that the overexpression of the anti-apoptotic Bcl-2 family proteins and dysregulation in Ca^{2+} homeostasis have both been reported to be responsible for MDR in cancer,[30−33] simultaneously regulating both pathways may provide an effective strategy to overcome drug resistance in cancer.

We previously reported the discovery of sHA 14-1 (Figure 1), a dual inhibitor of Bcl-2 and SERCA.[34] Recently, we developed

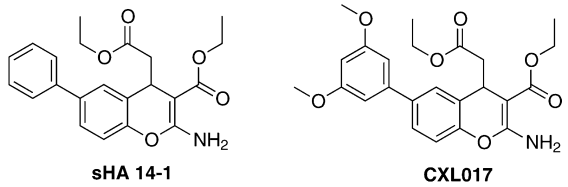

Figure 1. Chemical structures of sHA 14-1 and CXL017.

ethyl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (CXL017) (Figure 1), a more potent analogue of sHA 14-1, which demonstrates increased cytotoxicity toward a variety of MDR cancer cell lines as well as synergism with a wide range of standard therapies.[35,36] The improved potency observed in MDR cancer cell lines, also known as collateral sensitivity (CS), is a relatively rare phenomenon in cancer therapy, and the mechanisms leading to CS are largely unknown. Given its synergistic and CS properties, CXL017 harbors the potential to improve the treatment response of cancer patients.

In this report, we show that CXL017 demonstrates increased efficacy toward tumors inoculated from HL60/MX2, an MDR cell line, in a xenograft mouse model. Moreover, the HL60/MX2 cell line fails to develop stable resistance to CXL017, whereas it develops stable resistance (>2000-fold) to cytarabine (Ara-C) and ABT-737 (a representative Bcl-2 inhibitor). More significantly, upon long-term exposure to CXL017, the HL60/MX2 cell line becomes hypersensitive to standard therapies, such as cytarabine, doxorubicin, mitoxantrone, and etoposide. Molecular characterization of CXL017-treated HL60/MX2 cell lines reveals significant changes among several Bcl-2 family proteins, SERCA proteins, and ER calcium content. These changes may be responsible for CXL017-induced resensitization of drug-resistant cancer cell lines.

RESULTS AND DISCUSSION

CXL017 Selectively Targets Drug-Resistant Tumors *in Vivo*. Previously, we have reported the design and synthesis of 4*H*-chromene analogues based on sHA 14-1, a dual Bcl-2 and SERCA inhibitor, culminating in the development of CXL017 with an average IC_{50} value of 1 μM among NCI 60 cancer cell lines.[35,36] In addition, a variety of MDR cancer cell lines demonstrated collateral sensitivity toward CXL017 *in vitro*.[35,36] One example is HL60/MX2 MDR cancer cells, which were 4-fold more sensitive to treatment of CXL017 than the parent HL60 cells. Intrigued by CXL017's increased potency toward HL60/MX2 cells *in vitro*, we investigated its potential to selectively target drug resistant tumors *in vivo*.

For this purpose, we developed a xenograft mouse model by subcutaneously inoculating HL60/MX2 (right flank) and HL60 cells (left flank) into the same mouse. The tumors induced by HL60/MX2 cells were allowed to grow to a size of ∼40 mm^3, followed by treatment with CXL017 for a period of 3 weeks at a dose of 100 mg/kg/day for 6 days/week. The body weights of the mice were monitored during the course of the treatment. No significant differences were observed between control mice and mice with CXL017 treatment (Figure 2A). Our results show that CXL017 treatment significantly suppressed the growth of tumors derived from HL60/MX2 cells when compared to vehicle control (Figure 2C), whereas it had no effect on the growth of tumors derived from HL60 cells (Figure 2B). Thus, CXL017 demonstrates selective anticancer activity toward MDR cancer cell lines both *in vitro* and *in vivo*, supporting its potential as a candidate for further development. The lack of efficacy of CXL017 on HL60 derived tumors may be due to its limited serum concentration in mice, the peak of which was higher than the IC_{50} for HL60/MX2 but lower than the IC_{50} for HL60 (Supplementary Figure 1).

HL60/MX2 Overexpresses Bcl-2 Family Proteins and SERCAs but Not P-gp nor BCRP. The fact that CXL017 selectively eliminates HL60/MX2 MDR cells *in vitro* and *in vivo* led us to investigate the mechanism by which CXL017 may preferentially target HL60/MX2 cells. We first characterized HL60 and HL60/MX2 cell lines for the levels of Bcl-2 family proteins and ABC-transporter proteins because of their established functions in MDR. It has been previously reported that the HL60/MX2 cell line demonstrates an atypical MDR phenotype, *i.e.*, it does not overexpress P-gp. However, no positive control for P-gp was used in that study, and no other ABC members had been analyzed.[37] We therefore evaluated the levels of P-gp and BCRP in HL60 and HL60/MX2 cells *via* flow cytometry. As shown in Figure 3A and B, there were no significant differences in P-gp or BCRP expression between HL60/MX2 and HL60. We have also demonstrated that there is no significant difference of mitoxantrone cellular accumulation between these two cell lines (Supplementary Figure 2C and detailed later), suggesting that ABC transporter proteins do not mediate MDR in the HL60/MX2 cell line.

We had previously characterized the levels of three anti-apoptotic proteins in HL60 and HL60/MX2 cell lines, Bcl-2,

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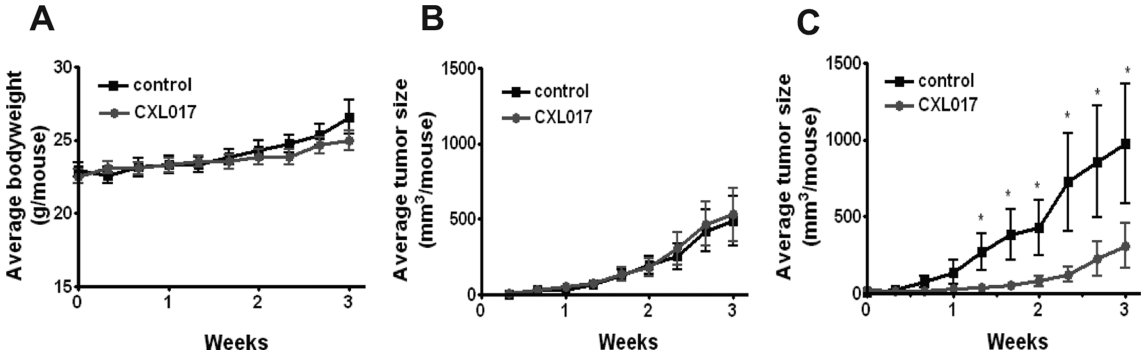

Figure 2. Effect of CXL017 (100 mg/kg/day i.p.) on the growth of HL60 and HL60/MX2 inoculated tumors in athymic nude mice. Each mouse was inoculated subcutaneously with HL60/MX2 (right flank) and HL60 cells (left flank). HL60/MX2 inoculated tumors were allowed to grow to the size of 40 mm^3, and mice were randomized into two groups. Control mice received vehicle, whereas treated mice received 100 mg/kg/day of CXL017 for 3 weeks, 6 days/week. (A) Bodyweight record of mice. Points, average body weight for each group (n = 10); bars, SEM. (B) Tumor size record induced by HL60. Points, average tumor volumes for each group (n = 10); bars, SEM. $P > 0.05$ for the comparison between treated and control groups. (C) Tumor size record induced by HL60/MX2. Points, average tumor volumes for each group (n = 10); bars, SEM; *, $P < 0.05$ for the comparison between treated and control groups.

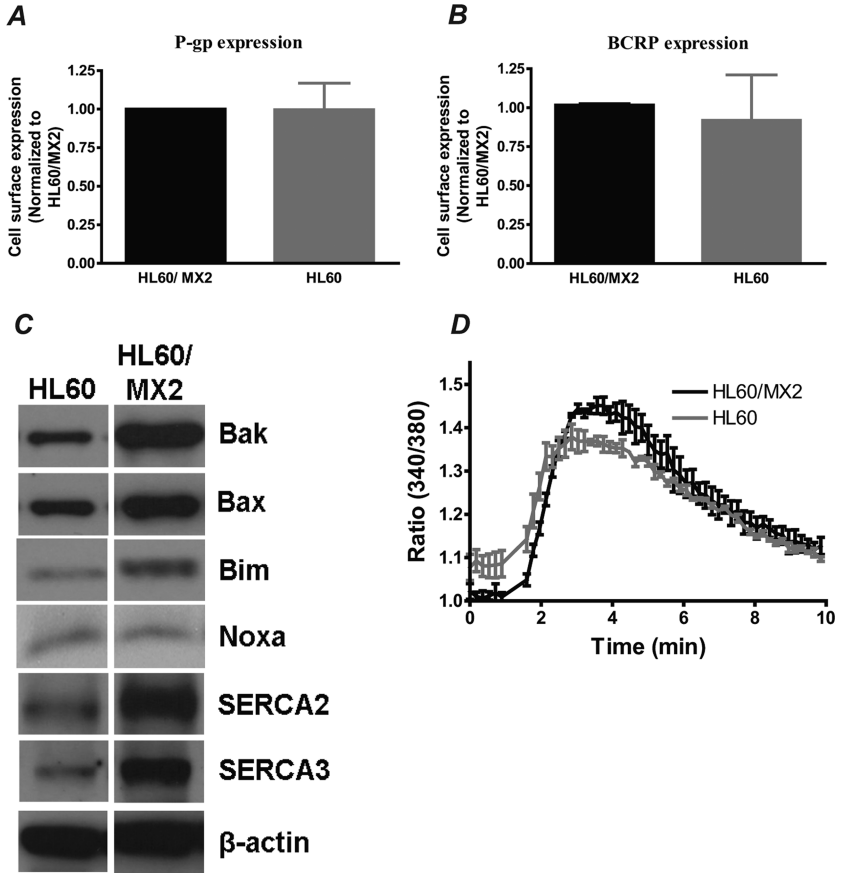

Figure 3. Characterization of HL60 and HL60/MX2 cell lines. (A) HL60 and HL60/MX2 cells were treated with PE conjugated anti-P-gp for 1 h. The cells were then washed, fixed, and analyzed using FACS caliber. Three independent experiments were averaged, and the figure depicts the fold difference of P-gp expression between HL60 and HL60/MX2. (B) HL60 and HL60/MX2 cells were treated with PE conjugated anti-BCRP for 1 h. The cells were then washed, fixed, and analyzed using FACS caliber. Three independent experiments were averaged, and the figure depicts the fold difference of BCRP expression between HL60 and HL60/MX2. (C) Total cell lysates from HL60 and HL60/MX2 were separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-SERCA2, SERCA3, Bax, Noxa or rabbit polyclonal anti-Bak and Bim. Mouse monoclonal anti-β-actin was used as a protein loading control, and the β-actin blot shown herein was from the SERCA3 experiment as a representative. Three independent experiments were conducted with similar results. (D) ER calcium content in HL60/MX2 cells and that in HL60. ER calcium levels were measured in HL60/MX2 and HL60 cells as described in Methods. Three independent experiments were averaged with SEM shown.

Bcl-X_L, and Mcl-1. Our results showed that HL60/MX2 cells greatly overexpress Mcl-1 protein, which may play a role in its MDR phenotype.[35] Here, we further characterized these cell lines for the expression levels of several pro-apoptotic Bcl-2 family proteins (Bax, Bak, Bim, and Noxa). Contrary to our expectation, pro-apoptotic proteins such as Bax, Bak, and Bim

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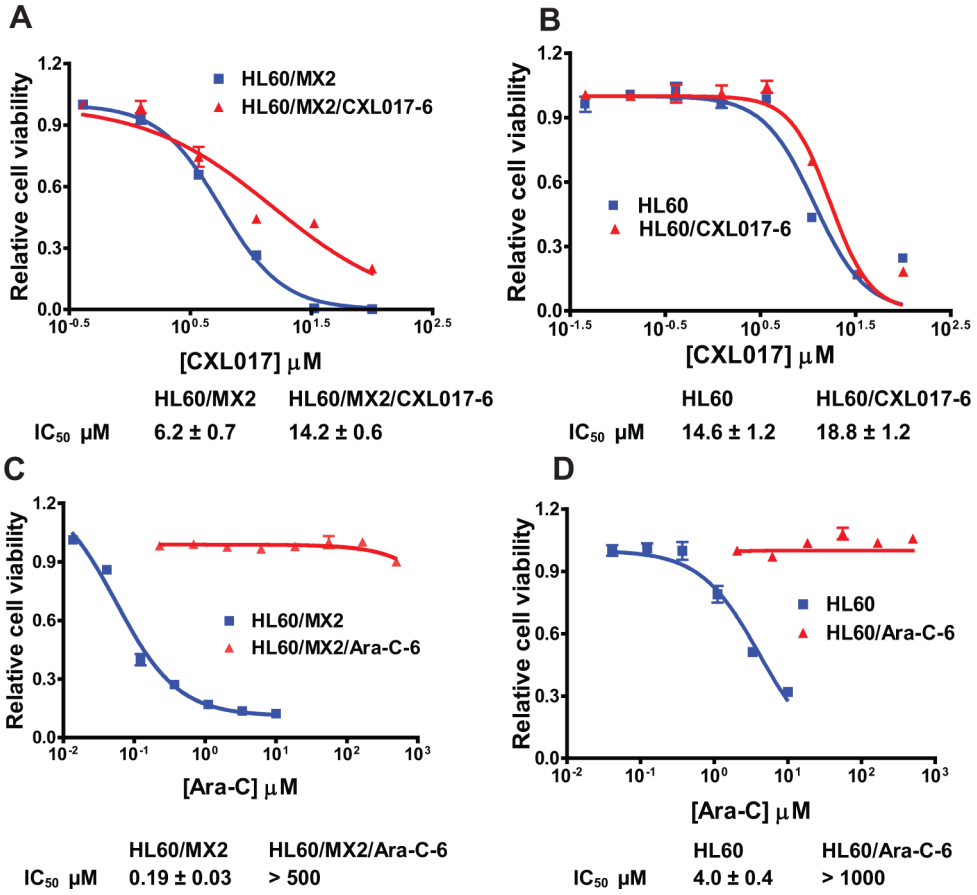

Figure 4. Long-term exposure of CXL017 and Ara-C to HL60 and HL60/MX2 cells. Drug resistance of HL60 and HL60/MX2 cells was developed as described in Methods. Briefly, HL60 and HL60/MX2 cells were treated with increasing concentrations of CXL017 and Ara-C, respectively, for 6 months, and their sensitivity to drugs was evaluated by using the CellTiter-blue assay. For drug sensitivity, three independent experiments were conducted with similar results. (A) Dose-dependent effect of CXL017 on the cell viability of HL60/MX2 and HL60/MX2/CXL017-6 cells. Points, mean (n = 3); bars, SEM. (B) Dose-dependent effect of CXL017 on the cell viability of HL60 and HL60/CXL017-6 cells. Points, mean (n = 3); bars, SEM. (C) Dose-dependent effect of Ara-C on the cell viability of HL60/MX2 and HL60/MX2/Ara-C-6 cells. Points, mean (n = 3); bars, SEM. (D) Dose-dependent effect of Ara-C on the cell viability of HL60 and HL60/Ara-C-6 cells. Points, mean (n = 3); bars, SEM.

were up-regulated in the MDR HL60/MX2 cell line (Figure 3C). The MDR nature of HL60/MX2 suggests that the overexpressed pro-apoptotic proteins are unable to overcome the protection against apoptosis imparted by the high level of Mcl-1.[38]

Taking into account the potential dual function of CXL candidates on Bcl-2 family proteins and SERCA proteins[34] and their reported physical interactions,[27−29] we also characterized the expression levels of SERCA2 and SERCA3 in these cell systems. It was found that HL60/MX2 has significantly elevated levels of SERCA2 and SERCA3 proteins (Figure 3C). As expected, HL60/MX2 also has higher ER calcium content than HL60 (Figure 3D), because SERCA proteins induce calcium ion influx into the ER. On the basis of the recent report of Bcl-2 family proteins regulating ER calcium content and homeostasis, these characteristics of HL60 and HL60/MX2 suggest that they are valuable systems to study MDR.

HL60/MX2 Fails To Develop Stable Resistance to CXL017 upon Prolonged Exposure. Although CXL017 demonstrates selectivity to HL60/MX2 both *in vitro* and *in vivo*, it is important to determine whether HL60/MX2 cells can develop resistance toward CXL017 upon chronic exposure. Therefore, HL60 and HL60/MX2 cells were cultured in the presence of increasing concentrations of CXL017 or Ara-C for a period of 6 months. Ara-C was used for comparison because it is the major first line therapy for treatment of AML. The newly derived cells, named as HL60/CXL017-6, HL60/MX2/CXL017-6, HL60/Ara-C-6, and HL60/MX2/Ara-C-6, were then evaluated for their sensitivity to CXL017 and Ara-C, respectively. HL60/MX2/CXL017-6 and HL60/MX2/Ara-C-6 cell lines were also cultured in the absence of drug for a period of 2 months and then re-evaluated for their drug sensitivity in order to determine whether the observed changes were stable. The cell lines thus obtained were named HL60/MX2/CXL017 and HL60/MX2/Ara-C, respectively. For additional comparison, HL60/MX2 cells were exposed to ABT-737 (a Bcl-2 inhibitor) for a period of 3 months, denoted as HL60/MX2/ABT-737.

Excitingly, even after 6-month exposure, HL60/CXL017-6 and HL60/MX2/CXL017-6 cells both failed to demonstrate stable resistance to CXL017. HL60/MX2/CXL017-6 cells only developed a 2.5-fold transient resistance to CXL017 (Figure 4A) that the resistance was lost upon removal of CXL017 (HL60/MX2/CXL017, Table 1, and Supplementary Figure 3A). HL60/CXL017-6 developed only 1.3-fold resistance toward CXL017 after a 6-month exposure (Figure 4B). In comparison, HL60/Ara-C-6 and HL60/MX2/Ara-C-6 cells developed >2000-fold stable resistance toward Ara-C (Figure 4C and D, Table 1, and Supplementary Figure 3B). Because no change was observed between HL60/MX2/Ara-C-6 and HL60/MX2/Ara-C cells,

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Table 1. IC_{50} Values of Various Anticancer Agents in HL60 and HL60/MX2 Cell Lines with/without Exposure to CXL017 or Ara-C[a]

	HL60	HL60/CXL017	HL60/MX2	HL60/MX2/CXL017-6	HL60/MX2/CXL017	HL60/MX2/Ara-C
mitoxantrone (nM)	16 ± 2	9.0 ± 0.5	720 ± 80	6.5 ± 0.8	4.1 ± 0.1	1900 ± 400
doxorubicin (μM)	0.14 ± 0.02	0.14 ± 0.01	1.2 ± 0.3	0.06 ± 0.01	0.06 ± 0.01	1.8 ± 0.4
cytarabine (μM)	4.0 ± 0.4	2.1 ± 0.3	0.36 ± 0.09	0.033 ± 0.004	0.013 ± 0.007	>500
etoposide (μM)	1.6 ± 0.1	1.3 ± 0.4	14 ± 1	0.36 ± 0.02	0.23 ± 0.02	19 ± 3
CXL017 (μM)	14 ± 1	19 ± 1	4.2 ± 0.7	14 ± 1	4.6 ± 0.5	3.4 ± 0.4
ABT-737 (μM)	1.4 ± 0.4	1.3 ± 0.2	2.7 ± 0.2	0.23 ± 0.15	0.31 ± 0.06	8.8 ± 0.5

[a]Cell viability was determined using the CellTiter-blue assay as described in Methods. Data represented are an average of at least three independent experiments with their SEM shown.

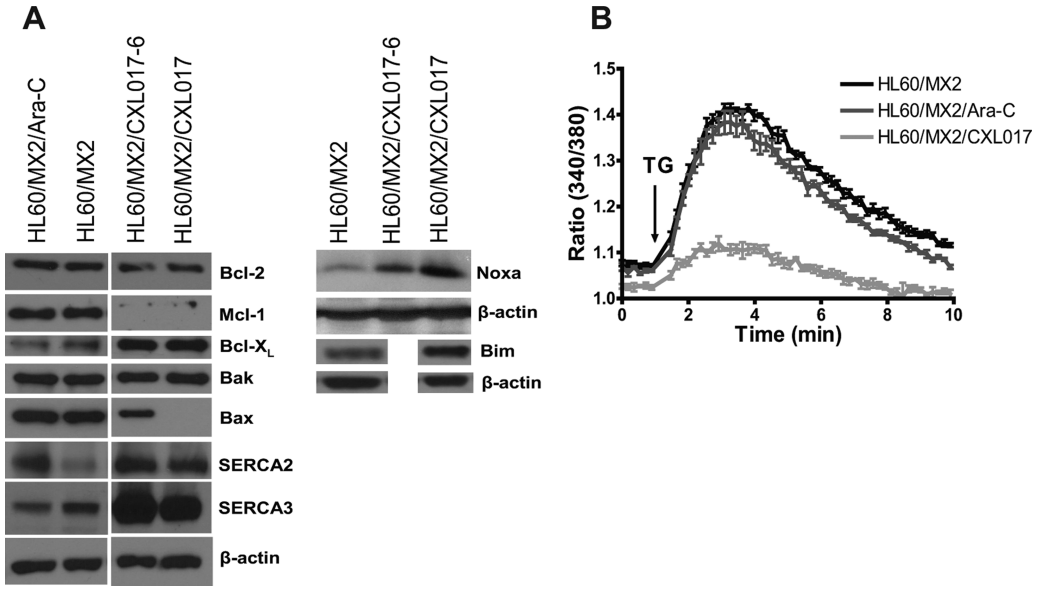

Figure 5. Characterization of molecular changes in CXL017 or Ara-C treated HL60/MX2 cells. (A) Total cell lysates from HL60/MX2/Ara-C, HL60/MX2, HL60/MX2/CXL017-6, and HL60/MX2/CXL017 were separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-SERCA2, SERCA3, Bcl-2, Bax, Noxa or rabbit polyclonal anti-Mcl-1, Bcl-X_L, Bak, or Bim. Mouse monoclonal anti-β-actin was used as a protein loading control, and the β-actin blot shown herein was from the Mcl-1 experiment as a representative. Three independent experiments were conducted with similar results. (B) ER calcium levels were measured in HL60/MX2, HL60/MX2/CXL017, and HL60/MX2/Ara-C cells as described in Methods. Three independent experiments were averaged, and the ratio of 340/380 nm was plotted with SEM shown.

HL60/MX2/Ara-C cells were used for later studies. These results show that both HL60 and HL60/MX2 cells fail to develop stable resistance toward CXL017 upon chronic exposure. As CXL017 derives from a Bcl-2 inhibitor, we were interested to determine whether other Bcl-2 inhibitors would show similar effects. For this purpose, we exposed HL60/MX2 cells to ABT-737 for 3 months. HL60/MX2 cells developed greater than 9-fold stable resistance to ABT-737 (Supplementary Figure 4A). These results show that the lack of resistance toward CXL017 observed in HL60 and HL60/MX2 cells is unique. The failure of HL60 and HL60/MX2 cells to develop resistance toward CXL017 is of clinical potential as rapid development of resistance to chemotherapy is a major obstacle in cancer treatment.

HL60/MX2 Cells Exposed to CXL017 Demonstrate Resensitization to Standard Therapies. Although HL60/MX2/CXL017-6 cells failed to develop stable resistance to CXL017, it remained to be determined whether these cells acquired additional MDR. Therefore, we evaluated the sensitivity of CXL017-exposed and Ara-C-exposed cells to a variety of standard therapies including mitoxantrone, Ara-C, doxorubicin, and etoposide, as well as ABT-737. CXL017-exposed HL60 and HL60/MX2 failed to acquire additional cross-resistance to the standard therapies tested. Surprisingly, they demonstrate increased sensitivity toward several standard therapies. In particular, HL60/MX2/CXL017-6 gained 10- to 100-fold increased sensitivity to mitoxantrone, doxorubicin, Ara-C, etoposide, and ABT-737 (Table 1). Resensitization was a unique feature observed with CXL017 as well because HL60/MX2/Ara-C demonstrated moderate cross-resistance to all standard therapies (Table 1), whereas HL60/MX2/ABT-737 retained its sensitivity toward mitoxantrone (Supplementary Figure 4B). Interestingly, HL60/MX2/CXL017 cells that have lost their resistance toward CXL017 still retain the resensitization toward standard agents (Table 1), suggesting that cells exposed to CXL017 do not require continuous exposure to maintain chemosensitization. These data further support the potential of CXL017 as a unique anticancer candidate to treat MDR cancers.

CXL017 Exposure Causes Changes in Bcl-2 Family Proteins, SERCAs, and ER Ca^{2+}. Because CXL017 was derived from a dual inhibitor of Bcl-2 family proteins and SERCA and our data show that these proteins are potentially involved in MDR in HL60/MX2 cells (Figure 3), we studied the involvement of these proteins in CXL017-induced resensitization in HL60/MX2 cells (Figure 5A) with HL60/MX2/Ara-C cells included for comparison. The levels of Bak protein remain unchanged among the HL60/MX2 cell lines. Bcl-X_L protein was significantly

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overexpressed in HL60/MX2/CXL017-6 and HL60/MX2/CXL017 cells (Figure 5A). The levels of Mcl-1 and Bax were vastly decreased among cells with exposure to CXL017, whereas pro-apoptotic proteins such as Noxa and Bim were up regulated (Figure 5A). Noxa and Bim are known to bind to Mcl-1,[39,40] and Noxa activity seems to be kept in complete check by Mcl-1.[41] A decrease in Mcl-1 level can lead to their release and thus activate Bak resulting in increased cell death.[42] There may be a slight decrease in the level of Bcl-2 protein in HL60/MX2/CXL017-6 cells, but its level recovered in HL60/MX2/CXL017 cells (Figure 5A). Given the similar drug sensitivity profiles among these two cell lines (Table 1), the change in Bcl-2 protein is likely less important relative to other changes.

In contrast, Ara-C exposure to HL60/MX2 had no effect on any of the Bcl-2 family proteins evaluated (Figure 5A), supporting that these changes are CXL017-specific and that the Bcl-2 family proteins are involved in the mechanism of action of CXL017. On the basis of the established functions of Bcl-2, Bcl-X_L, Noxa, Bim, Mcl-1, and Bax proteins, their expression levels, and the distinct drug sensitivity profiles among HL60/MX2 and its derived cells (Figure 5A and Table 1), the down-regulation of Mcl-1 and the up-regulation of Noxa and Bim in HL60/MX2/CXL017-6 and HL60/MX2/CXL017 are likely responsible for the increased sensitivity of these cell lines to standard therapies. Although the up-regulation of Bcl-X_L and down-regulation of Bax were expected to induce resistance, it is possible that the extent of changes for them was insufficient to counterbalance the loss of Mcl-1 and the increase in Noxa/Bim or that Bcl-X_L and Bax are functionally less important relative to the other Bcl-2 family proteins in these cell systems. In addition, the drug-resensitized HL60/MX2/CXL017-6 and HL60/MX2/CXL017 cell lines reveal further overexpression of SERCA2 and SERCA3, which again was absent in the HL60/MX2/Ara-C cell line (Figure 5A). These CXL017-specific changes among Bcl-2 family proteins and SERCA proteins support its dual targeting on these proteins or the communications among these proteins.

Because of the function of both Bcl-2 family proteins and SERCA proteins on calcium homeostasis, ER calcium content was also compared among HL60/MX2, HL60/MX2/CXL017, and HL60/MX2/Ara-C (Figure 5B). Surprisingly, HL60/MX2/CXL017 cells have significantly lower level of ER calcium compared to HL60/MX2 cells despite the increased levels of SERCA2 and SERCA3. This is in contrast to what was observed between the HL60 and HL60/MX2 cell lines where HL60/MX2 cells have increased levels of both SERCA and ER calcium content (Figure 3B). It is possible that in HL60/MX2/CXL017 other proteins, potentially the Bcl-2 family proteins, regulate the activities of these SERCA proteins such that the overexpressed SERCA proteins are less effective in fluxing calcium into ER. It is also possible that the Bcl-2 family proteins may regulate IP_3Rs or other proteins to increase ER calcium release. In addition, cells lacking Bax and Bak demonstrate decreased ER calcium content when compared to its wild type.[43] Therefore, the decrease in ER calcium content in HL60/MX2/CXL017 may be due to the down-regulation of Bax. Again, there was no difference in ER calcium content between HL60/MX2/Ara-C and HL60/MX2 cell lines. We finally characterized the potential contribution of ABC transporter proteins to the increased sensitivity of CXL017 exposed cells toward chemotherapy by measuring the levels of P-gp and BCRP in these cell lines. As we have observed in HL60 and HL60/MX2 cells, exposure to CXL017 or Ara-C did not introduce clear changes in P-gp and BCRP (Supplementary Figure 2A and 2B), arguing against their involvement in CXL017 induced resensitization. To further confirm that drug cellular uptake is not responsible for the observed drug sensitivity changes, we have quantified mitoxantrone accumulation among HL60, HL60/MX2, and HL60/MX2/CXL017 cells because of their biggest sensitivity differences to mitoxantrone (Table 1). As shown in Supplementary Figure 2C, there was no significant difference in mitoxantrone accumulation among these cell lines, supporting that the observed drug sensitivity differences among these cell lines were not regulated *via* differential cellular drug accumulation.

Overall, CXL017-induced resensitization toward standard therapies correlates well with the changes in the levels of Mcl-1, Noxa/Bim, and ER calcium content among these cell lines. It is well documented that overexpression of Mcl-1 can lead to MDR; therefore, reduced Mcl-1 expression in CXL017-exposed HL60/MX2 cells would cause the observed resensitization of the cells. In addition, there is increasing evidence that a balance between Noxa and Mcl-1 regulates the susceptibility of cells to apoptosis.[41,44] Therefore, down-regulation of Mcl-1 and up-regulation of Noxa might collectively lead to CXL017-induced resensitization. The calcium content changes among these cells indicate that levels of ER calcium content may also play a role in acquired drug resistance in HL60/MX2 cells and altering the ER calcium content may be an effective way to overcome drug resistance. The specific role of these changes is currently under investigation.

Conclusions. MDR is a complex but common phenomenon in cancer therapy with multiple mechanisms involved in its development, many of which are poorly understood. Therefore, anticancer agents that can resensitize MDR cancer cells to chemotherapy not only bring forth the opportunity to greatly improve cancer patient response but also provide valuable tools to help understand MDR mechanisms. In this study, we provide evidence that CXL017 is one such compound that not only has a strong potential to overcome drug resistance in the clinic but also provides us with a valuable tool to understand MDR cancers. In this study, we have demonstrated the ability of CXL017 to selectively target drug-resistant cancer cells both *in vitro* and *in vivo*. Moreover, we have shown that cancer cells fail to develop stable drug resistance to CXL017 upon chronic exposure, whereas they rapidly develop resistance to other therapies. In addition, we have revealed its unique ability to resensitize drug-resistant cancer cells to chemotherapy, possibly by down-regulating Mcl-1, up-regulating Noxa/Bim, and lowering ER calcium content. Taken together these results demonstrate the unique biological activity of CXL017 and strongly support the clinical potential of CXL017 scaffold-based cancer therapies with minimum risk of acquired drug resistance, particularly against MDR malignancies.

METHODS

Chemicals and Reagents. CXL017 [ethyl-2-amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate] was synthesized and characterized by NMR and mass spectrometry, as previously described.[35] ABT-737 was synthesized following published procedures.[45] Fura-2AM was obtained from Invitrogen. Thapsigargin was obtained from Acros Organics. All drugs including mitoxantrone, Ara-c, etoposide, and doxorubicin were obtained from commercial sources. Probenecid was obtained from Sigma-Aldrich. CellTiter-Blue Assay Kit was obtained from Promega. SERCA2 (1:300), SERCA3 (1:200), Mcl-1 (1:400), Bcl-X_L (1:750), Bax (1:200), Noxa (1:250 or 1:500), and Bim (1:200) antibodies were from Santa Cruz Biotechnology, Inc. Bak (1:300) antibody was from Millipore. Bcl-2 (1:1000), β-actin (1:40000), and anti-mouse secondary (1:3000) antibodies were from Sigma-Aldrich.

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Cell Culture. CCl-240 (HL60) and CRL-2257 (HL60/MX2) were purchased from ATCC. HL60/DNR was kindly provided by Dr. Tang.[46] HL60 cell line and its derivative cell lines were grown in IMDM glutamax media supplemented with 20% FBS. The HL60/MX2 cell line, its derivative cell lines, and HL60/DNR were grown in RPMI 1640 supplemented with 10% FBS. All cell lines were incubated at 37 °C with 5% CO_2 in air atmosphere.

Evaluation of Cell Viability. Leukemic cells were plated at a density of 1×10^4 cells/well in a 96-well round-bottom plate. Test compounds were added at varying concentrations in 0.5−1% DMSO and cells treated with medium containing the same amount of DMSO served as a control. After a 48 h treatment, relative cell viability in each well was determined using the CellTiter-Blue Cell Viability Assay. The IC_{50} of each compound was determined by fitting the relative viability of the cells to the drug concentration, using a dose−response model in Prism program from GraphPad Software, Inc.

Determination of ER Calcium Content. Cells at a density of 1×10^6 cells/mL were incubated in medium containing 5 μM Fura-2AM and 2.5 mM probenecid at RT in the dark for 1 h. The cells were then washed once with cold PBS and resuspended to a concentration of 2×10^6 cells/mL in medium containing 2.5 mM probenecid. To a cuvette with 735 μL of media containing 100 mM EGTA and 2.5 mM probenecid was added 750 μL of the cell suspension. The cell suspension was mixed by pipetting up and down in a slow controlled manner. Under this condition, the cell media contained a negligible amount of free calcium. Therefore, the cells had no extracellular calcium source. Fluorescence emission was measured for 1 min followed by adding 15 μL of DMSO solution of thapsigargin, and readings were continued for another 9 min. Readings were obtained on a dual wavelength fluorometer (Cary Eclipse, Varian) with excitation wavelengths alternating between 340 and 380 nm and an emission wavelength of 510 nm.

Western Blotting Analysis. Western blotting was conducted using standard chemiluminescent techniques, as previously described.[47] Following overnight incubation in primary antibody at 4 °C, the blot was washed, and secondary staining was accomplished using corresponding IgG conjugated to HRP. Protein loading was assessed using β-actin.

Flow Cytometry Analysis. To detect and quantify P-gp and breast cancer resistance protein (BCRP), cells (1×10^6) were blocked with normal mouse serum for 10 min, followed by incubation for 1 h with phycoerythrin (PE)-conjugated UIC2 (Anti-Human P-gp PE) (Ebiosciences), PE-conjugated 5D3 (Anti-Human BCRP PE) (Ebiosciences) with PE conjugated isotype IgG1 as control. After 1 h of incubation, the cells were washed and fixed in 50 μL of 10% formalin overnight at 4 °C. The fluorescence intensity was measured using a flow cytometer (BD FACS calibur, BD Biosciences). Protein level was determined by subtracting the mean fluorescence intensity of the control antibody from each specific antibody (P-gp-IgG1, BCRP-IgG1). For each sample, 25,000 events were collected. To quantify the cellular accumulation of mitoxantrone, the selected HL60 cell lines were incubated with mitoxantrone (5 μM) for 15 min. The fluorescence intensity was measured using the flow cytometer (BD FACS calibur, BD Biosciences). The relative level of mitoxantrone was determined by subtracting the mean fluorescence intensity of the corresponding control cells from the cells exposed to mitoxantrone and normalized to that in HL60 cells. For each sample, 20,000 events were collected.

Chronic Exposure of Cell Lines to CXL017, Ara-C, or ABT-737. We have followed the reported drug exposure regimen, which was developed by Harker *et al.* to acquire multidrug resistant cancer cell lines, with slight modifications.[37] Specifically, HL60 and HL60/MX2 cell lines were exposed to varying concentrations of CXL017, starting at their corresponding 48 h. The medium was replaced every 2−3 days with new compound replenished. The cells were then not treated for 2 days over the weekend. The cells were also continually monitored for their sensitivity to CXL017 so that CXL017 concentration was increased in a manner to cause 20−50% cell death during each treatment period. This treatment regimen continued for a period of 6 months with the final concentration of CXL017 being 2- to 4-fold higher than its original IC_{50}, which still caused significant cell death. A similar procedure was applied to Ara-C as well with a 6-month treatment period. For ABT-737, the treatment regimen was 3 months following the similar criteria stated above. To determine if the drug-exposed cell lines demonstrated stable resistance, they were cultured in the absence of drug for a period of 2 months and then re-evaluated for their drug sensitivity.

***In Vivo* Anticancer Efficacy Evaluation.** For *in vivo* anticancer potential evaluation, female athymic nude mice obtained from the Harlan Laboratories were maintained on standard diet in a laminar airflow cabinet under pathogen-free conditions and used at 6−12 weeks of age. The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee (IACUC) of the research animal resources facility at the University of Minnesota. After 1 week of acclimation, HL60 cells (5×10^6 in 0.1 mL of PBS/Matrigel (v/v 1:1)) were implanted subcutaneously into the left flank of each mouse. At the same time, HL60/MX2 cells (5×10^6 in 0.1 mLof PBS/Matrigel (v/v 1:1)) were implanted subcutaneously into the right flank of the same mouse. Formation of a bulla indicated a satisfactory injection. Tumors were measured three times a week with a caliper. Tumor volumes were calculated using the following formula: $1/2 \times w_1 \times w_2 \times w_2$ (w_1 the largest tumor diameter and w_2 the smallest tumor diameter). When tumors induced by HL60/MX2 reached ~40 mm^3, mice were randomized into two groups with similar distribution in HL60/MX2 induced tumor size and bodyweight, 10 mice in each group. Mice in Group 1 were given carrier (PEG400/EtOH (v/v 2:1), 0.1 mL) *via* i.p. injection. Mice in Group 2 were given CXL017 (100 mg/kg of body weight). Treatment was once a day and 6 days/week for 3 weeks. Mice were euthanized with CO_2, and tumors were removed and weighed.

Statistical Analysis. All biological experiments, including *in vitro* cytotoxicity assays, calcium assays, flow cytometry analyses, and Western blots, were performed at least three times. Representative results are depicted in this report. Data are presented as means ± standard error of the mean (SEM), and comparisons were made using Student's *t* test. A probability of 0.05 or less was considered statistically significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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