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A Novel SERCA Inhibitor Demonstrates Synergy with Classic SERCA Inhibitors and Targets Multidrug-Resistant AML

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Abstract

Drug resistance exists as a major obstacle in the treatment of cancer and drug molecules that retain effectiveness against resistant cancers are a high clinical priority. Ethyl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate (CXL017) was recently identified as a promising lead for the treatment of multidrug-resistant leukemia, which elicits its cytotoxic effect, in part, through inhibition of the sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA). Herein initial experiments with SERCA1a, CXL017 demonstrated no significant effect on calcium affinity, competed with ATP, and induced a dose-dependent decrease in ATPase activity. Among all CXLs tested, (–)-CXL017 exhibited the greatest SERCA inhibition with an IC₅₀ = 13.5 ± 0.5 μM. Inhibitor combination studies were used to assess potential interactions between (–)-CXL017 and well-known SERCA inhibitors: thapsigargin, cyclopiazonic acid, and 2,5-di-*tert*-butylhydroquinone. Surprisingly, (–)-CXL017 exhibited marked synergy with each of the known SERCA inhibitors whereas all combinations of the known inhibitors yielded additive effects, indicating that (–)-CXL017 may bind at a unique allosteric site. Treatment of parental (HL60) and multidrug-resistant (HL60/MX2) acute myeloid leukemia cells with the known SERCA inhibitors revealed that all of these inhibitors demonstrate selective cytotoxicity (7.7 to 400 fold) for the resistant cell line. Within the CXL series, a positive correlation exists between SERCA inhibition and cytotoxicity in HL60/MX2 but not HL60. (–)-CXL017 was also shown to enhance the cytotoxicity of thapsigargin in HL60/MX2 cells. Given the elevated SERCA levels and ER calcium content in HL60/MX2, SERCA likely plays a significant role in the collateral sensitivity of this multidrug-resistance cell line to CXL molecules as well as known SERCA inhibitors.

Keywords

SERCA; inhibitor; synergy; cancer; drug-resistance

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Supporting Information

A figure showing no effect on SERCA $V_{max(reg)}$ and $V_{max(cat)}$ upon varying ATP in the presence of 10 or 30 μM CXL017 is provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Drug resistance, either pre-existent or acquired, is a major obstacle for all cancer chemotherapeutics, as it limits the range of viable treatment options (1). In acquired chemoresistance, two mechanisms are most commonly associated with the multidrug-resistant (MDR) phenotype, namely the overexpression of drug-efflux pumps such as P-glycoprotein (P-gp) and the overexpression of anti-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins (2, 3). Indeed, both mechanisms strongly correlate with adverse clinical outcomes and are the focus of numerous drug discovery programs (4, 5). The role of P-gp is well-known to lower the accumulation of drug within the tumor cell, thereby attenuating any therapeutic benefit (6). The role of Bcl-2 family proteins, however, is more complex as both pro- and anti-apoptotic members comprise a dynamic equilibrium that control apoptotic signaling at both the endoplasmic reticulum (ER) and the mitochondria (7). The impact of anti-apoptotic Bcl-2 proteins at the ER is to regulate intracellular calcium homeostasis through interaction with calcium channels (i.e. inositol triphosphate receptor; IP₃R) and/or pumps (i.e. sarco/endoplasmic reticulum Ca²⁺-ATPase; SERCA) (8, 9), highlighting the importance of this tightly regulated and multi-functional signaling ion in the execution of cell death (10, 11). Thus, altered calcium flux across the ER via manipulation of IP₃R or SERCA has been linked to the efficiencies of various apoptotic stimuli (12, 13). Even with respect to P-gp mediated drug resistance, calcium plays a dynamic role exemplified by altered signaling events and expression of ER proteins upon P-gp over-expression (14). Together, these findings highlight the involvement of calcium in both mechanisms of cancer drug resistance. Given the pleiotropic nature of this critical cell messenger (15), small molecules that target calcium mobilization may provide a means to affect both mechanisms simultaneously and may lead to the development of novel therapies that maintain efficacy across a greater number of multidrug-resistant cancer types.

SERCA is the most-studied member of the P-type or E1–E2 family of ATPases that couples the energy derived from ATP hydrolysis to the transport of calcium against its concentration gradient, in order to maintain a >1000 fold excess of calcium within the ER relative to the cytosol (16). Inhibition of SERCA causes a depletion of the ER calcium storage pool and a subsequent rise in cytosolic calcium levels. While transient spikes in cytosolic calcium are often generated in normal cellular signaling events, prolonged or extreme elevation of cytosolic calcium results in both ER and mitochondrial damage, leading to apoptosis. Specifically, high cytosolic calcium elicits an ER stress response typified by the accumulation of unfolded protein, depolarization of the mitochondrial membrane, cytochrome C release, and caspase activation (17). Given the dynamic apoptotic functionality of calcium ions and the essential role of SERCA in maintaining calcium homeostasis, SERCA inhibitors provide a viable route to investigate the potential sensitivity of drug-resistant cancers to disrupted calcium transport.

A variety of SERCA inhibitors have been identified with diverse chemical structures and binding affinities (18). Several of the more potent inhibitors have evolved as tools to study the impact of calcium signaling on a number of cell processes and were pivotal to obtaining high-resolution crystal structures of the ATPase (Fig. 1) (19–22). Of these classic inhibitors, thapsigargin (TG), cyclopiazonic acid (CPA), and 2,5-di-*tert*-butylhydroquinone (BHQ) have submicromolar dissociation constants (23). TG is a sesquiterpene lactone that binds the E2 form of the enzyme in the transmembrane (TM) domain (24). CPA and BHQ also bind the E2 form of SERCA at a well-defined site in the TM domain, though with lower affinity than that of TG (25, 26). The CPA binding site is distinct from that of TG but partially overlaps with the BHQ site (23). Paired inhibitor studies have revealed that a change in conformation upon binding of a single inhibitor can result in enhanced affinity for a second inhibitor, suggesting an inhibitor-induced change in SERCA conformation that favors

binding of a second inhibitor (27). This cooperative binding decreases the concentration of each individual inhibitor needed to generate an equivalent inhibitory effect, an important consideration for the potential therapeutic value of SERCA inhibitors. To date, only TG and related derivatives have been implemented in the clinic (28). However, a recent discovery by Roti *et al.* demonstrating SERCA as an up-stream regulator of Notch1 and the ability of TG to successfully target *NOTCH1* mutated leukemia both *in vitro* and *in vivo*, validates further research into new and existing SERCA inhibitors for certain cancer types (29).

Structure-activity relationship studies of sHA 14-1, a dual inhibitor against Bcl-2 and SERCA, led to the discovery of ethyl 2-amino-6-(3,5-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate, CXL017, a novel lead for the treatment of multidrug-resistant leukemia (30, 31). Specifically, CXL compounds were evaluated in both parental (HL60) and multidrug-resistant (HL60/MX2) AML cell lines, where it was found that HL60/MX2 cells demonstrate collateral sensitivity to CXLs. CXL017 was also shown to retain activity in multidrug-resistant ALL (K562/HHT300) and CML (CCRF-CEM/VLB100), despite different resistance mechanisms than HL60/MX2 (32). These findings provide impetus for characterizing the inhibition of SERCA by CXLs in order to understand the role of disrupted calcium mobilization to the targeting of multidrug-resistant leukemia.

In this study, inhibition of SERCA by CXL compounds was characterized along with the contribution of SERCA inhibition to the selective cytotoxicity observed in multidrug-resistant AML. A uniquely synergistic relationship was uncovered between (–)-CXL017 and classic SERCA inhibitors in both biochemical and cell-based assays, suggesting a novel binding site for (–)-CXL017. Together, these results support the proposal that SERCA is indeed the target of CXLs in HL60/MX2, and that the resulting inhibition contributes to the unique ability of these compounds to target this resistant AML cell line.

MATERIALS AND METHODS

Chemicals and Reagents

All CXL molecules were synthesized as previously described (31). Thapsigargin was purchased from MP Biomedicals (Solon, OH). Cyclopiazonic acid and 2,5-di-*tert*-butylhydroquinone were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from reliable sources.

SERCA ATPase Measurements and Data Analysis

Ca²⁺-dependent ATPase activity was measured using light sarcoplasmic reticulum (LSR) vesicles purified from fast-twitch skeletal muscle of New Zealand white rabbits in a NADH-linked enzyme-coupled microtiter plate assay (200 μ L/well) with free calcium ([Ca²⁺]) tightly controlled using an EGTA buffering system as previously described (33). Briefly, reaction buffer for the assay consisted of 5 mM MgCl₂, 100 mM KCl, 1 mM EGTA, 0.4 mM NADH, 10 IU/mL lactate dehydrogenase, 10 IU/mL pyruvate kinase, 7 μ g/mL A23187 ionophore, and 50 mM MOPS at pH 7.0. The concentrations of lactate dehydrogenase and pyruvate kinase were varied to ensure that effects of compounds were not due to their effects on these enzymes. LSR protein was resuspended at 0.5 mg/mL. All reactions were conducted at 37 °C in duplicate or triplicate and results are reported as the mean of three independent experiments. ATP hydrolysis was determined as the rate of NADH oxidation, measured from the decay in absorbance at 340 nm using a Molecular Devices SpectraMax Plus spectrophotometer (Sunnyvale, CA). Compounds were introduced into the assay at their respective concentrations with 1% DMSO in the final assay volume. All data fitting was carried out using GraphPad Prism software (San Diego, CA). In experiments where

calcium was varied, ATPase activity was measured from pCa 5.0–7.5 while pATP was held constant. The data were plotted (V vs pCa) and fitted by the Hill equation:

$$V = \frac{V_{max}}{1 + 10^{-n(pK_{Ca} - pCa)}} \quad (1)$$

where V is the initial ATPase rate and n is the Hill coefficient. Data were normalized to the maximal rate, V_{max} , determined from the fit in the presence of 1% DMSO, and then replotted to assess changes in V_{max} and pK_{Ca} due to the added compound. In experiments where ATP was varied, ATPase activity was measured from pATP 2.6–6.4 while pCa was held constant. In this case, the data were plotted (V vs. pATP) and fitted by a Bi-Michaelis-Menten equation:

$$V = \frac{V_{max(cat)}}{1 + 10^{(-pK_{ATP(cat)} + pATP)}} + \frac{V_{max(reg)}}{1 + 10^{(-pK_{ATP(reg)} + pATP)}} \quad (2)$$

where $V_{max(cat)}$ and $pK_{ATP(cat)}$ describe the catalytic ATP binding site and $V_{max(reg)}$ and $pK_{ATP(reg)}$ describe the regulatory ATP binding site. Data were normalized to the maximal rate, $V_{max(cat)}$, determined in the presence of 1% DMSO, and then plotted to assess changes in $V_{max(cat)}$, $V_{max(reg)}$, $pK_{ATP(cat)}$, and $pK_{ATP(reg)}$. As no significant change in $V_{max(cat)}$ or $V_{max(reg)}$ was detected, these values were fixed to the values determined from control experiments and changes in $pK_{ATP(cat)}$ and $pK_{ATP(reg)}$ were ultimately determined. Lastly, in dose-response experiments where serial dilutions of each inhibitor were tested, pCa and pATP were held constant. Data were normalized to the enzyme rate determined in the presence of 1% DMSO, plotted (relative V vs. $\log[I]$) and fitted with GraphPad Prism (San Diego, CA) according to a 4-parameter dose-response equation:

$$Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{1 + 10^{n(pIC_{50} - p[X])}} \quad (3)$$

where Y_{min} and Y_{max} were fixed to 0.5 and 1 respectively (to accommodate comparison of the partial inhibitors), X is the inhibitor concentration, and n is the Hill coefficient. From the fit, the IC_{50} of each inhibitor was determined.

Evaluation of synergy in SERCA ATPase assay

Compounds (Fig. 1) were introduced individually and in combination at a constant molar ratio to the ATPase assay. Data were processed using CompuSyn Software (Paramus, NJ) to determine the combination index (CI) based on the fraction of enzyme affected (f_a) by the individual inhibitors and their combinations. From the CI value, each inhibitor pair was characterized as synergistic (CI<0.9), additive (CI=0.9–1.1), or antagonistic (CI>1.1) (34). In the actual experiment, inhibitor ratios were determined experimentally to give f_a values ranging from 0.1–0.9 for individual inhibitors. Inhibitor concentrations were as follows: (–)-CXL017, 6.25 μ M, 12.5 μ M, and 25 μ M; TG, 18.75 nM, 37.5 nM, and 75 nM; CPA, 0.16 μ M, 0.31 μ M, and 0.63 μ M; BHQ, 0.31 μ M, 0.63 μ M, and 1.25 μ M. Molar ratios were as follows: (–)-CXL017: TG, 667:1; (–)-CXL017: CPA, 80:1; (–)-CXL017: BHQ, 40:1; TG: CPA, 1:8.33; TG: BHQ, 1:16.7; CPA: BHQ, 1:2.

Cell Culture Techniques

HL60 cells were purchased from ATCC and grown in IMDM Glutamax medium (GIBCO, Carlsbad, CA) supplemented with 20% FBS. HL60/MX2 cells were also purchased from ATCC but grown in RPMI 1640 media (ATCC) supplemented with 10% FBS. Both cell lines were incubated at 37 °C under 5% CO₂ in air.

Cell Viability Measurement

Cytotoxicity was assessed via a growth inhibition assay as reported previously (31). Cells were plated in 96-well plates at 1×10^4 cells/well and treated with serial dilutions of each compound in the presence of 1% DMSO. Following 48-hour incubation, relative cell viability was determined using a CellTiter-Blue cell viability assay kit (Promega, Madison, WI). Data were plotted (relative cell viability vs. $\log[\text{drug}]$) and fit using GraphPad Prism (San Diego, CA) according to a 4-parameter dose-response equation (Eq. 3). Based on the fit, the IC_{50} of each compound was determined.

Evaluation of synergy in cell culture

HL60/MX2 cells were plated in 24-well plates at 7.5×10^5 cells/well and treated with (–)-CXL017, TG, or a combination thereof at a molar ratio of 667:1 in the presence of 1% DMSO. Following 16-hour incubation, 500 μL of each cell suspension was collected and centrifuged at $400 \times g$ for 4 minutes. After removing the media, cell pellets were resuspended in fresh media, transferred to individual wells of a 24-well plate, and allowed to incubate for additional 48 hours. Relative cell viability was assessed by trypan blue dye exclusion assay. Data were plotted as relative cell viability affected by each inhibitor or inhibitor combination.

RESULTS

Characterization of CXL017 as an inhibitor of SERCA

The catalytic mechanism of SERCA allows two Ca^{2+} ions to be translocated across the ER membrane per molecule of ATP hydrolyzed (35). This pumping action is facilitated by the movement of three cytoplasmic domains (A, actuator; P, phosphorylation; and N, nucleotide binding) in concert with 10 transmembrane helices. During the multi-step enzymatic cycle, ATP binds within the N domain leading to phosphorylation within the P domain and the ultimate translation of movement to afford the necessary conformational adjustments that result in active Ca^{2+} transport into the ER (36). Presumably, an inhibitor could disrupt the enzymatic action of SERCA by interfering with Ca^{2+} binding, ATP binding, or both. To test the potential effects of CXL017 on Ca^{2+} and ATP utilization, each substrate was varied (while the other was held constant) in the ATPase assay and CXL017 was introduced at either 10 or 30 μM . First, free Ca^{2+} was varied from pCa 5 to 7 and the resulting data set was fitted to the Hill equation to obtain normalized V_{max} and pK_{Ca} values. Although CXL017 displayed no significant effect on the apparent Ca^{2+} affinity (pK_{Ca}) of SERCA, a dose-dependent decrease in V_{max} was observed (Fig. 2A and B) with a normalized V_{max} of 0.80 ± 0.04 and 0.40 ± 0.01 in the presence of 10 μM and 30 μM CXL017 respectively.

Effects on ATP binding were measured in a similar manner, with pATP ranging from 2.6 to 6.4. In this case, the data were fitted to a Bi-Michaelis-Menten equation to accommodate two ATP binding sites; the first, a higher affinity catalytic site and the second, a lower affinity regulatory site (37). The V_{max} at both the catalytic and regulatory sites was well-defined by the two-site model in the absence of inhibitor and yielded no significant change in the presence of 10 or 30 μM CXL017 (Fig. S1). Since changes in $V_{\text{max}(\text{reg})}$ and $V_{\text{max}(\text{cat})}$ were not detected, data were reprocessed with the value for the catalytic and regulatory V_{max} fixed to the values obtained in the control experiment (Fig. 2C, circles), which provided equally good fits of the data ($r^2 = 0.99$). With these constraints, CXL017 showed apparent competition with ATP at both the high and low affinity sites in comparison to control ($\text{pK}_{\text{ATP}(\text{cat})} = 4.99 \pm 0.07$ and $\text{pK}_{\text{ATP}(\text{reg})} = 3.30 \pm 0.08$). Specifically, $\text{pK}_{\text{ATP}(\text{cat})}$ was 3.92 ± 0.02 and $\text{pK}_{\text{ATP}(\text{reg})}$ was 2.96 ± 0.07 in the presence of 10 μM CXL017, and $\text{pK}_{\text{ATP}(\text{cat})}$ was 3.99 ± 0.02 and $\text{pK}_{\text{ATP}(\text{reg})}$ was 2.6 ± 0.1 in the presence of 30 μM CXL017 (Fig. 2D). Thus,

the reduction of pK_{ATP} at the regulatory site responded in a dose-responsive manner, whereas the catalytic site was similarly affected by 10 and 30 μ M CXL017.

In order to compare the potency of CXL017 with other members of the CXL series, 5 other CXLs were selected and evaluated alongside CXL017 in the ATPase assay with the concentration of calcium and ATP held constant to maximize SERCA activity and assay sensitivity. CXLs demonstrated only partial SERCA inhibition with the most potent inhibitor providing a saturating amount of inhibition near 50%. A fit of the data using Eq. 3 was implemented to determine the IC_{50} of each inhibitor (Fig. 3). As predicted, based on the cytotoxicity of these inhibitors, CXL017 was the most potent among the racemic compounds evaluated with an IC_{50} of $25 \pm 1 \mu$ M. In addition, (–)-CXL017 ($IC_{50} = 13.5 \pm 0.5 \mu$ M) proved to be nearly 5 times more potent than (+)-CXL017 (IC_{50} of $68 \pm 11 \mu$ M), similar to the eudysmic ratio observed in cell viability experiments (31) and providing additional evidence that SERCA is the intracellular target of CXLs.

(–)-CXL017 synergizes with known SERCA inhibitors

Proving to be the most potent SERCA inhibitor among the CXLs evaluated, (–)-CXL017 was further investigated for potential interaction with three well-known SERCA inhibitors: TG, CPA, and BHQ. In this study, the Chou and Talalay method was employed by application of the CompuSyn program, which provides a mechanism-independent method for quantitative determination of drug interactions (calculated as the combination index (CI)), based on the mass-action law (38). A CI ranging from 0.9 to 1.1 is considered additive, while a $CI < 0.9$ indicates synergy, and a $CI > 1.1$ indicates antagonism. Employing this method, the four inhibitors were investigated for synergistic, additive, or antagonistic interaction by introduction into the SERCA activity assay both individually and in combination (6 total combinations) at appropriate fixed molar ratios. Quite surprisingly, (–)-CXL017 demonstrated marked synergy with each of the known SERCA inhibitors (Fig. 4A–C, $CI_s \ll 0.9$), whereas all combinations of the known inhibitors were mostly additive and did not synergize (Fig. 4D–F, $0.9 \leq CI_s < 1.2$). Among the interactions with (–)-CXL017, BHQ provided the strongest synergy ($CI_s = 0.15$ – 0.25), followed by CPA ($CI_s = 0.35$ – 0.45) and TG ($CI_s = 0.4$ – 0.65). These results suggest that (–)-CXL017 binds at a site within SERCA that is distinct from the binding sites of the known inhibitors in order to simultaneously occupy the SERCA structure and provide enhanced combinatorial inhibition.

SERCA Inhibitors Selectively Target Drug Resistant Leukemia

HL60/MX2 is well-characterized as a non-P-gp mediated multidrug-resistant AML cell line with respect to HL60, the parental AML line (32). Previously, HL60/MX2 cells demonstrated collateral sensitivity to CXLs, with 2–7 fold selectivity for the resistant line (31). In addition, HL60/MX2 cells exhibited elevated levels of SERCA2, SERCA3, and ER calcium content relative to HL60 cells (39). Taken together, these findings suggest the possibility that SERCA inhibition is the one mechanism for compounds selectively targeting HL60/MX2. To test this hypothesis, the selectivity ratio of each of the known SERCA inhibitors was evaluated in a cell viability assay in both HL60/MX2 and HL60. Remarkably, TG, CPA, and BHQ all demonstrated selectivity for the resistant cell line (Fig. 5A). TG was the most potent and selective with an IC_{50} of $0.007 \pm 0.001 \mu$ M in HL60/MX2 and an IC_{50} of $3 \pm 1 \mu$ M in HL60, giving a selectivity of 400 fold. Though considerably less potent, both CPA and BHQ also provided good selectivity, with values of 17 and 7.7 fold respectively. Given that the cytotoxic SERCA inhibitors, including (–)-CXL017 (Fig. 5B), all demonstrate selectivity towards HL60/MX2, SERCA inhibition may provide one strategy for targeting multidrug-resistance in cancer chemotherapy.

SERCA Inhibitors Synergize in HL60/MX2

Given the impressive synergy observed between (–)-CXL017 and the known SERCA inhibitors in the biochemical assay, it was hypothesized that this same synergy could be exploited to target HL60/MX2 by combining (–)-CXL017 with one of the other inhibitors in cells. TG was selected to test this hypothesis, as it demonstrated the weakest synergy with (–)-CXL017 in the enzymatic assay and would provide the most rigorous test of cell-based synergy. Accordingly, (–)-CXL017 and TG were evaluated as individual inhibitors and in combination at a constant molar ratio; while cell viability was assessed by cell counting based on the uptake of trypan blue. Two concentrations of (–)-CXL017 (12.5 and 6.25 μ M) and TG (18.75 nM and 9.375 nM) were evaluated to give four possible inhibitor combinations. Treatment with 6.25 μ M (–)-CXL017 and 9.375 nM TG reduced cell viability to 47% and 43% respectively, whereas the combination of the two inhibitors reduced cell viability to 17% (Fig. 6). A similar outcome was observed with the other combinations of (–)-CXL017 and TG evaluated. These results are in agreement with the synergy observed in the SERCA activity assay and provide additional support that the cytotoxicity of (–)-CXL017 in HL60/MX2 is related to SERCA inhibition.

DISCUSSION

The data presented herein demonstrate that CXL017 inhibits SERCA, the Ca^{2+} -ATPase responsible for maintaining the ER calcium load (and low cytoplasmic calcium concentration) (16). The kinetic data relating to SERCA activity in the presence of varied calcium or ATP provide insight into the mode of inhibition by CXL017. Though the apparent calcium affinity (K_{Ca}) of the enzyme is not affected by CXL017, V_{max} for ATPase activity at saturating calcium decreases in a dose-dependent manner (Fig. 2A and B). Furthermore, CXL017 competes with ATP, as indicated by the rightward shift in Fig. 2C, and demonstrates differential inhibition at the regulatory and catalytic binding sites, leading to a dose-dependent response exclusively at the regulatory site (Fig. 2D). Since the physiological range of ATP is in the range of 2–10 mM (pATP 2.7) (40), the regulatory site is probably more relevant to SERCA inhibition in cells. Although the competition observed at the regulatory site may be due to CXL017 occupying the nucleotide-binding domain directly, conformational changes within SERCA are often transmitted allosterically throughout the length of the protein, such that even binding in the TM domain can elicit effects on the flexibility of the cytoplasmic domains (41). For example, TG and CPA are both known to bind in the TM domain, but each induces unique changes to the cytoplasmic headpiece (23). Thus, further experimentation is needed to determine the binding site of CXL017 within the ER Ca^{2+} -ATPase.

In cytotoxicity experiments, CXL017 was established as a novel lead for the treatment of multidrug-resistant leukemia (31). Several analogues have been prepared that reveal a narrow structure-activity relationship within the CXL series (32). Similarly, five CXLS, evaluated in the SERCA activity assay, demonstrate low- to mid-micromolar inhibitory potencies despite modest changes in chemical structure (Fig. 3). Two observations are particularly interesting from these experiments. First, the eudysmic ratio observed in cytotoxicity experiments is maintained in SERCA inhibition such that (–)-CXL017 is the eutomer (31). In fact, (–)-CXL017 is thirteen and tenfold more cytotoxic than (+)-CXL017 in HL60 and HL60/MX2 respectively, while (–)-CXL017 is fivefold more potent than (+)-CXL017 in the SERCA activity assay (Fig. 3). Secondly, while no correlation exists between SERCA inhibition and the cytotoxicity recorded in HL60 cells, a good correlation exists between the former and the cytotoxicity recorded in HL60/MX2 cells (Fig. 7A and B). In light of the elevated SERCA levels and ER calcium content in HL60/MX2 compared to HL60 (39), this correlation strongly suggests that SERCA inhibition is responsible for the

collateral sensitivity of HL60/MX2 to CXL small molecules. The selective cytotoxicity of TG, CPA, and BHQ in HL60/MX2 provides further evidence that SERCA antagonists offer a viable strategy to mitigate the drug-resistant phenotype in HL60/MX2.

The scope of SERCA inhibition in overcoming drug resistance across multiple cancer types remains to be determined. However, among nine resistant leukemia cell lines, where a related CXL compound maintained efficacy, TG succumbs to resistance in five of the nine lineages (32). Of the cells displaying resistance to thapsigargin, four are reported to overexpress P-gp, suggesting that TG is subject to drug efflux. Yet this may also suggest that the different binding modes exerted by CXLs and TG are significant with respect to the resultant cellular response. Regardless of the molecular rationale, the utility of CXLs in treating multidrug-resistant leukemia has been demonstrated, and with respect to HL60/MX2, this can likely be attributed to SERCA inhibition. Future experimentation will be needed to determine the impact of P-gp on TG resistance and whether SERCA alone is responsible for the cytotoxicity of CXLs across various cancer models.

Drug combination studies have proven to be an invaluable strategy for understanding mechanisms of enzyme inhibition but also for therapeutic benefit (42). Indeed, drug cocktails makeup a considerable portion of clinical treatments and are especially valuable in cancer therapy where drug combinations are often more efficacious than medicines used individually (43). In this study, (–)-CXL017 demonstrated a synergy with three known SERCA inhibitors in the SERCA ATPase assay (Fig. 4). While all combinations involving (–)-CXL017 were markedly synergistic, combinations of the known inhibitors were merely additive (Fig. 4). This result was surprising given that TG, CPA, and BHQ bind at different sites within SERCA and exhibit different modes of inhibition. TG, CPA, and BHQ are known to lock SERCA in its E2 conformation upon binding in the TM domain (23), which lends to the notion that a small molecule that synergizes with one of these inhibitors likely synergizes with all of them. Since (–)-CXL017 seems to interfere at or near the nucleotide binding domain in the cytoplasmic region, it is plausible that the conformational changes induced by (–)-CXL017 in the cytoplasmic headpiece are complementary to those induced by the TM inhibitors, thus potentiating the overall effect. Beyond the biochemical assay, synergy is also observed in cell viability experiments, specifically between (–)-CXL017 and TG, thus confirming the contribution of SERCA inhibition to the cytotoxicity of (–)-CXL017 in multidrug-resistant HL60/MX2 cells.

CONCLUSION

Small molecules targeting drug resistant cancers are of great clinical utility. In particular, molecules that maintain efficacy across cancers with unique mechanisms of resistance would be ideal. CXL017 was recently identified as a novel lead for the treatment of multidrug-resistant leukemia that demonstrates efficacy in several chemoresistant leukemia cell lines, despite varied modes of resistance. From the work presented here, CXL017 is shown to inhibit the ER Ca^{2+} -ATPase. Notable results include a synergistic profile that is upheld in cell viability assays, and a good correlation between SERCA inhibition and cell viability that is evident in the resistant (HL60/MX2) but not the parental (HL60) cell line. These findings strongly suggest that SERCA is responsible for the susceptibility of HL60/MX2 to these inhibitors, and that inhibition of SERCA by CXLs may provide a novel route to target multidrug-resistant AML. Research into the scope of these findings in other cancer types as well as the involvement of other potential intracellular targets is underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
CML	chronic myeloid leukemia
SERCA	sarco/endoplasmic reticulum Ca^{2+} -ATPase
P-gp	permeability-glycoprotein
Bcl-2	B-cell lymphoma 2
IP₃R	inositol triphosphate receptor
TG	thapsigargin
CPA	cyclopiazonic acid
BHQ	2,5-di- <i>tert</i> -butylhydroquinone
CI	combination index
f_a	fraction of biological system affected

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Figure 1. Chemical structures of CXL small molecules and known SERCA inhibitors used in this study. Asterisk (*) denotes a chiral center.

Figure 2.

CXL017 inhibits SERCA V_{max} and ATP binding. (A) Ca^{2+} -ATPase activity, normalized to V_{max} of control, measured with varied $[\text{Ca}^{2+}]$ (pCa 7.5–5.0) and fixed $[\text{ATP}]$ ($\text{pATP} = 2.6$), in the presence of no inhibitor (●), 10 μM CXL017 (■), and 30 μM CXL017 (▲). Curves show fits to the Hill equation (Eq. 1). (C) Ca^{2+} -ATPase activity measured with varied $[\text{ATP}]$ (pATP 6.2–2.6) and fixed $[\text{Ca}^{2+}]$ ($\text{pCa} = 5.4$) in the presence of no inhibitor (●), 10 μM CXL017 (■), and 30 μM CXL017 (▲). Curves show fits to the Bi-Michaelis-Menten equation (Eq. 2). (B) Effect on V_{max} (black, left y-axis) and pK_{Ca} (gray, right y-axis) by 10 and 30 μM CXL017 as determined from a fit in A. (D) Effect on $\text{pK}_{\text{ATP}}(\text{cat})$ (black) and $\text{pK}_{\text{ATP}}(\text{reg})$ (gray) by 10 and 30 μM CXL017 as determined from fits in C. Data are expressed as mean $\pm$ SEM, $n = 3$. Figures are representative of three independent experiments. Comparisons of data in B and D were made using an unpaired t test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ compared to the control.

Figure 3.

(-)-CXL017 is the most potent SERCA inhibitor among the CXLs evaluated. CXL small molecules were introduced into the ATPase assay at various concentrations (0–60 μM) with fixed $[\text{Ca}^{2+}]$ and $[\text{ATP}]$. The resulting dose-response curve was fit by a 4-parameter dose-response equation (Eq. 3) to give the IC_{50} value of each inhibitor. Data are expressed as mean $\pm$ SEM, $n = 3$. Figure is representative of three independent experiments.

Figure 4.

(–)-CXL017 synergizes with several known SERCA inhibitors. (–)-CXL017, TG, CPA, and BHQ were introduced into the ATPase assay at the indicated concentrations both individually (see color key below) and in pairs (—) with fixed $[Ca^{2+}]$ and $[ATP]$. Inhibitor concentrations were as follows: (–)-CXL017 (—), 6.25 μ M, 12.5 μ M, and 25 μ M; TG (—), 18.75 nM, 37.5 nM, and 75 nM; CPA (—), 0.16 μ M, 0.31 μ M, and 0.63 μ M; BHQ (—), 0.31 μ M, 0.63 μ M, and 1.25 μ M. (A) (–)-CXL017 and TG dosed individually and in combination at a molar ratio of 667:1. (B) (–)-CXL017 and CPA dosed individually and in combination at a molar ratio of 80:1. (C) (–)-CXL017 and BHQ dosed individually and in combination at a molar ratio of 40:1. (D) TG and CPA dose individually and in combination at a molar ratio of 1:8.33. (E) TG and BHQ dose individually and in combination at a molar ratio of 1:16.7. (F) CPA and BHQ dosed individually and in combination at a molar ratio of 1:2. Data for generation of dose-effect (f_a) plots and calculation of the combination indices (indicated above data points) was facilitated by CompuSyn. $CI < 0.9$, $CI = 0.9-1.1$, and $CI > 1.1$ indicate synergy, additive effect, and antagonism, respectively. Data are expressed as mean $\pm$ SEM, $n = 9$ for individual inhibitor plots, $n = 3$ for inhibitor combination plots. Figures are representative of two independent experiments.

Figure 5. SERCA inhibitors demonstrate selective cytotoxicity in multidrug-resistant AML. (-)-CXL017, TG, CPA, and BHQ were evaluated in a cell viability assay at various concentrations in HL60 and HL60/MX2 cells. The IC₅₀ of each inhibitor was determined by a fit of the respective dose-response curves to the 4-parameter dose-response equation (Eq. 3). (A) IC₅₀ values (Y-axis) of SERCA inhibitors in HL60 (black) and HL60/MX2 (gray) with the selectivity ratio of each inhibitor shown above the bars. (B) Dose-response curves of (-)-CXL017 in HL60 (●) and HL60/MX2 (■). Data are expressed as mean ± SEM, n = 3. Figure is representative of three independent experiments.

Figure 6.

(-)-CXL017 demonstrates synergistic cytotoxicity with TG in HL60/MX2 cells. (-)-CXL017 and TG were evaluated in a cell viability assay individually and in combination at the indicated concentrations to give a molar ratio of 667:1. Relative cell viability was determined based on trypan blue dye exclusion in treated cells. (A) 6.25 μ M (-)-CXL017 (black), 9.375 nM TG (gray), and the combination thereof (white). (B) 12.5 μ M (-)-CXL017 (black), 18.75 nM TG (gray), and the combination thereof (white). Data are expressed as mean $\pm$ SEM, $n = 3$. Figure is representative of three independent experiments.

Figure 7. SERCA inhibition correlates with cytotoxicity in HL60/MX2 but not HL60 among CXL compounds. CXL small molecules were evaluated in the SERCA activity assay (described and illustrated in Figure 3) and their respective inhibition was correlated with their respective cytotoxicity in HL60 and HL60/MX2 as previously reported (31). The r^2 value describes the goodness of fit for the linear regression line. (A) Correlation of SERCA inhibition and cytotoxicity in HL60 cells. (B) Correlation of SERCA inhibition and cytotoxicity in HL60/MX2 cells.