Inhibition of Mnk kinase activity by cercosporamide and suppressive effects on acute myeloid leukemia precursors

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Key Points

- The Mnk inhibitor cercosporamide suppresses human leukemic progenitors and exhibits antileukemic effects in a xenograft mouse model.
- Cercosporamide enhances the antileukemic effects of cytarabine in vitro and in vivo.

Abstract

Mnk kinases regulate phosphorylation and activation of the eukaryotic initiation factor (eIF4E), a protein that plays key roles in the initiation of mRNA translation and whose activity is critical for various cellular functions. eIF4E is deregulated in acute myeloid leukemia (AML) and its aberrant activity contributes to leukemogenesis. We determined whether cercosporamide, an antifungal agent that was recently shown to act as a unique Mnk inhibitor, exhibits antileukemic properties. Treatment of AML cells with cercosporamide resulted in dose-dependent suppression of eIF4E phosphorylation. Such suppression of Mnk kinase activity and eIF4E phosphorylation by cercosporamide resulted in dose-dependent suppressive effects on primitive leukemic progenitors (CFU-L) from AML patients and enhanced the antileukemic properties of cytarabine (Ara-C) or mTORC1 inhibition (rapamycin). Similarly, the combination of cercosporamide with cytarabine resulted in enhanced antileukemic responses in a xenograft mouse model in vivo. Altogether, this work demonstrates that the unique Mnk inhibitor cercosporamide suppresses phosphorylation of eIF4E and exhibits antileukemic effects, in support of future clinical-translational efforts involving combinations of Mnk inhibitors with cytarabine and/or mTOR inhibitors for the treatment of AML.
Introduction

The need for novel therapies for acute myeloid leukemia (AML) remains urgent and of high clinical importance. Multiple signaling pathways that promote leukemic cell survival and proliferation are constitutively activated in AML cells, providing potential therapeutic targets. Among them, the mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways play central roles in leukemogenesis (1-3). MAPK pathways have been previously shown to be involved in the regulation of gene transcription, cell proliferation, and survival (4). There has been extensive evidence that these pathways regulate normal and malignant hematopoiesis and transduce signals generated by engagement of growth factor and cytokine receptors (1).

A family of kinases which are key effectors for MAPK pathways includes the Mnk1 and Mnk2 kinases, which regulate phosphorylation of the eukaryotic initiation factor 4E (eIF4E) in response to a variety of signals (5-13). eIF4E is a key component of the mRNA cap-binding complex (14). The phosphorylation of this protein by Mnk1/2 has important functional consequences for mRNA translation and the regulation of malignant cell proliferation (5, 6). Inhibition of eIF4E may be an important approach for the development of novel treatments for patients with various malignancies, as this protein appears to be critical for growth and survival of cancer cells (15,16) as well as malignant transformation (17,18). On the other hand, Mnk activity does not appear to be required for normal development (19).

In prior studies, we demonstrated that Mnk kinases may act as negative feedback regulators in response to antileukemic agents, including arsenic trioxide (As$_2$O$_3$) (11) and chemotherapy (cytarabine) (20). These studies demonstrated that pharmacological inhibition or siRNA
targeting of Mnk kinases suppresses leukemic progenitor growth and enhances the antileukemic properties of other antileukemia agents (11, 20). However, efforts to therapeutically target Mnk pathways for the treatment of leukemias have been limited by the lack of Mnk inhibitor compounds with potential for clinical development.

Cercosporamide was recently identified during a chemical screen for Mnk1 inhibitors (21). It was demonstrated that this known orally bioavailable anti-fungal agent, is a potent and selective Mnk inhibitor (21). Cercosporamide was found to suppress the growth of melanoma lung metastases and colon carcinoma xenograft tumors (21), but its potential activity against AML cells and other leukemias is unknown. In the present study we examined the effects of cercosporamide on different AML cell lines and primary leukemic progenitors from AML patients. Our data show that cercosporamide is a potent inhibitor of phosphorylation of eIF4E at Ser209 in AML cells and results in potent inhibitory effects on primitive leukemic progenitors (CFU-L) from AML patients. In addition, we found that combinations of low dose cytarabine with cercosporamide result in enhanced antileukemic responses, raising the potential of combinations of cercosporamide with other agents for the treatment of AML.
Materials and Methods

**Cells and Reagents** The U937, MM6, and K562 human leukemia cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and gentamycin. MV4-11 acute myelogenous leukemia cells were purchased from ATCC (American Type Culture Collection Manassas, VA) and cultured in Iscove's modified Dulbecco's medium w/ L-glutamine, 25mM HEPES with 10% FBS adjusted to contain 1.5 g/L sodium bicarbonate. Cytarabine was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against the phosphorylated form of eIF4E on Ser209 and eIF4E were obtained from Cell Signaling Technology (Danvers, MA). The antibody against GAPDH was purchased from Millipore Corporation (Billerica, MA). The mTOR inhibitor rapamycin was purchased from Calbiochem/EMD (San Diego, CA). Cercosporamide was from Eli Lilly and Company (Indianapolis, IN).

**Cell Lysis and Immunoblotting** For the immunoblotting experiments, cells were treated with cercosporamide or rapamycin for the indicated times and lysed in phosphorylation lysis buffer (22, 23). Immunoblotting using an enhanced chemiluminescence method was performed as in previous studies (22, 23).

**Animal Studies** All animal work was approved by Eli Lilly and Company Institutional Animal Care and Use Committee and performed in an Association for Assessment of Laboratory Animal Care (AALAC)-certified facility. MV4-11 cells were implanted at a density of 5 x 10^6 cells per mouse as described (21). Tumors were measured by caliper and tumor volume calculated as
described (21). Once tumors reached a group mean of 100 mm$^3$, animals were randomized to treatment groups- Ara-C (20 mg/kg qd dosed IP), cercosporamide (10 mg/kg BID, 20 mg/kg qd dosed orally by gavage), Ara-C + cercosporamide combinations (as above), or the relative vehicle controls (captisol for Cercosporamide, water for Ara-C). Cercosporamide was formulated as described previously (21). Tumor lysates were taken 2 hours after dosing and western blot analyses were performed as described with antibodies against eIF4E, peIF4E on serine 209 and β-actin, as described (21).

**Cell Proliferation/Viability Assays** Such assays using the MTT methodology were conducted as in our previous studies (24, 25).

**Hematopoietic Progenitor Cell Assays.** Peripheral blood was obtained from patients with AML after obtaining informed consent in accordance with the Declaration of Helsinki and approval from the Northwestern University Institutional Review Board. Clonogenic assays in methylcellulose were performed as in previous studies (20, 24).
Results

In initial studies, we examined the effects of cercosporamide on phosphorylation of eIF4E in AML cells. In experiments using the U937 acute myelomonocytic leukemia cell line, we found that cercosporamide potently inhibited phosphorylation of eIF4E on serine 209 (Fig. 1A). Similar results were obtained when the effects of cercosporamide were assessed on the AML line MM6 (Fig. 1B), and the acute erythroleukemia cell line K562 (Fig. 1C). The inhibitory effects of cercosporamide on eIF4E were dose-dependent with partial suppression of phosphorylation seen at concentration of cercosporamide of 1 μM and maximizing at 5-10 μM (Fig. 1D).

To determine whether cercosporamide exhibits negative regulatory effects on cell proliferation and viability of leukemia cells, MTT assays were conducted. When U937 cells were incubated in the presence or absence of the increasing doses of cercosporamide, we found dose-dependent suppression of cell growth (Fig. 2A). Similar experiments with comparable results were seen when the effects of cercosporamide on MM6 (Fig. 2B) and K562 (Fig. 2C) cells were examined. Altogether, these studies demonstrated that cercosporamide blocks phosphorylation of eIF4E in human AML lines, and such inhibitory effect correlates with decreased cell viability/suppression of leukemic cell proliferation.

In subsequent studies, the effects of Mnk inhibition on primitive leukemic progenitor colony formation were examined. When U937 cells were treated with increasing concentrations of cercosporamide in a methylcellulose culture system, there were dose-dependent suppressive effects on leukemic progenitor colony formation (CFU-L) (Fig. 2D). Also, when the effects of Mnk inhibition on the antileukemic effects of cytarabine were examined, we found that the
A combination of cercosporamide and cytarabine led to more potent inhibitory effects on CFU-L growth than with either alone (Fig. 2E).

We next sought to determine whether Mnk inhibition might similarly enhance the effects of cytarabine (Ara-C) \textit{in vivo} using MV-411 AML xenograft tumors. MV4-11 cells were used to generate the xenograft tumors, as in immunoblotting studies we found that these cells do not express the Jak3 protein (data not shown), whose kinase activity is also inhibited by cercosporamide (21). MV4-11 cells were injected subcutaneously into nude mice. Once palpable tumors reached a group mean size of \(~ 100 \text{ mm}^3\), mice were randomized to treatment groups-vehicle (captisol for cercosporamide or H\textsubscript{2}O for Ara-C), cercosporamide (10 mg/kg orally by gavage twice daily or 20 mg/kg once daily) alone, Ara-C alone (20 mg/kg IP), or cercosporamide plus cytarabine (10 mg/kg BID cercosporamide plus 20 mg/kg Ara-C or 20 mg/kg qd cercosporamide plus 20 mg/kg Ara-C). Treatment with cercosporamide or Ara-C alone significantly suppressed xenograft growth when compared to the respective vehicle (\(p < 0.011\) for 10 mg/kg BID cercosporamide, \(p < 0.006\) for cercosporamide 20 mg/kg qd, \(p < 0.0374\) for Ara-C). The combination of cercosporamide 10 mg/kg BID plus Ara-C was significantly more effective than either agent alone (\(p < 0.0009\) vs. cercosporamide, \(p = 0.005\) vs. Ara-C, \(p < 0.0001\) vs either vehicle) (Fig. 3A). Cercosporamide (20 mg/kg once daily) in combination with Ara-C showed similar effects with significant inhibition of tumor growth vs. captisol (\(p < 0.0001\)) or water (\(p = 0.0003\)) but did not show statistical significance vs. cercosporamide alone (20 mg/kg) or Ara-C alone. In each treatment group, mean body weights increased over the course of the study (Fig. 3B). Further, tumor volume data were evaluated in relation to body weight for each mouse during treatment and revealed no significant effects of body weight on tumor volume...
Finally, eIF4E serine 209 phosphorylation was reduced in all xenografted tumors treated with cercosporamide (Fig. 3C).

We have previously shown that treatment of AML cells with rapamycin results in phosphorylation of eIF4E on Ser 209, consistent with Mnk activation via a feedback loop (20). To determine whether cercosporamide can modulate the effects of antileukemic effects of rapamycin by blocking this negative feedback loop, its effects on rapamycin-dependent phosphorylation of eIF4E were first examined. As shown in Fig. 4A, treatment with rapamycin enhanced phosphorylation of eIF4E on Ser209 (Fig. 4A). Such phosphorylation was completely abrogated by concomitant treatment of the cells with cercosporamide (Fig. 4A). In addition, the combination of rapamycin with cercosporamide resulted in enhanced suppressive effects on CFU-L colony formation, as compared to each agent alone (Fig. 4B). Thus, cercosporamide enhances the antileukemic effects of mTORC1 inhibition by blocking Mnk/eIF4E phosphorylation.

In subsequent studies we examined the effects of cercosporamide on leukemic precursors from different AML patients. The effects of cercosporamide against leukemic CFU-L were assessed in clonogenic assays in methylcellulose. There was dose-dependent suppression of primary CFU-L colony formation (Fig. 5A). Importantly, combinations of cercosporamide with rapamycin (Fig. 5B) or with low doses of cytarabine (Fig. 5C) resulted in enhanced anti-leukemic activity when compared to either of these agents alone.
Discussion

eIF4E is a key eukaryotic initiation translation factor in mammalian cells and its function is critical for mRNA translation of genes whose protein products mediate mitogenic responses, such as c-Myc and cyclin D1 (5, 26). De-regulation of eIF4E promotes leukemogenesis, making it an attractive target for the treatment of leukemias (27-29). This has resulted in efforts to inhibit its function and/or expression for the treatment of AML (29). One approach to directly target eIF4E in the past has been the use of antisense compounds against eIF4E (30). LY2275796 is an antisense oligonucleotide against eIF4E that was developed and studied in solid tumors and found to decrease tumor growth \textit{in vitro} and \textit{in vivo} (30), prompting clinical trials in patients with advanced cancers. In a phase I trial, of the 30 patients who received at least one dose of the eIF4E antisense oligonucleotide, 7 patients had stable disease of at least 6 weeks duration and 2 patients had disease control for over 3 months (31). Other efforts have included the use of the ribavirin, an antiviral drug which may mimic the m$^7$G cap to block eIF4E activity (27). A clinical trial of ribavirin was conducted in patients with AML and among the 11 evaluable patients, there was 1 complete remission (CR) and 2 partial remissions (PRs) (32). Notably, response was found to be associated with re-localization of nuclear eIF4E to the cytoplasm and a decrease in the levels of eIF4E expression (32).

The clinical-translational efforts to target eIF4E by inhibiting its expression (30) or directly targeting its function (32) have been encouraging and underscore the importance of the Mnk pathway in tumorigenesis. As Mnk kinases are required for phosphorylation of eIF4E on Ser209 (5-13), efforts to target and block their kinase activities may provide an important approach for the treatment of malignancies. Such approaches may be particularly attractive because, although
necessary for malignant transformation (17, 18), Mnk kinase-mediated phosphorylation of eIF4E does not appear to be a requirement for normal cellular development and growth (19).

In the current study we examined the effects of cercosporamide, a relatively selective and orally bioavailable Mnk1 inhibitor (21), which was previously shown to have activity against experimental melanoma lung metastases and subcutaneous colon carcinoma xenograft tumors (21). Our data demonstrate that cercosporamide inhibits phosphorylation of eIF4E on Ser209 in AML cells and exhibits direct suppressive effects on leukemic progenitor cell growth, enhancing the antileukemic properties of mTORC1 inhibition by rapamycin and promoting the suppressive effects of cytarabine in vitro. In addition, it strongly enhances the antileukemic effects of cytarabine in MV4-11 xenografts in vivo. These data suggest that the antileukemic effects of cercosporamide are attributable to Mnk inhibition. Indeed, the kinase selectivity profiles of cercosporamide were previously tested in a panel of 76 kinases (21). The only kinase inhibited with potency similar to that on Mnk (i.e. an IC50 less than 100 nmol/L) was Jak3 (21). The AML xenograft chosen for these studies, MV411, does not express Jak3 protein (data not shown), further substantiating the notion that the anti-leukemic effects of cercosporamide reflect Mnk inhibitory activity. Nevertheless, to definitively establish the activity and mode of action, further preclinical studies in the future utilizing a bone marrow-residing model of AML involving use of immunocompetent animals would be useful.

Altogether, our findings underscore the relevance of targeting Mnk pathways for the treatment of AML (20) and raise the possibility of developing cercosporamide as an antileukemic agent. Also, as the mechanism of action of cercosporamide is distinct from ribavirin (32), these studies raise the potential of future combinations of cercosporamide with
ribavirin to target Mnk pathways in AML. In addition, efforts to combine cercosporamide or other Mnk targeting agents with dual mTORC1/mTORC2 catalytic inhibitors, which were recently shown to exhibit potent antileukemic properties against AML precursors (33) may be warranted. Such efforts may be particularly relevant as our data suggest that in response to mTORC1 inhibition in AML cells, there is engagement of a feedback loop involving Mnk/eIF4E (20 and current study). It is also known from prior work that in malignant cells there is S6K-PI3’K-Ras-dependent activation of Mek/Erk (34), which is upstream of Mnk/eIF4E (6). Taken together, our work suggests that targeting Mnk/eIF4E may provide a selective and potent approach to block effectors of MAPK cascades during mTOR inhibition and enhance antileukemic responses in AML and possibly other leukemias.
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Authorship

Contribution: JKA, performed research, designed research, analyzed data, wrote manuscript; AS, BWK, PWI, BK, HG, AS, EV, performed research; JRG, designed research, analyzed data and edited manuscript; LCP, designed research, analyzed data, wrote manuscript.

Conflict of interest disclosure: BWK, PWI and JRG are employees and stockholders of Eli Lilly and Company.
References

Figure Legends

**Figure 1:** Cercosporamide suppresses phosphorylation of eIF4E.  
A. U937 cells were incubated with cercosporamide (10 µM) for 24 hours. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E on serine 209. The same blot was stripped and re-probed with an antibody against eIF4E, as indicated.  
B. MM6 cells were incubated with cercosporamide for 24 hrs. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E on serine 209. The same blot was stripped and re-probed with an antibody against eIF4E, as indicated.  
C. K562 cells were incubated with cercosporamide for 24 hrs. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E on serine 209. The same blot was stripped and re-probed with an antibody against eIF4E, as indicated.  
D. Serum starved U937 cells were treated for one hour with increasing doses of cercosporamide, as indicated. Lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E on serine 209. The same blot was striped and re-probed with an antibody against eIF4E, as indicated.

**Figure 2:** Antileukemic effects of cercosporamide.  
A. U937 cells were incubated for 5 days in the presence or absence of the indicated doses of cercosporamide. Cell proliferation was assessed by an MTT assay. Data are expressed as means ± S.E. of 5 independent experiments.  
B. MM6 cells were incubated for 5 days in the presence or absence of the indicated doses of cercosporamide. Cell proliferation was assessed by an MTT assay. Data are expressed as means ± S.E. of 3 independent experiments.  
C. K562 cells were incubated for 5 days in the presence or absence of the indicated doses of cercosporamide. Cell proliferation was assessed by an MTT assay. Data are expressed as means ± S.E. of 4 independent experiments.  
D. U937 cells were plated in methylcellulose culture assay system with increasing concentrations of cercosporamide, as indicated. Data are expressed as percentage control of leukemic colonies for untreated cells and represent means ± S.E. of 5 experiments.  
E. U937 cells were plated in methylcellulose culture assay system with cytarabine (1ng/ml) and/or cercosporamide (10 µM), as indicated and CFU-L leukemic colony formation was assessed. Data are expressed as a percentage control of CFU-L for untreated cells. Means ± S.E. of the values from 5 independent experiments are shown. Paired t test analysis for the combinations of cytarabine plus cercosporamide, compared to cercosporamide alone, showed p = 0.011, and compared with cytarabine alone, p = 0.009.

**Figure 3:** Combining cercosporamide and Ara-C suppresses growth of MV4-11 AML xenograft tumors.  
A. Tumor-bearing mice were randomized to treatment groups and dosed daily (qd) with 20 mg/kg Ara-C, or with cercosporamide (20 mg/kg qd or 10 mg/ kg BID), or with Ara-C in...
combination with either cercosporamide treatment. The vehicle treated mice (captisol for cercosporamide, water for Ara-C) are shown. Relative tumor volumes measured by caliper are plotted at each time point divided by initial tumor volume for each animal (T/t0) ± SEM. All treatments showed statistically significant activity when compared to the appropriate vehicle treated controls* (cercosporamide 10 mpk BID, p < 0.011, cercosporamide 20 mg/kg qd, p < 0.006, Ara-C, p < 0.0374, cercosporamide 10 mg/kg BID plus Ara-C vs either vehicle, p < 0.0001). Cercosporamide 10 mg/kg BID plus Ara-C was also significantly more effective than cercosporamide, p < 0.0009**, or Ara-C alone, p = 0.0005**. Data are representative of 4 separate xenograft studies. B. Body weight distribution across all treatment groups was calculated by normalizing body weight for each animal at end-of-study (day 42) (W1) with that immediately prior to treatment (W0). C. Western blot analyses for p-eIF4E were run on tumor lysates harvested at the end of study. Cell lysates for the indicated treatment conditions were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E (upper panel). Equal amount of lysates from the same experiment shown in the upper panel were analyzed separately by SDS-PAGE and immunoblotted with an antibody against eIF4E (middle panel). The same blot shown in the middle panel was immunoblotted with an anti-actin antibody, as indicated (lower panel). Each lane represents an individual tumor.

Figure 4: Combined inhibition of mTOR and Mnk activity results in enhanced antileukemic effects. A. U937 cells were incubated for 2 hours with rapamycin, in the presence or absence of cercosporamide, as indicated. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E on serine 209 or with an antibody against total eIF4E as indicated. B. U937 cells were plated in methylcellulose culture assay system with rapamycin (20nM) and/or cercosporamide (10 µM), as indicated and CFU-L leukemic colony formation was assessed. Data are expressed as a percentage control of CFU-L for untreated cells. Means ± S.E. of the values from four independent experiments are shown. Paired t test analysis for the combination of rapamycin plus cercosporamide, compared with cercosporamide alone, showed p = 0.00087, and compared with rapamycin alone, p = 0.00024.

Figure 5: Antileukemic effects of cercosporamide on primary leukemic progenitors from AML patients. A. Dose-dependent suppression of primitive leukemic precursors from AML patients by cercosporamide. Effects were assessed in clonogenic assays in methylcellulose. Data are expressed as percentage control of leukemic colonies for untreated cells and represent means ± S.E. of 3 experiments, using cells from three different patients. B. Effects of the combination of rapamycin (20nM) and cercosporamide (1 µM) on primitive leukemic precursors from AML patients. Leukemic CFU-L colony formation was assessed in clonogenic assays in methylcellulose. Data are expressed as percentage control of leukemic colonies for untreated
cells and represent means ± S.E. of four experiments, using cells from 4 different patients. Paired $t$ test analysis of the combination of rapamycin and cercosporamide, compared with rapamycin alone, showed $p = 0.0188$, and compared with cercosporamide alone, $p = 0.0125$. C. Effects of the combination of Ara-C (1ng/ml) and cercosporamide (1 µM) on primitive leukemic precursors from AML patients. Leukemic CFU-L colony formation was assessed in clonogenic assays in methylcellulose. Data are expressed as percentage control of leukemic colonies for untreated cells and represent means ± S.E. of 4 experiments. Paired $t$ test analysis of the combination of Ara-C and cercosporamide, compared with Ara-C alone, showed $p = 0.0013$, and compared with cercosporamide alone, $p = 0.0193$. 
Figure 1
Figure 2
Figure 3
A

Rapamycin  -  +  -  +
Cercosporamide  -  -  +  +

Blot: anti-phospho-eIF4E (Ser 209)

Blot: anti-eIF4E

B

% Colony Formation

p = 0.00087
p = 0.00024

Figure 4
Figure 5
Inhibition of Mnk kinase activity by cercosporamide and suppressive effects on acute myeloid leukemia precursors