Negative Regulatory Effects of Mnk Kinases in the Generation of Chemotherapy-Induced Antileukemic Responses

Jessica K. Altman, Heather Glaser, Antonella Sassano, Sonali Joshi, Takeshi Ueda, Rie Watanabe-Fukunaga, Rikio Fukunaga, Martin S. Tallman, and Leonidas C. Platanias

Robert H. Lurie Comprehensive Cancer Center and Division of Hematology/Oncology, Northwestern University Medical School, and Jesse Brown VA Medical Center, Chicago, Illinois (J.K.A., H.G., A.S., S.J., M.S.T., L.C.P.); Campbell Family Institute for Breast Cancer Research, Princess Margaret Hospital, Toronto, Ontario, Canada (T.U.); and Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto, Japan (R.W.-F., R.F.)

Received March 12, 2010; accepted July 27, 2010

ABSTRACT

Mnk kinases are downstream effectors of mitogen-activated protein kinase pathways and mediate phosphorylation of the eukaryotic initiation factor (eIF4E), a protein that plays a key role in the regulation of mRNA translation and is up-regulated in acute myeloid leukemia (AML). We determined the effects of chemotherapy (cytarabine) on the activation status of Mnk in AML cells and its role in the generation of antileukemic responses. A variety of experimental approaches were used, including immunoblotting, apoptosis assays, small interfering RNA (siRNA)-mediated knockdown of proteins, and clonogenic hematopoietic progenitor assays in methylcellulose. Cytarabine induced phosphorylation/activation of Mnk and Mnk-mediated phosphorylation of eIF4E on Ser209, as evidenced by studies involving pharmacological inhibition of Mnk or experiments using cells with targeted disruption of *Mnk1* and *Mnk2* genes. To assess the functional relevance of cytarabine-inducible en-

gagement of Mnk/eIF4E pathway, the effects of pharmacological inhibition of Mnk on cytarabine-mediated suppression of primitive leukemic progenitors [leukemic colony forming unit (CFU-L)] were examined. Concomitant treatment of cells with a pharmacological inhibitor of Mnk or siRNA-mediated knockdown of Mnk1/2 strongly enhanced the suppressive effects of low cytarabine concentrations on CFU-L. It is noteworthy that the mammalian target of rapamycin (mTOR) inhibitor rapamycin also induced phosphorylation of eIF4E in a Mnk-dependent manner, whereas inhibition strongly enhanced its antileukemic effects. These data demonstrate that Mnk kinases are activated in a negative-feedback regulatory manner in response to chemotherapy and impair the generation of antileukemic responses. They also identify this pathway as a novel target for the design of new approaches to enhance the antileukemic effects of chemotherapy or mTOR inhibitors in AML.

There is significant interest in developing the means to increase the effectiveness of chemotherapeutic agents used for the treatment of acute myeloid leukemia (AML). Although chemotherapy results in significant remission rates, there are profound associated toxicities, and even after obtaining complete remission, most patient relapse and die (Farag et al., 2005; Büchner et al., 2006). AML results from mutations of different groups of genes that control differen-

tiation, proliferation/survival, and differentiation/apoptosis (Gilliland and Tallman; 2002). Various mutations lead to aberrant activation of cell survival pathways, including the mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways (Platanias, 2003; Hay, 2005; Bhaskar and Hay, 2007; Altman and Platanias, 2008; Martelli et al., 2009). Because the disease continues to have a very poor prognosis, novel therapeutic approaches are desperately needed to increase the effectiveness of current treatment strategies that traditionally include various chemotherapeutic regimens (Farag et al., 2005; Büchner et al., 2006).

MAPK pathways play important roles in the regulation of key cellular functions, including control of gene transcription, cell proliferation, and survival. There are three major

This work was supported by the National Institutes of Health National Center for Research Resources [Grant KL2-RR025740]; the National Institutes of Health National Cancer Institute [Grants CA121192, CA100579, CA77816]; and the Department of Veterans Affairs.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.110.064642.

ABBREVIATIONS: AML, acute myeloid leukemia; Ara-C, cytarabine; CFU-L, leukemic colony forming unit; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; Mnk1/2, mitogen-activated protein kinase-interacting kinases 1 and 2; p70 S6K, p70 S6 kinase; rpS6, S6 ribosomal protein; siRNA, small interfering RNA; PAGE, polyacrylamide gel electrophoresis; CGP57380, N3-(4-fluorophenyl)-1H-pyrazolo-[3,4-d]pyrimidine-3,4-diamine.

families of MAP kinases: p38 MAP kinase, the extracellular signal-regulated kinase (Erk), and the c-Jun NH₂-terminal kinase (Platanias, 2005; Raman et al., 2007). Among other things, MAP kinases play an important functional role in the regulation of normal and malignant hematopoiesis by growth factors and cytokines (Platanias, 2003). Mnk1 and the related Mnk2 kinases are known downstream effectors of p38 and ERK, and once activated, they phosphorylate the cap binding eukaryotic initiation factor 4E (eIF4E) in response to mitogens and stress signals (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997, 1999; Scheper et al., 2001). Because Mnks regulate the phosphorylation of eIF4E, a key element of the cap-binding complex, their function complements the mTOR pathway in promoting cap-dependent mRNA translation and malignant cell growth. Although extensive work has been focused in targeting mTOR (Bhaskar and Hay, 2007), there have been no substantial clinical-translational efforts to target Mnk kinases for the treatment of malignancies. In a previous work, we demonstrated that Mnk kinases negatively regulate the generation of arsenic trioxide (As₂O₃)-induced apoptosis and that antileukemic effects in response to As₂O₃ can be enhanced by pharmacological inhibition of Mnk (Dolniak et al., 2008). Although As₂O₃ is a unique antileukemic agent and induces responses to a large extent via generation of reactive oxygen species (Platanias, 2009), these findings have raised the possibility that negative-feedback regulatory mechanisms involving Mnk kinases may account for the development of leukemic cell resistance in response to chemotherapeutic drugs and other antileukemic agents.

Cytarabine (Ara-C) is among the most effective chemotherapeutic drugs for the treatment of AML and has been the key agent used in the treatment of AML for many years (Herzig et al., 1985). It is a pyrimidine analog that acts as an anti-metabolite, and its major use in oncology is in the treatment of AML both at diagnosis and relapse (van Prooijen et al., 1977). In the present study, we determined the effects of cytarabine on the activation of the Mnk/eIF4E pathway. Our data demonstrate that Mnk is phosphorylated/activated by low doses of cytarabine and that such phosphorylation leads to the downstream activation of eIF4E. Pharmacological or molecular targeting of Mnk in leukemic cell lines or primitive leukemic progenitors from patients with AML results in potent enhancement of chemotherapy-induced suppression. We also provide evidence that simultaneous targeting the mTOR and Mnk/eIF4E pathways leads to more potent antileukemic responses than selective targeting of each pathway alone, providing a rationale for the future development of clinical-translational efforts involving combinations of mTOR and Mnk inhibitors for the treatment of AML.

Materials and Methods

Cells and Reagents. The U937 and K562 human leukemia cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and gentamicin. Cytarabine was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against the phosphorylated or nonphosphorylated forms of Mnk, eIF4E, Akt, rpS6, and p70S6K were obtained from Cell Signaling Technology (Danvers, MA). The antibody against GAPDH was purchased from Millipore Corporation (Billerica, MA). The mTOR inhibitor rapamycin and the Mnk inhibitor *N*3-(4-fluorophenyl)-1*H*-pyrazolo-[3,4-*d*]pyrimidine-3,4-diamine (CGP57380) were purchased from Calbiochem/EMD (San Diego, CA). Immortalized mouse embryonic fibroblasts (MEFs)

from Mnk1(−/−)/Mnk2(−/−) mice were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics (Ueda et al., 2004; Dolniak et al., 2008). siRNAs against Mnk 1 and 2 and control siRNA were obtained from Dharmacon RNA Technologies (Lafayette, CO).

Cell Lysis and Immunoblotting. For the immunoblotting experiments, cells were treated with cytarabine (200 ng/ml) for the indicated times and lysed in phosphorylation lysis buffer (Lekmine et al., 2003). In some experiments, the cells were preincubated with the Mnk inhibitor CGP57380 (10 μM) or rapamycin (20 nM), as indicated before treatment with cytarabine. Immunoblotting using an enhanced chemiluminescence method was performed as in previous studies (Lekmine et al., 2003).

Evaluation of Apoptosis. Cells were exposed to cytarabine (50 ng/ml) for 24 h, as indicated. Flow cytometric assays to assess apoptosis by annexin and propidium iodide staining were performed as described previously (Kannan-Thulasiraman et al., 2006).

Hematopoietic Progenitor Cell Assays. Peripheral blood was obtained from patients with AML after obtaining consent approved by the Institutional Review Board of Northwestern University and used in clonogenic assays in methylcellulose (Giafis et al., 2006; Kannan-Thulasiraman et al., 2006). Primary peripheral blood-derived leukemic colonies were scored at approximately 14 days (range, 11–18). In brief, cells were separated over Ficoll-Hypaque and cultured with the indicated concentrations of cytarabine. In the experiments in which the effects of Mnk inhibition were determined, the cells were incubated in the presence or absence of very low concentrations of cytarabine with or without the indicated concentrations of rapamycin (10 nM) or the Mnk inhibitor CGP57380 (10 μM).

Results

In initial studies, we sought to determine whether treatment of acute leukemia cells with cytarabine results in activation of Mnk. U937 cells (Fig. 1A) were treated with cytarabine, and cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Mnk 1 on Thr197 and Thr202 (corresponding to Thr209 and Thr214 of human Mnk1, respectively). Treatment with cytarabine resulted in strong Mnk phosphorylation on Thr197/202 (Fig. 1A), a site that correlates with induction of Mnk kinase activity. In addition, in experiments to determine whether the downstream effector of Mnk, eukaryotic initiation factor eIF4E, is phosphorylated during treatment of cells with cytarabine, we found strong phosphorylation of the protein on serine 209 (Fig. 1B). To determine whether such eIF4E phosphorylation is Mnk-dependent, the effects of the Mnk inhibitor CGP57380 were assessed. As shown in Fig. 1B, treatment of cells with CGP57380 blocked the induction of cytarabine-dependent phosphorylation of eIF4E, establishing that this event is Mnk-dependent. Likewise, Ara-C-dependent phosphorylation of Mnk and eIF4E were seen when the K562 leukemia cell line was studied (Fig. 1, C and D).

We then sought to determine the functional relevance of phosphorylation/activation of Mnk and downstream engagement of eIF4E. Experiments were performed in which U937 cells were treated with low concentrations of cytarabine in the presence or absence of the Mnk inhibitor CGP57380, and CFU-L colony formation was assessed. As shown in Fig. 1, E and F, the addition of CGP57380 to the cultures significantly enhanced cytarabine-dependent suppression of CFU-L colony formation, suggesting that the activation of the Mnk/eIF4E pathway in response to cytarabine occurs in a negative-feedback regulatory manner to counteract the antileukemic effects

Fig. 1. Cytarabine-dependent phosphorylation/activation of Mnk and its downstream effector eIF4E. **A**, U937 acute myeloid leukemia cells were incubated with cytarabine (Ara-C) for 10 min. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Mnk on threonine 197/202 or with an anti-GAPDH antibody as indicated. **B**, U937 cells were incubated in the absence or presence of CGP57380, with or without cytarabine for the indicated times. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of eIF4E on serine 209, or against eIF4E or against GAPDH, as indicated (left). The signals for phosphorylated eIF4E and total eIF4E were quantitated by densitometry, and the intensity of eIF4E phosphorylation relative to the levels of total eIF4E expression was calculated (right). **C**, K562 cells were incubated with cytarabine for 10 min. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Mnk on threonine 197/202 or with an anti-Mnk antibody as indicated. **D**, K562 were incubated with cytarabine for the indicated times. 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 (left). The signals for the different bands shown in blot D were quantitated by densitometry and the intensity of eIF4E phosphorylation relative to the levels of eIF4E expression was calculated (right). **E**, U937 cells were plated in methylcellulose culture assay system with cytarabine (0.08 ng/ml) and/or CGP57380, as indicated, and CFU-L leukemic colony formation was assessed. Data are expressed as a percentage control of CFU-L for untreated cells. Means $\pm$ S.E. of the values from five independent experiments are shown. Paired *t* test analysis for the combinations of cytarabine plus CGP57380, compared with cytarabine alone, showed a *p* value of 0.022 and compared with CGP57380 alone, showed a *p* value of 0.037. **F**, U937 cells were plated in methylcellulose culture assay system with cytarabine (0.9 ng/ml) and/or CGP57380, as indicated, and CFU-L leukemic colony formation was assessed. Data are expressed as percent control of CFU-L colony formation for untreated cells. Means $\pm$ S.E. of the values from four independent experiments are shown. Paired *t* test analysis for the combinations of cytarabine plus CGP57380, compared with cytarabine alone, showed a *p* value of 0.038 and compared with CGP57380 alone, showed a *p* value of 0.021. **G**, U937 cells were then incubated in the absence or presence of CGP57380 for 60 min. Cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of rpS6 on serine 235/236 or against rpS6 (left) or with an antibody against the phosphorylated form of Akt on serine 473 or against Akt (middle) or with an antibody against the phosphorylated form of p70 S6K on threonine 421/serine 424 or against p70 S6K (right).

of cytarabine. As expected, CGP57380 did not inhibit the phosphorylation of rpS6, Akt, or p70S6K, indicating that the enhancing effects of CGP57380 do not result by the modulation of elements of the AKT/mTOR pathway (Fig. 1G).

To further establish the functional relevance of the Mnk/eIF4E pathway in the generation of cytarabine-induced responses, experiments were carried out using cells with targeted disruption of both the *Mnk1* and *Mnk2* genes (Ueda et al., 2004). Mnk1/2(+/+) or Mnk1/2(-/-) MEFs were treated with cytarabine, and eIF4E phosphorylation on Ser209 was assessed. As shown in Fig. 2A, cytarabine-induced phosphorylation of eIF4E on Ser209 was completely abrogated in the absence of Mnk1/Mnk2 (Fig. 2A). It is noteworthy that cytarabine-induced apoptosis was greatly enhanced in such cells (Fig. 2B), strongly suggesting that negative-feedback activation of the Mnk/eIF4E pathway results in generation of antiapoptotic, prosurvival signals.

In other studies, we determined whether siRNA-mediated knockdown of Mnk1 and Mnk2 enhances the antileukemic effects of low-dose cytarabine on U937-derived CFU-L progenitors (Dolnik et al., 2008). When U937 cells were transfected with siRNAs specifically targeting *Mnk1* and *Mnk2*, there was suppression of Mnk protein expression (Fig. 3A) compared with cells transfected with control siRNA. Knockdown of Mnk1/2 did not affect significantly baseline leukemic CFU-L colony formation (Fig. 3B). However, such knockdown strongly enhanced the inhibition of CFU-L formation in response to cytarabine (Fig. 3B).

Fig. 2. Mnk1 and Mnk2 are required for cytarabine-induced phosphorylation of eIF4E and targeted disruption of the *Mnk1/Mnk2* genes potentiates cytarabine-induced apoptosis. **A**, Mnk1/2(+/+) and Mnk1/2(-/-) MEFs were incubated in the absence or presence of cytarabine for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of eIF4E on serine 209 (top left). The same blot was then stripped and reprobed for eIF4E, and then GAPDH was used to assess for equivalent protein loading. The signals for the different bands shown in blot A were quantitated by densitometry, and the intensity of eIF4E phosphorylation relative to the levels of eIF4E expression was calculated (right). **B**, Mnk1/2(+/+) and Mnk1/2(-/-) MEFs were incubated in the absence or presence of cytarabine for 24 h. The total percentage of apoptotic cells was determined by flow cytometry using propidium iodide/annexin V staining. The data are expressed as the means $\pm$ S.E. of seven experiments.

All together, our studies with cell lines provided strong evidence that activation of the Mnk/eIF4E pathway abrogates chemotherapy-induced apoptosis and generation of antileukemic responses. To determine the functional relevance of this pathway in a more physiologically relevant system, experiments were performed using primary leukemic samples from patients with AML. Increasing concentrations of cytarabine resulted in a dose-dependent suppression of primitive CFU-L progenitors from patients with AML (Fig. 4A). Very low doses of cytarabine (0.08 ng/ml) or CGP57380 alone each had minimal effects on leukemic progenitor colony formation (Fig. 4B). However, the combination of cytarabine with CGP57380 resulted in potent antileukemic effects, far greater than each agent alone (paired p value = 0.0025 for cytarabine plus CGP57380 versus cytarabine alone, and paired p value = 0.033 for cytarabine plus CGP57380 versus CGP57380 alone) (Fig. 4B).

In previous studies, we have demonstrated that targeting of the mTOR pathway enhances the suppressive effects of arsenic trioxide on primary leukemic progenitors from patients with AML (Altman et al., 2008). Because both the mTOR and Mnk/eIF4E pathways converge at the level of eIF4E, we sought to determine whether combined targeting of mTOR and Mnk results in enhanced antileukemic effects. In experiments in which the effects of rapamycin on eIF4E phosphorylation on Ser209 were assessed in leukemia cells, we found rapamycin-dependent eIF4E phosphorylation on Ser209 (Fig. 5A), suggesting that Mnk kinases are activated in a negative-feedback manner during targeted inhibition of the mTOR pathway. On the other hand, and as expected, treatment of cells with rapamycin inhibited phosphorylation of the p70 S6K (Fig. 5B). When the effects of dual mTOR and

Fig. 3. Knockdown of Mnk1 and Mnk2 enhances the suppressive effects of low dose cytarabine on primitive leukemic progenitors. **A**, U937 cells were transfected with control siRNA or a mixture of siRNAs targeting Mnk1 and Mnk2, as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with antibody against Mnk (top). The same blot was then reprobed with anti-GAPDH antibody to control for protein loading (bottom). **B**, U937 cells were transfected with either control siRNA or a mixture of siRNAs specifically targeting Mnk1 and Mnk2. The cells were subsequently incubated in methylcellulose in the presence or absence of cytarabine. Means $\pm$ S.E. of four independent experiments are shown. Paired t test analysis comparing the effects of cytarabine in the presence of Mnk1 and Mnk2 siRNA versus its effects in the presence of control siRNA showed a p value of 0.0044.

Mnk pharmacological inhibition on CFU-L colony formation derived from either U937 cells (Fig. 5C) or primary peripheral blood samples from patients with AML (Fig. 5D) were examined, there was much more potent suppression of leukemic hematopoiesis than the ones seen in response to each agent alone. Thus, inhibition of the mTOR pathway results in feedback activation of Mnk/eIF4E, and simultaneous targeted inhibition of Mnk/eIF4E may provide an approach to enhance rapamycin-generated antileukemic responses in vitro and possibly in vivo.

Discussion

Although the mechanisms by which various chemotherapeutic agents induce cytotoxic effects and apoptosis of malignant cells have been studied extensively, very little is known on negative-feedback signaling pathways that are activated as “defense” mechanisms by malignant cells in response to cytotoxic agents. In previous work we had established that the p38 MAPK and its upstream effectors, Mitogen-activated protein kinase kinase 3 and 6, are activated in a negative-feedback regulatory manner during treatment of leukemia cells with arsenic trioxide (Verma et al., 2002; Giasis et al., 2006). Such engagement of the p38 MAPK pathway seems to exhibit negative effects on arsenic-inducible apoptosis and generation of antileukemic responses (Verma et al., 2002; Giasis et al., 2006). Likewise, others have shown that the p38 MAPK counteracts the induction of proapoptotic responses in multiple myeloma cells (Wen et al., 2008). Such findings have

raised the potential of concomitant targeting of p38 MAPK as a means to enhance the antileukemic effects of arsenic trioxide and other arsenicals in vitro and, possibly, in vivo (Platanias, 2003). It is noteworthy that Mnk kinases act as key effectors downstream of the arsenic-induced p38 MAPK pathway in the generation of such negative responses (Dolniak et al., 2008), whereas another p38 effector kinase that plays an important role in the control of arsenic-responses is the mitogen-activated stress kinase 1 (Kannan-Thulasiraman et al., 2006).

It is well established by previous extensive work that Mnk1 and Mnk2 are activated downstream of p38 MAPK and/or ERK pathways in different systems (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997) and are the key serine kinases that regulate serine phosphorylation of eIF4E on serine 209 (Ueda et al., 2004). There is evidence that such eIF4E engagement plays a positive role in the induction of mRNA translation and protein expression in other systems (Bonneau and Sonenberg, 1987; Pyronnet et al., 2001; Takei et al., 2001), suggesting a potentially important role for Mnk kinases in the regulation of key cellular functions. However, there is also evidence that in certain systems, Mnk1 and Mnk2 may play negative roles in protein translation and synthesis (Knauf et al., 2001; Shenberger et al., 2007), underscoring the complexity of the system and the difficulties in establishing the precise functional roles of these kinases in controlling cellular functions. Beyond targeting eIF4E, Mnks phosphorylate heterogeneous nuclear ribonucleoprotein A1 (Buxadé et al., 2005), a nuclear protein known to be involved in the control of alternating splicing (Mayeda and Krainer, 1992; Guil et al., 2006); and polypyrimidine tract-binding protein-associated splicing factor (Buxadé et al., 2008), an RNA-binding protein that forms a complex with 54-kDa nuclear RNA-binding protein, to regulate nuclear RNA processing, as well as nuclear retention and stability of edited RNA (Shav-Tal and Zipori, 2002). Thus, diverse signals are activated downstream of Mnks to regulate RNA translation, processing, and stability.

In the present study, we provide evidence that Mnk kinases are paradoxically activated during the treatment of cells with cytarabine chemotherapy. Our data demonstrate that treatment of acute leukemia cell lines with cytarabine results in phosphorylation/activation of Mnk1 and downstream engagement of the initiation factor eIF4E. It is noteworthy that pharmacological or molecular targeting of Mnk1/2 results in enhanced induction of apoptosis and cytarabine-induced suppression on primitive leukemic progenitors. These data suggest that engagement of Mnk kinases regulates mRNA translation of antiapoptotic and progrowth genes in AML cells and raise the potential for the design and development of novel therapeutic approaches in the future, involving combinations of chemotherapeutic agents with drugs that block the Mnk pathway or its effectors. The precise mechanism by which the treatment of AML cells with chemotherapy results in Mnk activation also remains to be established in future studies. It is likely that such engagement of Mnk occurs downstream of activation MAP kinases, because extensive previous work has shown that the Mnk pathway is activated downstream of the p38 MAPK and/or ERK in other systems (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997). It is also possible that generation of

Fig. 4. Pharmacological inhibition of Mnk enhances the suppressive effects of cytarabine on primitive leukemic progenitors (CFU-L) from patients with AML. A, peripheral blood mononuclear cells from three patients with AML were plated in methylcellulose culture assay system with increasing concentrations of Ara-C as indicated. Data are expressed as a percentage control of leukemic colonies for untreated cells and represent means $\pm$ S.E. of three experiments. B, peripheral blood mononuclear cells from patients with AML were plated in methylcellulose culture assay system with Ara-C and CGP57380, as indicated. Data are expressed as a percentage of control of CFU-L colony numbers for untreated cells. Means $\pm$ S.E. of the values from five experiments using different patient samples are shown. Paired *t* test analysis of the combination of Ara-C plus CGP57380, compared with Ara-C alone, showed $p = 0.0025$, and compared with CGP57380 alone, $p = 0.033$.

reactive oxygen species is involved in the process, but this remains to be directly examined in future studies.

There is extensive previous evidence that eIF4E has oncogenic activity (Wendel et al., 2004), whereas there is an absolute requirement for Mnk-mediated Ser209 phosphorylation for eIF4E's oncogenic action (Wendel et al., 2007). Moreover, there is evidence for eIF4E overexpression in various tumors, including leukemias (Topisirovic et al., 2003), and this has led to efforts to target eIF4E for the treatment of AML using either antisense oligonucleotides against eIF4E (Tamburini et al.,

2009) or the antiviral drug ribavirin (Assouline et al., 2009). It is of particular interest that a recent study demonstrated that ribavirin, a physical mimic of the m⁷G cap, induced 1 complete response and 2 partial responses, among 11 patients with M4/M5 AML who had refractory or relapsed disease (Assouline et al., 2009). Because Mnk inhibition provides an additional distinct approach to block eIF4E activity, these findings, taken together with our data, suggest that combinations of Mnk inhibitors with ribavirin and chemotherapy may result in more pronounced antileukemic activities than each agent alone.

Fig. 5. Combined inhibition of mTOR and Mnk activity results in enhanced antileukemic responses. **A**, U937 cells were treated for 60 min in the absence or presence of rapamycin as indicated. Subsequently, CGP57380 was added to the cultures as indicated, and cells were incubated for an additional 4 h. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of eIF4E on serine 209 or against eIF4E or against GAPDH as indicated. The signals for the different bands were quantitated by densitometry (below), and the intensity of eIF4E phosphorylation relative to the levels of eIF4E expression was calculated. **B**, U937 cells were treated for 60 min in the absence or presence of rapamycin, as indicated. Subsequently, CGP57380 was added to the cultures as indicated, and cells were incubated for an additional 4 h. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of p70S6 kinase (p70S6K) on threonine 421/serine 424 or against p70S6K, as indicated. The signals for the different bands were quantitated by densitometry (below), and the intensity of p70S6K phosphorylation relative to the levels of p70S6K expression was calculated. **C**, U937 cells were plated in methylcellulose culture assay system with CGP57380 and/or rapamycin, as indicated. Data are expressed as a percentage of control of leukemic colonies for untreated cells. Means $\pm$ S.E. of the values from five independent experiments are shown. Paired *t* test analysis of the combinations of CGP57380 and rapamycin, compared with CGP57380 alone, showed $p = 0.0015$ and compared with Rapa alone, showed p value 0.0014. **D**, peripheral blood mononuclear cells from patients with AML were plated in a methylcellulose culture assay system with CGP57380 and/or rapamycin, as indicated. Data are expressed as a percentage of control of CFU-L for untreated cells. Means $\pm$ S.E. of the values from six experiments using different patient samples are shown. Paired *t* test analysis of the combinations of CGP57380 and rapamycin, compared with CGP57380 alone, showed $p = 0.002$ and compared with rapamycin alone, showed $p = 0.018$.

Our studies also provide evidence that mTOR inhibition results in negative feedback up-regulation of Mnk activity and eIF4E phosphorylation and demonstrate that combined inhibition of mTOR- and Mnk-pathways results in more pronounced effects than targeting each pathway alone. Because there is a major interest toward targeting mTOR for the treatment of AML and other hematological malignancies (Récher et al., 2005; Altman and Platanias, 2008; Martelli et al., 2009), these findings also raise the prospect of future clinical translational efforts involving the combinations of Mnk and mTOR inhibitors for the treatment of AML, and this should be directly examined in future studies.

References

- Altman JK and Platanias LC (2008) Exploiting the mammalian target of rapamycin pathway in hematologic malignancies. *Curr Opin Hematol* **15**:88–94.
- Altman JK, Yoon P, Katsoulidis E, Kroczyńska B, Sassano A, Redig AJ, Glaser H, Jordan A, Tallman MS, Hay N, et al. (2008) Regulatory effects of mammalian target of rapamycin-mediated signals in the generation of arsenic trioxide responses. *J Biol Chem* **283**:1992–2001.
- Assouline S, Culjkovic B, Cocolakis E, Rousseau C, Beslu N, Amri A, Caplan S, Leber B, Roy DC, Miller WH Jr., and Borden KL (2009) Molecular targeting of the oncogene eIF4E in acute myeloid leukemia (AML): a proof-of-principle clinical trial with ribavirin. *Blood* **114**:257–260.
- Bhaskar PT and Hay N (2007) The two TORCs and Akt. *Dev Cell* **12**:487–502.
- Bonneau AM and Sonenberg N (1987) Involvement of the 24-kDa cap-binding protein in regulation of protein synthesis in mitosis. *J Biol Chem* **262**:11134–11139.
- Büchner T, Berdel WE, Schoch C, Haferlach T, Serve HL, Kienast J, Schnittger S, Kern W, Tchinda J, Reichle A, et al. (2006) Double induction containing either two courses or one course of high-dose cytarabine plus mitoxantrone and postremission therapy by either autologous stem-cell transplantation or by prolonged maintenance for acute myeloid leukemia. *J Clin Oncol* **24**:2480–2489.
- Buxadé M, Morrice N, Krebs DL, and Proud CG (2008) The PSF/p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha. *J Biol Chem* **283**:57–65.
- Buxadé M, Parra JL, Rousseau S, Shpiro N, Marquez R, Morrice N, Bain J, Espel E, and Proud CG (2005) The Mnk2s are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. *Immunity* **23**:177–189.
- Dolnik B, Katsoulidis E, Carayol N, Altman JK, Redig AJ, Tallman MS, Ueda T, Watanabe-Fukunaga R, Fukunaga R, and Platanias LC (2008) Regulation of arsenic trioxide-induced cellular responses by Mnk1 and Mnk2. *J Biol Chem* **283**:12034–12042.
- Farag SS, Ruppert AS, Mrózek K, Mayer RJ, Stone RM, Carroll AJ, Powell BL, Moore JO, Pettenati MJ, Koduru PR, et al. (2005) Outcome of induction and postremission therapy in younger adults with acute myeloid leukemia with normal karyotype: a cancer and leukemia group B study. *J Clin Oncol* **23**:482–493.
- Fukunaga R and Hunter T (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J* **16**:1921–1933.
- Giannis N, Katsoulidis E, Sassano A, Tallman MS, Higgins LS, Nebreda AR, Davis RJ, and Platanias LC (2006) Role of the p38 mitogen-activated protein kinase pathway in the generation of arsenic trioxide-dependent cellular responses. *Cancer Res* **66**:6763–6771.
- Gilliland DG and Tallman MS (2002) Focus on acute leukemias. *Cancer Cell* **1**:417–420.
- Gül S, Long JC, and Cáceres JF (2006) hnRNP A1 relocalization to the stress granules reflects a role in the stress response. *Mol Cell Biol* **26**:5744–5758.
- Hay N (2005) The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* **8**:179–183.
- Herzig RH, Lazarus HM, Wolff SN, Phillips GL, and Herzig GP (1985) High-dose cytosine arabinoside therapy with and without anthracycline antibiotics for remission induction of acute nonlymphoblastic leukemia. *J Clin Oncol* **3**:992–997.
- Kannan-Thulasiraman P, Katsoulidis E, Tallman MS, Arthur JS, and Platanias LC (2006) Activation of the mitogen- and stress-activated kinase 1 by arsenic trioxide. *J Biol Chem* **281**:22446–22452.
- Knauf U, Tschopp C, and Gram H (2001) Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. *Mol Cell Biol* **21**:5500–5511.
- Lekmine F, Uddin S, Sassano A, Parmar S, Brachmann SM, Majchrzak B, Sonenberg N, Hay N, Fish EN, and Platanias LC (2003) Activation of the p70 S6 kinase and phosphorylation of the 4E-BP1 repressor of mRNA translation by type I interferons. *J Biol Chem* **278**:27772–27780.
- Martelli AM, Evangelisti C, Chiarini F, Grimaldi C, Manzoli L, and McCubrey JA (2009) Targeting the PI3K/AKT/mTOR signaling network in acute myelogenous leukemia. *Expert Opin Investig Drugs* **18**:1333–1349.
- Mayed A and Krainer AR (1992) Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. *Cell* **68**:365–375.
- Platanias LC (2003) Map kinase signaling pathways and hematologic malignancies. *Blood* **101**:4667–4679.
- Platanias LC (2005) Mechanisms of type-I- and type-II-interferon-mediated signaling. *Nat Rev Immunol* **5**:375–386.
- Platanias LC (2009) Biological responses to arsenic compounds. *J Biol Chem* **284**:18583–18587.
- Pyronnet S, Dostie J, and Sonenberg N (2001) Suppression of cap-dependent translation in mitosis. *Genes Dev* **15**:2083–2093.
- Raman M, Chen W, and Cobb MH (2007) Differential regulation and properties of MAPKs. *Oncogene* **26**:3100–3112.
- Récher C, Dos Santos C, Demur C, and Payrastra B (2005) mTOR, a new therapeutic target in acute myeloid leukemia. *Cell Cycle* **4**:1540–1549.
- Scheper GC, Morrice NA, Kleijn M, and Proud CG (2001) The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. *Mol Cell Biol* **21**:743–754.
- Shav-Tal Y and Zipori D (2002) PSF and p54(nrb)/NonO—multi-functional nuclear proteins. *FEBS Lett* **531**:109–114.
- Shenberger JS, Zhang L, Hughlock MK, Ueda T, Watanabe-Fukunaga R, and Fukunaga R (2007) Roles of mitogen-activated protein kinase signal-integrating kinases 1 and 2 in oxidant-mediated eIF4E phosphorylation. *Int J Biochem Cell Biol* **39**:1828–1842.
- Takei N, Kawamura M, Hara K, Yonezawa K, and Nawa H (2001) Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes: comparison with the effects of insulin. *J Biol Chem* **276**:42818–42825.
- Tamburini J, Green AS, Chapuis N, Bardet V, Lacombe C, Mayeux P, and Bouscary D (2009) Targeting translation in acute myeloid leukemia: a new paradigm for therapy? *Cell Cycle* **8**:3893–3899.
- Topisirovic I, Guzman ML, McConnell MJ, Licht JD, Culjkovic B, Neering SJ, Jordan CT, and Borden KL (2003) Aberrant eukaryotic translation initiation factor 4E-dependent mRNA transport impedes hematopoietic differentiation and contributes to leukemogenesis. *Mol Cell Biol* **23**:8992–9002.
- Ueda T, Watanabe-Fukunaga R, Fukuyama H, Nagata S, and Fukunaga R (2004) Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. *Mol Cell Biol* **24**:6539–6549.
- van Prooijen R, van der Kleijn E, and Haanen C (1977) Pharmacokinetics of cytosine arabinoside in acute myeloid leukemia. *Clin Pharmacol Ther* **21**:744–750.
- Verma A, Mohindru M, Deb DK, Sassano A, Kambhampati S, Ravandi F, Minucci S, Kalvakolanu DV, and Platanias LC (2002) Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to arsenic trioxide. *J Biol Chem* **277**:44988–44995.
- Waskiewicz AJ, Flynn A, Proud CG, and Cooper JA (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J* **16**:1909–1920.
- Waskiewicz AJ, Johnson JC, Penn B, Mahalingam M, Kimball SR, and Cooper JA (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. *Mol Cell Biol* **19**:1871–1880.
- Wen J, Cheng HY, Feng Y, Rice L, Liu S, Mo A, Huang J, Zu Y, Ballon DJ, and Chang CC (2008) P38 MAPK inhibition enhancing ATO-induced cytotoxicity against multiple myeloma cells. *Br J Haematol* **140**:169–180.
- Wendel HG, De Stanchina E, Fridman JS, Malina A, Ray S, Kogan S, Cordon-Cardo C, Pelletier J, and Lowe SW (2004) Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* **428**:332–337.
- Wendel HG, Silva RL, Malina A, Mills JR, Zhu H, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Teruya-Feldstein J, Pelletier J, et al. (2007) Dissecting eIF4E action in tumorigenesis. *Genes Dev* **21**:3232–3237.

Address correspondence to: Dr. Jessica K. Altman, Robert H. Lurie Comprehensive Cancer Center, 303 East Superior Street, Lurie 5-111, Chicago, IL 60611. E-mail: j-altman@northwestern.edu