Activation of mammalian target of rapamycin and the p70 S6 kinase by arsenic trioxide in BCR-ABL–expressing cells

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Abstract

Arsenic trioxide (As_2O_3) exhibits important antitumor activities *in vitro* and *in vivo*, but the precise mechanisms by which it induces its effects are not known. We provide evidence that during treatment of BCR-ABL–expressing cells with As_2O_3, there is activation of a cellular pathway involving the p70 S6 kinase (p70S6K). Our data show that p70S6K is rapidly phosphorylated on Thr^{421} and Ser^{424} and is activated in an As_2O_3-inducible manner. The mammalian target of rapamycin (mTOR) is also phosphorylated/activated in an As_2O_3-inducible manner, and its activity is required for downstream engagement of p70S6K. p70S6K subsequently phosphorylates the S6 ribosomal protein on Ser^{235}/Ser^{236} and Ser^{240}/Ser^{244} to promote initiation of mRNA translation. Treatment of chronic myelogenous leukemia–derived cell lines with As_2O_3 also results in phosphorylation of the 4E-BP1 repressor of mRNA translation on Thr^{37}/Thr^{46} and Thr^{70}, sites required for its deactivation and its dissociation from the eukaryotic initiation factor 4E complex to allow cap-dependent mRNA translation. In studies to determine the functional relevance of this pathway, we found that inhibition of mTOR and downstream cascades enhances induction of apoptosis by As_2O_3. Consistent with this, the mTOR inhibitor rapamycin strongly potentiated As_2O_3-mediated suppression of primitive leukemic progenitors from the bone marrow of chronic myelogenous leukemia patients. Altogether, our data show that the mTOR/p70S6K pathway is activated in a negative feedback regulatory manner in response to As_2O_3 in BCR-ABL–transformed cells and plays a key regulatory role in the induction of anti-leukemic responses. [Mol Cancer Ther 2006;5(11):2815–23]

Introduction

Arsenic trioxide (As_2O_3) is an arsenic derivative, which exhibits potent growth inhibitory effects against malignant cells (1–3). The remarkable antitumor effects of As_2O_3 *in vitro* and *in vivo* have prompted the development of various clinical trials that established its activity in acute promyelocytic leukemia (1–3). As_2O_3 is part of the standard treatment for this leukemia and is highly effective in cases that have developed resistance to retinoic acid treatment (1–3). As As_2O_3 has potent effects *in vitro* and *in vivo* against a variety of neoplastic cells, it is also currently under clinical development for the treatment of other hematologic malignancies as well, in particular, chronic myelogenous leukemia (CML) and multiple myeloma (1–4).

Because of the important antitumor properties of As_2O_3, extensive efforts have been made by several research groups to understand its mechanisms of action in malignant cells. In acute promyelocytic leukemia cells, it has been shown that As_2O_3 induces degradation of the PML-RARα fusion protein that may account in part for its anti-leukemic effects in these cells (2, 5). However, there is also evidence that acute promyelocytic leukemia cell differentiation can be induced without concomitant degradation of the PML-RARα fusion protein (6), indicating the existence of additional mechanisms. Other cellular events that may contribute to arsenic-induced apoptosis include suppression of bcl-2 levels and decreased nuclear factor-κB translocation to the nucleus (7) and collapse of mitochondrial transmembrane potential, resulting in cytochrome *c* release and activation of caspase-3 (8). Generation of reactive oxygen species by As_2O_3 potentiates induction of cell killing, and an accumulating body of evidence points towards a role for reactive oxygen species, particularly H_2O_2, on arsenic-induced apoptosis (9, 10). Recent work has uncovered an additional mechanism by which As_2O_3 may generate its anti-leukemic responses, involving inhibition of nuclear receptor function via c-Jun NH_2-terminal kinase–mediated retinoid X receptor α phosphorylation (11). Thus, several cellular cascades seem to be engaged in the generation of arsenic-dependent growth inhibition and apoptosis of malignant cells. However, the role of pathways that may be activated in a negative feedback regulatory manner to counteract the induction of arsenic responses is unknown.

Received 5/9/06; revised 8/24/06; accepted 9/11/06.

Grant support: Department of Veterans Affairs (L.C. Platanias), NIH grants CA94079 and CA77816 (L.C. Platanias), and NIH/National Cancer Institute training grant T32 CA09560 (P. Yoon).

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doi:10.1158/1535-7163.MCT-06-0263

In the present study, we provide evidence for a novel signaling cascade activated in response to As_2O_3 in CML-derived cell lines, involving activation of the mammalian target of rapamycin (mTOR) and sequential downstream engagement of the p70 S6 kinase (p70S6K) and the S6 ribosomal protein. In addition, we show that the 4E-BP1 repressor of mRNA translation is phosphorylated in an As_2O_3-dependent manner on sites required for its deactivation and dissociation from the eukaryotic initiation factor 4E, to allow initiation of mRNA translation. Our data show that activation of mTOR and its downstream effectors results in negative regulatory effects on As_2O_3-induced cell death, as evidenced by the promotion of apoptosis during pharmacologic inhibition of mTOR in CML-derived cell lines. In addition, our data establish that inhibition of mTOR activation promotes the generation of the suppressive effects of As_2O_3 on primitive leukemic granulocyte-macrophage colony-forming unit (CFU-GM) progenitors from CML patients, supporting an important regulatory role for this pathway in the induction of the anti-leukemic effects of arsenic.

Materials and Methods

Cells and Reagents

The CML-derived KT-1, K562, and BV-173 cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. As_2O_3 was purchased from Sigma (St. Louis, MO). Antibodies against the phosphorylated forms of p70S6K, rpS6, mTOR, and 4E-BP1 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The FRAP/mTOR inhibitor rapamycin and the phosphatidylinositol 3′-kinase (PI3K) inhibitor LY294002 were obtained from Calbiochem, Inc. (La Jolla, CA).

Cell Lysis and Immunoblotting

Cells were stimulated with the indicated doses of As_2O_3 for the indicated times and subsequently lysed in phosphorylation lysis buffer as described previously (12). Immunoprecipitations and immunoblotting using an enhanced chemiluminescence method were done as previously described (12). In the experiments in which pharmacologic inhibitors of FRAP/mTOR or the PI3K were used, the cells were pretreated for 60 minutes with the indicated concentrations of the inhibitors and subsequently treated for the indicated times with As_2O_3, before lysis in phosphorylation lysis buffer. All immunoblotting experiments were highly reproducible, and each experiment shown is representative of at least three independent experiments.

p70S6K Assays

Assays to detect the arsenic-dependent activation of the p70S6K were done as previously described (13). Briefly, KT-1 cells were lysed in phosphorylation lysis buffer, and cell lysates were immunoprecipitated with an antibody against p70S6K or control non-immune rabbit immunoglobulin (RIgG). *In vitro* kinase assays were done using a synthetic peptide substrate (AKRRRLSSLRA), and p70S6K activity was measured using an S6K assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer's instructions. Values were calculated by subtracting nonspecific activity, detected in RIgG immunoprecipitates, from kinase activity detected in anti-p70S6K immunoprecipitates.

Cell Proliferation Assays

KT-1 or K562 cells were treated with the indicated doses of As_2O_3, in the presence or absence of rapamycin (20 nmol/L), for the indicated time periods. Cell proliferation assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide methodology were subsequently done as described previously (14).

Evaluation of Apoptosis

Cells were exposed to As_2O_3 in the presence or absence of rapamycin (20 nmol/L) or LY294002 (10 or 20 μmol/L). Flow cytometric assays to evaluate apoptosis by Annexin and propidium iodide staining were done essentially as previously described (15).

Human Hematopoietic Progenitor Cell Assays

Bone marrow or peripheral blood was obtained from patients with chronic myelogenous leukemia after obtaining consent, approved by the Institutional Review Board of Northwestern University. Bone marrow or peripheral blood mononuclear cells were used for clonogenic assays in methylcellulose as previously described (16). The cells were cultured in the presence or absence of As_2O_3 (1 μmol/L), with or without the indicated concentration of rapamycin (10 nmol/L) or LY294002 (10 μmol/L). Leukemic CFU-GM colonies were scored on day 14 of culture.

Results

We initially examined whether treatment of CML-derived cell lines with As_2O_3 results in phosphorylation and activation of the p70S6K. KT-1 or K562 cells were incubated for different times with As_2O_3, and total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of the p70S6K on Thr^{421} and Ser^{424}. Treatment of both BCR-ABL–expressing cell lines with As_2O_3 resulted in rapid phosphorylation of the p70S6K, whereas there was no change in the amounts of p70S6K protein detected after treatment with As_2O_3 (Fig. 1A and B). We subsequently sought to directly determine whether such As_2O_3-dependent phosphorylation of the p70S6K results in activation of its kinase domain. KT-1 cells were incubated in the presence or absence of As_2O_3, and after immunoprecipitation of cell lysates with an anti-p70S6K antibody, immunoprecipitates were subjected to an *in vitro* kinase assay (13). As shown in Fig. 1C, As_2O_3 treatment resulted in an activation of the catalytic domain of p70S6K (Fig. 1C), suggesting that this kinase may participate in a cellular cascade that regulates the induction of As_2O_3 responses in BCR-ABL–expressing cells.

Previous studies have established that mTOR regulates activation of the p70S6K, downstream of the PI3K and PDK-1 (reviewed in refs. 17–19). It is also established that activation of mTOR requires its phosphorylation on Ser^{2448}.

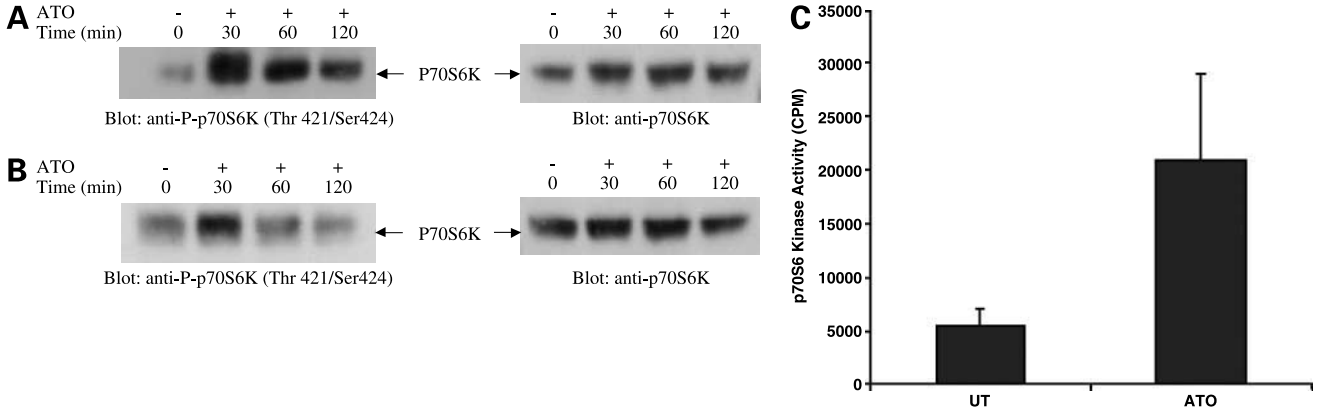

Figure 1. As_2O_3-dependent phosphorylation of the p70S6K in BCR-ABL–expressing cells. KT-1 (**A**) or K562 (**B**) cells were treated with As_2O_3 (1 μmol/L) for the indicated times. Equal amounts of total lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of the p70S6K on Thr^{421} and Ser^{424} (*left*). The same blots were stripped and reprobed with an anti-p70S6K antibody (*right*), to control for loading. **C,** KT-1 cells were treated with As_2O_3 for 20 min. The cells were subsequently lysed, and equal amounts of protein were immunoprecipitated with an anti-p70S6K antibody or non-immune rabbit immunoglobulin (RIgG). *In vitro* kinase assays to detect p70S6K activity were subsequently carried out on the immunoprecipitates. Kinase activity is expressed as counts per minute (*CPM*) after normalizing for nonspecific activity present in RIgG immunoprecipitates. *Columns,* mean of five experiments; *bars,* SE. *ATO,* As_2O_3; *UT,* untreated.

To determine whether mTOR is phosphorylated/activated in response to As_2O_3, KT-1 or K562 cells were treated with As_2O_3, and after cell lysis, total lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho mTOR antibody. Some baseline phosphorylation of mTOR was detectable before As_2O_3 treatment (Fig. 2A and B). However, As_2O_3 treatment of the cells strongly enhanced phosphorylation/activation of mTOR, showing that this protein is indeed engaged in an As_2O_3-activated cellular cascade on target cells (Fig. 2A and B). Pharmacologic inhibition of mTOR using rapamycin blocked the As_2O_3-inducible phosphorylation of p70S6K (Fig. 2C and D), suggesting that such phosphorylation is mTOR dependent. The phosphorylation of p70S6K was also blocked when cells were pretreated with the PI3K inhibitor LY294002 (Fig. 2C and D), suggesting that, as in other systems (17–19), activation of mTOR and p70S6K by As_2O_3 requires upstream PI3K activity.

In subsequent studies, we sought to identify functional downstream effectors of the As_2O_3-activated mTOR/

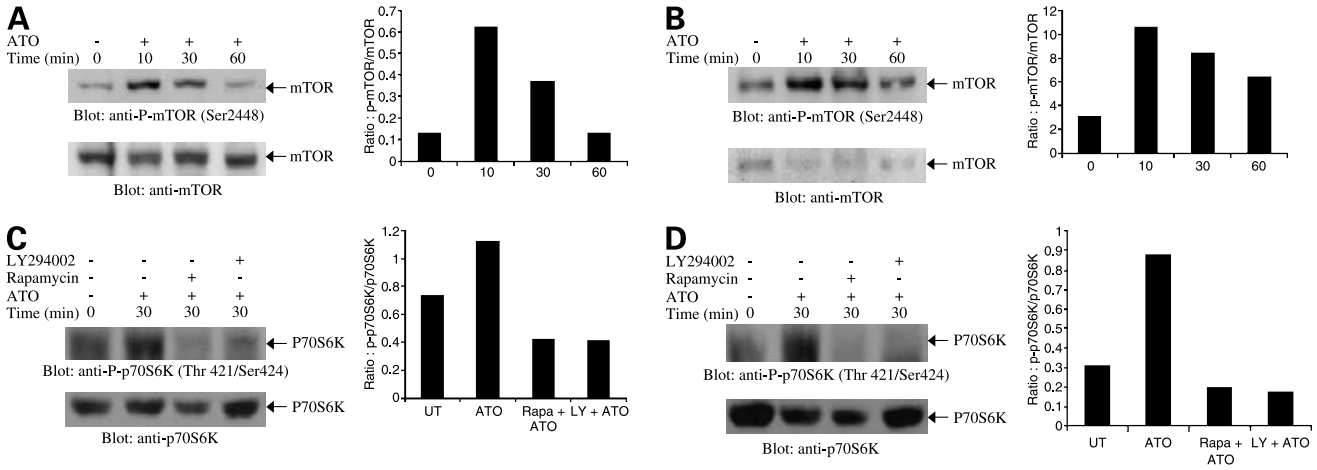

Figure 2. Activation of the p70S6K by As_2O_3 in BCR-ABL–expressing cells is PI3K and mTOR dependent. KT-1 (**A**) or K562 (**B**) cells were treated with As_2O_3 (1 μmol/L) for the indicated times. Total cell lysates were resolved on SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of mTOR on Ser^{2448} (*top left*). The same blots were stripped and reprobed with an anti-mTOR antibody to control for loading (*bottom left*). The signals for the different bands shown in blots **A** and **B** were quantitated by densitometry (*right*), and the intensity of mTOR phosphorylation relative to the levels of mTOR expression was calculated. KT-1 (**C**) or K562 (**D**) cells were pretreated for 1 h with either rapamycin (20 nmol/L) or LY294002 (50 μmol/L), before treatment with As_2O_3 for 30 min, as indicated. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho p70S6K antibody (*top left*). The same blots were stripped and reprobed with an anti-p70S6K antibody (*bottom left*). The signals for the different bands shown in blots **C** and **D** were quantitated by densitometry (*right*), and the intensity of p70S6K phosphorylation relative to the levels of p70S6K expression was calculated.

p70S6K cascade. One well-known effector of the p70S6K is the 40S ribosomal S6 protein (17–19). This protein is a direct substrate for the activity of p70S6K, and its phosphorylation results in initiation of translation for mRNAs that have oligopyrimidine tracts in the 5′ untranslated region (17–19). We determined whether ribosomal S6 protein is phosphorylated in response to treatment of BCR-ABL–expressing cells with As_2O_3. As_2O_3 treatment of

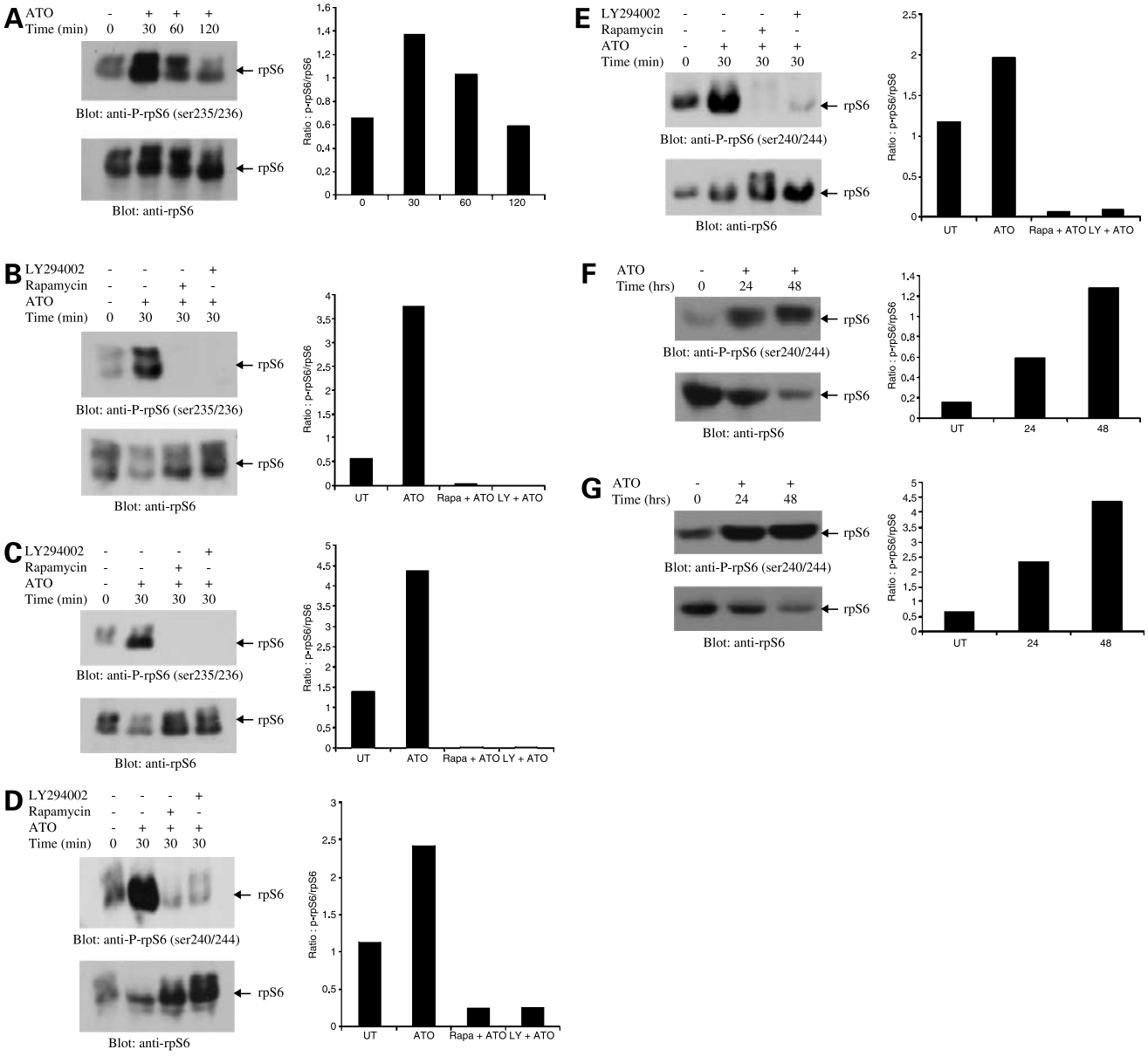

Figure 3. As_2O_3 induces phosphorylation of the S6 ribosomal protein (rpS6) on Ser^{235}/Ser^{236} and Ser^{240}/Ser^{244}. **A,** KT-1 cells were incubated with As_2O_3 As_2O_3 (1 μmol/L) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of rpS6 on Ser^{235}/Ser^{236} (*top left*). The blot shown in **A** was stripped and reprobed with an anti-rpS6 antibody, to control for protein loading (*bottom left*). The signals for the different bands shown in blots (**A**) were quantitated by densitometry (*right*), and the intensity of p-rpS6 expression relative to the levels of rpS6 expression were calculated. K562 (**B**), BV173 (**C** and **E**), or KT-1 (**D**) cells were preincubated with rapamycin (20 nmol/L) or LY294002 (50 μmol/L) for 60 min and were subsequently treated with As_2O_3, as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with antibodies against the phosphorylated form of rpS6 on Ser^{235}/Ser^{236} (*left*) or Ser^{240}/Ser^{244} (*top left*). The same blots were stripped and reprobed with an anti-rpS6 antibody, to control for protein loading (*bottom left*). The signals for the different bands shown in blots (**B** to **E**) were quantitated by densitometry (*right*), and the intensity of p-rpS6 expression relative to the levels of rpS6 expression was calculated. KT-1 (**F**) or K562 (**G**) cells were treated with As_2O_3 (2 μmol/L) for the indicated times. Equal amounts of total lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated/activated form of the rpS6 on Ser^{240}/Ser^{244} (*top left*). The same blots were stripped and reprobed with an anti-rpS6 antibody, to control for loading (*bottom left*). The signals for the different bands shown in blots (**F** and **G**) were quantitated by densitometry (*right*), and the intensity of rpS6 phosphorylation relative to the levels of rpS6 expression was calculated.

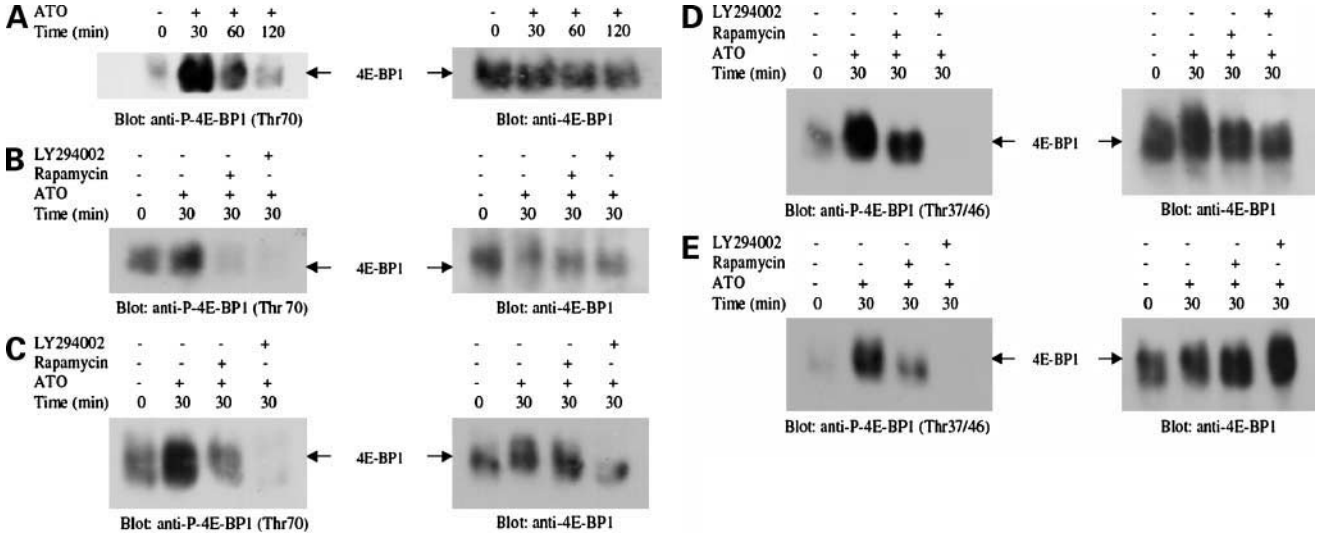

Figure 4. As_2O_3 induces phosphorylation of 4E-BP1. **A,** KT-1 cells were incubated with As_2O_3 (1 μmol/L) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of 4E-BP1 on Thr^{70} (*left*). **B,** equal amounts of total cell lysates from the experiment in **A** were resolved separately by SDS-PAGE and immunoblotted with an anti-4E-BP1 antibody to control for protein loading (*right*). K562 (**B** and **E**), BV173 (**C**), or KT-1 (**D**) were preincubated with rapamycin (20 nmol/L) or LY294002 (50 μmol/L) for 60 min and were subsequently treated with As_2O_3, as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of 4E-BP1 on Thr^{70} (*left*) or on Thr^{37}/Thr^{46} (*left*). The same blots were stripped and reprobed with an anti-4E-BP1 antibody, to control for protein loading (*right*).

several different BCR-ABL–expressing cell lines, including KT-1 (Fig. 3A), K562 (Fig. 3B), and BV-173 (Fig. 3C), resulted in strong phosphorylation of rpS6 on Ser^{235}/Ser^{236}, whereas there was no change in the total amounts of ribosomal S6 detected before and after As_2O_3 stimulation. As in the case of the phosphorylation of p70S6K, the phosphorylation of the rpS6 protein was abrogated by pretreatment of cells with either the mTOR inhibitor rapamycin or the PI3K inhibitor LY294002 (Fig. 3B and C). Treatment of different CML cell lines with As_2O_3 also resulted in phosphorylation of the S6 ribosomal protein on Ser^{240}/Ser^{244} (Fig. 3D and E). Such phosphorylation was also inhibited by pretreatment of cells with either rapamycin or LY294002 (Fig. 3D and E), further establishing that As_2O_3 engagement of the S6 ribosomal protein occurs downstream of mTOR and the p70S6K. When the phosphorylation of the S6 ribosomal protein was examined after prolonged treatment of the cells with As_2O_3, we found that such phosphorylation is prolonged and can be detected after 24 and 48 hours of treatment of the cells (Fig. 3F and G). Thus, it seems that the phosphorylation of S6 ribosomal protein in response to As_2O_3 is biphasic, with one early peak occurring early, within 30 minutes of treatment of the cells, and a second delayed peak occurring after prolonged treatment of the cells (24–48 hours).

Previous work has shown that in response to insulin, cytokines, and retinoids, the 4E-BP1 repressor of mRNA translation is phosphorylated in a PI3K- and mTOR-dependent manner (13, 20–22), and that such phosphorylation leads to its dissociation from the initiation factor eukaryotic initiation factor 4E, resulting in induction of mRNA translation (21). We sought to determine whether As_2O_3 treatment results in phosphorylation of 4E-BP1 to regulate initiation of translation. KT-1, K562, or BV-173 cells were treated with As_2O_3, and after cell lysis, total lysates were analyzed by SDS-PAGE and immunoblotted with antibodies against different phosphorylation sites in 4E-BP1. Treatment of all different cell lines with As_2O_3 resulted in phosphorylation of 4E-BP1 on sites required for its deactivation and dissociation from eukaryotic initiation factor 4E, including Thr^{70} (Fig. 4A–C) and Thr^{37}/Thr^{46} (Fig. 4D–E; data not shown). Inhibition of PI3K activity using the LY294002 inhibitor blocked phosphorylation of 4E-BP1 on both sites (Fig. 4B–E). On the other hand, phosphorylation on Thr^{70} (Fig. 4B and C) was completely blocked by rapamycin, whereas phosphorylation on Thr^{37}/Thr^{46} was not (Fig. 4D and E). These findings strongly suggest that the activation of 4E-BP1 by As_2O_3 in CML cell lines is functionally relevant, as it follows the hierarchical phosphorylation on sites essential for deactivation of the protein and its dissociation from eukaryotic initiation factor 4E.

In subsequent studies, we sought to determine the functional relevance of activation of the mTOR/p70S6K pathway in response to As_2O_3. Experiments were done to determine the effects of pharmacologic inhibition of mTOR on the generation of growth inhibitory responses by As_2O_3 in KT-1 and K562 cells. Cells were incubated for 5 days with As_2O_3, in the presence or absence of the mTOR inhibitor rapamycin, and cell proliferation was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. As expected, treatment of KT-1 or K562 cells with As_2O_3 suppressed cell growth (Fig. 5A and B). Such a growth inhibitory response was significant (>50%)

only at the high concentration of As_2O_3 (2 μmol/L; Fig. 5). Interestingly, concomitant treatment of the cells with rapamycin enhanced the effects of As_2O_3 in a dose-dependent manner and resulted in the generation of growth suppression at the lower (0.5–1 μmol/L) concentrations of As_2O_3 in KT-1 (Fig. 5A) and K562 cells (Fig. 5B). These findings prompted us to perform further studies, aimed to define whether rapamycin potentiates As_2O_3-induced apoptosis. KT-1 (Fig. 5C) or K562 (Fig. 5D) cells were incubated with As_2O_3, in the presence or absence of rapamycin or LY294002, and the percentage of cells undergoing apoptosis were determined by flow cytometry. Treatment of cells with As_2O_3 resulted in induction of apoptosis (Fig. 5C and D), whereas concomitant treatment of the cells with the mTOR inhibitor rapamycin or the PI3K inhibitor LY294002 further enhanced As_2O_3-induced cell death (Fig. 5C and D). Thus, pharmacologic inhibition of the mTOR pathway enhances induction of arsenic-dependent apoptosis and growth suppression of BCR-ABL–expressing cell lines, suggesting an important role for this pathway in the control of As_2O_3 responses.

To further explore the role of such activation in a more physiologically relevant system, we evaluated the effects of inhibition of this cascade on the induction of the suppressive effects of As_2O_3 on primary leukemia progenitors from a relatively large number of patients with CML. Bone marrow or peripheral blood mononuclear cells from 11 different CML patients were isolated, and leukemic

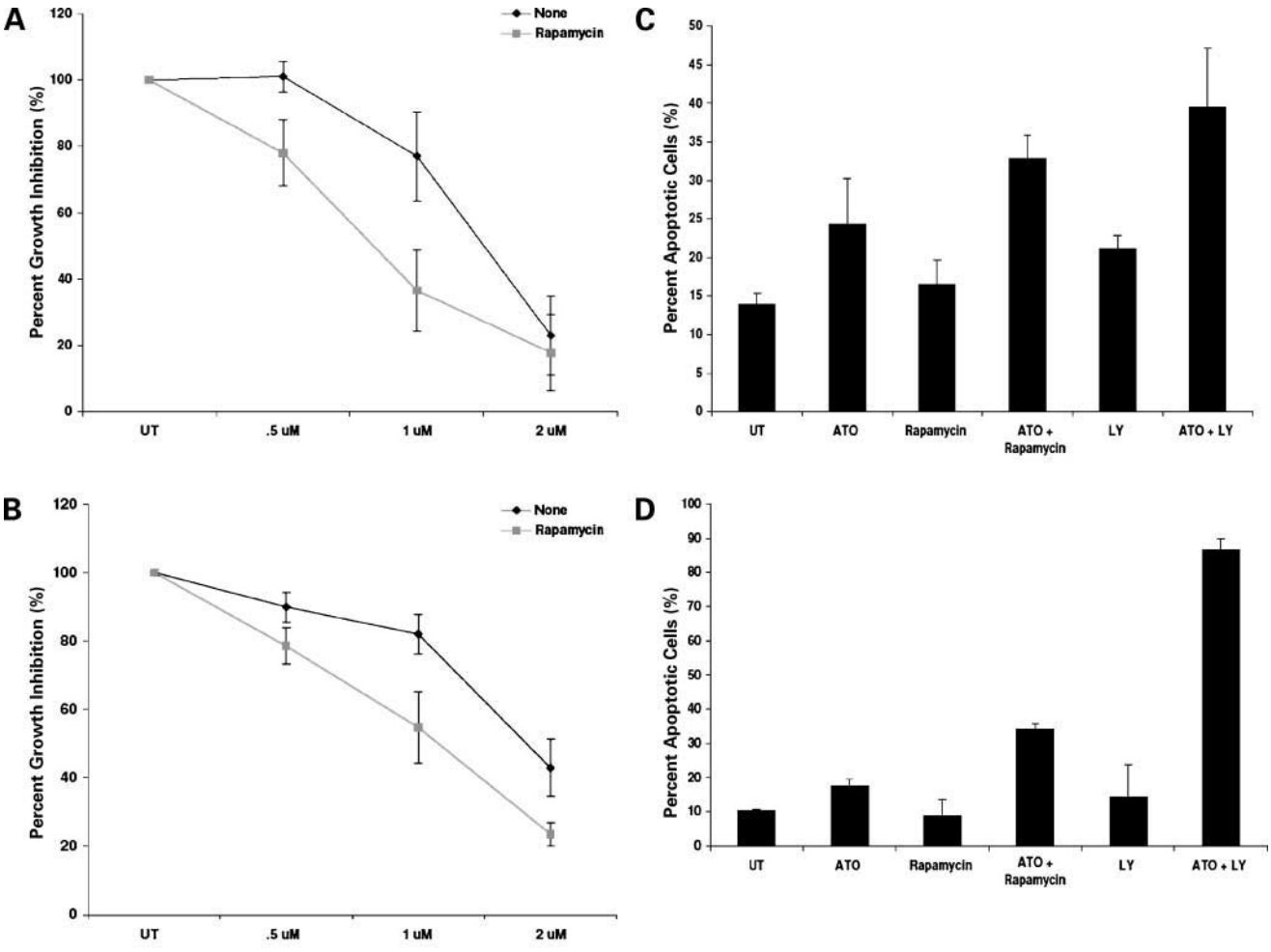

Figure 5. Pharmacologic inhibition of mTOR or PI3K promotes As_2O_3-induced growth inhibition and apoptosis. **A,** KT-1 cells were incubated for 5 d in the presence or absence of the indicated doses of As_2O_3, in the presence or absence of rapamycin. Cell proliferation was assessed by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Points,* mean of five experiments; *bars,* SE. **B,** K562 cells were incubated for 5 d in the presence or absence of the indicated doses of As_2O_3, in the presence or absence of rapamycin. Cell proliferation was assessed by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Points,* mean of four experiments; *bars,* SE. **C,** KT-1 cells were treated for 72 h in the absence or presence of As_2O_3 (1 μmol/L) in the presence or absence of rapamycin or LY294002, as indicated. Induction of apoptosis was evaluated by flow cytometry, after Annexin V and propidium iodide staining. *Columns,* mean % Annexin V – positive and propidium iodide – positive cells of three experiments; *bars,* SE. **D,** K562 cells were incubated for 120 h in the absence or presence of As_2O_3 (2 μmol/L), in the presence or absence of rapamycin or LY294002, as indicated. The cells were subsequently analyzed for apoptosis in the same manner as in **A**. *Columns,* mean of two experiments; *bars,* SE.

CFU-GM progenitor colony formation was determined by clonogenic assays in methylcellulose. Consistent with our previous studies (23), addition of As_2O_3 to the cultures suppressed leukemic CFU-GM progenitor growth (Fig. 6). However, concomitant addition of the mTOR inhibitor rapamycin strongly enhanced the suppressive effects of As_2O_3 on leukemic CFU-GM progenitor growth (two-tailed $P = 0.00001$), strongly suggesting that the mTOR pathway negatively regulates the generation of As_2O_3 responses in BCR-ABL cells.

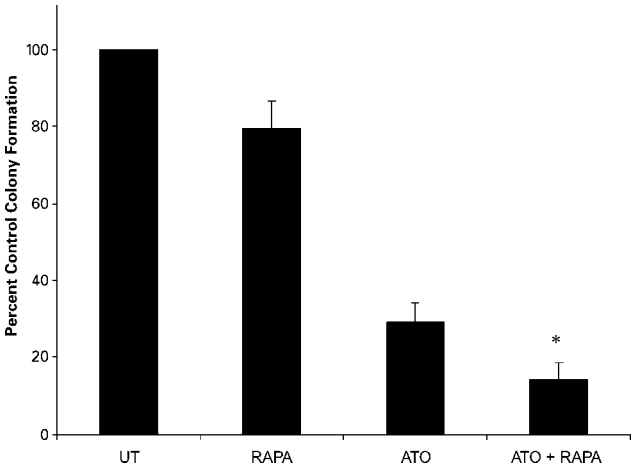

Figure 6. Pharmacologic inhibition of mTOR enhances the suppressive effects of As_2O_3 on leukemic CFU-GM progenitors from patients with CML. Bone marrow or peripheral blood mononuclear cells from 11 different patients with CML were plated in a methylcellulose culture assay system with As_2O_3 and rapamycin, as indicated. % Control of CFU-GM colony numbers for untreated cells. *Columns,* mean of the values from the experiments using different patient samples; *bars,* SE. *, $P = 0.0000101$ (two tailed), paired *t* test analysis of the combinations of As_2O_3 plus rapamycin compared with As_2O_3 alone.

Discussion

Despite the extensive work in the area of As_2O_3 signaling, the precise mechanisms required for the generation of As_2O_3-inducible apoptosis in different cellular backgrounds remain unclear. The well-established role of this agent in the treatment of acute promyelocytic leukemia and its potential applications to other hematologic malignancies have ignited extensive efforts to understand the mechanisms by which it regulates generation of anti-leukemic responses (1–3). The cellular effects of As_2O_3 are concentration dependent. As_2O_3 induces differentiation of acute promyelocytic leukemia blasts at low concentrations (<0.5 μmol/L), whereas for the induction of apoptosis, high final concentrations (>2 μmol/L) are required (1–3). Identifying ways to enhance the effects of As_2O_3 on malignant cells is of particular interest, as it may facilitate the development of novel therapeutic approaches using lower nontoxic doses of arsenic for the treatment of leukemias.

CML is characterized by the expression of the abnormal BCR-ABL oncoprotein. BCR-ABL is the protein product of the *bcr-abl* oncogene, which results from the reciprocal translocation between chromosomes 9 and 22, and the abnormal fusion of the *bcr* and *c-abl* genes (23). Extensive studies over the years have established that the constitutively activated tyrosine kinase activity of BCR-ABL promotes leukemic transformation by activating multiple downstream mitogenic cascades (24). Several signaling elements have been shown to be regulated by BCR-ABL, including the Ras-GAP (25), the Shc oncoprotein (26), the tyrosine phosphatase SHP-2 and phosphatidylinositol polyphosphate 5′-phosphatase SHIP (27), the c-CBL (28) and Vav (29) proto-oncogene products (28), the transcriptional activator Stat5 (30, 31), and the PI3K/Akt signaling pathway (32). In addition, mTOR has been recently implicated as a downstream effector of BCR-ABL (33–35). Consistent with this, the BCR-ABL kinase inhibitor imatinib mesylate (Gleevec) has been shown to block mTOR-dependent signals (33–35), suggesting that inhibition of mitogenic pathways activated downstream of mTOR may be important for the generation of the anti-leukemic properties of this agent.

Although imatinib mesylate is clearly the most potent agent available against CML cells *in vitro* and *in vivo*, resistance to its anti-leukemic properties develops in many instances (36, 37), underscoring the importance of developing novel therapeutic approaches to overcome such resistance. As_2O_3 exhibits potent inhibitory properties against BCR-ABL–expressing cells (4) and has been shown to exhibit synergistic effects with imatinib mesylate (38–41). Moreover, clinically relevant concentrations of As_2O_3 can induce apoptosis of BCR-ABL cells (38, 42) and suppress endogenous levels of BCR-ABL protein expression (43), further emphasizing the potential of As_2O_3 as an agent in the treatment of CML.

Despite the well-established anti-leukemic and proapoptotic effects of As_2O_3 in CML cells, the mechanisms that regulate induction of such effects are not known. In the present study, we show that As_2O_3 treatment regulates activation of the mTOR-p70S6K pathway in BCR-ABL–expressing cells, and we provide the first evidence for the existence of an As_2O_3-activated signaling cascade involved in the regulation of mRNA translation. Our data show that As_2O_3 induces phosphorylation/activation of mTOR in the KT-1 and K562 cell lines and downstream phosphorylation/activation of p70S6K. Such As_2O_3-dependent activation of p70S6K may play an important role in the regulation of mRNA translation, as it results in the downstream phosphorylation of the S6 ribosomal protein. The S6 ribosomal protein is known to participate in the initiation of translation of mRNAs with oligopyrimidine tracts in the 5′ untranslated region (44–46). Our data establish that As_2O_3 treatment results in phosphorylation of S6 on Ser^{235}/Ser^{236} and Ser^{240}/Ser^{44}, in an mTOR-dependent manner, directly implicating it in the regulation of arsenic-inducible responses. We also show that As_2O_3 induces phosphorylation and deactivation of the translational repressor 4E-BP1, an event required for its dissociation from the eukaryotic initiation factor eukaryotic initiation factor 4E and the start of cap-dependent translation (17, 20, 21).

It should be pointed out that a previous study (43) suggested that As_2O_3 decreases activation of p70S6K after prolonged treatment (48 hours) of K562 cells. Such down-regulation was proposed as a mechanism for the arsenic-induced decrease in BCR-ABL expression, as the *bcr-abl* mRNA possesses a 5′ untranslated region containing a TOP sequence. However, in that article (43), the authors did not examine the phosphorylation of the S6 ribosomal protein at earlier time points, as in our study. Independently of that, we failed to reproduce the findings of that study, as we have consistently observed that there is strong phosphorylation of the S6 ribosomal protein after 48 hours of As_2O_3 treatment of KT-1 and K562 cells, despite some decrease in the total rpS6 protein levels. We cannot account for the differences between our study and that from the study of Nimmanapalli et al., but a recent study showed that As_2O_3 induces phosphorylation/activation of the Akt kinase in HL-60 cells (47). The results of that study (47) are consistent with our findings, as the Akt kinase is a known regulator of the p70S6K.

Altogether, our studies show that two distinct cellular pathways, known to regulate translation, are activated in response to As_2O_3 downstream of mTOR. In efforts to understand the functional relevance of these pathways, we found that inhibition of mTOR activation enhances the induction of arsenic-mediated apoptosis and growth suppression in CML-derived cell lines. Moreover, the mTOR inhibitor rapamycin was found to promote the suppressive effects of As_2O_3 on primary leukemic CFU-GM progenitors from patients with CML. Thus, activation of mTOR-dependent pathways occurs in a negative feedback regulatory manner to counteract the anti-leukemic properties of As_2O_3, in a manner similar to the previously described activation of the p38 mitogen-activated protein kinase cascade (48). The precise sequence of upstream signaling events that lead to activation of mTOR remains to be determined. Our data raise the possibility of involvement of the PI3K in such regulation, as in addition to mTOR inhibition, PI3K blockade also promotes the suppressive effects of As_2O_3 on leukemic progenitor cell growth. However, it is also possible that the arsenic-inducible mTOR activation occurs in a PI3K-independent manner, possibly via engagement of tuberous sclerosis complex and Rheb (17), and future studies should address this issue. Independently of the precise upstream regulatory signals, our studies strongly suggest that combination of As_2O_3 with pharmacologic inhibitors of mTOR may be a novel approach to overcome arsenic resistance in leukemic cells. They also raise the potential of future clinical trials in CML-blast crisis and possibly other leukemias, involving combinations of As_2O_3 with mTOR inhibitors.

References

1. Douer D, Tallman MS. Arsenic trioxide: new clinical experience with an old medication in hematologic malignancies. J Clin Oncol 2005;23:2396–410.

2. Miller WH, Schipper HM, Lee JS, Singer J, Waxman S. Mechanisms of action of arsenic trioxide. Cancer Res 2002;62:3893–903.

3. Tallman MS, Nabhan C, Feusner JH, Rowe JM. Acute promyelocytic leukemia: evolving therapeutic strategies. Blood 2002;99:759–67.

4. Anderson KC, Boise LH, Louie R, Waxman S. Arsenic trioxide in multiple myeloma: rationale and future directions. Cancer J 2002;28:12–25.

5. Chen GQ, Zhu J, Shi XG, et al. *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As_2O_3 induces NB4 cell apoptosis with down-regulation of Bcl-2 expression and modulation of PML-RAR α/PML proteins. Blood 1996;88:1052–61.

6. Nervi C, Ferrara F, Fanelli M, et al. Caspases mediate retinoic acid-induced degradation of the acute promyelocytic leukemia PML/RARα fusion protein. Blood 1998;92:2244–51.

7. Kapahi P, Takahashi T, Natoli G, et al. Inhibition of NF-κ B activation by arsenite through reaction with a critical cysteine in the activation loop of Iκ B kinase. J Biol Chem 2000;275:36062–6.

8. Mahieux R, Pise-Masison C, Gessain A, et al. Arsenic trioxide induces apoptosis in human T-cell leukemia virus type 1- and type 2-infected cells by a caspase-3-dependent mechanism involving Bcl-2 cleavage. Blood 2001;98:3762–9.

9. Jing Y, Dai J, Chalmers-Redman RM, Tatton WG, Waxman S. Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood 1999;94:2102–11.

10. Park WH, Seol JG, Kim ES, et al. Arsenic trioxide-mediated growth inhibition in MC/CAR myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. Cancer Res 2000;60:3065–71.

11. Mann KK, Padovani AM, Guo Q, et al. Arsenic trioxide inhibits nuclear receptor function via SEK1/JNK-mediated RXRα phosphorylation. J Clin Invest 2005;115:2924–33.

12. Uddin S, Majchrzak B, Woodson J, et al. Activation of the p38 mitogen-activated protein kinase by type I interferons. J Biol Chem 1999;274:30127–31.

13. Lekmine F, Uddin S, Sassano A, et al. Activation of the p70 S6 kinase and phosphorylation of the 4E-BP1 repressor of mRNA translation by type I interferons. J Biol Chem 2003;278:27772–80.

14. Alsayed Y, Uddin S, Mahmud N, et al. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-*trans*-retinoic acid. J Biol Chem 2001;276:4012–9.

15. Verma A, Deb DK, Sassano A, et al. Cutting edge: activation of the p38 mitogen-activated protein kinase signaling pathway mediates cytokine-induced hemopoietic suppression in aplastic anemia. J Immunol 2002;168:5984–8.

16. Verma A, Deb DK, Sassano A, et al. Activation of the p38 mitogen-activated protein kinase mediates the suppressive effects of type I interferons and transforming growth factor-β on normal hematopoiesis. J Biol Chem 2002;277:7726–35.

17. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926–45.

18. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signaling. Nat Rev Immunol 2005;5:375–86.

19. Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 2005;37:19–24.

20. Gingras A-C, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. Genes Dev 1998;12:502–13.

21. Gingras AC, Raught B, Gygi SP, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev 2001;15:2852–64.

22. Lal L, Li Y, Smith J, et al. Activation of the p70 S6 kinase by all-*trans*-retinoic acid in acute promyelocytic leukemia cells. Blood 2005;105:1669–77.

23. Ben-Neriah Y, Daley GQ, Mes-Masson A, Witte ON, Baltimore D. The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. Science 1986;233:212–4.

24. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia 2004;18:189–218.

25. Druker B, Okuda K, Matulonis U, Salgia R, Roberts T, Griffin JD. Tyrosine phosphorylation of rasGAP and associated proteins in chronic myelogenous leukemia cell lines. Blood 1992;79:2215–20.

26. Matsuguchi T, Salgia R, Hallek M, et al. Shc phosphorylation in myeloid cells is regulated by granulocyte macrophage colony-stimulating

factor, interleukin-3, and steel factor and is constitutively increased by p210BCR/ABL. J Biol Chem 1994;269:5016 – 21.

27. Sattler M, Salgia R, Shrikhande G, et al. The phosphatidylinositol polyphosphate 5-phosphatase SHIP and the protein tyrosine phosphatase SHP-2 form a complex in hematopoietic cells which can be regulated by BCR/ABL and growth factors. Oncogene 1997;15:2379 – 84.

28. Sattler M, Salgia R, Okuda K, et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3′ kinase pathway. Oncogene 1996;12:839 – 46.

29. Bassermann F, Jahn T, Miething C, et al. Association of Bcr-Abl with the proto-oncogene Vav is implicated in activation of the Rac-1 pathway. J Biol Chem 2002;277:12437 – 45.

30. Gesbert F, Griffin JD. Bcr/Abl activates transcription of the Bcl-X gene through STAT5. Blood 2000;96:2269 – 76.

31. Horita M, Andreu EJ, Benito A, et al. Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL. J Exp Med 2000;191:977 – 84.

32. Skorski T, Bellacosa A, Nieborowska-Skorska M, et al. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. EMBO J 1997;16:6151 – 61.

33. Ly C, Arechiga AF, Melo JV, Walsh CM, Ong ST. Bcr-Abl kinase modulates the translation regulators ribosomal protein S6 and 4E-BP1 in chronic myelogenous leukemia cells via the mammalian target of rapamycin. Cancer Res 2003;63:5716 – 22.

34. Mohi MG, Boulton C, Gu TL, et al. Combination of rapamycin and protein tyrosine kinase (PTK) inhibitors for the treatment of leukemias caused by oncogenic PTKs. Proc Natl Acad Sci U S A 2004;101:3130 – 5.

35. Parmar S, Smith J, Sassano A, et al. Differential regulation of the p70 S6 kinase pathway by interferon α (IFNα) and imatinib mesylate (STI571) in chronic myelogenous leukemia cells. Blood 2005;106:2436 – 43.

36. Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood 2005;105:2640 – 53.

37. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer 2005;5:172 – 83.

38. Porosnicu M, Nimmanapalli R, Nguyen D, Worthington E, Perkins C, Bhalla KN. Co-treatment with As2O3 enhances selective cytotoxic effects of STI-571 against Brc-Abl-positive acute leukemia cells. Leukemia 2001;15:772 – 8.

39. La Rosee P, Johnson K, O'Dwyer ME, Druker BJ. *In vitro* studies of the combination of imatinib mesylate (Gleevec) and arsenic trioxide (Trisenox) in chronic myelogenous leukemia. Exp Hematol 2002;30:729 – 37.

40. La Rosee P, Johnson K, Corbin AS. *In vitro* efficacy of combined treatment depends on the underlying mechanism of resistance in imatinib-resistant Bcr-Abl-positive cell lines. Blood 2004;103:208 – 15.

41. Du Y, Wang K, Fang H, et al. Coordination of intrinsic, extrinsic and endoplasmic reticulum-mediated apoptosis by imatinib mesylate combined with arsenic trioxide in chronic myeloid leukemia. Blood 2006;107:1582 – 90.

42. Perkins C, Kim CN, Fang G, Bhalla KN. Arsenic induces apoptosis of multidrug-resistant human myeloid leukemia cells that express Bcr-Abl or overexpress MDR, MRP, Bcl-2, or Bcl-x(L). Blood 2000;95:1014 – 22.

43. Nimmanapalli R, Bali P, O'Bryan E, et al. Arsenic trioxide inhibits translation of mRNA of bcr-abl, resulting in attenuation of Bcr-Abl levels and apoptosis of human leukemia cells. Cancer Res 2003;63:7950 – 8.

44. Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 1999;68:913 – 63.

45. Jefferies HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G. Rapamycin suppresses 5′TOP mRNA translation through inhibition of p70s6k. EMBO J 1997;16:3693 – 704.

46. Terada N, Patel HR, Takase K, Kohno K, Nairn AC, Gelfand EW. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc Natl Acad Sci U S A 1994;91:11477 – 81.

47. Pelicano H, Carew JS, McQueen TJ, et al. Targeting Hsp90 by 17-AAG in leukemia cells: mechanisms for synergistic and antagonistic drug combinations with arsenic trioxide and Ara-C. Leukemia 2006;20:610 – 9.

48. Verma A, Mohindru M, Deb DK, et al. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to arsenic trioxide. J Biol Chem 2002;277:44988 – 95.