

RhoA/ROCK/PTEN signaling is involved in AT-101-mediated apoptosis in human leukemia cells *in vitro* and *in vivo*

G Li^{1,4}, L Liu^{1,4}, C Shan¹, Q Cheng¹, A Budhraj², T Zhou¹, H Cui^{*,3} and N Gao^{*,1}

R-(–)-gossypol acetic acid (AT-101) is a natural cottonseed product that exhibits anticancer activity. However, the molecular mechanism behind the antileukemic activity of AT-101 has not been well characterized. In this study, we investigated how AT-101 induces apoptosis in human leukemia cells. Exposure to AT-101 significantly increased apoptosis in both human leukemia cell lines and primary human leukemia cells. This increase was accompanied by the activation of caspases, cytochrome *c* release, Bcl2-associated X protein (Bax) translocation, myeloid cell leukemia-1 (Mcl-1) downregulation, Bcl-2-associated death promoter (Bad) dephosphorylation, Akt inactivation, and RhoA/Rho-associated coiled-coil containing protein kinase 1/phosphatase and tensin homolog (RhoA/ROCK1/PTEN) activation. RhoA, rather than caspase-3 cleavage, mediated the cleavage/activation of ROCK1 that AT-101 induced. Inhibiting RhoA and ROCK1 activation by C3 exoenzyme (C3) and Y27632, respectively, attenuated the ROCK1 cleavage/activation, PTEN activity, Akt inactivation, Mcl-1 downregulation, Bad dephosphorylation, and apoptosis mediated by AT-101. Knocking down ROCK1 expression using a ROCK1-specific siRNA also significantly abrogated AT-101-mediated apoptosis. Constitutively active Akt prevented the AT-101-induced Mcl-1 downregulation, Bad dephosphorylation, and apoptosis. Conversely, AT-101 lethality was potentiated by the phosphatidylinositol 3-kinase inhibitor LY294002. *In vivo*, the tumor growth inhibition caused by AT-101 was also associated with RhoA/ROCK1/PTEN activation and Akt inactivation in a mouse leukemia xenograft model. Collectively, these findings suggest that AT-101 may preferentially induce apoptosis in leukemia cells by interrupting the RhoA/ROCK1/PTEN pathway, leading to Akt inactivation, Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation, which culminate in mitochondrial injury and apoptosis.

Cell Death and Disease (2014) 5, e998; doi:10.1038/cddis.2013.519; published online 16 January 2014

Subject Category: Cancer

Members of the Rho GTPase family have important roles in regulating several aspects of cytoskeleton-based functions, including cell migration, proliferation, and apoptosis.^{1,2} The Rho-associated coiled-coil containing protein kinase (ROCK) serine/threonine kinase is a major downstream effector of Rho GTPases that controls actin–cytoskeleton assembly and actomyosin contractility via the phosphorylation of numerous downstream target proteins, including the myosin light chain (MLC) and actin-binding Lin11, Isl-1, and Mec-3 domain (LIM) kinases. Consequently, ROCK mediates membrane blebbing, enhances actin–myosin contraction, and activates caspase signaling cascades and cellular apoptosis.^{3–5}

Phosphatase and tensin homolog (PTEN) has been identified as a new ROCK substrate.⁶ ROCK phosphorylates PTEN, which stimulates its phosphatase activity. PTEN is a

negative regulator of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, which has important roles in cell survival and apoptosis.^{7,8} ROCK appears to be involved in both positive and negative regulation of PI3-K/Akt signaling, and the outcome may depend on cell type or stimulus. For instance, in HEK cells, PTEN decreases the Akt phosphorylation levels induced by ROCK activation.⁹ In contrast, a previous study has shown that ROCK interacts with and phosphorylates IRS1 at Ser632/635 to enhance PI3-K activation in adipocytes, muscle cell lines, and isolated soleus muscle *ex vivo*.¹⁰

R-(–)-gossypol acetic acid (AT-101) is a natural cottonseed product that has been identified as a small-molecule inhibitor of B-cell lymphoma 2/B-cell lymphoma-extra large/myeloid cell leukemia-1 (Bcl-2/Bcl-xL/Mcl-1) and potently induces apoptosis in various cancer cell lines.^{11,12} AT-101 is

¹Department of Pharmacognosy, College of Pharmacy, Third Military Medical University, Chongqing, China; ²Graduate Center for Toxicology, College of Medicine, University of Kentucky, Lexington, KY, USA and ³State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China

*Corresponding authors: N Gao, Department of Pharmacognosy, College of Pharmacy, Third Military Medical University, Chongqing 400038, China. Tel: +86 23 68753736; Fax: +86 23 68753046; E-mail: gaoning59@gmail.com or H Cui, State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, China. Tel: +86 23 68251713; Fax: +86 23 68251128; E-mail: hongjuan.cui@gmail.com

⁴These authors contributed equally to this work.

Keywords: AT-101; RhoA/ROCK1/PTEN signaling; apoptosis; leukemia; xenograft

Abbreviations: AT-101, *R*-(–)-gossypol acetic acid; ROCK, Rho-associated coiled-coil containing protein kinase; PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol 3-kinase; Mcl-1, myeloid cell leukemia-1; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; Bax, Bcl2-associated X protein; Bad, Bcl-2-associated death promoter; AML, acute myeloid leukemia; Akt-CA, Akt constitutive active form; C3, C3 exoenzyme; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay; GST-RBD, glutathione S-transferase linked to the RhoA-binding domain of Rhotekin

Received 20.8.13; revised 11.11.13; accepted 25.11.13; Edited by P Salomoni

one of the world's first small-molecule Bcl-2 inhibitors to enter clinical trials and is now in phase II clinical trials for hormone-refractory prostate, breast, and lung cancer as well as leukemia.^{13–16} Preclinical studies revealed that AT-101 can simultaneously antagonize several antiapoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL, Bcl-W, and Mcl-1, which interfere with mitochondrial outer membrane permeabilization.¹⁷ Several signaling pathways have been reportedly involved in AT-101-triggered apoptosis, including the ROS-ERK-CHOP pathway in colon cancer cells,¹⁸ the Akt-p53 pathway and Bcl2-associated X protein (Bax) translocation in ovarian cancer cells,¹⁹ the COX2/PGE₂ pathway in colorectal carcinoma cells,²⁰ and the downregulation of telomerase reverse transcriptase expression in leukemia cells.²¹ Currently, the mechanisms by which AT-101 induces apoptosis in leukemia cells have not yet been characterized.

In the present study, we investigated how AT-101 induces apoptosis in human leukemia cells. Specifically, we evaluated the role of the RhoA/ROCK1/PTEN signaling pathway in regulating the expression of Bcl-2 family proteins and the activation of the mitochondrial apoptotic pathway. We found that AT-101 induces apoptosis in both human leukemia cell lines and primary human leukemia cells, and also inhibits tumor growth of U937 xenograft mouse model via RhoA/ROCK1/PTEN signaling, Akt inactivation, Mcl-1

downregulation, Bcl-2-associated death promoter (Bad) dephosphorylation, and Bax translocation. These findings identify a novel mechanistic basis for AT-101 in leukemia treatment.

Results

Apoptosis and mitochondrial injury is induced by AT-101 in multiple leukemia cell lines and primary human leukemia cells but not in normal human peripheral blood mononuclear cells. We examined the effects of AT-101 on apoptosis and mitochondrial injury (loss of $\Delta\psi_m$) in U937 leukemia cells. Treating cells with various dosages and lengths of AT-101 markedly increased apoptosis and mitochondrial injury (Figure 1a). The same AT-101 concentrations and treatment lengths resulted in the cleavage of caspase-3, caspase-9, and PARP. These events were accompanied by the release of cytochrome *c* into the cytosol (Figure 1b).

Parallel studies were also performed using Jurkat T-lymphoblastic and HL-60 promyelocytic leukemia cells to determine whether these events were restricted to myeloid leukemia cells. These cells exhibited apoptotic effects when exposed to AT-101 that were similar to those of U937 cells, although the HL-60 cells were less sensitive to these effects

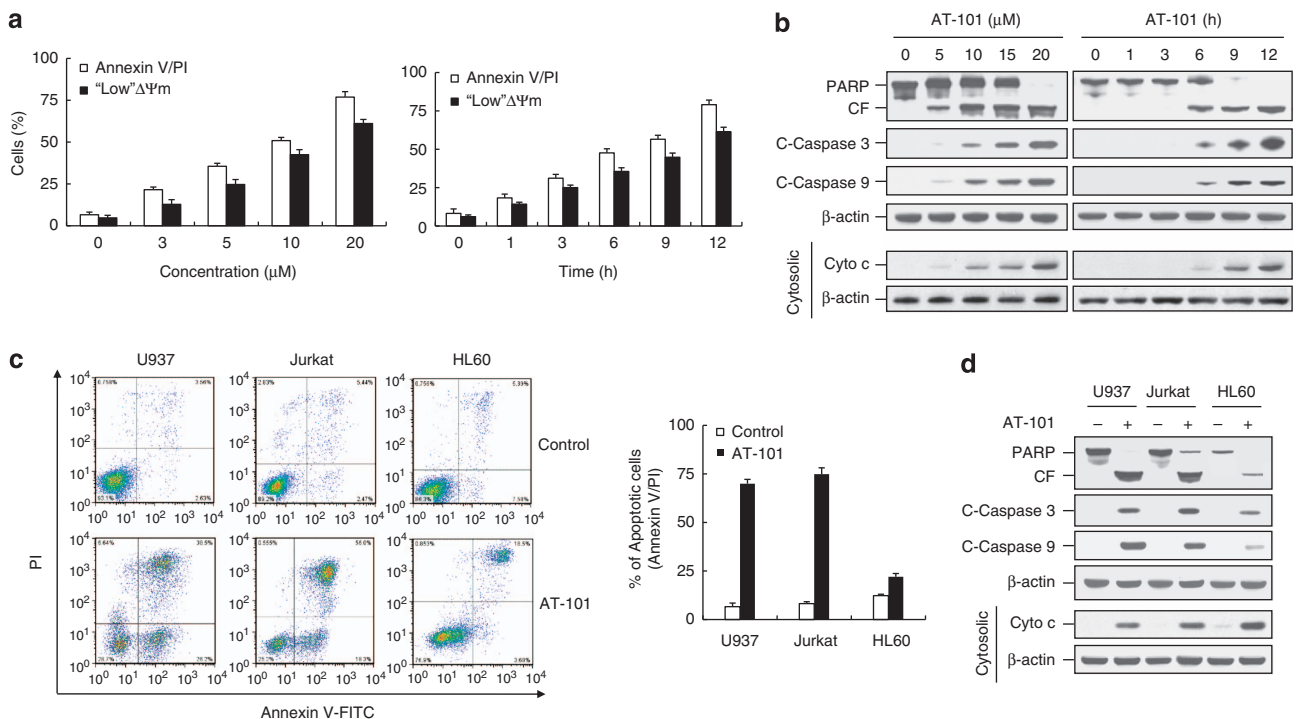


Figure 1 AT-101 selectively induced apoptosis and mitochondrial injury in human leukemia cell lines. **(a)** U937 cells were treated with or without various doses of AT-101 for 12 h or with 20 μ M AT-101 for different lengths as indicated. Apoptosis was measured by flow cytometry using Annexin V/propidium iodide (PI) staining. In a separate experiment, cells were stained with DiOC₆, and the decrease in $\Delta\psi_m$ was determined by monitoring DiOC₆ uptake using flow cytometry. 'Low' $\Delta\psi_m$ values are expressed as the percentage of cells exhibiting a diminished mitochondrial membrane potential. The results from the Annexin V/PI and DiOC₆ assays represent the mean \pm S.D. for five separate experiments. **(b)** Total cellular extracts and cytosolic fractions were analyzed by western blot analysis using antibodies against poly (ADP-ribose) polymerase (PARP), cleaved-caspase-3 (C-Caspase 3), cleaved-caspase-9 (C-Caspase 9), and cytochrome *c* (Cyto *c*). **(c)** U937, Jurkat, and HL-60 cells were treated with or without 20 μ M AT-101 for 12 h, and apoptosis was measured by flow cytometry. **(d)** Western blot analysis were used to determine PARP, C-Caspase 3, C-Caspase 9, and Cyto *c* expression. For each western blot analysis, each lane was loaded with 30 μ g of protein. Blots were subsequently stripped and reprobed with anti- β -actin to ensure equivalent loading. CF, cleaved fragment; FITC, fluorescein isothiocyanate

(Figure 1c). In addition, the Jurkat and HL-60 cells also exhibited comparable caspase-3 and caspase-9 activation, PARP cleavage, and cytochrome *c* release (Figure 1d).

Next, we examined whether AT-101 could also trigger apoptosis in primary human leukemia cells by using primary leukemia blasts isolated from 15 acute myeloid leukemia (AML) patients. Exposing AML blasts to AT-101 resulted in a marked increase in apoptosis (Figure 2a). In leukemia blasts from three AML patients, AT-101 treatment also resulted in caspase-3, caspase-9, and PARP cleavage and the release of cytochrome *c* (Figure 2b). In contrast, little AT-101 toxicity was observed in normal human peripheral blood mononuclear cells (Figure 2c). In these normal cells, AT-101 had little effect on caspase-3, caspase-9, and PARP cleavage (Figure 2d). These findings suggest that AT-101 selectively induces apoptosis and mitochondrial injury in human leukemia cell lines and primary human leukemia cells but not in normal human peripheral blood mononuclear cells.

Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation in leukemia cells treated with AT-101. AT-101 can simultaneously antagonize several antiapoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL, and Mcl-1, which interfere with mitochondrial outer membrane permeabilization.¹⁷ Thus, we examined the effects of AT-101 on the expression of antiapoptotic Bcl-2 family proteins in U937 cells. There was a marked dose- and time-dependent decrease in Mcl-1 expression in cells treated with AT-101. Treating cells with TW-37, a positive control of Mcl-1 inhibitor, also decreased Mcl-1 level (Figure 3a). In contrast, AT-101 had little effect on Bcl-2 and Bcl-xL expression (Figure 3a).

We also examined the effects of AT-101 on Bad phosphorylation and the translocation of Bax from the cytosol to the mitochondria. Bax and Bad are proapoptotic proteins that can

regulate mitochondria-dependent apoptosis.^{22,23} Treating cells with AT-101 decreased phospho-Bad levels and translocated Bax from the cytosol to the mitochondria in both dose- and time-dependent manner (Figure 3a). Immunofluorescence was used to investigate the subcellular localization of Bax during AT-101-induced apoptosis. Bax was largely distributed in the cytosol in untreated cells; when cells were treated with AT-101, the cellular localization of Bax clearly shifted from the cytosol to the mitochondria (Figure 3b).

Marked decreases in Mcl-1 and phosphorylated Bad and Bax translocation after AT-101 treatment were also observed in Jurkat and HL-60 cells (Figure 3c) and primary human leukemia blasts but not in normal human peripheral blood mononuclear cells (Figure 3d). These findings suggest that AT-101-induced apoptosis is associated with Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation in both human leukemia cell lines and primary human leukemia blasts.

AT-101 increased PTEN activity and decreased Akt phosphorylation in leukemia cells. PI3-K/Akt signaling directly affects the apoptosis by targeting Bcl-2 family proteins.²⁴ PTEN negatively regulates the PI3-K/Akt pathway,²⁵ and therefore we determined the effect of AT-101 on phosphorylated PTEN and Akt levels. Treating cells with various dosages and lengths of AT-101 markedly increased phospho-PTEN levels and slightly increased total PTEN expression (Figure 4a). AT-101 also significantly decreased phospho-Akt (p-Akt) levels. Similar results were observed in Jurkat and HL-60 cells and primary human leukemia blasts but not in normal human peripheral blood mononuclear cells (Figures 4b–d). These findings indicate that PTEN activation and Akt inactivation may be involved in the apoptosis induced by AT-101 in human leukemia cells.

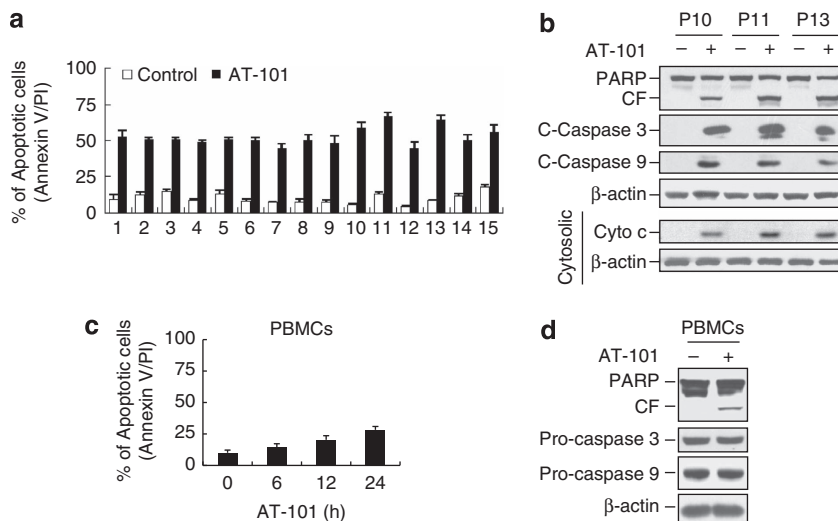


Figure 2 AT-101 selectively induced apoptosis in primary human leukemia cells but not in normal human peripheral blood mononuclear cells. (a) Primary leukemia blasts were isolated from the peripheral blood of 15 patients. After exposure to 20 μ M AT-101 for 24 h, apoptosis was measured using flow cytometry. The results from the Annexin V/propidium iodide (PI) assays represent the mean \pm S.D. for five separate experiments. (b) Whole-cell lysates and cytosolic fractions from blasts isolated from three AML patients were obtained and analyzed by western blot analysis using antibodies against poly (ADP-ribose) polymerase (PARP), cleaved-caspase-9 (C-Caspase 9), cleaved-caspase-3 (C-Caspase 3), and cytochrome *c* (Cyto *c*). (c) Normal human peripheral blood mononuclear cells (PBMCs) were exposed to 20 μ M AT-101 for 6, 12, and 24 h. Apoptosis was then measured by flow cytometry. (d) Normal human PBMCs were treated with 20 μ M AT-101 for 24 h, after which whole-cell lysates were prepared and subjected to western blot analysis using antibodies against PARP, Pro-caspase-3 and Pro-caspase-9. CF, cleaved fragment

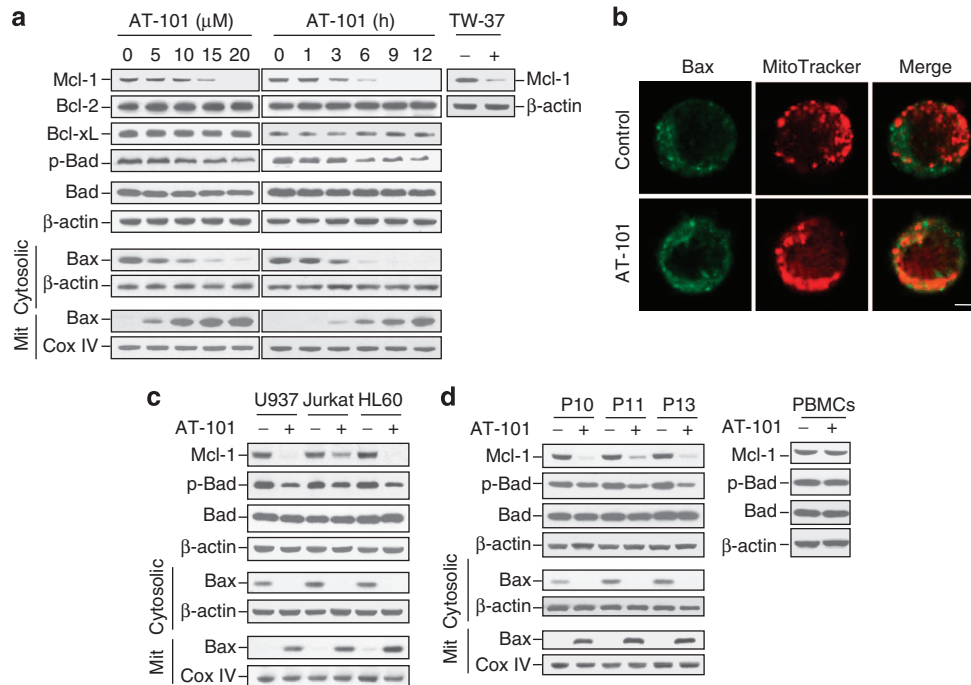


Figure 3 AT-101 downregulated Mcl-1, dephosphorylated Bad, and translocated Bax. **(a)** U937 cells were treated with or without AT-101 at the indicated concentrations and for the indicated lengths. Total cellular extracts and cytosolic and mitochondrial (Mit) fractions were analyzed by western blot analysis using antibodies against Mcl-1, Bcl-2, Bcl-xL, Bad, phospho-Bad (p-Bad), and Bax. U937 cells were also treated with 10 μM TW-37 for 24 h, total cellular extracts were analyzed by western blot analysis using antibodies against Mcl-1. **(b)** Immunostaining of mitochondria (red) and Bax (green). Scale bar represents 5 μm. **(c and d)** U937, Jurkat, and HL60 cells were treated with or without 20 μM AT-101 for 12 h, and three AML blasts and normal human peripheral blood mononuclear cells were treated with or without 20 μM AT-101 for 24 h. Total cellular extracts and cytosolic and Mit fractions were analyzed by western blot analysis. Blots were then stripped and reprobed with antibodies against β-actin (total cellular extract and cytosolic fraction) or complex IV (COX IV) (mitochondrial fraction) to ensure equivalent loading

AT-101 increased RhoA and ROCK1 activation in leukemia cells. Next, we examined the effect of AT-101 on ROCK1, ROCK2, and RhoA activation. ROCK1 is a major target of RhoA that regulates PTEN and helps control apoptosis.^{3,9} AT-101 caused a dose- and time-dependent increase in the GTP-activated form of RhoA, decrease in ROCK1 levels, and an increase in ROCK1 cleavage (Figure 4a). Modest decreases in RhoA levels were also noted in the cells treated with AT-101. In contrast, AT-101 had no effect on expression of ROCK2 (Figure 4a). Marked increases in RhoA and ROCK1 activation were also observed in Jurkat and HL-60 cells and primary leukemia blasts from three AML patients but not in normal human peripheral blood mononuclear cells (Figures 4b–d). In contrast, AT-101 had no effect on expression of ROCK2 in these cells (Figures 4b and c). Taken together, these results suggest that RhoA-ROCK1 signaling is essential for AT-101-mediated apoptosis in both human leukemia cell lines and primary leukemia blasts.

ROCK1 is activated by RhoA, rather than caspase-3, in AT-101-treated leukemia cells. As ROCK1 is a prominent caspase-3 substrate that is cleaved and activated during apoptosis,^{4,9} we also investigated the effects of z-VAD-fmk, a broad caspase inhibitor, on the cleavage and activation of ROCK1 during AT-101-induced apoptosis. Treating cells with both AT-101 and z-VAD-fmk (20 μM) significantly abrogated apoptosis and caspase-3 activation (Figures 5a and b).

However, pretreatment with z-VAD-fmk failed to prevent the cleavage/activation of ROCK1 (Figure 5b). Knocking down caspase-3 using siRNA also significantly diminished the apoptosis and caspase-3 activation, but not ROCK1 cleavage, induced by AT-101 (Figures 5c and d). These findings indicate that the cleavage/activation of ROCK1 caused by AT-101 exposure is not a secondary caspase-dependent event.

Our results indicate that caspase-3 is not involved in ROCK1 activation during AT-101-induced apoptosis. Therefore, we investigated the possibility ROCK1 cleavage/activation via RhoA using C3 exoenzyme (C3), a selective RhoA inhibitor. Treating cells with both C3 (5 μg) and AT-101 (20 μM) for 12 h significantly decreased apoptosis levels (Figure 6a). Cotreatment with C3 also abrogated caspase-3 and caspase-9 activation, PARP cleavage, cytochrome *c* release, and RhoA/ROCK1 activation that AT-101 induces (Figures 6b and c). These data indicate that the ROCK1 cleavage/activation involved in AT-101-mediated apoptosis occurs via RhoA, rather than caspase-3.

The roles of ROCK1 activation and Akt inactivation in AT-101-mediated apoptosis. We used Y27632, a ROCK1 inhibitor, to determine whether ROCK1 regulates PTEN activity and Akt inactivation during AT-101-induced apoptosis. Treating cells with both Y27632 (20 μM) and AT-101 (20 μM) for 12 h significantly decreased apoptosis levels (Figure 6d). Coadministration of Y27632 also reduced

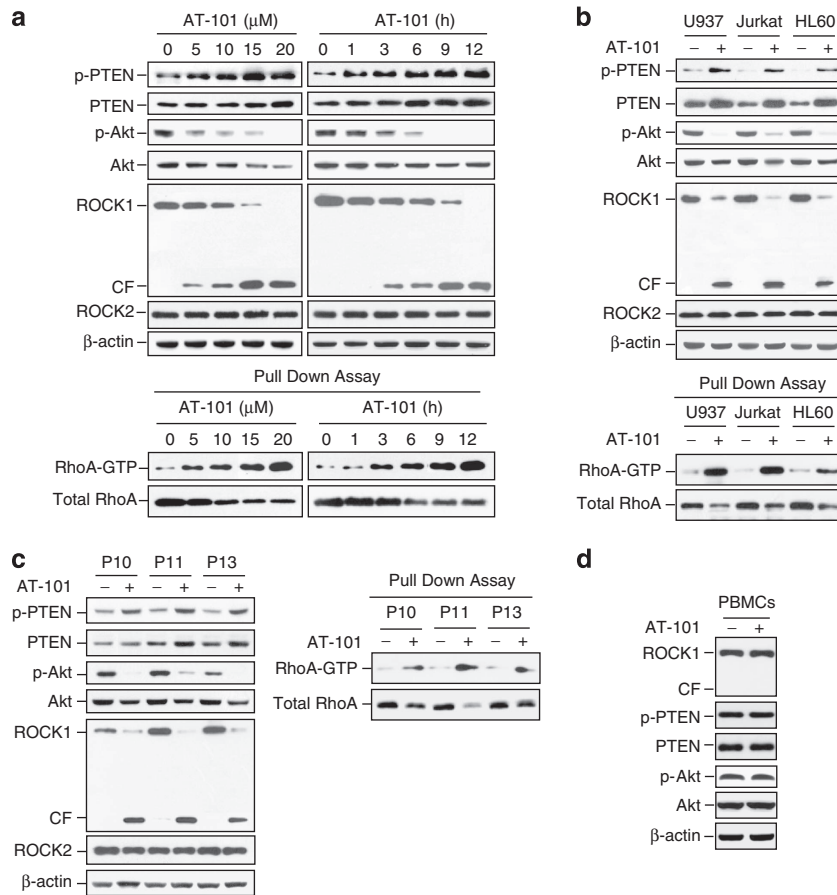


Figure 4 AT-101 activated RhoA, ROCK1, and PTEN and inactivated Akt. (a) U937 cells were treated with or without AT-101 at the indicated concentrations and for the indicated lengths. (b) U937, Jurkat, and HL60 cells were treated with or without 20 μ M AT-101 for 12 h. (c) Three AML blasts were treated with or without 20 μ M AT-101 for 24 h. (d) Normal human peripheral blood mononuclear cells were exposed to 20 μ M AT-101 for 24 h. Total cellular extracts were analyzed by western blot analysis using antibodies against phospho-PTEN (p-PTEN), PTEN, phospho-Akt (Ser473, p-Akt), Akt, and ROCK1. Active RhoA-GTP was pulled down by glutathione *S*-transferase linked to the RhoA-binding domain of Rhotekin (GST-RBD); bead/protein complexes and total RhoA were detected by immunoblotting with anti-RhoA, CF, cleaved fragment

caspase-3 and caspase-9 activation, PARP cleavage, and cytochrome *c* release and blocked ROCK1 cleavage/activation, PTEN activity, Akt inactivation, Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation (Figures 6e and f).

Next, we knocked down ROCK1 expression in U937 cells using a ROCK1-specific siRNA. Knocking down ROCK1 expression significantly abrogated AT-101-mediated apoptosis and diminished AT-101-induced PARP cleavage, caspase activation, and cytochrome *c* release (Figures 6g and h). Western blot analysis showed that U937 cells transfected with ROCK1 siRNA had reduced total ROCK1 and cleaved ROCK1 expression. Knocking down ROCK1 also blocked PTEN activity, Akt inactivation, Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation mediated by AT-101 (Figure 6i). Taken together, these findings suggest that ROCK1 activation has an important role in regulating PTEN activity in leukemia cells that are exposed to AT-101. This results in downstream Akt inactivation, Mcl-1 downregulation, Bad dephosphorylation, Bax translocation, and finally apoptosis.

These data also suggest that Akt inactivation may have an important role in the apoptosis induced by AT-101; therefore,

LY294002, a PI3-K inhibitor, was coadministered with AT-101 to test this possibility. Nontoxic concentrations of LY294002 (i.e., 20 μ M, ~11%) combined with modestly toxic concentrations of AT-101 (10 μ M, ~33%) significantly increased apoptosis levels by ~66% (Figure 7a). Coadministration of LY294002 also increased caspase-3 and caspase-9 activation, PARP cleavage, and cytochrome *c* release; potentiated Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation; and virtually abrogated Akt activation (Figures 7b and c).

To further characterize the functional role of Akt in AT-101-mediated apoptosis, U937 cells that ectopically expressed constitutively active forms of Akt (Akt-CA) were treated with AT-101. Two separate clones (Akt-CA6 and Akt-CA10) with variable degrees of constitutively active Akt were both less sensitive to AT-101 than a vector control (Figure 7d). Western blot analysis showed increases in both total and phospho-Akt expression in Akt-CA cells, and AT-101 failed to inhibit Akt phosphorylation in these cells (Figure 7f). The caspase-3 and caspase-9 activation, PARP cleavage, and cytochrome *c* release induced by AT-101 were also markedly attenuated in Akt-CA cells (Figure 7e). Furthermore, the constitutive activation of Akt blocked the Mcl-1

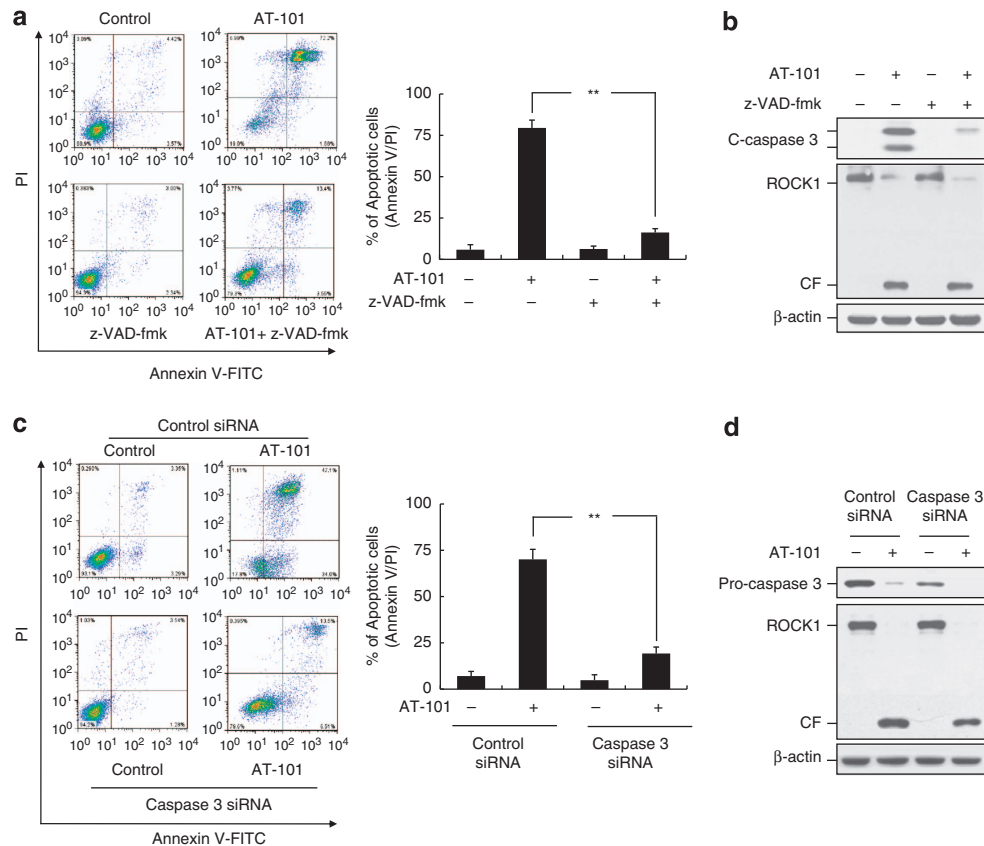


Figure 5 AT-101-mediated apoptosis requires caspase-3-independent ROCK1 activation. U937 cells were pretreated with z-VAD-fmk (20 μ M), a caspase inhibitor, for 2 h, followed by 20 μ M AT-101 for 12 h. **(a)** Apoptosis was measured by flow cytometry. Error bars represent the mean \pm S.D. ($n=5$). Values for cells treated with z-VAD-fmk and AT-101 were significantly lower than those for cells treated by AT-101 alone (** $P<0.01$). **(b)** Total cellular extract was analyzed by western blot analysis using antibodies against cleaved-caspase-3 (C-Caspase 3) and ROCK1. **(c)** and **(d)** U937 cells were infected with lentiviral vectors containing either control or caspase-3-specific siRNA. Cells were then treated with or without 20 μ M AT-101 for 12 h, and apoptosis was measured by flow cytometry. Error bars represent the mean \pm S.D. ($n=5$). Values for cells that received caspase-3 siRNA and treated with AT-101 were significantly lower than those for cells that received control siRNA and treated with AT-101 (** $P<0.01$). Total cellular extracts were analyzed by western blot analysis using antibodies against Pro-caspase-3 and ROCK1. CF, cleaved fragment; FITC, fluorescein isothiocyanate

downregulation, Bad dephosphorylation, and Bax translocation mediated by AT-101 (Figure 7f). These findings indicate that Akt inactivation has a critical role in the apoptosis induced by AT-101 and that Akt lies upstream of Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation.

AT-101 suppressed tumor growth in a U937 xenograft model. Finally, NOD/SCID mice were subcutaneously inoculated with U937 cells to determine whether these *in vitro* findings were applicable *in vivo*. After inoculation, mice received injections of either vehicle or AT-101 (50 mg/kg intraperitoneally) for 60 days. The median survival time of the control group ($n=20$) was approximately 35 days. Daily AT-101 treatment significantly prolonged the animals' survival compared with their vehicle-treated controls (** $P<0.01$). The median survival time of the AT-101-treated animals ($n=20$) was 53 days (Figure 8a).

We also examined the effects of AT-101 on the tumor volume of the U937 xenografts. AT-101 modestly, but significantly, suppressed tumor growth at 10 days after the

first drug exposure ($*P<0.05$). This effect became more apparent after 15 and 20 days of drug exposure and was quite extensive by 25 and 30 days (** $P<0.01$) (Figure 8b). No statistically significant changes in body weight were noted between the control and AT-101-treated mice (Figure 8c). Moreover, mice from the AT-101 group did not exhibit any other signs of toxicity, such as agitation, impaired movement and posture, indigestion or diarrhea, and areas of redness or swelling.

We then used hematoxylin and eosin (H&E), the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, and immunohistochemistry to examine morphological changes and the induction of apoptosis in U937 cells *in vivo*. U937 xenografts from mice treated with AT-101 had lower numbers of cancer cells and exhibited signs of necrosis, infiltration of inflammatory cells (i.e., phagocytic cells), fibrosis, and apoptosis (Figure 8d, top panels). Tumor sections from these mice also showed numerous dark brown-colored apoptotic cells (Figure 8d, middle panels). AT-101 exposure caused a rapid increase in immunoreactivity for the cleaved forms of caspase-3 and PARP, which are indicative of apoptosis

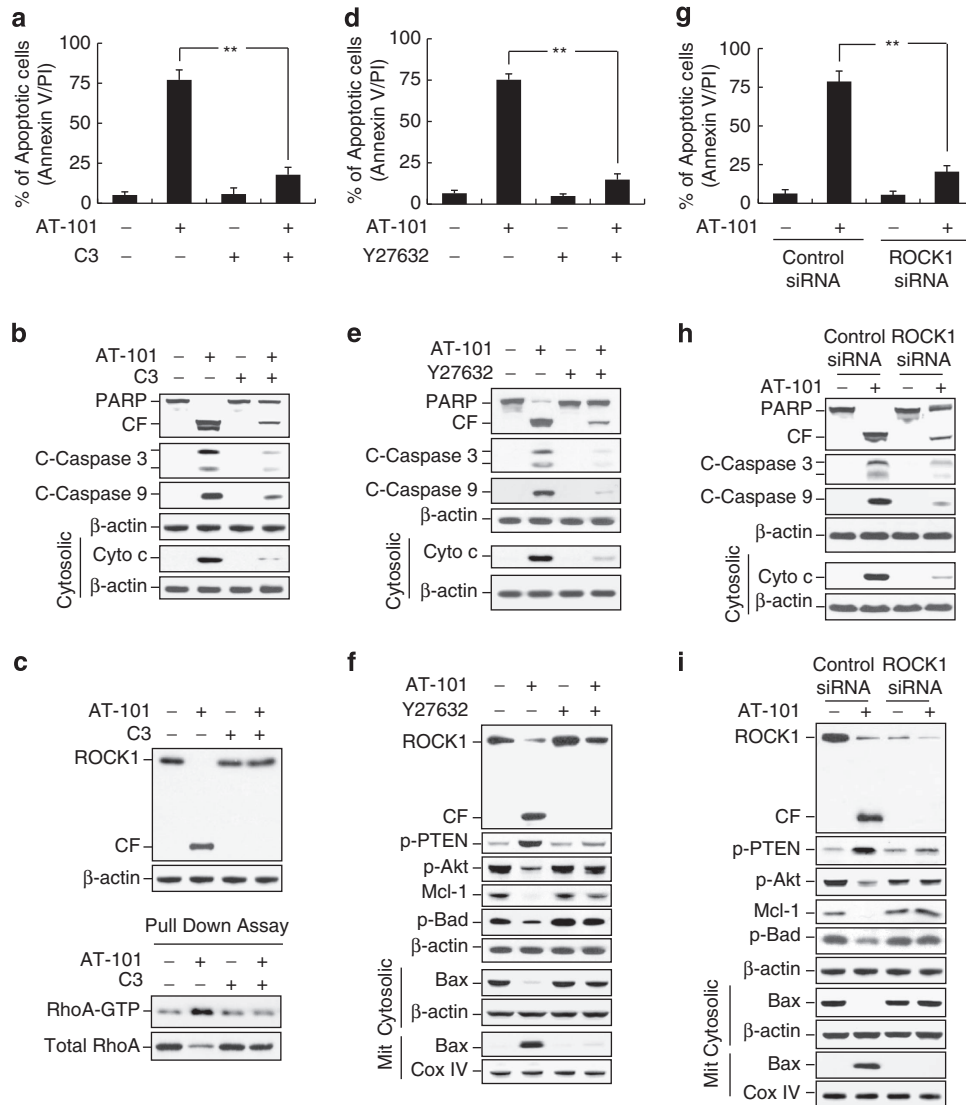


Figure 6 RhoA/ROCK1 activation has an important functional role in AT-101-induced apoptosis. U937 cells were transfected with 5 μ g C3 (premixed with FuGENE 6 reagent in RPMI 1640 medium). After 5 h incubation, cells were treated with 20 μ M AT-101 for 12 h. **(a)** Apoptosis was measured by flow cytometry. Error bars represent the mean \pm S.D. ($n = 5$). Values for cells treated with AT-101 and C3 were significantly lower than those for cells treated with AT-101 alone (** $P < 0.01$). **(b and c)** Whole-cell lysates and cytosolic fractions were analyzed by western blot analysis. Activated RhoA was pulled down by glutathione *S*-transferase linked to the RhoA-binding domain of Rhotekin (GST-RBD) and analyzed by immunoblotting. U937 cells were pretreated with Y-27632 (20 μ M), a specific ROCK1 inhibitor, for 2 h, followed by 20 μ M AT-101 for 12 h. **(d)** Apoptosis was measured by flow cytometry. Error bars represent the mean \pm S.D. ($n = 5$). Values for cells treated with AT-101 and Y27632 were significantly lower than those for cells treated with AT-101 alone (** $P < 0.01$). **(e and f)** Total cellular extracts and cytosolic and mitochondrial fractions were analyzed by western blot analysis. U937 cells were infected with lentivirus containing ROCK1-specific small interfering RNA (siRNA) (siROCK1) or a scrambled control siRNA. Cells were then treated with or without 20 μ M AT-101 for 12 h. **(g)** Apoptosis was measured by flow cytometry. Error bars represent the mean \pm S.D. ($n = 5$). Values for siROCK1 cells treated with AT-101 were significantly lower than those for control siRNA cells treated with AT-101 (** $P < 0.01$). **(h and i)** Total cellular extracts and cytosolic and mitochondrial fractions were analyzed by western blot analysis. C-Caspase 3, cleaved-caspase-3; C-Caspase 9, cleaved-caspase-9; CF, cleaved fragment; COX IV, complex IV; Cyto *c*, cytochrome *c*; p, phosphorylated; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide

(Figure 8d, bottom panels). These findings suggest that AT-101 significantly inhibited tumor growth of U937 xenografts by inducing apoptosis.

Western blot analysis and pull-down assays were used to further evaluate whether the RhoA/ROCK1/PTEN signaling pathway was involved in the antileukemic activity of AT-101 *in vivo*. In mice that were treated with AT-101, RhoA/ROCK1/PTEN activation and Akt inactivation occurred (Figure 8e). This suggests that interrupting the RhoA/ROCK1/PTEN

signaling pathway could contribute to the antileukemic activity of AT-101 *in vivo*.

Discussion

Recently, therapeutic strategies that target Bcl-2 have emerged as promising prospects for treating many types of cancer. AT-101, a natural cottonseed product, possesses anticancer activity via Bcl-2, Bcl-xL, and Mcl-1 inhibition²⁶ and

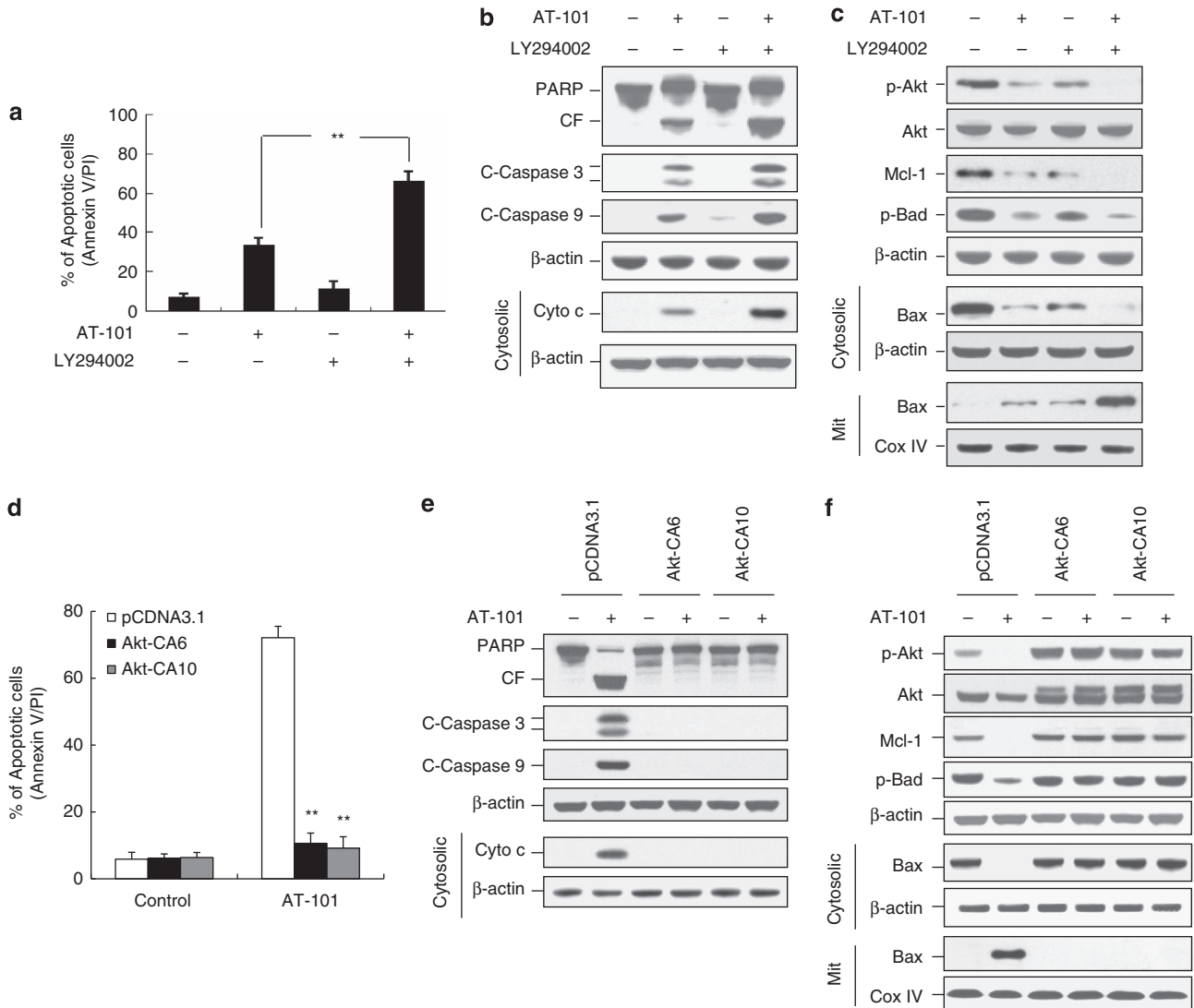


Figure 7 Interrupting Akt activation is involved in AT-101-mediated apoptosis. U937 cells were pretreated with 20 μ M of LY294002 for 1 h, followed by the addition of 10 μ M AT-101 for 12 h. (a) Apoptosis was measured using flow cytometry. Error bars represent the mean \pm S.D. ($n = 5$). Values for cells treated with AT-101 and LY294002 in combination were significantly higher than those for cells treated with AT-101 alone (** $P < 0.01$). (b and c) Total cellular extracts and cytosolic and mitochondrial fractions were analyzed by western blot analysis. U937 cells were stably transfected with constitutively active forms of Akt (Akt-CA6 and Akt-CA10) or an empty vector (pcDNA3.1). All cells were then treated with or without 20 μ M AT-101 for 12 h. (d) Apoptosis was measured using flow cytometry. Values for Akt-CA cells treated with AT-101 were significantly lower than those for pcDNA3.1 cells (** $P < 0.01$). (e and f) Total cellular extracts and cytosolic and mitochondrial fractions were analyzed by western blot analysis. C-Caspase 3, cleaved-caspase-3; C-Caspase 9, cleaved-caspase-9; CF, cleaved fragment; COX IV, complex IV; Cyto c, cytochrome c; p, phosphorylated; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide

has recently advanced into clinical trials for treating patients with advanced malignancies.¹⁷ Recent studies revealed that AT-101 induces apoptosis by targeting different anti- and proapoptotic Bcl-2 family proteins in various cancer cell types by distinct mechanisms that include downregulating Bcl-2, Bcl-xL, and XIAP,¹⁸ upregulating Puma and Noxa,¹² releasing Smac and translocating Bax,¹⁹ and releasing cytochrome c through conformational Bcl-2 changes.²⁷ In the present study, we demonstrated that AT-101 induces apoptosis and mitochondrial injury (cytochrome c release) in human leukemia cells by downregulating Mcl-1, dephosphorylating Bad, and translocating Bax. Our results are consistent with previously reported findings that show that AT-101 induces apoptosis by

downregulating Mcl-1 in chronic lymphocytic leukemia B cells,²⁸ dephosphorylating Bad in colorectal carcinoma cells,²⁹ and translocating Bax in ovarian cancer cells.¹⁹

Akt is a serine–threonine kinase that is intimately involved in the regulation of cell survival.³⁰ It is activated by PI3-K recruitment to the cell membrane and is negatively regulated by the PTEN phosphatase.³¹ Akt may directly or indirectly regulate Bcl-2 family members that control cell survival and death. The bulk of the evidence suggests that Akt directly phosphorylates Bad at Ser136, leading to its association with Bax and the inhibition of Bax-induced cell death.³² Akt can also inhibit the translocation of Bax to the mitochondria by Ser184 Bax phosphorylation.³³ Akt has also been reported to

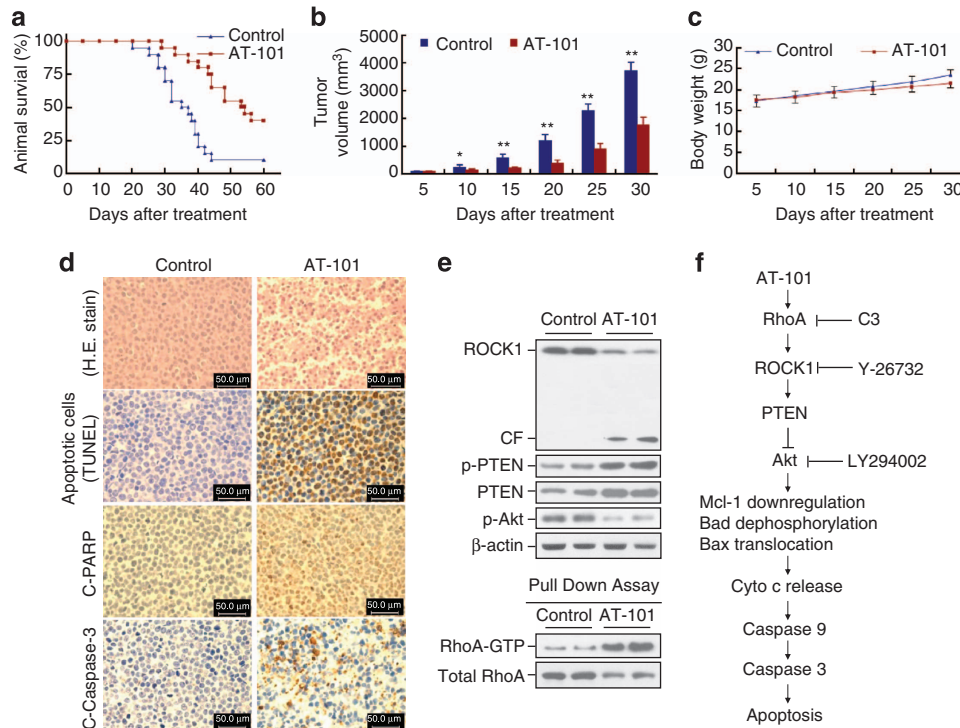


Figure 8 AT-101 inhibited tumor growth and induced apoptosis in a U937 xenograft animal model. (a) Survival comparison of the AT-101 and vehicle-treated groups (** $P < 0.01$). (b) Average tumor volumes of mice treated with vehicle or 50 mg/kg AT-101. Data are mean \pm S.D. ($n = 20$). Tumor volumes of mice treated with AT-101 were significantly lower than those of mice treated with vehicle (* $P < 0.05$ or ** $P < 0.01$). (c) Body weight changes of mice during the 30 study days. Statistical analyses of body weight changes showed no significant differences between the AT-101 and vehicle-treated groups. (d) After treating with AT-101 (50 mg/kg), tumor tissues were sectioned and subjected to H&E staining, TUNEL assay, and immunohistochemistry analysis to evaluate histological morphology, apoptosis, and cleaved-poly (ADP-ribose) polymerase (C-PARP) and cleaved-caspase-3 (C-Caspase 3) expression. (e) Tumors from two vehicle-treated mice and two AT-101-treated (50 mg/kg) mice were harvested and homogenized. Whole-tumor lysates were analyzed by western blot analysis or pull down assays. (f) A hypothetical model of AT-101-induced lethality in leukemia cells. In this model, AT-101 induces RhoA/ROCK1 activation, leading to PTEN activation and Akt inactivation, which in turn results in Mcl-1 downregulation, Bad dephosphorylation, Bax translocation, and finally in mitochondrial injury (cytochrome c (Cyto c) release), caspase activation, and apoptosis. CF, cleaved fragment; p, phosphorylated

block the phosphorylating ability of GSK3 β and to down-regulate Mcl-1.³⁴ Our findings revealed that AT-101 not only induces Mcl-1 downregulation, Bad dephosphorylation, and Bax translocation but also inhibits Akt phosphorylation. Constitutively active Akt largely blocked the Mcl-1 down-regulation, Bad dephosphorylation, and Bax translocation induced by AT-101.

In this study, we demonstrated that the AT-101-induced apoptosis in human leukemia cells occurs via the RhoA/ROCK1/PTEN signaling pathway and Akt inactivation. These processes have a critical role in regulating apoptosis in response to AT-101. RhoA is a member of the Ras super-family of small guanosine triphosphatases (GTPases), which shuttles between an inactive GDP-bound state and an active GTP-bound state and exhibits intrinsic GTPase activities. ROCK1 is a serine/threonine kinase that is cleaved/activated by binding of GTP-RhoA to its C-terminal coiled-coil domain.^{4,35} ROCK1-mediated apoptosis may proceed through actin cytoskeleton rearrangements, which in turn activate the caspase cascade.³⁶ The ROCK1 cleavage/activation can be regulated by several distinct mechanisms, including RhoA and caspase-3 activation. Caspase-3 is believed to be responsible for ROCK1 cleavage/activation in apoptotic cells; ROCK1 is not cleaved in MCF-7 breast carcinoma cells, which are caspase-3 deficient.⁵ Caspase-3

cleavage of ROCK1 can also be inhibited by caspase inhibitors in a variety of apoptotic cells.^{4,37} However, caspase-3-independent cleavage of ROCK1 has been observed in apoptosis induced by extracellular ATP and the P2 \times 7 ATP and in cancer cells subjected to a combined BGC9331 and SN-38 treatment.^{38,39} On the other hand, ROCK1 can also be cleaved/activated by RhoA-GTP by a conformational change that shifts the inhibitory carboxyl-terminal domain of ROCK1 away from its active kinase site.⁴⁰

In the present study, treating U937 cells with both z-VAD-fmk, a caspase inhibitor, and AT-101 abrogated the AT-101-induced caspase-3 activation and apoptosis and failed to prevent ROCK1 cleavage/activation. This strongly suggested that other factors mediated ROCK1 activation under these conditions. Our results strongly support the hypothesis that ROCK1 is cleaved/activated by GTP-bound RhoA based on the following findings. First, AT-101 treatment induced RhoA activation by GTP-bound RhoA. Second, pretreatment with C3, which inactivates RhoA, completely abolished the cleavage/activation of ROCK1. Finally, pretreatment with C3 also significantly abrogated the AT-101-mediated apoptosis. Taken together, these results suggest that RhoA, rather than caspase-3, activates ROCK1 during AT-101-induced apoptosis.

Recent evidence identified PTEN as a new ROCK1 substrate that is also involved in cell death and survival.⁶ PTEN is a negative regulator of the PI3-K/Akt pathway, and PTEN phosphorylation by ROCK1 stimulates its phosphatase activity.⁷ Several studies have found that RhoA/ROCK1 activation enhances PTEN activity and suppresses Akt activation. For instance, PTEN phosphorylation by ROCK1 decreases Akt phosphorylation in HEK cells.⁹ RhoA and ROCK1, its effector kinase, inhibit Akt activation by enhancing PTEN activity.⁴¹ Our findings are consistent with these reports and suggest that RhoA/ROCK1 activation contributes to the Mcl-1 downregulation and apoptosis induced by AT-101 via PTEN activation and Akt inactivation. Specifically, AT-101 activates RhoA/ROCK1 and PTEN and inactivates Akt. Inhibiting ROCK1 activation by both Y27632 and a ROCK1-specific siRNA attenuated the apoptosis mediated by AT-101 by inhibiting PTEN activity and Akt inactivation.

Previous reports indicate that inhibition of the RhoA/ROCK1/PTEN pathway and prolonged Akt activation are key regulatory steps in cell transformation and tumorigenesis.⁴² Because RhoA/ROCK1/PTEN activation and Akt inactivation have critical roles in AT-101-induced apoptosis in human leukemia cells, a better understanding of these pathways involved in regulating apoptosis is probably of therapeutic benefit for treating hematological malignancies. As AT-101 has been recently introduced into clinical trials,^{13–16} further attempts to explore this novel strategy seem warranted.

In summary, the present study found that AT-101 selectively induces apoptosis and mitochondrial injury in human leukemia cell lines and primary human leukemia cells, and also inhibits tumor growth of U937 xenografts by activating RhoA/ROCK1/PTEN signaling and inactivating Akt. Collectively, these findings support a hypothetical model of AT-101-induced lethality in leukemia cells (Figure 8f). In this model, AT-101 induces RhoA/ROCK1 activation, leading to PTEN activation and Akt inactivation, which in turn results in Mcl-1 downregulation, Bad dephosphorylation, Bax translocation, and finally in mitochondrial injury (cytochrome *c* release), caspase activation, and apoptosis. Further efforts to understand the mechanism(s) by which AT-101 induces apoptosis in human leukemia cells both *in vitro* and *in vivo* could improve treatment outcomes for leukemia and potentially other hematologic malignancies.

Materials and Methods

Cells and reagents. U937, HL-60, and Jurkat cells were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

Peripheral blood samples for the *in vitro* studies were obtained from 15 patients with newly diagnosed or recurrent AML after acquiring informed consent (four of those patients are M2, five are M4, and six are M5 according to the FAB classification system). Approval for these studies was obtained from the Southwest Hospital (Chongqing, China) Institutional Review Board. AML blasts were isolated by Histopaque-1077 density gradient centrifugation (Sigma-Aldrich Co., St. Louis, MO, USA) at 600 × *g* for 15 min. The isolated mononuclear cells were suspended at 8 × 10⁵/ml in RPMI 1640 medium. Fresh normal peripheral blood mononuclear cells were isolated from blood samples that were collected from healthy volunteers.

The constitutively active form of Akt (Akt-CA) was kindly provided by Dr. Richard Roth (Stanford University, School of Medicine, Stanford, CA, USA). Akt-CA was subcloned into pcDNA3.1, and U937 cells were stably transfected with Akt-CA using Amara Nucleofector (Koeln, Germany) according to the manufacturer's instructions.

Stable single-cell clones were selected by 400 μg/ml of geneticin. Thereafter, Akt expression from each cell clone was analyzed using western blots (see below).

AT-101 was obtained from Ascenta Therapeutics (San Diego, CA, USA), Y-27632 and TW-37 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), C3 exoenzyme from Calbiochem (San Diego, CA, USA), LY294002 and z-VAD-fmk were both purchased from EMD Biosciences (La Jolla, CA, USA). Antibodies against Akt, Bcl-2, Bcl-xL, cytochrome *c*, and β-actin were obtained from Santa Cruz Biotechnology; procaspase-3, procaspase-9, cleaved caspase-3, cleaved caspase-9, cleaved PARP, phospho-Akt (Ser473), phospho-PTEN, PTEN, phospho-Bad, Bad, COX IV, and RhoA from Cell Signaling Technology (Beverly, MA, USA); Mcl-1 and Bax from Pharmingen (San Diego, CA, USA); PARP from Biomol (Plymouth Meeting, PA, USA); and ROCK1 from Abcam (Burlingame, CA, USA).

Apoptosis analysis and mitochondrial membrane potential

($\Delta\psi_m$). The extent of apoptosis in the leukemia cells was evaluated by flow cytometry using the Annexin V/PI staining kit (Pharmingen) according to the manufacturer's instructions. To analyze $\Delta\psi_m$, 2 × 10⁵ cells were incubated with 40 nm 3,3-dihexyloxycarbocynine (DiOC₆; Molecular Probes Inc., Eugene, OR, USA) in PBS at 37 °C for 20 min. Then analyzed using a Becton-Dickinson FACScan cytofluorometer (Becton-Dickinson, San Jose, CA, USA) as previously described.⁴³

Western blots. Total cellular samples were washed two times ice with ice-cold PBS and then lysed in 1 × NuPAGE LDS sample buffer supplemented with 50 mM dithiothreitol. Protein concentrations were determined using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA), and 30 μg of sample proteins were separated using SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% fat-free dry milk in 1 × Tris-buffered saline and then incubated with antibodies. Protein bands were detected by incubating with horseradish peroxidase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), which were visualized with enhanced chemiluminescence reagent (Perkin-Elmer, Boston, MA, USA).

Cytosolic cytochrome *c* and Bax and mitochondrial Bax

analysis. Mitochondrial and cytosolic fractions were obtained as described previously.⁴³ These fractions were prepared and analyzed by western blots to monitor cytochrome *c* and Bax expression.

RNAi assay. Caspase-3 shRNA (h), ROCK1 shRNA (h), and control shRNA lentiviral particles were purchased from Santa Cruz Biotechnology. U937 cells were incubated with the lentiviral vectors and 5 μg/ml polybrene overnight. After treatment, apoptosis levels were measured by flow cytometry, and total cellular extracts were prepared and analyzed by western blots.

RhoA activity assay. The RhoA activity assays were performed according to the manufacturer's instructions (Cytoskeleton, Denver, CO, USA). Briefly, 5 × 10⁵ U937 cells were plated and cultured for 2 days. Samples were then rapidly lysed at 4 °C and incubated with sepharose-bound Rhotekin to pull down active RhoA. After washing, the bead/protein complexes were boiled in sample buffer and separated by SDS-PAGE. The blots were incubated with an antibody against RhoA.

Immunofluorescence. Cells were preincubated with 500 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C, washed two times with RPMI 1640 medium, and then treated with or without 20 μM AT-101 for 12 h. Cells were then collected via centrifugation, fixed with 4% paraformaldehyde for 15 min, permeabilized by 0.1% Triton X-100 for 10 min, and then blocked with 1% bovine serum albumin for 30 min. Cells were further incubated with a primary cofilin antibody at 4 °C overnight, followed by a secondary Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes) for 1 h at room temperature. After washing, images were captured using a Leica scanning confocal microscope (TCS SP5; Leica Microsystems, Mannheim, Germany).

Xenograft assays. NOD/SCID mice (5 weeks old) were purchased from Vital River Laboratories (VRL, Beijing, China). All animal studies were conducted according to protocols approved by the University's Institutional Animal Care and Use Committee (IACUC). Forty mice were inoculated with U937 cells (2 × 10⁶ cells per mouse, subcutaneously) and then randomly divided into two groups

($n = 20$ per group). Five days after tumor inoculation, mice received either AT-101 (50 mg/kg, intraperitoneally, five times per week) or an equal volume of vehicle. The mice were monitored daily for signs of tumor growth, body weight, and time of death. Tumor tissues from representative mice were sectioned, embedded in paraffin, and processed for H&E, TUNEL, and immunohistochemical analysis.

TUNEL, histological, and immunohistochemical evaluations.

The apoptotic cells in the tissue samples were detected using an *In Situ* Cell Death Detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Tumor tissues from representative mice were sectioned, embedded in paraffin, and prepared for histological and immunohistochemical evaluations as described previously.^{44,45}

Statistical analyses. All data are expressed as means \pm S.D. of five individual experiments. The statistical significance of the difference between control and AT-101-treated groups was evaluated using Student's *t*-test. The survival analysis was analyzed using the Kaplan–Meier method, and significance was calculated using the log-rank test. * $P < 0.05$ or ** $P < 0.01$ were considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by the Chongqing Natural Science Foundation (CSTC2013jjB10007) and the National Natural Science Foundation of China (No. 30971288).

- Schofield AV, Steel R, Bernard O. Rho-associated coiled-coil kinase (ROCK) protein controls microtubule dynamics in a novel signaling pathway that regulates cell migration. *J Biol Chem* 2012; **287**: 43620–43629.
- Shi J, Wei L. Rho kinase in the regulation of cell death and survival. *Arch Immunol Ther Exp* 2007; **55**: 61–75.
- Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003; **4**: 446–456.
- Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol* 2001; **3**: 339–345.
- Sebbagh M, Renvoize C, Hamelin J, Riche N, Bertoglio J, Breard J. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat Cell Biol* 2001; **3**: 346–352.
- Li Z, Dong X, Wang Z, Liu W, Deng N, Ding Y *et al*. Regulation of PTEN by Rho small GTPases. *Nat Cell Biol* 2005; **7**: 399–404.
- Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. *Cell* 2000; **100**: 387–390.
- Keniry M, Parsons R. The role of PTEN signaling perturbations in cancer and in targeted therapy. *Oncogene* 2008; **27**: 5477–5485.
- Chang J, Xie M, Shah VR, Schneider MD, Entman ML, Wei L *et al*. Activation of Rho-associated coiled-coil protein kinase 1 (ROCK-1) by caspase-3 cleavage plays an essential role in cardiac myocyte apoptosis. *Proc Natl Acad Sci USA* 2006; **103**: 14495–14500.
- Furukawa N, Ongusaha P, Jahng WJ, Araki K, Choi CS, Kim HJ *et al*. Role of Rho-kinase in regulation of insulin action and glucose homeostasis. *Cell Metab* 2005; **2**: 119–129.
- Paoluzzi L, Gonen M, Gardner JR, Mastrella J, Yang D, Holmlund J *et al*. Targeting Bcl-2 family members with the BH3 mimetic AT-101 markedly enhances the therapeutic effects of chemotherapeutic agents in *in vitro* and *in vivo* models of B-cell lymphoma. *Blood* 2008; **111**: 5350–5358.
- Meng Y, Tang W, Dai Y, Wu X, Liu M, Ji Q *et al*. Natural BH3 mimetic (–)-gossypol chemosensitizes human prostate cancer via Bcl-XL inhibition accompanied by increase of Puma and Noxa. *Mol Cancer Ther* 2008; **7**: 2192–2202.
- Liu G, Kelly WK, Wilding G, Leopold L, Brill K, Somer B. An open-label, multicenter, phase I/II study of single-agent AT-101 in men with castrate-resistant prostate cancer. *Clin Cancer Res* 2009; **15**: 3172–3176.
- Van Poznak C, Seidman AD, Reidenberg MM, Moasser MM, Sklarin N, Van Zee K *et al*. Oral gossypol in the treatment of patients with refractory metastatic breast cancer: a phase I/II clinical trial. *Breast Cancer Res Treat* 2001; **66**: 239–248.
- Baggstrom MQ, Qi Y, Koczywas M, Argiris A, Johnson EA, Millward MJ *et al*. A phase II study of AT-101 (Gossypol) in chemotherapy-sensitive recurrent extensive-stage small cell lung cancer. *J Thorac Oncol* 2011; **6**: 1757–1760.
- Masood A, Chitta K, Paulus A, Khan AN, Sher T, Ersing N *et al*. Downregulation of BCL2 by AT-101 enhances the antileukaemic effect of lenalidomide both by an immune dependant and independent manner. *Br J Haematol* 2012; **157**: 59–66.
- Azmi AS, Mohammad RM. Non-peptidic small molecule inhibitors against Bcl-2 for cancer therapy. *J Cell Physiol* 2009; **218**: 13–21.
- Sung B, Ravindran J, Prasad S, Pandey MK, Aggarwal BB. Gossypol induces death receptor-5 through activation of the ROS-ERK-CHOP pathway and sensitizes colon cancer cells to TRAIL. *J Biol Chem* 2010; **285**: 35418–35427.
- Hu W, Wang F, Tang J, Liu X, Yuan Z, Nie C *et al*. Proapoptotic protein Smac mediates apoptosis in cisplatin-resistant ovarian cancer cells when treated with the anti-tumor agent AT101. *J Biol Chem* 2012; **287**: 68–80.
- Chien CC, Ko CH, Shen SC, Yang LY, Chen YC. The role of COX-2/PGE2 in gossypol-induced apoptosis of colorectal carcinoma cells. *J Cell Physiol* 2012; **227**: 3128–3137.
- Moon DO, Kim MO, Choi YH, Lee HG, Kim ND, Kim GY. Gossypol suppresses telomerase activity in human leukemia cells via regulating hTERT. *FEBS Lett* 2008; **582**: 3367–3373.
- Pucci B, Indelicato M, Paradisi V, Reali V, Pellegrini L, Aventaggiato M *et al*. ERK-1 MAP kinase prevents TNF-induced apoptosis through bad phosphorylation and inhibition of Bax translocation in HeLa Cells. *J Cell Biochem* 2009; **108**: 1166–1174.
- Kazi A, Sun J, Doi K, Sung SS, Takahashi Y, Yin H *et al*. The BH3 alpha-helical mimic BH3-M6 disrupts Bcl-X(L), Bcl-2, and MCL-1 protein-protein interactions with Bax, Bak, Bad, or Bim and induces apoptosis in a Bax- and Bim-dependent manner. *J Biol Chem* 2011; **286**: 9382–9392.
- Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *Biochem J* 2008; **415**: 333–344.
- Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002; **296**: 1655–1657.
- Wang G, Nikolovska-Coleska Z, Yang CY, Wang R, Tang G, Guo J *et al*. Structure-based design of potent small-molecule inhibitors of anti-apoptotic Bcl-2 proteins. *J Med Chem* 2006; **49**: 6139–6142.
- Lei X, Chen Y, Du G, Yu W, Wang X, Qu H *et al*. Gossypol induces Bax/Bak-independent activation of apoptosis and cytochrome *c* release via a conformational change in Bcl-2. *FASEB J* 2006; **20**: 2147–2149.
- Balakrishnan K, Burger JA, Wierda WG, Gandhi V. AT-101 induces apoptosis in CLL B cells and overcomes stromal cell-mediated Mcl-1 induction and drug resistance. *Blood* 2009; **113**: 149–153.
- Ko CH, Shen SC, Yang LY, Lin CW, Chen YC. Gossypol reduction of tumor growth through ROS-dependent mitochondria pathway in human colorectal carcinoma cells. *Int J Cancer* 2007; **121**: 1670–1679.
- Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol* 2001; **21**: 893–901.
- Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1999; **96**: 4240–4245.
- Masters SC, Yang H, Datta SR, Greenberg ME, Fu H. 14-3-3 Inhibits Bad-induced cell death through interaction with serine-136. *Mol Pharmacol* 2001; **60**: 1325–1331.
- Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* 2001; **20**: 7779–7786.
- Maurer U, Charvet C, Wagman AS, Dejaradin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 2006; **21**: 749–760.
- Xiaorong L, Wei W, Liyuan Q, Kaiyan Y. Underexpression of deleted in liver cancer 2 (DL2) is associated with overexpression of RhoA and poor prognosis in hepatocellular carcinoma. *BMC Cancer* 2008; **8**: 205.
- Lai JM, Wu S, Huang DY, Chang ZF. Cytosolic retention of phosphorylated extracellular signal-regulated kinase and a Rho-associated kinase-mediated signal impair expression of p21(Cip1/Waf1) in phorbol 12-myristate-13-acetate-induced apoptotic cells. *Mol Cell Biol* 2002; **22**: 7581–7592.
- Parent N, Sane AT, Droin N, Bertrand R. Procasase-2S inhibits procaspase-3 processing and activation, preventing ROCK-1-mediated apoptotic blebbing and body formation in human B lymphoma Namalwa cells. *Apoptosis* 2005; **10**: 313–322.
- Morelli A, Chiozzi P, Chiesa A, Ferrari D, Sanz JM, Falzoni S *et al*. Extracellular ATP causes ROCK I-dependent bleb formation in P2 \times 7-transfected HEK293 cells. *Mol Biol Cell* 2003; **14**: 2655–2664.
- Coudray AM, Louvet C, Kornprobst M, Raymond E, Andre T, Tournigand C *et al*. Increased anticancer activity of the thymidylate synthase inhibitor BGC9331 combined with the topoisomerase I inhibitor SN-38 in human colorectal and breast cancer cells: induction of apoptosis and ROCK cleavage through caspase-3-dependent and -independent mechanisms. *Int J Oncol* 2005; **27**: 553–561.
- Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M *et al*. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J* 1996; **15**: 2208–2216.
- Papakonstanti EA, Ridley AJ, Vanhaesebroeck B. The p110delta isoform of PI 3-kinase negatively controls RhoA and PTEN. *EMBO J* 2007; **26**: 3050–3061.
- Man JH, Liang B, Gu YX, Zhou T, Li AL, Li T *et al*. Gankyrin plays an essential role in Ras-induced tumorigenesis through regulation of the RhoA/ROCK pathway in mammalian cells. *J Clin Invest* 2010; **120**: 2829–2841.
- Li G, Cheng Q, Liu L, Zhou T, Shan CY, Hu XY *et al*. Mitochondrial translocation of cofilin is required for allyl isothiocyanate-mediated cell death via ROCK1/PTEIN/PI3K signaling pathway. *Cell Commun Signal* 2013; **11**: 50.

44. Zhou T, Li G, Cao B, Liu L, Cheng Q, Kong H *et al*. Downregulation of Mcl-1 through inhibition of translation contributes to benzyl isothiocyanate-induced cell cycle arrest and apoptosis in human leukemia cells. *Cell Death Dis* 2013; 4: e515.
45. Li G, Zhou T, Liu L, Chen J, Zhao Z, Peng Y *et al*. Ezrin dephosphorylation/downregulation contributes to ursolic acid-mediated cell death in human leukemia cells. *Blood Cancer J* 2013; 3: e108.



Cell Death and Disease is an open-access journal published by *Nature Publishing Group*. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>