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MEK inhibitor CI-1040 induces apoptosis in acute myeloid leukemia cells *in vitro*

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Abstract. – OBJECTIVE: MEK1/2 (mitogen-activated protein kinase 1 and 2)/ERK1/2 (extracellular signal-regulated kinase 1 and 2) is important transducers of external signals for cell growth, survival, and apoptosis in acute myeloid leukemia cells (AML). In this study, we analyzed the effect of MEK inhibitor CI-1040 on the survival of AML cells.

MATERIALS AND METHODS: Using ELISA and MTT we studied the cytotoxic effects of CI-1040 on AML U-937 cells. We studied the changes induced by CI-1040 on PUMA and p53 expression in U-937 cells by Western blotting assay. Moreover, we analyzed the cytotoxic effect of CI-1040 in U-937 cells with deleted PUMA, wt-p53 by wt-p53 siRNA and PUMA siRNA transfection.

RESULTS: CI-1040 induced apoptosis and inhibited proliferation in U-937 cells in a dose and time-dependent manner. CI-1040 induced a significant increase in PUMA mRNA and protein levels. Importantly, we show that knockdown of PUMA by PUMA siRNA transfection inhibited CI-1040-induced apoptosis and proliferation inhibition in U-937 cells. Moreover, CI-1040 induced apoptosis and proliferation inhibition was irrespective of wt-P53 status.

CONCLUSIONS: These results demonstrate that CI-1040 induce apoptosis of U-937 cells and might be a new therapeutic option for the treatment of AML.

Key Words:
Acute myeloid leukemia, Mitogen-activated protein kinase, Extracellular signal-regulated kinase, Apoptosis, PUMA.

Introduction

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation, and apoptosis[1]. The extracellular signal-regulated kinase (ERK) is a subfamily member of MAPKs, which is activated by an upstream kinase called MAPK/ERK kinase (MEK) in response to growth stimuli. The ERK pathway mediates a number of cellular fates including growth, proliferation, and survival[2]. ERK1/2, which is one of the members of the RAS/MEK/ERK pathway, is known to contribute to tumorigenesis in many cancers[3-8], including leukemia cells [9-12].

The active phosphorylated form of ERK1/2 (p-ERK) translocates to the nucleus and phosphorylates transcription factors that control cell proliferation and differentiation[13]. Past studies have shown that constitutive activation of MEK/ERK pathway is a common cause for the resistance of cells to death receptor-mediated or mitochondria-mediated apoptosis[7]. Recently, blocking MAPK via small-molecule MEK inhibitors has come to be the exciting approach in cancer therapeutics.

The importance of ERK1/2 in survival signaling in cancer will be determined by the remodelling of survival signaling in cancer cells. ERK1/2 signaling can also promote cell survival by other mechanisms. For example, ERK1/2 activation can inhibit apoptotic signaling by Fas, TNF, and TRAIL receptors[14], suggesting that ERK1/2 inhibition might be effective in combination with recombinant ligands or agonistic mAbs for these receptors in tumours with de-regulated ERK1/2 signaling[14].

In this study we examined the effect of CI-1040 on apoptosis of AML U-937 cells and investigated its mechanisms. It was demonstrated that pharmacologic MEK inhibitor CI-1040 promoted PUMA expression, playing a central role in apoptosis induced by CI-1040.

Materials and Methods

Cell Culturel

The U-937 leukemic cells were maintained in Roswell Park Memorial Institute (RPMI)-1640

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culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) (Sigma-Aldrich), 2 mM of glutamine, and 1% sodium pyruvate at 37°C in 95% humidified atmosphere containing 5% CO_2. The cells were sub-cultured with an initial concentration of 5×10^4 cells/ml and used in the logarithmic growth phase in whole.

Reagents

CI-1040 was purchased from Selleckchem (Houston, TX, USA). Mouse monoclonal antibodies against pERK1/2, wt-p53, PUMA and rabbit polyclonal antibodies against ERK1/2 were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Inhibitor Treatments

The MEK inhibitor CI-1040 was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions and used in cell culture at final concentration 50 μg/ml. U-937 cells were treated with 0, 1, 5 and 20 μm, CI-1040 for 24 hrs. Cells were lysed and whole extracts were analyzed by Western blot.

siRNA Transfection

Small interfering RNA (siRNA) targeting the wt-p53 and PUMA were purchased from Santa Cruz Biotechnology (Shanghai, China). U-937 cells were plated into 35-mm 6-well trays and allowed to adhere for 24 hours. In all, 8 mL siPORT Amine transfection reagent (Ambion, Inc., Austin, TX, USA) per well were added to the serum-free medium for a final complexing volume of 200 mL, vortexed, and incubated at room temperature for 15 minutes. The transfection reagent/siRNA complexes were added to the wells containing 800 mL media with 10% fetal bovine serum and incubated in normal cell culture conditions for 6 hours, after which 1 mL Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS was added.

Western Blotting

Cell lysates were prepared by homogenization in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH8.0), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40 and 0.1% SDS, supplemented with 1 mmol/L phenylmethylsulfonyl fluoride]. The homogenates were incubated on ice for 30 min, and were then centrifuged at 15,000 x g for 15 min at 4°C, and the pellets were discarded. The protein concentration of the supernatants was measured using the detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA). Aliquots of lysates equivalent to 20 μg of protein were subjected to SDS-PAGE, followed by transfer to Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat powdered milk before incubation with the primary antibody. Then, the blots were washed and incubated with the appropriate secondary antibody coupled to horseradish peroxidase. The antigen-antibody complexes were detected using the enhanced chemiluminescence plus reagent (Amersham Biosciences). The following primary antibodies were used: anti-pERK1/2, p-ERK1/2, wt-p53, PUMA antibody and anti-actin antibody.

Quantification of Apoptosis by ELISA

The Cell Apoptosis ELISA Detection Kit (Roche, Beijing, China) was used to detect apoptosis in U-937 cells with different treatments according to the manufacturer's protocol. Briefly, U-937 cells were treated with 5 and 20 μM CI-1040 for 24-72 hours. U-937 cells were pretreated for 24 h with 5 and 20 μM CI-1040, then transfected with wt-p53 siRNA or PUMA siRNA for 48 hrs. After treatment, the cytoplasmic histone-DNA fragments from U-937 cells with different treatments were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone-DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined using

ULTRA Multifunctional Microplate Reader (TECAN, Mannedorf, Switzerland) at 405 nm.

Cell Growth Inhibition by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay

U-937 cells were seeded at a density of 3 ×103 cells per well in 96-well microtiter culture plates. After overnight incubation, the medium was removed and replaced with fresh medium containing different concentrations of CI-1040 (5 and 20 μM) for 24-72 hours of incubation. U-937 cells were pretreated for 24 hrs with 5 and 20 uM CI-1040, then transfected with wt-p53 siRNA or PUMA siRNA for 48 hrs. Then 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) were added to each well and incubated further for 2 hours. Upon termination, the supernatant was aspirated

and the MTT formazan formed by metabolically viable cells was dissolved in 100 μL of isopropanol. The plates were mixed for 30 minutes on a gyratory shaker, and absorbance was measured at 595 nm using a plate reader.

Statistical Analysis

Statistics were conducted by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean±SD. One-way ANOVA was used for comparisons between groups; the *t*-test was used to compare the mean values of the samples from different groups. $p<0.05$ value was considered statistically significant.

Results

CI-1040 Inhibits ERK1/2 Phosphorylation of U-937 Cells

To assess the effect of CI-1040 on ERK1/2 activity in U-937 cells, we examined the phosphorylation status of ERK1/2 or ERK1/2 substrates which are used to assess the activation status of ERK1/2. After 24 h of *in vitro* incubation of the U-937 cells with CI-1040 at concentrations of 0, 1,5 and 20 μM, we observed a dose-dependent down regulation of pERK1/2 (Figure1A). When the U-937 cells were treated with 100 μM for 6,12,18 and 24 h we observed a time-dependent down regulation of pERK1/2 (Figure1B).

CI-1040 I upregulates PUMA, but not wt-p53 in U-937 leukemic cells

U-937 cells were treated with CI-1040 at concentrations of 0, 1,5 and 20 μM for 24 hrs. We observed a dose-dependent PUMA upregulation in the U-937 cells (Figure 2A). When the U-937 cells were treated with 100 μM for 6, 12, 18 and 24 hrs, we observed a time-dependent PUMA upregulation in the U-937 cells (Figure 2B). No matter U-937 cells were treated with CI-1040 at concentrations of 0, 1, 5 and 20 μM for 24 hrs or treated with100 μM for 6, 12, 18 and 24 h, wt-p53 was without affecting in the U-937 cells (Figure 2A-B).

CI-1040 Inhibits Proliferation in U-937 Leukemic Cells

U-937 cells were treated with CI-1040 at concentrations of 5 and 20 μM for 24 hs-72 hs. After 24 h incubation, we detected 7.6±4.5 and 16.3±6.4 inhibition of U-937 cell proliferation at concentrations of 5 and 20 μM. After 48 h incubation, we detected 27±8.3 and 43.5±11.4 inhibition at concentrations of 5 and 20 μM. After 72 h incubation, we detected 36.2±10.4 and 72.5±14.1 inhibition at concentrations of 5 and 20 μM (Figure 3).

CI-1040 Induces Apoptosis in U-937 Leukemic Cells

U-937 cells were treated with CI-1040 at concentrations of 5 and 20 μM for 24 hs-72 hrs. Treatment of U-937 cells with 5 μM and 20 μM CI-1040 for 24 h induced few apoptotic cells (Figure 4). Apoptosis appeared later with significant detection at 48 h with 5 μM and 20 μM CI-1040. At this point, 5 μM showed 15.4% apoptosis with respect to the total cell count, and 20 μM showed 37.5% apoptosis with respect to the total cell count (Figure 4). After 72 h incubation, 5 μM showed 28.6% of apoptotic cells, and 20 μM showed 61.7% of apoptotic cells respectively (Figure 4).

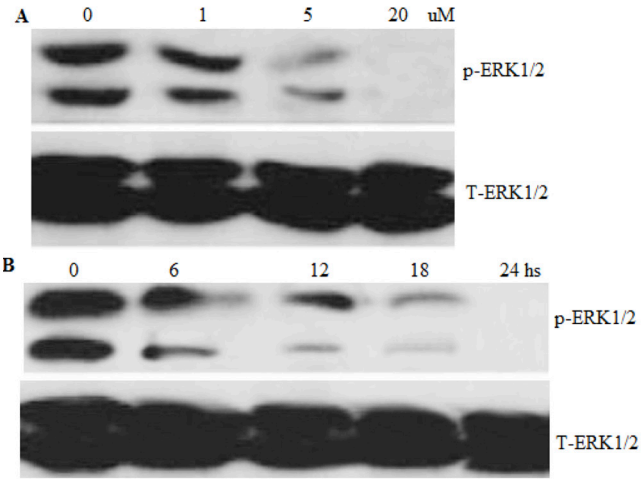

Figure 1. CI-1040 treatment decreased phosphorylated ERK1/2 in U-937 cells. *A,* Cells were exposed to **CI-1040** (1-20 μM) for 24 h. After treatment, the cell extracts were subjected to Western blot analysis. ***B,*** Cells were exposed to CI-1040 (20 μM) for 6, 12, 18 and 24 h. After treatment, the cell extracts were subjected to Western blot analysis. Dose-dependent and time-dependent downmodulation of the phosphorylation status of ERK1/2 was showed in U-937 cells.

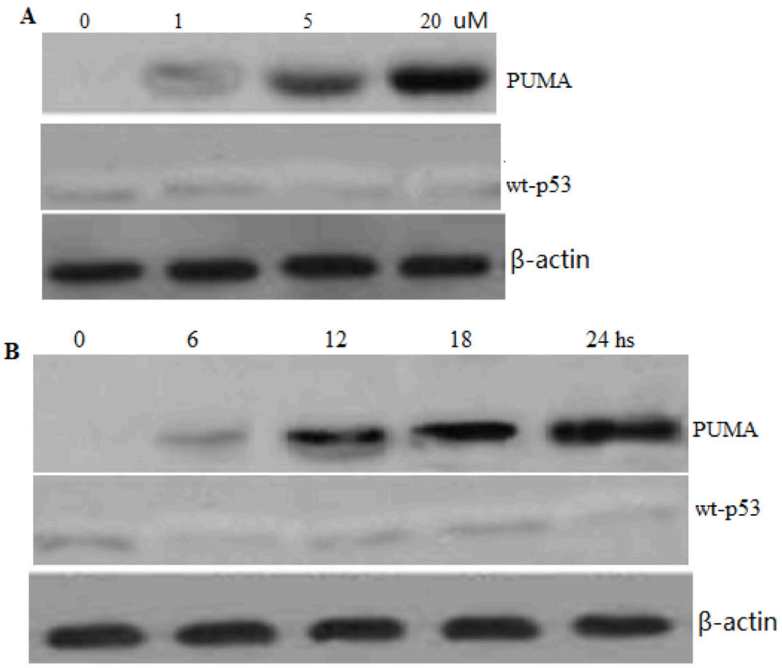

Figure 2. CI-1040 treatment induced PUMA expression in U-937 cells. ***A,*** Cells were exposed to CI-1040 (1-20 μM) for 24 h. After treatment, the cell extracts were subjected to Western blot analysis. ***B,*** Cells were exposed to CI-1040 (20 μM) for 6, 12, 18 and 24 h. After treatment, the cell extracts were subjected to Western blot analysis. Dose-dependent and time-dependent upregulation of PUMA was showed in U-937 cells. wt-p53 was without affecting in the U-937 cells by CI-1040 treatment.

CI-1040 Inhibits Proliferation and Induces Apoptosis by PUMA Upregulation in U-937 Leukemic Cells

Pretreatment with 20 μM CI-1040, followed by PUMA siRNA transfection effectively attenuated the phosphorylation of ERK1/2 and PUMA expression in U-937 cells (Figure 5A). Knockdown of PUMA by PUMA siRNA transfection inhibited CI-1040-induced proliferation inhibition (Figure 5B). Furthermore, CI-1040-induced apoptosis was also decreased with PUMA siRNA transfection (Figure 5C). Knockdown of wt-p53 by wt-p53 siRNA did not affect CI-1040-induced proliferation and apoptosis (data not shown). These results suggested that activation of ERK1/2 pathway may represent a major intracellular mechanism involved in resistance to PUMA-induced apoptosis of U-937cells. ERK1/2 signal inhibition could be free of PUMA, which induces cell apoptosis.

Discussion

AML is a complex disease with a diverse genetic landscape. The field is rapidly expanding with increased understanding of the biology as well as potential gene targets. PRAME (Prefer-

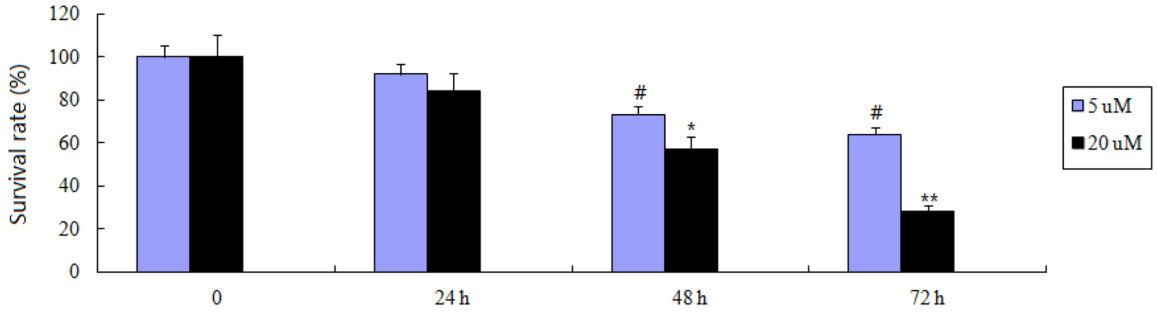

Figure 3. CI-1040 treatment induced apoptosis in U-937 cells. Cells were exposed to CI-1040 (1,5 20 μM) for 24 h or exposed to CI-1040 (20 μM) for 6, 12, 18 and 24 h. After treatment, the cell survival was detected by MTT assay. *Vs.* control, $^{*}p<0.05$, $^{\#}p<0.05$, $^{**}p<0.01$.

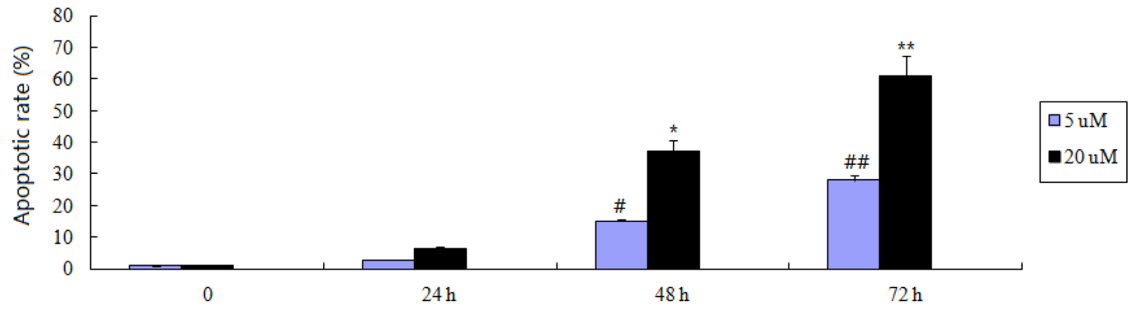

Figure 4. CI-1040 treatment induced apoptosis in U-937 cells. Cells were exposed to CI-1040 (1, 5 20 μM) for 24 h or exposed to CI-1040 (20 μM) for 6, 12, 18 and 24 h. After treatment, the cell apoptosis was detected by ELISA assay. *Vs.* control, $^{*}p<0.05$, $^{\#}p<0.01$, $^{**}p<0.001$.

entially Expressed Antigen in Melanoma) is a tumor-associated antigen recognized by immunocytes, and it induces cytotoxic T cell-mediated responses in melanoma. In leukemia cells, PRAME could be a target for promoting *in vitro* leukemia cells death[34], as well as sensitizing K562 cells to chemotherapy[35]. In addition, targeting livin gene was also an effective method to induce apopto-

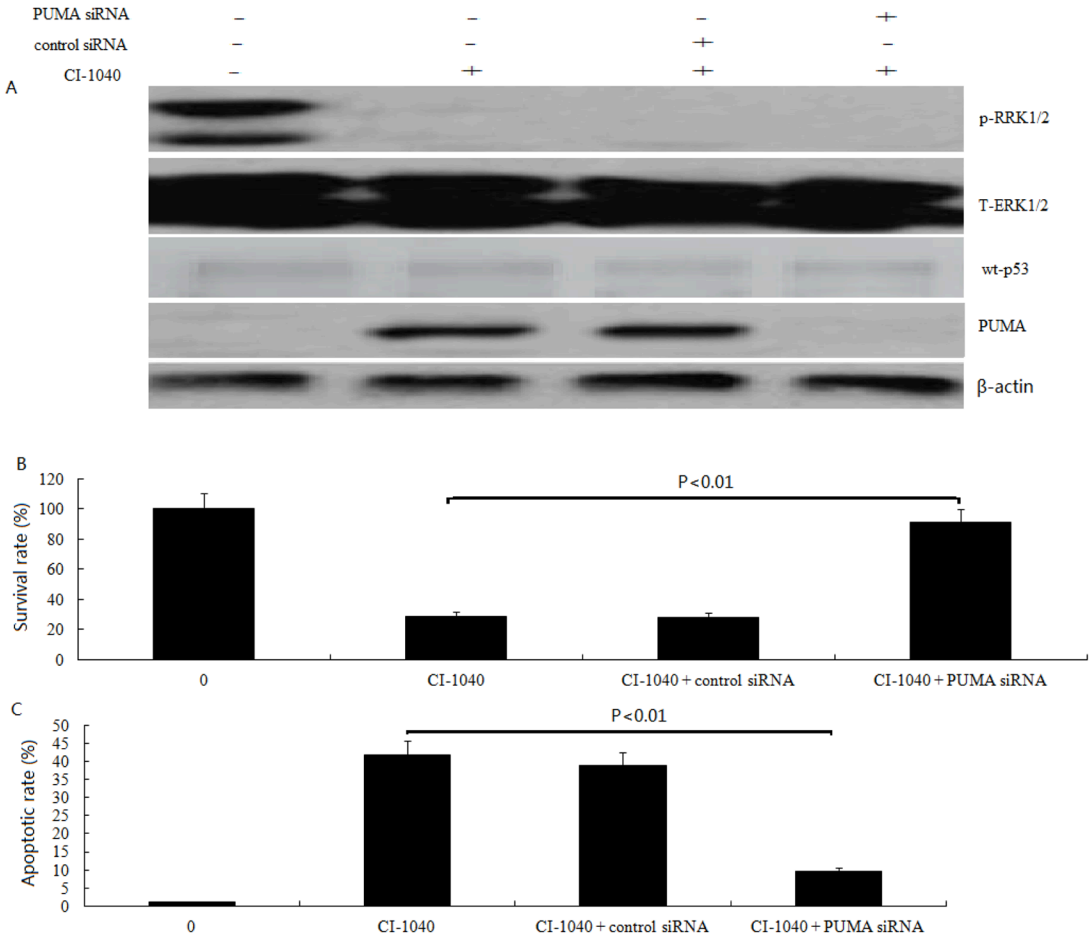

Figure 5. CI-1040 treatment induced apoptosis and inhibited proliferation by PUMA upregulation in U-937 leukemic cells. Cells were Pretreated with 20 μM CI-1040 for 24 hrs, followed by PUMA siRNA or control siRNA transfection for 48 hrs. After treatment, the cell extracts were subjected to Western blot analysis (A), the cell survival was detected by MTT assay and the cell apoptosis was detected by ELISA assay.

sis and enhance the sensitivity of leukemia cells to VP-16[36]. Targeted therapy with small molecule inhibitors (SMIs) represents a new therapeutic intervention that has been successful for the treatment of multiple tumors, such as gastrointestinal stromal tumors, chronic myelogenous leukemia (CML)[37]. However, unlike in CML and APL, the identification of potential targets in AML has been limited by the heterogeneous clonal architecture of non-M3 AML and by the contribution of numerous driver mutations in its onset and progression.

In this study, we investigated the effect of MEK inhibitor CI-1040 on the viability and apoptosis of U-937 cells. CI-1040 induced apoptosis in U-937 cells. These results are in agreement with a previous report describing that introduction of MEK1/2/ERK1/2 activity increases the viability of AML cells[38]. It was also in agreement with a previous report describing that the primary effect of ERK downmodulation was a cell cycle arrest followed by the apoptosis of a significant percentage of the leukemic blasts[39]. Time-course experiments also shown that CI-1040 induces a low decrease of U-937 cell proliferation and low levels of apoptotic death during the first 24 h of *in vitro* incubation. After 48 and 72 h of incubation, a strong decrease of blast cell survival and increase of apoptosis is detectable.

Recently, it has found that inhibition of RAF/MEK/ERK signaling could only induce BH-3 only protein PUMA expression, but not other BH-3 only protein Bim, and induce intrinsic apoptosis in melanoma cells[40]. Rambal et al[41] have found that PUMA upregulation by CI-1040 may not be essential for apoptosis induced by CI-1040 in acute lymphoblastic leukemia cells, however, Bim was essential for apoptosis induced by CI-1040. Pellicano et al[42] have reported that CI-1040 increased Annexin-V levels, caspase-3, -8 and -9 activation and potentiated mitochondrial damage, which is associated with decreased levels of anti-apoptotic BCL-2 family protein MCL-1. It is clear that activation of PUMA proteins by apoptotic stimuli initiates mitochondria-dependent cell death pathway. It cause cytochrome c release by activating BAX and/or BAK, and the anti-apoptotic BCL-2 family of proteins prevents this process.

In the present study, we found that MEK inhibitor CI-1040 inhibits ERK1/2 and induce PUMA upregulation in a time and dose-dependant way. Upregulation of PUMA is correlated with apoptosis induction and proliferation inhibition in U-937 cells. However, induction of *PUMA* by CI-1040 was blocked in U-937 cells by targeting PUMA. Furthermore, apoptosis induction and proliferation inhibition by CI-1040 was decreased with PUMA downregulation, suggesting PUMA is necessary for CI-1040-induced apoptosis in U-937 cells.

Chen et al[43] have reported that regorafenib, a multikinase inhibitor targeting the Ras/Raf/MEK/ERK pathway, induces PUMA in colorectal cancer cells irrespective of p53 status through the NF-κB pathway following ERK inhibition and glycogen synthase kinase 3β activation. Jou et al[44] have found that γ-bisabolene-induced apoptosis in oral squamous cell carcinoma cells by ERK1/2-p53-PUMA pathway. In the present study, we found that CI-1040 induced PUMA upregulation without affecting p53. Knockdown of p53 did not affect CI-1040-induced apoptosis, suggesting CI-1040-induced apoptosis was irrespective of p53 status.

Conclusions

Our studies showed the MEK inhibitor CI-1040 inhibits ERK1/2 activity and promotes PUMA- mediated apoptosis. The PUMA-induced proapoptotic function was independent of wt-p53. The study encourages the use of MEK inhibitor in clinical trials for the treatment of leukemia, either as single agents or in association with other compounds.

Conflicts of interest

The authors declare no conflicts of interest.

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