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Obatoclax Induces G1/G0-Phase Arrest via p38/p21$^{waf1/Cip1}$ Signaling Pathway in Human Esophageal Cancer Cells

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ABSTRACT

Pan-Bcl-2 family inhibitor obatoclax has been demonstrated to be effective against various cancers, of which the mechanism of action is not fully understood. In this study, we demonstrate that obatoclax suppressed esophageal cancer cell viability with concomitant G1/G0-phase cell cycle arrest. At the tested concentrations (1/2 IC_{50} and IC_{50}), obatoclax neither induced PARP cleavage nor increased the Annexin V-positive population, suggesting G1/G0-phase arrest rather than apoptosis accounts for most of the reduction of cell viability produced by obatoclax. Double knockdown of Bak and Bax by small interference RNA failed to block obatoclax-induced G1/G0-phase arrest, implying its role in cell cycle progression is Bak/Bax-independent. The cell cycle arresting effect of obatoclax was associated with up-regulation of p21$^{waf1/Cip1}$. Knockdown of p21$^{waf1/Cip1}$ significantly attenuated obatoclax-induced G1/G0-phase arrest. Although obatoclax stimulated phosphorylation of Erk, p38, and JNK, pharmacological inhibition of p38 but not Erk or JNK blocked obatoclax-induced G1/G0-phase arrest. Moreover, knockdown of p38 abolished the cell cycle arresting effect of obatoclax. In consistent with this finding, inhibition of p38 blocked obatoclax-induced p21$^{waf1/Cip1}$ expression while inhibition of Erk or JNK failed to exert similar effect. To conclude, these findings suggest that obatoclax induced cell cycle arrest via p38/p21$^{waf1/Cip1}$ signaling pathway. This study may shed a new light on the anti-cancer activity of obatoclax in relation to cell cycle arrest. J. Cell. Biochem. 115: 1624–1635, 2014.

KEY WORDS: ESOPHAGEAL CANCER; OBATOCLAX; p38; p21$^{waf1/Cip1}$; APOPTOSIS; CELL CYCLE

Esophageal cancer is the ninth most common malignancy and sixth leading cause of cancer-related death in the world [Siersema, 2008]. Mortality rates are close to incidence rates, due to the relatively late stage of diagnosis and the poor efficacy of treatment [Jemal et al., 2011]. Better knowledge of the signaling pathways connected to carcinogenesis, tumor growth and metastasis can probably provide potential novel molecular-targeted therapy in esophageal cancer treatment.

Evasion from apoptosis is one of the hallmarks of cancer and contributes to tumor progression, resistance to chemotherapy, and treatment failure [Hanahan and Weinberg, 2011]. Many different signals for apoptosis converge on permeabilizaiton of the outer mitochondrial membrane, which is orchestrated by the Bcl-2 family proteins. The Bcl-2 family consists of antiapoptotic proteins (e.g., Bcl-2, Mcl-1, Bcl-X_L) and two fractions of proapoptotic proteins that include multidomain Bak and Bax and the BH3-only proteins (e.g., Bad, Bim, Bid, Puma, Noxa). BH3-only proteins cause cytochrome *c* release and the activation of apoptosis by activating Bax and/or Bak, while the anti-apoptotic Bcl-2 family of proteins prevents this process [Shamas-Din et al., 2011; Davids and Letai, 2012]. Remarkably, aberrant expression of Bcl-2 family members such as downregulation of Bax and upregulation of Bcl-2 and Bcl-X_L has been found in esophageal cancer [Azmi et al., 2000; van der Woude et al., 2002; Kang et al., 2007]. Owing to their important function in regulating cell death, pharmacological inhibition of antiapoptotic Bcl-2 proteins represents an attractive strategy for therapies in esophageal cancer.

Abbreviations: BH3, Bcl-2 homology 3; CDK, cyclin-dependent kinase; Erk, extracellular signal-regulated kinase; PBS, phosphate buffered saline; PARP, poly (ADP-ribose) polymerase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; siRNA, small interference RNA.

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Recently, a new series of small molecules that mimetic BH3-only proteins have been developed, constituting a new class of potentially useful anticancer drugs. Among the BH3-mimetics, obatoclax (GX15-070) is an indole bipyrrole compound that can inhibit all known antiapoptotic Bcl-2 family members. This agent is proposed to operate like a BH3 mimetic, competitively inhibiting the binding of proapoptotic proteins to the hydrophobic groove of antiapoptotic proteins [Joudeh and Claxton, 2012]. Although obatoclax has been under investigation in phase I and II clinical trials for the treatment of hematological malignancies and solid tumors [Parikh et al., 2010; Paik et al., 2011; Oki et al., 2012; Coard and Shimmer, 2013], the molecular mechanisms of its anti-tumor activity have still not been fully resolved. Obatoclax was shown to induce apoptosis at concentrations that resulted in disruption of Bak from Mcl-1 and cytochrome c release [Nguyen et al., 2007; Pérez-Galán et al., 2007]. However, obatoclax was also cytotoxic in cells deficient for the apoptosis effectors Bax and Bak, suggesting the existence of additional target mechanisms [Konopleva et al., 2008; Vogler et al., 2009; Bonapace et al., 2010].

Many of the compounds under investigation as anti-tumor agents act at multiple steps in the cell cycle [Stewart et al., 2003], however, the relationship between cell cycle progression and inhibitory effect of obatoclax on cell viability remains obscure. While obatoclax was reported to induce an S-phase arrest in acute leukemia cells [Konopleva et al., 2008; Urtishak et al., 2013], the precise mechanisms have not been explored. In this study, we sought to investigate the effects of obatoclax on cell viability in relation to cell cycle progression in esophageal cancer cells. Moreover, we explored the mechanisms underlying the action of obatoclax on cell cycle progression. We anticipate that this study will bring new insight into the anti-cancer mechanism of obatoclax.

MATERIALS AND METHODS

REAGENTS AND ANTIBIOTICS

The Bcl-2 inhibitor obatoclax was purchased from Selleck Chemicals (Houston, TX). U0126, SB203580, and JNK inhibitor II were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies for $p21^{waf1/Cip1}$, CDK2, Erk, phospho-Erk, p38, phospho-p38, JNK, phospho-JNK, PARP, and β-actin were purchased from Cell Signaling Technology (Beverley, MA). Other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Control siRNA, Bak siRNA, Bax siRNA, and HiPerFect Transfection Reagent were from QIAGEN GmbH (Hilden, Germany), while $p21^{waf1/Cip1}$ siRNA and p38 siRNA were from Sigma–Aldrich.

Cell culture and cell viability assay. The human esophageal squamous cell carcinoma cell line HKESC-1 was kindly provided by Prof. G. Srivastava (Department of Pathology, The University of Hong Kong, Hong Kong, China) [Hu et al., 2000]. Other two esophageal squamous cell carcinoma cell lines, EC109 and CaES-17, were obtained from the Cancer Institute Chinese Academy of Medical Sciences (Beijing, China) and China Center for Type Culture Collection (Wuhan, China), respectively. HKESC-1 cells were maintained in MEM medium (Corning Cellgro, Manassas, VA) while EC109 and CaES-17 cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA), both supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cell viability was determined by CellTiter-Glo Luminescent Cell Viability Assay according to manufacturer's instructions (Promega, Madison, WI).

Western blot analysis. Cells were harvested in radioimmunoprecipitation buffer containing proteinase and phosphatase inhibitors as previously described [Yu et al., 2008]. Equal amounts of protein were resolved by SDS-PAGE, and transferred to PVDF membranes (Roche, Indianapolis, IN). The membranes were probed with primary antibodies overnight at 4°C and incubated for 1 h with secondary peroxidase-conjugated antibodies. Chemiluminescent signals were then developed with Lumiglo reagent (Cell Signaling Technology) and exposed to X-ray film (Fujifilm Europe GmbH, Dusseldorf, Germany).

Cell cycle analysis. Cells were fixed with ice-cold 70% ethanol in phosphate buffered saline (PBS) followed by incubation with 50 μg/ml propidium iodide and 0.5 μg/ml RNase A at 37 °C for 0.5 h and analyzed by BD FACSCanto™ II flow cytometry (BD Biosciences, CA). The resultant DNA histograms were generated and analyzed using FlowJo 7.6 software (Treestar, Inc., Ashland, OR).

Annexin V labeling. After drug treatment, adherent cells were detached from culture dishes by treating with trypsin (Invitrogen) for 3–5 min and combined with floating cells. Apoptosis was measured using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences). The extent of apoptosis was quantified as percentage of Annexin V-positive cells.

RNA Interference. The expression of Bak, Bax, $p21^{waf1/Cip1}$, and p38 was lowered using predesigned target-specific siRNA oligonucleotides, a method employed in our previous study [Yu et al., 2009]. Cells were seeded in 6-well plate. At 50–80% confluence, 150 ng of gene-specific or control siRNA was transfected into cells using 12 μl HiPerFect Transfection Reagent.

Statistical analysis. Results were representative of multiple experiments and expressed as means ± SEM. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Turkey's *t*-test. *P* values less than 0.05 were considered statistically significant. IC_{50} of obatoclax was calculated by Prism 5.

RESULTS

OBATOCLAX REDUCED CELL VIABILITY OF HUMAN ESOPHAGEAL CARCINOMA CELLS

The effects of obatoclax on cell viability was investigated in three human esophageal carcinoma cell lines including EC109, CaES-17, and HKESC-1 by determination of the number of viable cells in culture based on quantization of the ATP present, which signals the presence of metabolically active cells. The IC_{50} values of obatoclax in different cell lines were calculated based on the data collected from three times respective assays. As shown in Fig. 1, obatoclax reduced cell viability in EC109, CaES-17, and HKESC-1 cells, with IC_{50} values of 0.9 ± 0.1, 0.3 ± 0.1, and 0.9 ± 0.2 μM, respectively.

OBATOCLAX INDUCED G1/G0-PHASE ARREST

Results from flow cytometry-based cell cycle analysis showed that obatoclax at indicated concentrations (1/4 IC_{50}, 1/2 IC_{50}, and IC_{50} for each cell line) induced a substantial accumulation of cells at the G1/

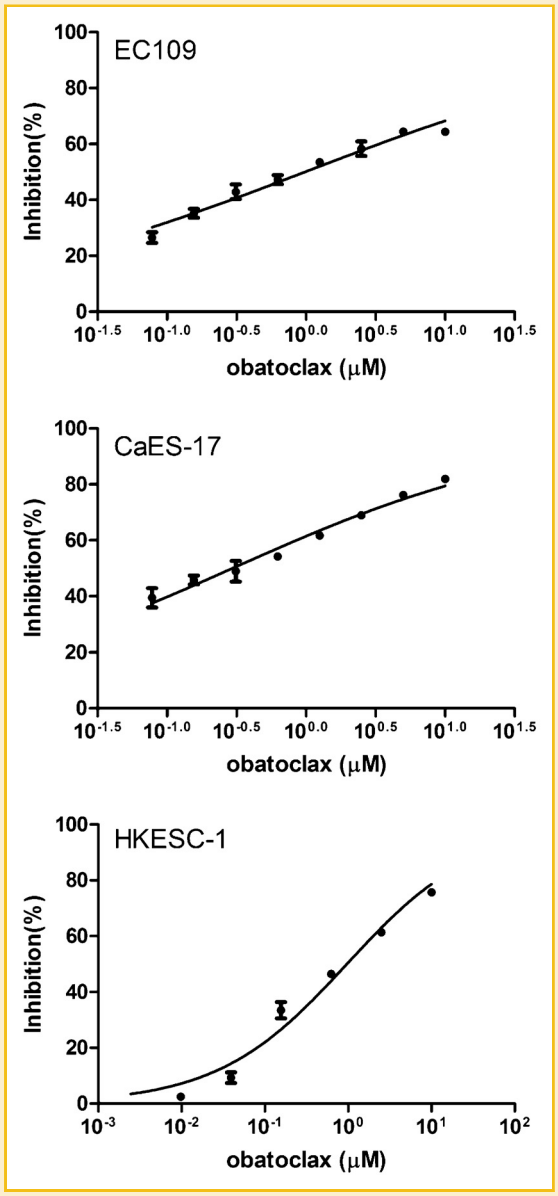

Fig. 1. Obatoclax reduces cell viability of esophageal cancer cells. Cells (EC109, CaES-17, and HKESC-1) were exposed to increasing concentrations of obatoclax for 24 h. Cell viability was determined by CellTiter-Glo Luminescent Cell Viability Assay.

G0-phase in tested three cell lines. A reciprocal reduction of proportion of cells in S-phase and G2/M phase was also observed in obatoclax-treated cells. Statistical analysis of G1/G0-phase arrest induced by obatoclax was shown (Fig. 2).

OBATOCLAX FAILED TO INDUCE APOPTOSIS

To determine if apoptosis contributes to the reduced cell viability induced by obatoclax, we tested the ability of obatoclax to induce apoptosis using Annexin V labeling. In this respect, obatoclax at indicated concentrations (1/2 IC_{50} and IC_{50} for each cell line) did not increase apoptosis in EC109, CaES-17, and HKESC-1 cells, with no significant difference compared with the control group (Fig. 3A). Moreover, obatoclax failed to induce PARP cleavage in the tested three cell lines, which is indicative of apoptosis. In contrast, cisplatin, which was used as a positive control for apoptosis induction, obviously induced PARP cleavage in the tested three cell lines (Fig. 3B). These findings suggest that G1/G0-phase arrest rather than apoptosis accounts for most of the reduction of cell viability induced by obatoclax.

DOUBLE KNOCKDOWN OF Bak AND Bax FAILED TO BLOCK OBATOCLAX-INDUCED G1/G0-PHASE ARREST

Obatoclax has been reported to trigger apoptosis by inducing the formation of an active Bak/Bax complex which permeabilized the outer mitochondrial membrane to release apoptogenic factors [Konopleva et al., 2008]. We therefore determined if the targets that mediate cell cycle arrest were different from those mediating apoptosis by downregulating both Bak and Bax in EC109, CaES-17 and HKESC-1 cells. The efficacy of Bak and Bax depletion was verified by Western blot analysis, in which the results showed that Bak siRNA and Bax siRNA respectively down-regulated Bak and Bax protein levels (Fig. 4A). Exposure to obatoclax resulted in a significant increase in the percentage of cells at the G1/G0-phase. However, double knockdown of Bak and Bax did not block obatoclax-induced G1/G0-phase arrest, suggesting other targets rather than Bak/Bax contributed to cell cycle arrest induced by obatoclax (Fig. 4B).

OBATOCLAX INCREASED $p21^{waf1/Cip1}$ PROTEIN EXPRESSION

To further characterize the mechanism of action of obatoclax, given its ability to induce G1/G0 cell cycle arrest, we investigated the effect of obatoclax on the regulatory mechanisms involved in the G1-to-S transition in EC109, CaES-17, and HKESC-1 cells. In this regard, obatoclax did not alter the expression of cyclin-dependent kinases (CDK) including CDK2, CDK4, and CDK6, whereas it substantially promoted the protein level of the CDK inhibitor $p21^{waf1/Cip1}$. Given that the phosphorylation status of $p21^{waf1/Cip1}$ has been proposed to be critical for the regulation of $p21^{waf1/Cip1}$ function [Zhou et al., 2001], the effect of obatoclax on $p21^{waf1/Cip1}$ phosphorylation was further determined. In this respect, the phosphorylation of $p21^{waf1/Cip1}$ remained quite stable (Fig. 5A). The results of the quantification analysis of the expression of phospho-$p21^{waf1/Cip1}$, CDK2, CDK4, and CDK6 were shown in Fig. S1 of Supporting information.

KNOCKDOWN OF $p21^{WAF1/CIP1}$ ATTENUATED OBATOCLAX-INDUCED G1/G0-PHASE ARREST

Given that $p21^{waf1/Cip1}$ was dramatically increased in response to obatoclax treatment in EC109, CaES-17, and HKESC-1 cells, whether $p21^{waf1/Cip1}$ was involved in obatoclax-induced G1/G0-phase arrest was further investigated by RNA interference experiments. The knockdown efficacy of $p21^{waf1/Cip1}$ was confirmed by Western blot analysis in the tested three cell lines (Fig. 5B). As showed in Fig. 5C, obatoclax significantly increased the proportation of cells at G1/G0-phase in tested three cell lines. Moreover, downregulation of $p21^{waf1/Cip1}$ significantly attenuated obatoclax-induced G1/G0-phase arrest in EC109 cells, whereas it completely blocked obatoclax-induced G1/G0-phase arrest in CaES-17 and HKESC-1 cells. These findings suggested that $p21^{waf1/Cip1}$ was implicated in the cell cycle arresting effect of obatoclax.

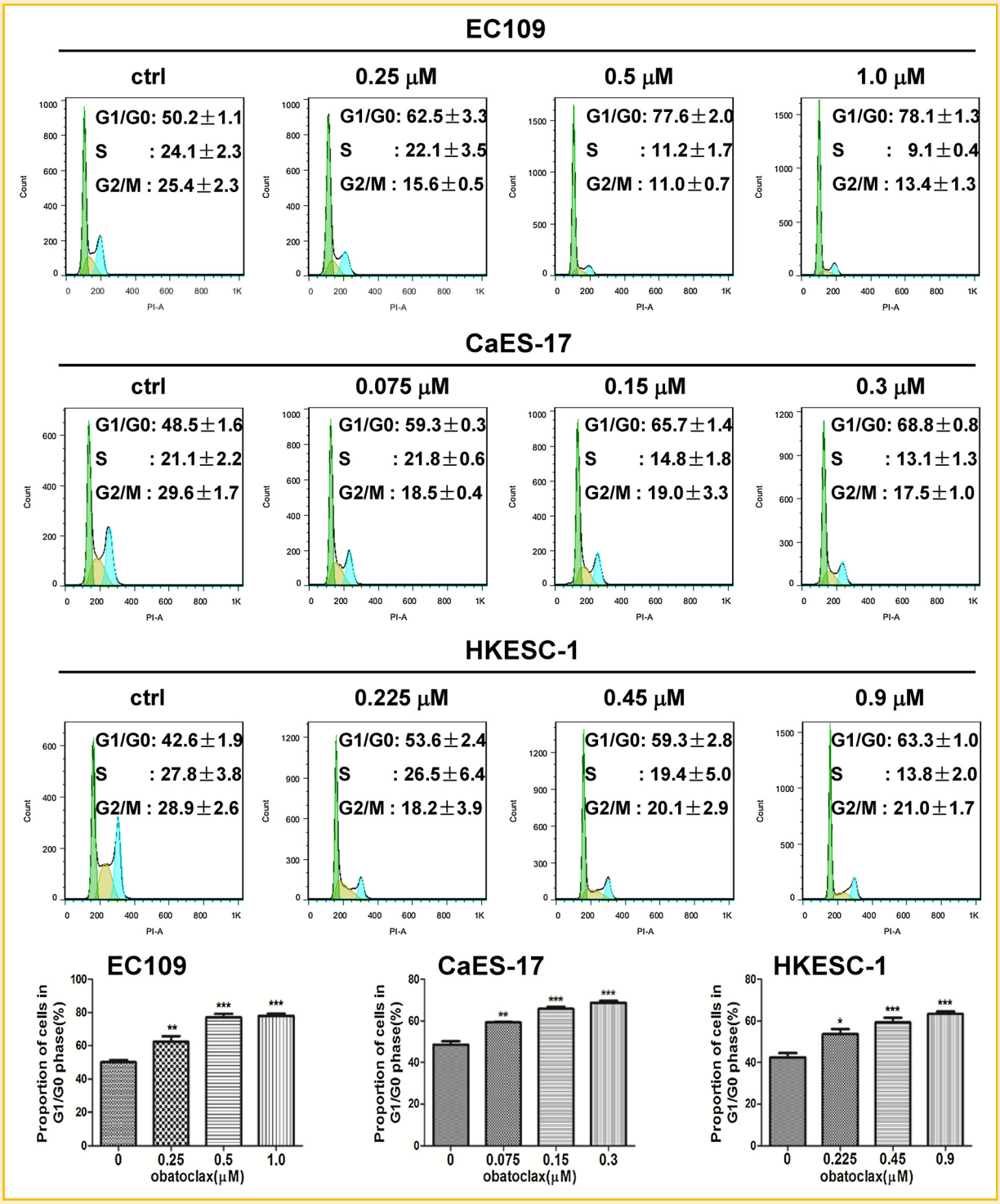

Fig. 2. Obatoclax inhibits cell cycle progression in esophageal carcinoma cells. DNA histogram shows the accumulation of G1/G0-phase cells induced by obatoclax. Cells (EC109, CaES-17, and HKESC-1) were exposed to the indicated concentrations of obatoclax for 24 h, and their DNA contents were determined by flow cytometry analysis. Data are presented as mean ± SEM from three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to the control.

THE p38 PATHWAY MEDIATED OBATOCLAX-INDUCED G1/G0-PHASE ARREST

Mitogen-activated protein kinases (MAPKs), comprising a family of serine and threonine kinases of Erk, p38, and JNK, are a family of signal transduction proteins that convert extracellular stimuli into a wide range of cellular responses including proliferation, survival, and death [Plotnikov et al., 2011]. We therefore examined the direct effects of obatoclax on the phosphorylation

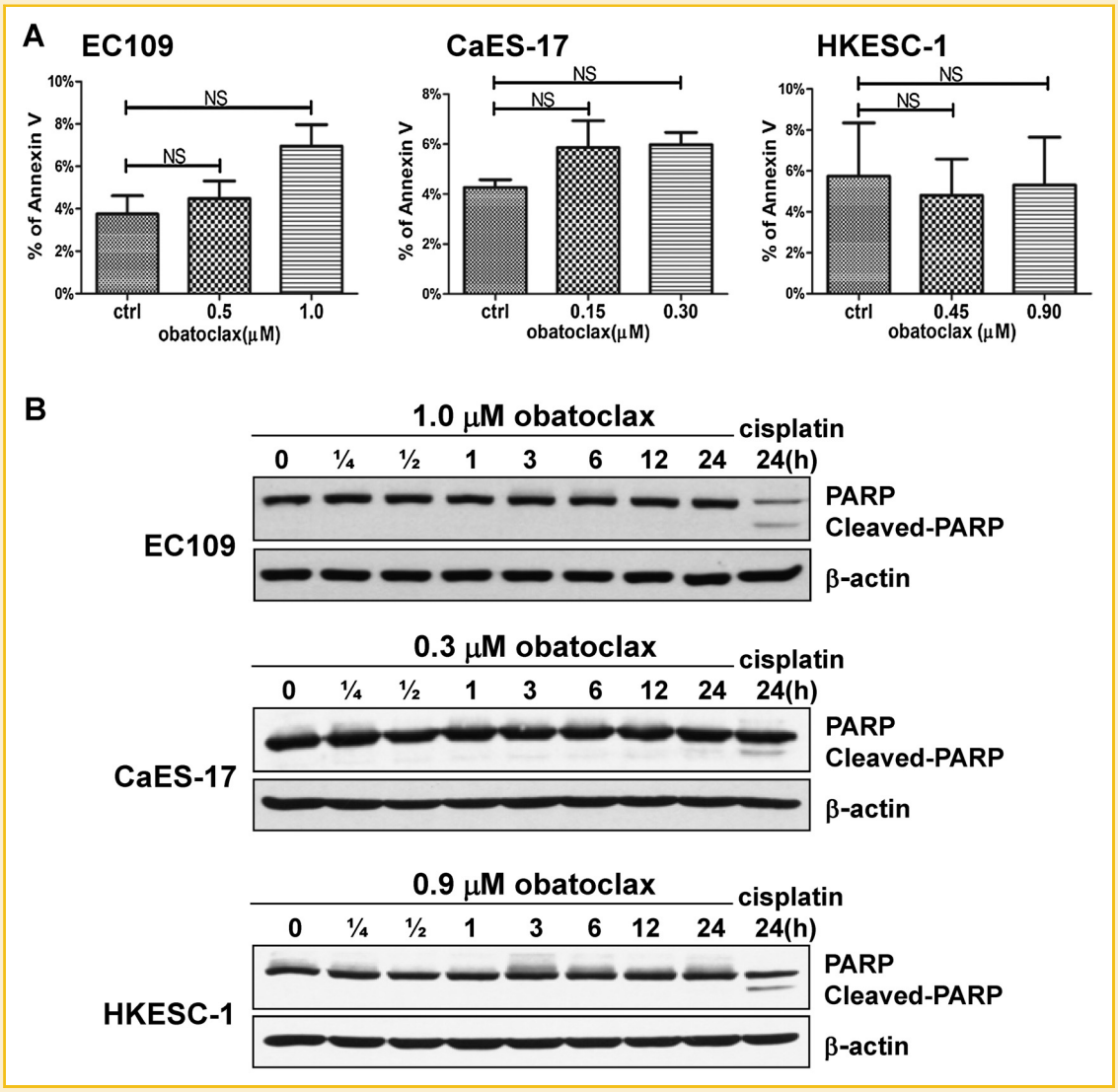

Fig. 3. Obatoclax fails to induce cellular apoptosis in esophageal cancer cells. (A) Cells (EC109, CaES-17, and HKESC-1) were exposed to the indicated concentrations of obatoclax for 24 h. Cell apoptosis was assessed by phosphatidylserine (PS) externalization and binding of Annexin V-FITC. Data are presented as mean ± SEM from three independent experiments. NS indicates no significant difference ($P > 0.05$). (B) EC109, CaES-17, and HKESC-1 cells were treated with obatoclax for the indicated time points. PARP cleavage was examined by Western blot. Cisplatin (10 μM) was used as a positive control. β-actin was used to evaluate protein loading.

of these protein kinases. As shown in Figure 6A, treatment of obatoclax obviously stimulated the phosphorylation of Erk1/2, p38, and JNK. The results of the quantification analysis of the phosphorylation of Erk1/2, p38, and JNK are shown in Fig. S2 of Supporting information. To further examine whether phosphorylation of Erk1/2, p38, or JNK was involved in modulating obatoclax-induced G1/G0-phase arrest, MAPK/Erk kinase (MEK) inhibitor U0126, p38 inhibitor SB203580, and JNK inhibitor II were used to inhibit Erk1/2, p38, and JNK phosphorylation, respectively. Exposure to obatoclax led to a significant increase in the percentage of cells at the G1/G0-phase, whereas p38 inhibitor SB203580 completely reversed G1/G0-phase arrest induced by obatoclax in tested three cell lines. However, inhibition of either Erk1/2 or JNK did not affect obatoclax-induced cell cycle arrest in EC109 and CaES-17 cells. With regard to HKESC-1 cells, inhibition of JNK showed no effect on obatoclax-induced G1/G0-phase arrest. Unexpectedly, MEK inhibitor U0126 alone significantly increased proportion of cells at G1/G0-phase ($P < 0.01$). Moreover, the combination of U0126 and obatoclax further led to a significant increase in the percentage of cells at G1/G0-phase compared with obatoclax-treated cells ($P < 0.001$) (Fig. 6B). In addition to pharmacological approach, siRNA-mediated knockdown of p38 was used. As shown in Fig. 6C, the knockdown efficacy of p38 siRNA was confirmed by Western blot. Notably, exposure to obatoclax resulted in significant increase in the percentage of cells at the G1/G0-phase, whereas downregulation of p38 completely blocked obatoclax-induced G1/G0-phase arrest in EC109, CaES-17, and HKESC-1 cells (Fig. 6D). These findings

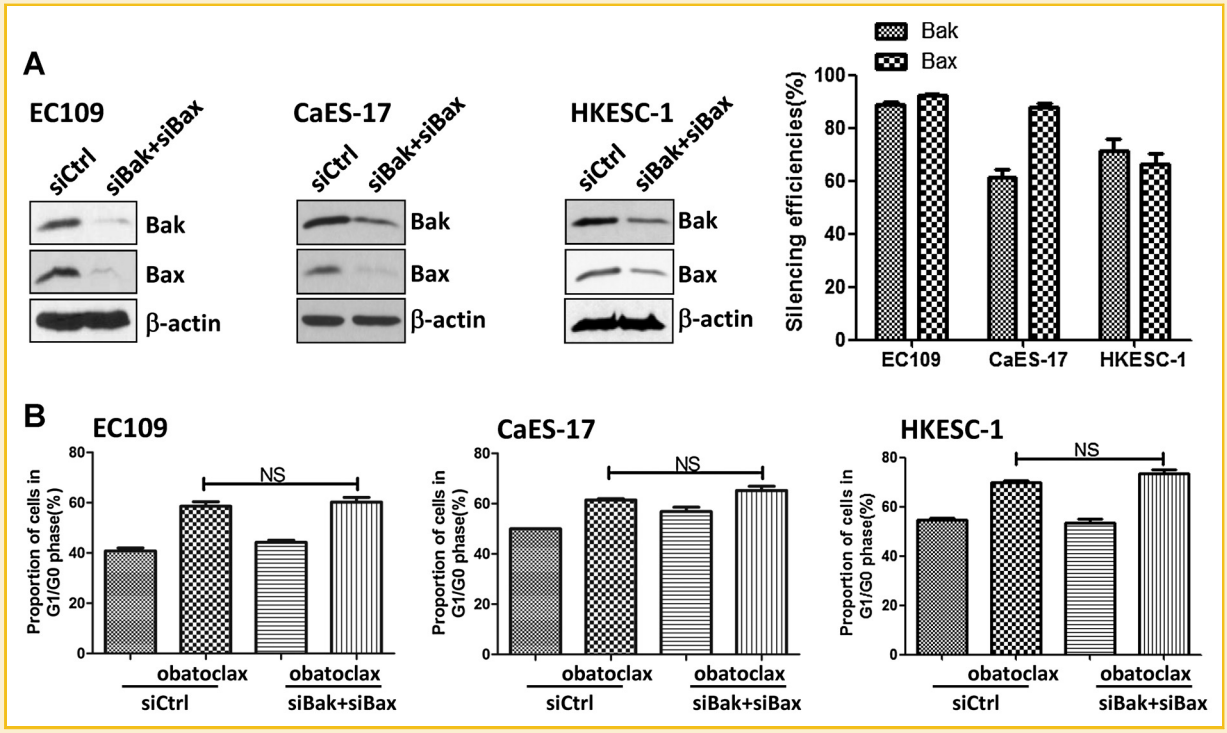

Fig. 4. Double knockdown of Bak and Bax fails to block obatoclax-induced G1/G0-phase arrest in EC109, CaES-17, and HKESC-1 cells. (A) The efficacy of Bak and Bax depletion by siRNA was verified by Western blot analysis. Non-targeting siRNA was used as control siRNA. β-actin was used to evaluate protein loading. (B) Cells were transfected with control siRNA, Bak siRNA, and Bax siRNA. At 24 h post-transfection, EC109, CaES-17, and HKESC-1 cells were treated with obatoclax (0.25, 0.075, and 0.225 μM, respectively) for 24 h, and their DNA were determined by flow cytometry analysis. Data are presented as mean ± SEM from three independent experiments. Comparisons between groups are indicated by brackets. NS indicates no significant difference ($P > 0.05$).

collectively suggested that the p38 pathway mediated the cell cycle arresting effect of obatoclax.

OBATOCLAX INDUCED G1/G0-PHASE ARREST VIA p38/p21$^{waf1/Cip1}$ SIGNALING PATHWAY

Given the fact that obatoclax promoted p21$^{waf1/Cip1}$ protein level after the activation of p38, this finding raised question as to whether p38 phosphorylation contributed to increased p21$^{waf1/Cip1}$ expression. In this regard, inhibition of p38 completely blocked obatoclax-induced p21$^{waf1/Cip1}$ expression in EC109, CaES-17, and HKESC-1 cells. There was no significant difference in p21$^{waf1/Cip1}$ expression between cells treated with obatoclax combined with SB203580 and control cells ($P > 0.05$), indicating inhibition of p38 suppressed obatoclax-induced p21$^{waf1/Cip1}$ expression to the basal level. On the contrary, inhibition of neither Erk1/2 nor JNK attenuated the effect of obatoclax on p21$^{waf1/Cip1}$ expression. These findings suggested that obatoclax induced G1/G0-phase arrest through p38/p21 $^{waf1/Cip1}$ signaling pathway. Interestingly, MEK inhibitor U0126 alone increased p21$^{waf1/Cip1}$ expression in HKESC-1 ($P < 0.05$). Moreover, the combination of obatoclax and U0126 led to a further increase in p21$^{waf1/Cip1}$ expression compared with cells treated with obatoclax alone in HKESC-1 cells ($P < 0.05$). Given the fact that U0126 increased the proportion of cells at G1/G0-phase in HKESC-1 cells (Fig. 6B), it is possible that this increase was due to elevated p21$^{waf1/Cip1}$ expression induced by U0126 (Fig. 6E).

DISCUSSION

The development of BH3 mimetics has provided a novel therapeutic method for the treatment of cancer [Letai, 2006]. BH3 mimetics such as obatoclax have been proved to be effective against various hematological malignancies, such as acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, lymphomas, and myelomas [Chauhan et al., 2007; Konopleva et al., 2008; Kuroda et al., 2008], and a variety of solid tumors including lung, prostate, colon, and cervival cancer [Oltersdorf et al., 2005]. In addition to its proapototic effect, obatoclax has been shown to exert anti-proliferative effect by blocking cell cycle progression [Konopleva et al., 2008; Urtishak et al., 2013]; however, the underlying mechanism was unclear. Here, we show that obatoclax lowers the cell viability and induces G1/G0- phase arrest concomitant with activation of p38 followed by upregulation of the CDK inhibitor p21$^{waf1/Cip1}$ in human esophageal cancer cells. In our view the most significant finding of the present study is that treatment of cells with obatoclax did not induce overt cellular apoptosis, a response commonly associated with Bcl-2 inhibition, but rather induced G1/G0-phase arrest. In this respect, our experimental findings reveal a hitherto unreported effect of obatoclax on cell cycle control in esophageal cancer cells.

Of noted frequently in the literatures, obatoclax has been proposed to act by inducing the formation of an active Bak/Bax

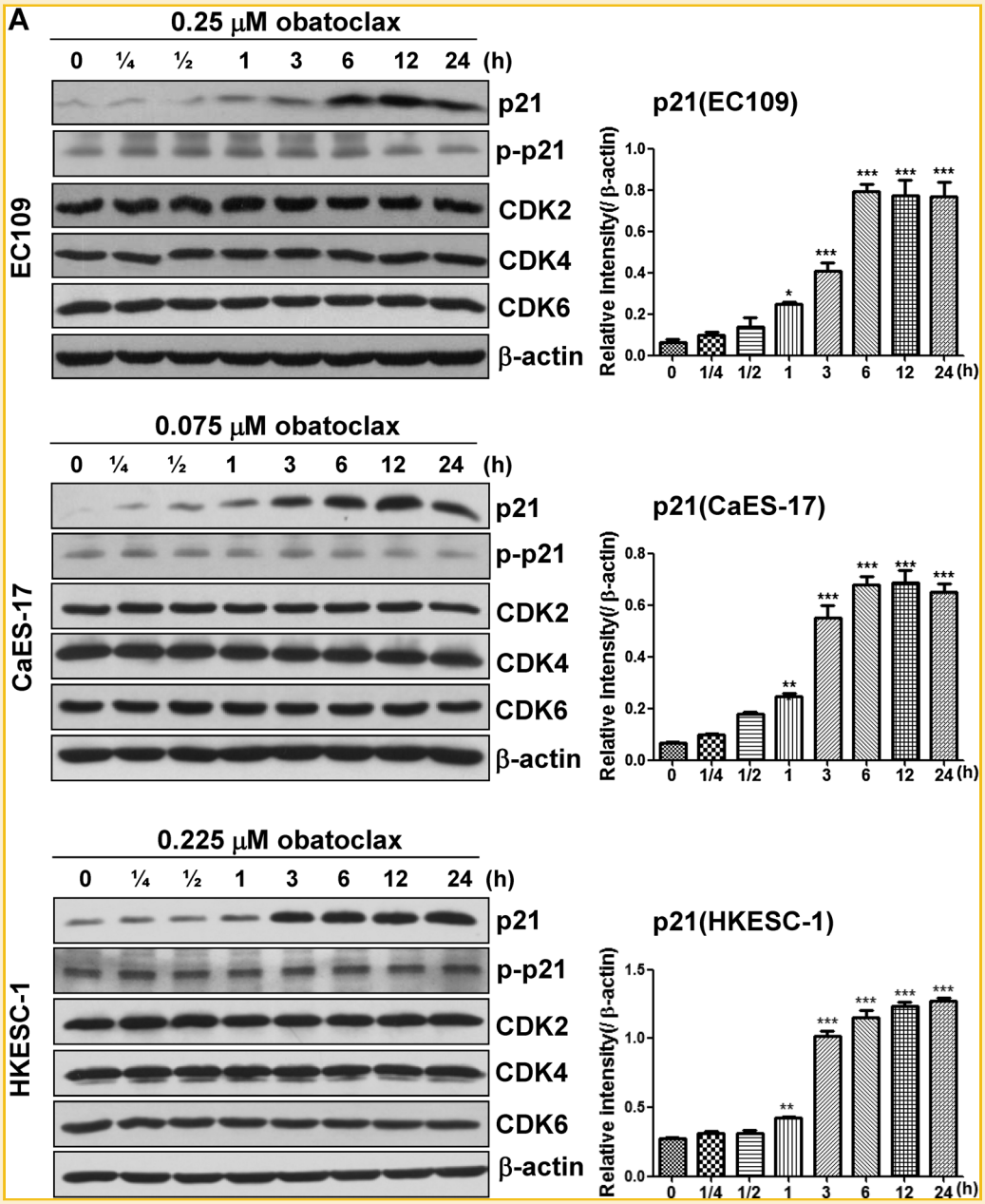

Fig. 5. Effects of siRNA-mediated knockdown of $p21^{waf1/Cip1}$ on obatoclax-induced G1/G0-phase arrest in EC109,CaES-17 and HKESC-1 cells. (A) EC109, CaES-17 and HKESC-1 cells were treated with obatoclax for the indicated time points. Cells were collected for the determination of protein expression by Western blot. The protein expression was evaluated by the graphic quantification of the band density. (B) The knockdown efficacy of $p21^{waf1/Cip1}$ was verified by Western blot analysis at 48 h post-transfection. Non-targeting siRNA was used as control siRNA. β-actin was used to evaluate protein loading. (C) After transfection with control siRNA and $p21^{waf1/Cip1}$ siRNA, EC109, CaES-17, and HKESC-1 cells were treated with obatoclax (0.25, 0.075, and 0.225 μM, respectively) for 24 h before flow cytometry analysis for DNA contents. Data are presented as mean ± SEM from three independent experiments. Comparisons between groups are indicated by brackets. NS indicates no significant difference ($P > 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to the control.

complex with permeabilized the outer mitochondrial membrane, which leads to the release of cytochrome *c* and the activation of apoptosis [Konopleva et al., 2008]. For instance, obatoclax was reported to induce apoptosis in a subset of lung cancer cell lines and the induction of apoptosis based on PARP cleavage correlated with the IC_{50} identified in cell viability assays [Li et al., 2008]. On the contrary, our present study showed that obatoclax at the concentration approximate its IC_{50} failed to induce obvious apoptosis in tested three esophageal cancer cell lines. This conclusion is made because there is no significant increase in Annexin V-positive cells in obatoclax-treated cells as compared with those cultured in medium alone and this agent fails to induce PARP cleavage. We noted that the treatment time used by the above study was 48 and 72 h, while the treatment time employed in our

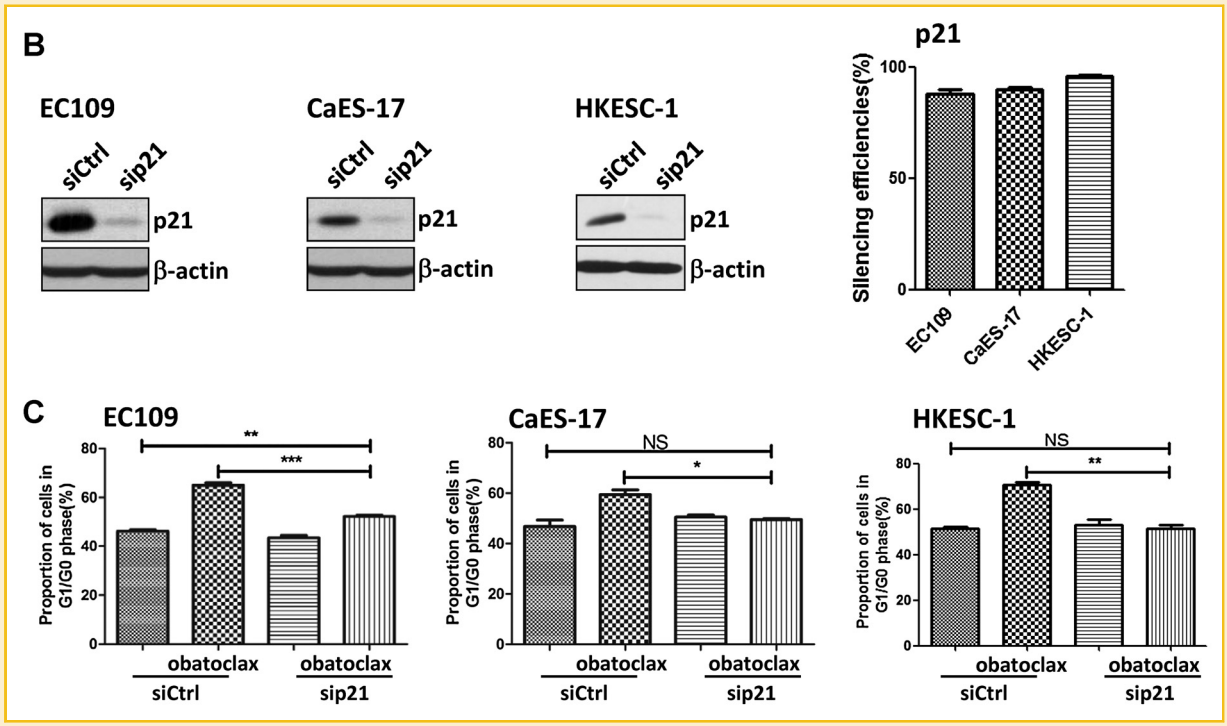

Fig. 5. (Continued)

present study is shorter (24 h). Based on this consideration, we prolonged the drug treatment time to 48 h. In this regard, obatoclax substantially induced apoptosis in esophageal cancer cells (data not shown here). Although cell cycle arrest may result in activation of pathways leading to apoptosis [Shapiro and Harper, 1999; Pietenpol and Stewart, 2002], whether G1/G0-phase arrest induced by obatoclax contributes to apoptosis occurring later needs further exploration. Based on our current data, we propose that obatoclax-induced cell cycle arrest contributes to its anti-cancer activity and occurs prior to apoptosis.

In addition to the antiapoptotic effects, Bcl-2 and Bcl-X_L have well documented cell cycle activities. Studies in mice and in cell culture have demonstrated that the most pronounced cell cycle effect of Bcl-2 and Bcl-X_L is delay of progression to S phase from G0/G1 phase [Huang et al., 1997; Greider et al., 2002; Janumyan et al., 2003]. For instance, quiescent T cells overexpressing Bcl-2 or Bcl-X_L are delayed in activation-induced cell cycle entry, while $bcl2^{-/-}$ knockout cells enter S phase more quickly [Linette et al., 1996; Mazel et al., 1996]. Therefore, the effects of obatoclax on G1/G0-phase arrest raise intriguing questions of whether inhibition of Bcl-2 and Bcl-X_L contributes to its action of cell cycle arrest. This hypothesis is unlikely because direct inhibition of Bcl-2 or Bcl-X_L would facilitate G1/G0 to S phase transition instead of G1/G0-phase arrest based on the anti-proliferative action of Bcl-2 and Bcl-X_L. Accumulating evidence has supported many different mechanisms of action, some of which do not require Bax and Bak to kill cells, suggesting off-target effects of obatoclax [Tang et al., 2012; Basit et al., 2013; Schwartz-Roberts et al., 2013; Sharma et al., 2013; Yu and Liu, 2013]. Consistent with these findings, our data showed that obatoclax-induced cell cycle arrest is independent of Bak/Bax activation for downregulation of both Bak and Bax does not block obatoclax-induced G1/G0-phase arrest. Thus, we favor the hypothesis that obatoclax induces G1/G0 cell cycle arrest in a manner which is not related to Bcl-2 and Bcl-X_L inhibition and subsequent Bax/Bak activation. Intriguingly, obatoclax has been reported to induce an S-phase arrest instead of G1/G0-phae arrest in acute myeloid leukemia cells and acute lymphoblastic leukemia cells [Konopleva et al., 2008; Urtishak et al., 2013]. These findings, combined with our study presented here, suggest that the effect of obatoclax on cell cycle progression may be cell type-specific.

In light of G1/G0-phase arrest induced by obatoclax, we investigated the mechanism of action of obatoclax by defining its effect on pathways that involve G1-to-S transition. Obatoclax treatment did not affect the expression of CDK-2, -4, and -6 in EC109, CaES-17, and HKESC-1 cells. Because CDKs are inhibited by CDK inhibitors such as $p21^{waf1/Cip1}$, we next examined the effect of obatoclax on the expression of this protein. In this respect, obatoclax treatment substantially increased the protein level of $p21^{waf1/Cip1}$. Moreover, knockdown of $p21^{waf1/Cip1}$ significantly attenuated obatoclax-induced G1/G0-phase arrest. This raises the question of how obatoclax increases $p21^{waf1/Cip1}$ expression in relation to cell cycle arrest.

Since obatoclax has been reported to activate MAPKs signaling pathways including Erk, p38, and JNK [Martin et al., 2009; Cruickshanks et al., 2012; Dasmahapatra et al., 2012; Tang et al., 2012], we therefore determined the effect of obatoclax on

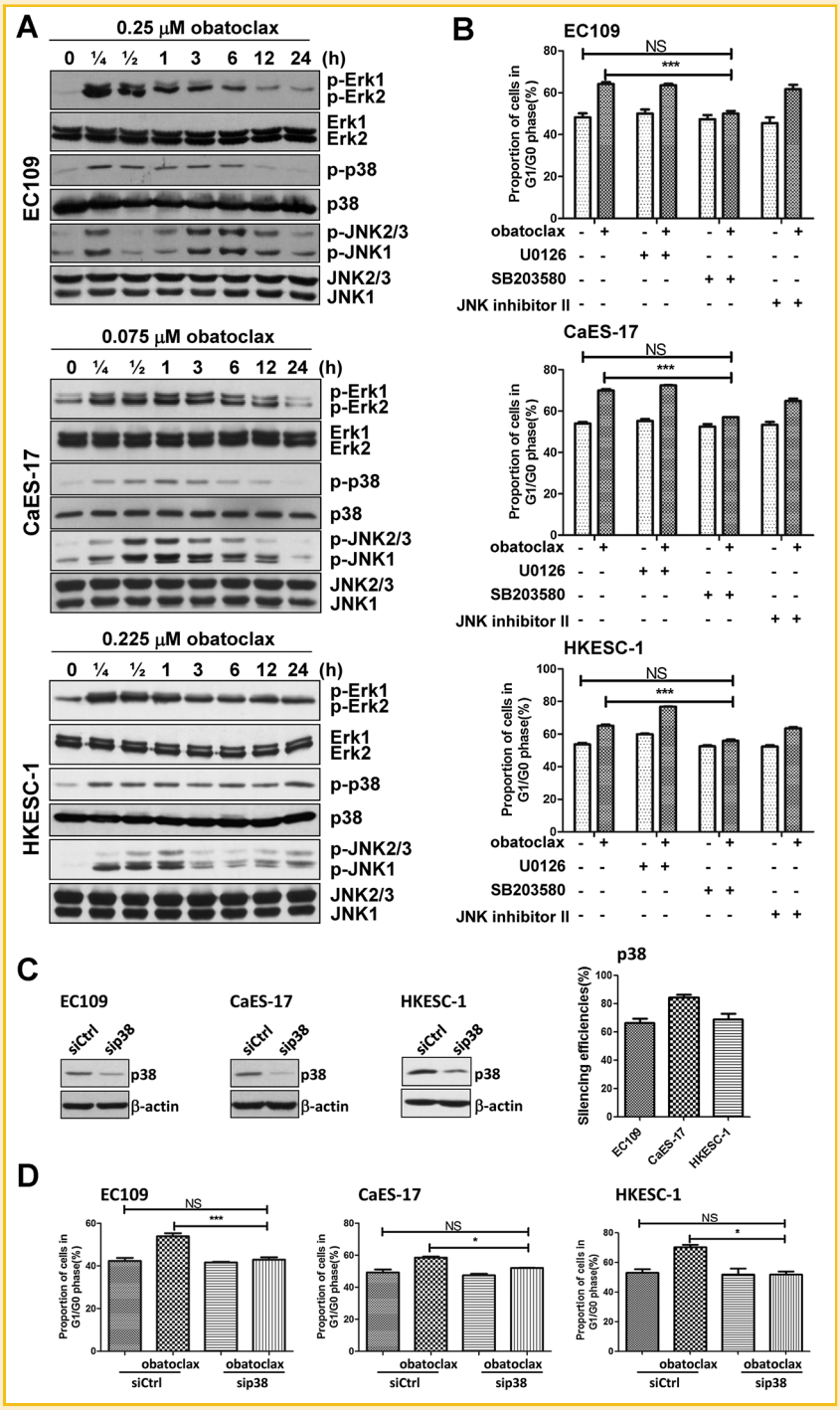

Fig. 6. Effects of p38 on obatoclax-induced G1/G0-phase arrest. (A) EC109, CaES-17, and HKESC-1 cells were treated with obatoclax for the indicated time points. Cells were collected for the determination of protein expression by Western blot. (B) EC109, CaES-17, and HKESC-1 cells were treated with obatoclax (0.25, 0.075, and 0.225 μM, respectively) alone or in combination with MEK inhibitor U0126 (10 μM), p38 inhibitor SB203580 (10 μM), or JNK inhibitor II (10 μM) for 24 h, their DNA contents were determined by flow cytometry analysis. (C) The knockdown efficacy of p38 was verified by Western blot analysis at 48 h post-transfection. Non-targeting siRNA was used as control siRNA. β-actin was used to evaluate protein loading. (D) After transfection with control siRNA and p38 siRNA, EC109, CaES-17, and HKESC-1 cells were treated with obatoclax (0.25, 0.075, and 0.225 μM, respectively) for 24 h before flow cytometry analysis for DNA contents. (E) EC109, CaES-17, and HKESC-1 cells were treated with obatoclax (0.25, 0.075, and 0.225 μM, respectively) alone or in combination with U0126 (10 μM), SB203580 (10 μM), or JNK inhibitor II (10 μM) for 24 h. Cells were collected for the determination of protein expression by Western blot. The protein expression was evaluated by the graphic quantification of the band density. Data are presented as mean ± SEM from three independent experiments. Comparisons between groups are indicated by brackets. NS indicates no significant difference ($P > 0.05$). *$P < 0.05$, ***$P < 0.001$ as compared to the control. #$P < 0.05$, ##$P < 0.01$ as compared to obatoclax-treated group.

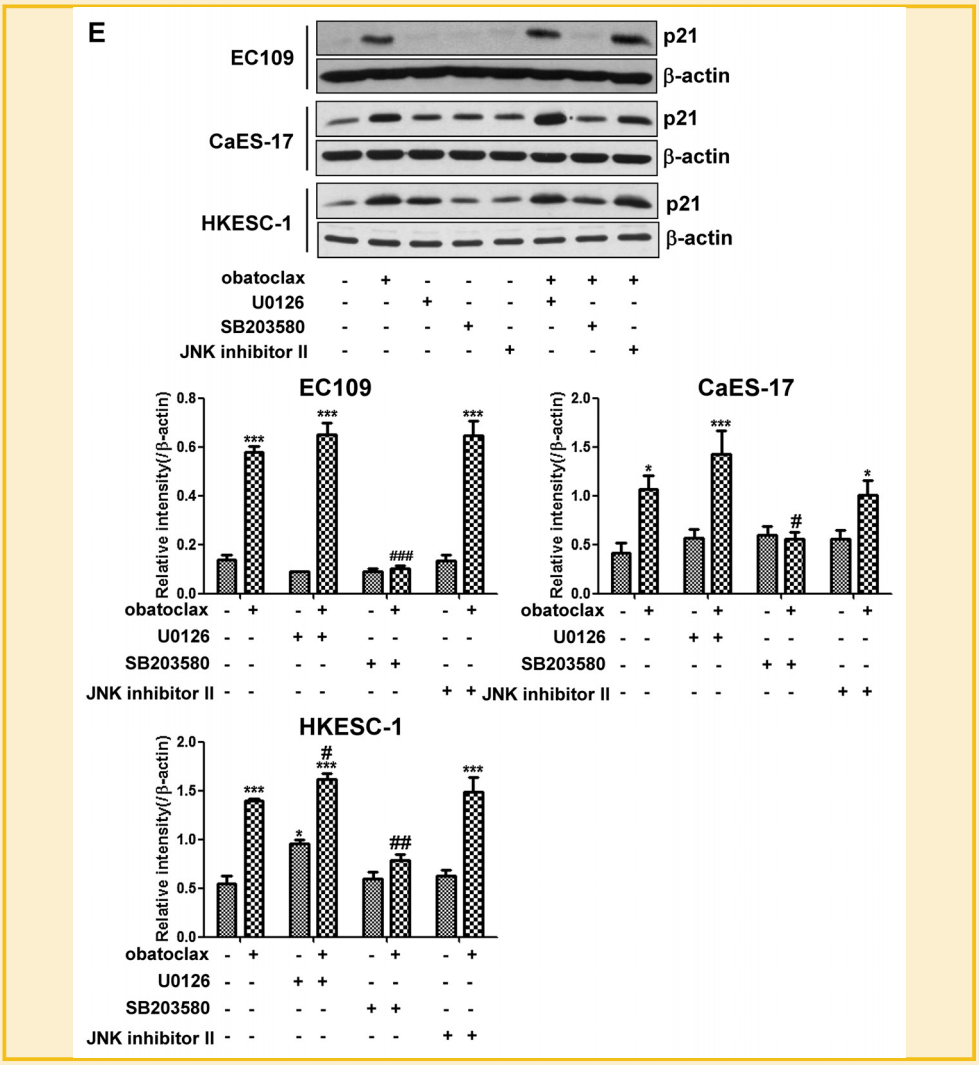

Fig. 6. (Continued)

MAPKs activation. Although obatoclax increased phosphorylation of Erk, p38, and JNK, only inhibition of p38 completely reversed obatoclax-induced G1/G0-phase arrest. In consistent with this finding, inhibition of p38 completely blocked obatoclax-promoted $p21^{waf1/Cip1}$ expression, whereas inhibition of Erk or JNK did not attenuated obatoclax-induced $p21^{waf1/Cip1}$ expression. Consistent with our findings, it has been reported that p38 activation contributes to G1/G0-phase arrest through increasing $p21^{waf1/Cip1}$ expression [Ambrosino and Nebreda, 2001; Thornton and Rincon, 2009]. Notably, the $p21^{waf1/Cip1}$ expression in EC109 cells treated with SB203580 was undetectable, which was comparable to the basal level of $p21^{waf1/Cip1}$ in the control cells. However, there was substantial expression of $p21^{waf1/Cip1}$ in CaES-17 and HKESC-1 cells treated with SB203580, indicating inhibition of p38 was unable to completely abolish basal $p21^{waf1/Cip1}$ expression. Our findings suggest that p38 mediated obatoclax-induced $p21^{waf1/Cip1}$ expression in the tested three cell lines, whereas the basal expression of $p21^{waf1/Cip1}$ relied on other mechanisms rather than p38 in CaES-17 and HKESC-1 cells.

In conclusion, our studies show that obatoclax induces G1/G0-phase arrest via p38/$p21^{waf1/Cip1}$ signaling pathway in esophageal cancer cells. Moreover, the inhibitory effect of obatoclax on cell viability is independent of Bak/Bax function. Of note, obatoclax at the concentrations as low as 0.25, 0.075, and 0.225 μM obviously induces G1/G0-phase arrest in EC109, CaES-17, and HKESC-1 cells, respectively, which are pharmacologically achievable and are clinically relevant, as the approximate peak plasma concentration of obatoclax is 100 ng/ml (~250 nM) [McCoy et al., 2010]. Obatoclax as single agent or in combination regimens is currently under clinical evaluation for the treatment of various cancers [Parikh et al., 2010; Paik et al., 2011; Oki et al., 2012; Coard and Shimmer, 2013]. Since many chemotherapies are cell cycle-dependent and cell cycle determines chemosensitivity [Hochhauser, 1997], it is possible that increased G1/G0-phase cells with obatoclax may promote synergy with cell cycle-dependent chemotherapy. Our study may shed new light on the anti-cancer activity of obatoclax and its potential application in clinic.

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