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Sorafenib Induces Endometrial Carcinoma Apoptosis by Inhibiting Elk-1-Dependent Mcl-1 Transcription and Inducing Akt/GSK3β-Dependent Protein Degradation

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ABSTRACT

Endometrial carcinoma (EC) is one of the main gynecologic malignancies affecting women, but effective treatments are currently lacking. In the present study, we investigated the effect of sorafenib, a general kinase inhibitor, on several EC cell lines (HEC1A, HEC1B, and RL95-2). Sorafenib induced cell death in EC cells with the following order of sensitivity: HEC1A > HEC1B > RL95-2. Sorafenib suppressed several anti-apoptotic proteins in HEC1A cells, including myeloid cell leukemia 1 (Mcl-1). Ectopic overexpression of Mcl-1 prevented the cell killing effect of sorafenib. Sorafenib suppressed Mcl-1 at the gene transactivation level by inactivating the ERK/Elk-1 pathway. Accordingly, the inhibitory effect of sorafenib on Mcl-1 expression decreased following knockdown of Elk-1 using short-hairpin RNA (shRNA). Elk-1 overexpression rescued both the inhibitory effect of sorafenib on Mcl-1 expression and the cell killing effect of sorafenib. Furthermore, sorafenib reduced the stability of the Mcl-1 protein by enhancing its ubiquitination and degradation by the proteasome via the AKT/GSK3β and the ERK pathways. Similar results were detected in other EC cell lines. These results indicate that sorafenib induces apoptosis in EC cells by down-regulating the anti-apoptotic protein Mcl-1 via transcriptional inhibition and protein degradation. Our results thus support the notion that sorafenib may be used in endometrial cancer therapy. J. Cell. Biochem. 114: 1819–1831, 2013.

KEY WORDS: AKT; APOPTOSIS; ELK-1; ENDOMETRIAL CARCINOMA; MCL-1; SORAFENIB

Abbreviations: Bcl-2, B-cell lymphoma 2; ChIP, chromatin immunoprecipitation; EC, endometrial carcinoma; ERK, extracellular-signal regulated kinase; FBS, fetal bovine serum; FBW7, F-box and WD repeat domain containing 7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; Luc, luciferase; Mcl-1, myeloid cell leukemia 1; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NTC, nitrilotriacetic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene fluoride; qPCR, quantitative real-time polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; shRNA, short-hairpin RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGFR, vascular endothelial growth factor receptor.

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In Western countries, endometrial carcinoma (EC) represents the most common gynecologic malignancy in women. Worldwide, EC accounts for the fourth most common type of cancer [Society, 2010]. Alterations in several genes are important for the development of type I EC, including PTEN, PIK3CA, K-ras, b-catenin, and DNA mismatch repair genes. Type II EC is associated with mutations in p53, amplification of STK15 and HER2/neu, overexpression of p16, down-regulation or loss of E-cadherin, and loss of heterozygosity [Llaurado et al., 2012]. Despite the striking molecular differences between type I and type II EC, most clinical trials have been conducted without taking this diversity into account. Drugs currently under clinical investigation for the treatment of EC and their target molecules which are part of the PI3K/AKT/mTOR and VEGF signaling pathways have been extensively reviewed [Dedes et al., 2011]. Resistance to apoptosis also plays an important role in the development and progression of EC. Cells that become resistant to apoptosis are likely to escape immune surveillance, and are also prone to develop resistance to chemotherapy. Given that the mechanisms underlying the progression to aggressive EC are largely unknown, the development of rational therapies to treat this disease and the design of novel interventions to limit chemoresistance remain important challenges.

One of the major modes of action of anti-cancer drugs is to induce apoptosis in cancer cells. Apoptosis can be induced by two primary mechanisms: the intrinsic pathway, which has its origin in the mitochondria, and the extrinsic pathway, which is triggered by the activation of death receptors situated on the cell membrane. The two apoptotic signaling pathways are inter-connected [Dolcet et al., 2005; Llobet et al., 2009]. In contrast, activation of the PI3K/AKT pathway suppresses apoptosis induced by various stimuli. In addition, members of the B-cell lymphoma 2 (Bcl-2) gene family and other proteins involved in regulating apoptosis (like survivin) have been shown to be abnormally regulated in EC [Pallares et al., 2005]. NF-kB activation is also frequent in EC, and this may explain the resistance to apoptosis since this transcription factor induces several target genes like c-FLIP and Bcl-xL [Pallares et al., 2004; Dolcet et al., 2005; Llobet et al., 2008, 2010]. Moreover, the gene encoding kinase suppressor of Ras 1 (KSR1), which is overexpressed in EC, also regulates cell sensitivity to TRAIL by regulating c-FLIP levels [Llobet et al., 2011]. Elucidation of the signaling pathways involved in resistance to apoptosis will help us to prevent this process during EC treatment.

The myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic member of the Bcl-2 family of apoptosis-regulating proteins, plays a pivotal role in protecting cells against apoptosis, and is overexpressed in a variety of human cancers. Mcl-1 plays an important role in many cell death and cell survival regulatory pathways [Yang-Yen, 2006]. Targeting Mcl-1 activity in these cancer cells, using genetic or pharmacological approaches, not only represents a promising novel cancer therapy but also a way to reverse resistance to conventional cancer treatments [Quinn et al., 2011]. The N-terminus of Mcl-1 is unique amongst the Bcl-2 family proteins in that it is rich in several confirmed and putative regulatory residues and motifs. These amino acid residues include sites for ubiquitination, protein cleavage, and phosphorylation, which can influence the stability, localization, dimerization, and cellular function of Mcl-1 [Thomas et al., 2010].

Sorafenib represents an inhibitor of several kinases present either in the cytoplasm (CRAF, BRAF, and mutant BRAF) or on the cell surface (KIT, FLT-3, VEGFR-2, VEGFR-3, and PDGFR-β). Several of these kinases are involved in cell proliferation and angiogenesis, thus sorafenib is thought to reduce tumor growth mainly by inhibiting these processes. Sorafenib has been shown to improve survival rates in human cancer patients [Yau et al., 2010]. This drug decreases cell proliferation and increases apoptosis in cancer cells by inhibiting the Raf/MEK/ERK [Liu et al., 2006; Huynh et al., 2009; Peng et al., 2009] and PI3K/Akt pathways [Chai et al., 2010]. p38/MAPK is also a target of sorafenib in kinase assays performed in vitro [Wilhelm et al., 2006]. By inhibiting these kinases, genetic transcription involved in cell proliferation and angiogenesis is inhibited. Moreover, knockdown of Raf using siRNA leads to mitotic spindle abnormality and early mitotic exit in human somatic cells [Borysova et al., 2008]. Depletion of p38/MAPK also stops the cell cycle at the G2/M phase in HeLa cells, and later induces apoptosis. These results suggest that sorafenib may perturb mitotic spindle formation as well as chromosome alignment and congression by inhibiting protein kinases, and possibly also by depleting anti-apoptotic proteins such as Mcl-1. While sorafenib has been shown to induce apoptosis of EC cells via proteasome-mediated Mcl-1 down-regulation [Llobet et al., 2010]. The mechanism of sorafenib-induced Mcl-1 down-regulation has not been examined and whether additional proteins regulate this process is unknown. In addition, whether sorafenib may be beneficial for EC treatment is unclear. This possibility prompted us to study the effect of sorafenib on EC cells, and to determine its mechanism of action in this context.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

The cells used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml, Gibco), and streptomycin (100 μg/ml, Gibco) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, except for the RL95-2 cells which were cultured in a 1:1 mixture of DMEM/nutrient F-12 Ham (Gibco). The human EC cell lines HEC1A, HEC1B, and RL95-2 were obtained from the American Tissue Type Collection (Manassas, VA). The reagents used included antibodies against cleaved caspase-3, phosphorylated-AKT, phosphorylated-Elk-1, phosphorylated-GSK3β (Cell Signaling, Danvers, MA), AKT, Bax, Bcl-2, Bcl-xL, phosphorylated and total Elk-1, phosphorylated and total ERK1/2, GAPDH, GSK3β, Mcl-1, PARP, survivin, ubiquitin, and VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA). The proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA). The kinase inhibitors used in this study included PD98059 and U0126 (Calbiochem). The chemotherapeutic drug sorafenib is marketed as Nexavar (Bayer HealthCare AG, Berlin, Germany). Unless indicated otherwise, the other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All reagents were used according to the instructions provided by the supplier.

GENE KNOCKDOWN USING SHORT-HAIRPIN RNA

pLKO.1 plasmid expressing short-hairpin RNA shRNA to knock-down Elk-1 (shElk-1) was purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The most effective shElk-1 plasmid (TRCN0000237875) was used in the present study. A plasmid expressing shRNA to down-regulate luciferase (TRCN0000072244; shLuc) was used as a negative control. Transient transfection of shRNA plasmids was performed by adding 2 μg/well of plasmid and 5 μl/well of lipofectamine (Invitrogen, Carlsbad, CA) in cells cultured in 6-well plates (1.5×10^4 cells/well). Three days following plasmid transfection, Elk-1 mRNA level was determined by quantitative real time-polymerase chain reaction (qPCR) as described below. Recombinant lentivirus constructs were incubated with the cells for 2 weeks in puromycin-containing selection medium according to the procedure provided by the supplier (National RNAi Core Facility).

QUANTITATIVE REAL-TIME PCR

qPCR was performed on total RNA extracted with Trizol (Invitrogen) and 200 nM of primers as before [Sun and Chao, 2005]. Primers for Mcl-1 (GenBank sequence number NM_021960) and GAPDH (NM_000996) were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA). The resulting primers consisted of Mcl-1, forward, 5′-TGATCCATGTTTTCAGCGAC-3′, reverse, 5′-AATG GTTCGATGCAGC-TTTC-3′, and GAPDH, forward, 5′-TCCTGCAC-CACCAACTGCTT-3′, reverse, 5′-GAGGGGGCCATCCACGTCTT-3′. All samples and controls were prepared in triplicate on the same plate. Relative quantification was calculated using the $\Delta\Delta C_t$ method with normalization against GAPDH [Wu and Chao, 2010].

PLASMIDS, TRANSFECTION, CELL EXTRACTS, AND IMMUNOBLOT ANALYSIS

Cells were transfected with plasmid cDNA to express Mcl-1 (pcDNA3-Mcl-1, provided by Dr. Hsin-Fang Yang-Yen, Academia Sinica), or Elk-1 (pCMV2-Elk-1, provided by Dr. Jer-Yuh Liu, Chinese Medical University, Taiwan). Fifty micrograms of total protein extract was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by transfer to polyvinylidene fluoride (PVDF) membrane, and incubation with primary antibodies raised against the indicated proteins. The membranes were then incubated with the following secondary antibodies: goat anti-mouse, goat anti-rabbit, or donkey anti-goat IgG HRP (Santa Cruz Biotechnology). The resulting signal was visualized by enhanced chemiluminescence according to specifications from the supplier (Pierce, Rockford, IL). Protein band intensity was determined by scanning X-ray films with a densitometer (Personal Densitometer SI; Amersham Biosciences, Sunnyvale, CA). Western blotting experiments were performed in triplicate.

APOPTOTIC CELL ANALYSIS

Apoptotic cells following drug treatment for 24 h were determined by nuclear phenotype [Kamarajan et al., 2001]. To evaluate drug-induced apoptosis, we used cell extracts for immunoblotting experiments with antibodies specific for the apoptotic markers cleaved caspase-3 and PARP. To confirm apoptosis, sub-G1 cells were also measured by flow cytometry as before [Tsai et al., 2007]. Three independent experiments were performed.

ANALYSIS OF Mcl-1 PROTEIN STABILITY

EC cells (5×10^5) plated on 60-mm tissue culture dishes were grown for 24 h, and cycloheximide was then added at 50 μg/ml. At various times after the addition of cycloheximide, the cells were harvested and lysed in lysis buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail; BD Biosciences, San Jose, CA), and detected by Western blot with anti-Mcl-1 antibody.

UBIQUITINATION ASSAY

Cells were transfected for 24 h with expression plasmids (2 μg) encoding for histidine-tagged ubiquitin plasmid (provided by Prof. R.-H. Chen, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan). Cells were treated with either sorafenib or DMSO as a control in the presence of MG132 (20 μM) for 8 h. Cells were harvested in PBS and then lysed in lysis buffer A (6 M guanidine–HCl, 10 mM imidazole, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0), and sonicated. Equal amounts of total protein lysates (1,000 μg) were incubated with 20 μl of Ni-NTA resin (50% v/v; Novagen, Merck KGaA, Darmstadt, Germany) for 4 h at 4°C. Next, the resin was washed twice with buffer A/TI (1 vol buffer A:3 vol buffer TI) and three times with buffer TI (25 mM Tris–HCl, pH 6.8, 20 mM imidazole). The ubiquitinated Mcl-1 was analyzed by Western blot using an anti-Mcl-1 antibody.

STATISTICAL ANALYSIS

The data were reported as means ± standard deviation (SD). Statistical significance (P-value) was assessed by a two-tailed Student's t-test for single comparison. The symbol * denotes $P < 0.05$; ** denotes $P < 0.01$.

RESULTS

SORAFENIB INDUCES EC CELL DEATH

The effect of sorafenib on EC cell lines was examined using the MTT cell viability assay. HEC1A cells were highly sensitive to sorafenib (with an estimated IC50 of 4.2 μM), whereas RL95-2 and HEC1B cells were less sensitive (IC50 of 8.6 and 7.3 μM, respectively; Fig. 1).

SORAFENIB INHIBITS THE ANTI-APOPTOTIC PROTEIN Mcl-1 IN EC CELLS

Inhibition of anti-apoptotic proteins is an important mechanism of several anti-cancer drugs. To assess the effect of sorafenib on apoptosis in EC cells, we used HEC1A cells since they showed high sensitivity to this drug. The protein levels of all the tested anti-apoptotic members of the Bcl-2 family increased after 1 h of sorafenib treatment (Fig. 2A). Among the representative anti-apoptotic proteins tested, sorafenib inhibited Mcl-1 in a time-dependent manner (Fig. 2A). In contrast, the anti-apoptotic proteins survivin, Bcl-xL, and Bcl-2 were only transiently induced by sorafenib (Fig. 2A, lanes 1–5). While a dose-dependent inhibition of Mcl-1 by sorafenib was also detected, the other anti-apoptotic proteins tested were not affected by low concentrations of the drug

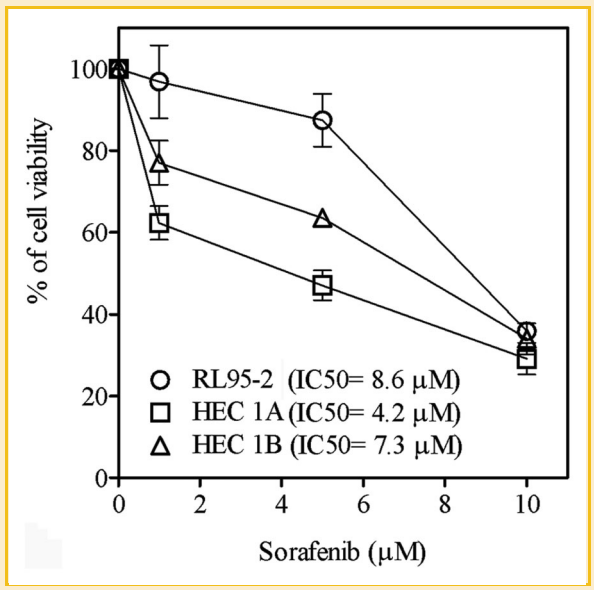

Fig. 1. Sorafenib effect on cell proliferation inhibition in EC cells. Cell viability of EC cells exposed to sorafenib estimated by the MTT assay.

(Fig. 2A, lanes 6–9). In addition, the pro-apoptotic proteins such as Bax were only slightly induced, if any, by sorafenib (Fig. 2A). HEC1A cells treated with sorafenib also showed increased cleavage and activation of caspase-3 and its PARP substrate (Fig. 2B, compare lanes 1 and 2). To rule out possible off-target effects, we overexpressed Mcl-1 and observed that this treatment considerably prevented the cleavage of caspase-3 and PARP induced by sorafenib (Fig. 2B, compare lanes 2 and 4). Sorafenib induced apoptosis in a dose-dependent manner and this effect was inhibited by ectopic expression of Mcl-1 (Fig. 2C). Sorafenib also induced accumulation of sub-G1 cells at higher concentration (10 μM), and this process was inhibited by ectopic expression of Mcl-1 (Fig. 2D). Similarly, the cell-death inducing effect of sorafenib was significantly rescued by ectopic expression of Mcl-1 in HEC1A cells (Fig. 2E). These results indicate that sorafenib induces cell death and apoptosis in EC cells by inhibiting the anti-apoptotic protein Mcl-1.

SORAFENIB SUPPRESSES THE Mcl-1 GENE VIA THE ERK/Elk-1 PATHWAY

To assess how Mcl-1 is suppressed by sorafenib, we examined the transcriptional regulation of this protein. Mcl-1 mRNA level in HEC1A cells was suppressed by sorafenib after 4–16 h of treatment (Fig. 3A). Since sorafenib is a powerful inhibitor of kinases involved in cell proliferation, we investigated the activation of MAPKs in the treated cells. As expected, activation, and phosphorylation of ERK and its target Elk-1, a transcription factor that potentially binds to the Mcl-1 gene promoter (see below), were rapidly inhibited by both sorafenib and the MEK1 inhibitor PD98059 (Fig. 3B). Phosphorylation of ERK and Elk-1 was inhibited to a lesser degree by U0126, which was used as an inhibitor of MEK1 and MEK2 (Fig. 3B, lanes 10–12). Following prolonged incubation with sorafenib, the protein levels of both Elk-1 and Mcl-1 were suppressed (Fig. 3C). Interestingly, while the protein level of Elk-1 remained unchanged following treatment with both MEK inhibitors, Mcl-1 protein was considerably reduced (Fig. 3C, lanes 7–12). These results suggest that sorafenib, in addition to inhibiting ERK-dependent transcription of Mcl-1, also causes a decrease of the Elk-1 protein. To confirm that Mcl-1 is transcriptionally regulated by sorafenib, we performed ChIP assays to assess the binding of Elk-1 to the Mcl-1 promoter in sorafenib-treated EC cells. A schematic presentation of the Mcl-1 promoter and the putative Elk-1 binding site is shown in Fig. 3D. PCR primers used for the ChIP assay are also indicated. We observed a decrease of the PCR product of Elk-1 bound to the Mcl-1 promoter in sorafenib-treated cells (Fig. 3E, compare lanes 4–6 with lane 3). A similar suppression pattern was detected in PD98059-treated cells (Fig. 3E, lanes 7–9). This observation is unlikely to represent off-target binding since no PCR product was detected when the negative binding site sequence of exon 3 was used. Statistical analysis indicated that the binding of Elk-1 to the Mcl-1 promoter was inhibited ($P < 0.005$) after 16 h of incubation with either sorafenib or the ERK inhibitor PD98059 (Fig. 3F). These findings reveal that the MKK1/2-ERK1/2 signaling pathway is an upstream signal that maintains the stability of the Mcl-1 protein.

To assess whether the decrease of Mcl-1 by ERK inhibitor PD98059 may also be due to enhanced protein degradation, we examined the kinetic changes of protein level in cells treated with the inhibitor of protein synthesis, cycloheximide. Mcl-1 protein level was reduced by PD98059 in a time-dependent manner (Fig. 4A, lanes 5–8). The rate of protein degradation in PD98059-treated cells appeared to be faster than in the control (Fig. 4A, compare with lanes 1–4). Indeed, quantitative analysis indicated a greater degradation rate in the PD98059-treated cells than the control DMSO group (Fig. 4B, slopes: −0.84 vs. −0.52). To investigate this possibility, we prepared protein extracts from cells treated with PD98059 and MG132 for purification of His-tagged ubiquitin modified Mcl-1 using Ni-NTA resin affinity chromatography. The eluted proteins were resolved in SDS–PAGE and further processed for immunoblot with an anti-Mcl-1 antibody. Interestingly, the PD98059 treatment caused dramatic poly-ubiquitination of the Mcl-1 protein (Fig. 4C). This observation was not due to protein overloading since no more protein was observed in this case when compared to the input control. PD98059 also induced mono-ubiquitination of Mcl-1 protein. As such, the effect of ubiquitination on Mcl-1 may also be important for the regulation of Mcl-1 expression by ERK signaling.

Similar to the results found in HEC1A cells, sorafenib suppressed the protein level of Mcl-1 and enhanced caspase activation in RL95-2 cells (Supplementary Fig. S1A,B). The phosphorylation and protein levels of Elk-1 and ERK were also reduced by sorafenib in these cells (Supplementary Fig. S1C,D). Interestingly, knockdown of Elk-1 (by shElk-1) enhanced caspase activation and Mcl-1 suppression by sorafenib (Supplementary Fig. S1E,F), and this effect was associated with increased sub-G1 cells and inhibition of cell proliferation (Supplementary Fig. S1G,H). Taken together, these results show that sorafenib induces apoptosis through ERK/Elk-1-dependent transcriptional suppression of the anti-apoptotic Mcl-1 in EC cells.

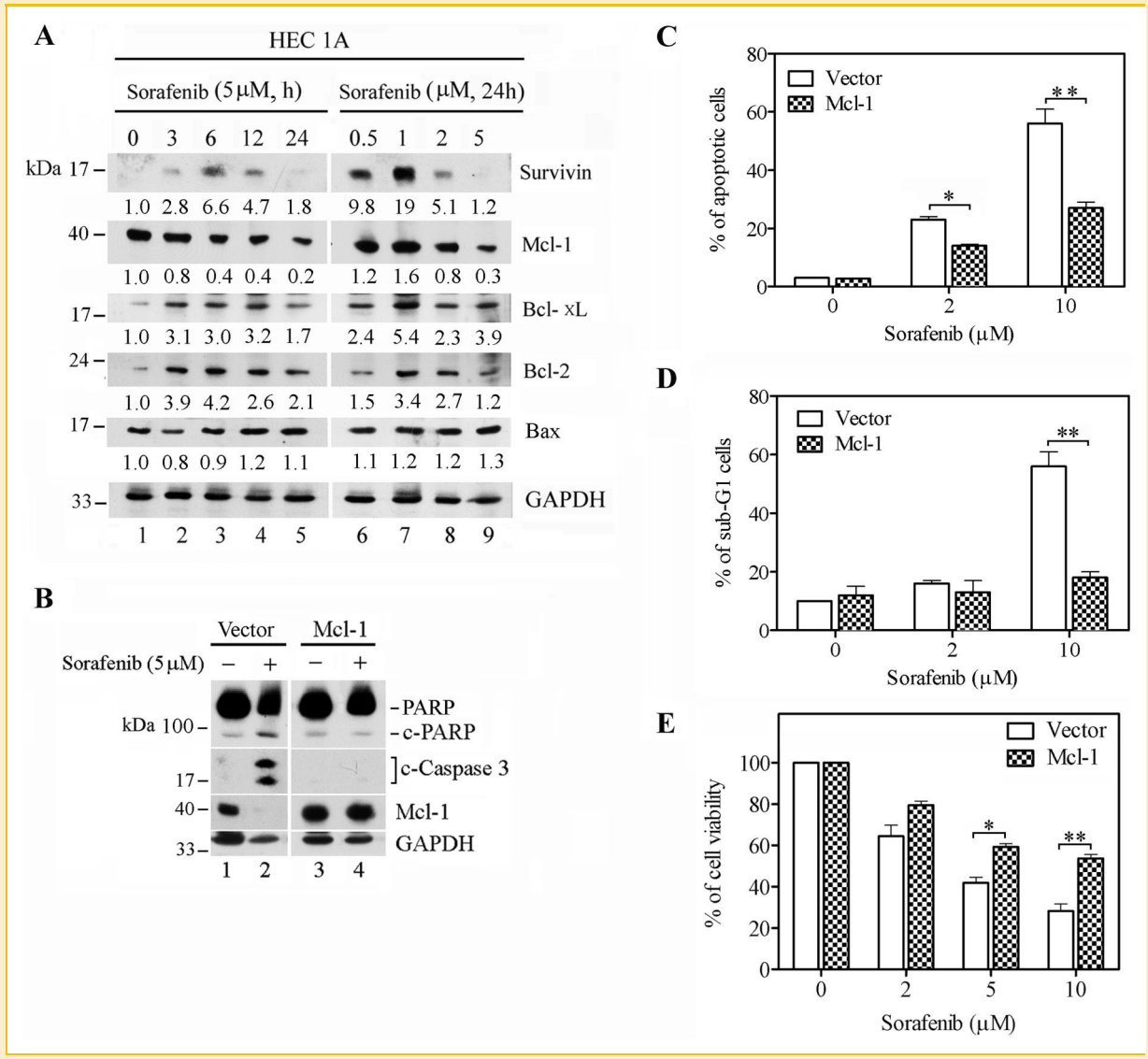

Fig. 2. Suppression of Mcl-1 protein level and enhancement of apoptosis in HEC1A cells treated with sorafenib. A: Inhibition of anti-apoptotic proteins by sorafenib. B: Induction of caspase activity by sorafenib is rescued by ectopic expression of Mcl-1. Fifty micrograms of cell extracts were used in A and B. Relative protein levels were determined by densitometry analysis of Western blot bands. C: Sorafenib-induced apoptosis was partly inhibited by ectopic expression of Mcl-1. D: Sorafenib-induced sub-G1 cells were partly inhibited by ectopic expression of Mcl-1. E: Sorafenib-induced inhibition of cell proliferation was partly rescued by ectopic expression of Mcl-1.

KNOCKDOWN OF Elk-1 REDUCES Mcl-1 PROTEIN LEVEL AND ENHANCES SORAFENIB-INDUCED CELL DEATH AND APOPTOSIS

To determine the importance of Elk-1 in Mcl-1 expression, we down-regulated Elk-1 by using shRNA (shElk-1). In the absence of sorafenib, Mcl-1 protein level was significantly reduced by shElk-1 ($P < 0.01$) and this observation was associated with increased caspase and PARP cleavage (Fig. 5A, compare lanes 1 and 4; Fig. 5B). While the level of Elk-1 was completely suppressed by shElk-1 in the presence of sorafenib, the level of Mcl-1 decreased and was associated with even higher caspase and PARP cleavage (Fig. 5A, lanes 5–6; Fig. 5B,C). While sub-G1 cells were induced by sorafenib in a dose-dependent manner, accumulation of sub-G1 cells was enhanced by shElk-1 only when the cells were exposed to sorafenib (Fig. 5D). Similarly, cell death was induced by sorafenib in a dose-dependent manner. Enhancement of cell proliferation inhibition by shElk-1 was detected only when the cells were exposed to sorafenib (Fig. 5E). These results suggest that the shElk-1-induced suppression of Mcl-1 and activation of caspase-3 may be pro-apoptotic.

OVEREXPRESSION OF Elk-1 INCREASES Mcl-1 PROTEIN LEVEL AND RESCUES SORAFENIB-INDUCED CELL DEATH AND APOPTOSIS

To rule out possible off-target effects of Elk-1, we overexpressed Elk-1 and examined the effects of this treatment on apoptotic markers. Overexpression of Elk-1 considerably inhibited sorafenib-induced cleavage of caspase-3 and PARP (Fig. 6A). In the absence of

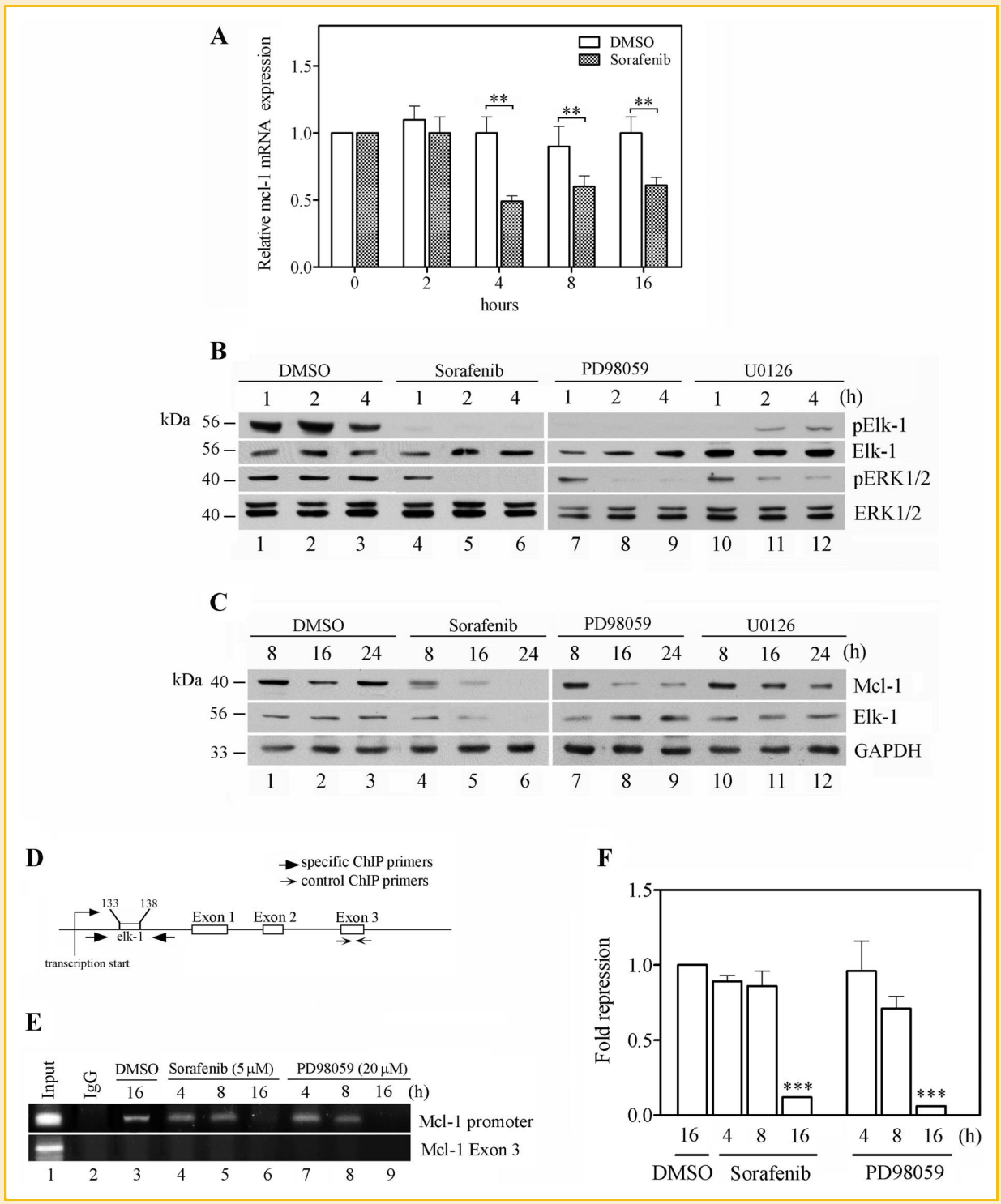

Fig. 3. Transcriptional suppression of Mcl-1 gene by sorafenib via the ERK/Elk-1 pathway. A: Suppression of Mcl-1 mRNA level by sorafenib. B: Inhibition of ERK/Elk-1 phosphorylation by sorafenib. C: Suppression of Mcl-1 and Elk-1 protein level by sorafenib. D: Schematic presentation of the Mcl-1 promoter and putative Elk-1 binding site. PCR primers used for the ChIP assay were indicated. E: Inhibition of Elk-1 binding to Mcl-1 promoter by sorafenib (ChIP assay). F: Fold repression of Elk-1 binding to Mcl-1 promoter by sorafenib. The results were calculated from data of ChIP assay as in E and repeated three times. The repression by an ERK inhibitor (PD98059) was also shown in E and F. $^{***}P < 0.005$.

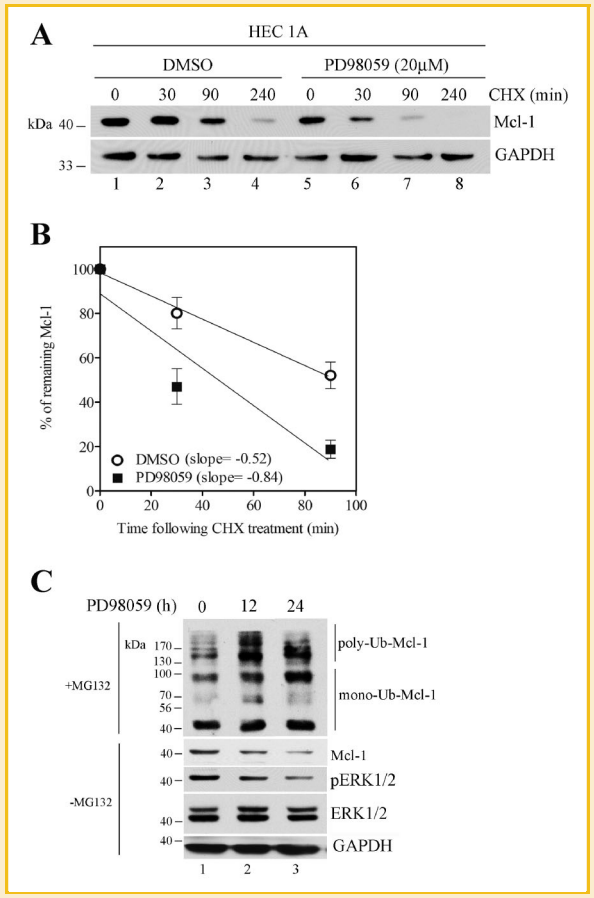

Fig. 4. Enhanced ubiquitination and degradation of Mcl-1 by PD98059 treatment. A: Enhanced degradation of the Mcl-1 protein in PD98059-treated HEC1A cells. Cells were pre-incubated with cycloheximide (CHX), an inhibitor of protein synthesis, followed by the indicated incubation. B: Increase in the degradation rate of Mcl-1 protein by PD98059 shown in A. The slope of the linear regression representing the Mcl-1 protein level of PD98059 group and DMSO control was indicated. C: Increased ubiquitination of Mcl-1 protein in HEC1A cells following PD98059 treatment. His-ubiquitin tagged Mcl-1 protein was eluted followed by immunoblotting with anti-ubiquitin antibody. Results of panel B are expressed as mean values ± SD for experiments performed in triplicate.

sorafenib, the protein level of Mcl-1 slightly increased following Elk-1 overexpression, and this observation was associated with decreased PARP cleavage (Fig. 6A). While the level of Elk-1 remained higher than that of the control vector following Elk-1 overexpression in the presence of sorafenib, the level of Mcl-1 increased and was associated with even less caspase and PARP cleavage (Fig. 6A, compare lanes 2–3 with 5–6; Fig. 6B, $P < 0.01$). Sorafenib induced the accumulation of sub-G1 cells in a dose-dependent manner, and this process was inhibited by ectopic expression of Elk-1 (Fig. 6C). Similarly, the cell death-inducing effect of sorafenib was significantly rescued by ectopic expression of Mcl-1 in HEC1A cells (Fig. 6D). These results indicate that sorafenib inhibits Mcl-1 expression and induces cell death in EC cells at least in part by inhibiting the transcription factor Elk-1.

SORAFENIB ENHANCES THE UBIQUITINATION AND DEGRADATION OF Mcl-1

To assess whether the decrease of Mcl-1 by sorafenib may also be due to enhanced protein degradation, we examined the kinetic changes of protein level in cells treated with the inhibitor of protein synthesis, cycloheximide. Mcl-1 protein level was reduced by sorafenib in a time-dependent manner (Fig. 7A, lanes 5–8). The rate of protein degradation in sorafenib-treated cells appeared to be faster than in the control (Fig. 7A, compare with lanes 1–4). Indeed, quantitative analysis indicated a greater degradation rate in the sorafenib-treated cells than the control DMSO group (Fig. 7B, slopes: −0.82 vs. −0.75). Suppression and slightly enhanced degradation of Mcl-1 by sorafenib was also found in HEC1B and RL95-2 cells (Supplementary Fig. S2). To determine the mechanism underlying the enhanced degradation of Mcl-1 by sorafenib, we also investigated the possibility that it is degraded by the proteasome. HEC1A cells treated with the proteasome inhibitor MG132 displayed an accumulation of Mcl-1 (Fig. 7C, compare lanes 1 and 3; Fig. 7D). Mcl-1 protein level, which was almost completely suppressed by sorafenib, increased in cells treated with MG132 (Fig. 7C, compare lanes 2 and 4; Fig. 7D), suggesting that ubiquitination may regulate the stability of Mcl-1 in response to sorafenib. To investigate this possibility, we prepared protein extracts from cells treated with sorafenib and MG132 for purification of His-tagged ubiquitin modified Mcl-1 using Ni-NTA resin affinity chromatography. The eluted proteins were resolved in SDS–PAGE and further processed for immunoblot with an anti-ubiquitin antibody. Interestingly, the sorafenib treatment caused dramatic poly-ubiquitination of the Mcl-1 protein compared to the DMSO control (Fig. 7E). This observation was not due to protein overloading since no more protein was observed in this case when compared to the input control (see the immunoblot performed without MG132 shown in the bottom panel of Fig. 7D). Sorafenib also induced more mono-ubiquitination of Mcl-1 protein than the control. As such, the effect of mono-ubiquitination on Mcl-1 may also be important for the regulation of Mcl-1 expression.

ENHANCED DEGRADATION OF Mcl-1 BY SORAFENIB VIA THE AKT/GSK3β PATHWAY

To assess the pathways involved in the enhanced degradation of Mcl-1 by sorafenib, we examined the protein kinases responsive to the drug in HEC1A cells. While the AKT protein level was increased by sorafenib in a dose-dependent manner, the phosphorylation level of AKT decreased in these cells (Fig. 8A). The phosphorylation of GSK3β, a downstream target of AKT, was also investigated. Although the protein level of GSK3β remained unchanged following treatment with sorafenib, the drug suppressed GSK3β phosphorylation. Notably, the protein level of Mcl-1 was reduced by sorafenib whereas its phosphorylation increased (Fig. 8A). Mcl-1 mRNA level was not suppressed by Wortmannin, a potent inhibitor of PI3Ks (Fig. 8B). These results suggest that the phosphorylation of Mcl-1 and its protein stability may be related to the activity of GSK3β. To test this possibility, we used the inhibitor II of GSK3. While the phosphorylation of GSK3β was suppressed by the inhibitor, the protein level of GSK3β considerably increased (Fig. 8B, compare lanes 1 and 3; Fig. 8C). Interestingly, the protein level of Mcl-1 was

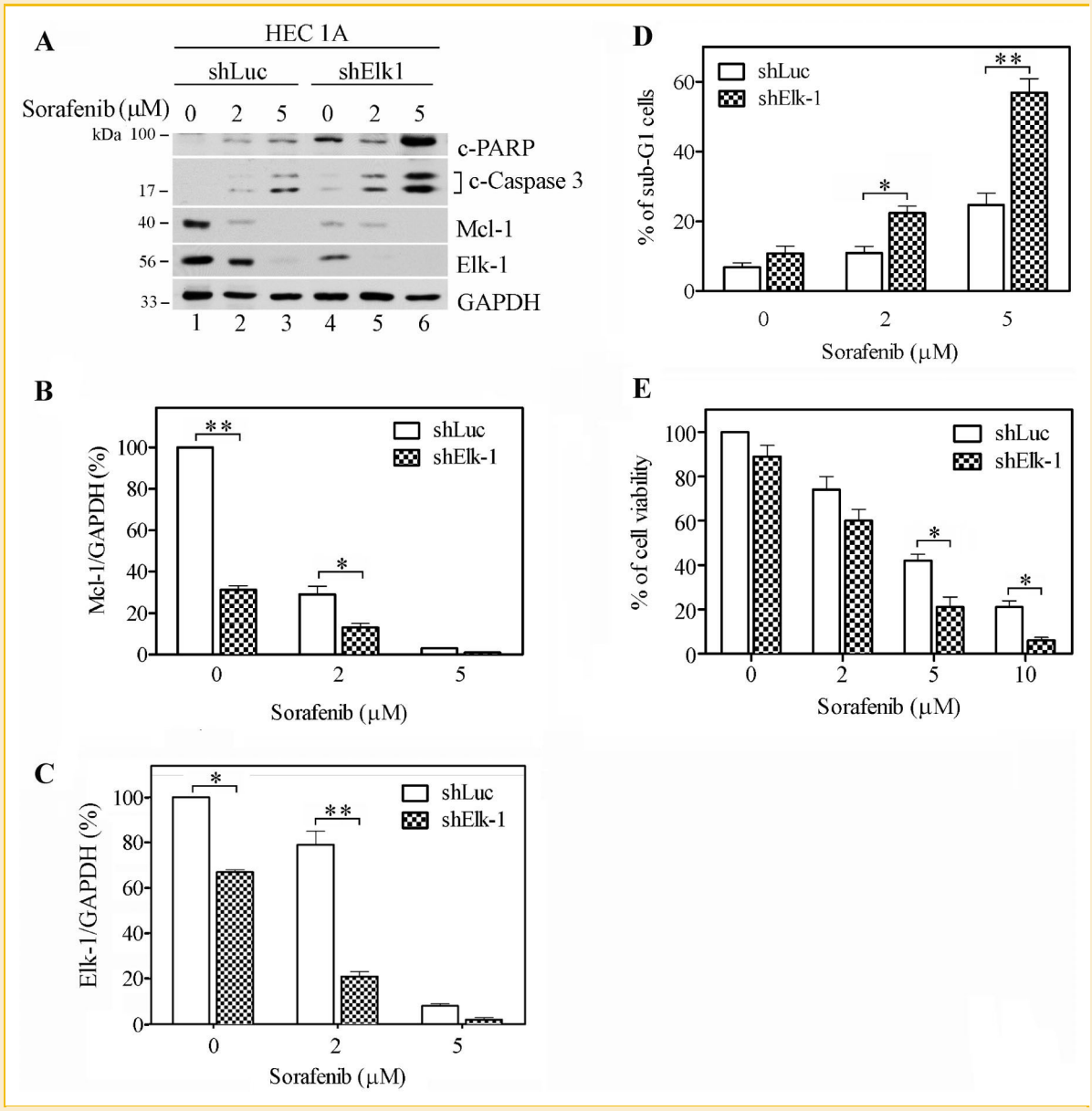

Fig. 5. Knockdown of Elk-1 reduces Mcl-1 protein level and potentiates apoptosis induction and growth inhibition by sorafenib. A: Reduction of Mcl-1 protein level by Elk-1 knockdown (shElk-1) is associated with reduced inhibition of caspase activity. B: Calculation of the reduction of Mcl-1 protein level by Elk-1 knockdown in A. The average was calculated from experiments repeated three times. C: Quantitative analysis of Elk-1 protein levels following Elk-1 knockdown as in A. D: Potentiation of sorafenib-induced sub-G1 cells by shElk-1. E: Reduction of sorafenib-induced growth inhibition by shElk-1. Symbol: $^{*}P < 0.05$; $^{**}P < 0.01$.

reduced by 50% by the GSK3 inhibitor. Furthermore, while the protein level of Mcl-1 was reduced by 70% by 5 μM of sorafenib (Fig. 8B, compares lane 1 and 2; Fig. 8C), reduction of this protein was partially recovered in cells treated with sorafenib and the GSK3 inhibitor (Fig. 8B, compare lanes 2 and 4; Fig. 8C). In addition, the induction of Mcl-1 phosphorylation was partially reversed by the GSK3 inhibitor. Similar to HEC1A cells, AKT/GSK3β-dependent protein degradation of Mcl-1 was also observed in RL95-2 cells following sorafenib treatment (data not shown). These results suggest that AKT and GSK3 regulate the stability of the Mcl-1 protein in EC cells following exposure to sorafenib.

DISCUSSION

In the present study, we found that sorafenib effectively inhibits the proliferation of EC cell lines. The IC50 of this drug varied for the three EC cell lines tested. We also found that the inhibition of cell proliferation by sorafenib is associated with induced apoptosis via down-regulation of the anti-apoptotic protein Mcl-1 in EC cells. Overexpression of Mcl-1 rescued cellular hypersensitivity to sorafenib, indicating that the protein level of Mcl-1 determines the response of EC cells to this drug. Among the anti-apoptotic Bcl-2 family proteins observed, only Mcl-1 was time- and

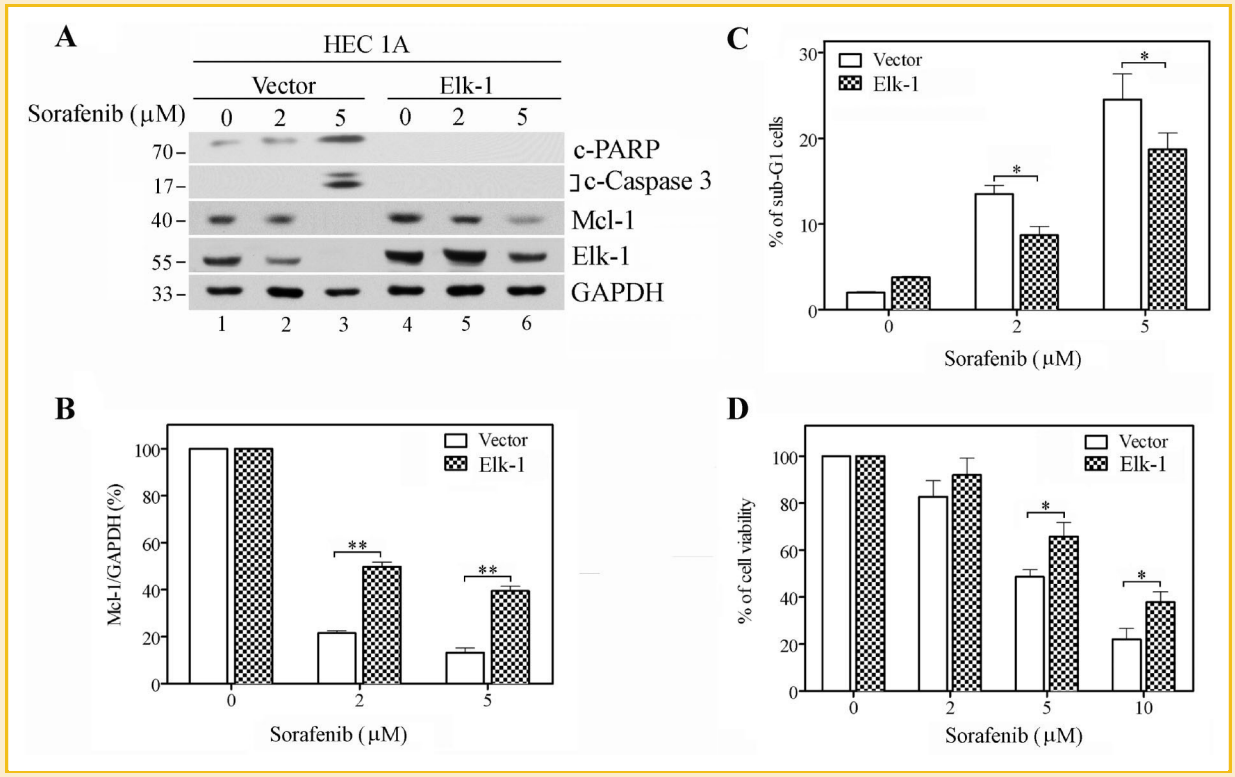

Fig. 6. Overexpression of Elk-1 increases Mcl-1 protein level and rescues sorafenib-induced cell death and apoptosis. A: Induction of Mcl-1 protein level by Elk-1 overexpression (Elk-1) is associated with increased inhibition of caspase activity. B: Quantitative analysis of Mcl-1 protein levels following Elk-1 overexpression as in A. Average protein levels were determined from experiments repeated three times. C: Reduction of sorafenib-induced sub-G1 cells by Elk-1 overexpression. D: Potentiation of sorafenib-induced cell growth inhibition by Elk-1 overexpression. $^{*}P < 0.05$; $^{**}P < 0.01$.

dose-dependently suppressed by sorafenib in this study. Several studies have shown that sorafenib is an Mcl-1 antagonist in melanoma and chronic lymphocytic leukemia [Panka et al., 2008; Huber et al., 2011]. Although the signaling pathway is not fully characterized, the importance of the down-regulation of Mcl-1 and c-FLIP$_L$ by sorafenib has been reported in human leukemia cells treated with TRAIL [Rosato et al., 2007]. These results support the notion that anti-apoptotic proteins such as Mcl-1 can be highly suppressed by sorafenib in cancer cells, and that these proteins may be used as biomarkers to predict the efficacy of cancer therapy in this context.

Our results also indicate that sorafenib inhibits both the transcriptional and post-translational pathways of Mcl-1 in EC cells. Poly-ubiquitination of Mcl-1 was enhanced by sorafenib, and was associated with enhanced protein degradation, which was in turn abolished by MG132, suggesting that the induced reduction of Mcl-1 may occur through proteasome degradation in HEC1A cells. Although ubiquitination was not investigated in HEC1B and RL95-2 cells due to low plasmid transfection efficiency, accumulation of Mcl-1 following treatment with a proteasome inhibitor suggests that sorafenib also causes Mcl-1 degradation in these cells. Furthermore, the level of Mcl-1 mRNA was down-regulated by sorafenib and an ERK1/2 inhibitor (PD98059; Fig. 3). Phosphorylation of Elk-1, which activates the ERK1/2-regulated transcription factor, was also down-regulated by sorafenib. These results suggest that suppression of Mcl-1 by sorafenib may occur through inhibition of Elk-1 transactivation activity in EC cells. This observation is supported by the ChIP assay which showed a reduction of Elk-1 binding to the Mcl-1 promoter in sorafenib-treated cells (Fig. 3D,E). However, Mcl-1 mRNA was suppressed by sorafenib in HEC1A cells after 4 h of incubation (Fig. 3A). Given that Elk-1 was bound to the Mcl-1 promoter starting at 4 h, and that this binding was suppressed by sorafenib at 16 h (Fig. 3E,F), it is unlikely that the suppression of mRNA is due to Elk-1 leaving the promoter. One possibility is that not all the bound Elk-1 is active in transactivation. Interestingly, the protein level of Elk-1 was also down-regulated by sorafenib in EC cells, but not by the ERK1/2 inhibitor (Fig. 3C). It is known that Elk-1 phosphorylation occurs at specific residues by MAPKs, including ERK. The phosphorylated Elk-1 translocates to the nucleus where, in association with serum response factor at the SRE site, it is critical for remodeling chromatin and triggering SRE-dependent transcription [Besnard et al., 2011]. Although MAPKs are also fundamental factors for cell response to chemotherapy, and extensive investigations of their downstream signaling partners have been conducted, no data have previously implicated Elk-1 in these processes. Mcl-1 protein was also down-regulated by PD98059 through enhanced poly-ubiquitination (Fig. 4). Our findings provide new insights to understand the downstream signaling partners of MAPKs in EC cells in response to sorafenib. We hypothesize that active Elk-1 may be suppressed by sorafenib at the transactivation step through reduced

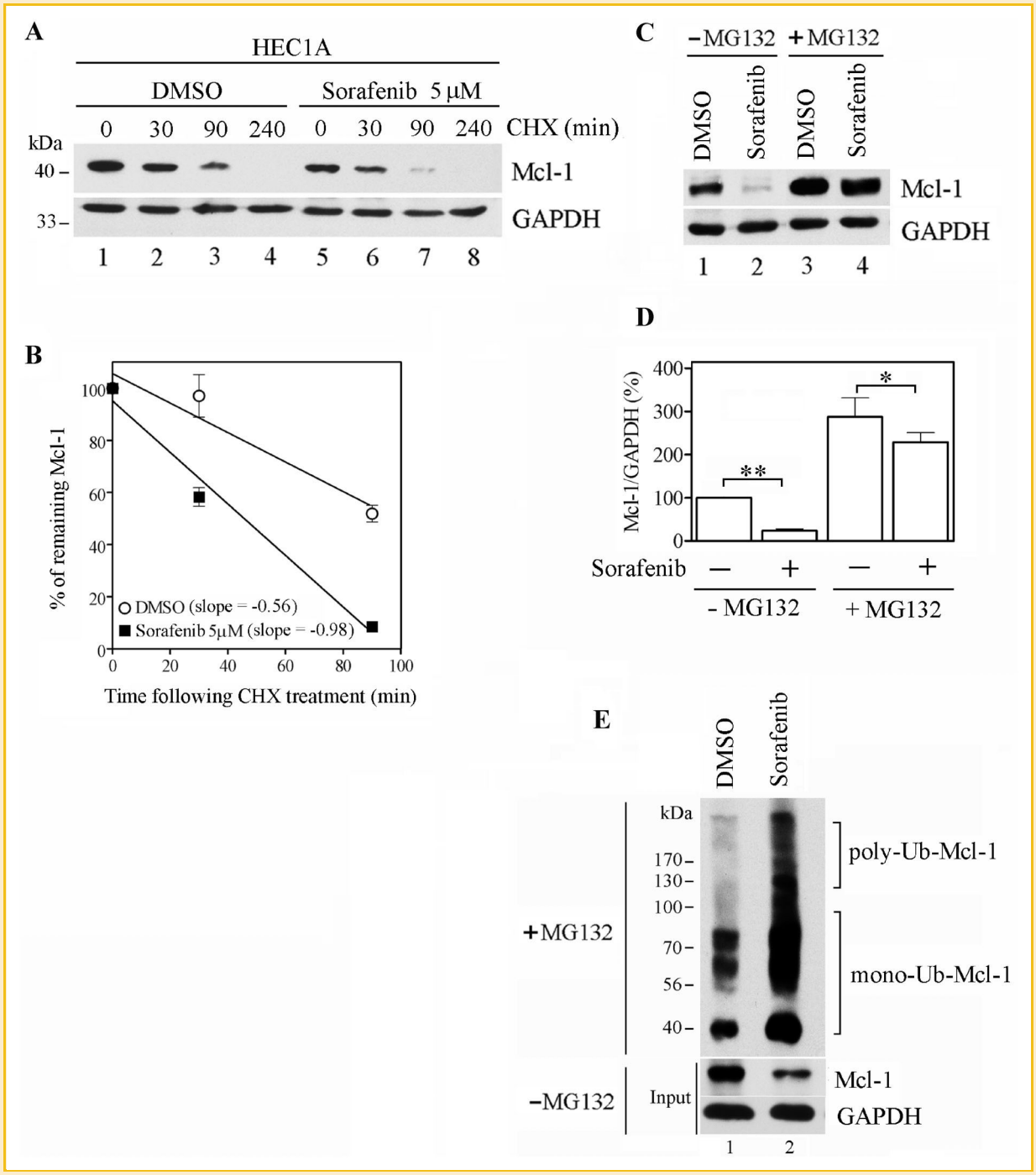

Fig. 7. Enhanced ubiquitination and degradation of Mcl-1 by sorafenib treatment. A: Enhanced degradation of the Mcl-1 protein in sorafenib-treated HEC1A cells. Cells were pre-incubated with cycloheximide (CHX), an inhibitor of protein synthesis, followed by the indicated incubation. B: Increase in the degradation rate of Mcl-1 protein by sorafenib shown in A. The slope of the linear regression representing the Mcl-1 protein level of sorafenib group and DMSO control was indicated. C: Immunoblot of Mcl-1 protein level in HEC1A cells following sorafenib treatment in the presence or absence of MG132. D: Quantitative analysis of Mcl-1 protein levels in response to sorafenib and MG132 as in C. Average protein levels were determined from experiments repeated three times. E: Increased ubiquitination of Mcl-1 protein in HEC1A cells following sorafenib treatment. Mcl-1 protein was eluted followed by immunoblotting with anti-ubiquitin antibody. Results of panel B are expressed as mean values ± SD for experiments performed in triplicate.

Elk-1 protein level and reduced active ERK, and the reduced ERK may also destabilize Mcl-1 protein through proteasome, leading to identification of a novel pathway that can be targeted by sorafenib in cancer therapy (Fig. 9, right path).

A previous study has shown that phosphorylated Mcl-1 is degraded by the proteasome [Maurer et al., 2006]. Using inhibition assay, our data indicate that GSK3β is involved in the regulation of Mcl-1 phosphorylation in an AKT-dependent manner (Fig. 8C,D). In

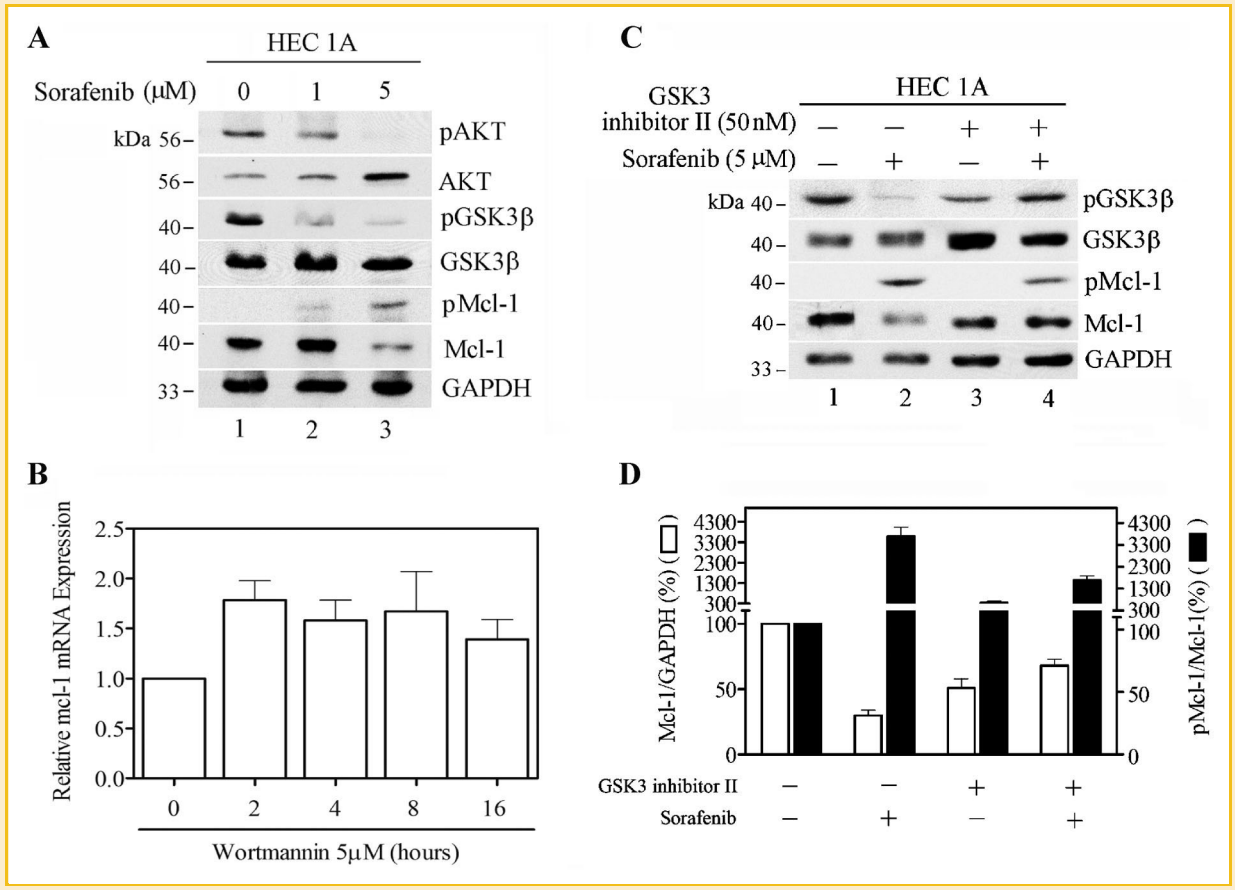

Fig. 8. Enhanced degradation of Mcl-1 protein by sorafenib via the AKT/GSK3β pathway. A: Inhibition of AKT/GSK3β phosphorylation and suppression of Mcl-1 protein level by sorafenib. B: Lack of suppression of Mcl-1 mRNA level by Wortmannin, a potent inhibitor of PI3Ks. C: Partial reversal of sorafenib-induced inhibition of GSK3β and Mcl-1 phosphorylation, and reversal of the inhibition of Mcl-1 protein level by GSK3 inhibitor II. D: Quantitative analysis of relative level of sorafenib-induced inhibition of Mcl-1 in the presence of GSK3 inhibitor II, and reversal of the induction of Mcl-1 phosphorylation by GSK3 inhibitor II as in B. Average protein levels were determined from experiments performed in triplicate. Relative levels of Mcl-1 protein are indicated.

addition, the active and phosphorylated form of AKT is inhibited by sorafenib in EC cells, and this is associated with reduced GSK3β phosphorylation (Fig. 8A). These results suggest that the PI3K/AKT/GSK3β pathway may mediate sorafenib-induced Mcl-1 degradation (Fig. 9, left path). The results of the level of Mcl-1 in sorafenib-treated HEC1A cells pretreated with MG132 are revealing. The Western blot experiments shown in Figure 7C reveal that the Mcl-1 protein is not fully stable when proteasome activity is inhibited. While Mcl-1 protein instability is enhanced by sorafenib through PI3K/AKT/GSK3β, the transcriptional suppression of Mcl-1 gene through inhibition of the ERK/Elk-1 pathway also appears to play a role in this context (Fig. 9, right path). Our results clearly show that sorafenib also down-regulates Mcl-1 protein level in other cell lines (Supplementary Fig. S2). However, the rate of Mcl-1 protein degradation is less affected by sorafenib in this context, suggesting that both the transcriptional suppression of Mcl-1 gene expression and the protein degradation of Mcl-1 are important in response to sorafenib. Taken together, the decrease of Mcl-1 protein induced by sorafenib in EC cells appears to occur through two pathways: ERK/Elk-1-dependent transcriptional suppression and AKT/GSK3β- (also ERK-) dependent protein degradation (Fig. 9).

Few ubiquitin ligases have been found to be responsible for Mcl-1 degradation (e.g., FBW7, b-TrCP, and MULE) while de-ubiquitinases (e.g., USP9X) are involved in preventing degradation of Mcl-1 [Vucic et al., 2011]. For example, a body of literature revealed that the F-box and WD repeat domain containing seven protein (FBW7) is involved in drug resistance in human cancers (Inuzuka et al., 2011a, b; Wertz et al., 2011). There is an intimate correlation between the loss of FBW7 and drug resistance, and the E3 activity of FBW7 targets a variety of substrate proteins for ubiquitination and degradation, including Mcl-1 (Inuzuka et al., 2011b; Wang et al., 2011; Wertz et al., 2011). It will be interesting to see whether the proteasomal degradation of Mcl-1 induced by sorafenib is mediated by FBW7 or b-TrCP in EC cells. Profiling the E3 ligase (FBW7 or b-TrCP) and Mcl-1 status of tumors, in terms of protein levels, mRNA levels and genetic status may thus be useful to predict the response of patients to chemotherapy.

Sorafenib also possesses anti-angiogenic properties, and is currently used in combination with other traditional chemotherapeutic drugs in hepatocellular carcinoma [Wilhelm et al., 2006; Llovet and Bruix, 2008; Whittaker et al., 2010]. These results strongly suggest that vascular endothelial growth factor receptors

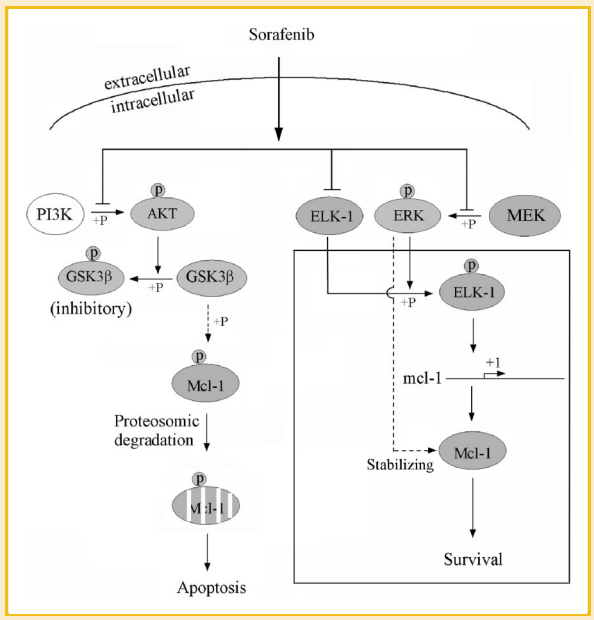

Fig. 9. Hypothetic model of illustrating the way sorafenib suppresses Mcl-1 expression and induces apoptosis. The underlying mechanism includes two pathways: potentiation of Akt/GSK3-dependent protein degradation and blockage of Elk-1-dependent transactivation of Mcl-1.

(VEGFRs), which have been found to be highly expressed in endometrioid cancer, are key targets for anticancer therapy. To assess this possibility, we examined the protein level of VEGFR in EC cell lines. Multiple VEGFRs were detected in these cells by Western blot with an anti-VEGFR2 antibody (Supplementary Fig. S3). The protein level of VEGFRs observed was in the following order: HEC1A > RL95-2 > HEC1B. Furthermore, the epidermal growth factor receptor (EGFR) protein was extremely abundant in RL95-2, compared to the other cell lines (data not shown). Given that HEC1A and RL95-2 display, respectively the most sensitive response and the most resistant response to sorafenib in this study, it appears that the protein level of VEGFRs may not be the only critical factor responsible for cell sensitivity to the drug.

Given that various kinase pathways are associated with these oncogene products or anti-apoptotic proteins, we hypothesize that the biological significance of the signal pathways associated with these proteins may be treatable with sorafenib, depending on the types of cancer cells involved. Our finding that Mcl-1 is targeted by sorafenib in EC cells appears to be important for the development of an alternative drug regimen for cancer cell therapy. Our findings together with those of others groups also support the notion that the mechanism of action of sorafenib on cancer cells involves multiple pathways.

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