Regorafenib induces extrinsic and intrinsic apoptosis through inhibition of ERK/NF-κB activation in hepatocellular carcinoma cells

JAI-JEN TSAI[1-3], PO-JUNG PAN[3,4] and FEI-TING HSU[5-7]

[1]Division of Gastroenterology, Department of Medicine, National Yang-Ming University Hospital, Yilan 260; [2]Institute of Traditional Medicine, School of Medicine, National Yang-Ming University, Taipei 112; [3]Cancer Medical Care Center and [4]Department of Physical Medicine and Rehabilitation, National Yang-Ming University Hospital, Yilan 260; [5]Department of Medical Imaging, Taipei Medical University Hospital, Taipei 110; [6]Research Center of Translational Imaging (TIRC) and [7]Department of Radiology, College of Medicine, Taipei Medical University, Taipei 110, Taiwan, R.O.C.

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Abstract. The aim of the present study was to investigate the role of NF-κB inactivation in regorafenib-induced apoptosis in human hepatocellular carcinoma SK-HEP-1 cells. SK-HEP-1 cells were treated with different concentrations of the NF-κB inhibitor 4-N-[2-(4-phenoxyphenyl)ethyl] quinazoline-4,6-diamine (QNZ) or regorafenib for different periods. The effects of QNZ and regorafenib on cell viability, expression of NF-κB-modulated anti-apoptotic proteins and apoptotic pathways were analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, western blotting, DNA gel electrophoresis, flow cytometry and NF-κB reporter gene assay. Inhibitors of various kinases including AKT, c-Jun N-terminal kinase (JNK), P38 and extracellular signal-regulated kinase (ERK) were used to evaluate the mechanism of regorafenib-induced NF-κB inactivation. The results demonstrated that both QNZ and regorafenib significantly inhibited the expression of anti-apoptotic proteins and triggered extrinsic and intrinsic apoptosis. We also demonstrated that regorafenib inhibited NF-κB activation through ERK dephosphorylation. Taken all together, our findings indicate that regorafenib triggers extrinsic and intrinsic apoptosis through suppression of ERK/NF-κB activation in SK-HEP-1 cells.

Correspondence to: Dr Fei-Ting Hsu, Department of Medical Imaging, Taipei Medical University Hospital, 252 Wu Hsing Street, Taipei 110, Taiwan, R.O.C.
E-mail: sakiro920@gmail.com

Abbreviations: MMP, mitochondrial membrane potential; C-FLIP, cellular FLICE-like inhibitory protein; XIAP, X-linked inhibitor of apoptosis protein; Mcl-1, myeloid leukemia cell differentiation protein; NF-κB, nuclear factor-κB; AKT, protein kinase B; ERK, extracellular signal-regulated kinase; QNZ, NF-κB inhibitor; JNK, Jun amino-terminal kinases; P38, P38 mitogen-activated protein kinase

Key words: regorafenib, ERK/NF-κB, hepatocellular carcinoma

Introduction

Apoptosis, or programmed cell death, occurs through both extrinsic and intrinsic pathways, which are modulated by apoptotic proteins including cytochrome *c*, caspase-8 and -3 (1). Morphological and biochemical hallmarks of apoptosis include cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (2). Anticancer agents and radiation are able to induce apoptotic protein-mediated signal transduction pathways and consequent inhibition of tumor growth (1,3). Anticancer agents and radiation-induced apoptosis can be blocked by overexpression of anti-apoptotic proteins in cancer cells leading to treatment failure (4). Human hepatocellular carcinoma (HCC) is endemic in Asia and among the deadliest types of cancers (5). Overexpression of anti-apoptotic proteins such as cellular FLICE-like inhibitory protein (c-FLIP), myeloid cell leukemia-1 (Mcl-1), and X-linked inhibitor of apoptosis protein (XIAP) has been identified in HCC and is associated with the poor prognosis of HCC patients (6-8).

Nuclear factor-κB (NF-κB) is a transcription factor of a number of oncogenes which modulate tumorigenesis (9). Cancer hallmarks that include self-sufficiency in proliferative growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis have been related to NF-κB-modulated expression of downstream effector proteins (10). Various anticancer agents and radiation not only trigger apoptosis, but also activate expression of NF-κB-induced anti-apoptotic proteins resulting in the reduction of therapeutic efficacy in HCC both *in vitro* and *in vivo* (11,12). Constitutive NF-κB activation is observed in patients with advanced HCC and may be used as a negative prognostic biomarker (13). Therefore, development of NF-κB signal inhibitors may facilitate the treatment of HCC patients.

Regorafenib (Stivarga®) is a multi-kinase inhibitor with a similar chemical structure to sorafenib (Nexavar®), but has an additional functional group, which produces more potent activity to inhibit oncogenic receptor tyrosine and cytoplasmic signaling kinases (14). Regorafenib has been approved to treat colorectal cancer and gastrointestinal stromal tumors. A recent update of an ongoing phase III clinical trial reported that regorafenib was effective in patients with sorafenib-resistant HCC (15). In our previous study, sorafenib, as an inhibitor of NF-κB signaling, was found to reduce the expression of NF-κB-modulated anti-apoptotic proteins in HCC both *in vitro* and *in vivo* (12). However, whether regorafenib, an analogue of sorafenib, can induce apoptosis through blockage of NF-κB activation in HCC cells remains obscure. The aim of the present study was to investigate the role of NF-κB inactivation on regorafenib-induced apoptosis in SK-HEP-1 cells using MTT assay, flow cytometry, DNA gel electrophoresis, western blotting and NF-κB reporter gene assay. ERK, AKT, JNK and P38 inhibitors were used to determine the mechanism of regorafenib-induced NF-κB inactivation in HCC.

Materials and methods

Agents and antibodies. Regorafenib was provided by Bayer Health Care Pharmaceuticals (Whippany, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin (PS) were purchased from Gibco/Life Technologies (Carlsbad, CA, USA). Propidium iodide (PI) and $DiOC_6$ were purchased from BioVision (Mountain View, CA, USA) and Enzo Life Sciences (Farmingdale, NY, USA), respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and RNase were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fermentas (St. Leon-Rot, Baden-Württemberg, Germany), respectively. Primary antibodies of cleaved-caspase-3, cellular FADD-like IL-1β-converting enzyme (FLICE)-inhibitory protein (c-FLIP) and pAKT (Ser473) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies of caspase-8 and X-linked inhibitor of apoptosis protein (XIAP) were obtained from Thermo Fisher Scientific (Fremont, CA, USA). Primary antibodies of ERK, AKT, NF-κB p65, β-actin and cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies of MCL-1 and pERK were purchased from BioVision (Milpitas, CA, USA) and Merck Millipore (Billerica, MA, USA), respectively. Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Nuclear and Cytoplasmic Extraction and Genomic DNA Miniprep kits were obtained from Chemicon (Temecula, CA, USA) and Axygen Biosciences (Union City, CA, USA), respectively. NF-κB inhibitor 4-N-[2-(4-phenoxyphenyl)ethyl]quinazoline-4,6-diamine (QNZ), AKT inhibitor LY294002, c-Jun N-terminal kinase (JNK) inhibitor SP600125, P38 inhibitor SB203580 and extracellular signal-regulated kinase (ERK) inhibitor PD98059 were purchased from Selleckchem (Houston, TX, USA). NF-κB-luciferrase2 vector (pNF-κB/*luc2*) and D-luciferin were obtained from Promega (Madison, WI, USA) and Caliper Life Science (Hopkinton, MA, USA), respectively. Hygromycin B was purchased from Santa Cruz Biotechnology.

Cell culture. SK-HEP-1 cells were gifted by Professor Jing-Gung Chung of the Department of Biological Science and Technology, China Medical University, (Taichung, Taiwan). Cells were cultured in DMEM and supplemented with 10% FBS, 2 mM L-glutamine and PS (100 U/ml and 100 μg/ml) at 37˚C in an atmosphere of 5% CO_2 (16).

Plasmid transfection and stable clone selection. SK-HEP-1 cells were transfected with pNF-κB/*luc2* using JetPEI™. Cells ($1x10^6$) were seeded into 10-cm dish and incubated overnight. DNA solution (10 μg NF-κB/luc2 plasmid dissolved in 250 μl of 150 mM NaCl) was mixed with 250 μl JetPEI solution (20 μl of JetPEI reagent diluted in 230 μl of 150 mM NaCl), and then incubated for 30 min at room temperature to make 500 μl DNA/JetPEI mixture. The DNA/JetPEI mixture was added to the SK-HEP-1 cells in a 10-cm diameter dish and incubated for 24 h. After transfection, the cells were cultured in medium containing 200 μg/ml of hygromycin B for two weeks. The surviving clones were subsequently subcultured into 96-well plates. Function of the NF-κB reporter gene in each clone was assayed using the IVIS 200 Imaging System (Xenogen, Alameda, CA, USA). Cells with functional NF-κB reporter gene product were renamed as SK-HEP-1/NF-κB-*luc2* cells (12).

MTT assay. SK-HEP-1 cells were seeded into 96-well plates at a density of $3x10^4$ cells/well and incubated overnight. Cells were treated with different concentrations of QNZ (0-0.4 μM in 0.1% DMSO) or regorafenib (0-50 μM in 0.1% DMSO) for different periods, and then the change in cell viability was determined with the MTT assay as previously described (17).

Detection of mitochondrial membrane potential (MMP). SK-HEP-1 cells were seeded into 12-well plates at a density of $2x10^5$ cells/well and incubated overnight. Cells were treated with 0.4 μM QNZ or 20 μM regorafenib for different periods. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS), and then stained with $DiOC_6$ solution (4 μM $DiOC_6$ in 500 μl PBS) for 30 min at 37˚C. Detection of MMP was performed using flow cytometry (FACS101; FACScan; Becton-Dickinson, Franklin Lakes, NJ, USA) as described by Wang *et al* (18).

Analysis of the sub-G_1 population. SK-HEP-1 cells were seeded into 12-well plates at a density of $2x10^5$ cells/well and incubated overnight. Cells were treated with 0.4 μM QNZ or 20 μM regorafenib for different periods. The cells were harvested by centrifugation and fixed with 70% ethanol and incubated overnight at -20˚C. Cells were washed twice with PBS and then stained with 500 μl of PI buffer (40 μg/ml PI, 100 μg/ml RNase and 1% Triton X-100 in PBS) for 1 h in darkness at room temperature. Detection of the sub-G_1 cell population was performed using flow cytometry (FACS101; FACScan) as described by Huang *et al* (19).

Western blot assay. SK-HEP-1 cells ($3x10^6$) were seeded into 10-cm diameter dishes and incubated overnight. Then, the cells were treated with 0.4 μM QNZ or 20 μM regorafenib for different periods. Lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40 and 1 mM phenylmethanesulfonyl

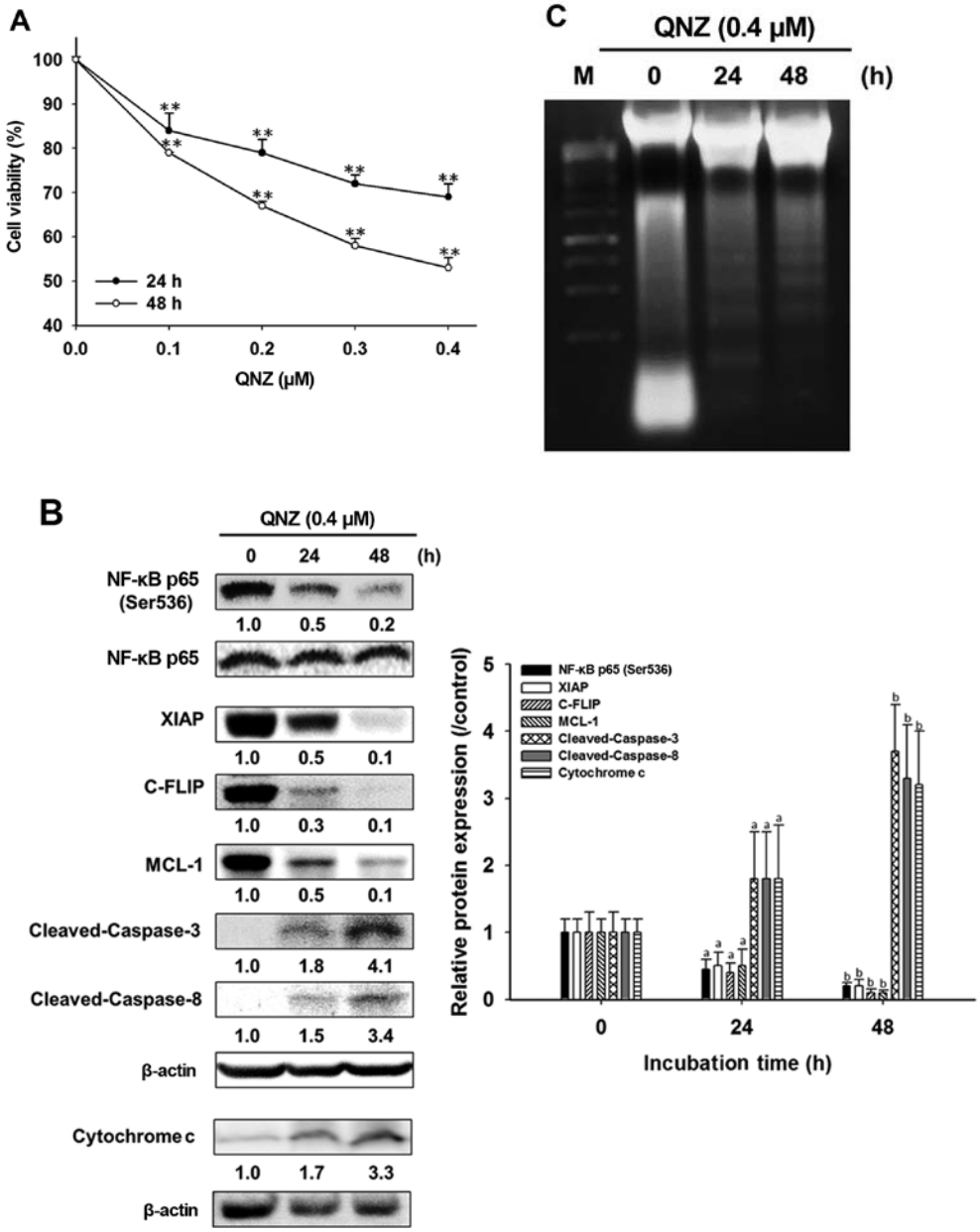

Figure 1. Effects of the NF-κB inhibitor QNZ on cell viability, expression of NF-κB-modulated anti-apoptotic proteins and apoptosis pathways in SK-HEP-1 cells. SK-HEP-1 cells were treated with different concentrations (0, 0.1, 0.2, 0.3 and 0.4 μM in 0.1% DMSO) of QNZ for 24 and 48 h. (A) Change in cell viability was determined with the MTT assay. **p<0.01. (B) Protein levels of NF-κB p65 (Ser536), XIAP, c-FLIP, MCL-1, cleaved-caspase-3 and -8, and cytochrome *c* were evaluated using western blot assay. ^{a}p<0.05 and ^{b}p<0.01 as compared with the control. (C) Detection of DNA fragmentation was performed using gel electrophoresis.

fluoride) was used for total protein extraction from the cells in the different treatment groups. Cytosolic proteins from cells in each group were extracted using a cytosol extraction kit following the instructions provided by the manufacturer. Protein expression of NF-κB p65, NF-κB p65 (Ser536), XIAP, Mcl-1, c-FLIP, cleaved-caspase-3, caspase-8, cytochorme *c*, ERK, pERK, AKT and pAKT were evaluated with western blot assay as described by Ting *et al* (20). Quantification of protein bands was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Detection of DNA fragmentation. SK-HEP-1 cells were seeded into 6-well plates at a density of 1x10^6 cells/well and incubated overnight, and then treated with 0.4 μM QNZ or 20 μM regorafenib for different periods. Genomic DNA from the cells was purified using the GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) following the instructions provided by the manufacturer. Analysis of DNA fragmentation was performed using 1.5% agarose gel electrophoresis (12).

NF-κB reporter gene assay. SK-HEP-1 cells were seeded into 96-well plates at a density of 3x10^4 cells/well and incubated overnight. The detailed conditions for the different treatment groups are provided in detail in the figure legends. D-luciferin solution (500 μM D-luciferin in 100 μl PBS) was added to each well, and photon signal was acquired for 1 min using the IVIS

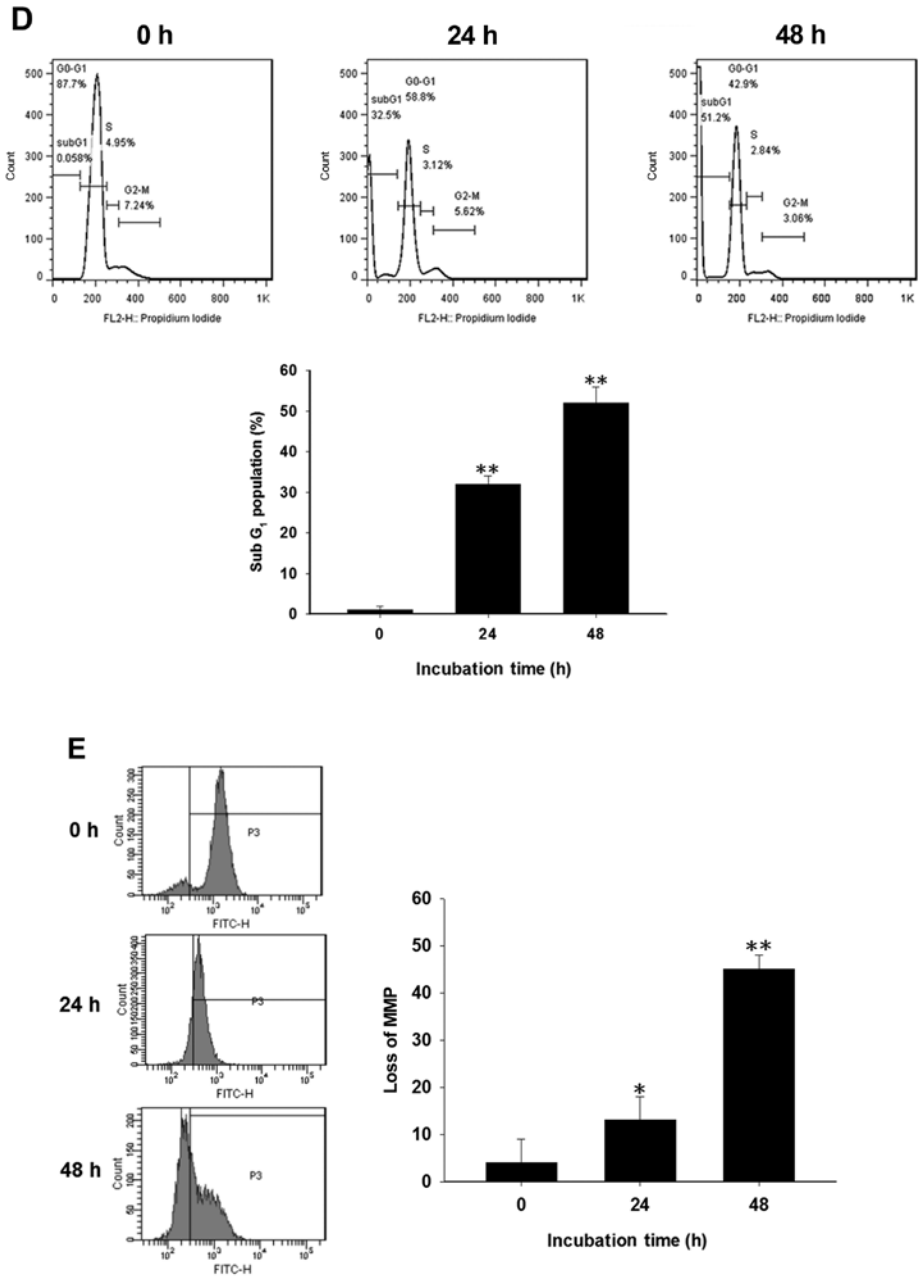

Figure 1. Continued. (D) Determination of the sub-G_1 cell population was carried out using flow cytometry. $^{**}p<0.01$ as compared with the control. (E) The change in MMP was investigated using flow cytometry. $^{*}p<0.05$ and $^{**}p<0.01$ as compared with the control.

200 Imaging System. Relative NF-κB activity was corrected by cell viability which was evaluated by the MTT assay as previously described (17).

Statistical analysis. Results are all representative of at least three independent experiments. Statistical significance was determined using the Student's t-test. p-values of <0.05 were considered statistically significant.

Results

NF-κB inhibitor diminishes the expression of anti-apoptotic proteins and induces both extrinsic and intrinsic apoptosis in the SK-HEP-1 cells. In order to verify the effects of NF-κB inactivation on pro-apoptotic and anti-apoptotic signal transduction, SK-HEP-1 cells were initially treated with different concentrations of QNZ for different periods. Subsequently, cell viability, expression of NF-κB p65 (Ser536), anti-apoptotic and pro-apoptotic proteins, and the effects of apoptosis were evaluated with MTT assay, western blotting, DNA gel electrophoresis and flow cytometry. Fig. 1A indicates that QNZ significantly reduced cell viability in a dose- and time-dependent manner as compared to that noted in the control cells (vehicle treatment with 0.1% DMSO). Fig. 1B shows that QNZ not only inhibited expression of NF-κB p65 (Ser536) and anti-apoptotic proteins (XIAP, MCL-1 and c-FLIP), but also increased levels of pro-apoptotic proteins (cleaved-caspase-3 and -8, and cytochrome *c*). DNA fragmentation

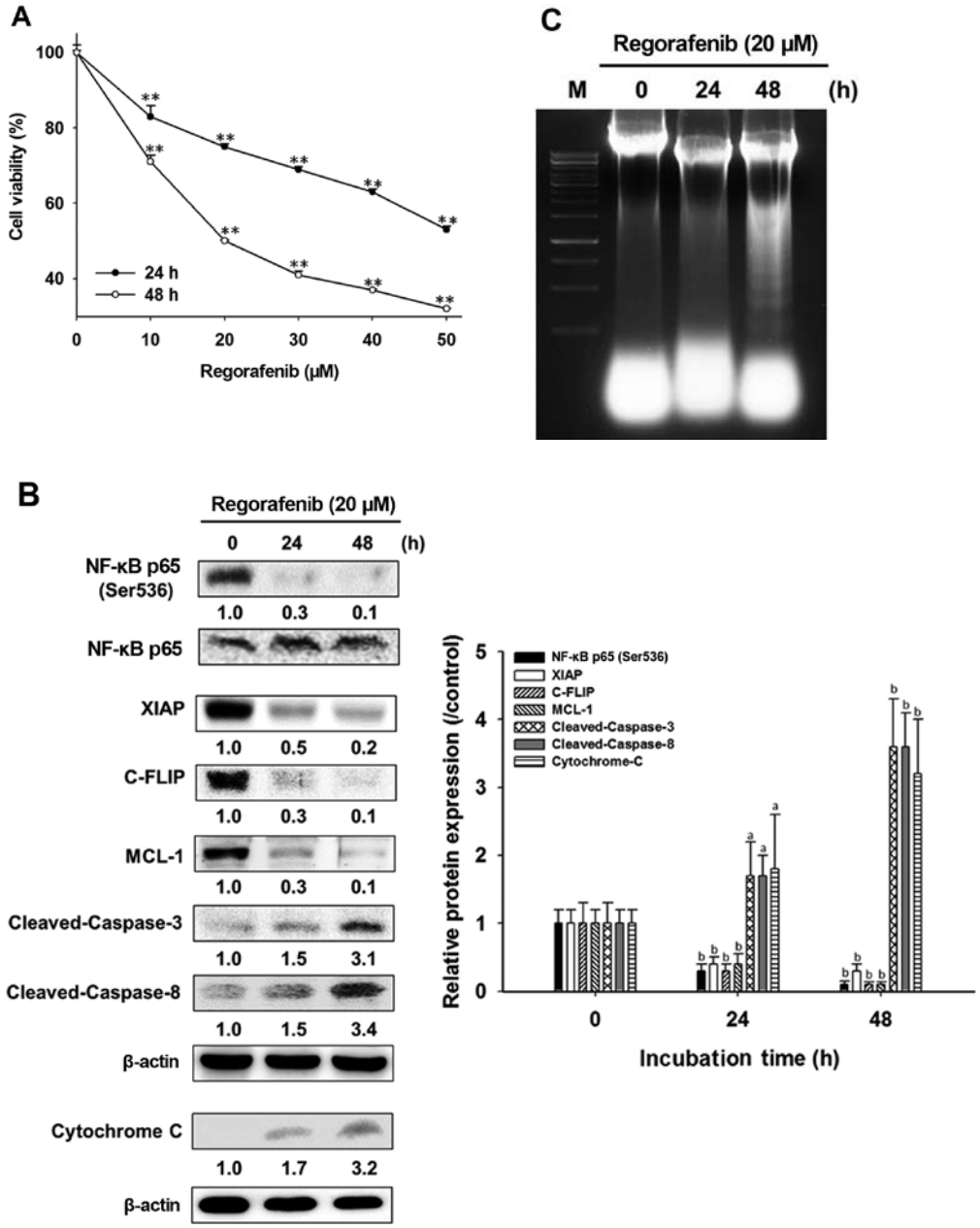

Figure 2. Effects of regorafenib on cell viability, expression of NF-κB-modulated anti-apoptotic proteins and apoptosis pathways in SK-Hep1 cells. Cells were treated with different concentration (0, 10, 20, 30, 40 and 50 μM in 0.1% DMSO) of regorafenib for 24 and 48 h. (A) Change in cell viability was determined with MTT assay. **p<0.01. (B) Protein levels of NF-κB p65 (Ser536), XIAP, c-FLIP, MCL-1, cleaved-caspase-3 and -8, and cytochrome *c* were evaluated by western blot assay. ^{a}p<0.05 and ^{b}p<0.01 as compared with the control. (C) Detection of DNA fragmentation was performed using gel electrophoresis.

is one of the apoptotic hallmarks and QNZ-induced DNA fragmentation is demonstrated in Fig. 1C. Apoptosis also can be measured by flow cytometry to detect the sub-G_1 cell population and MMP. The sub-G_1 cell population and loss of MMP were significantly enhanced by regorafenib treatment in a time-dependent manner as compared to the control (Fig. 1D and E).

Regorafenib inhibits expression of NF-κB-modulated anti-apoptotic proteins and induces both extrinsic and intrinsic apoptosis in the SK-HEP-1 cells. The SK-HEP-1 cells were treated with different concentrations of regorafenib for different periods. Cell viability, expression of NF-κB p65 (Ser536), expression of anti-apoptotic and pro-apoptotic proteins, and regorafenib effects on apoptosis were evaluated with MTT assay, western blotting, DNA gel electrophoresis, and flow cytometry. Regorafenib significantly decreased cell viability in a dose- and time-dependent manner as compared to that noted in the control cells (Fig. 2A). Regorafenib also significantly inhibited expression of NF-κB p65 (Ser536) and anti-apoptotic proteins (XIAP, MCL-1 and c-FLIP) while increased the levels of pro-apoptotic proteins (cleaved-caspase-3 and -8, and cytochrome *c*) in a time-dependent manner as compared to the control group (Fig. 2B). Fig. 2C shows that regorafenib induced DNA fragmentation and significantly induced the sub-G_1 cell population and loss of MMP in a time-dependent manner as compared to the control (Fig. 2D and E).

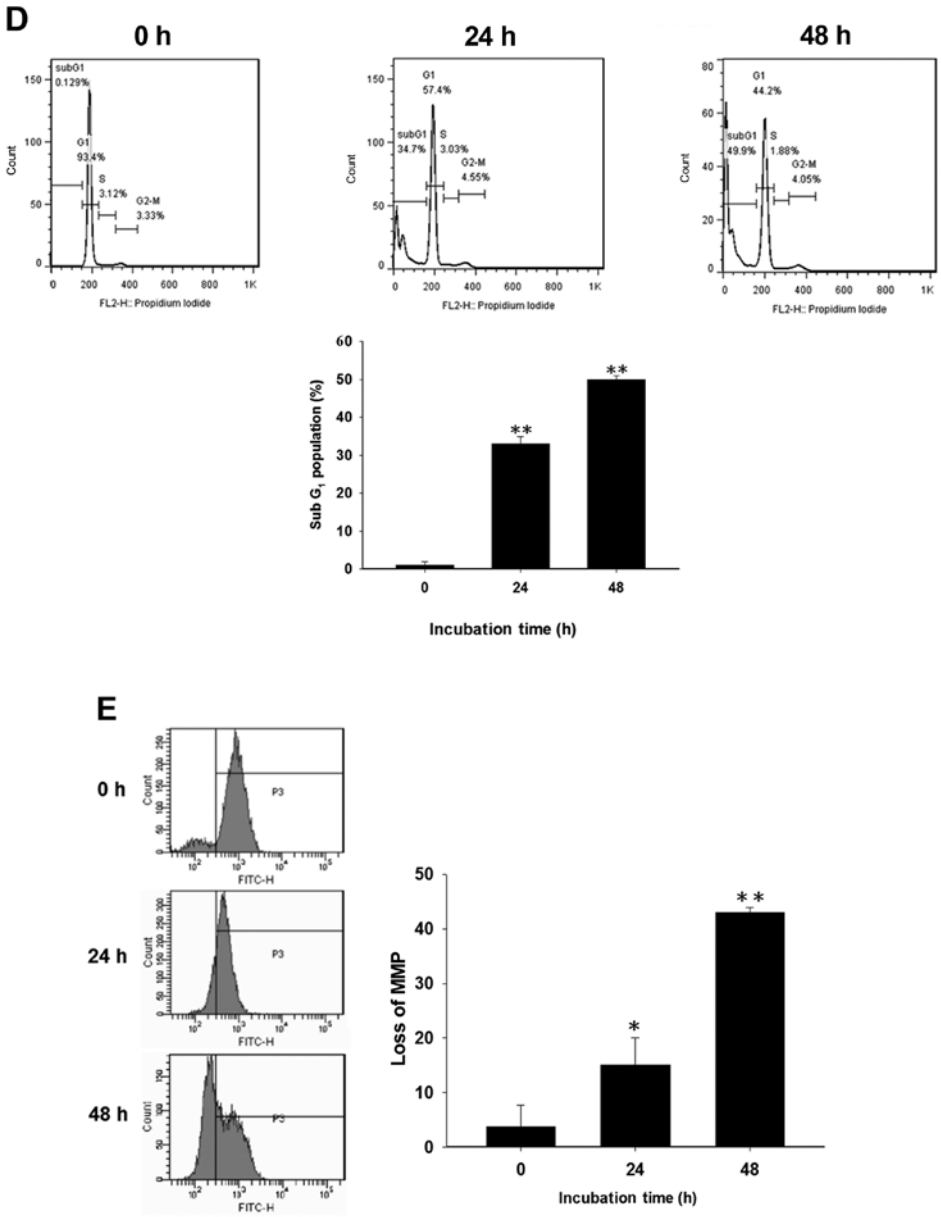

Figure 2. Continued. (D) Determination of Sub G_1 population was performed using flow cytometry. $^{**}p<0.01$ as compared with the control. (E) Change of MMP was investigated using flow cytometry. $^{*}p<0.05$ and $^{**}p<0.01$ as compared with the control.

Regorafenib inhibits NF-κB activation through ERK dephosphorylation in the SK-HEP-1 cells. We found that regorafenib reduced NF-κB activation and this turns regorafenib into an inhibitor of NF-κB signaling. In the next step, we used different kinase (AKT, JNK, P38 and ERK) inhibitors to investigate the mechanism of regorafenib-induced NF-κB inactivation in the SK-HEP-1 cells. Fig. 3A and B shows that regorafenib, QNZ (NF-κB inhibitor) and the ERK inhibitor (PD98059) significantly reduced NF-κB activation. Fig. 3C indicates that regorafenib also inhibited ERK and AKT phosphorylation in a time-dependent manner in the SK-HEP-1 cells.

Discussion

Regorafenib, a sorafenib analogue, has been approved for the treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumors (15). Sorafenib, as an inhibitor of NF-κB signaling, was indicated in our previous study to reduce the expression of NF-κB-modulated anti-apoptotic proteins and trigger the apoptotic pathway in HCC both *in vitro* and *in vivo* (12). However, whether regorafenib induces apoptosis through inhibition of NF-κB activation in HCC cells requires elucidation. Therefore, we evaluated the effects of regorafenib on NF-κB inhibition-related apoptosis and the mechanism in HCC SK-HEP-1 cells *in vitro*. First, we found that the NF-κB inhibitor QNZ reduced NF-κB activation and anti-apoptotic protein levels (XIAP, c-FLIP and MCL-1) while triggered extrinsic and intrinsic apoptotic pathways (Fig. 1A-E). Secondly, regorafenib as inhibitor of NF-κB signaling also suppressed NF-κB activation and anti-apoptotic protein levels, while induced extrinsic and intrinsic apoptotic pathways (Fig. 2A-E). Finally, we found that the ERK inhibitor

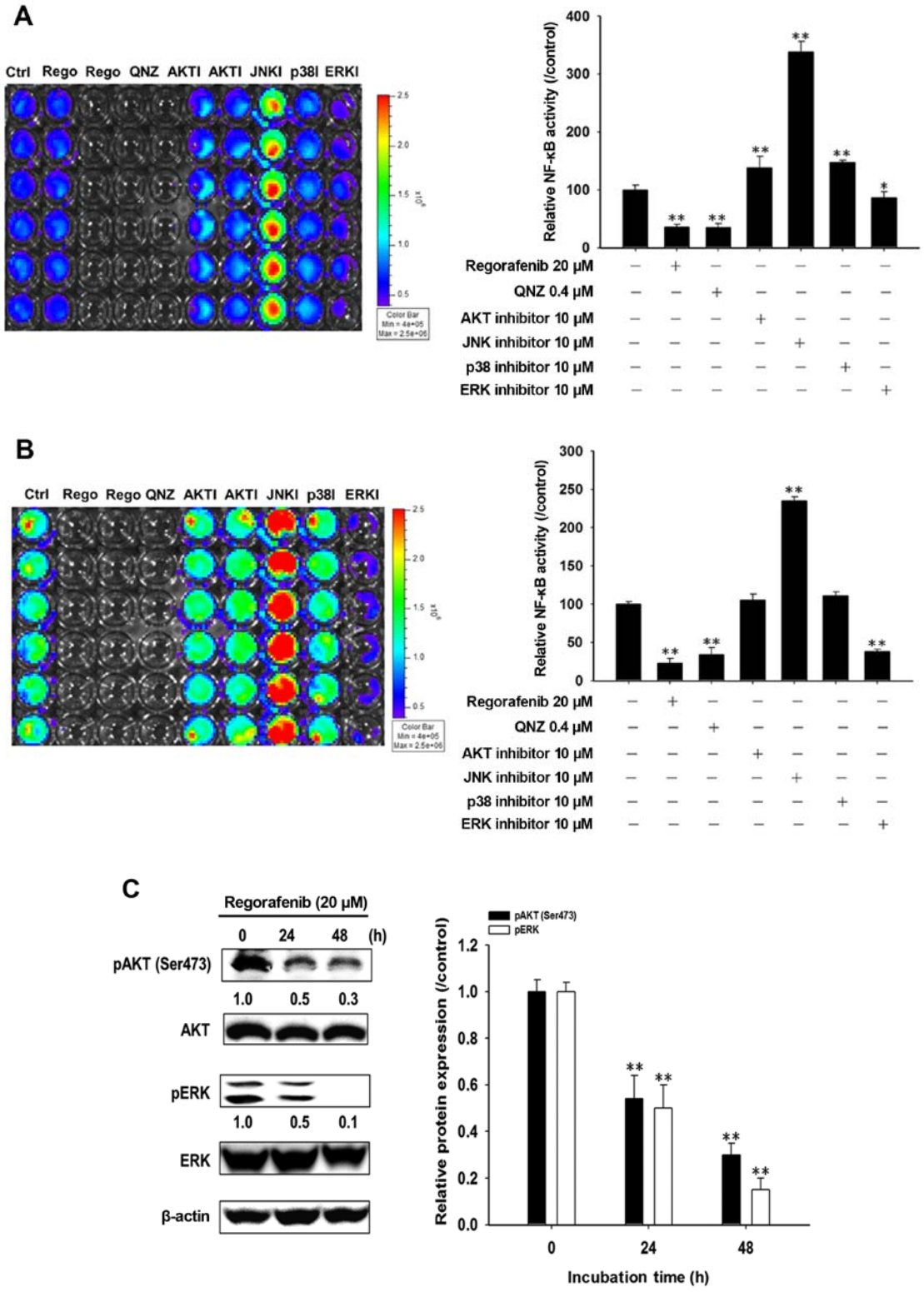

Figure 3. Effects of different kinase inhibitors on NF-κB activation and the effects of regorafenib on ERK and AKT phosphorylation in SK-Hep1 cells. SK-Hep1 cells were treated with 20 μM regorafenib, 0.4 μM QNZ and 10 μM various kinase (AKT, JNK, P38 and ERK) inhibitors for 24 and 48 h. (A) NF-κB activation was evaluated with NF-κB reporter gene assay after treatment for 24 h. Images were acquired by IVIS 200. (B) NF-κB activation was evaluated with NF-κB reporter gene assay after treatments for 48 h. Images were acquired by IVIS 200. (C) Protein expression of phosphorylated ERK and AKT (Ser473) was determined with western blot assay. *p<0.05 and **p<0.01 as compared with the control.

reduced NF-κB activation and regorafenib diminished ERK phosphorylation (Fig. 3A and B).

Expression of anti-apoptotic proteins such as XIAP, c-FLIP and MCL-1 is linked to constitutive NF-κB activation in cancer cells (12,21). XIAP can interact with the active site of caspase-3 resulting in inhibition of caspase-3-mediated apoptosis (22). c-FLIP, a caspase-8 inhibitor, disrupts caspase-8 and prevents initiation of the extrinsic

apoptotic pathway (23). MCL-1 suppresses loss of MMP and cytochrome *c* release from mitochondria that subsequently leads to inhibition of the intrinsic apoptotic pathway (24,25). The present study results demonstrated that both the NF-κB inhibitor and regorafenib inhibited NF-κB activation, reduced anti-apoptotic protein (XIAP, c-FLIP and MCL-1) expression, and activated extrinsic and intrinsic apoptotic pathways. Chen *et al* suggested that regorafenib activates NF-κB-regulated expression of p53-upregulated modulator of apoptosis (PUMA) and inhibits colorectal tumor growth (26). RAF/mitogen-activated protein kinase kinase (MEK)/ERK and phosphoinositide 3-kinase (PI3K)/AKT signaling transduction are the most critical pathways in the development and progression of HCC. Activation of ERK and AKT can be used as biomarkers to predict poor prognosis in HCC (27). Sorafenib induces apoptosis and inhibits angiogenesis in HCC via blockage of the RAF/MEK/ERK pathway. However, AKT activation is not inhibited by sorafenib (28). Fig. 3C shows that regorafenib significantly reduced both ERK and AKT phosphorylation. NF-κB can be activated through different kinases, such as AKT, JNK, P38 or ERK in different types of cancer cells (12,29-30). We used inhibitors of AKT, JNK, P38 and ERK to verify the mechanism of regorafenib-induced NF-κB inactivation in the SK-HEP-1 cells. We found that the ERK inhibitor revealed similar effects in the inhibition of NF-κB activation as regorafenib or QNZ (Fig. 3A and B). Therefore, we suggest that regorafenib inhibits NF-κB activation via dephosphorylation of ERK. In previous studies, we also found that sorafenib inhibited NF-κB-modulated tumor progression through suppression of ERK activation in HCC Huh7 cells (12,17).

In conclusion, the present study demonstrated that regorafenib triggered extrinsic and intrinsic apoptotic pathways through blockage of ERK/NF-κB activation in SK-HEP-1 cells *in vitro*. We propose that regorafenib may be a potential anticancer agent for the treatment of advanced HCC.

Acknowledgements

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