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Growth Inhibition by the Farnesyltransferase Inhibitor FTI-277 Involves Bcl-2 Expression and Defective Association with Raf-1 in Liver Cancer Cell Lines

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ABSTRACT

Farnesyltransferase inhibitors (FTIs) block the growth of tumor cells in vitro and in vivo with minimal toxicity toward normal cells. In general, inhibition of protein farnesylation results in G_0/G_1 cell cycle block, G_2/M cell cycle arrest, or has no effect on cell cycle progression. One aspect of FTI biology that is poorly understood is the ability of these drugs to induce cancer cell growth arrest at the G_2/M phase of cell cycle. In the present study, we investigated the effects of the farnesyltransferase inhibitor FTI-277 on two human liver cancer cell lines, HepG2 and Huh7. Treatment of these cells with FTI-277 inhibited Ras farnesylation in a dose-dependent manner. Both HepG2 and Huh7 cell growth was inhibited by FTI-277 and cells accumulated at the G_2/M phase of the cell cycle. In HepG2 and Huh7 cells, FTI-277 induced an up-regulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ without affecting the cellular levels of p53 and $p21^{Waf1}$. This event correlated with reduced activity of the cyclin-dependent kinase 2 and cyclin-dependent kinase 1. Moreover, increased expression of Bcl-2 protein was observed in HepG2 and Huh7 cells treated with FTI-277, and this was coincidental with reduced association between Raf-1 and Bcl-2. Finally, transient transfection of a dominant-negative Ras allele induced Bcl-2 expression and reduced Bcl-2/Raf-1 association demonstrating a requirement for Ras. Taken together, these findings show that increased expression of $p27^{Kip1}$ and Bcl-2 is concomitant with altered association between Ras, Raf-1 and Bcl-2 and suggest that this is responsible for the growth-inhibitory properties of FTI-277.

Farnesyltransferase inhibitors (FTIs) are a new class of anticancer drugs that block the growth of tumors with minimal toxicity in normal cells (Gibbs and Oliff, 1997). These compounds inhibit protein farnesyltransferase, an enzyme that catalyzes the farnesylation of a number of proteins, including the small GTP-binding protein Ras (Adjei, 2001). Ras is a key regulator of cell growth in all eukaryotic cells. Genetic and biochemical studies have shown the central role played by Ras in signal transduction pathways that respond to diverse extracellular stimuli, including growth factors, cytokines, and extracellular matrix proteins. Ras proteins must be localized at the plasma membrane after a series of post-translational modifications to function normally. The first and obligatory step in this series is farnesylation of the cysteine residue located at the Ras COOH-terminal CAAX motif, where C is cysteine, A is any aliphatic amino acid, and X is methionine or serine. Previous studies have demonstrated that inhibition of Ras farnesylation interferes with its membrane localization and blocks Ras-mediated cellular transformation. Therefore, the discovery of the critical role of Ras farnesylation has led to the development of farnesyltransferase inhibitors (FTIs), which are able to block the growth of tumor cells with minimal toxicity in normal cells in vitro and in vivo (Mangues et al., 1998, Suzuki et al., 1998).

Several FTIs have been described and exert growth inhibition by distinct mechanisms (Miquel et al., 1997; Vogt et al., 1997; Du et al., 1999). In general, inhibition of protein farnesylation can result in G_0/G_1 cell cycle block, G_2/M cell cycle arrest, or no effect on cell cycle progression (Sun et al., 1995; Law et al., 1999; Carloni et al., 2000). A poorly understood feature of FTI biology is the ability of these drugs to induce cancer cell growth arrest at the G_2/M phase of the cell cycle. In this study, we investigated the mechanism of action

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ABBREVIATIONS: FTI, farnesyltransferase inhibitor; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

of FTI-277, a peptidomimetic farnesyltransferase inhibitor that causes G_2/M cell cycle arrest in two liver cancer cell lines, HepG2 and Huh7. We evaluated the involvement of the p53 tumor suppressor gene because it is an essential mediator of cellular responses to toxic stress (Lowe et al., 1993). In response to such stresses, p53 accumulates and activates the transcription of several genes whose products are involved in cell cycle arrest and apoptosis (e.g., $p21^{Waf1}$ and Bcl-2/Bax)(El-Deiry et al., 1993). It is well documented that Ras functions as a molecular switch for entry into the G_1 phase of cell cycle. Here, Ras functions to regulate the level of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Specifically, Ras down-regulates $p27^{Kip1}$ through mechanisms involving both translational and post-translational control (Aktas et al., 1997; Takuwa and Takuwa 1997). In contrast, the role of Ras at the G_2/M phase of the cell cycle is less well understood.

Expression of $p27^{Kip1}$ is regulated by cell contact inhibition and transforming growth factor-β. In addition, $p27^{Kip1}$ is a regulator of drug resistance in solid tumors and acts as a safeguard against inflammatory injury (Ophascharoensuk et al., 1998). The levels of $p27^{Kip1}$ protein decrease during tumor development and progression in certain epithelial and lymphoid tissues (Lloyd et al., 1999; Tannapfel et al., 2000). Proliferation and apoptosis are events that are tightly linked during cell function; in the past few years, it has been shown that Bcl-2 exhibits a potent cell cycle inhibitory effect, in addition to its role in the suppression of apoptosis (O'Reilly et al., 1996; Korsmeyer, 1999). Bcl-2 is a protein anchored via its carboxyl-terminal hydrophobic tail to the outer membranes of mitochondria, nuclei, and the endoplasmic reticulum. Although a cytosolic Bcl-2 mutant retains partial function, membrane localization is required for full activity. Bcl-2 has been reported to physically associate with Ras and Raf-1, and this association is linked to the phosphorylation status of Bcl-2 (Chen and Faller, 1996; Wang et al., 1996). Therefore, Bcl-2 could function by targeting the Ras/Raf-1 complex to the membranes of mitochondria, nuclei, and the endoplasmic reticulum (Kinoshita et al., 1995; Wang et al., 1996).

Here, we provide evidence that inhibition of protein farnesylation induces $p27^{Kip1}$ in a p53-independent manner. Furthermore, we demonstrate that protein farnesylation as well as Ras function regulate Bcl-2 expression and the association between Bcl-2 and Raf-1.

Materials and Methods

Antibodies and Reagents. The affinity-purified polyclonal antibody to $p27^{Kip1}$ was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibodies to p53, Bcl-2, cyclin B1, and polyclonal antibodies to cyclin-dependent kinase (CDK2), Raf-1, Rap1A, and $p21^{Waf1}$ were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to Ras was from BD Transduction Laboratories (Lexington, KY). Histone H1 was from Roche (Mannheim, Germany).

The plasmid constructs used for transfection experiments were the dominant-negative Ras (pEXV N17ras) and Rac (pEXV N17Rac) provided by Dr. A. Hall (University College, London, UK).

Cell Lines. HepG2 cells, derived from a human hepatoblastoma expressing wild-type p53, and Huh7 cells, derived from a hepatocellular carcinoma expressing high levels of mutated p53 (point mutation at codon 220), were used. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical, St. Louis, MO) and supplemented with 10% fetal bovine serum, 5 mM sodium pyruvate, and 5 mM nonessential amino acids at 37°C in a humidified incubator containing 5% CO_2.

Ras and Rap1A Processing Assay. Cells were seeded on day 0 in 100-mm dishes; different doses of FTI-277 or vehicle [10 mM dithiothreitol in dimethyl sulfoxide (DMSO)] were employed on days 1 and 2. On day 1, cell treatment with FTI-277 was performed in DMEM with 10% FBS; on day 2, cells were incubated with FTI-277 in DMEM without serum. On day 3, cells were washed, harvested, and lysed in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM Na_3VO_4, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml leupeptin, 5 μM pepstatin, and 2 mM phenylmethylsulfonyl fluoride. Lysates were cleared (13,000 rpm, 4°C, 15 min), and equal amounts of protein were resolved on SDS-PAGE 6 to 20% gradient gel, transferred to polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA), and immunoblotted using an anti-panRas mouse monoclonal antibody or an anti-Rap1A polyclonal antibody. Antibody reactions were visualized using peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Cell Growth Analysis. Cell growth inhibition assays were performed by plating 2×10^4 cells on 12-well plates, in DMEM with 10% FBS. Cells were treated with FTI-277 (10 μM) or DMSO every 48 h for 6 days. The number of cells was determined at each time point by counting with a hemocytometer every 2 days. Cell viability was measured by trypan blue dye exclusion.

Flow Cytometry and DNA Fragmentation Analysis. Cells were plated on 100-mm Petri dishes and treated with FTI-277 or DMSO as indicated. Briefly, cells were harvested using 3 ml of trypsin-EDTA and washed twice with phosphate-buffered saline (PBS). Cells were resuspended at 1×10^6 ml in a solution containing 25 μg/ml propidium iodide, 0.02% Nonidet P-40, and 0.5 mg ribonuclease A in PBS. Samples were incubated in the dark at room temperature for 30 min and stored at 4°C. Cell cycle phases were determined in a FACScan flow cytometer (BD Biosciences, San Jose, CA). The proportion of apoptotic cells (A_0) corresponding to cells with a DNA content less than 2 N and cells in G_1, S, and G_2/M phase were calculated from the respective DNA histograms using the CellFit software (BD Biosciences). To detect DNA fragmentation, cellular DNA was prepared using the blood and cell culture mini DNA kit (QIAGEN, Valencia, CA). Purified DNA was then analyzed on 1.5% agarose gel. DNA was visualized by ethidium bromide staining.

Immunoprecipitations and Immunoblotting. After treatment with FTI-277, cells were harvested and lysed in HEPES lysis buffer as described above. The cellular extracts were centrifuged for 10 min at 13,000 rpm and the supernatant was used for immunoprecipitation or immunoblotting. For immunoprecipitations, antibodies were added to cell lysates and incubated overnight at 4°C, and antibodies collected on protein A Sepharose beads. Protein complexes were washed in a immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Tween-20) before direct analysis by SDS-PAGE or in vitro ^{32}P-labeling. Proteins were resolved by 12% SDS-PAGE and transferred to polyvinylidene fluoride. The membranes were blocked for 1 h at room temperature in 2% gelatin PBS solution and subsequently probed in the same solution with antibodies against $p21^{Waf1}$ (C-19; Santa Cruz Biotechnology), Raf-1 (C-20; Santa Cruz Biotechnology), $p27^{Kip1}$ (Upstate Biotechnology, Lake Placid, NY), p53 (Pab240; Santa Cruz Biotechnology), and Bcl-2 (100; Santa Cruz Biotechnology). The membranes were then washed with PBS/0.1% Triton X-100, and enhanced chemiluminescence was used for detection.

Cyclin-Dependent Kinase Assays. To measure the activity of cyclin-dependent kinase 2 (CDK2), histone H1 was used as substrate. CDK2 was immunoprecipitated using a rabbit polyclonal anti-CDK2 (M2; Santa Cruz Biotechnology) in a 30-μl reaction mixture containing 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol, 10 μCi of [γ^{32}P]ATP and 100 μg/ml histone H1, which was incubated for 30 min at 30°C. The reaction was termi-

nated by addition of an equal volume of SDS-PAGE sample buffer. The samples were fractionated by SDS-PAGE, and phosphorylated proteins visualized by autoradiography. Cyclin-dependent kinase 1 (CDK1) activity was performed by immunoprecipitating with a monoclonal antibody anti-cyclin B1 (GNS1; Santa Cruz Biotechnology). The kinase reaction was initiated by addition of 40 μl of kinase buffer containing 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol, 10 μCi of [γ-^{32}P]ATP, and 100 μg/ml histone H1. After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS-PAGE. The gel was stained, dried, and exposed to Kodak X-Omat AR film at −70°C. Intensity of bands was quantitated using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA).

Antisense Oligonucleotides and Transfection Conditions. Oligodeoxynucleotides with a phosphorothioate backbone were synthesized and purified by gel filtration on an Applied Biosystems 380B automated DNA synthesizer (Foster City, CA). The sequences of oligonucleotides were as follows: G3139, targeted to the first six codons of the human bcl-2 mRNA open reading frame, 5′-TCTCCCAGCGTGCGCCAT-3′; Isis4559, control, 5′- GGTTTTACCATCGGTTCTGG-3′ (Benimetskaya et al., 2001). Huh7 were seeded the day before the experiment in 60-mm dishes to be 60 to 70% confluent on the day of experiment. Cells were washed with Opti-MEM I medium (Invitrogen, Carlsbad, CA). G3139, anti-Bcl-2 (100 nM), or Isis4559 control (100 nM) was mixed with 10 μg/ml of LipofectAMINE (Invitrogen) in Opti-MEM I medium and added to the cells for 5 h then replaced with normal growth medium for 24 h. Thereafter, the cells were treated with 10 μM FTI-277 for 48 h and processed to further experiments.

In experiments with cDNA, HepG2 and Huh7 were plated on 100-mm diameter tissue culture dishes 24 h before transfection. Transfections were carried out using 10 μg of DNA and 50 μl of LipofectAMINE, as recommended by the manufacturer. After 48 h, cells were starved overnight and harvested in lysis buffer as described above.

Statistical Analysis. Results are expressed as means ± S.D. Statistical analysis of results was performed by analysis of variance.

Results

Effects of FTI-277 on Ras and Rap1A Processing. The ability of FTI-277 to selectively inhibit protein farnesylation of HepG2 and Huh7 cells was tested by treating cells with DMSO (vehicle) or increasing doses of FTI-277 (5, 10, 20 μM). The resulting cell lysates were immunoblotted with antibodies against Ras and Rap1A (Carloni et al., 2000). As shown in Fig. 1, the panRas antibody detected Ras protein in both cell types. Treatment of HepG2 (A) and Huh7 (B) with concentrations as low as 5 μM resulted in inhibition of Ras processing as indicated by the mobility shift of Ras on SDS-PAGE.

In contrast, FTI-277 was unable to alter the electrophoretic mobility of Rap1A, a geranylgeranylated protein (Fig. 1, A and B). Taken together, these results indicate that FTI-277 is a specific inhibitor of Ras farnesylation and does not interfere with the processing of geranylgeranylated proteins.

FTI-277 Inhibits Cell Growth and Arrests Liver Cancer Cells at the G_2/M Phase of the Cell Cycle. To evaluate the effects of FTI-277 on cell growth, we used two liver cancer cell lines, HepG2 and Huh7. Cells were treated with DMSO or FTI-277 (10 μM) and the growth rate was evaluated after 2, 4, and 6 days. As shown in Fig. 2, the growth rate of HepG2 and Huh7 cells treated with FTI-277 was significantly lower compared with control cells treated with vehicle alone.

Next, we evaluated the effect of FTI-277 on HepG2 and

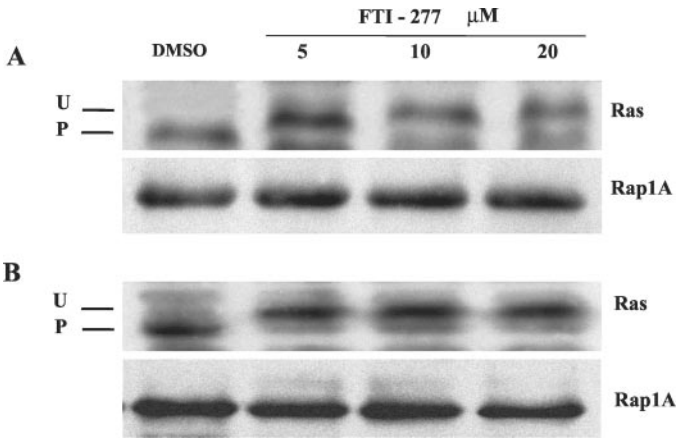

Fig. 1. FTI-277 selectively inhibits Ras processing. HepG2 (A) and Huh7 (B) cells were treated with DMSO or FTI-277 for 48 h and serum-starved overnight. Cell lysates were prepared and proteins were separated by SDS-PAGE on a 6 to 20% gradient gel, followed by immunoblotting with panRas or Rap1A antibodies, as indicated. The data are representative of four independent experiments. U, unprocessed; P, processed.

Huh7 cell cycle distribution. Cells were seeded on days 0 and 1, cells were treated with DMSO or FTI-277 (10 μM) in DMEM plus 10% FBS. On day 2, cells were incubated with DMSO or FTI-277 in DMEM without serum. DNA content was analyzed by flow cytometry after staining with propidium iodide. As shown in Table 1, HepG2 cells treated with DMSO displayed a distribution of 55% in G_1, 33% in the S phase, and 3% in G_2/M. Cell-cycle distribution of Huh7 cells treated with DMSO was 67% in G_1 phase, 25% in the S phase, and 8% in G_2/M. HepG2 treated with FTI-277 showed 29% in the G_1 phase, 38% in the S phase, and 25% in G_2/M phase. Cell cycle distribution of Huh7 cells treated with FTI-277 was 46% in G_1 phase, 33% in S phase, and 20% in G_2/M phase.

We also investigated, using flow cytometry, whether FTI-277 was able to induce apoptosis. The addition of 10 μM FTI-277 did not lead to the appearance of a significant fraction of cells in the region corresponding to cells with a DNA content less than 2N, a characteristic aspect of apoptotic cells (Table 1). To further confirm these findings, we performed the biochemical analysis of the internucleosomal DNA cleavage. HepG2 and Huh7 were treated 48 h with etoposide (100

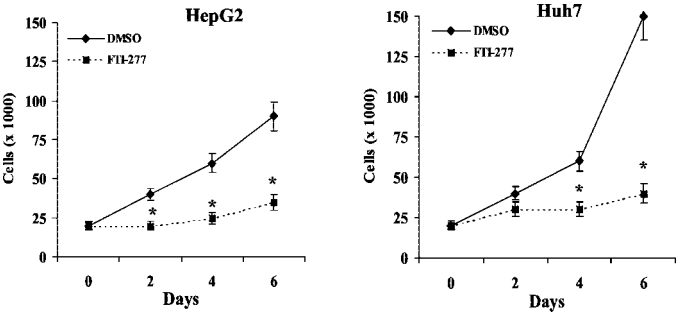

Fig. 2. Inhibition of HepG2 and Huh7 cell proliferation by FTI-277. Cell growth inhibition assays were performed by plating 2×10^4 cells on 12-well plates, in DMEM with 10% FBS. Cells were treated with FTI-277 (10 μM) or DMSO every 48 h for 6 days, and the number of cells was determined at 2, 4, or 6 days by counting with a hemocytometer. The viability of cells was measured by trypan blue dye exclusion. Results are expressed as cells per well and each value is the mean of three separate experiments performed in triplicate. *, $p < 0.05$ or higher degree of significance versus control cells.

TABLE 1

Effects of FTI-277 on cell cycle distribution and apoptosis in liver cancer cells

HepG2 and Huh7 cells were seeded on days 0 and 1, and cells were treated with DMSO or FTI-277 (10 μM) in DMEM plus 10% FBS. On day 2, cells were incubated with DMSO or FTI-277 in DMEM without serum. On day 3, cells were harvested and analyzed by propidium iodide staining and flow cytometry. Apoptotic cell populations (A_0) were evaluated by using Cellfit software (BD Biosciences). At least two separate experiments were carried out with results similar to those shown here.

HepG2		Huh7	
DMSO	FTI-277	DMSO	FTI-277
A_0 = 1% G_1 = 55% S = 33% G_2-M = 3%	A_0 = 5% G_1 = 29% S = 38% G_2-M = 25%	A_0 = 3% G_1 = 67% S = 25% G_2-M = 8%	A_0 = 8% G_1 = 46% S = 33% G_2-M = 20%

μM) an apoptosis-inducing drug or 10 μM FTI-277. DNA genomic was extracted and separated by 1.5% agarose gel. As shown in Fig. 3 the treatment of cells with FTI-277 was unable to cause DNA fragmentation. In aggregate, these results suggest that FTI-277 does not induce apoptosis at the dose employed.

Treatment of FTI-277 Results in Increased Levels of $p27^{Kip1}$ but Does Not Alter p53 and $p21^{Waf1}$ Expression. To investigate the potential mechanisms by which FTI-277 interferes with cell cycle progression in liver cancer cell lines, we treated cells with various concentrations of FTI-277 (5, 10, and 20 μM) and immunoblotted whole cell lysates with antibodies against $p27^{Kip1}$, $p21^{Waf1}$, and p53. As shown in Fig. 4, HepG2 cells did not express significant levels of both p53 and $p21^{Waf1}$ (top and middle). However, treatment of these cells with FTI-277 resulted in increased expression of $p27^{Kip1}$ (Fig. 4, bottom). In contrast to HepG2 cells, Huh7 cells (Fig. 5) showed high constitutive expression of p53 and no significant alteration in p53 levels after FTI-277 treatment. There was no detectable expression of $p21^{Waf1}$ in these cells.

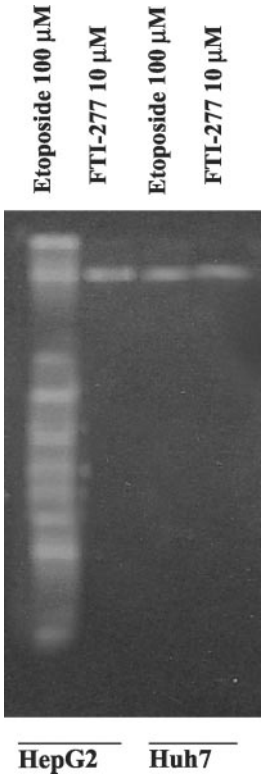

Fig. 3. DNA fragmentation assay. Cells were seeded on day 0 in 100-mm dishes, and FTI-277 or etoposide was added. On day 1, cell treatment was performed in DMEM with 10% FBS, whereas on day 2, cells were incubated in DMEM without serum. On day 3, cells were washed and subjected to DNA extraction. DNA was electrophoresed in a 1.5% agarose gel and visualized by UV fluorescence after staining with ethidium bromide. A representative experiment of three is shown.

Effects of FTI-277 on the Activity of CDK2 and Cyclin B-Associated Kinase. The finding that treatment of cells with FTI-277 results in increased levels of $p27^{Kip1}$ prompted us to evaluate the ability of FTI-277 to affect the activity of the cyclin-dependent kinase 2 (CDK2), a kinase whose activity is controlled by $p27^{Kip1}$. In resting or growth-arrested cells, $p27^{Kip1}$ is expressed at high levels, suggesting that up-regulation of $p27^{Kip1}$ may play a role in cell cycle arrest. Although the pool of CDK2 does not fluctuate significantly during the cell cycle, its kinase activity oscillates, with two major peaks of activity during DNA synthesis and before mitosis. In both cases, the majority of this activity is attributable to the association of CDK2 with cyclin A. Therefore, cells were treated with FTI-277 (10 μM) and the resulting lysates were immunoprecipitated with anti-CDK2 antibody. Figure 6 shows that CDK2 from DMSO-treated HepG2 and Huh7 cells was active and able to phosphorylate histone H1 in an immune complex kinase assay in vitro. Prior treatment of cells with FTI-277 blocked CDK2 activity.

We next investigated whether FTI-277 could suppress also the activity of CDK1, which is a ubiquitously expressed serine/threonine kinase. CDK1 binding to the subunit cyclin B is essential during the G_2/M cell cycle progression. CDK1 was difficult to immunoprecipitate directly, as also described by others, but could be precipitated through bound cyclin B. We assayed kinase activity associated with the cyclin B immunoprecipitates using histone H1 as a substrate. Both HepG2 and Huh7 were treated with 10 μM FTI-277 and showed a reduction in cyclin B-associated kinase activity, even after normalizing for the amount of cyclin B immunoprecipitated (Fig. 6).

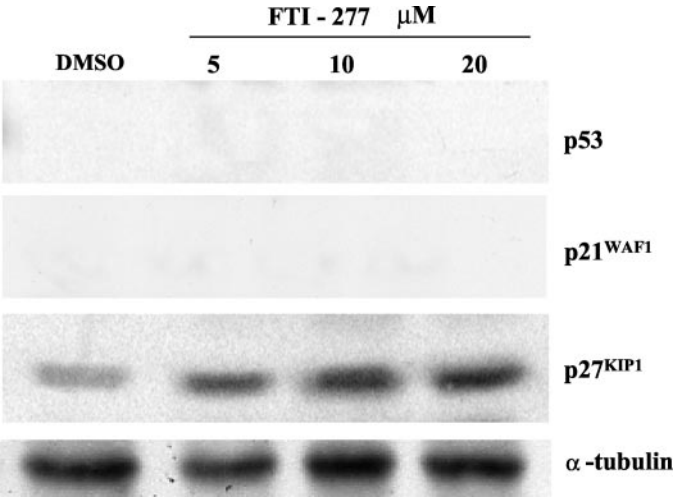

Fig. 4. FTI-277 treatment of HepG2 cells results in increased levels of $p27^{Kip1}$ but does not alter p53 and $p21^{Waf1}$ expression. Cells were seeded on day 0 in 100-mm dishes, and different doses of FTI-277 or vehicle (DMSO) were used. On day 1, cell treatment with FTI-277 was performed in DMEM with 10% FBS, whereas on day 2, cells were incubated with FTI-277 in DMEM without serum. On day 3, cells were washed, harvested, and lysed in buffer. Proteins were resolved by 12% SDS-PAGE and immunoblotted with antibodies against p53, $p21^{Waf1}$, and $p27^{Kip1}$ as indicated. α-Tubulin served as control for sample loading. The data are representative of three independent experiments.

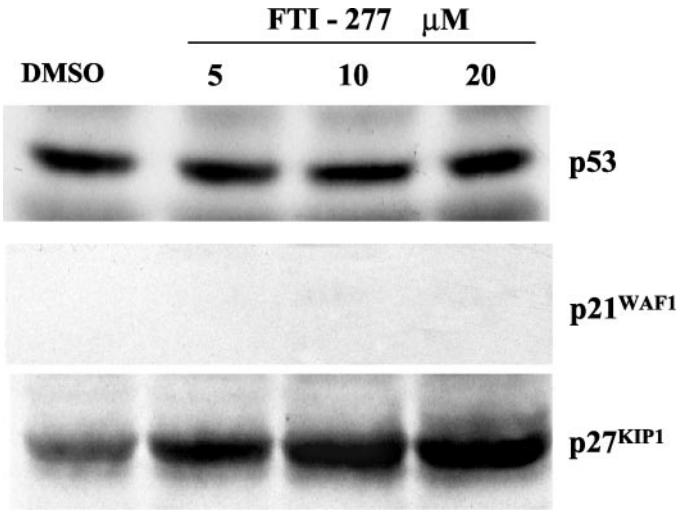

Fig. 5. FTI-277 treatment of Huh7 cells results in increased levels of $p27^{Kip1}$ but does not alter p53 and $p21^{Waf1}$ expression. Cells were seeded on day 0 in 100-mm dishes, and different doses of FTI-277 or DMSO were used. On day 1, cell treatment with FTI-277 was performed in DMEM with 10% FBS, whereas on day 2, cells were incubated with FTI-277 in DMEM without serum. On day 3, cells were washed, harvested, and lysed in buffer. Proteins were resolved by 12% SDS-PAGE and immunoblotted with antibodies against p53, $p21^{Waf1}$, and $p27^{Kip1}$ as indicated. The data are representative of three independent experiments.

FTI-277 Treatment Up-Regulates Bcl-2 Protein Content and Inhibits Bcl-2/Raf-1 Association. In addition to its role in apoptosis, several studies have demonstrated that Bcl-2 can also delay cell cycle entry. One mechanism that is generally considered to exert this function is increased cellular levels of Bcl-2. We therefore studied the expression of Bcl-2 in HepG2 and Huh7 cells treated with DMSO or FTI-277. Equal amounts of protein were immunoprecipitated with an anti–Bcl-2 antibody separated by SDS-PAGE and immunoblotted with the same antibody. As shown in Fig. 7, there was a dose-dependent increase in Bcl-2 in cells treated with FTI-277 compared with control-treated cells. This effect was unrelated to the cell type because it was detected in both HepG2 and Huh7 cells (Fig. 7). Moreover, several reports have demonstrated an association of Bcl-2 with Ras and Raf-1. We then evaluated whether protein farnesylation affected this association. Cell lysates were immunoprecipitated with antibodies against Bcl-2 followed by and immunoblotting with anti-Raf-1. Treatment of cells with FTI-277 led to decreased association between Bcl-2 and Raf-1 in a dose-dependent manner both in HepG2 and Huh7 cells (Fig. 7).

To further address the significance of these results, we transiently transfected HepG2 and Huh7 cells with a dominant-negative Ras allele (N17Ras), dominant-negative Rac (N17Rac), or empty vector (C). As shown in Fig. 8, transfection with N17Ras induced increased expression of Bcl-2 compared with the N17Rac or empty vector controls. Furthermore, N17Ras-transfected cells exhibited reduced association between Bcl-2 and Raf-1 (Fig. 8).

We next wished to determine whether Bcl-2 affected the growth inhibitory property of FTI-277. Therefore, we used an antisense strategy to reduce cellular levels of Bcl-2 protein. Because Huh7 express higher levels of Bcl-2 protein than HepG2, we transfected these cells with oligonucleotides Isis 4559 as control and G3139 as anti-Bcl-2. Interestingly, the down-regulation of Bcl-2 protein (Fig. 9B) led to a decreased cell number (Fig. 9A) and this effect was amplified by 10 μM FTI-277 cell treatment. Furthermore, flow cytometry analysis of FTI-277–treated Huh7 with defective expression of

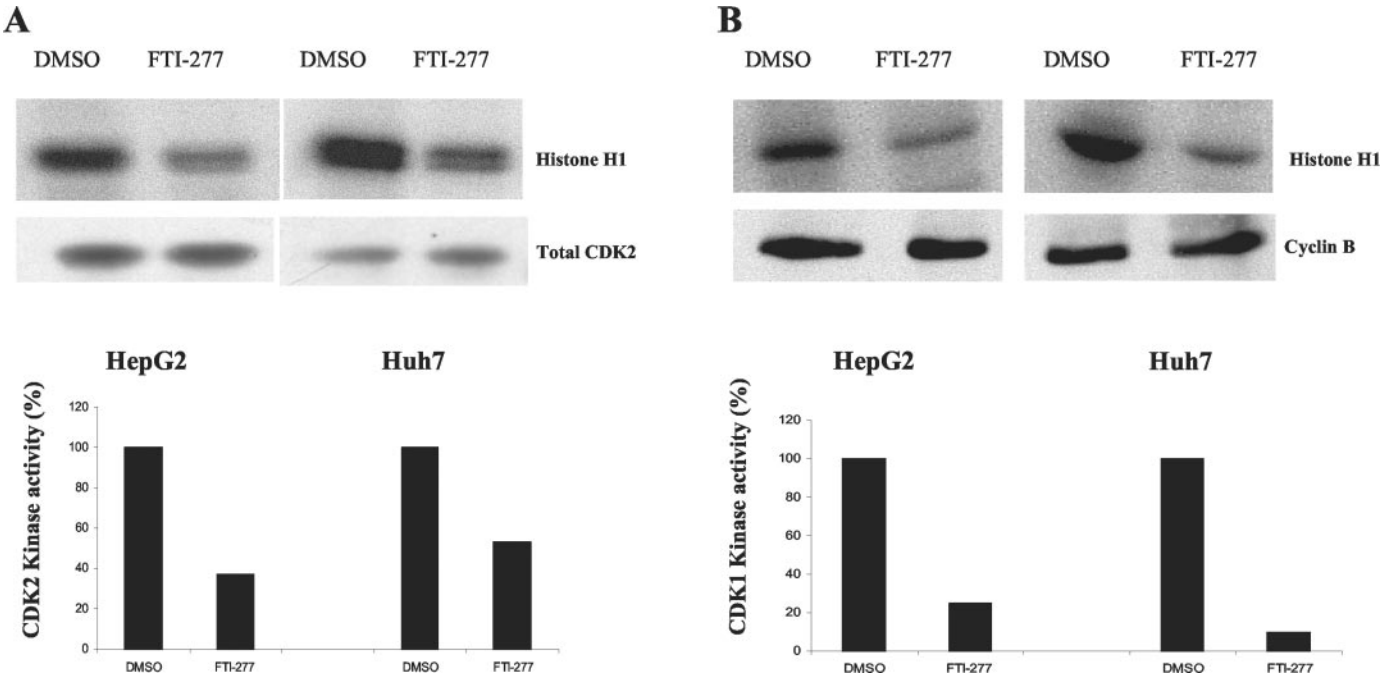

Fig. 6. Effects of FTI-277 on the activity of cyclin-dependent kinase 2 (CDK2) and cyclin B-associated kinase. Cells were seeded on day 0 in 100-mm dishes and 10 μM FTI-277 or DMSO was added. On day 1, cell treatment with FTI-277 was performed in DMEM with 10% FBS, whereas on day 2, cells were incubated with FTI-277 in DMEM without serum. On day 3, cells were washed, harvested, and lysed in buffer. CDK2 was immunoprecipitated using a rabbit polyclonal anti-CDK2 antibody and histone H1 was used as substrate in an immunocomplex kinase assay, as described under *Materials and Methods*. The samples were separated by SDS-PAGE, and visualized by autoradiography (Fig. 6 A). Kinase activity associated with the normalized cyclin B immunoprecipitates was assayed using histone H1 as a substrate (Fig. 6B). Histone H1 phosphorylation was quantitated on a densitometer to calculate the percentage of kinase activity inhibition. The data are representative of two independent experiments.

Bcl-2 protein showed that the reduced cell number was caused by an increased rate of apoptosis (Fig. 9C). The augmented cell death was associated with a low number of cells arrested in G_2/M, 13% versus 23% of control. The findings obtained suggest that Bcl-2 plays an essential role in controlling both G_2/M cell cycle progression and apoptosis of this cell type.

Discussion

In this study, we have investigated the effects of the farnesyltransferase inhibitor FTI-277 in two human liver cancer cell lines, HepG2 and Huh7. In both cell types, FTI-277 inhibits growth and cells accumulate at the G_2/M phase of the cell cycle. It is well established that under normal conditions, exposure of cells to toxic stress can lead to a marked elevation of p53 protein within a relatively short period of time, resulting in cell cycle arrest and apoptosis (Oren, 1999). Furthermore, the activity of a wide range of chemotherapeutic agents in many cell types has been shown to be mediated by p53-dependent apoptosis (Muller et al., 1997). However, our results show that in HepG2 cells, FTI-277 is unable to induce p53 expression. Similarly, FTI-277 did not alter the levels of $p21^{Waf1}$, a cyclin-dependent kinase inhibitor that blocks cell cycle progression and whose transcription can be induced by p53 (El-Deiry et al., 1993). Therefore, the ability of FTI-277 to induce cell cycle arrest is not caused by an up-regulation of $p21^{Waf1}$. On the contrary, our results support the notion that cell cycle arrest is caused by increased levels of another CDK inhibitor, $p27^{Kip1}$. $p27^{Kip1}$ levels are highest in quiescent cells and decline as cells re-enter the cell cycle. Many antiproliferative signals, including mitogen/cytokine withdrawal, cell-to-cell contact, and agents such as cAMP and rapamycin lead to an accumulation of $p27^{Kip1}$ (Kato et al., 1994). This implies that reduced expression of $p27^{Kip1}$ may predispose cells to abnormal cell cycle and tumor progression (Rosenblatt et al., 1992; Fero et al., 1998). Hence, increased expression of $p27^{Kip1}$ induced by FTI-277 may provide a protective effect on tumor progression. Growth factor-activation of Ras (or constitutively active Ras) can reduce $p27^{Kip1}$ levels by decreasing its translation and stability (Aktas et al., 1997). The present results indicate that Ras farnesylation regulates $p27^{Kip1}$ expression and is therefore required at the G_2/M transition. Progression through the cell cycle is governed by the cyclin-dependent kinases. In mammalian cells, CDK2 is a member of the CDK family and exhibits histone H1 kinase activity, which oscillates during the cell cycle. CDK2 activity shows a complex pattern of activation that includes peaks coinciding with the S and G_2 phases of the cell cycle (Rosenblatt et al., 1992). This complex pattern of activation is consistent with a role for CDK2 in multiple cell cycle processes ranging from S phase initiation or maintenance to the preparation for mitosis. The results provided in this study suggest that the ability of FTI-277 to arrest liver cancer cells in the G_2 phase, and blocking entrance into mitosis is probably related to the inhibition of CDK2 by $p27^{Kip1}$. Several works have demonstrated the contribution of CDK2 in G_2/M cell cycle progression. In a study by Furuno et al. (1999), microinjection of purified cyclin A-CDK2 complexes in G_2-phase HeLa cells was found to accelerate entry in mitosis. Others reports have described the requirement of CDK2 kinase as a positive regulator of CDK1-cyclin B kinase activity. In particular, CDK2 would be able to modulate cyclin B-CDK1 complex through phosphorylation of CDK1 (Guadagno and Newport, 1996; Hu et al., 2001). Accordingly, in our study, reduced CDK2 kinase activity was found to be associated with a reduced cyclin B-dependent kinase activity. In aggregate, these results reinforce the concept of a key role for CDK2 and $p27^{Kip1}$ in controlling G_2/M cell cycle progression.

The association of cell survival with cell-cycle progression has been suggested by numerous studies. Bcl-2 is the proto-

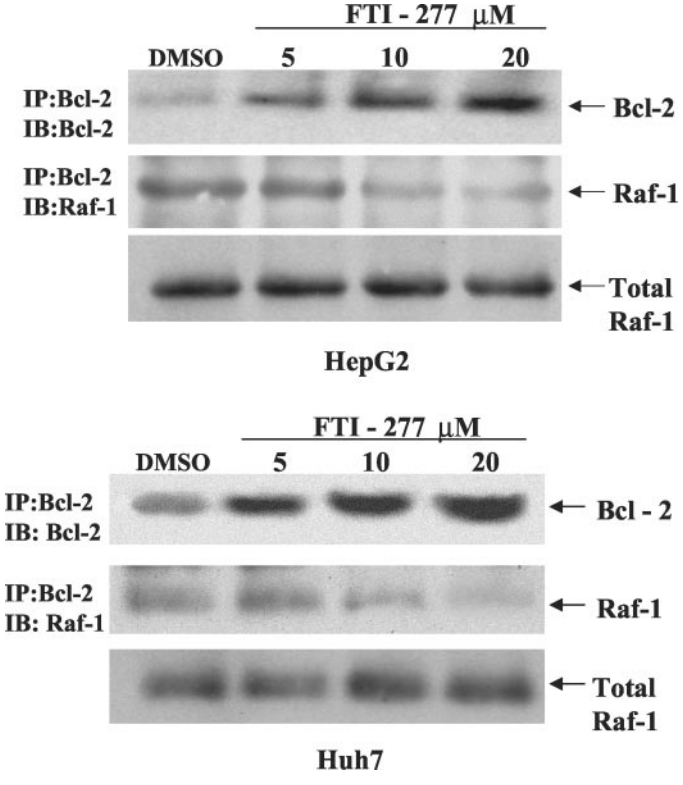

Fig. 7. Bcl-2 expression and Raf-1/Bcl-2 association in FTI-277–treated HepG2 and Huh7. Cells were seeded on day 0 in 100-mm dishes, and different doses of FTI-277 or DMSO were added. On day 1, cell treatment with FTI-277 was performed in DMEM with 10% FBS, whereas on day 2, cells were incubated with FTI-277 in DMEM without serum. On day 3, cells were washed, harvested, and lysed in buffer; equal amounts of proteins were immunoprecipitated with antibodies against Bcl-2 and separated by 12% SDS-PAGE followed by immunoblotting against Bcl-2 or Raf-1. The data are representative of three independent experiments.

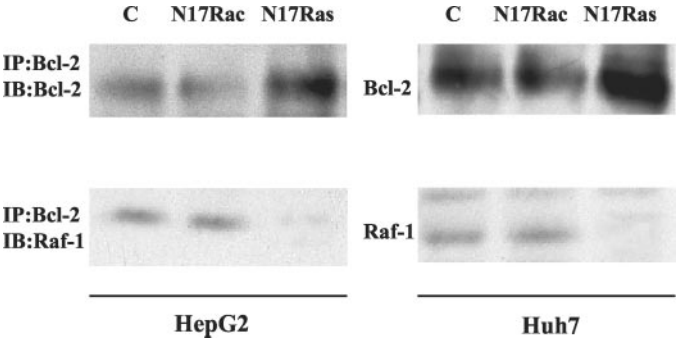

Fig. 8. Transient transfection of dominant-negative N17Ras up-regulates Bcl-2 and interferes in Raf-1/Bcl-2 association. HepG2 and Huh7 were transiently transfected with pEXV N17Ras, pEXV N17Rac, or pEXV empty vector (C). After 48 h, cells were maintained in serum-free medium overnight. Cells were then lysed, and equal amounts of proteins were immunoprecipitated with antibodies against Bcl-2, separated by 12% SDS-PAGE, and immunoblotted against Bcl-2 or Raf-1. The data are representative of three independent experiments.

type of a large family of proteins that serve to oppose the cell death process and to promote cell survival (Korsmeyer, 1999). Aside from regulating apoptosis, several studies have shown that Bcl-2 can also regulate the cell cycle (Mazel et al., 1996; Huang et al., 1997). Overexpression of Bcl-2 slows down cell-cycle progression and induces retardation of cell-cycle entry from quiescence (Lind et al., 1999; Vairo et al., 2000). In endometrial carcinoma cells, Bcl-2 overexpression results in reduced cell proliferation rates, decreased cloning efficiency, and G_2/M cell cycle arrest (Crescenzi and Palumbo, 2001). In hemopoietic cells, transforming growth factor-β1–induced growth inhibition is accompanied by increased levels of Bcl-2 and the induction of Bcl-2 is concomitant with increased expression of $p27^{Kip1}$ (Mahmud et al., 1999). In breast cancer, Bcl-2 inhibits cancer cell growth despite its antiapoptotic effect (Knowlton et al., 1998). The ability of Bcl-2 to retard cell cycle progression has also been described in primary cells; constitutive Bcl-2 expression impairs the activation of primary lymphocytes, as measured by entry into S phase or by interleukin-2 production (Brady et al., 1996). The protein domains of Bcl-2 that are responsible for the cell cycle regulatory effect are not the same as those required for antiapoptotic activity (Vairo et al., 1996; Huang et al., 1997). This latter finding raises the possibility that Bcl-2 may influence cell cycle progression independently of the antiapoptotic effects. However, the exact nature of the mechanisms by which Bcl-2 regulates the cell cycle control have yet to be described. The results provided in this study highlight an important role for protein farnesylation and Ras signaling in Bcl-2 expression. Furthermore, our results provide evidence for the association of Bcl-2 with Raf-1 and the contribution of Ras in regulating this event. Ras isoforms vary in their ability to activate Raf-1; K-Ras recruits Raf-1 to the membrane more efficiently than does H-Ras. Raf-1 is not only activated after growth factor stimulation, but also during mitosis. In mitosis, activated Raf-1 is localized primarily

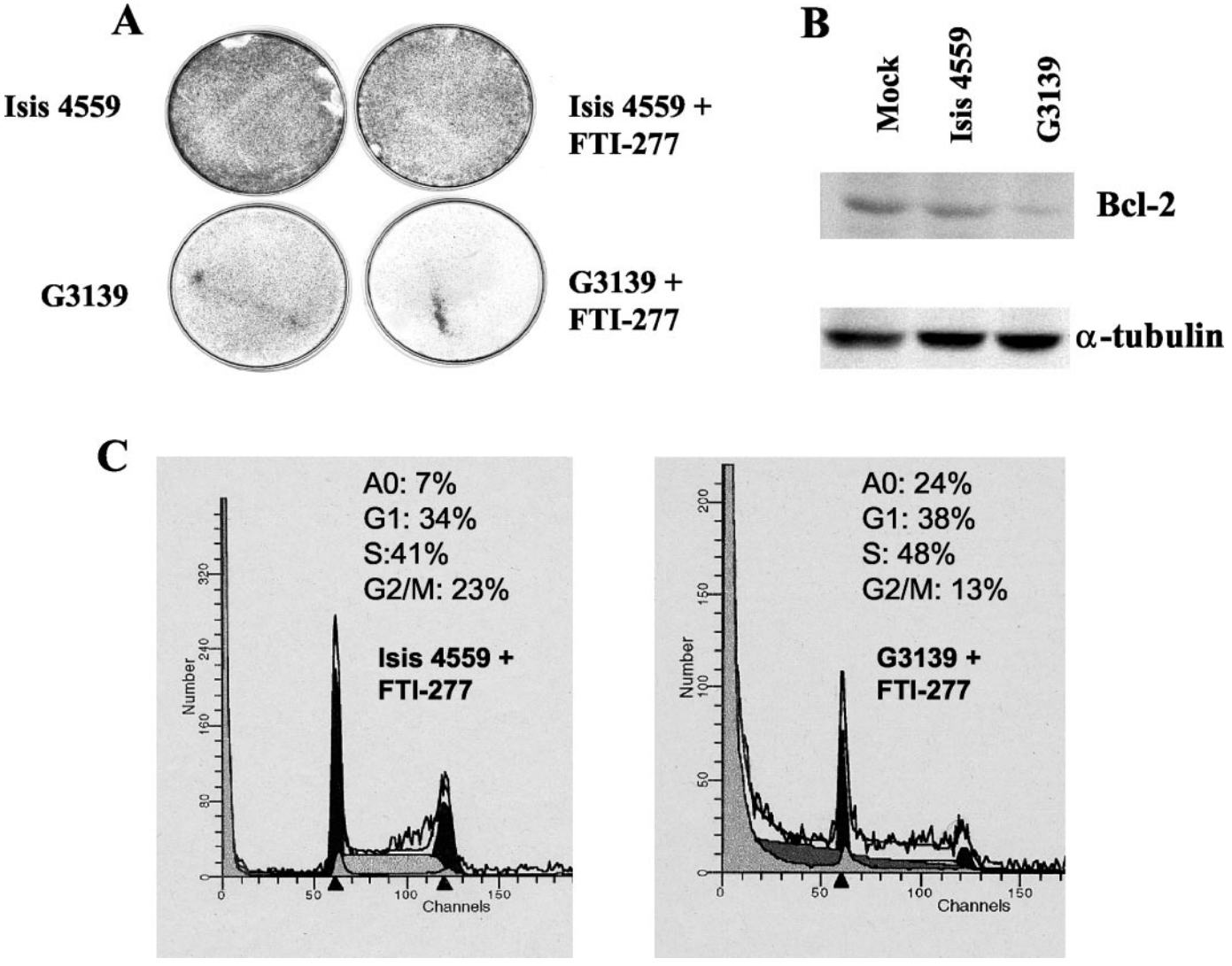

Fig. 9. Analysis of cell cycle and apoptosis after Bcl-2 down-regulation and FTI-277 treatment in Huh7. Huh7 were seeded in 60-mm dishes, cells were washed with Opti-MEM I medium and transfected with oligonucleotides G3139, anti-Bcl-2 (100 nM), or Isis4559, control (100 nM) complexed with 10 μg/ml of LipofectAMINE. After transfection (24 h), the cells were treated for 48 h with 10 μM FTI-277. Dishes were washed with PBS, and the cells fixed in 3% formaldehyde and then stained with crystal violet (A). B, cells were harvested after 72 h from transfection and lysed. Protein lysates were quantified and separated by SDS-PAGE and blotted anti-Bcl-2 or reprobed anti-α-tubulin to confirm that the differences did not reflect variations in loading or transfer. Cell cycle after down-regulation of Bcl-2 and treatment with FTI-277 was evaluated by flow cytometry. DNA fluorescence histograms of propidium iodide-stained Huh7. The percentage of apoptotic cells represented by a subG1 peak is indicated as A0 (C).

in the cytoplasm, whereas mitogen-activated Raf-1 is bound to the plasma membrane. Mitotic activation of Raf-1 is partially dependent on tyrosine phosphorylation and does not signal via the MAP kinase pathway. Our study indicates that protein farnesylation, including that of Ras, is necessary for G_2/M cell cycle progression. Moreover, we identify a functional role for the Ras/Raf-1/Bcl-2 interaction in regulating the entry of liver cancer cells into mitosis, whereby disruption of this interaction contributes to arrest in G_2/M.

Although FTIs clearly inhibit Ras farnesylation, it is unclear whether the antiproliferative effects of these compounds are mediated exclusively through their effects on Ras. This study and future studies directed toward determining the pathways inhibited by FTIs during cancer cell growth may lead to identification of new molecular targets for the next generation of mechanism-based anticancer drugs.

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