Cytoreductive Effects of Farnesyl Transferase Inhibitors on Multiple Myeloma Tumor Cells[1]

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Abstract

Farnesyl transferase inhibitors (FTIs) are anticancer agents designed to target ras processing and ras-dependent signal pathways. Because oncogenic ras mutations are found in up to 50% of multiple myeloma (MM) specimens, these agents may be effective in this disease. However, some preclinical studies suggest that FTI antitumor responses are unrelated to effects on ras. To address this issue in myeloma, we used the ANBL-6 myeloma cell line where interleukin (IL)-6-dependent cells are stably transfected with mutated N-ras or K-ras genes. Because expression of mutated ras allows for IL-6-independent growth, this is a good model to test whether FTIs specifically target growth-promoting ras-activated pathways in myeloma. Although they had little effect in 10% serum, two separate FTIs induced apoptosis of myeloma cells when cultured in low serum, and mutated ras-expressing cells were more sensitive than wild-type (WT) ras-expressing cells. However, induction of apoptosis did not correlate with inhibition of ras processing. Although they had no effect on AKT activity, under low serum conditions FTIs inhibited constitutive activation of the p70S6kinase and nuclear factor κB signal proteins in both mutated ras-expressing MM lines and extracellular signal-regulated kinase (ERK) activity in mutated N-ras-expressing cells. However, in studies where p70, nuclear factor κB, and ERK were comparably inhibited by other inhibitors or by gene transfer, we could not identify effects on these pathways as participating in the apoptotic response. FTIs were also able to abrogate the IL-6 proliferative response of WT ras-expressing MM cells, and this was associated with inhibition of IL-6-induced activation of ERK, AKT, and p70. The induction of apoptosis and prevention of the IL-6 response in MM cells containing mutated or WT ras provide support for the therapeutic potential of FTIs in this disease.

Introduction

FTIs[3] are newly developed antitumor drugs that inhibit farnesylation of ras proteins, thus preventing their membrane localization and function (reviewed in Refs. 1–3). This inhibition theoretically interrupts ras-dependent growth-promoting cascades that are overactive in many tumor types. Because oncogenic mutations in both N-ras and K-ras genes are very common in MM tumor cells [up to 40–50% of cases (4–7)], FTIs may be very effective in this disease. Indeed, several recent early reports in abstract form (8, 9) suggest effectiveness against MM cells *in vitro*, and an early Phase I clinical study demonstrates some efficacy (10). However, other studies in different tumor models suggest that FTIs may induce antitumor effects by mechanisms independent of ras inhibition (1), and, in fact, the presence of ras mutations and overactive ras function may not identify tumor cells overly sensitive to FTIs (11, 12). To address this issue in myeloma, we used the ANBL-6 MM cell model (13–15) where IL-6-dependent cells are stably transfected with oncogenic mutated N-ras or K-ras genes or empty vector. Ectopic expression of mutated ras genes in this model allows for cytokine-independent growth expansion. Thus, this is a good model to test whether FTIs can specifically target growth-promoting ras-activated pathways in MM cells. Our results indicate that FTIs can induce apoptosis of myeloma cells in low serum concentrations. Although the sensitivity of mutated ras-containing MM cells to FTIs was significantly greater than that of WT ras-expressing cells, induction of apoptosis did not correlate with inhibitory effects on ras processing. In addition, the induction of apoptosis could not be explained by FTI inhibitory effects on ERK, AKT, p70S6K, or NF-κB.

Materials and Methods

Cell Lines and ras Transfections. The ANBL-6 parent and its transfected lines have been described previously in several publications (13–15). Briefly, ANBL-6 was stably transfected by retroviral transfection with virus expressing N-ras or K-ras genes mutated at codon 12 as described previously

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[3] The abbreviations used are: FTI, farnesyl transferase inhibitor; MM, multiple myeloma; IL, interleukin; NF-κB, nuclear factor κB; ERK, extracellular signal-regulated kinase; p70S6K, p70S6kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IKBsr, IKB super repressor; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift analysis; WT, wild-type; MEK, mitogen-activated protein/ERK kinase; PI3K, phosphatidylinositol 3′-kinase; STAT, signal transducers and activators of transcription; GSK, glycogen synthase kinase.

(14). ANBL-6 cells were also identically transfected with retrovirus lacking the transgene to serve as a control. G418-resistant populations arose after approximately 1 month of *in vitro* selection. To avoid artifacts of clonal selection, all studies were performed on mixed polyclonal cell populations. Expression of retroviral ras transcripts was confirmed by Northern analysis (14). The three ANBL-6 cell lines (which expressed WT ras, mutant N-ras, and mutant K-ras) were maintained in culture with continuous exposure to IL-6 added at 100 units/ml. While they were cultured in IL-6, the three cell lines demonstrated comparable growth rates and viabilities. To test cytoreductive or apoptotic effects of FTIs, IL-6 was depleted from all three cell lines, and MTT or flow cytometric assays were performed at 24, 48, or 72 h. During this time frame, viabilities and recoveries of all three cell lines in the absence of FTIs were comparable (see "Results").

Reagents. Recombinant IL-6 was purchased from R&D Systems (Minneapolis, MN). Phospho-specific antibodies were obtained from New England Biolabs. Rapamycin was a kind gift from Wyeth-Ayerst (Pearl River, NY). It was diluted in 100% ethanol. FTIs FTI-277 and FPT III were purchased from Calbiochem, Inc. (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO), unless otherwise described. In all experiments using inhibitors, final concentrations of all solvents were maintained at $<0.1\%$.

Western Blot Analysis. Protein was extracted and separated by SDS-PAGE as described previously (16). Proteins were transferred to polyvinylidene difluoride membranes and phosphorylated, and total proteins were detected as described previously (16). Relative expression was determined by densitometry.

Ras Processing Assay. The processing of different ras family members was performed as described previously (17) using immunoblot assays with a pan-ras antibody (catalogue number SC-35; Santa Cruz Biotechnology) or antibodies specific for K-ras (catalogue number SC-30; Santa Cruz Biotechnology) or N-ras (catalogue number SC-31; Santa Cruz Biotechnology).

AKT Kinase Assay. The *in vitro* kinase assay used a nonradioactive kit purchased from New England Biolabs. AKT was first immunoprecipitated from cell extracts and then incubated with GSK-3 fusion protein in the presence of ATP and kinase buffer. AKT-dependent GSK-3 phosphorylation was then measured by immunoblotting using a phospho-GSK-3 antibody that recognizes GSK-3 when phosphorylated.

MTT Assay. The MTT assay was performed as described previously (16). Briefly, $1–2 \times 10^4$ IL-6-depleted targets in 0.1 ml of complete medium were dispensed into a 96-well microtiter plate. Drugs were added to appropriate concentrations in 100 μl of media. After 48 h of culture, 100 μl of medium were removed, and 20 μl of MTT (5 mg/ml stock) were added to each well. The plates were incubated overnight to solubilize the formazan dye. The $A_{570\ nm}$ of each well was measured the next day with a microplate ELISA reader equipped with a 570 nm filter. Quadruplicate wells were run for each group, and the SD of each group was always $<5\%$ of the mean. To test the effects of FTIs on IL-6-stimulated proliferation in WT ras-expressing ANBL6 cells, the WT cells were first depleted of IL-6 for 24 h and then stimulated with or without IL-6 (100 units/ml) for 48 h in the presence or absence of different concentrations of FTIs. MTT assay was then performed.

Flow Cytometry for Cell Cycle Analysis and Apoptosis. For cell cycle analysis, myeloma cells were stained with hypotonic propidium iodide (50 μg/ml in 0.1% sodium citrate) and 0.1% Triton X-100 for 1 h at 4°C. Cells were kept in the dark at 4°C before analysis. Cell cycle distribution was determined by analyzing 10,000–15,000 events on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The DNA data were fitted to a cell cycle distribution analysis by use of the MODFIT program for MAC V2.0. The percentage of apoptotic cells was determined by enumeration of a sub-G_1 peak or by neoexpression of membrane annexin-V.

Transfection with IKBsr. An adenoviral vector that expresses an IKBsr (Ad-IKBsr) in which serines 32 and 36 of IKB-α have been mutated to alanines, thus preventing phosphorylation and dissociation from NF-κB and its subsequent degradation (18), and the control vector (Ad-CMV) were amplified and titered in 293 cells. Transgene expression in the Ad-IKBsr vector is driven by the CMV early/intermediate promoter/enhancer. The control vector, Ad-CMV, is identical to Ad-IKBsr but lacks a transgene insert. All adenoviral vectors were expanded and purified from 293 cells and subsequently titered. Adenoviral infection of myeloma cells was accomplished as described previously (19). Briefly, mutant N-ras-containing cells were transduced with adenovirus at a multiplicity of infection of 100 for 2 h. Adenovirus was then washed away, and cells were resuspended in media with low FCS overnight to minimize proliferation. Cells were studied by flow cytometry 48 h after infection.

EMSAs. WT and mutant κB oligonucleotide probes were purchased from Santa Cruz Biotechnology. Fifteen μg of nuclear protein were combined with end-labeled, double-stranded κB oligonucleotide probe, 1 μg of poly(deoxyinosinic-deoxycytidylic acid) (Amersham Biotech, Piscataway, NJ), 1 μg of BSA, and 5 mM spermidine in a final reaction volume of 20 μl for 20 min at room temperature. The DNA-protein complex was run on a 4% nondenaturing polyacrylamide gel with 0.4× Tris-borate EDTA running buffer before subsequent autoradiography.

Statistics. The *t* test was used to determine the significance of differences between groups. Western blot experiments that compared expression of phosphorylated signal proteins were performed at least two and often three separate times.

Results

FTIs Induce Myeloma Cell Cytoreduction and Apoptosis in Low Serum Conditions. To test the ras specificity of FTI antitumor effects against myeloma cells, we used the ANBL-6 myeloma cell line that has been stably transfected with oncogenic N-ras or K-ras genes or with an empty vector. Although the ANBL-6 parental cell line ceases growth when depleted of exogenous IL-6 (20), both mutant N-ras-expressing cells (N-ras cells) and K-ras-expressing myeloma cells (K-ras cells) are IL-6 independent and demonstrate continuous growth and survival in the absence of added

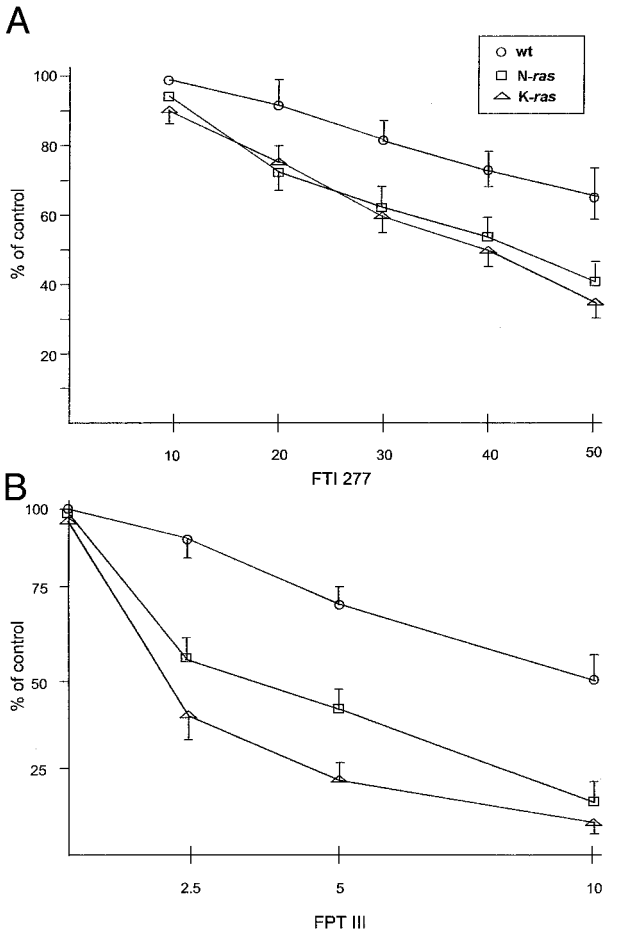

Fig. 1. Cytoreductive effects of FTIs against MM cells under low serum conditions. Forty-eight-h MTT assays were performed in 0.5% FCS with increasing concentrations (shown in μM) of FTI-277 (*A*) or FPT III (*B*). Results shown for WT ras (*wt*)-, mutated N-ras (*N-ras*)-, or K-ras (*K-ras*)-expressing MM cells are the percentage of control (relative to cultures without any FTIs present at 0.5% FCS; means $\pm$ SD of four separate experiments). The cytoreductive effects on WT cells are significantly less than those on mutated ras cells ($P < 0.05$) at all FTI doses.

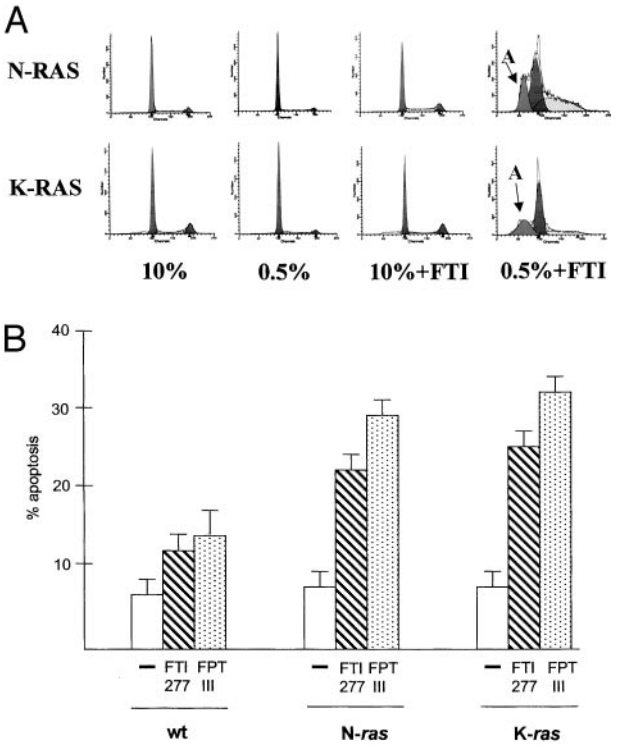

Fig. 2. FTIs induce MM cell apoptosis in low serum. In *A,* a representative cell cycle analysis is shown for mutated N-ras (*N-RAS*)- or K-ras (*K-RAS*)-expressing MM cells cultured in 10% or 0.5% FCS in the presence or absence of 30 μM FTI-277. *A*, sub-G_1 peak represents apoptotic cells. In *B,* the percentage of apoptosis induced in WT ras (*wt*)-, mutated N-ras (*N-ras*)-, or K-ras (*K-ras*)-expressing cells is shown when cells were cultured for 48 h in 0.5% FCS alone (–) or in 0.5% FCS with FTI-277 (30 μM) or FPT III (10 μM). Results are the means $\pm$ SD of three experiments.

cytokines (13–15). Our preliminary experiments confirmed the results of these previous studies. When maintained in 100 units/ml recombinant IL-6, viability and growth of N-ras, K-ras, and WT ras-expressing cells (WT cells, ANBL-6 transfected identically with an empty vector) was equivalent. After depletion of IL-6, cell numbers and viabilities were comparable for 72 h, but thereafter, both became inhibited in WT cells, and, by 12 days, few viable WT cells were present.

Because there were no significant differences in cell growth between the three lines during the first 72 h of IL-6 depletion, we first tested the effects of two FTIs, FTI-277 and FPT III, in 24–72 h MTT assays in the presence of 10% FCS and absence of IL-6. There was no significant effect on viable recovery of WT or K-ras cells and only a very modest effect on N-ras cells, which was only seen at the highest FTI concentrations. At 30 μM FTI-277 and 15 μM FPT III, a 14 $\pm$ 4% (mean $\pm$ SD of four separate experiments) and 16 $\pm$ 3% decrease in survival was observed in N-ras cells, which was significant ($P < 0.05$). This cytoreductive effect was optimal by 48 h of incubation and was due to G_1 arrest. After treatment with 30 μM FTI-277, the percentage of N-ras cells in S phase decreased from 18 $\pm$ 4% to 12 $\pm$ 3% (mean $\pm$ SD of four separate experiments). After treament with 15 μM FPT III, the percentage of cells in S phase decreased to 13 $\pm$ 3%. There was no significant induction of apoptosis as assayed by sub-G_1 peak analysis or annexin-V staining (data not shown). In addition, there was no significant alteration in S-phase distribution at lower concentrations of FTI-277 (1–20 μM) or FPT III (1–10 μM).

In contrast to the above-mentioned results, FTIs were significantly more effective when used with relative serum deprivation. When the MTT assay was performed in 0.5% FCS instead of 10% FCS in the absence of FTIs, there was no significant alteration in absorbance values over the first 48 h of incubation (data not shown). However, when exposed to FTIs in 0.5% FCS, a marked cytoreductive effect was observed (Fig. 1). This growth inhibition was concentration dependent and more marked in N-ras and K-ras cells than in WT cells (Fig. 1). Furthermore, growth inhibition was associated with an induction of apoptosis (Fig. 2). As shown in Fig. 2*B*, the detected apoptosis (with FTI-277 at 30 μM or FPT III at 10 μM) was significantly ($P < 0.05$) greater in N-ras and K-ras cells than in WT cells. When

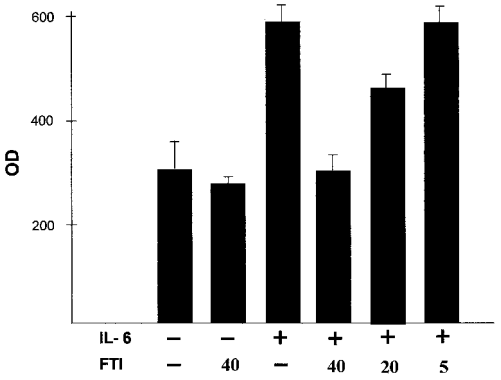

Fig. 3. FTIs prevent an IL-6 proliferative response. WT ras-expressing ANBL-6 MM cells were depleted of IL-6 for 24 h and then cultured for 48 h in 10% FCS with or without IL-6 (100 units/ml) ± FTI-277 at 0, 5, 20, or 40 μM. MTT assays were performed, and results are presented as absorbance (mean ± SD of four separate experiments). FTI-277 at 40 and 20 μM significantly ($P < 0.05$) inhibited the IL-6-induced increase in cell growth.

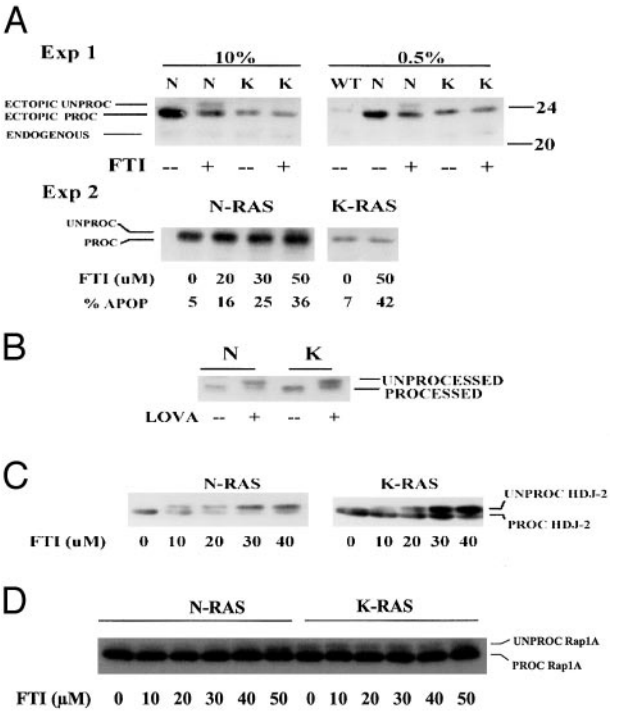

Fig. 4. FTI-induced apoptosis does not correlate with effects on ras processing. In *A, Exp 1*, WT ras (*WT*)-, mutated N-ras (*N*)-, or K-ras (*K*)-expressing MM cells were cultured in 10% or 0.5% FCS in the absence (−) or presence (+) of FTI-277 at 30 μM. After 48 h, immunoblot was performed with a pan-ras antibody. *UNPROC,* unprocessed; *PROC,* processed. A second identical experiment gave similar results (data not shown). In *Exp 2*, mutated N-ras (*N-RAS*)- or K-ras (*K-RAS*)-expressing cells were cultured in 0.5% FCS for 48 h with varying concentrations (in μM) of FTI-277 (shown *below* the blots). Immunoblot was then performed with N-ras- or K-ras-specific antibodies, and the percentage of apoptosis was determined [mean of three separate culture wells, shown *below* FTI concentrations (% *APOP*)]. In *B,* mutant N-ras (*N*)- or K-ras (*K*)-expressing cells were cultured for 48 h in 10% FCS in the presence (+) or absence (−) of lovastatin (*LOVA*) at 20 μM. Extracts were immunoprecipitated with anti-EE antibody and immunoblotted with a pan-ras antibody. In *C* and *D,* mutated ras-expressing cells (N-ras or K-ras) were cultured for 48 h with increasing concentrations of FTI-277 (in μM), and immunoblot was performed with anti-HDJ-2 antibody or anti-Rap1A antibody.

used at 15 μM, FTI-277 also induced a greater degree of apoptosis in N-ras and K-ras cells (15 ± 3% and 19 ± 4%, mean ± SD of three experiments; data not shown in Fig. 2) than in WT cells (9 ± 2%).

Although the FTIs had little effect on *in vitro* growth of WT cells in 10% FCS, they were capable of preventing IL-6-induced stimulated proliferation (Fig. 3). This inhibition was dose dependent because 40 μM FTI-277 completely abrogated IL-6-induced proliferation of WT cells, 20 μM partially inhibited stimulation, and 5 μM had no significant effect.

Effects of FTI on ras Processing. To test whether the cytoreductive/apoptotic effects of FTIs were due to inhibition of ras processing, we first treated N-ras or K-ras cells with or without 30 μM FTI-277 in 10% or 0.5% FCS. After 48 h, cell extracts were immunoblotted with a pan-ras antibody. The ectopically expressed mutated ras proteins in N-ras or K-ras cells were present as approximately M_r 24,000 proteins (Fig. 4*A, Exp 1*, compare N-ras and K-ras cells with WT cells in the *right panel*). Endogenous p21 ras was expressed as a faster migrating band and at much lower levels than transfected ras. The differential migration between ectopic and endogenous ras proteins was confirmed by immunoblot with anti-EE antibody to detect the epitope tag of the transgene (data not shown). When we tested the effects of FTIs on N-ras cells, ectopically expressed N-ras became expressed as a doublet with a very small amount of slower migrating protein representing nonprenylated, unprocessed N-ras [Refs. 17 and 21 (Fig. 4*A, Exp 1*). This decreased ras processing was comparable in 10% or 0.5% FCS (Fig. 4*A, Exp 1*). In contrast, little effect was seen on the processing of ectopically expressed K-ras in K-ras cells. In the same experiment, 30 μM FTI-277 induced apoptosis in 27% of N-ras cells and 30% of K-ras cells when low serum conditions were used. This effective apoptotic induction in the absence of effects on K-ras processing and with minimal effects on N-ras processing suggested a cytotoxic mechanism independent of effects on ras. This was confirmed in a second experiment (Fig. 4*A, Exp 2*) where N-ras or K-ras cells were again treated with or without different concentrations of FTI-277 in 0.5% FCS (concentrations shown *below* the blot). In this experiment, extracts from N-ras or K-ras cells were immunoblotted with N-ras- or K-ras-specific antibodies, respectively. The percentage of apoptosis induced by the various concentrations of FTI-277 is also shown below the blots (Fig. 4*A, Exp 2, % APOP*). Once again, effective apoptosis was induced in K-ras cells incubated with 50 μM FTI-277 without concomitant effects on K-ras processing. In N-ras cells, there was an inhibitory effect on N-ras processing at 50 μM FTI concentration that was associated with 36% apoptosis. However, at 30 and 20 μM, there were minimal effects on processing but significant induction of apoptosis (25% and 16% apoptosis, respectively).

To ensure that effects on ras processing could be detected by immunoblot assay if effectively induced, we also treated both mutant ras-containing cell lines with lovastatin, effectively blocking the production of mevalonate, a critical component in the synthesis of isoprenoids that are needed to

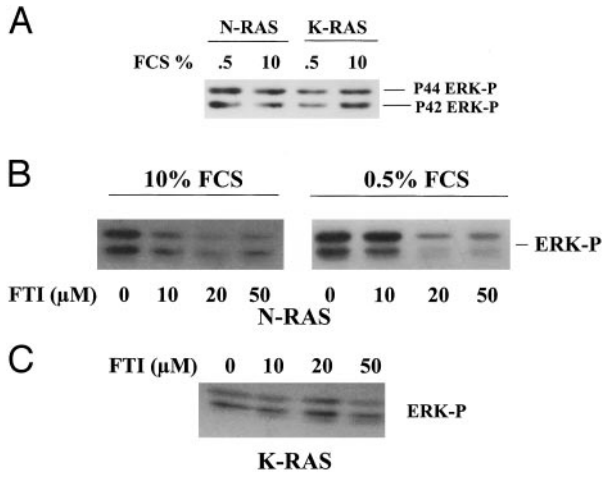

Fig. 5. Effects of FTI-277 and low serum on ERK phosphorylation. In *A,* mutated ras (N-ras or K-ras)-expressing cells were cultured for 48 h in 0.5% or 10% FCS, and immunoblot was performed with phospho-specific anti-ERK antibody. Equal amounts of total ERK were present in each lane (data not shown). In *B,* mutant N-ras-expressing cells were cultured for 48 h in 10% or 0.5% FCS with increasing concentrations of FTI-277, and immunoblot was performed with phospho-specific anti-ERK antibody. Equal amounts of total ERK were present in each lane (data not shown). In *C,* mutant K-ras-expressing cells were cultured in 0.5% FCS with increasing concentrations (μM) of FTI-277, and immunoblot was performed with phospho-specific anti-ERK antibody. Equal amounts of total ERK were present in each lane (data not shown).

process ras (22). After treatment with 20 μM lovastatin for 48 h, we immunoprecipitated transfected N-ras or K-ras with anti-EE antibody and immunoblotted with a pan-ras antibody. As shown in Fig. 4*B*, lovastatin was comparably effective in inhibiting processing of both N-ras and K-ras. Finally, to demonstrate that FTI-277 was capable of inhibiting farnesylation of some proteins, we immunoblotted extracts for the chaperone protein HDJ-2, which is normally farnesylated (23). As shown in Fig. 4*C*, FTI-277 was effective in inhibiting farnesylation of HDJ-2, and this was comparable in N-ras *versus* K-ras cells. Thus, FTIs can inhibit farnesylation of some proteins in N-ras and K-ras cells, but at concentrations that induce apoptosis, processing of ectopically expressed K-ras is unaffected, and processing of N-ras is minimally affected, indicating that apoptosis is mediated by independent effects.

Because geranylgeranyltransferase inhibitors can induce apoptosis under certain conditions, we also asked whether the efficacy of FTI-277 may have been due to its ability to inhibit geranylgeranylation. To test this issue, we immunoblotted extracts for the Rap1A protein, which is geranylgeranylated. Fig. 4*D* demonstrates very little effect on prenylation of this geranylgeranylated protein in N-ras or K-ras cells. There is only a slight increase in unprocessed Rap1A in N-ras cells exposed to 50 μM FTI-277. In K-ras cells, a small amount of unprenylated Rap1A is expressed, which does not increase with exposure to up to 50 μM FTI-277. Thus, the significant apoptotic effect of FTI-277 seen with concentrations below 50 μM cannot be due to inhibiting effects on geranylgeranylation.

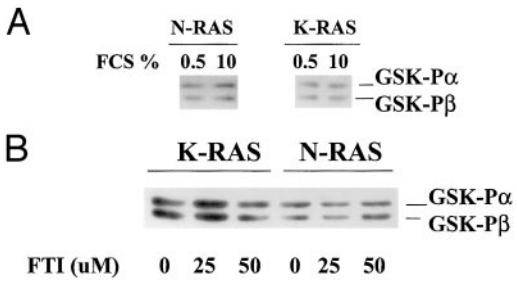

Fig. 6. FTI-277 or low serum does not affect AKT activity. In *A,* mutant ras-expressing cells (N-ras or K-ras) were cultured for 48 h in 0.5% or 10% FCS, and AKT *in vitro* kinase assay was performed where AKT was immunoprecipitated and tested for its ability to phosphorylate the substrates GSK-α or GSK-β. Immunoblot is shown with phospho-specific anti-GSK antibody. This experiment was repeated once with identical results. In *B,* mutated ras-expressing cells were cultured for 48 h in 0.5% FCS with increasing concentrations of FTI-277 (in μM), and AKT *in vitro* kinase assay was performed. This experiment was repeated with identical results.

Effects of FTIs on Signaling Proteins as a Possible Explanation for Induction of Apoptosis in Low Serum. In previous work (24), we identified up-regulated signaling through MEK/ERK, PI3K/AKT, mTOR/p70S6K, and NF-κB in mutant ras-expressing myeloma cells. Because these pathways can promote viability in some models (25–28), we next investigated whether FTIs could inhibit these signal cascades in attempts to gain insight into the mechanism of FTI-induced apoptosis. Because the combination of serum deprivation and FTI exposure was required to induce MM cell apoptosis, we also asked whether low serum conditions, alone, inhibited these signal cascades or allowed for a significantly greater inhibition induced by FTIs.

We first assayed ERK because it is downstream of ras signaling and regulates survival responses of certain cell types (25). N-ras and K-ras cells were first treated with FTI-277 in the presence of 10% or 0.5% FCS, and protein extracts were obtained. The presence of low serum concentration had little effect on the degree of ERK phosphorylation in both N- and K-ras mutation-containing cells (Fig. 5*A*). In additional experiments, N-ras cells were sensitive to FTI-induced effects on levels of phosphorylated ERK (Fig. 5*B*), and ERK inhibition was comparable in 10% *versus* 0.5% FCS. In contrast, ERK phosphorylation was unaffected in K-ras cells in 0.5% FCS (shown in Fig. 5*C*) or in 10% FCS (data not shown). This was expected because FTIs did not affect K-ras processing in K-ras cells (see above).

In experiments with a similar design, we examined AKT kinase activity in immune kinase assays using GSK as a substrate. As shown in Fig. 6*A*, low serum had little effect on basal AKT kinase activity in both cell populations. Furthermore, FTI-277 used in concentrations of up to 50 μM had no effect on AKT kinase activity in either cell line in the presence of low serum (Fig. 6*B*). Because neither low serum nor FTI-277 could inhibit AKT activity, it appears unlikely that effects on AKT contribute to the apoptotic effect of FTI-277 in low serum.

We next looked at FTI effects on p70S6K activation. p70S6K activation is a result of hierarchical phosphorylation

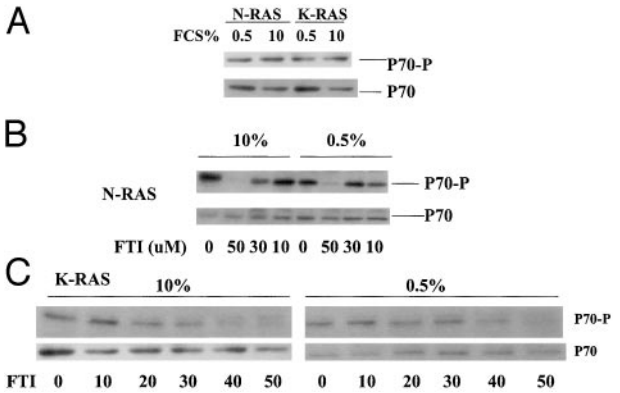

Fig. 7. Effects of FTI-277 on phosphorylation of p70S6K. In *A,* mutated ras-expressing cells were cultured in 0.5% or 10% FCS for 48 h, and immunoblot assay was performed for phosphorylated (on threonine 389) p70 (*P70-P*) or total p70 (*P70*). In *B,* mutated N-ras-expressing cells were cultured in 10% or 0.5% FCS with increasing concentrations of FTI-277 (in μM), and immunoblot was performed for phosphorylated or total p70. In *C,* mutated K-ras-expressing cells were cultured in 10% or 0.5% FCS with increasing concentrations of FTI-277, and immunoblot assay was performed for expression of phosphorylated p70 (*P70-P*) or total p70. Each of the experiments shown in *A–C* was repeated once with identical results.

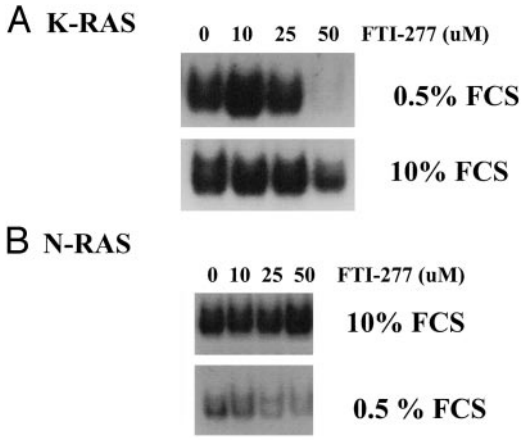

Fig. 8. Effects of FTI-277 on NF-κB activation. In *A,* mutant K-ras-expressing cells were cultured in 0.5% or 10% FCS with increasing concentrations of FTI-277 for 48 h, and EMSA was performed using κB oligonucleotide probe. In *B,* a similar experiment was performed for mutant N-ras-expressing cells. Both experiments (*A* and *B*) were repeated with identical results.

events culminating in phosphorylation on threonine 389 and 229 (29). Immunoblot assays with antibody specific for p70S6K phosphorylated on threonine 389 demonstrated constitutive phosphorylation in both N-ras and K-ras cells that was unaffected by low serum concentrations (Fig. 7*A*). FTI-277 was capable of inhibiting p70S6K phosphorylation in both cell lines, but low serum concentration did not enhance this suppression (Fig. 7, *B* and *C*). When N-ras cells were incubated in FTI-277 (Fig. 7*B*), p70S6K phosphorylation was completely abrogated in 50 μM FTI-277 in both 0.5% and 10% FCS and was modestly decreased (approximately 50% by densitometric analysis) in 10% serum when treated with 30 μM FTI-277. In contrast, 30 μM FTI had little effect in 0.5% FCS. In K-ras cells (Fig. 7*C*), p70S6K phosphorylation was significantly inhibited by 40 or 50 μM FTI-277, and inhibition was comparable in 10% *versus* 0.5% FCS. Inhibition of p70S6K phosphorylation was very rapid with a significant decrease detected by 2 h of incubation with FTI-277 (data not shown). These accelerated kinetics suggest that the effects on p70S6K may be independent of effects on farnesylation (see "Discussion").

To test potential inhibitory effects on NF-κB activation, we performed EMSAs. A previous study (24), using competition with cold WT *versus* mutant κB probes and supershift analysis, confirmed the presence of two DNA-binding complexes that were specific for NF-κB members and represented p65/p50 heterodimers and p50/p50 homodimers. In these experiments, the presence of low serum significantly inhibited NF-κB activation and enhanced the inhibitory effect of FTI-277. In K-ras cells, for example (Fig. 8*A*), 50 μM FTI-277 only modestly inhibited NF-κB activation in 10% FCS but completely prevented it in 0.5% FCS. Likewise, in N-ras cells (Fig. 8*B*), the presence of low serum itself significantly inhibited activation, and a further inhibition occurred with 25 and 50 μM of drug. In contrast, FTI-277 was ineffective in 10% FCS (Fig 8*B*). These results suggested that the enhanced inhibition of NF-κB in low serum might account for FTI-induced apoptosis.

Inhibition of ERK Phosphorylation, AKT Activity, and p70S6K Phosphorylation by Other Agents Do Not Induce Apoptosis. The results above indicate that FTIs can inhibit ERK phosphorylation in N-ras cells (Fig. 5) and p70S6K phosphorylation in both cell types (Fig. 7). To test whether the inhibitory effects of FTI-277 on ERK or p70 might participate in the apoptotic response, we similarly inhibited ERK phosphorylation by incubation with the MEK inhibitor PD98059 and inhibited p70 phosphorylation with the mTOR inhibitor rapamycin. Preliminary experiments demonstrated that 50 μM PD98059 and 10 μM rapamycin completely abrogated ERK and p70 phosphorylation, respectively, in mutated ras-containing ANBL-6 MM cells (data not shown). MM cells were then incubated with these drugs for 48 h in 10% or 0.5% FCS. As shown for PD98059 in Fig. 9*A* and rapamycin in Fig. 9*B*, neither drug could induce any apoptosis under these conditions, whereas FTI-277 consistently induced apoptotsis in 0.5% FCS. Thus, it is unlikely that a similar inhibition of these signal proteins induced by FTI-277 in low serum participates in the apoptotic response. Furthermore, the combination of PD98059 or rapamycin with FTI-277 in 10% FCS did not induce apoptosis. This indicates that serum-induced activation of ERK or p70 is not masking the apoptotic response of FTI.

The results of AKT *in vitro* kinase assays (Fig. 6) demonstrated that neither low serum nor FTI-277 could inhibit AKT activity, suggesting that effects on AKT do not participate in FTI-induced apoptosis in low serum. However, because this conclusion is contrary to a previous report (30), we also tested this question by inhibiting AKT activation with the PI3K inhibitor wortmannin. As shown in Fig. 9*C*, wortmannin by itself (at 0.1 μM, which completely prevented AKT activity, data not shown) did not induce apoptosis in either 0.5% or 10% FCS. Importantly, inhibiting AKT activity with wortman-

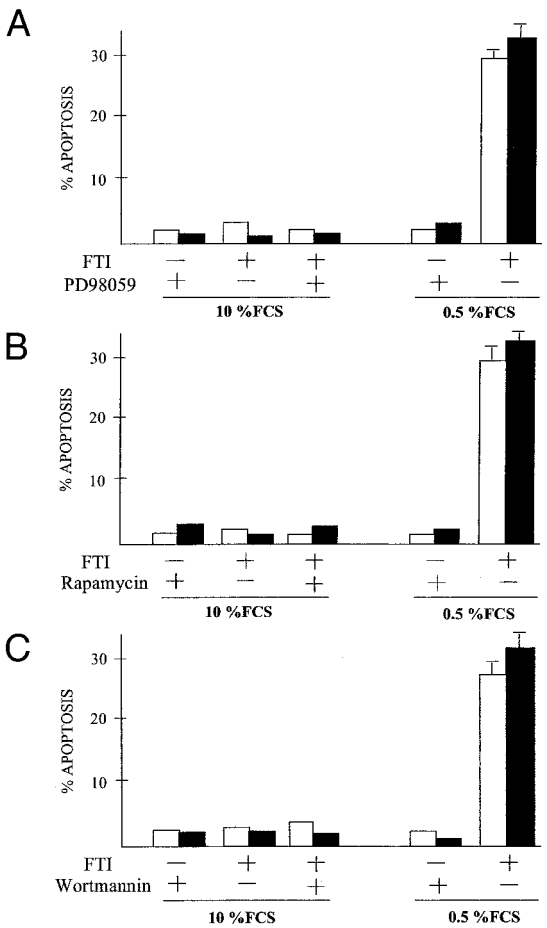

Fig. 9. Inhibition of ERK, p70, or AKT by other inhibitors does not result in apoptosis. Mutated N-ras (□)- or K-ras (■)-expressing cells were cultured for 48 h in 10% or 0.5% FCS in the presence or absence of FTI-277 (30 μM) and in the presence or absence of PD98059 (*A*; 50 μM), rapamycin (*B*; 10 μM), or wortmannin (*C*; 0.1 μM). The percentage of apoptosis was determined by sub-G_1 peak on cell cycle analysis and is presented as the mean ± SD of three separate experiments.

nin in 10% FCS did not synergize with FTI-277 for significant induction of apoptosis. These data further support the notion that effects on AKT do not participate in FTI-induced apoptosis.

Inhibition of NF-κB Activation by IKBsr. Because EMSA experiments indicated that FTI-277 was a more effective inhibitor of NF-κB in 0.5% FCS than in 10% FCS (Fig. 8), we investigated a possible role in the apoptotic response by acutely expressing the IKBsr gene with an adenoviral vector. The IKBsr protein contains serine-to-alanine mutations that prevent its phosphorylation and dissociation from NF-κB (18). For unknown reasons, the IKBsr is readily expressed by infection of mutated N-ras-containing ANBL6 MM cells (>85% transfection efficiency with multiplicity of infection = 100), but K-ras cells are resistant. Thus, we could only test N-ras cells in these experiments. As in our previous study (24), expression of the IKBsr completely abrogated NF-κB activation in N-ras cells (Fig. 10*A*), and the degree of inhibition was comparable in 10% *versus* 0.5% FCS. Cell cycle analysis demonstrated that inhibition of NF-κB in IKBsr-expressing N-ras cells induced significant apoptosis (27% apoptosis (*A*) in the *top third panel* of Fig. 10*B*) when the incubation was run in 10% FCS. However, although IKBsr expression completely abrogated NF-κB activation by EMSA assay, its induction of apoptosis was considerably less impressive than that achieved by FTI-277 in low serum. Fig. 10*B* also shows that inhibition of NF-κB activation induced by expression of the IKBsr in combination with presence of low serum concentration did not result in enhanced apoptosis (21% apoptosis, *bottom right panel*). These results argue against the notion that FTI-induced NF-κB inhibition can completely explain the resulting apoptosis in low serum.

FTI-277 Inhibits IL-6-induced Signaling in WT ANBL6 MM Cells. As shown above in Fig. 3, FTI-277 successfully prevented the ability of IL-6 to stimulate growth of WT ras-expressing MM cells. To test effects on IL-6 signal pathways, we pretreated these ANBL6 cells with or without FTI-277 for 48 h and then stimulated them with IL-6 (100 units/ml) for 15 min. As shown in Fig. 11, IL-6 activated AKT activity (*in vitro* kinase assay) as well as ERK, p70S6K, and STAT3 phosphorylation. Pretreatment with FTI-277 inhibited the activation of ERK, AKT, and p70 but had no effect on IL-6-induced tyrosine phosphorylation of STAT3.

Discussion

Several studies indicate that activation of ras-dependent signal pathways in MM cells promotes tumor cell proliferation and viability. These pathways may be hyperactive due to continual stimulation with the IL-6 myeloma growth factor that signals through ras (31, 32) or the presence of constitutively active mutated ras genes (24). Thus, targeting ras with inhibitors of ras farnesylation may lead to effective therapy in patients. Our results are supportive of this strategy. Two FTIs, FTI-277 and FPT III, induced apoptosis of myeloma cells containing ras mutations when cultured under low serum conditions. In addition, FTI-277 abrogated an IL-6 proliferative effect in 10% serum when used in the IL-6-dependent ANBL-6 myeloma line containing WT p21 ras.

Although FTIs were initially developed to target ras, several studies support the notion that FTIs induce antitumor effects via mechanisms independent of effects on ras processing (11, 12). Our results are consistent with apoptotic effects of FTIs that are independent of alterations in ras processing. There was no effect of apoptotic concentrations of FTI-277 on K-ras processing, and there were very minimal effects on N-ras processing. A very modest inhibition of N-ras processing was present with concentrations of FTI-277 that could still induce significant apoptosis (*i.e.*, 20–30 μM; Fig. 4*A, Exp 2*). In previous studies (17, 33, 34), K-ras processing has been extremely resistant to inhibition by FTIs, whereas N-ras processing has been variably sensitive. Presumably, in our MM cells, oncogenic N-ras and K-ras continue to be predominantly processed by geranylgeranylation during treatment with FTI-277. In fact, processing of both ras oncoproteins was extremely sensitive to inhibition with lovastatin, a drug that inhibits both farnesylation as well as geranylgeranylation (Fig. 4*B*).

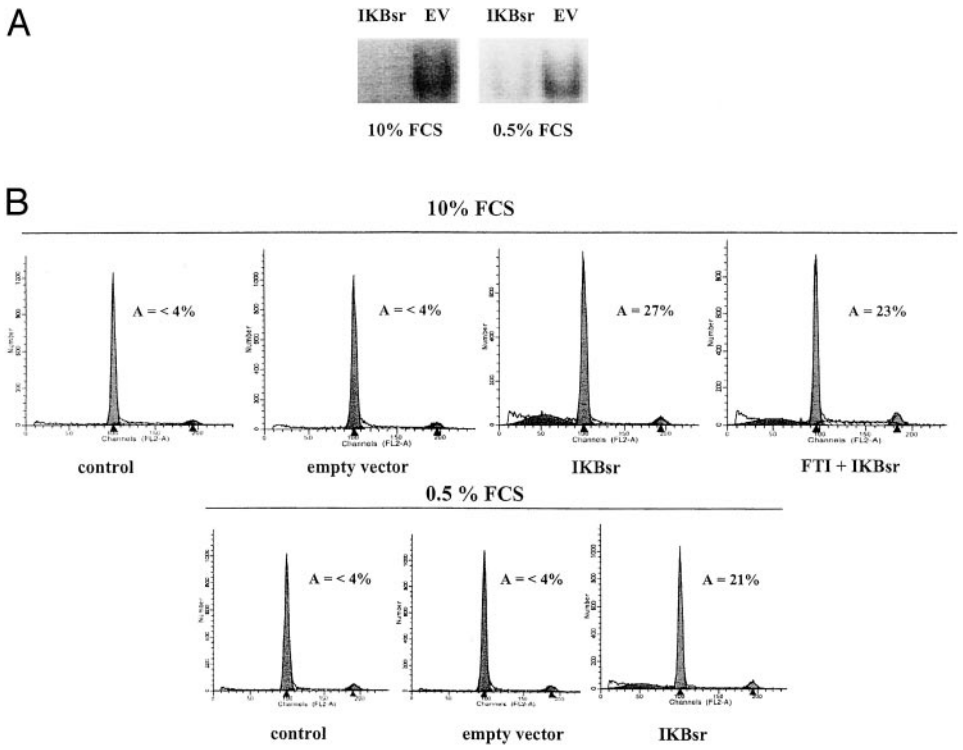

Fig. 10. In *A,* mutant N-ras-expressing cells were infected with adenoviruses expressing the IKBsr gene or empty vector (*EV*). After culture in 10% or 0.5% FCS for 48 h, EMSA was performed using κB oligonucleotide probe. In *B,* mutant N-ras-expressing cells were infected with virus expressing IKBsr or empty vector and then cultured for 48 h in 10% or 0.5% FCS with or without FTI-277 (30 μM). Cell cycle analysis was performed, and the percentage of cells in sub-G_1 peak (apoptosis = *A*) is shown. The experiment in *B* was repeated once with identical results.

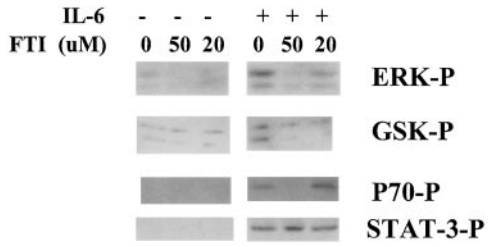

Fig. 11. FTI inhibits IL-6-induced signaling in WT ras-expressing MM cells. WT ANBL-6 MM cells were cultured with or without FTI-277 at increasing concentrations for 48 h in the absence of IL-6. Cells were washed and then stimulated without or with IL-6 (100 units/ml) for 15 min. At that time, immunoblot assay was performed for expression of phosphorylated ERK (*ERK-P*), p70S6K (*P70-P*), and STAT3 (*STAT-3-P*). AKT was also immunoprecipitated from extracts, and *in vitro* kinase assay was performed for phosphorylation of GSK substrate.

In previous studies (30, 35), ras-transformed cells were susceptible to FTI-induced apoptosis in low serum, but non-transformed cells remained viable. Our results are similar in that the cytoreductive/apoptotic effect of FTIs was more marked in myeloma cells containing mutated ras than in control WT ras-expressing cells that were transfected with empty vector. Our WT ras-expressing cells may be considered nontransformed because they do not survive indefinitely if depleted of IL-6. However, although less sensitive, these latter cells were significantly killed by FTIs, so the correlation to the previous studies (30, 35) is not perfect. By inhibiting farnesylation of a still unknown protein, apoptosis may be triggered, and the activity of this protein may be higher in the mutant ras-containing myeloma cells or a more critical determinant of survival in these cells. Alternatively, by changing the prenylation pattern of Rho B from farnesylation to geranylgeranylation, FTIs may induce apoptosis [as suggested by Du *et al.* (30)], and geranylgeranylated Rho B may be more active in mutant ras-containing myeloma cells than in WT-expressing cells.

FTI-277 had a potent inhibitory effect in preventing IL-6-induced growth of WT ras-expressing MM cells. This antitumor effect may be clinically relevant because IL-6 is clearly an important tumor growth factor *in vivo* in patients (36, 37). In conjunction with this antitumor effect, FTI-277 significantly inhibited the ability of IL-6 to stimulate ERK, AKT, and p70S6K but had no effect on STAT3 tyrosine phosphorylation (Fig. 11). This is consistent with an inhibitory effect on p21 ras because the known IL-6 signal pathways diverge proximally from the gp130 IL-6 receptor (38) into either STAT activation pathways or ras-dependent pathways that include MEK/ERK (31, 32), PI3K/AKT (39), or mTOR/p70S6K (40). The inhibition of the ras-dependent MEK/ERK pathway may be particularly important because prior work implicated this cascade in the IL-6-induced proliferation of MM cells (32). The sensitivity of the IL-6-stimulated, WT ras-expressing cells may be explained by the ability of IL-6 to induce GTP binding to H-ras. In experiments not shown, H-ras was found to be strongly expressed in WT ANBL-6 cells. A recent study in astrocytoma cell lines (41) indicates that increased H-ras GTP levels predict for FTI efficacy.

We have previously identified (24) constitutive activation of four signal pathways in MM cells containing ras mutations: (*a*) MEK/ERK; (*b*) PI3K/AKT; (*c*) mTOR/P70S6K; and (*d*) NF-κB. We thus examined the effects of apoptotic concentra-

tions of FTIs on these signal proteins in attempts to dissect the mechanism of apoptosis of N-ras and K-ras cells in low serum. FTI-277 was capable of inhibiting ERK phosphorylation when used in high concentrations in N-ras cells but had little effect on K-ras cells. Because these concentrations had an effect on oncogenic N-ras processing but no effect on K-ras processing, the inhibition of ERK phosphorylation was probably due to accumulation of cytoplasmic nonprenylated N-ras that sequestered raf-1 in a nonactivated form as described previously (42). Inhibited raf-1 activation would result in inhibited downstream signaling through ERK. The absence of effects on ERK on K-ras cells clearly rules out a role for this kinase in FTI-induced apoptosis of K-ras cells. To test whether FTI-induced ERK inhibition in N-ras cells played a role in induction of apoptosis, we similarly inhibited ERK phosphorylation with the MEK inhibitor PD98059. Inhibition did not induce significant apoptosis, even in 0.5% FCS. These data rule out the notion that inhibitory effects on ERK phosphorylation in N-ras cells play a role in the apoptotic response.

The regulation of AKT phosphorylation and activity by FTIs is controversial, with some models demonstrating inhibition (43, 44), and some showing an absence of effect (30, 45, 46). In our N-ras and K-ras cells, the lack of AKT inhibition in FTI-treated or low serum-treated cells strongly argues against a role for effects on AKT in FTI-mediated apoptosis. However, because this is in contrast to prior work that suggested that serum-induced AKT activation masked the proapoptotic effects of FTIs (30), we further tested this issue by inhibiting AKT with the PI3K inhibitor wortmannin. Wortmannin was unable to induce apoptosis in 10% or 0.5% FCS, nor was it able to unmask a possible FTI apoptotic effect in 10% FCS.

In contrast to ERK and AKT, FTIs significantly prevented p70S6K phosphorylation in both N-ras and K-ras cells, and this was detected by 2 h of treatment. These results are similar to those obtained by Law *et al.* (47). As described by these authors, effects of FTI on farnesylation should only be manifested when a significant percentage of previously farnesylated protein is degraded. Because most farnesylated proteins are long-lived, this rapid inhibition of p70 phosphorylation suggests that the FTI effect is not due to effects on a farnesylated protein. In contrast, the previous work of Law *et al.* (47) suggested that FTI induced rapid activation of a p70S6K phosphatase. The inability of p70 dephosphorylation to induce apoptosis when achieved by rapamycin indicates that the effects on p70 phosphorylation also do not mediate apoptosis.

The combination of low serum conditions and FTI-277 successfully synergized for marked inhibition of NF-κB activation. Because NF-κB is an important antiapoptotic protein (28), its inhibition during exposure to FTI-277 under low serum conditions suggested a role in FTI-induced apoptosis. However, FTI-277 used at 25 μM has no effect on NF-κB activation in K-ras cells, even in 0.5% FCS (Fig. 8*A*), and is successful in inducing apoptosis (Fig. 2*B*). Experiments in which we abrogated NF-κB activation in N-ras cells by acute expression of the IKBsr also argue against a role in FTI-induced apoptosis. Although abrogation of NF-κB activation by the IKBsr induces significant apoptosis in 10% FCS, there is no further increase in apoptosis when the experiment is run in 0.5% FCS (in fact, the degree of apoptosis decreases from 27% to 21%). Furthermore, the combination of FTI-277 and expression of IKBsr in 10% FCS (*top right panel* in Fig. 10*B*) does not increase the level of apoptosis over that of IKBsr expression alone (*top third panel* in Fig. 10*B*), indicating that serum-induced NF-κB activation does not prevent FTI-induced apoptosis.

In summary, mutated ras-expressing myeloma cells are sensitive to apoptosis induced by FTIs when cultured in relative serum deprivation. In addition, WT ras-expressing myeloma cells are also sensitive to FTI-induced prevention of an IL-6 proliferative response. Although not well correlated with inhibitory effects on ras processing or on individual inhibitory effects of ERK, AKT, p70S6K, or NF-κB, these results indicate that a therapeutic potential of FTIs in patients with myeloma should be further explored.

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Cytoreductive Effects of Farnesyl Transferase Inhibitors on Multiple Myeloma Tumor Cells [1]

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