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Characterization of a Human Carcinoma Cell Line Selected for Resistance to the Farnesyl Transferase Inhibitor 4-(2-(4-(8-Chloro-3,10-dibromo-6,11-dihydro-5*H*-benzo-(5,6)-cyclohepta(1,2-*b*)-pyridin-11(*R*)-yl)-1-piperidinyl)-2-oxo-ethyl)-1-piperidinecarboxamide (SCH66336)

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ABSTRACT

Farnesyl protein transferase inhibitors (FTIs) have demonstrated clinical activity in certain solid tumors and hematological malignancies. Little is known about mechanisms of resistance to these agents. To provide a basis for better understanding FTI resistance, the colorectal carcinoma cell line HCT 116 was selected by stepwise exposure to increasing 4-(2-(4-(8-chloro-3,10-dibromo-6,11-dihydro-5*H*-benzo-(5,6)-cyclohepta(1,2-*b*)-pyridin-11(*R*)-yl)-1-piperidinyl)-2-oxo-ethyl)-1-piperidinecarboxamide (SCH66336) concentrations. The resulting line, HCT 116R, was 100-fold resistant to SCH66336 and other FTIs, including methyl {*N*-[2-phenyl-4-*N*[2(*R*)-amino-3-mercaptopropylamino] benzoyl]}-methionate (FTI-277), but was less than 2-fold resistant to the standard agents gemcitabine, cisplatin, and paclitaxel. Accumulation of the unfarnesylated forms of prelamin A and HDJ-2, two substrates that reflect farnesyl transferase inhibition, was similar in FTI-treated parental and HCT 116R cells, indicating that alterations in drug uptake or inhibition of farnesyl protein transferase is not the mechanism of resistance. Changes in signal-transduction pathways that might account for this resistance were examined by immunoblotting and confirmed pharmacologically. There was no difference in activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase pathway or sensitivity to the MEK1/2 inhibitor 2′-amino-3′-methoxyflavone (PD98059) in HCT 116R cells. In contrast, increased phosphorylation of the molecular target of rapamycin (mTOR) and its downstream target p70 S6 kinase and increased levels of Akt1 and Akt2 were demonstrated in HCT 116R cells. Further experiments demonstrated that the mTOR inhibitor rapamycin selectively sensitized HCT 116R cells to SCH66336 but not to gemcitabine, cisplatin, or paclitaxel. These findings provide evidence that alterations in the phosphatidylinositol-3 kinase/Akt pathway can contribute to FTI resistance and suggest a potential strategy for overcoming this resistance.

Several polypeptides involved in signal transduction undergo prenylation (Zhang and Casey, 1996). This covalent addition of either a farnesyl (15-carbon) or a geranylgeranyl (20-carbon) group to the cysteine residue located in a tetrapeptide CAAX (A = aliphatic, X = methionine or serine) sequence at the carboxyl terminus facilitates membrane attachment of several G proteins that are synthesized in the cytoplasm but perform their functions when anchored in the plasma membrane (Clarke, 1992). One such protein is Ras, which ordinarily requires farnesylation before it can mediate its proliferative functions (Kato et al., 1992). As a conse-

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ABBREVIATIONS: PI-3, phosphatidylinositol-3; ERK, extracellular signal-regulated kinase; FT, farnesyl protein transferase; FTI, farnesyltransferase inhibitor; GSK, glycogen synthase kinase; MAPK, mitogen activated protein kinase; mTOR, molecular target of rapamycin; MEK, mitogen-activated protein kinase kinase; topo, topoisomerase; PAGE, polyacrylamide gel electrophoresis; SCH6636, 4-(2-(4-(8-chloro-3,10-dibromo-6,11-dihydro-5*H*-benzo-(5,6)-cyclohepta(1,2-*b*)-pyridin-11(*R*)-yl)-1-piperidinyl)-2-oxo-ethyl)-1-piperidinecarboxamide; PD98059, 2′-amino-3′-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; R115777, (*R*)-6-(amino(4-chlorophenyl)(1-methyl-1*H*-imidazol-5-yl)methyl)-4-(3-chlorophenyl)-1-methyl-2(1*H*)-quinolinone; BMS-214662, (*R*)-7-cyano-2,3,4,5-tetrahydro-1-(1*H*-imidazole-4-ylmethyl)-3-(phenylmethyl)-4-(2-thienylsulfonyl)-1*H*,4-benzodiazepine; FTI-277, methyl {*N*-[2-phenyl-4-*N*[2(*R*)-amino-3-mecaptopropylamino] benzoyl]}-methionate, TFA; GGTI-286, *N*-4-[2(*R*)-amino-3-mercaptopropyl]amino-2-phenylbenzoyl-(L)-leucine methyl ester, TPA.

quence of mutations in *Ras* genes, Ras proteins are constitutively activated in 30% of human tumors. These mutations result in constitutive activation of several Ras-activated intracellular signaling pathways, including the PI-3 kinase/Akt pathway, which is critical for cell survival, and the Raf/MEK/ERK pathway, which has been implicated in cell proliferation (Lange-Carter et al., 1993). Thus, tumors bearing *Ras* mutations are relatively more aggressive and have a poor clinical outcome (Samowitz et al., 2000).

FTIs were originally developed to inhibit the aberrant Ras-mediated signals that stimulate cell proliferation, apoptosis, invasion, and angiogenesis (Downward, 1998). At least three FTIs discovered through high-throughput screening are currently undergoing clinical testing. These include SCH66336 (Lonafarnib, Sarasar), R115777 (Tipifarnib, Zarnestra), and BMS-214662 (Adjei, 2003). As a class, these agents have shown clinical activity in breast cancer, glioma, head and neck cancer, and a wide range of hematological malignancies, including acute myelogenous leukemia, chronic myelogenous leukemia, and myelodysplastic syndrome (Adjei, 2003).

Despite this potentially promising clinical activity, several questions about the action of these agents remain unanswered. First, the cellular target responsible for the biological activity of FTIs has remained uncertain. Although early studies in Ras-transfected fibroblasts supported the idea that FTIs target Ras, more recent studies suggest that Ras proteins might not be the exclusive target of FTIs. Instead, FTI-induced cytotoxicity has been postulated to involve inhibition of the small GTPase RhoB (Du and Prendergast, 1999), the centromere-binding proteins CENP-E and CENP-F (Ashar et al., 2000), an unidentified polypeptide that acts upstream of the serine/threonine kinases Akt1 and Akt2 (Jiang et al., 2000; Chun et al., 2003), and/or other polypeptides that are yet to be identified (Haluska et al., 2002). Second, the mechanisms that influence the differential sensitivity of various tumors to FTIs are poorly understood. Previous studies have demonstrated that deliberate mutations of FT can produce an enzyme with diminished drug sensitivity (Del Villar et al., 1999); but the occurrence of these mutations in intact cells has not been demonstrated. More recently, Smith et al. (2002) described an R115777-selected cell line but were unable to establish a mechanism for its resistance.

In the present study, we sought to address these issues by selecting a mixed population of HCT 116 colorectal carcinoma cells for resistance to FTIs. This HCT 116R line exhibited 100-fold resistance to various FTIs. Inhibition of protein farnesylation was similar in the parental and resistant cell lines, suggesting that altered drug uptake or FT sensitivity was not the underlying mechanism of resistance. Instead, resistant HCT 116 cells exhibited elevated levels of Akt1 and Akt2 and increased phosphorylation of downstream targets, including the kinase mTOR and its substrate p70 S6 kinase. Further experiments demonstrated that the mTOR inhibitor rapamycin selectively sensitized the resistant cells to SCH66336. These findings not only suggest that resistance of HCT 116R cells is caused by changes in Akt isoforms, but they also provide a potential strategy for overcoming this resistance.

Materials and Methods

Materials. SCH66336 was supplied by Schering Plough Research Institute (Kenilworth, NJ). FTI-277, LY294002, and rapamycin were from Calbiochem (San Diego, CA). Paclitaxel and cisplatin were from Sigma Chemical (St. Louis, MO). PD98059 was from Alexis Biochemicals (San Diego, CA). Gemcitabine was from Eli Lilly & Co. (Indianapolis, IN). A rabbit polyclonal serum that recognizes the precursor peptide at the carboxyl terminus of human lamin A and a chicken serum that recognizes mature lamin A were generated as described previously (Kaufmann, 1989; Adjei et al., 2000b). Histone H1 antibody was from James Sorace at the Veteran's Affairs Medical Center (Baltimore, MD). Murine monoclonal antibodies that recognize poly(ADP-ribose) polymerase and topo IIα were gifts from Drs. Guy Poirier (Laval University, Ste-Foy, QC, Canada) and Udo Kellner (Magdeburg, Germany), respectively. E10 monoclonal phospho-(Thr^{202}/Try^{204})-ERK; affinity-purified epitope-specific rabbit antisera that recognize phospho-(Ser^{473})-Akt, phospho-GSK-3α/β (Ser^{21}/Ser^{9}), phospho-(Ser^{2448})-mTOR, phospho-(Thr^{389})-p70 S6 kinase, and phospho-(Thr^{183}/Tyr^{185}) c-Jun N-terminal kinase; and antisera to Akt, GSK-3α/β, mTOR, c-Jun N-terminal kinase, and ERK1/2 were from Cell Signaling Technology (Beverly, MA). Antibodies to actin, Akt2, and p70 S6 kinase were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to H-Ras, HDJ-2, and PTEN were from ViroMed Biosafety Laboratories (Camden, NJ), Neomarkers (Fremont, CA), and Oncogene Research Products (Cambridge, MA), respectively. Horseradish peroxidase-conjugated secondary antibodies were from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Enhanced chemiluminescence reagents were from Amersham Biosciences Inc. (Piscataway, NJ).

Cell Culture. HCT 116 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine. HCT 116R were developed by exposing HCT 116 cells (IC_{50} = 50 nM) to increasing concentrations of SCH66336 at each passage until concentrations of 1 μM were reached. To begin with, the cells were exposed to 25 nM, and the concentration was doubled approximately once per week until 400 nM was reached. The concentration was then increased by 50 or 100 nM each week until concentrations of 1 μM were achieved. Cells were passed two times per week or when subconfluent monolayers formed.

Colony-Forming Assays. After subconfluent monolayers were trypsinized, 500 cells in 2 ml of medium were added to 35-mm dishes and incubated for 24 h at 37°C to allow cells to attach. Increasing drug concentrations that spanned the range of at least 0.5 to 2.0 times the individual IC_{50} values or equivalent volumes of diluent were added to triplicate plates, which were then incubated for 7 days to simulate the prolonged drug exposure achieved clinically (Adjei et al., 2000b; Eskens et al., 2001). The resulting colonies were stained with Coomassie blue and counted manually.

Sample Preparation and Immunoblotting. HCT 116 and HCT 116R cells were plated at 1.5×10^6 cells per 100-mm plate and incubated for 72 h. Whole-cell lysates were prepared by washing cells three times with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM β-glycerophosphate, 50 mM NaF, 10 mM $Na_4P_3O_7 \cdot 10H_2O$, 0.1 mM Na_3VO_4, 0.1 mg/ml leupeptin, 0.01 mg/ml aprotinin, 0.001 mg/ml pepstatin, and 0.02 μM microcystin), collecting cells into 200 μl of lysis buffer, and sonicating. After protein concentration was estimated using the Bradford method (Bradford, 1976), serial dilutions of lysates were combined with 4× SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 4 M urea, 1 mM EDTA, 8% SDS, and 20% 2-mercaptoethanol), heated to 65°C for 20 min, and separated by SDS-PAGE. Otherwise, 100-mm plates containing cells at 50 to 60% confluence were washed with serum-free RPMI 1640 medium containing 10 mM HEPES (pH 7.4 at 21°C) and solubilized in 3 ml of alkylation buffer (6 M guanidine HCl, 250 mM Tris-HCl, pH 8.5 at 21°C, 10 mM EDTA, 1 mM

phenylmethylsulfonyl fluoride, and 1% 2-mercaptoethanol). After the cells were alkylated and dialyzed as described previously (Kaufmann, 1989; Kaufmann et al., 1997), aliquots containing of 50 μg of protein, as determined by the bicinchoninic acid method (Smith et al., 1985), were subjected to SDS-PAGE. After polypeptides were transferred to nitrocellulose, blots were blocked with 5 or 10% (w/v) powdered milk in buffer consisting of 150 mM NaCl, 10 mM Tris-HCl (pH 7.4 at 21°C), and 0.5% (w/v) Tween 20 as described previously (Kaufmann, 2001).

TABLE 1
Primer sequences used for sequencing of FT subunits

Primer	Sequence
FTA-F1	CATGGACGACGGGTTTGTGA
FTA-F2	CTGTCCTGCAGCGTGATGAA
FTA-F3	TGCCTGGCAGCATCGACAAT
FTA-F4	GATCGTGCTGTATTGGAGAG
FTA-B1	CTGTCCCTGTACAGGACATA
FTB-F1	GCTTCTCCGAGTTCTTTCAC
FTB-F2	GTTCCTGGAGCTGTGTCAGA
FTB-F3	CGCGCTGGTAATCCTCAAGA
FTB-F4	GAGCCATGTTGCATGATGTG
FTB-B1	CCTGTTCTATGGACGTGACT

Immunoprecipitation. Cells were plated in 100-mm dishes and incubated for 48 h followed by a 24-h incubation in serum-free medium and then stimulated with 20% (v/v) fetal bovine serum for 1 h before harvesting. After release by trypsinization, cells were sedimented, washed twice in ice-cold calcium- and magnesium-free Dulbecco's phosphate-buffered saline, and lysed by incubation at 4°C in lysis buffer containing 1% (w/v) Triton X-100. Insoluble material was sedimented at 12,000*g* for 5 min. For immunoprecipitation, 200 μl of lysate, adjusted to yield equal protein concentrations, and 800 μl of lysis buffer were added to 50 μl of a slurry of 3.2 μg of primary antibody covalently bound to Protein A-Sepharose (Harlow and Lane, 1988). After incubation with gentle rotation at 4°C for 1.5 h, the bound antibody-antigen complexes were sedimented, washed three times with lysis buffer, and disrupted by boiling for 5 min in 1× SDS-PAGE sample buffer (4 M urea, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 1 mM EDTA, and 10% 2-mercaptoethanol). Serial dilutions of immunoprecipitated lysates were separated on 10% gels, transferred to nitrocellulose, and probed for the appropriate proteins.

Sequencing of FT. Total RNA was isolated from HCT 116 and HCT 116R cells using the RNeasy Mini Kit from QIAGEN (Valencia,

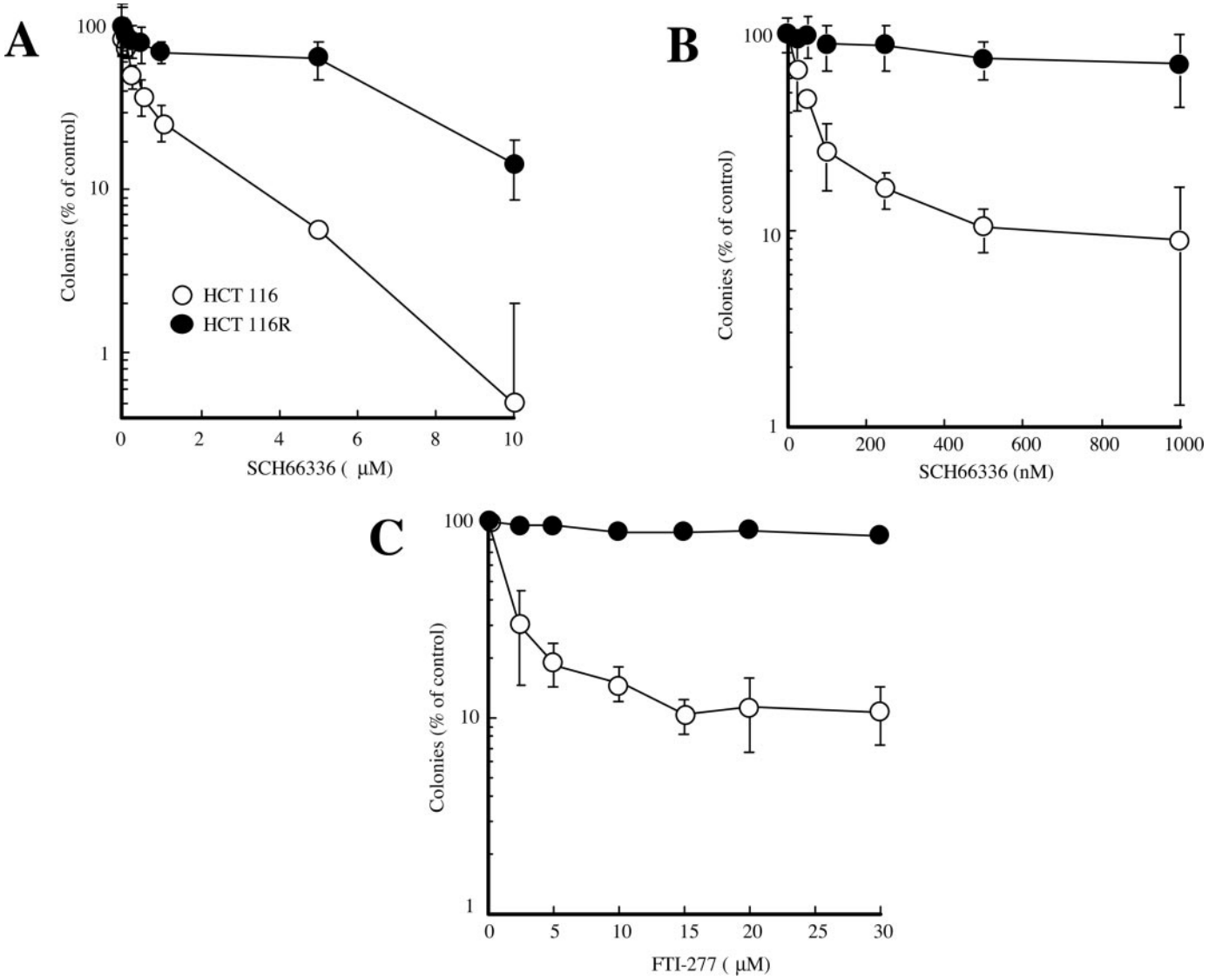


Fig. 1. HCT 116R cells are resistant to FTIs. HCT 116 cells (○) and HCT 116R cells (●) grown for 7 days in the presence of continuous exposure to SCH66336 (A) or FTI-277 (C). B, in a pilot experiment, HCT 116R cells were cultured for 3 months in drug-free medium and then subjected to 7 days of continuous exposure to SCH66336 in a colony-forming assay. Some error bars in C are smaller than the symbols.

CA). FT α- and β-subunit cDNA were synthesized using the SuperScript III First-Strand Synthesis Kit from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions and using the following primers: forward, 5′-CTGTCCTGCAGCGTGATGAA-3′; and reverse, 5′-ACCACTCTCGTGTGAAACTC-3′ for FTα; and forward, 5′-CTGCTGCTCTTCCTGATCATGGCTTCTCC-3′; and reverse, 5′-TCTAGTCGGTTGCAGGCTCTGCCGATGT-3′ for FTβ. Amplification of the sequences from the total cDNA was achieved using the Expand High-Fidelity polymerase chain reaction system from Roche. The amplification cycles were 94°C for 2 min, 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min, followed by 72°C for 7 min. Sequencing was carried out using the ABI PRISM rhodamine dye terminator reaction kit and analyzed using the ABI PRISM 3700 DNA Analyzer from Applied Biosystems (Foster City, CA). Primers for sequencing are listed in Table 1.

Transcriptional Profiling Analysis. Labeled complementary DNA was synthesized from total RNA and hybridized to human U133A chips (Affymetrix, Santa Clara, CA). Gene Chip 5.0 (Affymetrix) was used to scan and quantitatively analyze the scanned image. Gene Chip software was used to calculate intensity values for each probe cell. The data were imported into Microsoft Excel (Microsoft, Redmond, CA) and further analyzed with the help of Steve Iturria in the Mayo Clinic Biostatistics Department (Rochester, MN).

Results

Selection of a Cell Line Resistant to FTIs. The HCT 116 cell line was chosen for development of resistance to SCH66336 because it represents a solid tumor cell line that is sensitive to FTIs. After 6 months of continuous exposure to increasing SCH66336 concentrations, a mixed population of HCT 116 cells were able to grow and form colonies in the presence of 1 μM SCH66336. There were no apparent differences in the growth kinetics of the parental cells growing in the absence of SCH66336 and the resistant cells, termed HCT 116R, growing in its presence. Further analysis revealed that the HCT 116R cell line does not differ significantly in population doubling time or cell-cycle distribution

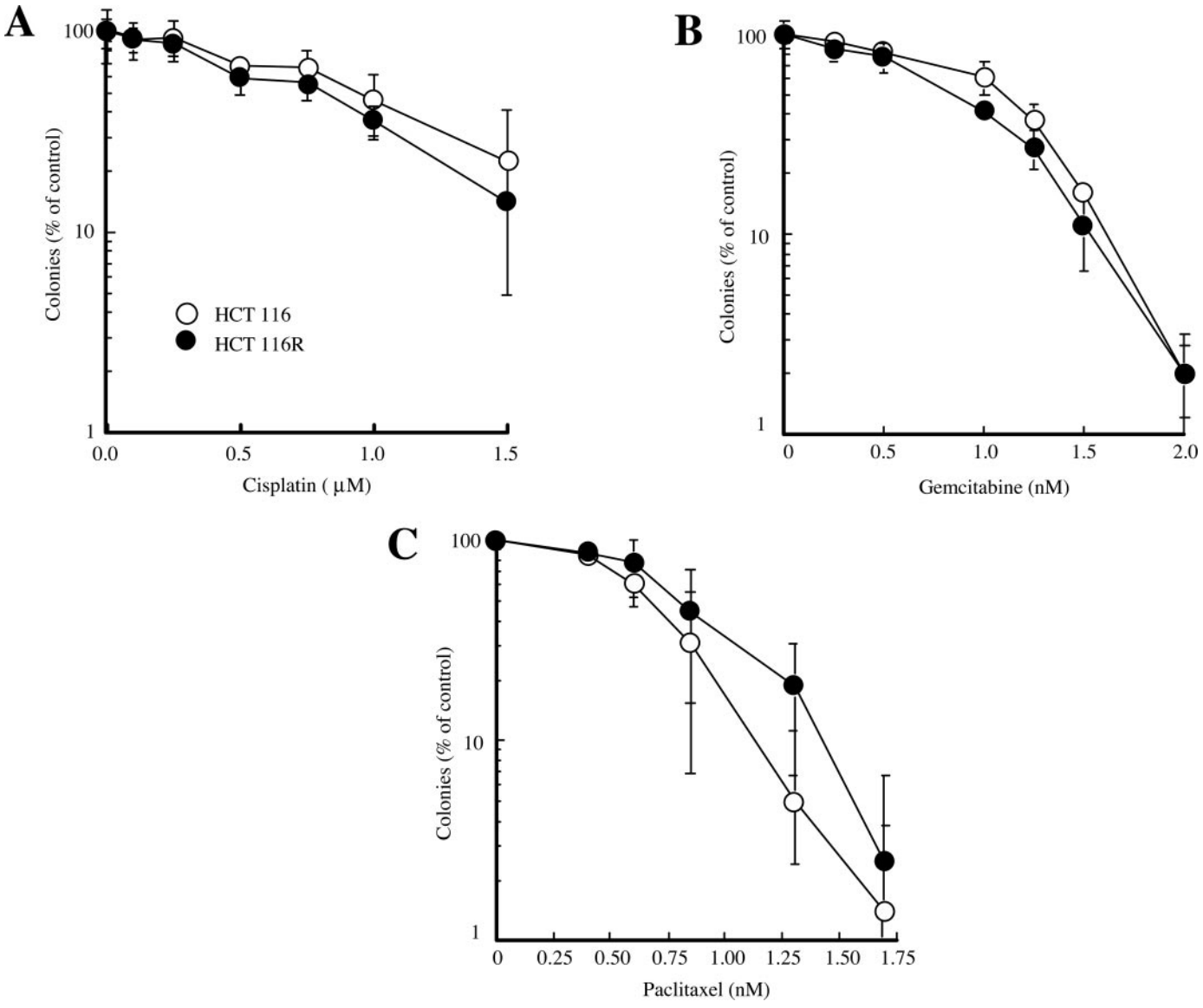


Fig. 2. HCT 116R cells are minimally cross-resistant to other chemotherapeutic agents. HCT 116 cells (○) and HCT 116R cells (●) were grown for 7 days in the presence of continuous exposure to cisplatin (A), gemcitabine (B), and paclitaxel (C). Graphs are representative of three independent experiments.

from the parental HCT 116 cell line (data not shown). The IC_{50} values for parental and resistant cells were 50 nM and 5 μM, respectively (Fig. 1A). A pilot experiment in which the HCT 116R cells were cultured for 3 months in drug-free medium revealed that the resistance is stable (Fig. 1B). Resistance to several other FTIs was of the same order of magnitude as illustrated in Fig. 1C using the commercially available inhibitor FTI-277. The IC_{50} values for FTI-277 in the parental and resistant cells were 1 and >30 μM, respectively (Fig. 1C).

Lack of Cross-Resistance of HCT 116R to Standard Cytotoxic Agents. To rule out the possibility that the HCT 116R cells had acquired resistance to a wide array of anticancer agents, colony-forming assays were used to examine the sensitivity of the parental and resistant cells to 7 days of continuous exposure to the cross-linking agent cisplatin, the antimetabolite gemcitabine, and the microtubule poison paclitaxel. As can be seen in Fig. 2, there was much less difference in sensitivity to these agents, suggesting that HCT 116R cells had acquired relatively specific resistance to FTIs.

Accumulation of Prelamin A and Unfarnesylated HDJ-2 in Parental HCT 116 and HCT 116R Cells. In subsequent experiments, parental and resistant cells were treated with varying SCH66336 concentrations and then subjected to SDS-PAGE followed by immunoblotting with reagents raised against the FT substrates prelamin A (Adjei et al., 2000a,b) and HDJ-2 (Sinensky et al., 1994; Britten et al., 1999; Adjei et al., 2000a). Increased amounts of a slower migrating species of HDJ-2 were observed after SCH66336 treatment in both parental and resistant cell lines (Fig. 3A, third row). Moreover, a small amount of this slower migrating unfarnesylated HDJ-2 was present in HCT 116R cells before FTI exposure. When these same blots were probed with antiserum that specifically recognizes the precursor peptide at the carboxyl terminus of prelamin A (Adjei et al., 2000b), inhibition of farnesylation-dependent prelamin A processing could be detected in a dose-dependent manner in HCT 116 and HCT 116R cells over roughly the same concentration range (Fig. 3A, top row). Similar results were observed when cells were treated with FTI-277 and probed for HDJ-2 (Fig. 3B). Because inhibition of protein farnesylation occurred over the same SCH66336 or FTI-277 concentration range in both cell lines, these data suggest that the uptake of FTIs into HCT 116R cells is unimpaired and that FT within these cells remains sensitive to drug-mediated inhibition.

Sequencing and Expression of FT. To further address the possibility that changes in the FT enzyme might contribute to the SCH66336 resistance observed in HCT 116R cells, we sequenced FT cDNA from both the parental and resistant cell lines using the approach outlined by Smith et al. (2002). For FTα, the full-length cDNA could not be amplified because of its high GC content. Therefore, a primer starting at base pair 302 was designed. Full-length FTβ was sequenced. No difference in the sequence of either the α- or β-subunit was observed when results obtained in HCT 116R and parental cells were compared with each other and to the consensus sequence published in GenBank (accession no. L10413 for α-subunit and L10414 for β-subunit; data not shown).

In additional experiments, expression of the FTα and FTβ mRNAs was compared in HCT 116R and parental cells. Once again, no differences were seen (data not shown). Likewise, there were no detectable differences in expression of mRNAs encoding farnesyl dipshophate synthase and a variety of FT substrates, including H-Ras, N-Ras, RhoB, and CENP-F. These observations prompted us to search for alternative explanations for the 100-fold resistance in HCT 116R cells.

Expression of Ras/Raf/ERK Signaling Pathway Components in HCT 116 and HCT 116R Cells. As indicated in the Introduction, FTIs were initially designed to inhibit the post-translational modification of Ras proteins. The mechanism of cytotoxicity of FTIs is currently unknown but could potentially be related to the interruption of H-ras processing (Gibbs et al., 1993; Kohl et al., 1993; Lerner et al., 1995). Inhibition of MAPK signaling downstream of Ras has been described after treatment with FTIs (Lerner et al., 1995; Karp et al., 2001). To investigate possible alterations involving H-Ras or the MAPK pathway, we initially evaluated the sensitivity of the parental and resistant cell lines to the MEK1/2 inhibitor PD98059 (Dudley et al., 1995). Both cell lines were equally sensitive to this agent (Fig. 4A), suggesting that there were potentially no alterations in MEK or proteins upstream of MEK in the resistant cells. Further-

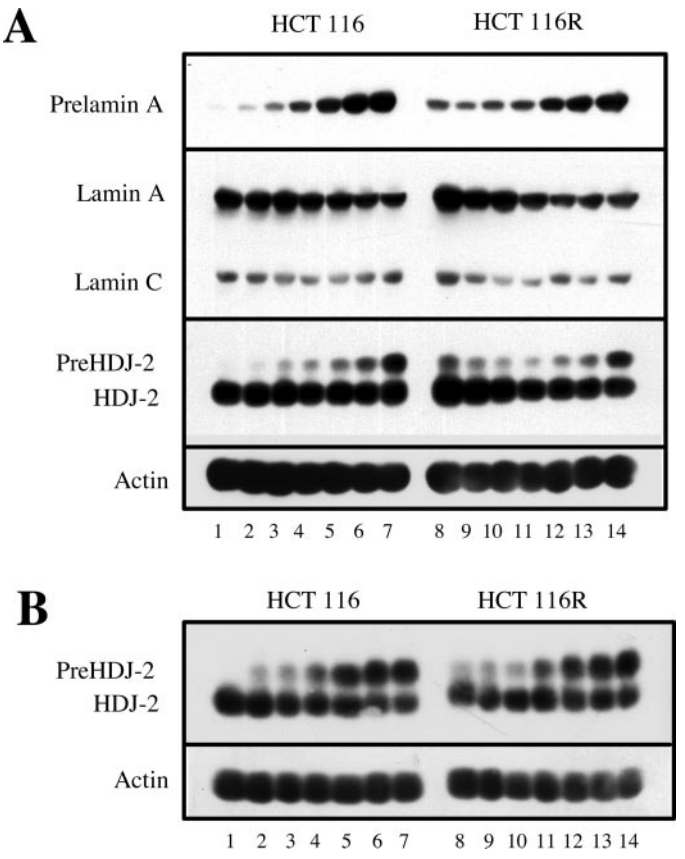
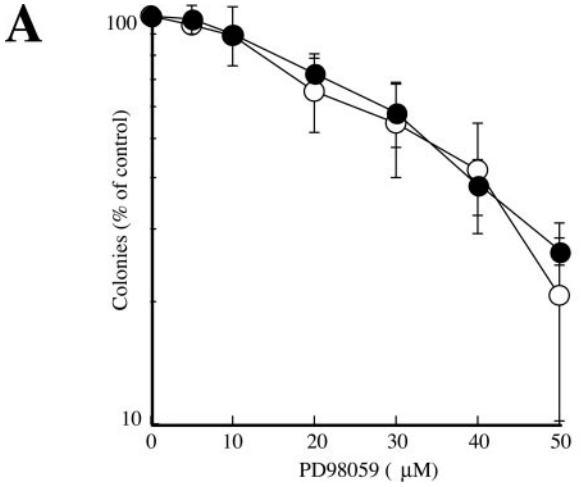


Fig. 3. Resistance of HCT 116R cells to FTIs is not caused by a change in farnesyltransferase. A, HCT 116 or HCT 116R cells were treated with diluent (lanes 1 and 8) or SCH66336 at a concentration of 3.125 nM (lanes 2 and 9), 6.25 nM (lanes 3 and 10), 25 nM (lanes 4 and 11), 50 nM (lanes 5 and 12), 100 nM (lanes 6 and 13), or 200 nM (lanes 7 and 14) for 24 h. Whole-cell lysates (50 μg/aliquot) were then subjected to SDS-PAGE followed by blotting with reagents that react with prelamin A (first), anti-lamin A (second), or anti-HDJ-2 (third). Lamin C, which is not farnesylated and actin (fourth) serve as a loading controls. B, HCT 116 or HCT 116R cells were treated with diluent (lanes 1 and 8) or FTI-277 at a concentration of 125 nM (lanes 2 and 9), 250 nM (lanes 3 and 10), 500 nM (lanes 4 and 11), 1 μM (lanes 5 and 12), 2 μM (lanes 6 and 13), or 4 μM (lanes 7 and 14) for 24 h. Whole-cell lysates (50 μg/aliquot) were then subjected to SDS-PAGE followed by blotting with reagents to that react anti-HDJ-2 (top) and actin as a loading control (bottom).

more, when whole-cell lysates prepared from parental and resistant HCT 116 cells were subjected to SDS-PAGE followed by immunoblotting with reagents that recognize H-Ras, phospho-ERK, and ERK, there were no differences between HCT 116 and HCT 116R cells (Fig. 4B). These results seem to rule out the possibility that altered the expression of H-Ras or enhanced signaling through the Raf/MEK/ERK pathway contributes to resistance in the HCT 116R cells.

Differential Activation of the Akt Pathway in HCT 116R Cells. Activated H-ras is known to signal through the PI-3 kinase/Akt pathway. In addition, several studies have implicated Akt in the cytotoxicity of FTIs (Jiang et al., 2000;



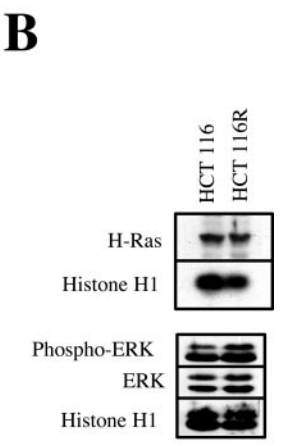
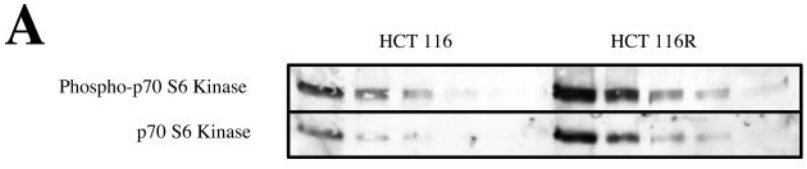
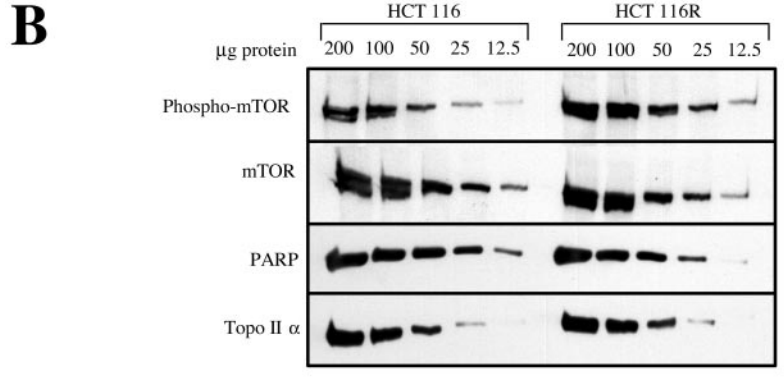


Fig. 4. Members of the Ras/Raf/ERK pathway do not contribute to HCT 116R resistance to FTIs. A, HCT 116 cells (○) and HCT 116R cells (●) were treated with PD98059, a MEK inhibitor. B, alkylated and dialyzed lysates of HCT 116 and HCT 116R cells were probed with antibodies to H-Ras, phospho-ERK, and ERK. Histone H1 served as a loading control.





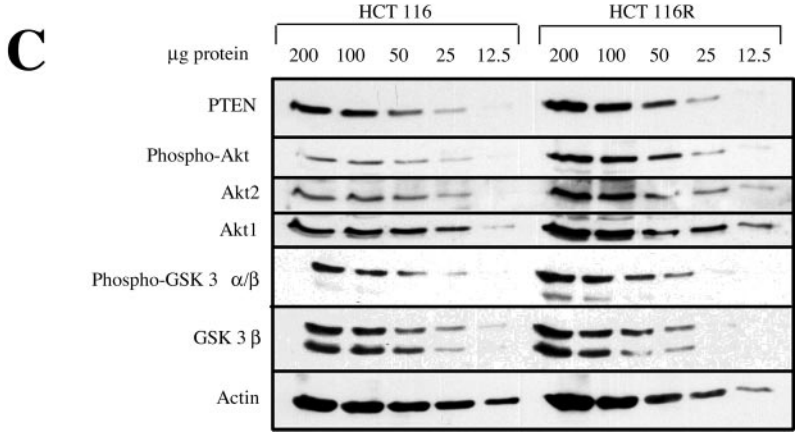


Fig. 5. Differential expression and activation of Akt pathway components is observed in HCT 116R cells. A, immunoprecipitates of p70 S6 kinase in HCT 116 and HCT 116R cells were probed with anti-phospho-p70 S6 kinase and p70 S6 kinase as a loading control. B, serial dilutions of whole-cell lysates of HCT 116 and HCT 116R cells were probed on duplicate blots with anti-phospho-mTOR and anti-mTOR. Poly(ADP-ribose) polymerase and topo II α served as loading controls. C, serial dilutions of whole-cell lysates were probed on duplicate blots with anti-PTEN, anti-phospho-Akt, anti-Akt2, and anti-Akt1. The same blots were also probed for phospho-GSK-3α/β and GSK-3α/β, a downstream target of the PI-3 kinase/Akt pathway. Actin served as a loading control.

Chun et al., 2003). To assess the activation of the Akt pathway in parental and resistant HCT 116 cells, whole-cell lysates were analyzed by immunoprecipitation followed by immunoblotting (Fig. 5A) or by direct immunoblotting (Fig. 5, B and C). These analyses demonstrated an increased signal for phospho-Thr^{389}-p70 S6 kinase in HCT 116R cells compared with parental cells (Fig. 5A, top) and an increase in the amount of activating phosphorylation (Nave et al., 1999; Reynolds et al., 2002) on Ser^{2443} of the intermediary kinase mTOR (Fig. 5B). Although the increased amount of phopsho-Thr^{389}-p70 S6 kinase reflected, at least in part, increased p70 S6 kinase content (Fig. 5A, bottom), the increase in phosphorylated mTOR occurred without any change in mTOR content (Fig. 5B).

Increased Akt Expression in HCT 116R Cells. In an attempt to further delineate components of the Akt pathway contributing to FTI resistance, we performed immunoblotting for polypeptides upstream of mTOR. Akt1 and Akt2 were both increased 2- to 4-fold in the HCT 116R cells (Fig. 5C). Reactivity with anti-phospho-Ser^{473}-Akt, which detects an activating phosphorylation of Akt1 and Akt2, was increased a similar amount in the resistant cells compared with the parental cells (Fig. 5C). In addition, enhanced phosphorylation of GSK-3α/β, another downstream target of Akt, was detected in the HCT 116R cells. In contrast, the lipid phosphatase PTEN upstream of Akt was unchanged in these cells. Together, these results indicate that Akt isoforms are increased and activated in the HCT 116R cells.

Partial Sensitization of HCT 116R Cells by Rapamycin. Because there were multiple changes in the resistant cells, the role of these changes could not be readily assessed by down-regulating a single polypeptide using antisense or RNA silencing technology. As an alternative, to further explore the relationship between overexpression of Akt and FTI resistance, we initially set out to transfect parental HCT 116 cells with an expression vector encoding myristilated, constitutively active Akt. Pilot studies demonstrated that this construct was expressed for only 2 to 3 days after transient transfection, making it difficult to assess effects on colony-forming assays under these conditions. In four separate transfections, we subsequently attempted to generate stable HCT 116 transfectants. None of the more than 100 clones that grew in selective medium expressed the transfected Akt alleles. Therefore, a pharmacological approach was used to assess the potential importance of the observed changes.

The hypothesis that FTI resistance in the HCT 116R cells results from increased expression of Akt isoforms and a concomitant increase in Akt/mTOR signaling leads to two predictions: 1) depending on the nature of the signaling network, the two cell lines might exhibit similar responses to agents that act upstream of Akt; but 2) mTOR inhibition should diminish the resistance. Further experiments were performed to test these hypotheses.

To assess their sensitivity to agents that act upstream of Akt, cells were treated with increasing concentrations of LY294002, which inhibits PI-3 kinase. The lipid product of PI-3 kinase, phosphatidylinositol-3,4,5-trisphosphate, activates several lipid-dependent kinases. Only part of this signaling impinges on Akt. If the signaling from PI-3 kinase is not the critical input for the resistance, then exposure to LY294002, a PI-3 kinase inhibitor, will not affect HCT 116R cells differentially. Consistent with this hypothesis, there was no difference in sensitivity to this agent (Fig. 6A).

To test the prediction that interruption of mTOR signaling would restore FTI sensitivity, HCT 116 and HCT 116R cells were treated with SCH66336 in the absence or presence of rapamycin. In the presence of 10 μM rapamycin, which is the LD_{30} dose for HCT 116R cells, the LD_{50} value of SCH66336 was reduced from 5 μM to 250 nM (Fig. 6C), confirming the ability of rapamycin to at least partially revert the resistant phenotype. In contrast, rapamycin did not sensitize HCT 116 cells to SCH66336 (Fig. 6B), nor did it sensitize HCT 116R cells to cisplatin (Fig. 6D), gemcitabine, or paclitaxel (data not shown).

In a final series of experiments, we explored whether the effect of rapamycin resulted from increased inhibition of FT. HCT 116 and HCT 116R cells treated with 100 nM SCH66336 in the absence or presence of increasing concentrations of rapamycin were immunoblotted for HDJ-2. Rapamycin had no discernible effect on the ability of SCH66336 to inhibit FT as manifested by the appearance of unfarnesylated HDJ-2 (Fig. 6E). Thus, it seems that rapamycin sensitizes the HCT 116R cells downstream of FT inhibition rather than at the level of FT.

Discussion

The present study evaluated the mechanism underlying the resistance of a colon cancer cell line to FTIs. The HCT 116R line was established by stepwise selection with increasing concentrations of SCH66336, an FTI that is currently being tested in the clinic. It is important to note that the resulting HCT 116R line, which was 100-fold resistant to SCH66336, exhibited stable resistance after drug withdrawal (Fig. 1B) and was cross-resistant to other FTIs (Fig. 1C) but not to standard cytotoxic agents (Fig. 2) or the geranylgeranyltransferase inhibitor GGTI-286 (data not shown). These observations suggest that the resistance observed in HCT 116R cells is selective for FTIs.

To further delineate the cause of this resistance, the effects of SCH66336 on protein farnesylation in parental and HCT 116R were compared. Previous studies have demonstrated that the prenylated protein HDJ-2 demonstrates a shift in mobility upon treatment with FTIs (Britten et al., 1999; Adjei et al., 2000a; Karp et al., 2001). In addition, work from several laboratories demonstrated previously that prelamin A is a farnesylated polypeptide (Adjei et al., 2000a; Sinensky et al., 1994). The present study demonstrated that farnesylation of prelamin A and HDJ-2 was inhibited at similar SCH66336 concentrations in both parental and resistant HCT 116 cells. HDJ-2 farnesylation was also inhibited at similar concentrations by FTI-277 in the parental and resistant cells. These observations suggest that neither alterations in drug transport nor alterations in the sensitivity of FT to inhibition by SCH66336 would explain the observed resistance. These results are consistent with the observations of Smith et al. (2002), who also failed to detect an alteration in FT in an R115777-selected colorectal carcinoma cell line.

Even though changes in FT sensitivity do not seem to explain the resistance, a slight alteration in protein farnesylation was observed in the HCT 116R cells (Fig. 3). In particular, the presence of unfarnesylated polypeptides in the

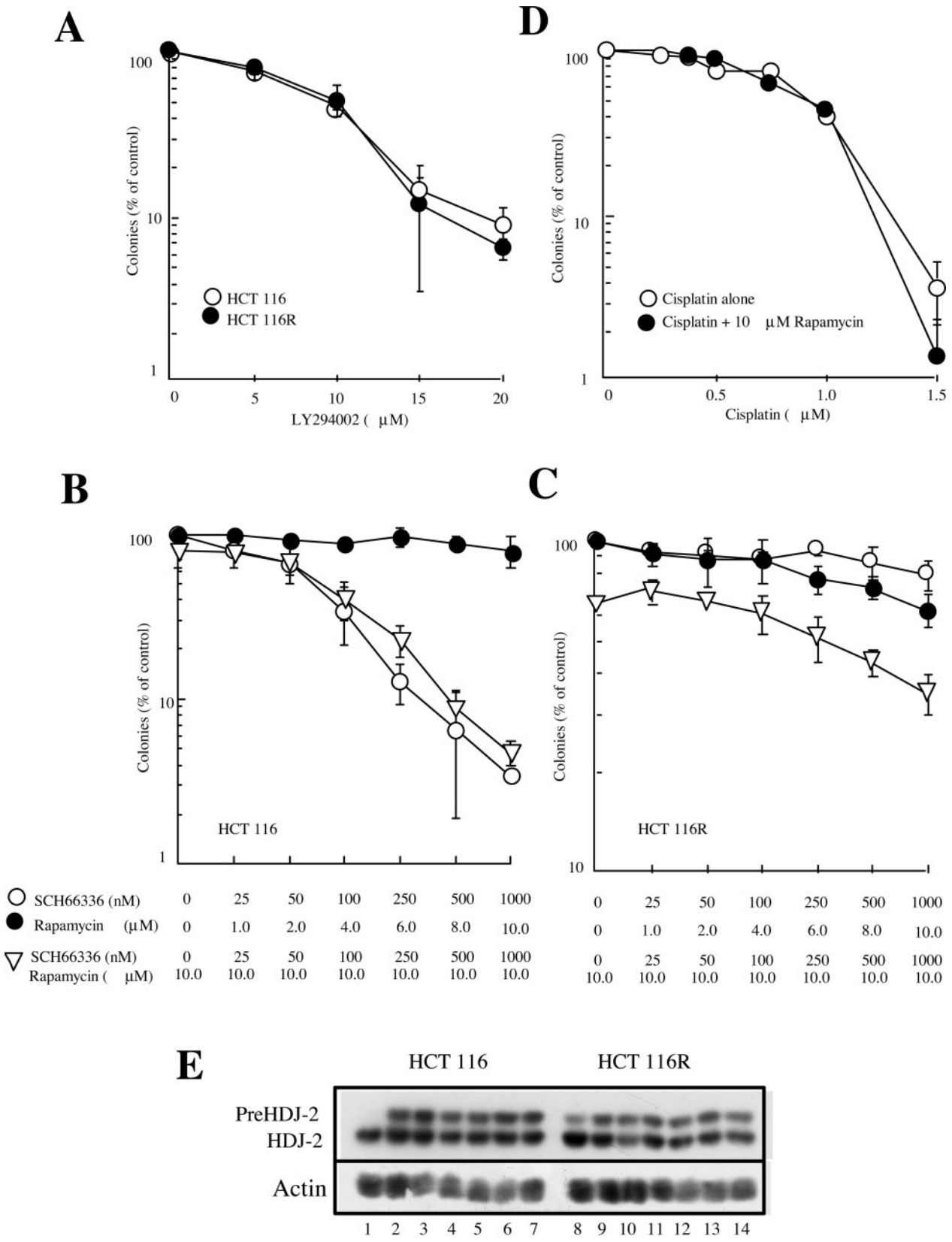


Fig. 6. Pharmacological assessment of the importance of the Akt/mTOR pathway up-regulation. A, HCT 116 cells (○) and HCT 116R cells (●) were subjected to continuous exposure to LY294002, a PI-3 kinase inhibitor. HCT 116 (B) or HCT 116R (C) cells were treated with SCH66336 (○), rapamycin (●), or SCH66336 and 10 μM rapamycin (△). D, HCT 116R cells were treated with cisplatin (○) or cisplatin and 10 μM rapamycin (●). E, HCT 116 or HCT 116R cells were treated with diluent (lanes 1 and 8), 100 nM SCH66336 (lanes 2 and 9), or 100 nM SCH66336 in combination with rapamycin at 1 nM (lanes 3 and 10), 10 nM (lanes 4 and 11), 100 nM (lanes 5 and 12), 1 μM (lanes 6 and 13), or 10 μM (lanes 7 and 14) for 24 h. Whole-cell lysates (50 μg/aliquot) were then subjected to SDS-PAGE followed by blotting with reagents to that react with anti-HDJ-2 (top) and actin as a loading control (bottom).

resistant cells before FTI exposure raised the possibility that the catalytic ability of FT may be slightly reduced in these cells. Further experiments designed to explore this possibility failed to demonstrate any change in the sequence or expression of either FT subunit. Even if this analysis missed a small difference in FT activity, it is important to emphasize that decreased FT activity in HCT 116R cells would be expected to sensitize the cells to FT catalytic inhibition rather than convey resistance.

In further studies, we focused on the Ras/MAPK and PI-3 kinase/Akt pathways that have been implicated in the cytotoxicity of FTIs (Gibbs et al., 1993; Kohl et al., 1993; Lerner et al., 1995; Jiang et al., 2000; Chun et al., 2003). The results in Fig. 4 fail to implicate alterations in the Ras/Raf/MAPK pathway in the resistance of HCT 116R cells. In particular, the parental and resistant HCT 116R cells were equally sensitive to the cytotoxicity of the MEK inhibitor PD98059. Moreover, the expression of H-ras and the phosphorylation of ERK, a readout of pathway activation, were indistinguishable in the two cell lines. Therefore, the cause of the FTI resistance was believed to lie elsewhere.

We next examined the PI-3 kinase/Akt pathway. Signaling through this pathway inhibits apoptosis by phosphorylation of substrates such as Bad and forkhead transcription family members (Datta et al., 1999). Akt activity, which is constitutive and PI-3 kinase–dependent in a number of tumor cells, does not correlate directly with Ras status (Brognard et al., 2001). Instead, Akt also mediates survival signals from polypeptide growth factor receptors and other tyrosine kinases as well as focal adhesion kinase, which is activated by integrin-mediated signaling. It is interesting to note that several previous studies have indicated that engagement of adhesion and growth factor receptors can rescue cells from FTI-induced apoptosis (Lebowitz et al., 1997; Suzuki et al., 1998), providing indirect evidence that Akt pathway activation might cause resistance to FTIs. This conclusion was further strengthened by subsequent results showing that forced overexpression of Akt1 or Akt2 results in resistance to FTI-induced apoptosis (Jiang et al., 2000; Chun et al., 2003).

In the present study, comparison of the HCT 116 and HCT 116R cells revealed multiple changes in the Akt signaling pathway. Phosphorylation of p70 S6 kinase, a downstream target of the Akt pathway, was elevated (Fig. 5A). This change, which reflected enhanced mTOR activity, occurred without any change in mTOR levels. Instead, increased phosphorylation of mTOR was detected (Fig. 5B). In addition, increased phosphorylation of GSK-3α and -β was observed in HCT 116R cells (Fig. 5C). Increased mTOR and GSK-3α/β phosphorylation in turn reflected increased levels of phosphorylated Akt1 and Akt2, as well as increased levels of total Akt1 and Akt2 protein in the resistant cells (Fig. 5C). These changes are summarized in Fig. 7.

To our knowledge, this is the first report showing Akt/mTOR pathway alterations in FTI-selected cells. The cause of the increased Akt1 and Akt2 activation and expression in the resistant cells is not currently known and is under investigation. Further studies in additional FTI-selected cell lines and in clinical material are also required to determine how commonly alterations in this pathway contribute to FTI resistance.

If enhanced signaling downstream of Akt were contributing to the FTI resistance, then inhibition of this signaling should sensitize the resistant cells. Because mTOR is activated in the HCT 116R cells (Fig. 5B), and previous studies have demonstrated that cells with enhanced Akt/mTOR signaling as a consequence of PTEN deletion are particularly sensitive to mTOR inhibitors (Neshat et al., 2001; Bjornsti and Houghton, 2004), we hypothesized that inhibition of mTOR might reverse the resistance of HCT 116R cells. Consistent with this prediction, rapamycin treatment enhanced SCH66336 sensitivity in HCT 116R cells (Fig. 6C). It is important that the effect of rapamycin seemed to occur downstream of FT inhibition (Fig. 6E). Moreover, rapamycin failed to sensitize HCT 116R cells to cisplatin (Fig. 6D), gemicitabine, or paclitaxel (data not shown), demonstrating the specificity of this effect. Nonetheless, rapamycin did not completely restore SCH66336 sensitivity to that of parental cells (compare Figs. 6C and 1A), perhaps because Akt signals through other pathways or additional alterations also contribute to FTI resistance. Even with this limitation, the IC_{50} values of the resistant cells in the presence of rapamycin falls well within therapeutically achievable SCH66336 plasma levels, which exceed 1 μM for weeks at a time on the twice-daily oral-dosing schedule (Eskens et al., 2001). Thus, the results in Fig. 6C not only provide pharmacological confirmation that the observed changes in the Akt pathway con-

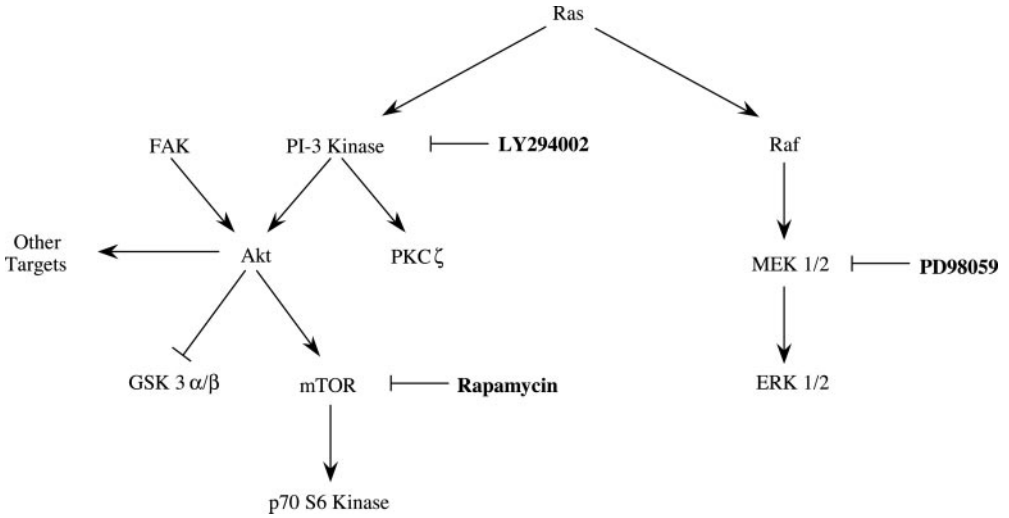


Fig. 7. Ras-activated pathways examined in the present work. Note that activating phosphorylations of Akt and several downstream molecules are enhanced in the HCT 116R cells (Fig. 5), whereas activation of the Raf/MEK/ERK pathway is unaltered (Fig. 4). Consistent with these results, rapamycin enhances the sensitivity of the resistant cells (Fig. 6B).

tribute to resistance in the HCT 116R cells but also suggest that the combination of FTIs with rapamycin analogs or Akt inhibitors currently under development might warrant further preclinical and possible clinical testing.

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