Establishment and Characterization of Acquired Resistance to the Farnesyl Protein Transferase Inhibitor R115777 in a Human Colon Cancer Cell Line[1]

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ABSTRACT

R115777 (Zarnestra) is a farnesyl protein transferase inhibitor currently undergoing worldwide clinical trials. As acquired drug resistance may limit the efficacy of the drug, a model of acquired resistance has been established *in vitro* by continuous drug exposure of the human colon cancer cell line KM12. A stably resistant cell line possessing 13-fold resistance to R115777 was generated. The resistant cells showed cross-resistance to another, structurally different farnesyl transferase inhibitor-277, but not to GGTI-298. A lack of cross-resistance was observed to a variety of other agents, which included clinically used drugs, such as doxorubicin, etoposide, cisplatin, and paclitaxel, as well as signal transduction blockers, such as the mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor UO126, the phosphatidylinositol 3′-kinase inhibitor LY294002, and the epidermal growth factor receptor tyrosine kinase inhibitor PD153035. Resistance did not appear to be related to differences in drug efflux pumps, such as P-glycoprotein or in drug accumulation. Total levels of farnesyl transferase protein subunits were similar in the parent and resistant cells, but, notably, the enzyme activity was markedly reduced in the resistant cell line compared with the parent cells. This was not because of a mutation in the enzyme or a difference in activation of the α-subunit of farnesyl transferase by phosphorylation. Hence, resistance to R115777 was generated; the mechanism of resistance in this model may be associated with the enzyme target of the inhibitor. The results suggest that the development of clinical resistance may occur with farnesyl protein transferase inhibitors.

INTRODUCTION

A variety of proteins are post-translationally modified by prenylation (1). This process covalently attaches a prenyl group to the protein. Two types of prenyl groups are known: (*a*) the 15-carbon farnesyl group; and (*b*) the 20-carbon geranylgeranyl group. Prenylation is catalyzed by farnesyl transferase, which transfers a farnesyl group, and/or geranylgeranyl transferase I, which transfers a geranylgeranyl group to the protein substrate. The donors of the prenyl group are the respective prenylpyrophosphates.

Proteins that are modified by prenylation often contain a CAAX box motif at their COOH terminus, where C is cysteine, A is any aliphatic amino acid, and X can be any amino acid, but determines which of the enzymes preferentially modifies the protein (1). The prenyl group is added to the cysteine of the CAAX box; then the three terminal amino acids are removed, and a carboxymethyl group is added. Altogether, these alterations enable the protein to bind to the plasma membrane, which is required to exert its biological function. Examples of protein substrates of farnesyl transferase are the *ras* family of oncoproteins, rhoB, nuclear lamins, rhodopsin kinase, CENP-E, and CENP-F (2, 3).

FTIs[3] are a class of inhibitors that affect prenylation (4, 5). It was originally thought that they would predominantly inhibit the growth of *ras*-dependent tumors. However, studies since have suggested that *ras* may not be the sole or even the main target of these compounds, and, certainly, responses to these agents do not appear to correlate with *ras* status (2, 5, 6).

Because FTIs are a new class of compounds that are currently undergoing clinical trials, little is known about possible mechanisms of resistance to these agents. As resistance occurs to most commonly used anticancer drugs and also occurs with new agents acting on novel "molecular targets," such as flavopiridol (7) and STI-571 (8), it seems likely that it will also occur with novel agents, such as R115777 (9). The present study set out to investigate the chemosensitivity to R115777 of a small panel of human cancer cell lines, to generate a resistant cell line, and to examine possible mechanisms of resistance in this line. The approaches taken were based on both known mechanisms of resistance and the mechanism of action of the agent investigated. Hence, the roles of drug efflux pumps, drug accumula-

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[3] The abbreviations used are: FTI, farnesyl transferase inhibitor; SRB, sulforhodamine B; GGTI, geranylgeranyl transferase I inhibitor; RF, resistance factor; wt, wild type.

tion, farnesyl transferase protein expression, and enzyme activity were determined.

MATERIALS AND METHODS

Drugs and Chemicals. Doxorubicin and etoposide were purchased from Sigma Chemicals (Poole, Dorset, United Kingdom) and Bristol Myers Squibb Pharmaceuticals (Hounslow, United Kingdom), respectively. R115777 (Janssen Research Foundation, Titusville, NJ) was dissolved in acidified water; cisplatin (Sigma Chemicals) was dissolved in 0.9% saline; FTI-277, GGTI-298 (both kindly provided by Dr. S. Sebti, H. Lee Moffitt Cancer Center and Research Institute; Ref. 10), UO126, LY294002, and PD153035 (all Calbiochem, Nottingham, United Kingdom) were dissolved in DMSO; and paclitaxel (Calbiochem) was prepared in ethanol. Other chemicals were obtained from Sigma Chemicals, unless otherwise stated.

Cell Culture. All cells (human colon cancer cell lines BE, COLO205, DLD1, HCT116, HT29, KM12, and KM12/R115 possessing acquired resistance to R115777), human breast cancer cell line MCF7, human ovarian carcinoma cell line CH1/doxR (11), A2780 A1, A2780 E6 (12), SKOV3-puro, and SKOV3-S2 (13) were grown as monolayers in DMEM (Life Technologies, Inc., Paisley, Scotland, United Kingdom) augmented with 10% heat-inactivated FCS, 2 mM L-glutamine, MEM nonessential amino acids (both Life Technologies, Inc.), and 0.5 μg/ml hydrocortisone in a 6.5% CO_2, 93.5% air atmosphere.

Growth Inhibition Assay. The SRB assay was used to determine growth inhibition (14). Briefly, cells were seeded at 4×10^4 cells/ml into 96-well microtiter plates in 160 μl of growth medium. After overnight incubation, serial dilutions of drug were added to quadruplicate wells, and cells were exposed for 96 h. Quantitation of cell growth was assessed using 0.4% SRB dissolved in 1% acetic acid. IC_{50}s were determined, and RFs were calculated for each individual experiment by the ratio of IC_{50} of KM12:R115 cells over IC_{50} of KM12 cells. A mean and SD was then determined.

Doubling Times. Cells were seeded at a concentration of 1×10^5 into 25-cm^2 flasks. The cells were harvested by trypsinization, and the number was determined using a hemocytometer after 24-, 48-, 72-, and 96-h incubation. The doubling times were calculated for the exponential growth phase.

Western Blot. Western blot analysis of proteins was carried out as described previously (13) using asynchronous cells in exponential growth phase with detection by enhanced chemiluminescence (NEN Life Sciences, Boston, MA). Antibodies were obtained from Santa Cruz Biotechnology [FTase α (C-19), FTase β (X-28), hsp40 (N-19), Raf-1 (E-10), rhoB (119), and lamin B (C-20)], Centicor Diagnostics [Malvern, PA; PgP (C219)], Transduction Laboratories (Lexington, KY; pan-ras), New England Biolabs [phospho-raf (ser259)], and Sigma Chemicals (actin). Prelamin A antibody was kindly provided by Drs. A. Adjei and S. Kaufmann (Mayo Clinic, Rochester, NY; Ref. 15). Secondary antibody conjugated to horseradish peroxidase was obtained from Amersham Pharmacia Biotech (Amersham, United Kingdom).

Immunoprecipitation. Cell lysates [50 mM HEPES, 250 mM NaCl, 0.1% NP40, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM EDTA, 1 mM DTT (Roche, Lewes, United Kingdom), 0.2 mM $NaVO_3$, and protease inhibitor tablets (Roche)] were prepared from exponentially growing cells exposed to equimolar or equitoxic doses of R115777 for 24 h before harvesting. Protein (300–400 μg) was immunoprecipitated using 2.4 μg of antibody to farnesyl transferase α and bound to protein A Sepharose beads. After washing, Western blot analysis was carried out as above, and the blot was probed for phospho-thr and phospho-ser (both Zymed, San Francisco, CA).

Uptake of R115777. The accumulation of R115777 was measured in exponentially growing cells exposed to medium containing 2.5 or 30 μM ^{14}C-R115777 provided by Dr. D. End, Janssen Research Foundations (specific activity 1.43 Gbq.mmol, 5.98 MBq/ml) for 4 h at 37°C. To determine drug efflux, cells were washed twice in PBS after the 4-h incubation and maintained for an additional 4 h. Cells were washed and lysed in NaOH overnight, and the radioactivity was counted in the lysate and supernatant by scintillation counting (2200CA; Packard).

Measurement of Prenyl Transferase Activity. Prenyl transferase activity was measured based on a method published previously (16). Briefly, exponentially growing cells were lysed in 50 mM Tris, 200 mM KCl, 20 μM $ZnCl_2$, 5 mM DTT, 100 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride by sonication. Homogenates were centrifuged at 178,000 × *g* for 90 min at 4°C using a fixed angle rotor (Centrikon, T-2060 Ultracentrifuge; Kontron, St. Albans, Herts, United Kingdom). The supernatant was concentrated in Millipore Biomax filters by centrifugation at 120 × *g* for 30 min at room temperature. An aliquot was taken to determine protein levels by the bicinchoninic acid protein assay (Pierce, Rockford, IL). Prenyl transferase (or geranylgeranyl transferase I) activity was measured in duplicate by mixing 10 μl of cytosol homogenate and 15 μl of FTase buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 20 μM $ZnCl_2$, 50 μM DTT, 6.8 mM n-octylglucoside, 0.5 μM [1-^{3}H] farnesylpyrophosphate (specific activity 20.5 Ci/mmol; NEN Life Sciences) or [1-^{3}H] geranylgeranylpyrophosphate (both NEN Life Sciences), 200 μM Na_3VO_4, 5 μM H-ras wt for farnesyl transferase, or H-*ras*-CVLL (both Panvera Corp., Madison, WI) for geranylgeranyl transferase I. Samples were incubated for times ≤60 min at 37°C, and the reaction was stopped by the addition of 65 μl of 10% hydrochloric acid in ethanol. Samples were transferred to Whatman GFC filters, which were washed four times with ethanol, dried filters were mixed with 4 ml of scintillant (Ultima Gold), and tritium levels were counted.

Sequencing. The two subunits were amplified using the extensor HI-Fidelity PCR kit (ABgene; Epsom, Surrey, United Kingdom) according to the manufacturer's instructions using the following primers: forward 5′ CTGTCCTGCAGCGTGATGAA 3′, reverse: 5′ ACCACTCTCGTGTGAAACTC 3′ for farnesyl transferase α and forward: 5′ CTGCTGCTCTTCCTGATCATGGCTTCTCC 3′, reverse: 5′ TCTAGTCGGTTGCAGGCTCTGCCGATGT 3′ for farnesyl transferase β. The amplification cycles for farnesyl transferase α were: 94°C for 5 min, 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min to yield a product of 902 bp in length; for farnesyl transferase β, were: 30 cycles were run at 94°C for 1 min, 65°C for 1 min, and 72°C for 3 min to yield a product of 1334 bp in length. DNA was extracted using the QIaAMP gel extraction kit

Table 1 Primer sequence used for sequencing of the FTase subunits

Primer	Sequence
FTAFOR2	CTGTCCTGCAGCGTGATGAA
FTAFOR3	TGCCTGGCCAGCATCGACAAT
FTAFOR4	GATCGTGCTGTATTGGAGAG
FTABAK1	ACCACTCTCGTGTGAAACTC
FTABAK2	GGAACTATGACTTGGTTGTA
FTABAK3	GCTGCCAGGCATGATAATTC
FTABAK4	AGGACAGCTCGGAAGTAATC
FTBFOR2	GTTCCTGGAGCTGTGTCAGA
FTBFOR3	CGCGCTGGTAATCCTCAAGA
FTBFOR4	GAGCCATGTTGCATGATGTG
FTBBAK1	GGAGATGGGTGAGCAAAGAG
FTBBAK2	TGGCAGCACATCAGGATGTA
FTBBAK3	CTACGGAGGCAGCACAGTAT

Table 2 Mean 96-h IC_{50} and resistance factors to a variety of anticancer drugs and signal transduction inhibitors[a]

	KM12	KM12/R115	Resistance factor
R115777 (μM)	0.23 ± 0.16	3.26 ± 1.88	13.00 ± 2.55*
FTI-277 (μM)	0.615 ± 0.61	7.40 ± 3.22	14.51 ± 9.58*
GGTI-298 (μM)	3.36 ± 1.11	2.11 ± 0.44	0.76 ± 0.31
Doxorubicin (μM)	0.043 ± 0.021	0.004 ± 0.0048	0.08 ± 0.06*
Cisplatin (μM)	43.00 ± 22.38	11.68 ± 7.30	0.34 ± 0.34*
Paclitaxel (nM)	2.95 ± 1.45	3.14 ± 0.97	1.42 ± 0.94
Etoposide (μM)	0.066 ± 0.026	0.061 ± 0.030	0.85 ± 0.42
UO126 (μM)	3.97 ± 0.35	5.10 ± 0.85	1.30 ± 0.26
LY294002 (μM)	4.30 ± 0.61	4.60 ± 0.79	1.10 ± 0.37
PD153035 (μM)	4.13 ± 0.21	4.73 ± 0.76	1.15 ± 0.25

[a] Data are given as mean ± SD; the resistance factor was calculated by the IC_{50} of KM12-R115/IC_{50} of KM12 for each experiment; $n \geq 3$; * = statistically significant ($P < 0.05$).

(Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. Sequencing was carried out using the rhodamine dye terminator reaction kit (PE Biosystems, Warrington, United Kingdom) according to the manufacturer's instructions and analyzed using ABI PRISM 377 (PE Biosystems). Primers for sequencing are listed in Table 1.

Statistics. Where appropriate, statistical significance was determined using an unpaired, two-tailed Student *t* test. All values shown are means of at least three experiments with the corresponding SD given, unless otherwise stated.

RESULTS

Initially, a small panel of six human colorectal tumor cell lines and one breast cancer line was used to investigate the growth inhibitory properties of R115777. A comparison between the seven cell lines examined showed that KM12 colon cells were by far the most sensitive to this compound with a 96-h IC_{50} of 0.24 μM ± 0.01 (SD, $n = 3$). COLO205 colon cells were the least sensitive cell line in the panel investigated with an IC_{50} of 4.7 μM ± 0.44. Other IC_{50}s were BE, 3.44 μM ± 0.72; DLD1, 4.13 μM ± 0.55; HCT116, 2.77 μM ± 0.55; HT29, 3.4 μM ± 0.44; and MCF-7, 4.33 μM ± 0.6. As part of the initial screening, the effects of p53 and MRP1 were investigated in isogenic pairs of cells that differed in either p53 function or the expression of MRP1. In the cells differing in p53 status, no difference was observed in the sensitivity to R115777 between the wt p53 cells and those lacking p53 [96-h IC_{50} 1.57 ± 0.28 μM for A2780A1 (empty vector control; wt p53)] and 2.03 ± 0.39 μM for A2780 E6 (lacking p53 function as a result of transfection of the human papillomavirus *E6* gene (12). The role of MRP1 was investigated in a pair of cell lines that were transfected with either empty vector or vector containing the gene encoding MRP1. The MRP1-overexpressing cells were more sensitive to R115777, compared with the cells transfected with empty vector [96-h IC_{50} 14 ± 2.94 μM for SKOV3-puro (empty vector control)] and 4.18 ± 0.33 μM for SKOV3-S2 (MRP1-overexpressing cells; Ref. 13). These results indicate that the growth inhibitory effects of R115777 are independent of both p53 status and that the expression of MRP1 pump results in an increase rather than a decrease in drug sensitivity.

As KM12 cells were the most sensitive cell line in the panel investigated, these were chosen to generate a cell line with acquired resistance to R115777. Over a period of 4 months, KM12 cells were continuously exposed to increasing concentrations of R115777. Concentrations started at 0.2 μM (96-h IC_{50}) and were doubled each time the cells grew to confluency until a concentration of 1.6 μM (8 × original 96-h IC_{50}) was achieved. Cells viable at this concentration showed a 13-fold increase in resistance to R115777 at the IC_{50} level (Table 2). The resistant subline was designated KM12/R115. The resistance was stable for ≥3 months (data not shown). Doubling times were assessed for the parent and the resistant lines and found not to be significantly different (11.8 ± 2.5 h for KM12 and 13.1 ± 2.6 h for KM12/R115, $P = 0.468$).

Using the 96-h exposure SRB assay, the sensitivity of the parent and the resistant subline was determined for a series of standard anticancer drugs (doxorubicin, cisplatin, paclitaxel, and etoposide) together with another structurally dissimilar FTI (FTI-277), GGTI-298 (5, 10), as well three signal transduction blockers, namely, the mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor UO126 (17), the phosphatidylinositol 3′-kinase inhibitor LY294002 (18), and the epidermal growth factor receptor tyrosine kinase inhibitor PD153035 (Ref. 19; Table 2). Notably, no significant cross-resistance was seen with doxorubicin, cisplatin, paclitaxel, or etoposide. In fact, significant collateral sensitivity was observed after exposure to doxorubicin and cisplatin. Additionally, no significant difference in the IC_{50} was detected after exposure to UO126, LY294002, or PD153035. Interestingly, significant cross-resistance was found to the structurally different FTI-277, but no cross-resistance was found to the GGTI. The cross-RF to FTI-277 was 14.5-fold and was thus very similar to the RF to R115777 (13-fold; Table 2).

Reduced drug accumulation is often associated with resistance to anticancer agents (20). It frequently involves the overexpression of drug efflux pumps, such as PgP (21). The protein expression of PgP was studied in KM12 and KM12/R115 cells using Western blotting (Fig. 1*A*). No protein was detectable for PgP as compared with the positive control CH1doxR. The screening results mentioned earlier showed that MRP1 expression did not reduce sensitivity to R115777. Besides PgP and MRP1, other drug efflux or uptake mechanisms may potentially be involved in the resistance to R115777. Altered activity of any

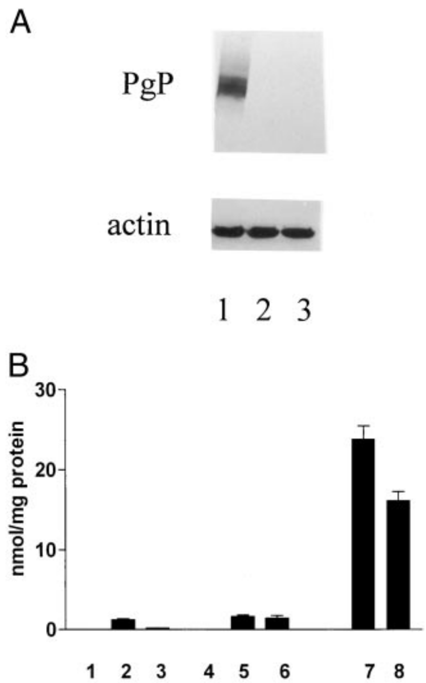

Fig. 1 *A,* PgP protein expression levels. Blots show one representative of at least two independent experiments. *Lane 1,* positive control (CH1doxR); *Lane 2,* KM12; *Lane 3,* KM12/R115; actin was used as a loading control. *B,* mean concentration of R115777 in cells after 240-min exposure to the compound or after 240-min exposure and 240 min in drug-free medium as measured using radiolabelled R115777. Data are given as mean ± SD. *Lane 1,* KM12 control; *Lane 2,* KM12 exposed to 2.5 μM R115777; *Lane 3,* KM12 exposed to 2.5 μM R115777 and drug-free medium; *Lane 4,* KM12/R115 control; *Lane 5,* KM12/R115 exposed to 2.5 μM R115777; *Lane 6,* KM12/R115 exposed to 2.5 μM R115777 and drug-free medium; *Lane 7,* KM12/R115 exposed to 30 μM R115777; *Lane 8,* KM12/R115 exposed to 30 μM R115777 and drug-free medium; $n \geq 3$.

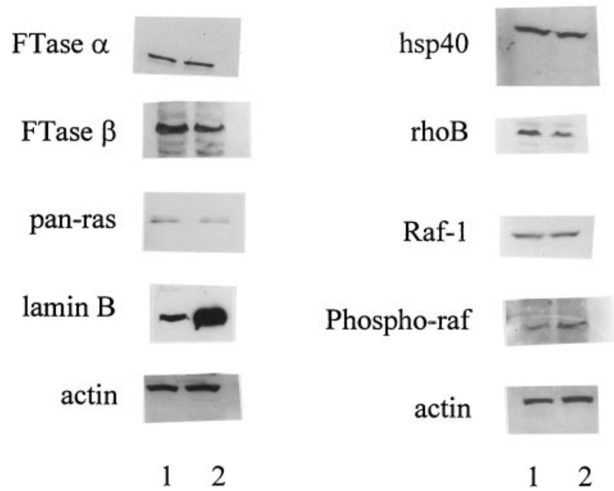

Fig. 2 Western blot analysis of proteins involved in farnesylation or substrates of the *ras* signaling pathway. *Lane 1,* KM12; *Lane 2,* KM12/R115; actin was used as a loading control. Blots shown are representatives of at least two independent experiments.

of these mechanisms could lead to decreased cellular accumulation of drugs. To investigate this, drug accumulation of radiolabelled R115777 was determined in the parent KM12 and the resistant KM12/R115 cells. Levels of R115777 were measured in both these cell lines after 4-h exposure to the drug and 4-h after removal of the drug. The results (Fig. 1*B*) show that when treated at 2.5 μM (10 × 96-h IC_{50} of KM12), parent and resistant cells showed similar levels of drug accumulation. Treatment of KM12/R115 cells with 30 μM (*i.e.,* at an equitoxic dose) showed a 12-fold increase in drug accumulation compared with the cells treated at 2.5 μM. Four h after drug removal, the amount of R115777 measured in the cells decreased. Interestingly, KM12/R115 cells exposed to 2.5 μM the drug retained more of the compound (1.47 ± 0.073 nmol/mg protein) than the parent cells (0.20 ± 0.05 nmol/mg protein) treated at the same concentration, although this difference was not statistically significant ($P = 0.08$). KM12/R115 cells exposed to 30 μM R115777 decreased levels by 1.5-fold (23.8–16.1 nmol/mg protein).

Expression of Proteins Involved in Farnesylation. Alterations in the level of target proteins can lead to drug resistance. As R115777 inhibits farnesylation, the expression of some proteins that are farnesylated was investigated. In addition, selected proteins involved in the *ras* signaling pathway were studied. Western blot analysis revealed no difference in the constitutive protein expression of the two subunits of farnesyl transferase, rhoB, hsp40, pan-ras, raf-1, and phosphorylated raf (Fig. 2). Only an increase in the protein expression of lamin B was observed.

Activity of Prenyl Transferase Enzymes. To determine whether a difference in the activity of prenyl transferases might be involved in the mechanism of resistance to this drug, the effect on farnesylation after treatment of parent and resistant cells with R115777 was determined. For this, an antibody to the COOH-terminal end of prelamin A was used where inhibition of farnesyl transferase leads to an accumulation of prelamin A in cells (15, 22, 23). KM12 and KM12/R115 cells were exposed to R115777 at equimolar (2.5 μM, 10 × 96-h IC_{50} for KM12 cells) and equitoxic (30 μM, 10 × 96-h IC_{50} for KM12/R115 cells) doses for 24 h before immunoblotting the proteins for prelamin A expression. Both parent and resistant nontreated controls showed barely detectable levels of prelamin A (Fig. 3*A*). Exposure to 2.5 μM R115777 in KM12 cells led to a large increase in prelamin A accumulation. KM12/R115 cells treated with the same concentration also revealed an increase in prelamin A signal but to a lesser extent than for KM12 cells. This is indicative of a lower degree of inhibition of farnesyl transferase in the resistant line compared with the parental cells. Interestingly, the accumulation of prelamin A did not increase any further when KM12/R115 cells were exposed to 30 μM.

These results, together with the data obtained from the cross-resistance studies (Table 2), indicated that farnesyl transferase enzyme itself may be involved in the resistance mechanism to R115777. The protein levels of the two subunits, α and β, did not differ between the parent and the resistant cell line (Fig. 2). However, changes in the activity of this enzyme could cause resistance to occur. Therefore, the activity of the enzyme was measured in cytosolic extracts of both the parent and the

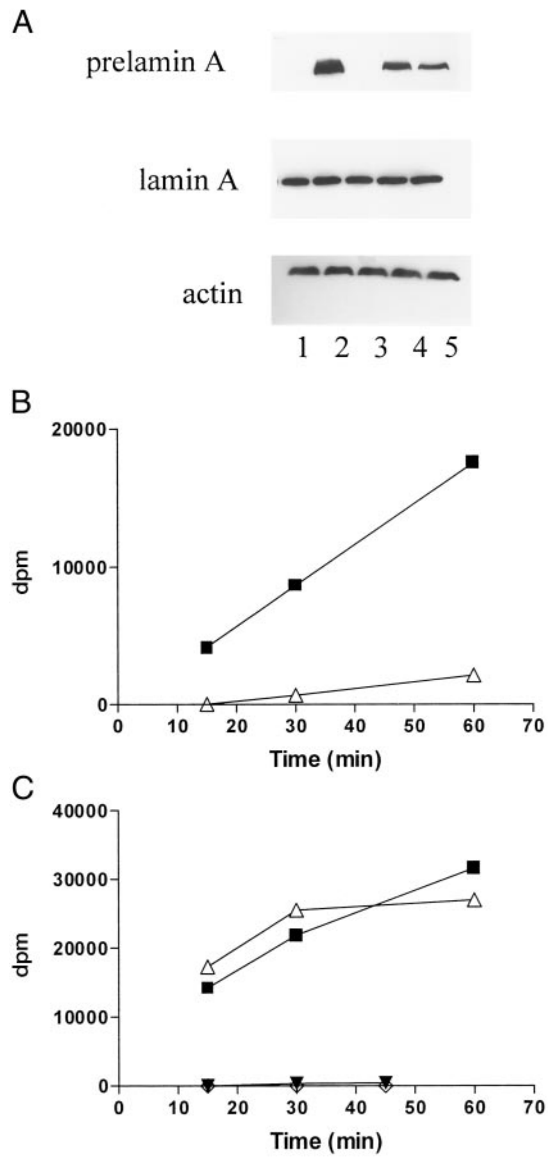

Fig. 3 Prenyl transferase activity. *A*, prelamin A expression after exposure to R115777. *Lane 1*, KM12 control; *Lane 2*, KM12 exposed to 2.5 μM R115777 for 24 h; *Lane 3*, KM12/R115 control; *Lane 4*, KM12/R115 exposed to 2.5 μM R115777 for 24 h, *Lane 5*, KM12/R115 exposed to 30 μM R115777 for 24 h; actin was used as a loading control. Blots shown are representatives of at least two independent experiments. *B*, FTase activity in cytosolic extracts. Curve shown is one example of three independent experiments carried out in duplicate. *C*, GGTase I activity. Curve shown is one example of three independent experiments carried out in duplicate. ■ KM12, Δ KM12/R115, ▼ KM12 + wt ras, ◇ KM12/R115 + wt ras; $n \geq 3$.

resistant cells. The activity of the enzyme was monitored over a period of ≤60 min. In the parent cell line, the amount of product formed by the enzyme activity of farnesyl transferase increased in a linear fashion over the time course investigated. However, in the resistant cells, the enzyme activity was barely detectable (>1000-fold lower than in the parent cells; 0.066 ± 0.028 *versus* <0.00006 pmol/min/mg protein) under the same conditions (Fig. 3*B*). For extracts of the parent cell line, an IC_{50} for R115777 against the enzyme was determined and found to be 11.2 ± 1.04 nM. As enzyme activity was barely detectable in the resistant cells, this could not be determined for KM12/R115 cells. The geranylgeranyl transferase-I enzyme is a similar enzyme to farnesyl transferase. The two enzymes share the α-subunit and only differ in their β-subunit (24). Additionally of note, proteins can become geranylgeranylated when farnesylation is blocked (25). To determine whether geranylgeranyl transferase I was active in both cell lines, the activity of this enzyme was also determined in cell extracts. Similar levels of geranylgeranyl transferase I activity could be observed in both cell lines (0.127 ± 0.052 pmol/min/mg protein in KM12 *versus* 0.121 ± 0.075 in KM12/R115; Fig. 3*C*). Moreover, the addition of 500 nM R115777 (a concentration of the drug 50-fold above the IC_{50} of farnesyl transferase) revealed that geranylgeranyl transferase I activity in cell extracts was inhibited by <10% (91.1 ± 5.8% in KM12 and 98.3 ± 7.2% in KM12/R115). Geranylgeranyl transferase activity was also measured when wt-*ras* was used instead of ras-CVLL as a substrate; neither cell line showed any detectable activity.

Sequencing of Farnesyl Transferase. The results described earlier in this article indicated that the resistance mechanism to R115777 may involve the target enzyme farnesyl transferase. Studies on another FTI (FTI-277) showed that a mutation in the *Dpr1* gene encoding the β-subunit of the yeast FTase enzyme could lead to resistance to FTIs (26). Therefore, sequence analysis of the two subunits of the enzyme was carried out by PCR sequencing of cDNAs from the sensitive and resistant lines. For the α-subunit, the full-length cDNA could not be amplified because of its GC richness at the beginning of the sequence. Thus, a primer starting at 320 bp was used. For the β-subunit, the full-length sequence was determined. No differences in sequence were observed in either the α- or the β-subunit when the sequences were compared between the two cell lines and also when the sequences were compared with those published in GenBank (accession no. L10413 for α-subunit and L10414 for β-subunit; data not shown). Of particular note, the β-subunit mutations associated previously with conversion of substrate specificity of farnesyl transferase to that of geranylgeranyltransferase (amino acid positions Ser-159, Tyr-362, and Tyr-366; Ref. 26) were not detected in KM12/R115.

Regulation of Farnesyl Transferase α. As shown above, although the levels of the two subunits are similar on Westerns and there are no mutations, the activity of farnesyl transferase is markedly reduced in extracts from the resistant cells in comparison to extracts from the parent line. There is some evidence that the farnesyl transferase α-subunit may be activated by phosphorylation at either Thr and/or Ser; possibly (27) by transforming growth factor β1 receptor (27, 28). Thus, activation of the enzyme could be affected by phosphorylation of the α-subunit. Cells were treated for 24 h with equimolar (2.5 μM) or equitoxic (2.5 μM for KM12 and 30 μM for KM12/R115) concentrations of R115777. Farnesyl transferase α was immunoprecipitated, and Western blot analysis was performed using phospho-ser and phospho-thr antibodies. The results (Fig. 4) indicate no difference in phosphorylation between KM12 and KM12/R115 *(Lane*s *1* and *3*). In addition, no difference was

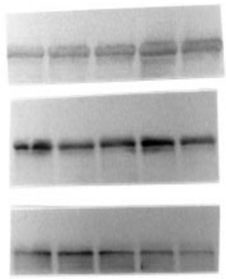

Fig. 4 Immunoprecipitation of farnesyl transferase α-subunit and Western blot analysis of phospho-thr (*A*), phospho-ser (*B*), and farnesyl transferase α (*C*). *Lane 1,* KM12 control; *Lane 2,* KM12 exposed to 2.5 μM R115777 for 24 h; *Lane 3,* KM12/R115 control; *Lane 4,* KM12/R115 exposed to 2.5 μM R115777 for 24 h, *Lane 5,* KM12/R115 exposed to 30 μM R115777 for 24 h. Blots shown are representatives of at least two independent experiments.

observed between samples from control cells and those treated with R115777.

DISCUSSION

Growth inhibition by the FTI R115777, a drug currently undergoing extensive clinical study (29), was investigated in a panel of six human colon and one human breast cancer cell line. The colon cancer cell line KM12 was the most sensitive line in the panel studied, whereas COLO205 were the most resistant cells with 96-h IC_{50}s of 0.24 and 4.7 μM for the two cell lines, respectively.

A cell line was generated that showed acquired resistance to R115777. This resistance was found to be stable for ≥3 months in drug-free medium. The fold resistance was 13-fold as measured by SRB growth inhibition assays. No difference in doubling times was observed between the two cell lines.

The chemosensitivity to several other compounds, including one structurally different FTI (FTI-277) and also GGTI-298, were compared between the parent KM12 cells and the resistant KM12/R115 cells. No statistically significant cross-resistance was observed to the commonly used anticancer agents doxorubicin, cisplatin, paclitaxel, and etoposide or to the signal transduction inhibitors UO126, LY294002, and PD153035. Actually, collateral sensitivity was observed after exposure to doxorubicin and cisplatin. The mechanism underlying this intriguing observation is presently unknown but may relate to the involvement of farnesylation in many cellular processes, such as cell cycle control and apoptosis (2, 6, 30). A 14.5-fold increase in 96-h IC_{50} was observed after treatment with FTI-277. Interestingly, the 96-h IC_{50} of GGTI-298 did not differ between KM12 and KM12/R115 cells. These results indicate that the mechanism of resistance is probably specific to the molecular target enzyme farnesyl transferase. The two FTIs used for cross-resistance both compete with the CAAX box peptide for its binding site (5). Additional investigations using a competitive inhibitor of the farnesyl donor, α-hydroxyfarnesylphosphonic acid, did not show any growth inhibitory effects at concentrations ≤100 μM (data not shown). Thus, attempts to determine whether the CAAX box binding site may be involved in the mechanism of resistance in this model could not be carried out using this approach. The lack of cross-resistance to the signaling inhibitors indicates that the mechanism of resistance probably does not involve alterations elsewhere in the *ras* signaling pathway (although *ras* does affect pathways other than phosphatidylinositol 3′-kinase/akt and mitogen-activated protein/extracellular signal-regulated kinase kinase).

It was important to rule out other potential mechanisms of resistance. Decreased drug accumulation is often associated with drug resistance and can involve overexpression of drug efflux pumps (20). One of the most studied efflux pumps is PgP (21). However, no protein expression of PgP was observed in either the parent or resistant cell line. The studies using the isogenic pair of cells differing only in the expression of MRP1 also indicated that MRP1 was not involved in resistance. Additionally, studies on the KM12 and KM12/R115 cells were carried out using buthionine sulfoximine, which leads to inhibition of MRP1 activity (13). These confirmed the above results that MRP1 is not involved in the mechanism of resistance to R115777 (data not shown). Determining the concentration of radiolabelled R115777 in KM12 and KM12/R115 cells revealed that after exposure to an equimolar concentration of R115777, similar amounts of the drug were measured in the two cell lines. When the resistant cells were exposed to an equitoxic concentration of the inhibitor (*i.e.,* 12-fold more than the parent cells), a 12-fold increase in R115777 measured in the resistant cells was found. After removing the drug from the medium, a decrease in the amount of drug found in the cells was observed in both the parent and resistant lines. Interestingly, more residual compound was measured in the resistant cells exposed to 2.5 μM compared with the parent cells exposed to the same concentration. Taken together, these results indicate that reduced drug accumulation does not play a role in the resistance mechanism in this model.

Alterations in the level of target proteins can lead to drug resistance, *e.g.,* in the case of topoisomerase inhibitors. Thus, the expression of the two subunits of farnesyl transferase, and various proteins known to be farnesylated (rhoB, hsp40, and raf-1, including phosphorylated raf), pan-*ras* and lamin B were studied (1). Except for lamin B, no marked difference in expression of any of these proteins was found. Lamin B is a nuclear protein that forms filaments at the inner surface of the nuclear membrane and is involved in the disassembly of the nucleus during mitosis (1). The increased expression of lamin B could be a consequence of the decreased activity of farnesyl transferase in the resistant cells, *e.g.,* to compensate for less farnesylation, more protein is being produced. However, it is not clear why lamin B is increased in the resistant line when other proteins that are farnesylated (*e.g., ras* and rho B) are unaffected. Of potential relevance, proteins normally farnesylated have been found to be geranylgeranylated when farnesylation is inhibited (25). This switch from one type of prenylation to another might be sufficient for the activity of the respective proteins.

As already mentioned, the activity of farnesyl transferase was measured in KM12 and KM12/R115 cells. This was first performed indirectly by measuring the accumulation of prelamin A after exposure to R115777 (15, 22). In both cell lines, inhibition of farnesyl transferase by 2.5 μM R115777 resulted in an increase in prelamin A accumulation consistent with reduced farnesylation and decreased conversion to lamin A. However, in the resistant cells, this increase was not as pronounced as in the parent cells. Additionally, no further increase in prelamin A accumulation was observed when the resistant cells were treated at 30 μM. These results indicate that a decrease in farnesyl transferase activity may be involved in the mechanism of resistance in this model. Therefore, farnesyl transferase activity was measured in cytosolic extracts of the cells. Farnesyl transferase activity was found to be barely detectable in the extracts of resistant cells, whereas it was clearly detectable (>1000-fold higher) in the extracts of parent cells under the same conditions. The parent cells showed an IC_{50} of 11.2 nM for R115777 against FTase, which is similar to reported values (9). Western blotting showed that expression of both α and β subunits of the farnesyl transferase enzyme was very similar at the protein level. Hence, this could be ruled out as an explanation for the reduced enzyme activity. The decreased activity of farnesyl transferase in the resistant cells was not attributed to inhibitory substances in the cytosolic extracts, as mixing pure farnesyl transferase with the extract did not change the activity of the enzyme (data not shown). In addition, the farnesyl transferase enzyme did not alter its substrate specificity, as no activity could be measured when either ras-CVLL or geranylgeranylpyrophophate were used. The activity of geranylgeranyl transferase I did not differ between the two cell lines and was not affected by 500 nM R115777, a concentration 50-fold above the IC_{50} for farnesyl transferase in the parent cells. It is interesting that the resistant line does not attempt to compensate for the reduced activity of farnesyl transferase by elevating geranylgeranyltransferase I.

It seemed possible from the above results and also from data published previously (26) that the mechanism of resistance could involve a mutation of the enzyme. A previous study in yeast found that mutations of Ser-159, Tyr-362, or Tyr-366 in the β-subunit of farnesyl transferase led to an alteration of substrate specificity (26). Therefore, the cDNAs for the two subunits of farnesyl transferase were sequenced in the parent and resistant lines. In neither of the two subunits was a mutation found when the sequences were compared either to each other or to GenBank entries (although the first 320 bp of the α-subunit were not analyzed). Thus, a mutation in the sequenced fragments can be ruled out as the cause of the resistance to R115777 in this model, including the Ser-159, Tyr-362, and Tyr-366 β-subunit sites described in the above yeast study. It is unlikely that a mutation in the unsequenced fragment of the α-subunit of farnesyl transferase plays a role in the resistance mechanism to R115777, because the first 51 amino acid residues of this subunit do not affect the catalytic activity of the enzyme. Moreover, a mutation in this region would also lead to a decrease in the activity of geranygeranyl transferase activity, as both enzymes share the same α-subunit (24), and this was not seen.

Another potential explanation for the reduced farnesyl transferase enzyme activity in the resistant cells could be a difference in posttranslational modification or cellular localization. The α-subunit of farnesyl transferase has been shown in some studies to be phosphorylated after stimulation by insulin or interaction with the transforming growth factor-β receptor (27, 28). This phosphorylation is associated with an increase in farnesyl transferase activity. However, the exact residues that are phosphorylated or the kinase involved in this process are currently not known. It has been suggested that the α-subunit is phosphorylated by a serine/threonine kinase (27). If this phosphorylation site lies within the region not sequenced, it is possible that a mutation could have occurred there that would reduce the activity of the enzyme. Alternatively, the activity of the kinase(s) responsible for phosphorylating the farnesyl transferase α-subunit could be reduced. Analysis of the phosphorylation state of the α-subunit of farnesyl transferase by Western blotting showed no difference between parent and resistant cell line. In addition, no difference was observed when cells were exposed to R115777. Thus, these results indicate that activation of the α-subunit of farnesyl transferase by phosphorylation is unlikely to be altered between the two cell lines. However, how the β-subunit of farnesyl transferase is regulated is currently unknown and, hence, this is a possible area of further investigation.

In conclusion, the work presented herein describes the first example of the generation of a cell line that is resistant to a FTI, in this case, to the drug R115777, which is currently undergoing clinical trial. Evidence was presented that shows that the resistance to R115777 was specific for the class of FTIs. A number of potential mechanisms for the resistance was ruled out, including those involving altered drug transport or changes in the level of α or β enzyme subunits. Several approaches pointed to a resistance mechanism involving an alteration of the molecular target enzyme itself. Farnesyl transferase enzyme activity was markedly reduced in extracts of the resistant cells, and this was consistent with increased expression of lamin B, which provides a cellular readout for farnesyl transferase activity. However, this reduced activity was not because of mutation or to differences in overall phosphorylation of the α-subunit at either thr or ser. Overall, however, it remains unclear as to how exactly the measured reduced enzyme activity causes resistance to R115777. Additional experiments are required such as isolating FTase from the resistant line and demonstrating it is less sensitive to R115777 and/or using additional quantitative methods showing that protein farnesylation in the resistant cells is affected to a lesser degree by R115777 (*e.g.,* labeling with $[^3H]$farnesyl PP_I and immunoprecipitating farnesylated proteins). Regardless, the resistant cell line described here shows a mechanism of resistance that appears to be specific to farnesyl transferase inhibition and indicates that resistance to this class of compounds may also occur in a clinical setting.

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