Characterization of a R115777-Resistant Human Multiple Myeloma Cell Line with Cross-Resistance to PS-341

Robert Buzzeo,[1,2] Steven Enkemann,[1] Rama Nimmanapalli,[1] Melissa Alsina,[1] Mathias G. Lichtenheld,[3] William S. Dalton,[1] and Darrin M. Beaupre[1]

Abstract The farnesyl transferase inhibitor R115777 has been found to have clinical activity in diverse hematopoietic tumors. Clinical efficacy, however, does not correlate with Ras mutation status or inhibition of farnesyl transferase. To further elucidate the mechanisms by which R115777 induces apoptosis and to investigate drug resistance, we have identified and characterized a R115777-resistant human myeloma cell line. 8226/R5 cells were found to be at least 50 times more resistant to R115777 compared with the parent cell line 8226/S. K-Ras remained prenylated in both resistant and sensitive cells after R115777 treatment; however, HDJ-2 farnesylation was inhibited in both lines, implying that farnesyl transferase (the drug target) has not been mutated. Whereas many 8226 lines that acquire drug resistance have elevated expression of P-glycoprotein, we found that P-glycoprotein expression is not increased in the 8226/R5 line and intracellular accumulation of R115777 was not reduced. In fact, 8226/R5 cells were insensitive to a diverse group of antitumor agents including PS-341, and multidrug resistance did not correlate with the expression of heat shock proteins. Comparison of gene expression profiles between resistant and sensitive cells revealed expression changes in several genes involved in myeloma survival and drug resistance. Future experiments will attempt to identify genes that are directly linked to the resistant phenotype. Identification of molecules associated with R115777 and PS-341 resistance is clinically relevant because both compounds are being tested in solid tumors and hematopoietic malignancies.

Multiple myeloma is a plasma cell malignancy with no known curative therapy. RAS mutations occur frequently in myeloma (1, 2) and have been linked at least in some studies to a poor prognosis (3–5). Farnesyl transferase inhibitors (FTI) inhibit Ras function by preventing its posttranslational prenylation, a modification done by the enzyme farnesyl transferase (FTase). The FTI R115777 was designed as a highly selective inhibitor of FTase (6) and has been clinically tested in several hematopoietic tumors. This compound has shown activity in acute myelogenous leukemia, chronic myelogenous leukemia, and myelodysplastic syndrome (7–10).

Preclinical studies have reported that FTIs have antitumor activity in myeloma cell lines and primary isolates (11–13). Based on these observations, we have recently completed a phase II clinical trial testing R115777 in patients with relapsed myeloma (14). Forty-three patients with a median of four prior treatment regimens entered our study. R115777 was well tolerated and 64% of patients achieved disease stabilization. Of importance, RAS mutation and inhibition of farnesyl transferase did not correlate with clinical efficacy consistent with our prior observation that R115777 can induce apoptosis via a Ras-independent mechanism (15). In myeloma cells, R115777 activates multiple intrinsic proapoptotic cascades (15). However, the molecules and/or signaling pathways that trigger these events remain elusive.

To further elucidate the mechanisms by which R115777 induces apoptosis and to investigate drug resistance, we have established and characterized a R115777-resistant human multiple myeloma cell line (8226/R5). This line is unlike a previously described R115777-resistant colon cancer line (16) for resistance is unrelated to the prenylation activity of the enzyme FTase. This finding correlates with our observation that 8226/R5 cells are insensitive to a diverse group of antitumor agents, including PS-341. In this study, we investigate and exclude several potential mechanisms of R115777 resistance. Using comparative gene expression profiling (between sensitive and resistant cells), we have identified expression changes in several genes implicated in myeloma survival and drug resistance. Further evaluation of these genes may lead to the identification of novel FTI targets or resistance mechanisms that are clinically relevant.

Materials and Methods

Cell lines and reagents

The RMPI 8226 human myeloma cell line was obtained from the American Type Culture Collection (Manassas, VA). 8226/LR5 and

Authors' Affiliations: [1]Department of Interdisciplinary Oncology, Malignant Hematology and Experimental Therapeutics Division, H. Lee Moffitt Cancer Center and Research Institute; [2]Department of Biology, University of South Florida, Tampa, Florida; and [3]Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Florida

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Requests for reprints: Darrin M. Beaupre, H. Lee Moffitt Cancer Center and Research Institute, SRB4, 12902 Magnolia Drive, Tampa, FL 33612-9497. Phone: 813-745-8090; Fax: 813-745-6510; E-mail: BeauprDM@moffitt.usf.edu.

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8226/Dox40 lines were developed in our laboratory and have been previously described (17, 18). Sensitive and resistant 8226 cells were grown in RPMI 1640 (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA). The 8226/R5 cell line was established by continuous exposure of 8226 parental cells (8226/S) to increasing concentrations of R115777 for over 6 months. The established 8226/R5 line is maintained in 5 μmol/L R115777. 8226/R5 cells were grown in R115777-free supplemented medium for 2 weeks before experimentation.

R115777 and [^{14}C]R115777 (specific activity 1.43 GBq/mmol, 3.68 MBq/mL) were kindly provided by David End (Johnson and Johnson Pharmaceutical Research and development, LLC, Titusville, NJ). PS-341 was provided by Millennium Pharmaceuticals, Inc. (Cambridge, MA) and FTI277 from Said M. Sebti (Moffitt Cancer Center and Research Institute, Tampa, FL). Additional drugs were purchased from the following vendors: doxorubicin hydrochloride, melphalan, and perillic acid (Sigma Chemical, St. Louis, MO); staurosporine and etoposide (A.G. Scientific, San Diego, CA); and tunicamycin (Calbiochem, San Diego, CA).

Antibodies and Western blotting

Antibodies were purchased from the following vendors: anti–K-Ras-2B (C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HDJ-2 (NeoMarkers, Freemont, CA); anti-hsp90, anti-hsp70, anti-hsp27, and anti–glyceraldehyde-3-phosphate dehydrogenase (Stressgen Biotechnologies, San Diego, CA); and anti–α-tubulin (BD Biosciences, San Diego, CA). Western blotting was done as previously described (11). For the majority of experiments, lysates were harvested using radioimmunoprecipitation assay lysis buffer [150 μmol/L NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, and 50 μmol/L Tris (pH 8.0)]. For evaluation of hsp27, hsp70, hsp90, and glyceraldehyde-3-phosphate dehydrogenase, lysates were harvested using Baverian lysis buffer [150 μmol/L NaCl, 1% Triton X-100, 30 μmol/L Tris (pH 7.5), and 10% glycerin].

Analysis of cell growth, cell cycle arrest, and cell death

Degree of resistance was determined using the tetrazolium salt 3,4,5-dimethylazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT; Sigma) reduction assay. 8226/S and 8226/R5 cells were plated into 96-well microtiter plates at a density of 5×10^4 cells/mL in 200 μL of supplemented media. Cells were exposed to a broad range of drug concentrations (R115777 and PS-341) in replicates of four. After a 72-hour (R115777) or 48-hour (PS-341) incubation period at 37°C, 50 μL of 2 mg/mL MTT was added to each well and cells were incubated for an additional 4 hours. Plates were centrifuged for 5 minutes at 1,200 rpm in a Sorvall RT6000D table top centrifuge (Sorvall, Asheville, NC), supernatants were removed, and water-insoluble product was dissolved in 100 μL of 100% DMSO (Sigma). Plates were shaken for 30 seconds and absorbance read at 540 nm on a Wallac Victor-2 1420 Multilabel Counter (Perkin-Elmer, Torrance, CA). The number of surviving cells was expressed as a percentage: absorbance of the experimental sample/absorbance of the control × 100. The IC_{50} of the drug was calculated by linear regression analysis using Excel software. Flow cytometric cell cycle analysis was done after propidium iodide staining as previously described (11). The presented histograms represent gating on live cells only. Apoptosis and cell death were evaluated by flow cytometry after Annexin V-FITC and propidium iodide staining as per the recommendations of the manufacturer (BioVision Research Products, Mountain View, CA). TO-PRO-3 (Molecular Probes, Eugene, OR) was substituted for propidium iodide in experiments where cells were treated with doxorubicin. Cell death was calculated as the sum of Annexin V-FITC and propidium iodide or TO-PRO-3–positive cells. Specific cell death was determined by subtracting background death in untreated samples.

Determination of P-glycoprotein expression

8226/S, 8226/Dox40, and 8226/R5 cells were washed in PBS [0.8% NaCl, 0.02% KCl, 0.14% NA_2HPO_4, 0.02% KH_2PO_4 (pH 7.4)] and resuspended in 200 μL of ice-cold PBS containing 0.5 μg/mL anti–P-glycoprotein-FITC antibody (BD Biosciences) or 0.5 μg/mL isotype control antibody anti-dansyl $IgG2_{b,\kappa}$-FITC (BD Biosciences). Samples were incubated on ice for 1 hour in the dark, washed twice in PBS, and analyzed by flow cytometry.

R115777 accumulation and efflux

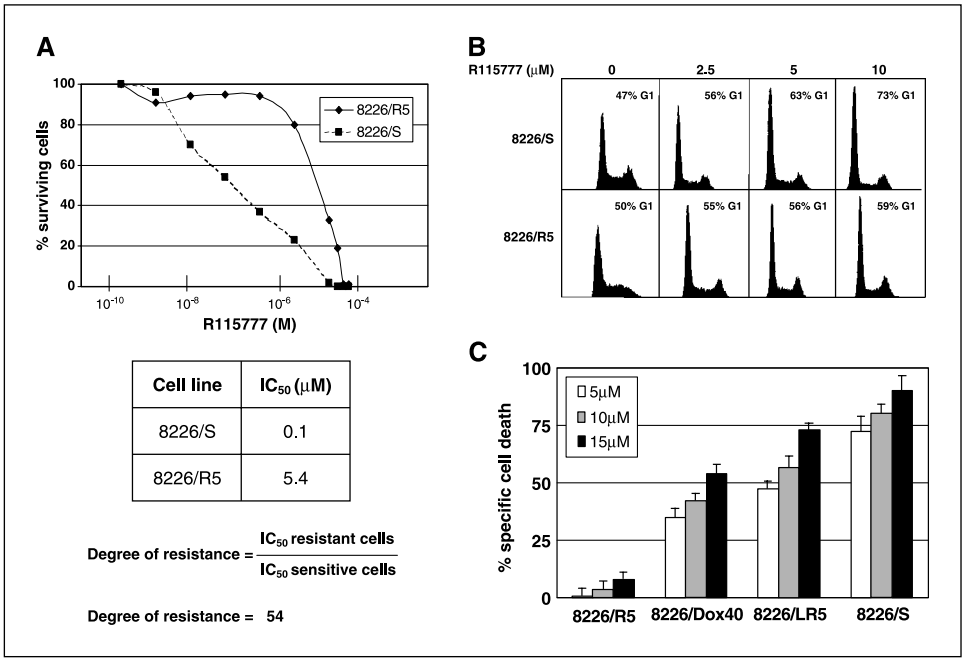
To quantitate cellular accumulation of [^{14}C]R115777, 8226/S and 8226/R5 cells were washed once in PBS and 1×10^6 cells were resuspended in 1 mL supplemented media containing 5 μmol/L R115777 (1:2.5 dilution of [^{14}C]R115777 to cold R115777). Cells were incubated at 37°C for increasing time periods and were then washed three times in 10 mL ice-cold PBS. Cell pellets were resuspended in 1 mL PBS and transferred to scintillation vials containing 10 mL Scintisafe 30% scintillation fluid (Fisher Scientific, Pittsburgh, PA). Samples were analyzed on a L56500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA). Because drug accumulation occurred rapidly, cells were incubated in 5 μmol/L R115777 at decreasing dilutions (1:10, 1:2.5, 1:1) of [^{14}C]R115777 for 1 hour and samples were processed and analyzed as described above to confirm a dose-dependent accumulation of [^{14}C]R115777 in each cell line. For efflux experiments, 8226/S and 8226/R5 cells were washed once in PBS and 1×10^6 cells were resuspended in 1 mL supplemented media containing 5 μmol/L R115777 (1:2.5 dilution of [^{14}C]R115777 to cold R115777). Cells were incubated for 1 hour at 37°C and then washed thrice in 10 mL PBS and placed in R115777-free supplemented media for increasing time periods. Samples were then processed and analyzed as described above. Because efflux of R115777 also occurred rapidly, cells were incubated with 5 μmol/L R115777 at increasing dilutions (1:1, 1:2.5, 1:10) of [^{14}C]R115777 for 1 hour and then washed and placed in R115777-free media for an additional hour. Samples were processed and analyzed as above to confirm a dose-dependent efflux of [^{14}C]R115777. Experiments with 8226/S and 8226/R5 lines were done in parallel.

Microarray analysis

Probe arrays. The oligonucleotide probe arrays were the Affymetrix U133A human arrays. These arrays consist of 22,215 probe sets, which target known and suspected genes as well as a number of suspected splice variants. The U133A chips detect an estimated 15,000 well-characterized human genes.

Sample processing for microarray analysis. Five micrograms of total RNA derived from 8226/S, 8226/LR5, and 8226/R5 cells served as the mRNA source for microarray analysis. The polyadenylate RNA was specifically converted to cDNA and then amplified and labeled with biotin following the procedure initially described by Van Gelder et al. (19). Hybridization with the biotin-labeled RNA, staining, and scanning of the chips followed the proscribed procedure outlined in the Affymetrix technical manual and has been previously described (20).

Data analysis. Scanned output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix Microarray Suite 5.1 software. Signal intensity was scaled to an average intensity of 500 before comparison analysis. The MAS 5.1 software uses a statistical algorithm to assess increases or decreases in mRNA abundance in a direct comparison between two samples (20, 21). This analysis is based on the behavior of 11 different oligonucleotide probes designed to detect the same gene. The software generates a *P* value for the likelihood that any perceived difference was due to chance. The *P* values for all probe sets were exported to a text file and all pairwise comparisons were then aligned in Excel. For the comprehensive analysis, $P < 0.05$ was identified as changed (increased or decreased) for each individual comparison. Two independent samples from 8226/S, 8226/LR5, and 8226/R5 cells were collected. The samples generated from the resistant cell line were compared with the sensitive lines in all possible combinations. Genes were ultimately selected if they were identified as increased in all eight comparisons or decreased in all eight comparisons.



Cell line	IC_{50} (μM)
8226/S	0.1
8226/R5	5.4

$$\text{Degree of resistance} = \frac{IC_{50}\ \text{resistant cells}}{IC_{50}\ \text{sensitive cells}}$$

Degree of resistance = 54

Fig. 1. 8226/R5 cells are highly resistant to R115777. *A*, degree of resistance was determined using the MTT reduction assay. 8226/S and 8226/R5 cells were treated with increasing concentrations of R115777 for 72 hours. Percentage surviving cells was calculated relative to cells treated with control media only (see Materials and Methods). IC_{50} determinations were done by linear regression analysis. *B*, cell cycle analysis after R115777 treatment. Sensitive and resistant 8226 lines were treated with increasing concentrations of R115777 for 24 hours. Cell cycle arrest was determined by flow cytometry after propidium iodide staining. Gating was on live cells only. *C*, evaluation of cell death after R115777 treatment. 8226/S, 8226/Dox40, 8226/LR5, and 8226/R5 cells were treated with increasing concentrations of R115777 for 72 hours. Cell death was determined by flow cytometry after Annexin V-FITC and propidium iodide staining. Specific cell death was calculated by subtracting background death in untreated samples. The presented data is representative of three independent experiments.

Statistical analysis

Unless otherwise stated, statistical data are expressed as mean ± SD using descriptive statistics.

Results

8226/R5 cells are resistant to R115777-induced growth arrest and cell death. Continuous culture of 8226/S cells with increasing concentrations of R115777 established the 8226/R5 line. In cytotoxicity assays, 8226/R5 cells were at least 50 times more resistant to R115777 compared with parental 8226/S cells (Fig. 1A). The IC_{50} for the 8226/S line was 0.1 μmol/L and that for the 8226/R5 line was 5.4 μmol/L. Cell cycle analysis revealed that R115777 induced G_1 growth arrest in 8226/S cells and this effect was largely abolished in the 8226/R5 line (Fig. 1B). 8226/R5 cells were also protected from apoptosis at concentrations of R115777 as high as 20 μmol/L (Fig. 1C; data not shown). The observed resistance was stable

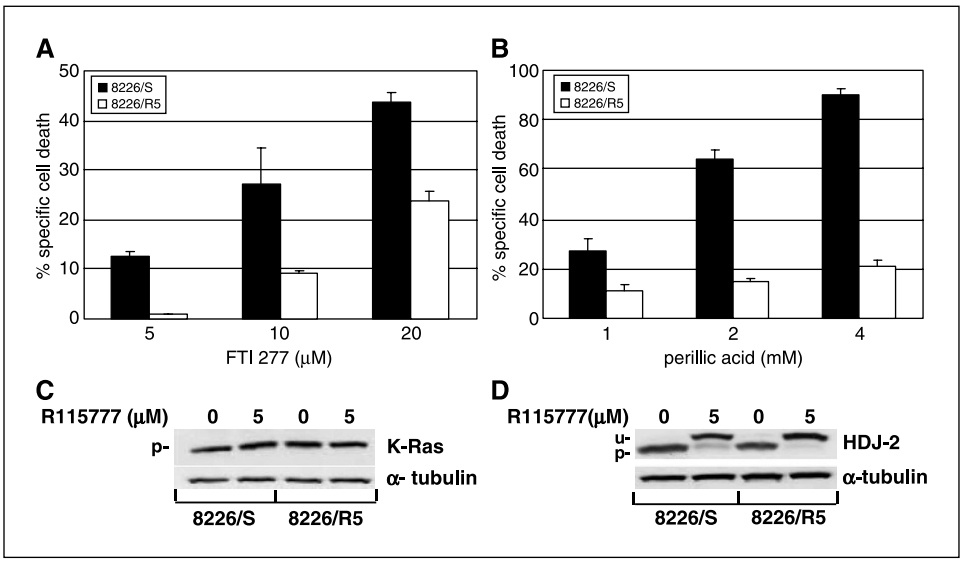


Fig. 2. Resistance in 8226/R5 cells is not linked to prenylation. *A*, 8226/R5 cells are resistant to the FTase-specific inhibitor FTI-277. 8226/S and 8226/R5 cells were treated with increasing concentrations of FTI-277 for 72 hours. Cell death was determined by flow cytometry after Annexin V-FITC and propidium iodide staining. Specific cell death was calculated by subtracting background death in untreated samples. *B*, 8226/R5 cells are resistant to perillic acid, an inhibitor of both FTase and GGTase I. Sensitive and resistant cells were treated with increasing concentrations of perillic acid for 72 hours. Samples were analyzed as in (*A*). *C*, K-Ras remains prenylated in sensitive and resistant cells after R115777 treatment. 8226/S and 8226/R5 cells were treated with control media or 5 μmol/L R115777 for 72 hours. Cell lysates were harvested and evaluated by Western blotting using the indicated antibodies. *D*, HDJ-2 farnesylation is inhibited in both 8226/S and 8226/R5 cell lines. Sensitive and resistant cells were treated and analyzed as in (*C*) using the indicated antibodies. u, unprocessed form; p, processed form. (*A*) and (*B*) are representative of three independent experiments; (*C*) and (*D*) are representative of two independent experiments.

because cells cultured in R11577-free conditioned media for several months continued to display the resistant phenotype. Relative to doxorubicin- (8226/Dox 40) and melphalan-resistant (8226/LR5) isogenic lines, 8226/R5 cells were the most resistant to R115777 (Fig. 1C).

Resistance to R115777 does not correlate with K-Ras prenylation or farnesyl transferase activity. To determine whether 8226/R5 cells were cross-resistant to other compounds that inhibit prenylation, cells were exposed to inhibitors of FTase and geranylgeranyl transferase I (GGTase I). Similar to our results with R115777, 8226/R5 cells were resistant to the specific FTase inhibitor FTI 277 (22) when compared with parental 8226/S cells (Fig. 2A). In addition, resistance was maintained in the presence of perillic acid, a compound that inhibits both FTase and GGTase I (Fig. 2B; ref. 23). 8226/S cells are known to express K-Ras and harbor a codon 12 K-RAS mutation. Similar to our prior observation in U266 cells (15), K-Ras remained prenylated in both sensitive and resistant cells after R115777 treatment (Fig. 2C). Moreover, farnesylation of HDJ-2 (a protein that can be farnesylated but not geranylgeranylated) was inhibited in both lines (Fig. 2D). These results indicate that R115777 resistance does not correlate with the prenylation status of K-Ras or HDJ-2. Furthermore, the fact that HDJ-2 farnesylation can be inhibited in 8226/R5 cells implies that mutation of FTase (the drug target) is not responsible for the development of resistance.

Resistance is not related to decreased accumulation of R115777. Many 8226 lines that acquire drug resistance have elevated expression of P-glycoprotein. An example of this is the 8226/Dox 40 cell line that expresses high levels of membrane P-glycoprotein that participates in the doxorubicin-resistant phenotype (18). As expected, 8226/Dox 40 cells were found to have a marked increase in surface P-glycoprotein expression when compared with parent 8226/S cells (Fig. 3A). 8226/R5 cells, however, had membrane levels that were similar to that noted for the 8226/S line (Fig. 3A). Because increased expression or activity of other membrane pumps may also produce a resistant phenotype, we investigated the influx and efflux of R115777 in both 8226/S and 8226/R5 cells. Influx of R115777 occurred rapidly in both lines and reached steady state within 15 minutes of incubation with radiolabeled R115777 (Fig. 3B). A dose-dependent increase in R115777 uptake was also observed (Fig. 3C) and both time- and concentration-dependent experiments revealed that R115777 uptake was increased in 8226/R5 cells compared with the parental line. With regard to R115777 efflux, intracellular

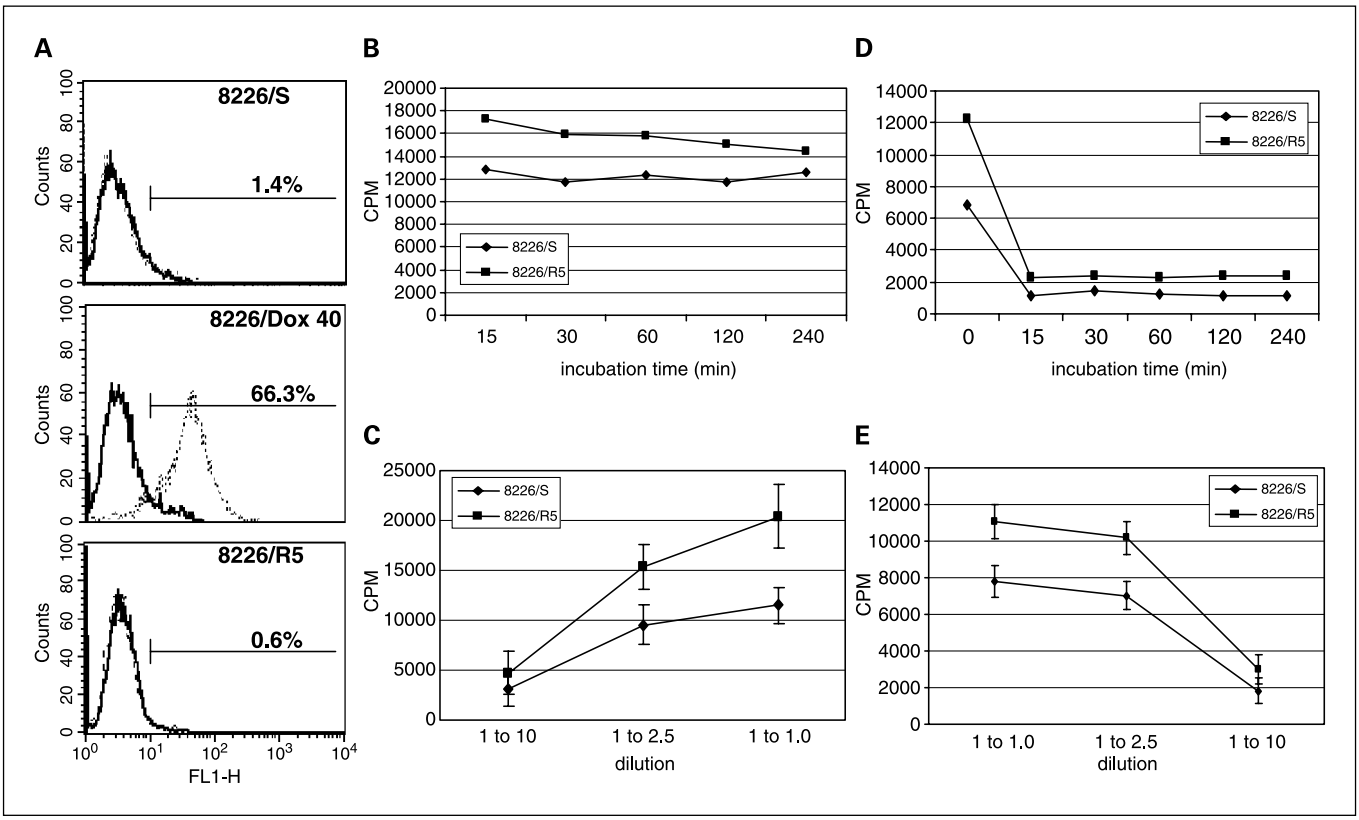


Fig. 3. Resistance is not related to decreased influx or increased efflux of R115777. *A*, P-glycoprotein surface expression is not increased in 8226/R5 cells. 8226/S, 8226/Dox40, and 8226/R5 cells were stained with anti – P-glycoprotein (*dashed line histograms*) or isotype control antibody (*solid line histograms*) as described in Materials and Methods. Samples were analyzed by flow cytometry. Decreased accumulation and/or increased efflux of R115777 is not responsible for resistance in the 8226/R5 line (*B-E*). *B*, 8226/S and 8226/R5 cells were exposed to 5 μmol/L R115777 (1:2.5 dilution of [^{14}C]R115777 to cold R115777) in supplemented media for increasing time periods. Cells were washed and accumulation of [^{14}C]R115777 was quantitated by scintillation counting (see Materials and Methods). *C*, 8226/S and 8226/R5 cells were incubated in supplemented media containing 5 μmol/L R115777 at decreasing dilutions (1:10, 1:2.5, 1:1) of [^{14}C]R115777 for 1 hour. Samples were processed and analyzed as in (*B*) to confirm a dose-dependent accumulation of [^{14}C]R115777 in each line. *D*, sensitive and resistant lines were treated with 5 μmol/L R115777 (1:2.5 dilution of [^{14}C]R115777 to cold R115777) for 1 hour, then washed and placed in R115777-free supplemented media for increasing time periods. Samples were processed and analyzed as in (*B*). *E,* 8226/S and 8226/R5 cells were treated with 5 μmol/L R115777 at increasing dilutions (1:1, 1:2.5, 1:10) of [^{14}C]R115777 for 1 hour and then washed and placed in R115777 free media for an additional hour. Samples were processed and analyzed as in (*B*) to confirm a dose-dependent efflux of [^{14}C]R115777. (*A*), (*B*), and (*D*) are representative of two independent experiments; (*C*) and (*E*) are representative of three independent experiments.

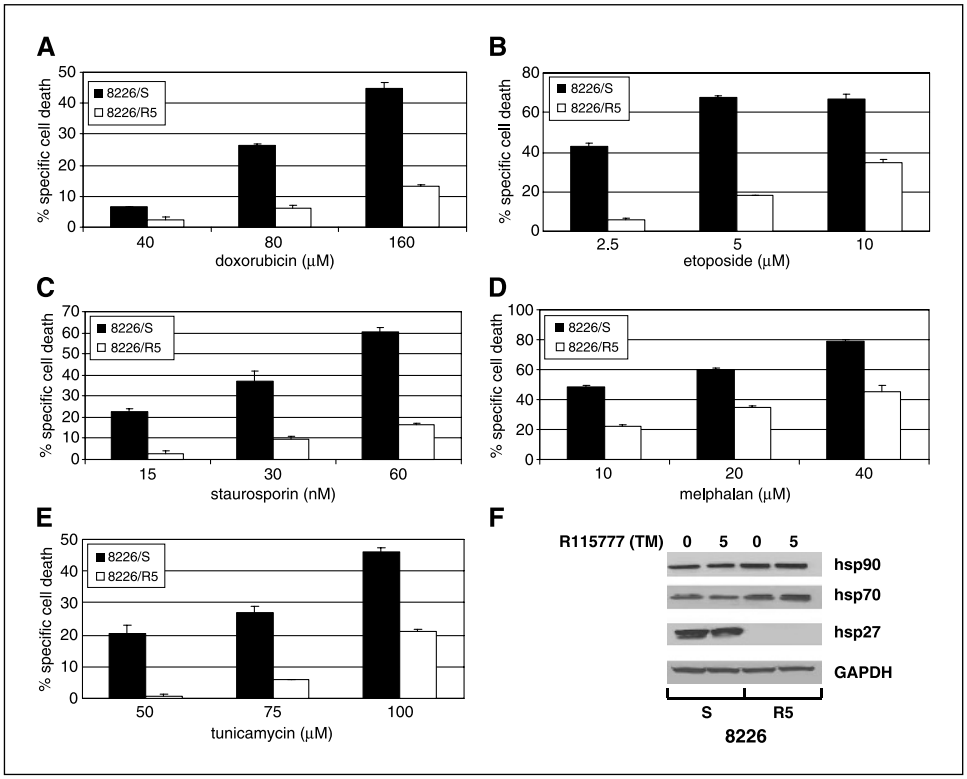


Fig. 4. 8226/R5 cells harbor a multidrug-resistant phenotype and resistance does not correlate with the expression of heat shock proteins. 8226/R5 cells display multidrug resistance (*A-E*). 8226/S and 8226/R5 lines were treated with the indicated compounds for 48 hours (*A* and *B*) or 24 hours (*C-E*). Cell death was determined by flow cytometry after Annexin V-FITC and propidium iodide staining. Specific cell death was calculated by subtracting background death in untreated samples. The presented data is representative of three independent experiments. *F*, resistance in 8226/R5 cells is not associated with increased expression of heat shock proteins. 8226/S and 8226/R5 cells were treated with control media or 5 μmol/L R115777 for 72 hours. Cell lysates were harvested and analyzed by Western blotting using the indicated antibodies (see Materials and Methods). The presented data is representative of two independent experiments.

R115777 decreased rapidly (within 15 minutes) in both sensitive and resistant cells in time course experiments (Fig. 3D). Furthermore, a dose-dependent efflux of R115777 was noted with resistant cells retaining more R115777 compared with sensitive 8226/S cells (Fig. 3E). These results indicate that 8226/R5 resistance is not related to decreased influx or increased efflux of R115777.

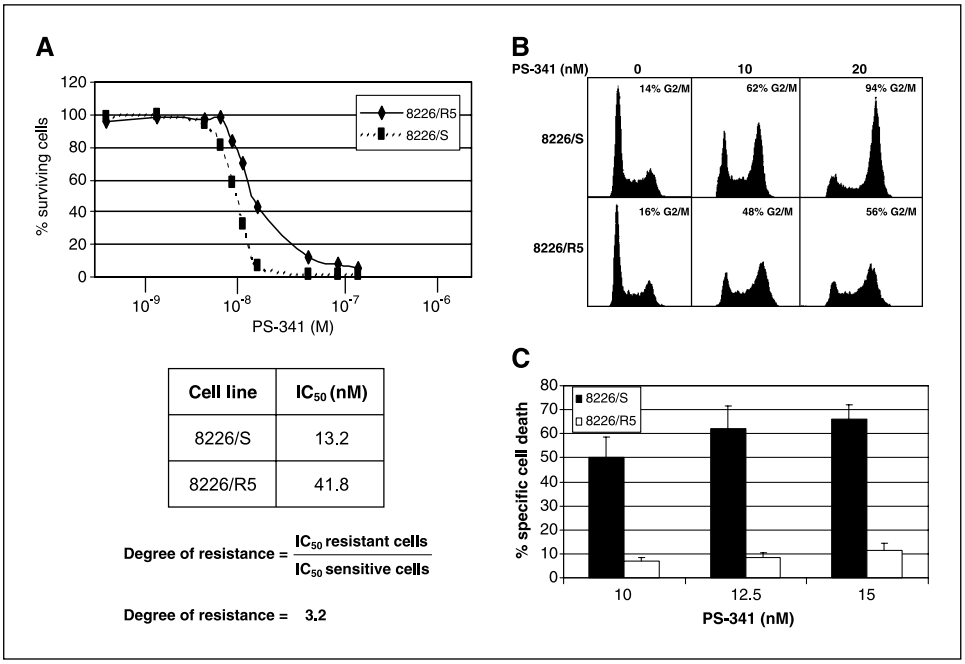
8226/R5 cells display a multidrug-resistant phenotype and resistance is not associated with increased expression of heat shock proteins. In an attempt to further categorize resistance, 8226/R5 cells were exposed to agents that induce apoptosis with diverse mechanisms of action. Compounds that have been reported to promote mitochondrial dysfunction, endoplasmic reticulum stress, and nuclear stress were tested (Fig. 4A-E). Surprisingly, 8226/R5 cells were resistant to all evaluated agents when compared with the parent 8226/S line. The proteosome inhibitor PS-341 has been shown to have cytotoxic activity in several chemoresistant myeloma lines (24). In cytotoxicity assays, 8226/R5 cells were three times more resistant to PS-341 when compared with 8226/S cells with an IC_{50} of 41.8 and 13.2 nmol/L, respectively (Fig. 5A). Unlike our findings with R115777, PS-341 induced G_2-M arrest in both sensitive and resistant cells with 8226/R5 cells being relatively protected (Fig. 5B). 8226/R5 cells were also protected from PS-341–induced apoptosis at concentrations as high as 20 nmol/L (Fig. 5C; data not shown). 8226/R5 cells maintained a PS-341–resistant phenotype when cultured in the absence of R11577 for several months, indicating stable resistance.

Heat shock proteins have antiapoptotic properties that can protect cells from stressful conditions. It has been previously reported that up-regulation of hsp70 can protect ovarian cancer cells from FTI-induced apoptosis (25). In addition, blockade of hsp27 expression has been shown to overcome PS-341 resistance in lymphoma cell lines (26, 27). To determine whether multidrug resistance was related to overexpression of heat shock proteins, sensitive and resistant 8226 lines were evaluated for heat shock protein expression (Fig. 4F). Expression levels of hsp70 and hsp90 were similar in sensitive and resistant lines and treatment with R115777 (drug-induced stress) had no appreciable effect. Hsp27 expression was also not effected in 8226/S cells after R115777 treatment but was undetectable in the 8226/R5 line. These results indicate that resistance does not correlate with the expression of heat shock proteins hsp27, hsp70, and hsp90.

Transcriptional profile of 8226/R5 cells. To identify potential targets of R115777 and/or molecules associated with R115777 resistance, we compared gene expression profiles between sensitive and resistant variants of 8226 cells. Direct comparisons were made between the 8226/S and 8226/R5 cell lines. In an attempt to reduce the number of nonspecific gene expression changes identified, comparisons were also made between 8226/LR5 (which possessed an intermediate degree of resistance to R115777; Fig. 1C) and 8226/R5 cells. This approach produced 2,064 probe sets (representing 1,666 genes) with changes unique to the 8226/R5 line. A selected list of genes involved in cell survival and growth, maintenance of cell cytostructure, cell-microenvironment contact, cholesterol biosynthesis, and protein degradation are presented in Table 1.

Discussion

FTIs were designed as specific inhibitors of Ras intended to interfere with a crucial posttranslational processing step. Ras requires the addition of a 15-carbon farnesyl group to its carboxyl-terminal cysteine, which permits its localization to the plasma membrane (a requirement for function). This



Cell line	IC_{50} (nM)
8226/S	13.2
8226/R5	41.8

$$\text{Degree of resistance} = \frac{IC_{50}\ \text{resistant cells}}{IC_{50}\ \text{sensitive cells}}$$

Degree of resistance = 3.2

Fig. 5. 8226/R5 cells are resistant to PS-341. *A*, degree of resistance was determined by the MTTreduction assay. 8226/S and 8226/R5 cells were treated with increasing concentrations of PS-341 for 48 hours. Percentage surviving cells and IC_{50} determinations were done as in Fig. 1. *B*, analysis of cell cycle after PS-341 treatment. Sensitive and resistant 8226 lines were treated with increasing concentrations of PS-341 for 24 hours. Cell cycle arrest was determined by flow cytometry after propidium iodide staining. Gating was on live cells only. *C*, evaluation of cell death after PS-341 treatment. 8226/S and 8226/R5 cells were treated with increasing concentrations of PS-341 for 48 hours. Cell death was determined by flow cytometry after Annexin V-FITC and propidium iodide staining. Specific cell death was calculated by subtracting background death in untreated samples. The presented data is representative of three independent experiments.

modification is done by the enzyme FTase, the purported target of R115777. It has been well established that alternate prenylation of Ras may occur in the presence of FTIs (28). This has been most notably described for K-Ras but also applies to the N-Ras protein. These reports are consistent with our prior observation in U266 cells (N-Ras–expressing line; ref. 15) and our present finding that K-Ras remains prenylated in 8226/S cells after R115777 treatment. These results suggest a Ras-independent mechanism of cell death. This is further supported by the clinical observation that responses to R115777 do not correlate with Ras mutation status or inhibition of farnesyl transferase measured *ex vivo* (7, 10, 14).

We hypothesized that the isolation of a R115777-resistant human myeloma cell line (8226/R5) might provide insight concerning the potential targets of R115777 or identify novel mechanisms of FTI resistance. R115777 resistance was not associated with an increase in the surface expression of P-glycoprotein nor was it associated with decreased influx or increased efflux of R115777. In addition, the expression of heat shock proteins (hsp27, hsp70, hsp90) did not correlate with the drug-resistant phenotype. We therefore undertook molecular profiling of sensitive and resistant 8226 lines and identified expression changes in several genes implicated in cell survival and drug resistance. They included genes involved in cell signaling, cholesterol biosynthesis, and protein degradation. It remains possible that molecules associated with one of these genes is a major target of R115777 and/or directly participates in FTI resistance.

Our microarray data identified increased expression of several proteins associated with Jak-Stat signaling, including Jak2. In primary isolates, constitutive Stat3 activation has been observed in the majority of patients with multiple myeloma (29, 30). Stat3 homodimers and Stat1:Stat3 heterodimers seem to be the predominant DNA-binding forms (29). It has been reported that inhibition of Stat3 can sensitize resistant myeloma cells to chemotherapy mediated apoptosis (31). These results imply that Jak-Stat signaling may contribute to the drug-resistant phenotype. Stat3 activity has been linked to up-regulation of Bcl-X_L (29) and consistent with this, Bcl-X_L expression was increased in 8226/R5 cells. Elevated expression of Bcl-X_L has been observed in several hematopoietic tumors, including blast crisis chronic myelogenous leukemia and non-Hodgkin's lymphoma (32), and one study suggested that it was an indicator of chemoresistance in multiple myeloma (33). We have previously reported that R115777 can partially overcome drug resistance in U266 cells that maintain high levels of stable Bcl-X_L expression (15). These findings imply that R115777 resistance may only in part be regulated by increased Jak-Stat signaling or Bcl-X_L expression in 8226/R5 cells.

Our comparative gene expression profiling also identified other genes potentially involved in R115777 resistance. Phosphatidylinositol 3-kinase is a heterodimer consisting of p85 and p110 subunits (34, 35). The p110 δ isoform is preferentially expressed in cells of hematopoietic origin (36, 37) and elevated expression of the catalytic δ polypeptide was observed in the 8226/R5 line. An important downstream target of phosphatidylinositol 3-kinase is Akt/protein kinase B, a protein that is known to play a role in myeloma survival and drug resistance (38–40). Interestingly, it has been reported that FTI-induced apoptosis can be prevented by a constitutively activated form of Akt-2 (41). Therefore, it remains possible that phosphatidylinositol 3-kinase/Akt signaling also contributes to R115777 resistance and the multidrug-resistant phenotype. Because the p110 δ subunit is mainly expressed in hematopoietic cells, it potentially represents a novel therapeutic target particularly for resistant tumors of hematopoietic origin.

Our analysis also identified increased expression of mevalonate kinase, an enzyme associated with cholesterol biosynthesis. Influx and efflux experiments suggest that R115777 is retained in 8226/R5 cells compared with the parent 8266/S line. This is relevant because an increase in cholesterol-rich microdomains, such as lipid rafts, have been associated with the development of multidrug resistance (42). R115777 is a lipophilic molecule that could potentially be

Table 1. Gene expression changes in 8226/R5 cells

Expression increased	Expression decreased
Janus kinase 2	Interleukin-6 receptor
Signal transducer and activator of transcription 1	Insulin-like growth factor 2 receptor
Bcl-XL	Mcl-1
Phosphoinositide-3-kinase, catalytic, δ polypeptide	Heat shock protein 27
Fibroblast growth factor receptor activating protein 1	Retinoblastoma 1
Fibroblast growth factor 20	Transforming growth factor α
Mevalonate kinase	Tumor protein p53
Rap 2A	Farnesyl diphosphate synthetase
Rab GGTase, β subunit	Lamin A/C
Rab 4A	Ras protein activator like-1
Ras and Rab interactor 3	Ras and Rab interactor 2
Mitogen-activated protein kinase 1	Rab 36
Mitogen-activated protein kinase 9	Rho GTPase-activating protein 6
Mitogen-activated protein kinase 14	Mitogen-activated protein kinase phosphatase
Cyclin D3	Cyclin D1
Fas apoptotic inhibitory molecule	Syndecan 1
Calmodulin 3	X-box – binding protein 1
Calpain 2	Actin, β
S100 calcium binding protein A13 and A14	Fibronectin-1
Collagen type XXI α	Integrin β5
Collagen IV, α3	Integrin β7
Paxillin	Intercellular adhesion molecule-1
Integrin α-4	Ubiquitin-conjugating enzymes
Integrin α-6	Proteosome subunit B-4 type
Ubiquitin-specific proteases 15	Proteosome 26S subunit
Ubiquitin-specific proteases 24	S100 calcium binding protein (A11 pseudogene)
Proteosome inhibitor subunit 1	S100 calcium-binding protein (A11 calgizzarin)

sequestered and compartmentalized in such microdomains; however, in 8226/R5 cells, R115777 efficiently inhibits FTase. Nevertheless, it remains possible that subcompartmentalization of R115777 influences its interaction with unforeseen targets. Also of importance was the observation that 8226/R5 cells were resistant to the proteosome inhibitor PS-341. It has been previously reported that increased expression of hsp27 produces a PS-341–resistant phenotype in multiple myeloma cells (26). In 8226/R5 cells, however, hsp27 protein expression was undetectable. Our microarray analysis revealed decreased expression of the 26S proteosome subunit (the target of PS-341) in 8226/R5 cells when compared with the parental 8226/S line. This perhaps represents a novel mechanism of proteosome inhibitor resistance.

In conclusion, we have identified a myeloma cell line with resistance to both R115777 and PS-341. Further characterization of this line may lead to identification of novel drug targets or resistance mechanisms that are clinically relevant.

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