

R115777 induces Ras-independent apoptosis of myeloma cells via multiple intrinsic pathways

Darrin M. Beaupre,^{1,2} Enrique Cepero,³
Esther A. Obeng,³ Lawrence H. Boise,^{2,3} and
Mathias G. Lichtenheld^{2,3}

¹ Department of Medicine, Division of Hematology and Oncology,
² Sylvester Cancer Center, and ³ Department of Microbiology and
Immunology, University of Miami School of Medicine, Miami, FL

Abstract

Ras activation is frequently observed in multiple myeloma either by mutation or through interleukin-6 receptor signaling. Recently, drugs designed to inhibit Ras have shown promise in preclinical myeloma models and in clinical trials. In this report, we characterize the pathways by which the clinically tested farnesyl transferase inhibitor (FTI) R115777 induces apoptosis in multiple myeloma cells. Contrary to the proposed mechanistic action of FTIs, we found that R115777 induces cell death despite Ras prenylation implying participation of Ras-independent mechanism(s). Apoptosis proceeded via an intrinsic cascade and was associated with an increase in the expression and activity of Bax. Bax activation correlated with a loss of mitochondrial membrane integrity and activation of the endoplasmic reticulum (ER) stress response. These pathways activate caspase-9 and consistent with this, cell death was prevented by caspase-9 blockade. Interestingly, cells overexpressing Bcl-X_L remained partially sensitive to R115777 despite suppression of mitochondrial membrane dysfunction and ER-related stress. Taken together, these results indicate that R115777 induces apoptosis in a Ras-independent fashion via multiple intrinsic pathways. [Mol Cancer Ther. 2004;3(2):179–186]

Introduction

Multiple myeloma is an incurable plasma cell malignancy in which Ras activation is frequently observed either secondary to *ras* mutations (1, 2) or through interleukin-6 receptor signaling (3). Since Ras activation has been linked to myeloma cell growth (3) and Ras signaling may also

contribute to myeloma cell survival (4), we and others have tested the efficacy of several classes of farnesyl transferase inhibitors (FTIs) in multiple myeloma models. Initial preclinical investigations revealed that FTIs could effectively induce growth arrest and cell death in diverse myeloma cell lines regardless of Ras mutation status, P-glycoprotein overexpression, or chemotherapy and steroid resistance (5–7). More importantly, in primary myeloma isolates, FTIs induced cell death with relative sparing of host mononuclear cells (5, 6). A phase II clinical trial testing the efficacy of R115777 has recently been completed in myeloma patients with refractory disease and the results indicate that the majority achieve at least disease stabilization (8). Taken together, these observations imply that FTIs may serve as a useful therapeutic strategy in multiple myeloma and further understanding of their molecular targets and mechanisms of action are warranted.

Previously we reported that the FTI perillidic acid inhibits Ras farnesylation and induces cell death via an intrinsic cascade in myeloma cell lines (5). To further advance on these observations, we tested the clinically promising and pharmacologically superior molecule R115777. Similar to perillidic acid, R115777 induced cell death via intrinsic pathways. However, growth arrest and cell death occurred despite Ras prenylation implying a Ras-independent mechanism(s) of cell death. This report delineates several of the involved pathways.

Materials and Methods

Cell Lines, Antibodies, and Reagents

The U266 cell line was obtained from the American Type Culture Collection (Manassas, VA) and the U266 Bcl-X_L line was kindly donated by William S. Dalton (Moffitt Cancer Center, Tampa, FL). U266 lines were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 15% fetal bovine serum, 25 mM HEPES (pH 7.4), and 50 µg/ml gentamicin (culture reagents from Life Technologies, Inc., Grand Island, NY). FL5.12 lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 mM HEPES (pH 7.4), WEHI-3B supernatant (source of interleukin-3), penicillin and streptomycin, and 1 mg/ml G418 (culture reagents from Life Technologies). Antibodies were purchased from the following vendors: anti-pan Ras (Oncogene Research Products, Boston, MA); anti-Bcl-2 (Dako Corporation, Carpinteria, CA); anti-Bcl-X_L, anti-Mcl-1, anti-Bak, anti-Bax, and anti-GADD153 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-β-actin (Sigma Chemical, St. Louis, MO); anti-cytochrome *c* (Biovision, Palo Alto, CA); anti-Smac (Upstate Biotechnology, Lake Placid, NY); anti-cytochrome oxidase subunit IV (Molecular Probes, Eugene, OR); and anti-BiP/GRP78 (BD Pharmingen, San Diego, CA). Annexin-V FITC, propidium iodide (PI), 500X protease inhibitor cocktail, and z-LEHD-fmk were obtained from

Received 9/18/03; revised 11/3/03; accepted 11/26/03.

Grant support: National Institute of Health Grants: K12 Miami AIDS Oncology Scientist Program CA77824-03 (D.M. Beaupre), R01 CA55811 (M.G. Lichtenheld), F31 GM20435 (E. Cepero); a Howard Hughes Medical Institute Fellowship (E.A. Obeng); a Senior Research Grant from the Multiple Myeloma Research Foundation (L.H. Boise); and an award from The Woman's Cancer Association of the University of Miami (M.G. Lichtenheld).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for Reprints: Mathias G. Lichtenheld, University of Miami School of Medicine, RMSB 3014, 1600 NW 10th Avenue, Miami, FL 33136. Phone: (305) 243-3301; Fax: (305) 243-4623. E-mail: mlichten@med.miami.edu

Biovision. Tetramethylrhodamine ethyl ester (TMRE) was purchased from Molecular Probes and U0126 from Promega (Madison, WI). R115777 was kindly provided by David End (Johnson & Johnson Pharmaceutical Research and Development, LLC, Titusville, NJ) and was dissolved in DMSO (Sigma) with sonication for 10 min at room temperature before its use. Melphalan was purchased from Sigma and tunicamycin was obtained from Calbiochem (San Diego, CA).

Analysis of Cell Cycle, Cell Death, and Mitochondrial Membrane Potential

U266 and U266 Bcl-X_L lines were plated at 5×10^5 cells/ml for the desired time periods in the presence of 0.4% DMSO containing medium (control medium) or R115777. Cell cycle analysis along with quantitative and qualitative analysis of apoptotic and live cells was performed by flow cytometry as previously described (5). Otherwise 20,000 events were analyzed to determine the percentage of annexin-V FITC and PI-positive cells. Evaluation of mitochondrial membrane potential was performed using TMRE staining as described previously (9).

Isolation of Cytoplasmic and Mitochondrial Fractions

Isolation of cytoplasmic and mitochondrial fractions was performed according to Khaled *et al.* (10). Briefly, U266 and U266 Bcl-X_L cells were plated at 5×10^5 cells/ml for 72 h in the presence of control media or R115777. Cells (5×10^7) were harvested, washed with PBS, and then pellets were resuspended in 500 μ l of isotonic buffer [200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES (pH 6.8), 1 \times protease inhibitor cocktail]. Samples were incubated on ice for 10 min and homogenized by douncing with a tight pestle. Cellular debris was removed by centrifugation at 3500 rpm in an Eppendorf centrifuge and supernatants were collected and spun at 12,000 rpm to recover the cytoplasmic fraction (supernatant). The mitochondrial pellet was washed once with isotonic buffer and then resuspended and solubilized in 100 μ l of RIPA lysis buffer (1 \times PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 \times protease inhibitor cocktail).

Western Blotting

U266 and U266 Bcl-X_L cell lysates were harvested after the indicated treatments and evaluated by Western blotting as previously described (5). For Western blotting of cytoplasmic and mitochondrial fractions, protein concentrations from isolated cellular fractions were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of cytoplasmic and mitochondrial protein (100 μ g) were then evaluated by Western blotting as referenced above. Densitometric quantitation of bands was performed using Alpha Ease FC software.

Flow Cytometric Detection of Bax Activation

Cell staining for Bax activation was performed according to Bellosillo *et al.* (11). Briefly, U266 cells were plated at 5×10^5 cells/ml for 72 h in the presence of increasing concentrations of R115777 or control media. Samples were harvested for staining with annexin-V FITC and PI as described above and aliquots of 1×10^6 cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Phar-

Mingen) as per the manufacturer's instructions. Samples were then stained with the anti-Bax antibody YTH-6A7 (Trevigen, Gaithersburg, MD) at 0.125 μ g/l for 20 min at room temperature, washed twice in 1 \times Perm/Wash buffer before staining with a phycoerythrin (PE)-conjugated goat anti-mouse antibody (Dako) at 1:20 dilution. After 20 min at room temperature, samples were washed as above and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Statistical Analysis

Statistical data are expressed as mean \pm SD using descriptive statistics.

Results

R115777 Induces Growth Arrest and Cell Death Despite Ras Prenylation

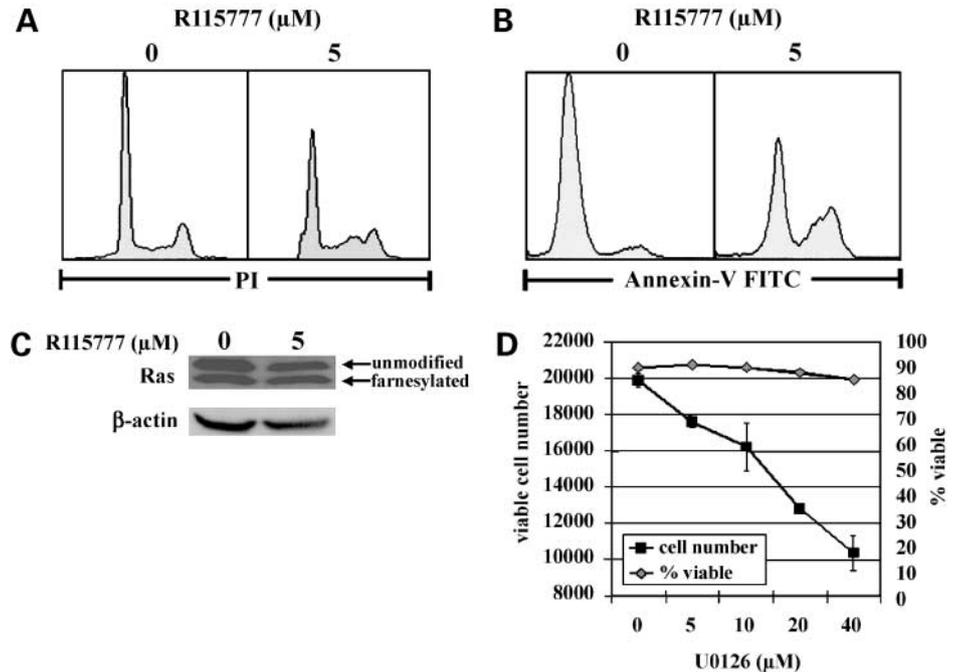
Previously, we observed that the nonselective FTI perillc acid [which inhibits both farnesyl transferase (FTase) and geranylgeranyl transferase I (GGTase I)] inhibits Ras prenylation and triggers G₁ growth arrest and cell death in multiple myeloma lines (5). Similarly, our laboratory and others have noted that the more selective FTI R115777 (which inhibits mainly FTase) also induces growth arrest and cell death in myeloma lines and primary isolates (5, 6). In contrast to our results with perillc acid, however, treatment of U266 cells with R115777 was associated with an accumulation of cells in the late S phase of the cell cycle (Fig. 1A). As noted above, R115777 also efficiently induced cell death as determined by annexin-V FITC staining (Fig. 1B). To address the similarities and differences between perillc acid and R115777, the status of Ras farnesylation was determined. Unlike our observations with perillc acid, R115777 did not inhibit Ras prenylation (Fig. 1C). This led us to resolve whether U266 cell survival required Ras-MAP kinase signaling. Treatment of U266 cells with the selective MEK inhibitor U0126 resulted in decreased proliferation (Fig. 1D, see viable cell number); however, there was no appreciable evidence of apoptosis as per morphological evaluation (data not shown) or by annexin-V FITC and PI staining (Fig. 1D, see % viability). Taken together, these observations imply that R115777 targets survival pathways that are independent of Ras in multiple myeloma cells.

Inhibition of Caspase-9 Activity Blocks R115777-Induced Apoptosis

Caspase-9 is an important regulator of apoptosis whereby its activity is known to increase in response to mitochondrial membrane dysfunction (12) and endoplasmic reticulum (ER)-related stress (13). To functionally determine if cell death induced by R115777 involved caspase-9 activation, we investigated a pro B lymphocyte cell line (FL5.12) that ectopically expresses dominant-negative caspase-9 (DN-C9).⁴ Treatment with increasing concentrations of R115777 resulted in a dose-dependent

⁴E. Cepero, A. M. King, and L. H. Boise. Caspase-9 and effector caspases have sequential and distinct effects on mitochondria, submitted for publication.

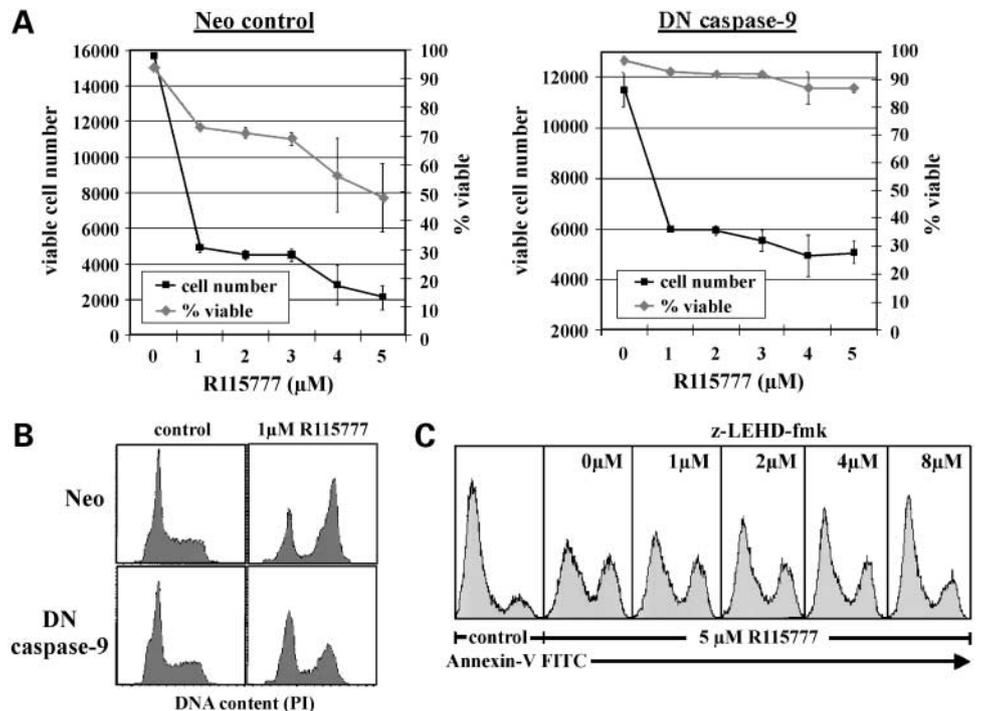
Figure 1. Growth arrest and cell death induced by R115777 occur without alterations in Ras prenylation. **A**, U266 cells were maintained with control media or 5 μM R115777 for 48 h to encompass one doubling time. Cells were then fixed and stained with PI for cell cycle analysis by flow cytometry. Forward scatter and side scatter gating was on viable cells only. **B**, U266 cells were maintained with either control media or 5 μM R115777 for 72 h. Cells were harvested and stained with annexin-V FITC for analysis by flow cytometry. **C**, U266 cells were maintained as in **B** and harvested lysates were analyzed by Western blotting using the indicated antibodies. U266 cells express the N-Ras isoform (7). **D**, U266 cells were treated with either control media or the indicated concentrations of the MEK inhibitor U0126 for 72 h. After annexin-V FITC and PI staining, the absolute number and percentage of viable cells was determined as described in Materials and Methods. The data presented in **A–D** are from three independent experiments.



decrease in cell growth in both the control (neomycin vector) and DN-C9-expressing line (Fig. 2A), see viable cell number). As seen in U266 cells (Fig. 1A), growth inhibition was associated with cell cycle arrest (Fig. 2B). Evaluation of cell death, however, revealed that cells ectopically expressing DN-C9 were protected (Fig. 2A, see % viable). To determine whether cell death was associated with caspase-9 activation in our myeloma model, U266

cells were treated with 5 μM R115777 in the absence or presence of the caspase-9 inhibitor z-LEHD-fmk. Compared to cells treated with R115777 alone, increasing concentrations of the caspase-9 inhibitor resulted in a dose-dependent increase in the number of live cells and a reciprocal decrease in cell death (Fig. 2C). These results imply that death induced by R115777 requires caspase-9 activity.

Figure 2. Blockade of caspase-9 activity prevents R115777-induced apoptosis. **A**, FL5.12 cells harboring either a neomycin control vector (*Neo control*) or ectopically expressing dominant-negative caspase-9 (*DN caspase-9*) were treated with either control media or increasing concentrations of R115777 for 48 h. The viable cell number and percentage of viable cells were determined by flow cytometry as described in Materials and Methods. **B**, the FL5.12 control line (*Neo*) and dominant-negative caspase-9-expressing line (*DN caspase-9*) were treated with control media or 1 μM R115777 for 48 h. Cell cycle analysis of viable cells was determined by PI staining as described in Materials and Methods. **C**, U266 cells were treated with either control media or 5 μM R115777 for 48 h in the absence or presence of increasing concentrations of the caspase-9 inhibitor z-LEHD-fmk. *Histograms*, annexin-V FITC-stained cells. The results of **A–C** are from two independent experiments.



R115777 Activates an Intrinsic Cascade That Promotes the Loss of Mitochondrial Membrane Integrity

Perillic acid induces apoptosis in U266 cells independent of death receptor signaling and activation of caspase-8 (5). Similarly, R115777 induced cell death in the presence of a caspase-8 inhibitor (*z*-IETD-fmk, data not shown) under conditions that we found abolishes death receptor-mediated apoptosis (5). These results imply that R115777 triggers apoptosis via an intrinsic cascade. Intrinsic cell death can be initiated via mitochondrial membrane dysfunction whereby leakage of mitochondrial proteins including cytochrome *c* and Smac can result in caspase-9 activation (12). Indeed, we observed that treatment with R115777 was associated with mitochondrial membrane depolarization (Fig. 3A). Furthermore, an increase of cytochrome *c* and Smac was detected in cytoplasmic fractions with a reciprocal decrease from mitochondrial fractions under standardized conditions using β -actin (cytoplasmic fraction) and cytochrome oxidase IV (mitochondrial fraction) as controls (Fig. 3B) (10). These results indicate that cell death induced by R115777 involves alterations in mitochondrial membrane integrity and release of proapoptotic molecules.

R115777 Increases the Expression and Activity of Bax

Antiapoptotic proteins including Mcl-1, Bcl-X_L, and Bcl-2 serve to stabilize mitochondrial membrane integrity (12) and are frequently overexpressed in multiple myeloma (14–20). Therefore, we investigated whether treatment with

R115777 was associated with a decline in their expression. Western blotting revealed no decrease in the expression of these Bcl-2 family members (Fig. 4A). To address an alternate trigger for mitochondrial membrane dysfunction, we also evaluated the proapoptotic proteins Bak and Bax (21). There was no significant change in the expression of Bak (as compared to β -actin by densitometry); however, Bax expression increased in a dose-dependent fashion with a 2.6-fold increase at the 4- μ M dose (Fig. 4B). Because an increase in the expression of Bax is not necessarily related to an increase in Bax activity, its activation was determined using an antibody that recognizes only its active conformation (11). As R115777 induced a dose-dependent increase in cell death (Fig. 5A), a parallel increase in Bax activation was detected (Fig. 5B, see 1^o + 2^o) when compared to background for the secondary antibody (Fig. 5B, see 2^o only). These results imply that R115777 triggers loss of mitochondrial membrane integrity by increasing the expression and activity of Bax.

Intrinsic Cell Death Induced by R115777 Involves Pathways That Are Independent of Mitochondria-Mediated Apoptosis

The activation of Bax coupled with loss of mitochondrial membrane integrity suggested that the mitochondria play an essential role in R115777-induced apoptosis (22). To address this, we evaluated the U266 Bcl-X_L line that ectopically overexpresses Bcl-X_L (5, 7). Treatment of U266 Bcl-X_L cells with R115777 resulted in mitochondrial membrane depolarization and apoptosis as determined by TMRE and annexin-V FITC staining (Fig. 6A). To identify whether the loss of mitochondrial membrane potential was associated with an alteration in the localization of Bcl-X_L, cytoplasmic and mitochondrial fractions were tested for their Bcl-X_L content. A shift of Bcl-X_L from cytoplasmic to mitochondrial fractions was noted (Fig. 6B) consistent with prior observations in thymocytes where apoptosis resulted in redistribution of Bcl-X_L to membrane fractions (23). Compatible with the ability of Bcl-X_L to prevent the release of mitochondrial proteins, we observed that neither cytochrome *c* nor Smac was released (Fig. 6B). These results indicate that cell death induced by R115777 also involves additional intrinsic pathways that may cooperate with but are independent of mitochondria-mediated apoptosis.

The ER stress response (unfolded protein response) has been shown to activate caspase-9 directly independent of the release of mitochondrial proteins (13). Because potential targets of FTIs may be localized to the ER, we evaluated U266 cells for evidence of ER-related stress. This was accomplished by monitoring the expression of GRP78, an ER chaperon protein the expression of which increases on triggering of the ER stress response (24, 25). Treatment of U266 cells with increasing concentrations of R115777 resulted in a dose-dependent increase in GRP78 protein levels with a 3.6-fold increase noted at the 15- μ M dose (Fig. 7A). Similarly, the expression of another ER stress-related protein, GADD153 (26), was also increased although to a lesser extent than that induced by the

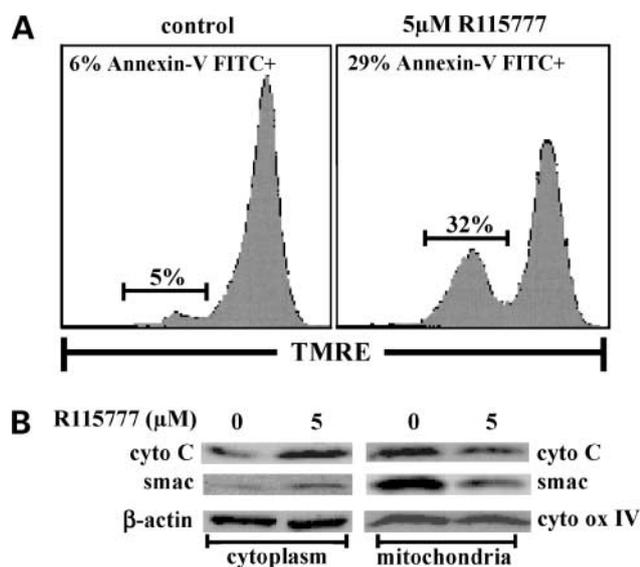


Figure 3. Treatment with R115777 is associated with mitochondrial membrane depolarization and release of cytochrome *c* and Smac. **A**, U266 cells were maintained with control media or 5 μ M R115777 for 72 h. Cells were stained with TMRE to monitor mitochondrial membrane potential (histograms). Percent cell death was determined by annexin-V FITC staining. **B**, U266 cells were treated as in **A** and cytoplasmic (cytoplasm) and mitochondrial (mitochondria) fractions were analyzed by Western blotting using the indicated antibodies at the time of maximal mitochondrial membrane dysfunction (72 h). Cytochrome oxidase IV was not detected in cytoplasmic fractions (data not shown). *cyto C*, cytochrome *c*; *cyto ox IV*, cytochrome oxidase IV. The data presented in **A** and **B** are from two independent experiments.

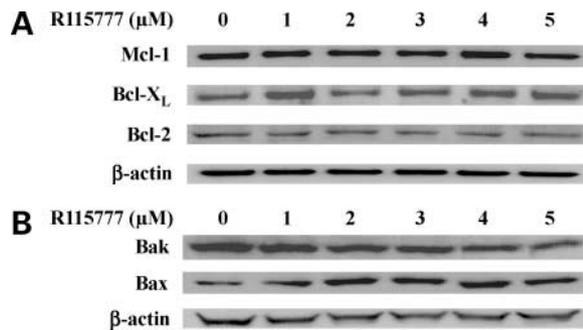


Figure 4. Expression of antiapoptotic and proapoptotic proteins after treatment with R115777. U266 cells were treated with control media or increasing concentrations of R115777 for 72 h and cell lysates were analyzed by Western blotting for expression of antiapoptotic proteins (**A**) or proapoptotic proteins (**B**). The data presented in **A** and **B** are from three independent experiments.

ER stressor tunicamycin (Fig. 7B). Treatment of U266 cells with the alkylating agent melphalan did not result in an increase in GADD153 expression (Fig. 7B). Interestingly, both GRP78 and GADD153 did not significantly increase when Bcl-X_L was overexpressed, suggesting that Bcl-X_L may confer ER protection (Fig. 7, B and C). Of note, U266 Bcl-X_L cells remained partially sensitive to R115777 (Fig. 7D) despite suppression of mitochondrial membrane dysfunction and ER stress. These results indicate that additional intrinsic pathway(s) also cooperate in R115777-induced apoptosis.

Discussion

Due to the prominent role of Ras in multiple myeloma (27–29), we and others have evaluated the efficacy of FTIs

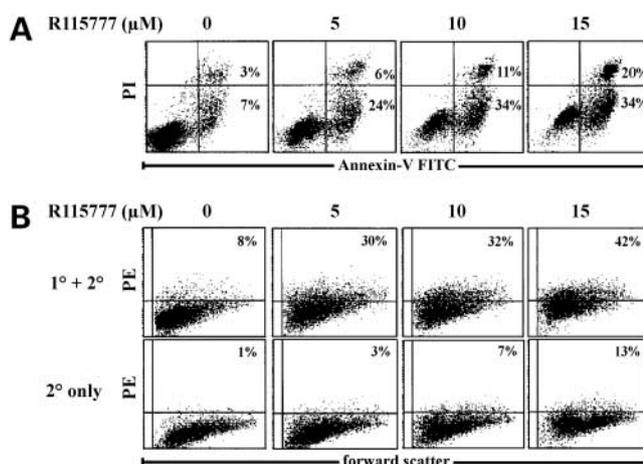


Figure 5. Bax activity is increased after treatment with R115777. U266 cells were maintained with control media or 5–15 μM R115777 for 72 h and cells were evaluated by flow cytometry. **A**, annexin-V FITC- and PI-stained samples with percentages indicated in quadrants. **B**, samples in **A** were stained with primary anti-Bax antibody followed by a phycoerythrin-conjugated secondary antibody ($1^{\circ} + 2^{\circ}$) or with secondary antibody alone (2° only). Percentages indicate Bax activation ($1^{\circ} + 2^{\circ}$) versus nonspecific background staining (2° only). The data presented in **A** and **B** are from three independent experiments.

in myeloma cell lines and primary isolates (5–7, 30). This class of drugs, which includes R115777, has been designed to inhibit Ras processing leading to its inactivation (31). Here, however, we found that R115777 induced apoptosis independent of Ras prenylation (Fig. 1C). U266 cells express wild-type N-Ras (7) which can be alternatively processed in cells exposed to FTIs (32, 33). The fact that perillidic acid (which inhibits both FTase and GGTase I) reverses the ratio of farnesylated to unmodified Ras (5) implies that R115777 permits N-Ras geranylgeranylation. Because death proceeded despite Ras processing, our results indicate that R115777 targets survival and/or death pathways that are independent of Ras in myeloma cells. This is supported by the observation that inhibition of MEK (a major downstream kinase) had no significant effect on myeloma cell viability (Fig. 1D and data not shown). Intrigued by these findings, we set out to discern the mechanisms by which R115777 triggers apoptosis.

Two major pathways promote programmed cell death in mammalian cells (12). Extrinsic pathways are initiated by death receptor signaling leading to activation of caspase-8. Because R115777 induced cell death in the presence of a caspase-8 inhibitor, we focused our attention on intrinsic mechanisms of cell death. The best characterized of these is triggered by activation of proapoptotic Bcl-2 family members (such as Bax) resulting in mitochondrial membrane dysfunction. Indeed, the expression and activity of

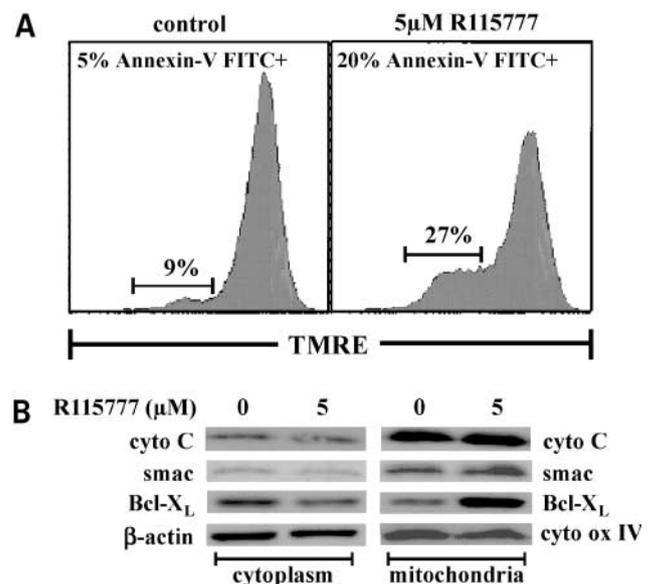


Figure 6. R115777 induces apoptosis in U266 Bcl-X_L cells independent of Bcl-X_L redistribution and the release of cytochrome *c* and Smac. **A**, U266 Bcl-X_L cells were maintained with control media or 5 μM R115777 for 72 h. Cells were stained with TMRE to evaluate mitochondrial membrane potential (histograms). Percent cell death was determined by annexin-V FITC staining. **B**, U266 Bcl-X_L cells were treated as in **A** and cytoplasmic (cytoplasm) and mitochondrial (mitochondria) fractions were analyzed by Western blotting for their Bcl-X_L, Smac, cytochrome *c* (cyto *C*), cytochrome oxidase IV (cyto *ox IV*), or β-actin content at the time of maximal mitochondrial membrane dysfunction (72 h). Cytochrome oxidase IV was not detected in cytoplasmic fractions (data not shown). The data presented in **A** and **B** are from two independent experiments.

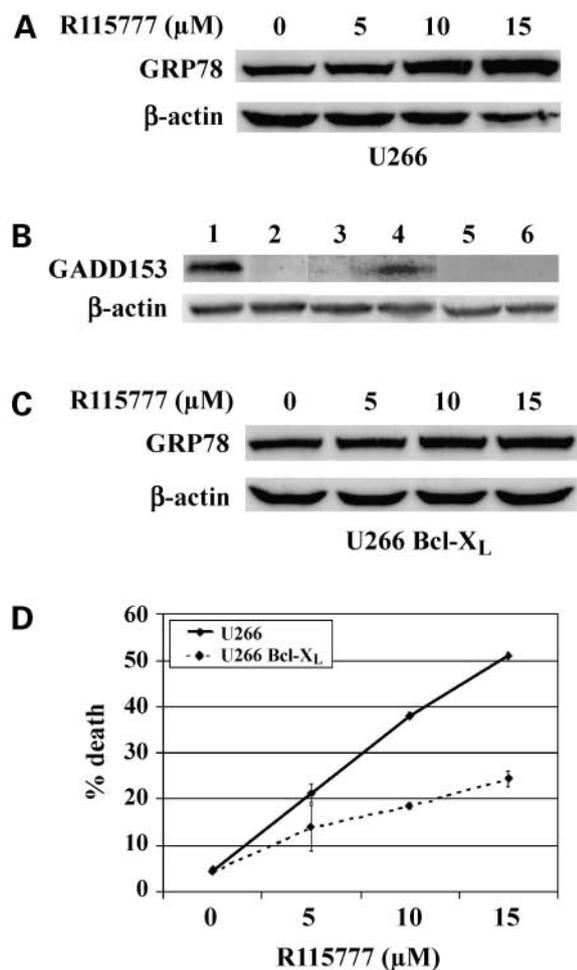


Figure 7. R115777 triggers a Bcl-X_L-sensitive ER stress response in U266 cells. **A**, U266 cells were maintained with control media or 5–15 μM R115777 for 72 h and cell lysates were analyzed by Western blotting for expression of GRP78 and β-actin. **B**, U266 cells were maintained with 50 μM tunicamycin (*lane 1*), 20 μM melphalan (*lane 2*), control media (*lane 3*), or 5 μM R115777 (*lane 4*) for 24 h. U266 Bcl-X_L cells were maintained with either control media (*lane 5*) or 5 μM of R115777 (*lane 6*) for 24 h. Expression of GADD153 and β-actin was determined by Western blotting. **C**, U266 Bcl-X_L cells were maintained with control media or 5–15 μM R115777 for 72 h and cell lysates were analyzed by Western blotting for expression of GRP78 and β-actin. **D**, U266 and U266 Bcl-X_L cells were maintained as in **A** and **C**. Percent cell death was evaluated by annexin-V FITC and PI staining. **A–C** are from two independent experiments. **D** is from three independent experiments.

Bax increased in our myeloma model (Figs. 4B and 5B) and was associated with a loss of mitochondrial membrane potential and release of cytochrome *c* and Smac (Fig. 3); the prototypical outcome of mitochondria-mediated apoptosis (34). Furthermore, death required caspase-9 activity (Fig. 2) which increases on release of these mitochondrial proteins (12). However, U266 cells ectopically expressing Bcl-X_L remained susceptible to R115777 despite their inability to release these proapoptotic molecules (Fig. 6B). These results implied involvement of additional pathways that function independent of the release of mitochondrial proteins.

Another intrinsic pathway that triggers caspase-9 activity is that associated with ER stress which also occurred in our myeloma model (Fig. 7, A and B). ER stress can result in activation of caspase-12 (35) with subsequent activation of caspase-9 and 3 (13). This pathway can proceed independent of the release of mitochondrial proteins (13) exactly as observed in U266 Bcl-X_L cells (Fig. 6B). It has been reported that Bax can localize to the ER (36, 37) where it promotes apoptosis by triggering ER calcium release (38, 39). Elevation of intracellular free calcium can result in opening of the mitochondrial permeability transition pore (40) which may explain the loss of mitochondrial membrane potential seen in the U266 Bcl-X_L line. Calcium also activates calpains that promote apoptosis through proteolytic cleavage of pro-caspase-12 (41). We are currently designing experiments to address alterations in ER calcium stores as well as assessing potential functional analogs of caspase-12 in myeloma lines.

Of particular interest was our observation that ER stress was suppressed in cells ectopically expressing Bcl-X_L (Fig. 7, B and C). Nevertheless, despite suppression of both mitochondrial membrane dysfunction and ER stress, R115777 partially retained its ability to induce apoptosis (Fig. 7D). These results imply cooperation of yet another intrinsic pathway that is Bcl-X_L insensitive. To that end, caspase-2 is a nuclear resident protein (42) involved in nuclear stress the ability of which to induce apoptosis requires caspase-9 activity (43). Nuclear stress can be triggered via inhibition of DNA synthesis which results in caspase-2 activation (44–46). Coincidentally, we found that R115777 arrested cells in the late S phase of the cell cycle with an apparent inability to complete the 2N complement of DNA (Fig. 1A). Another potential link between FTIs and nuclear stress may reside within the nuclear cytostructure because these compounds also inhibit the farnesylation of nuclear lamins (47). Further efforts in our laboratory will attempt to more clearly define nuclear events and the potential role of caspase-2 in FTI-induced apoptosis.

R115777 has shown clinical promise in trials conducted for hematologic disorders including multiple myeloma (8), acute leukemia (48), chronic myeloid leukemia (49, 50), and myelodysplasia (51). Interestingly, a correlation between Ras inactivation and response has not been established using surrogate biological markers (8, 48) nor has there been an association between response and Ras mutation status (48, 49, 51). These clinical observations are supported by our molecular results and imply that other molecules and/or pathways targeted by FTIs execute tumor cell death. Continuing to uncover the mechanisms by which FTIs trigger apoptosis in multiple myeloma may ultimately lead us to more predictable clinical markers or even novel therapeutic targets.

Acknowledgment

We thank William J. Harrington Jr. for his support of this project.

References

1. Kalakonda N, Rothwell DG, Scarffe JH, Norton JD. Detection of N-Ras codon 61 mutations in subpopulations of tumor cells in multiple myeloma at presentation. *Blood*, 2001;98:1555–60.

2. Bezieau S, Devilder MC, Avet-Loiseau H, Mellerin MP, Puthier D, Pennarun E, et al. High incidence of N and K-Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. *Hum Mutat*, 2001;18:212–24.
3. Ogata A, Chauhan D, Teoh G, Treon SP, Urashima M, Schlossman RL, et al. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. *J Immunol*, 1997;159:2212–21.
4. Downward J. Ras signalling and apoptosis. *Curr Opin Genet Dev*, 1998;8:49–54.
5. Beaupre DM, McCafferty-Grad J, Bahlis NJ, Boise LH, Lichtenheld MG. Farnesyl transferase inhibitors enhance death receptor signals and induce apoptosis in multiple myeloma cells. *Leuk Lymphoma*, 2003;44:2123–34.
6. Le Gouill S, Pellat-Deceunynck C, Harousseau JL, Rapp MJ, Robillard N, Bataille R, et al. Farnesyl transferase inhibitor R115777 induces apoptosis of human myeloma cells. *Leukemia*, 2002;16:1664–7.
7. Bolick SC, Landowski TH, Boulware D, Oshiro MM, Ohkanda J, Hamilton AD, et al. The farnesyl transferase inhibitor, FTI-277, inhibits growth and induces apoptosis in drug-resistant myeloma tumor cells. *Leukemia*, 2003;17:451–7.
8. Alsina M, Fonseca R, Wilson EF, Belle AN, Gerbino E, Price-Troska T, et al. The farnesyltransferase inhibitor Zarnestra is well tolerated, induces stabilization of disease and inhibits farnesylation and oncogenic/tumor survival pathways in patients with advanced multiple myeloma. *Blood*, 2004; in press.
9. Grad JM, Bahlis NJ, Reis I, Oshiro MM, Dalton WS, Boise LH. Ascorbic acid enhances arsenic trioxide-induced cytotoxicity in multiple myeloma cells. *Blood*, 2001;98:805–13.
10. Khaled AR, Kim K, Hofmeister R, Muegge K, Durum SK. Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc Natl Acad Sci USA*, 1999;96:14476–81.
11. Bellosillo B, Villamor N, Lopez-Guillermo A, Marce S, Bosch F, Campo E, et al. Spontaneous and drug-induced apoptosis is mediated by conformational changes of Bax and Bak in B-cell chronic lymphocytic leukemia. *Blood*, 2002;100:1810–6.
12. Hengartner MO. The biochemistry of apoptosis. *Nature*, 2000;407:770–6.
13. Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome *c*-independent activation of caspase-9 by caspase-12. *J Biol Chem*, 2002;277:34287–94.
14. Harada N, Hata H, Yoshida M, Soniki T, Nagasaki A, Kuribayashi N, et al. Expression of Bcl-2 family of proteins in fresh myeloma cells. *Leukemia*, 1998;12:1817–20.
15. Miguel-Garcia A, Orero T, Matutes E, Carbonell F, Miguel-Sosa A, Linares M, et al. bcl-2 expression in plasma cells from neoplastic gammopathies and reactive plasmacytosis: a comparative study. *Haematologica*, 1998;83:298–304.
16. Pettersson M, Jernberg-Wiklund H, Larsson LG, Sundstrom C, Givol I, Tsujimoto Y, et al. Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. *Blood*, 1992;79:495–502.
17. Egle A, Villunger A, Marschitz I, Kos M, Hittmair A, Lukas P, et al. Expression of Apo-1/Fas (CD95), Bcl-2, Bax and Bcl-x in myeloma cell lines: relationship between responsiveness to anti-Fas mab and p53 functional status. *Br J Haematol*, 1997;97:418–28.
18. Zhang B, Gojo I, Fenton RG. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. *Blood*, 2002;99:1885–93.
19. Zhang B, Potyagaylo V, Fenton RG. IL-6-independent expression of Mcl-1 in human multiple myeloma. *Oncogene*, 2003;22:1848–59.
20. Spets H, Stromberg T, Georgii-Hemming P, Siljason J, Nilsson K, Jernberg-Wiklund H. Expression of the bcl-2 family of pro- and anti-apoptotic genes in multiple myeloma and normal plasma cells: regulation during interleukin-6 (IL-6)-induced growth and survival. *Eur J Haematol*, 2002;69:76–89.
21. Wei MC, Zong WX, Cheng EH, Lindsen T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, 2001;292:727–30.
22. Suzuki N, Urano J, Tamanoi F. Farnesyltransferase inhibitors induce cytochrome *c* release and caspase 3 activation preferentially in transformed cells. *Proc Natl Acad Sci USA*, 1998;95:15356–61.
23. Hsu YT, Wolter KG, Youle RJ. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci USA*, 1997;94:3668–72.
24. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev*, 1999;13:1211–33.
25. Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci*, 2001;26:504–10.
26. Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, et al. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol*, 1996;16:4273–80.
27. Neri A, Murphy JP, Cro L, Ferrero D, Tarella C, Baldini L, et al. Ras oncogene mutation in multiple myeloma. *J Exp Med*, 1989;170:1715–25.
28. Liu P, Leong T, Quam L, Billadeau D, Kay NE, Greipp P, et al. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. *Blood*, 1996;88:2699–706.
29. Portier M, Moles JP, Mazars GR, Jeanteur P, Bataille R, Klein B, et al. Ras gene mutations in multiple myeloma. *Oncogene*, 1992;7:2539–43.
30. Shi Y, Gera J, Hsu JH, Van Ness B, Lichtenstein A. Cyto-reductive effects of farnesyl transferase inhibitors on multiple myeloma tumor cells. *Mol Cancer Ther*, 2003;2:563–72.
31. End DW, Smets G, Todd AV, Applegate TL, Fuery CJ, Angibaud P, et al. Characterization of the antitumor effects of the selective farnesyl protein transferase inhibitor R115777 *in vivo* and *in vitro*. *Cancer Res*, 2001;61:131–7.
32. Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, et al. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J Biol Chem*, 1997;272:14459–64.
33. Zhang FL, Kirschmeier P, Carr D, James L, Bond RW, Wang L, et al. Characterization of Ha-ras, N-ras, Ki-Ras4A, and Ki-Ras4B as *in vitro* substrates for farnesyl protein transferase and geranylgeranyl protein transferase type I. *J Biol Chem*, 1997;272:10232–9.
34. Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. *Nat Cell Biol*, 2001;3:E255–63.
35. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- β . *Nature*, 2000;403:98–103.
36. Gajkowska B, Motyl T, Olszewska-Badarczuk H, Godlewski MM. Expression of BAX in cell nucleus after experimentally induced apoptosis revealed by immunogold and embedment-free electron microscopy. *Cell Biol Int*, 2001;25:725–33.
37. Godlewski MM, Motyl MA, Gajkowska B, Wareski P, Koronkiewicz M, Motyl T. Subcellular redistribution of BAX during apoptosis induced by anticancer drugs. *Anticancer Drugs*, 2001;12:607–17.
38. Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, et al. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca²⁺ stores. *J Biol Chem*, 2002;277:9219–25.
39. Pan Z, Bhat MB, Nieminen AL, Ma J. Synergistic movements of Ca(2+) and Bax in cells undergoing apoptosis. *J Biol Chem*, 2001;276:32257–63.
40. Ferrari D, Pinton P, Szabadkai G, Chami M, Campanella M, Pozzan T, et al. Endoplasmic reticulum, Bcl-2 and Ca(2+) handling in apoptosis. *Cell Calcium*, 2002;32:413–20.
41. Nakagawa T, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol*, 2000;150:887–94.
42. Paroni G, Henderson C, Schneider C, Brancolini C. Caspase-2 can trigger cytochrome *c* release and apoptosis from the nucleus. *J Biol Chem*, 2002;277:15147–61.
43. Paroni G, Henderson C, Schneider C, Brancolini C. Caspase-2-induced apoptosis is dependent on caspase-9, but its processing during UV- or tumor necrosis factor-dependent cell death requires caspase-3. *J Biol Chem*, 2001;276:21907–15.
44. Robertson JD, Enoksson M, Suomela M, Zhivotovsky B, Orrenius S. Caspase-2 acts upstream of mitochondria to promote cytochrome *c* release during etoposide-induced apoptosis. *J Biol Chem*, 2002;277:29803–9.
45. Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Distinct pathways for stimulation of cytochrome *c* release by etoposide. *J Biol Chem*, 2000;275:32438–43.
46. Lässig P, Opitz-Araya X, Lazebnik Y. Requirement for caspase-2 in

stress-induced apoptosis before mitochondrial permeabilization. *Science*, 2002;297:1352–4.

47. Cox AD, Der CJ. Farnesyltransferase inhibitors and cancer treatment: targeting simply Ras? *Biochim Biophys Acta*, 1997;1333:F51–71.

48. Karp JE, Lancet JE, Kaufmann SH, End DW, Wright JJ, Bol K, et al. Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase 1 clinical-laboratory correlative trial. *Blood*, 2001;97:3361–9.

49. Cortes J, Albitar M, Thomas D, Giles F, Kurzrock R, Thibault A,

et al. Efficacy of the farnesyl transferase inhibitor R115777 in chronic myeloid leukemia and other hematologic malignancies. *Blood*, 2003; 101:1692–7.

50. Keating A. Chronic myeloid leukemia: current therapies and the potential role of farnesyltransferase inhibitors. *Semin Hematol*, 2002;39: 11–7.

51. Kurzrock R, Cortes J, Kantarjian H. Clinical development of farnesyltransferase inhibitors in leukemias and myelodysplastic syndrome. *Semin Hematol*, 2002;39:20–4.

Molecular Cancer Therapeutics

R115777 induces Ras-independent apoptosis of myeloma cells via multiple intrinsic pathways

Darrin M. Beaupre, Enrique Cepero, Esther A. Obeng, et al.

Mol Cancer Ther 2004;3:179-186.

Updated version Access the most recent version of this article at:
<http://mct.aacrjournals.org/content/3/2/179>

Cited articles This article cites 50 articles, 29 of which you can access for free at:
<http://mct.aacrjournals.org/content/3/2/179.full#ref-list-1>

Citing articles This article has been cited by 13 HighWire-hosted articles. Access the articles at:
<http://mct.aacrjournals.org/content/3/2/179.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://mct.aacrjournals.org/content/3/2/179>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.