

HDJ-2 as a Target for Radiosensitization of Glioblastoma Multiforme Cells by the Farnesyltransferase Inhibitor R115777 and the Role of the p53/p21 Pathway

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Abstract

Resistance of glioblastoma multiforme to radiotherapy poses a major clinical challenge. Farnesyltransferase inhibitors (FTI), such as R115777, have potential to increase radiotherapeutic benefit in this disease, although their mechanism of action is unclear. In our study with eight glioblastoma multiforme cell lines, the most sensitive ones underwent cell cycle arrest in response to FTI treatment. Radiosensitization by FTIs, however, seemed to involve other pathways. If R115777 treatment was initiated <6 hours before irradiation, all eight glioblastoma multiforme lines were radiosensitized. However, if the time between drug and radiation was extended to 24 hours, cells harboring wild type but not mutated p53 were able to counteract drug-induced radiosensitization. The involvement of the p53/p21 pathway in the development of resistance was confirmed by showing that U87 cells transfected with human papillomavirus E6 to block p53 or interfering RNA to inhibit p21 stayed radiosensitive for 24 hours after drug treatment. The time dependency of R115777-induced radiosensitization suggested that the initial FTI target for early radiosensitization was short-lived, and that a p21-directed pathway restored resistance. Consideration of prenylated molecules that could potentially be involved led us to consider HDJ-2, a co-chaperone of heat shock protein 70. This hypothesis was strengthened by finding that cellular radiosensitivity was increased by genetic inhibition of HDJ-2, whereas overexpression conferred radioresistance. Importantly, irradiation of cells caused HDJ-2 to migrate from the cytoplasm to the nucleus, and this migration was inhibited by prior FTI treatment. These results have clinical relevance in that they help explain the variability in responses to FTIs that occurs following radiotherapy and elucidate some of the reasons for the complexity underlying FTI-induced radiosensitization. (Cancer Res 2006; 66(13): 6756-62)

Introduction

Glioblastoma multiforme is the most common primary malignant brain tumor in adults and has dismal outcomes. Its formation and progression has been associated with upstream lesions that drive chronic activation of key intracellular signaling pathways,

such as phosphatidylinositol 3-kinase and Ras/mitogen-activated protein kinase (1). These same pathways may also cause intrinsic cellular resistance of glioblastoma multiforme to radiation therapy, its prime treatment modality. These dysregulated pathways are therefore potential targets through which tumor aggression might be moderated and radiotherapeutic benefit might be increased.

Farnesyltransferase inhibitors (FTI) were introduced in preclinical and clinical trials to target aberrant Ras in the hope of interfering with signaling of tumor cell proliferation, invasion, and angiogenesis (2). Addition of an isoprenyl (farnesyl or geranylgeranyl) lipid group to the cysteine residue in the COOH-terminal CAAX box of proteins like Ras confers on them a membrane address required for correct function (3, 4). Clinical trials of R115777 (Tipifarnib, Zarnestra), a nonpeptidomimetic FTI, gave promising results in hematologic malignancies (5, 6), but phase II and III studies in patients with lung cancer, colorectal cancer, or pancreatic cancer did not convincingly show single-agent efficacy (7–10). This may be because FTIs are more cytostatic than cytotoxic (11), and because of this, several preclinical and clinical trials combined them with radiation therapy with some success (12–14).

Remarkably, the cellular target of FTIs remains controversial and may differ depending on whether radiosensitization or growth inhibition is the end point. It is unlikely that Ras is the sole or prime target for growth inhibition. R115777 inhibits farnesylation of K-Ras4B peptides with an IC₅₀ value of 7.9 nmol/L (15), but N-Ras, K-Ras4A, and K-Ras4B can also be geranylgeranylated by geranylgeranyltransferase to allow membrane attachment and function (16). Similarly, whereas early studies suggested that FTIs radiosensitize cells with activated but not wild type or a K-Ras mutation (17), R115777 was recently shown to radiosensitize glioblastoma multiforme cells with wild-type Ras by a Ras-independent pathway (18). The number of farnesyltransferase substrates and the number of proteins that have been shown to contain CAAX motifs continue to grow, expanding the pool of potential FTI targets. RhoB, Rheb, IP (prostacyclin receptor), and CENP have been proposed as targets (19–21), but there is no consensus as to their involvement in tumor cell growth inhibition or radiosensitization.

Our studies indicate that R115777 can radiosensitize glioma cell lines, but this is counteracted over time by a p53/p21-dependent pathway in cells with wild-type p53. Time course observations suggested that the target for FTI radiosensitization is short-lived. Examination of the range of potential FTI targets led us to hypothesize that HDJ-2 (homologue of DNAJ-2), which is a human DNAJ member of the heat shock protein 40 (HSP40) family, may be involved, and this study was established to investigate this possibility.

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Materials and Methods

Cell lines. Eight cell lines representing general characteristics of glioblastoma multiforme tumors in patients except for the status of epidermal growth factor receptor (EGFR), which is known to be modulated *in vitro* (22), were used in the study. U87, A172, and T98 glioma cell lines were purchased from the American Type Culture Collection (Manassas, VA). U251, U343, SF188, SF763, and SF767 were obtained from University of California San Francisco/Neurosurgery Tissue Bank courtesy of Dr. D. Deen. The cells were cultured in DMEM (Mediatech, Herndon, VA) supplemented with 10% FCS and 1% antibiotic and antimycotic solution in a humidified atmosphere containing 5% CO₂ at 37°C. All cell lines were characterized for EGFR, MDM2, p53, cyclin-dependent kinase 4 (CDK4), cyclin D1, and RB expression by Western blot (Fig. 1). Genetic data were obtained from the literature (23), with additional verification of p53 status by sequencing exons 5 to 8. EGFR expression was high for the U343 and SF763 cell lines, although this was only about 25% of that in A431 (data not shown). With the exception of SF188, cell lines with p53 mutation (T98, SF188, U251, and SF763) had high protein levels of p53. Two cell lines (U343 and SF767) with wild-type p53 also had high p53 expression, as has been reported in several types of cancers, including gliomas (24). Interestingly, the two cell lines with wild-type p16 (SF188 and SF767) also had very high levels of CDK4 expression, indicating genetic alterations in the p16/cyclin D/CDK4 pathway.

Materials. R115777 [(B)-6-[amino(4-chlorophenyl)(1-methyl-1H-imidazo-5-yl)-methyl]-4-(3-chlorophenyl)-1-methyl-2(1H)-quinolinone; Johnson and Johnson, Spring House, PA] was stored as a 2 mmol/L stock solution in DMSO at room temperature. All other analytic grade reagents were purchased from Sigma (St. Louis, MO) or Fisher (Springfield, NJ). Monoclonal antibodies against human HDJ-2, p21, MDM2, Rb, EGFR, and actin were obtained from Neomarkers (Union City, CA). Polyclonal antibodies against human CDK4 and cyclin D were purchased from Cell Signaling Technology (Beverly, MA) and against human p53 from Santa Cruz Biotechnology (Santa Cruz, CA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and clonogenic assays. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

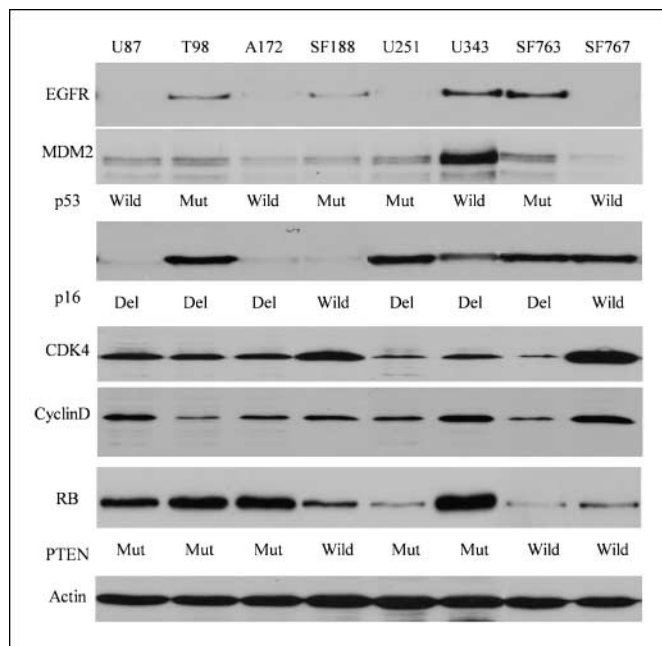


Figure 1. Characteristics of the eight glioblastoma multiforme cell lines. Protein levels of EGFR, MDM2, p53, CDK4, cyclin D, and RB were examined by Western blots. Actin was used as an endogenous standard to ensure equal loading. Genetic data were derived from literature.

bromide (MTT) assay determined the effect of R115777 on the growth of each cell line. Cells were plated in a 96-well plate, incubated overnight, and then treated with various concentrations (0.5-500 nmol/L) of R115777. After 6 days, the surviving cells were stained with 200 µg/mL MTT for 4 hours at 37°C. The resulting crystals were dissolved in DMSO, and the absorbance was read at 590 nm. The IC₅₀ (dose that inhibited growth to 50% of controls) was determined by a four-variable logistic regression function (SigmaPlot, Systat Software, Inc., Richmond, CA). IC₅₀ values were also determined using clonogenic cell survival as an end point, as was drug radiosensitization. For the former, cells were incubated in the continuous presence of the drug, whereas for the latter, the drug was added for varying periods of time before irradiation, and cells were washed before plating. Irradiation was delivered by a MARK-I irradiator (Cs-137 source, J.L. Shepherd, San Fernando, CA) at a dose rate of ~4.5 Gy/min. Cells in logarithmic growth phase were harvested and treated in suspension before seeding in 100-mm plates at concentrations predetermined to give 25 to 200 colonies at each dose. Ten to 14 days later, the cultures were fixed and stained with 0.5% crystal violet in methanol. Colonies containing >50 cells were counted. IC₅₀ values were derived for drug-only treatment, and radiosensitivity was quantified by the surviving fraction = (number of colonies formed) / (number of cells plated × plating efficiency). Clonogenic survival curves were plotted after normalization for cytotoxicity caused by R115777 alone with data pooled from at least two independent experiments.

Cell cycle analysis by flow cytometry. For cell cycle analysis, cells were treated with R115777 for 24 hours, washed, trypsinized, and fixed with ice-cold ethanol at -20°C overnight. After washing thrice with PBS, they were resuspended in Isoton II, treated with RNase A (0.5 mg/mL) for 15 minutes, and stained with propidium iodide (0.05 mg/mL) for 30 minutes at room temperature. Fluorescence was assessed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cells were gated by forward and side scatter and a secondary gate set to rule out doublet cells using FL-2 pulse area versus FL-2 pulse width (pulse processing). Data were analyzed by ModFit version 3.1 (Verity Software House, Inc., Topsham, ME).

Expression plasmids. Human HDJ-2 cDNA was amplified from an RNA extract of SF763 cells using QIAGEN One-step Reverse Transcription-PCR kit (Qiagen, Valencia, CA) with forward 5'-GCCACCATGGTGAAGAAA-CAACTACT-3' and reverse 5'-TGGCCATTAAGAGGTCTGACA-3' primers. The amplified cDNA of HDJ-2 was verified by sequencing and subcloned into pcDNA 3.1 V5 His-TOPO (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Cells were transfected with vectors using Superfectant (Qiagen). The construction of U87 cells transfected with human papilloma virus (HPV) E6 (U87-E6) was done as reported before (25).

RNA interference. Two sets of primers to interfere with the expression of HDJ-2 and p21 were designed using pSilencer 3.1-H1 hygro (Ambion, Austin, TX). pSilencer vector produces a hairpin small interfering RNA (siRNA) that induce RNA interference of the target gene. The hairpin siRNA inserts for HDJ-2 were 5'-GATCCGAAAGGAGCAGTAGAGTGCTTCAAGAGAGCACTCTACTGCTCCTTTCTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAA-GAAAGGAGCAGTAGAGTGCTCTCTTGAAGCACTCTACTGCTCCTTTTCG-3'. For p21, they were 5'-GATCCGCTTCGACTTTGTCACCGAGTTCAGAGACTCGGTGACAAAAGTCGAAGTTTTTTGGAAA-3' and 5'-AGCTTTTC-CAAAAAACTTCGACTTTGTCAACCGAGTCTCTTGAACCTCGGTGACAAAGTCGAAGCG-3'. The manufacturer provided the negative control vector with a scramble sequence.

Western blotting. Treated cells were washed in ice cold PBS and lysed on ice in M-Per buffer (Pierce, Rockford, IL) with protease inhibitor cocktail (Complete, Roche, Indianapolis, IN) for 20 minutes. Extracts were clarified by centrifugation at 13,000 rpm for 20 minutes at 4°C. Protein concentration was determined by micro-BCA (Pierce). The lysates were boiled for 5 minutes in the presence of SDS sample buffer, resolved by 10% (for HDJ2, p53, and other proteins) or 15% (for p21) SDS-PAGE, and electrophoretically transferred to polyvinylidene fluoride membranes (Immun-Blot Polyvinylidene Difluoride, Bio-Rad, Hercules, CA). The membranes were incubated with blocking buffer [5% nonfat dry milk, TBS-T (TBS with 0.1% Tween 20)] for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG)

or anti-mouse IgG antibody (Pierce). Finally, the reaction was visualized with a SuperSignal chemiluminescence Western blotting kit and CLXposure films (Pierce).

Confocal microscopy and laser scanning cytometry. To determine intracellular localization of HDJ-2, 10^4 SF763 cells per well were cultured on eight-well chamber slides (Lab-Tek, Naperville, IL), fixed in 4% formaldehyde for 15 minutes, permeabilized in 0.2% Triton X-100/PBS for 10 minutes, blocked with 10% goat serum for 1 hour at room temperature, and anti-HDJ-2 antibody was added for 30 minutes. After washing with PBS, cells were immunostained with FITC-conjugated anti-mouse IgG (Pharmingen, San Diego, CA), mounted with Prolong Gold Antifade Reagents with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen), and HDJ-2 visualized using confocal laser microscopy (Leica TCSSP, courtesy of Carol Moss, Spivak Cell Imaging Facility, University of California at Los Angeles). To quantify the signal, the cells were rescanned by laser scanning cytometry (CompuCyte, Cambridge, MA) and analyzed by WinCyte version 3.6. The contour of a nucleus was defined by DAPI staining. The signal intensity of the same area for HDJ-2 was measured and recorded. The integral value represents the sum of the fluorescent values for each pixel in the contour.

Results

Sensitivity of glioblastoma multiforme cell lines to R115777.

As a prelude to further studies and to allow comparison with published data, the sensitivity of the eight glioblastoma multiforme cell lines to the continuous presence of R115777 was determined by both MTT and clonogenic assays. The IC_{50} values are depicted in Table 1. The results were similar for both assays. The major discrepancy was with A172, which has a slow growth rate and is relatively drug resistant. U87, T98, U343, SF763, and SF767 were sensitive with IC_{50} values of <10 nmol/L. A172 (39.5 nmol/L) and SF188 (17.6 nmol/L) were of intermediate sensitivity, as judged primarily by clonogenic assays. U251 was most resistant. This, and other pilot data, formed the basis for our decision to use treatment for ≤ 1 day with a dose of 10 nmol/L R115777 as a standard protocol for radiation experiments. For most cell lines, this effectively inhibits farnesylation with modest effects on cell growth and clonogenicity.

Effect of R115777 on cellular radiosensitivity. The ability of 24-hour treatment with R115777 to sensitize glioblastoma multiforme cells to a radiation dose of 2 Gy was tested by clonogenic cell survival assay (Fig. 2A). U87, A172, U343, and SF767 cells were not radiosensitized, whereas treatment of T98, SF188, U251, and SF763 with R115777 reproducibly reduced the fraction surviving 2 Gy (SF2Gy) by about 15%. Radiosensitization was independent of the IC_{50} values for the cell lines. However, examination of their

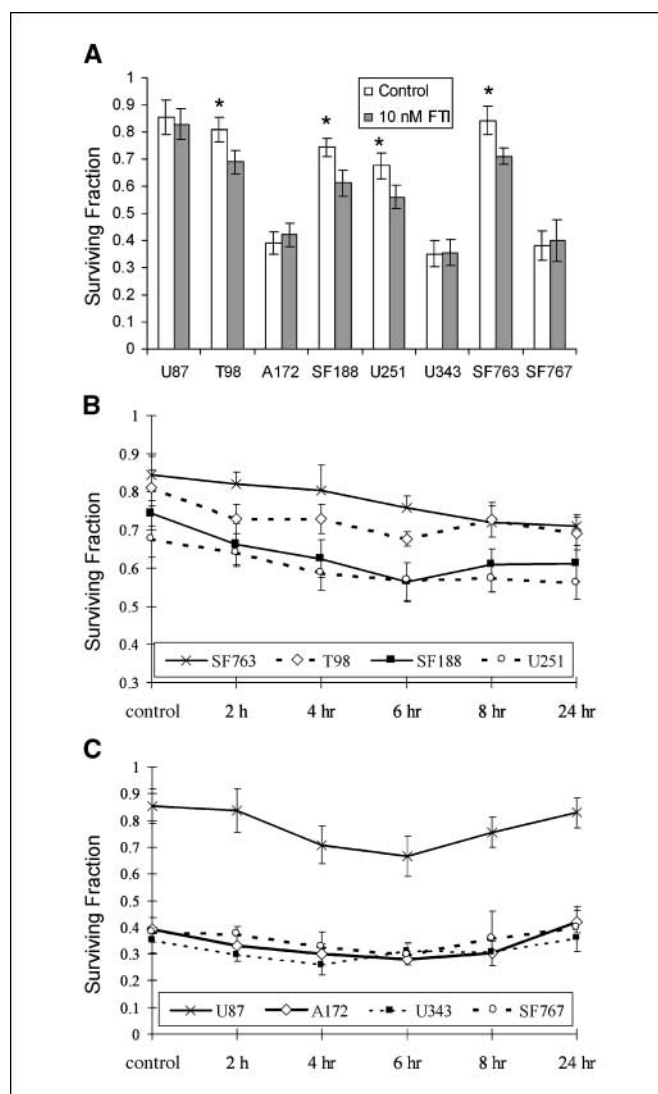


Figure 2. A, effect of vehicle (open columns) or 10 nmol/L R115777 (gray columns) treatment of glioblastoma multiforme cells for 24 hours on the clonogenic response to 2 Gy irradiation. Tested cells were seeded into 10-cm plates and incubated for 12 to 14 days. Importance of time of treatment by R115777 in inducing radiosensitization in (B) p53 mutant cells and (C) p53 wild-type cells. The SF2 value of each cell line was determined following exposure of the cells to 10 nmol/L R115777 for 0, 2, 4, 6, 8, and 24 hours. Bars, SE. *, $P < 0.05$, significant difference (Student's *t* test).

molecular characteristics (Fig. 1) showed that 24 hours treatment with R115777 radiosensitized only those glioblastoma multiforme cell lines that had mutant p53.

FTIs cause cell cycle redistribution (26), which could be influenced by p53 status. Because cellular radiosensitivity varies markedly with cell cycle phase, we asked whether cells that showed FTI-induced radiosensitivity had redistributed into G_2 -M at the time of irradiation. The results of three independent experiments are summarized in Table 1. U343 and SF767 responded to R115777 by accumulating in the G_1 phase with a concurrent decrease in S-phase cells. U87, T98, and SF763 accumulated in G_2 -M phase. A172, SF188, and U251 were unaffected by R115777 treatment. It can be concluded that cell lines that showed no cell cycle arrest were most resistant to growth inhibition by R115777, but there was no correlation between drug-induced cell cycle arrest and

Table 1. Cell cycle arrest and growth inhibition by R115777 for each glioblastoma multiforme cell line

	Cell cycle effect	IC_{50} by MTT assays (nmol/L)	IC_{50} by clonogenic assays (nmol/L)
U87	G_2 -M arrest	3.1	3.8
T98	G_2 -M arrest	4.3	2.5
A172	No effect	>500	39.5
SF188	No effect	15.9	17.6
U251	No effect	>500	101
U343	G_1 arrest	4.6	8.3
SF763	G_2 -M arrest	1.9	1.0
SF767	G_1 arrest	5.0	6.8

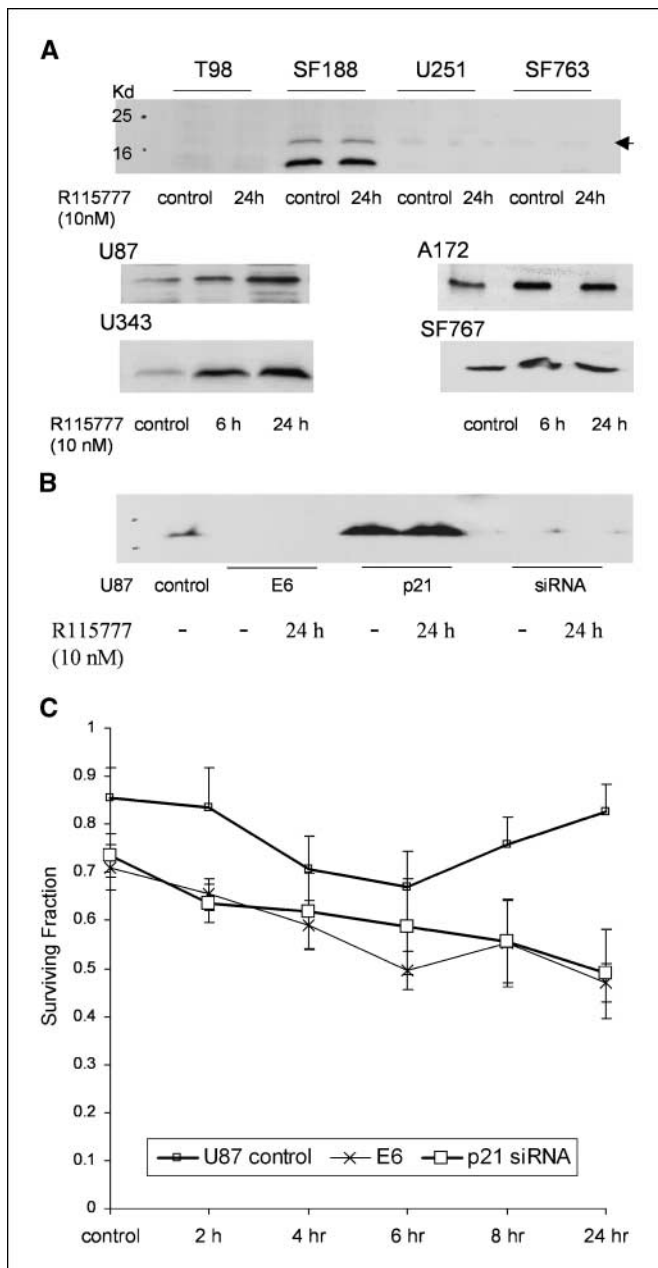


Figure 3. A, Western blot analysis of p21 protein. The cells were plated in 60-mm plates and treated with R115777. Total protein extract of the cells was obtained, and Western blot analysis was done as described in Materials and Methods. B, U87 cells transfected with HPV E6 (E6) and p21 siRNA. "p21" is a U87 cell line overexpressing p21, as an internal control. C, effect of p21 siRNA and E6 expression on radiosensitization. The SF2 value of each cell line was determined following exposure of the cells to 10 nmol/L R115777 for 0, 2, 4, 6, 8, and 24 hours.

radiosensitization by R115777. For example, only two of the three cell lines undergoing G₂-M arrest after 24 hours of FTI treatment were radiosensitized. Another two cell lines (SF188 and U251) were radiosensitized by R115777 and showed no cell cycle arrest.

p53- and p21-directed pathways and R115777-induced radiosensitization. p53-dependent R115777-induced radiosensitization could be caused by any of multiple pathways but through activation of p21 expression seemed a likely possibility (27) because glioblastoma multiforme cells with wild type, but not mutant, p53

up-regulated p21 following 10 nmol/L R115777 treatment (Fig. 3A). The role of the p53/p21 pathway in resistance to R115777-induced radiosensitization was explored in U87 cells by suppressing p21 expression using RNA interference and p53 using HPV E6. In stable transfectants, the basal level of p21 was decreased by both treatments, and R115777 could not induce p21 (Fig. 3B).

Because p53/p21 responses are time dependent, time course radiosensitization experiments were done. Cells were exposed to 10 nmol/L of R115777 for 0, 2, 4, 6, 8, and 24 hours before 2 Gy irradiation and clonogenic assays were done (Fig. 2B and C). Surprisingly, all eight glioblastoma multiforme cell lines showed increased radiosensitivity after 4 to 6 hours FTI treatment, whereas after 24 hours, only the four cell lines with mutant p53 were still radiosensitive, as before. Furthermore, U87 cells that were unable to express p21 after R115777 treatment for 24 hours had decreased survival (Fig. 3C). These results imply that R115777 initially

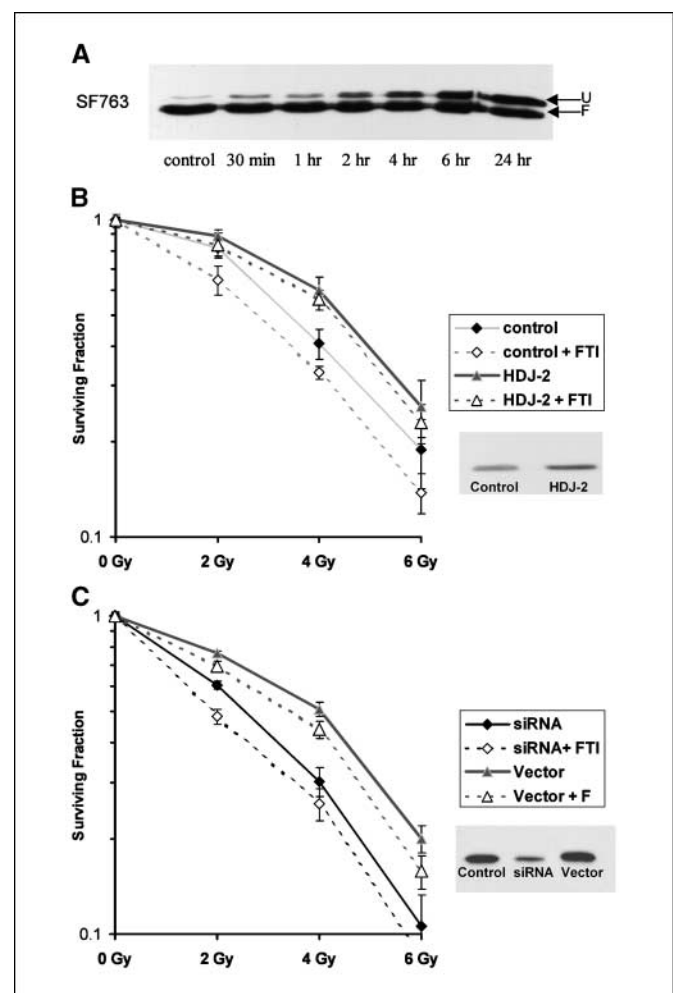


Figure 4. A, time course of inhibition of farnesylation by R115777. SF763 cells were treated with 10 nmol/L R115777 for the indicated times. Inhibition of farnesylation was shown by a band shift to a higher apparent molecular weight. Arrows indicate the position of unprocessed (U) and farnesylated (F) HDJ-2. B, effect of HDJ-2 level on radiosensitivity. The expression of HDJ-2 in HDJ-2 (HDJ) and HDJ-2 siRNA (HDJ siRNA) transfected SF763 cells as assessed by Western blots. Cells were pretreated with 10 nmol/L R115777 or with a carrier (DMSO) before plating for clonogenic survival determination. Dishes were irradiated with 2 to 6 Gy as indicated and cultured with drug-free medium and allowed to grow for additional 12 days before staining for colony formation. Bar, SE.

radiosensitizes glioma cells but that a p53/p21 pathway is induced in cells with wild-type p53 that subsequently protects against irradiation. They also suggest that a farnesylated protein with a short half-life is involved in FTI-mediated radiosensitization of glioblastoma multiforme cell lines.

HDJ-2 is involved in R115777-induced radiosensitization.

Consideration of the range of short-lived prenylated proteins that might interact with p53 pathways led us to hypothesize that the heat shock protein HDJ-2, which is prenylated exclusively by farnesyltransferase (28), might be involved in R115777-mediated radiosensitization. As shown in Fig. 4A, R115777 inhibited HDJ-2 farnesylation in SF763 cells after 1 hour of treatment. After 6 hours, almost half HDJ-2 was unfarnesylated. Other cell lines showed the same results (data not shown). This confirmed that farnesylated HDJ-2 has a half-life in keeping with the time course of FTI-induced radiosensitization. To investigate further the role of HDJ-2 in R115777-induced radiosensitization, its cellular levels were genetically altered in SF763 cells. Stable overexpression of HDJ-2 (Fig. 4B) increased resistance to irradiation, even after treatment with R115777. Furthermore, HDJ-2 siRNA, which suppressed HDJ-2 expression, increased cellular radiosensitivity to levels similar to control cells treated with R115777 (Fig. 4B). These results indicate

that the cellular level of HDJ-2 determines intrinsic cellular radiosensitivity, and that HDJ-2 may be a target for FTI-induced radiosensitization.

Localization of HDJ-2 after radiation. Because HDJ-2 is a chaperone protein that has been reported to redistribute from cytoplasm to the Golgi complex, nuclear membrane, and nucleus after heat shock treatment (29), we examined if radiation causes a similar redistribution and whether R115777 affected its relocation. Immunofluorescence with confocal microscopy was used to identify HDJ-2 in SF763 cells 24 hours after treatment with 0 or 10 Gy of radiation (Fig. 5A). In controls, most HDJ-2 was in the cytoplasm. Irradiation caused nuclear translocation, and this was inhibited by prior treatment with 10 nmol/L R115777. Laser scanning cytometry confirmed that HDJ-2 in the nucleus as defined by DAPI staining (Fig. 5B) increased after irradiation, and this was blocked by prior FTI treatment.

Discussion

Treatment of cells with FTIs, like R115777, has many effects on cellular behavior, including growth inhibition, cell cycle arrest, and radiosensitization. Importantly, these responses are not found with

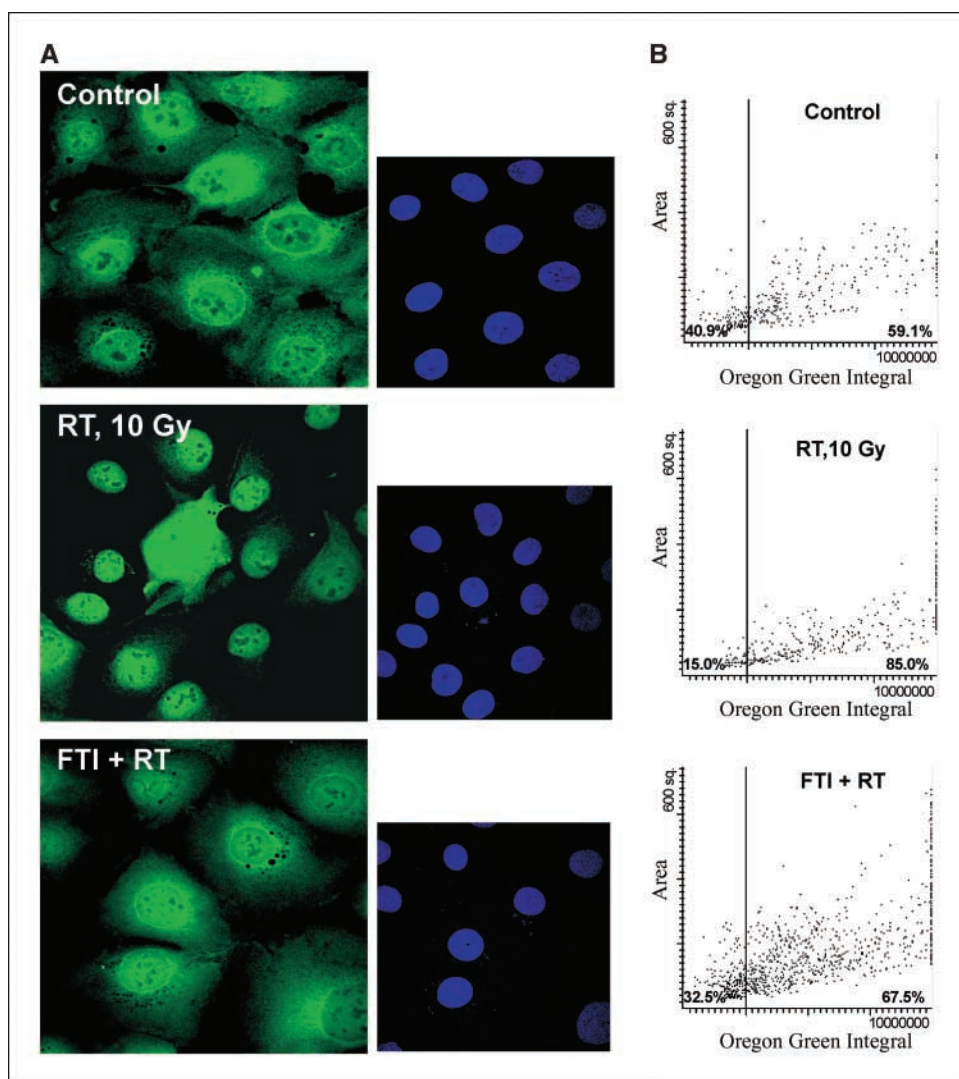


Figure 5. Redistribution of HDJ-2 after irradiation. A, immunofluorescent staining of HDJ-2 after irradiation and R115777 treatment. SF763 cells were incubated with 10 nmol/L R115777 for 24 hours (FTI) or irradiated for 10 Gy (RT). After fixation, cells were stained with the anti-HDJ-2 antibody, and a FITC-conjugated goat anti-mouse IgG. DNA was visualized with DAPI stain (right). B, laser scanning cytometry study of the same slides in (A).

every cell line, and this variability is a source of considerable concern because it may underlie the variable response to FTIs observed in the clinic (30). In agreement with Ashar et al., who showed that tumor cells sensitive to the FTI SCH66336 underwent G₁ or G₂-M cell cycle arrest (31), we found that those glioblastoma multiforme cell lines that were most sensitive to R115777 underwent G₂-M (U87, T98, and SG763) or G₁ (U343 and SF767) arrest. The three cell lines that did not arrest (A172, SF188, and U251) were relatively resistant to R115777.

Although the pathways leading to cell cycle arrest are multiple, p53-induced expression of p21, which is a universal inhibitor of CDK-cyclin complexes, is a mechanistic pathway commonly activated by many signals, including FTIs. For example, L-744,832 caused p53-dependent p21 expression in HCT116 and MCF-7 cells (32), and disruption of this pathway by HPV E6 suppressed p21 induction and the G₁ block. In our study, two of the glioblastoma multiforme cell lines with wild-type p53, U343, and SF767 produced p21 in response to R115777 and had G₁ arrest. In a third line with wild-type p53 (U87), R115777 activated p21 and caused G₂-M block, as has been observed in HCT116 and NCI-H460 cells treated with the FTI SCH66336 (31). Furthermore, T98 and SF763 cells that have mutant p53 and do not induce p21 still undergo G₂-M arrest in response to R115777. Other cells with mutant p53 have also been reported to accumulate in G₂-M phase after FTI treatment (21, 33).

Whereas cell cycle arrest after FTI treatment seemed to be important for sensitivity to the drug in terms of growth inhibition, it was not for radiosensitivity, although 24 hours of R115777 treatment radiosensitized only cells with mutated p53 status. The possibility that FTIs radiosensitized through redistribution of cells into a more radiosensitive phase of the cell cycle was excluded as a universal mechanism in our study, which is consistent with an early report by Bernhard et al. (12). This also implies that the mechanism of cell cycle arrest and radiosensitization by FTIs may involve different farnesylated molecules. Interestingly, time course experiments showed that R115777 radiosensitized all glioblastoma multiforme lines provided irradiation was given <6 hours after drug treatment, but that cells with wild-type p53 overcame this sensitization as the time between treatments was extended. The p53/p21 pathway was implicated in the development of the resistant state by targeting p53/p21 with HPV E6 or p21 with siRNA.

The concept that p21 can cause growth arrest that allows repair of DNA damage is well established. However, the role of p21 in radioresistance may extend beyond this and act through other functions of p21, such as in regulation of nuclear import, transcriptional activation, or regulation of apoptosis (34). For example, Izumaru et al. showed that conditionally expressed p21 rescued HeLa cells from radiation-induced apoptosis (35). Murrison-induced p21 expression also protected H1299 lung cancer cells against the irradiation and doxorubicin (27), and several studies have shown that p21-deficient tumors are more sensitive to radiation (36, 37). These results imply that FTI-induced p21 can increase resistance to radiation with time, and that R115777 might have a superior therapeutic benefit in glioblastoma multiforme with mutant p53 as surrounding normal tissues may activate p53/p21-mediated resistance.

The nature of the molecular target of FTI that is involved in initial radiosensitization is still debated. Ras was implicated by several studies (12, 14, 15). However, the kinetics of early FTI radiosensitization outpaces those for elimination of Ras, which has a half-life of about 24 hours (12), suggesting that Ras is not the sole

and perhaps not even the most important target. RhoB has been suggested as a possible alternative (18). RhoB has a short-half life and is rapidly up-regulated by various growth and stress stimuli (38, 39). However, it can be both geranylgeranylated (RhoB-GG) and farnesylated (RhoB-F; ref. 40), and whereas FTIs inhibit RhoB-F, they increase RhoB-GG (41). One report showed that a dominant-negative RhoB increased radiosensitivity of U87 cells (18), but this would block both RhoB-F and RhoB-GG, which would not happen with FTI treatment. These studies, therefore, only implicate RhoB in a cellular radioresistance mechanism that may not be related to FTI sensitization.

We studied the possible contribution of the short-lived farnesylated protein HDJ-2 in cellular radiosensitivity by genetically modulating its expression levels in SF763 cells. Cells overexpressing HDJ-2 survived irradiation better while suppressing expression by siRNA made SF763 cells more radiosensitive. HDJ-2 is readily farnesylated and is frequently used as an index of FTI efficacy (42). As a member of the HSP40 family of proteins that co-chaperone HSP70, HDJ-2 is involved in protein folding and mitochondrial protein import (43). It has been shown to block apoptosis, possibly by interfering with the trafficking of Bax from the cytosol to mitochondria (44). On the other hand, it accumulates within the nucleus in several neurodegenerative diseases and migrates there after heat shock that may be important for its cytoprotective role (29, 45). Our observation that HDJ-2 migrates to the nucleus following irradiation, and that R115777 prevents this may therefore be linked to rapid radiosensitization of glioblastoma multiforme cells by this drug.

The role of HSPs in the cellular response to radiation therapy remains ambiguous, most likely because multiple molecular pathways are involved. However, HSP70 is induced by γ -radiation (46) and has recently been shown in several studies to protect cells against radiation exposure (47, 48). Although the mechanism is unclear, HSP70 has p53 as a client protein (49) and has been postulated to play a role in the induction of DNA damage repair (50, 51). The p53/p21 dependency of FTI radioresistance found in this study has yet to be shown to have anything to do with HSP70-HDJ-2 interactions, but it is an interesting hypothesis that will be pursued. In any event, given the link between HSP70 and its co-chaperones with survival after irradiation, the fact that the co-chaperone HDJ-2 is a short-lived farnesylated HSP, and the evidence that it is a radiation response modulator, we believe that HDJ-2 is a reasonable candidate as a target protein for FTI-induced early radiosensitization, although several other farnesylated proteins and multiple pathways may also be involved. Differential expression of HDJ-2 in normal and cancerous tissues has not, to our knowledge been reported, but this has been observed for other HSPs, and it is not unreasonable to think that a therapeutic differential may be obtained by FTIs for cellular radiosensitization based on differences in HSP levels and distribution between tumor and normal cells, in addition to that which may be obtained from differences in p53 status.

Finally, throughout these experiments, we observed radiosensitization of glioblastoma multiforme cells by R115777. The improvement was only 15% after 2Gy, but these small differences can amplify over a course of 30 or so fractions into large, clinically significant increases in tumor cell kill. Phase I/II clinical trials of R115777 in glioblastoma multiforme patients proved that this drug is able to cross the blood-brain barrier and is effective in blocking the growth of some tumors (52). Preliminary results of FTI treatment in combination with radiation therapy for non-small cell

lung cancer are promising (53). To advance this further, clinical research is required to optimize tumor response and minimize toxic side effects of this combination therapy, and more basic research is required into the cellular and molecular variables that dictate whether or not the outcome will be successful.

References

1. Chakravarti A, Dicker A, Mehta M. The contribution of epidermal growth factor receptor (EGFR) signaling pathway to radioresistance in human gliomas: a review of preclinical and correlative clinical data. *Int J Radiat Oncol Biol Phys* 2004;58:927–31.
2. Maity A, Kao GD, Muschel RJ, McKenna WG. Potential molecular targets for manipulating the radiation response. *Int J Radiat Oncol Biol Phys* 1997;37:639–53.
3. Cox AD, Der CJ. Protein prenylation: more than just glue? *Curr Opin Cell Biol* 1992;4:1008–16.
4. Marshall CJ. Protein prenylation: a mediator of protein-protein interactions. *Science* 1993;259:1865–6.
5. Karp JE, Lancet JE, Kaufmann SH, et al. Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase I clinical-laboratory correlative trial. *Blood* 2001;97:3361–9.
6. Santucci R, Mackley PA, Sebti S, Alsina M. Farnesyltransferase inhibitors and their role in the treatment of multiple myeloma. *Cancer Control* 2003;10:384–7.
7. Adjei AA, Mauer A, Bruzek L, et al. Phase II study of the farnesyl transferase inhibitor R115777 in patients with advanced non-small-cell lung cancer. *J Clin Oncol* 2003;21:1760–6.
8. Heymach JV, Johnson DH, Khuri FR, et al. Phase II study of the farnesyl transferase inhibitor R115777 in patients with sensitive relapse small-cell lung cancer. *Ann Oncol* 2004;15:1187–93.
9. Rao S, Cunningham D, de Gramont A, et al. Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. *J Clin Oncol* 2004;22:3950–7.
10. Van Cutsem E, van de Velde H, Karasek P, et al. Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J Clin Oncol* 2004;22:1430–8.
11. Kelland LR, Smith V, Valenti M, et al. Preclinical antitumor activity and pharmacodynamic studies with the farnesyl protein transferase inhibitor R115777 in human breast cancer. *Clin Cancer Res* 2001;7:3544–50.
12. Bernhard EJ, McKenna WG, Hamilton AD, et al. Inhibiting Ras prenylation increases the radiosensitivity of human tumor cell lines with activating mutations of ras oncogenes. *Cancer Res* 1998;58:1754–61.
13. Song SY, Meszoely IM, Coffey RJ, Pietenpol JA, Leach SD. K-Ras-independent effects of the farnesyl transferase inhibitor L-744,832 on cyclin B1/Cdc2 kinase activity, G₂/M cell cycle progression and apoptosis in human pancreatic ductal adenocarcinoma cells. *Neoplasia* 2000;2:261–72.
14. Cohen-Jonathan E, Muschel RJ, Gillies McKenna W, et al. Farnesyltransferase inhibitors potentiate the antitumor effect of radiation on a human tumor xenograft expressing activated HRAS. *Radiat Res* 2000;154:125–32.
15. End DW, Smets G, Todd AV, 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.
16. Zhang FL, Kirschmeier P, Carr D, 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.
17. Bernhard EJ, Kao G, Cox AD, et al. The farnesyltransferase inhibitor FTI-277 radiosensitizes H-ras-transformed rat embryo fibroblasts. *Cancer Res* 1996;56:1727–30.

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18. Delmas C, Heliez C, Cohen-Jonathan E, et al. Farnesyltransferase inhibitor, R115777, reverses the resistance of human glioma cell lines to ionizing radiation. *Int J Cancer* 2002;100:43–8.
19. Tamanoi F, Gau CL, Jiang C, Edamatsu H, Kato-Stankiewicz J. Protein farnesylation in mammalian cells: effects of farnesyltransferase inhibitors on cancer cells. *Cell Mol Life Sci* 2001;58:1636–49.
20. Clark GJ, Kinch MS, Rogers-Graham K, et al. The Ras-related protein Rheb is farnesylated and antagonizes Ras signaling and transformation. *J Biol Chem* 1997;272:10608–15.
21. Ashar HR, James L, Gray K, et al. Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with the microtubules. *J Biol Chem* 2000;275:30451–7.
22. Humphrey PA, Wong AJ, Vogelstein B, et al. Amplification and expression of the epidermal growth factor receptor gene in human glioma xenografts. *Cancer Res* 1988;48:2231–8.
23. Ishii N, Maier D, Merlo A, et al. Frequent coalterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol* 1999;9:469–79.
24. Pardo FS, Hsu DW, Zeheb R, et al. Mutant, wild type, or overall p53 expression: freedom from clinical progression in tumours of astrocytic lineage. *Br J Cancer* 2004;91:1678–86.
25. Wang Y, Zhu S, Cloughesy TF, Liao LM, Mischel PS. p53 disruption profoundly alters the response of human glioblastoma cells to DNA topoisomerase I inhibition. *Oncogene* 2004;23:1283–90.
26. Tamanoi F, Kato-Stankiewicz J, Jiang C, Machado I, Thapar N. Farnesylated proteins and cell cycle progression. *J Cell Biochem Suppl* 2001;Suppl:64–70.
27. Wang Y, Blandino G, Givol D. Induced p21^{waf} expression in H1299 cell line promotes cell senescence and protects against cytotoxic effect of radiation and doxorubicin. *Oncogene* 1999;18:2643–9.
28. Kanazawa M, Terada K, Kato S, Mori M. HSDJ, a human homolog of Dnaj, is farnesylated and is involved in protein import into mitochondria. *J Biochem (Tokyo)* 1997;121:890–5.
29. Davis AR, Alevy YG, Chellaiah A, Quinn MT, Mohanakumar T. Characterization of HDJ-2, a human 40 kD heat shock protein. *Int J Biochem Cell Biol* 1998;30:1203–21.
30. Zhu K, Hamilton AD, Sebti SM. Farnesyltransferase inhibitors as anticancer agents: current status. *Curr Opin Investig Drugs* 2003;4:1428–35.
31. Ashar HR, James L, Gray K, et al. The farnesyl transferase inhibitor SCH 66336 induces a G(2) -> M or G(1) pause in sensitive human tumor cell lines. *Exp Cell Res* 2001;262:17–27.
32. Sepp-Lorenzino L, Rosen N. A farnesyl-protein transferase inhibitor induces p21 expression and G₂ block in p53 wild type tumor cells. *J Biol Chem* 1998;273:20243–51.
33. Crespo NC, Ohkanda J, Yen TJ, Hamilton AD, Sebti SM. The farnesyltransferase inhibitor, FTI-2153, blocks bipolar spindle formation and chromosome alignment and causes prometaphase accumulation during mitosis of human lung cancer cells. *J Biol Chem* 2001;276:16161–7.
34. Coqueret O. New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends Cell Biol* 2003;13:65–70.
35. Izumaru S, Arima N, Toyozumi Y, et al. Down-regulation of p21^{Waf-1} protein facilitates IR- and UV-induced apoptosis in human squamous carcinoma cells. *Int J Oncol* 2004;24:1245–55.
36. Waldman T, Lengauer C, Kinzler KW, Vogelstein B. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* 1996;381:713–6.
37. Wouters BG, Giaccia AJ, Denko NC, Brown JM. Loss of p21^{Waf1/Cip1} sensitizes tumors to radiation by an apoptosis-independent mechanism. *Cancer Res* 1997;57:4703–6.
38. Jahner D, Hunter T. The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Mol Cell Biol* 1991;11:3682–90.
39. Fritz G, Kaina B, Aktories K. The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments. *J Biol Chem* 1995;270:25172–7.
40. Adamson P, Marshall CJ, Hall A, Tilbrook PA. Post-translational modifications of p21^{rho} proteins. *J Biol Chem* 1992;267:20033–8.
41. Du W, Lebowitz PF, Prendergast GC. Cell growth inhibition by farnesyltransferase inhibitors is mediated by gain of geranylgeranylated RhoB. *Mol Cell Biol* 1999;19:1831–40.
42. Adjei AA, Davis JN, Erlichman C, Svingen PA, Kaufmann SH. Comparison of potential markers of farnesyltransferase inhibition. *Clin Cancer Res* 2000;6:2318–25.
43. Terada K, Kanazawa M, Bukau B, Mori M. The human Dnaj homologue dj2 facilitates mitochondrial protein import and luciferase refolding. *J Cell Biol* 1997;139:1089–95.
44. Gotoh T, Terada K, Oyadomari S, Mori M. hsp70-Dnaj chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. *Cell Death Differ* 2004;11:390–402.
45. Qiao Y, Ouyang YB, Giffard RG. Overexpression of HDJ-2 protects astrocytes from ischemia-like injury and reduces redistribution of ubiquitin staining *in vitro*. *J Cereb Blood Flow Metab* 2003;23:1113–6.
46. Sierra-Rivera E, Voorhees GJ, Freeman ML. Gamma irradiation increases hsp-70 in Chinese hamster ovary cells. *Radiat Res* 1993;135:40–5.
47. Hunt CR, Dix DJ, Sharma GG, et al. Genomic instability and enhanced radiosensitivity in Hsp70.1- and Hsp70.3-deficient mice. *Mol Cell Biol* 2004;24:899–911.
48. Brondani Da Rocha A, Regner A, Grivicich I, et al. Radioresistance is associated to increased Hsp70 content in human glioblastoma cell lines. *Int J Oncol* 2004;25:777–85.
49. King FW, Wawrzynow A, Hohfeld J, Zyllicz M. Co-chaperones Bag-1, Hop and Hsp40 regulate Hsc70 and Hsp90 interactions with wild-type or mutant p53. *EMBO J* 2001;20:6297–305.
50. Pandita TK, Higashikubo R, Hunt CR. HSP70 and genomic stability. *Cell Cycle* 2004;3:591–2.
51. Mendez F, Kozin E, Bases R. Heat shock protein 70 stimulation of the deoxyribonucleic acid base excision repair enzyme polymerase beta. *Cell Stress Chaperones* 2003;8:153–61.
52. Cloughesy TF, Kuhn J, Wen P. Phase II trial of R115777 (Zarnestra) in patients with recurrent glioma not taking enzyme inducing antiepileptic drugs (EIAED): a North American Brain Tumor Consortium (NABTC) report. *Proc Am Soc Clin Oncol* 2002;21:317.
53. Hahn SM, Bernhard EJ, Regine W, et al. A phase I trial of the farnesyltransferase inhibitor L-778,123 and radiotherapy for locally advanced lung and head and neck cancer. *Clin Cancer Res* 2002;8:1065–72.