Proapoptotic ability of oncogenic H-Ras to facilitate apoptosis induced by histone deacetylase inhibitors in human cancer cells

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Abstract
More than 35% of human urinary bladder cancers involve oncogenic H-Ras activation. In addition to tumorigenic ability, oncogenic H-Ras possesses a novel proapoptotic ability to facilitate the induction of apoptosis by histone deacetylase inhibitors (HDACI). HDACIs are a new class of anticancer agents and are highly cytotoxic to transformed cells. To understand the connection between the selectivity of HDACIs on transformed cells and the proapoptotic ability of oncogenic H-Ras to facilitate HDACI-induced apoptosis, we introduced oncogenic H-Ras into urinary bladder J82 cancer cells to mimic an acquisition of the H-ras gene activation in tumor development. Expression of oncogenic H-Ras promoted J82 cells to acquire tumorigenic ability. Meanwhile, oncogenic H-Ras increased susceptibility of J82 cells to HDACIs, including FR901228 and trichostatin A, for inducing apoptosis. The caspase pathways, the B-Raf and extracellular signal-regulated kinase pathways, p21Cip1 and p27Kip1, and core histone contents are regulated differently by FR901228 in oncogenic H-Ras–expressed J82 cells than their counterparts in parental J82 cells, contributing to the increased susceptibility to the induction of selective apoptosis. Our results lead us to a suggestion that HDACIs activate the proapoptotic ability of oncogenic H-Ras, indicating a potential therapeutic value of this new class of anticancer agents in the control of human urinary bladder cancer that has progressed to acquire oncogenic H-Ras. [Mol Cancer Ther 2007;6(3):1099–111]

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ability of oncogenic H-Ras to increase cell susceptibility to selected anticancer agents, such as FR901228, should be seriously considered in developing anticancer therapeutics against Ras-related cancers. However, the values of the ERK, PI3K, p38/SAPK, and caspase pathways as well as cyclin-dependent kinase inhibitors as therapeutic targets need to be validated in all types of human cancer cells subjected to FR901228 treatment.

Here, we present evidence that expression of oncogenic H-Ras resulted in increased susceptibility of human cancer J82 cells to both FR901228- and TSA-induced apoptosis. The J82 human urinary bladder transitional carcinoma cell line hosts wild-type ras and the inactive mutant Rb and p53 genes with deletion of the pTEN gene (15, 16). Expression of oncogenic H-Ras promoted J82 cells to acquire tumorigenicity, mimicking an acquisition of H-ras gene activation in tumor development. Meanwhile, expression of oncogenic H-Ras facilitated the induction of apoptosis by FR901228 and reduced clonogenic resistance to FR901228. Our studies revealed both commonality and discrepancy in modulation of signaling pathways associated with cell growth, survival, growth arrest, and apoptosis between oncogenic H-Ras–expressed and parental J82 cells. The discrepancies, which may contribute to the ability of FR901228 to selectively induce apoptosis in oncogenic H-Ras–expressed cells, should be considered in the selection of therapeutic targets for Ras-related human urinary bladder cancers.

Materials and Methods

Cell Cultures, Transfection, and Reagents

Human urinary bladder transitional carcinoma J82 and T24 cell lines (American Type Culture Collection, Rockville, MD) and oncogenic H-Ras–expressed J82 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C. To generate oncogenic H-Ras–expressed J82 cells, we used a previously constructed pcDNA4/TO-E-H-ras plasmid (17), which carries the oncogenic H-ras (V12) gene. Cells in a 35-mm culture dish were transfected with 0.5 μg of pcDNA4/TO-E-H-ras plasmid DNA using PolyFect Transfection Reagent (Qiagen, Valencia, CA). After 48 h of transfection, cells were subcultured and selected in 100 μg/mL zeocin (Invitrogen, Carlsbad, CA). Resistant cell clones were established as candidate J82-Ras cell lines. Stock aqueous solutions of FR901228 (obtained through a collaboration with Dr. K.K. Chan, Ohio State University, Columbus, OH), TSA (ICN, Aurora, OH), U0126 (Cell Signaling Technology, Beverly, MA), LY294002, SB203580, SP600125, and Ac-Asp-Glu-Val-Asp-COOH (Ac-DEVD-CHO; Alexis, San Diego, CA) were prepared in DMSO and diluted in culture medium for assays.

Flow Cytometry

Attached cells were trypsinized from cultures, rinsed with Ca2+- and Mg2+-free PBS, fixed in ethanol, and stained with propidium iodide for flow cytometric analysis as done previously (13). Analysis of DNA content and determination of the percentage of apoptotic cells and cells in each phase of the cell cycle were done on Multicycle software (Phoenix Flow System, San Diego, CA).

Cell Growth and Survival Rate Assay

Cells were seeded in 12-well culture plates and treated with FR901228. Every 24 h, attached cells were trypsinized, washed, and then resuspended in culture medium containing 0.2% trypan blue to stain dead cells. Live cells were counted in a hemocytometer to determine relative cell growth and survival rate (12).

Cell Growth Inhibition Assay

Inhibition of cell proliferation was determined by the inhibition of bromodeoxyuridine (BrdUrd) incorporation into cellular DNA using the BrdUrd cell proliferation ELISA kit (Roche, Indianapolis, IN). Cells (5 × 104) were seeded into each well of 96-well culture plates for 24 h. After treatment with FR901228, cells were labeled with BrdUrd for 12 h, fixed, incubated with peroxidase-conjugated BrdUrd-specific antibodies, and stained with peroxidase substrate. Quantification of BrdUrd-labeled cells was determined with an ELISA reader (Bio-Tek, Winooski, VT).

Cell Viability Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (American Type Culture Collection, Manassas, VA) was used to measure cell viability in cultures. Cells (5 × 104) were seeded into each well of 96-well culture plates for 24 h. After treatments with HDACIs and/or inhibitors, cells were incubated with MTT reagent for 4 h followed by incubation with detergent reagent for 24 h. Reduced MTT reagent in cultures was quantified with an ELISA reader.

Clonogenic Assay

Triplicates of 1 × 104 cells were seeded in 100-mm culture dishes for 24 h. After FR901228 treatment, cultures were rinsed and replaced with fresh medium. Growing colonies (>30 cells) were identified and counted under an anatomic microscope. Adherent colonies in untreated cultures were stained with crystal violet after 7 days, and FR901228-treated cultures were stained after 14 days.

Anchorage-Independent Cell Growth Assay

The base layer consisted of 2% low-gelling SeaPlaque agarose (Sigma, St. Louis, MO) in complete J82 culture medium. Soft agar consisting of 0.4% SeaPlaque agarose in a mixture (1:1) of complete J82 culture medium with 3-day conditioned medium prepared from J82 culture was mixed with 3 × 105 cells and plated on top of the base layer in 60-mm-diameter culture dishes. Soft agar cultures were maintained at 37°C and observed microscopically for the appearance of colonies. Growing colonies were identified, and the number of colonies that reached a diameter of 0.5 mm by 14 days was recorded.

Tumorigenic and Histopathologic Studies

Cells were prepared with Matrigel basement membrane matrix (13.35 mg/mL; BD Biosciences, San Jose, CA), and 1 × 107 cells in 100 μL were injected s.c. into flanks of 5-week-old female athymic NCr-nu/nu mice (National
Cancer Institute, Frederick, MD). Four mice were used per group and maintained under pathogen-free conditions. Animals were monitored weekly until tumors were visible; then, tumor growth was monitored every 3 days. Xenograft tumor tissues were immediately harvested after euthanasia by exposure to carbon dioxide. Tumor tissues were fixed in neutral-buffered formalin and embedded in paraffin for histopathologic evaluation of 5-μm H&E-stained sections.

**Western Immunoblotting**

To prepare cell lysates, cultured cells were lysed in a buffer [10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 10% glycerol, 0.1% Na3VO4, 50 mmol/L NaF (pH 7.4); ref. 12]. Tumor tissues were sliced into 1 mm³ cubes, incubated with FR901228 in culture medium, and lysed in the buffer above with 30 strokes of a loose-fitting Dounce homogenizer. Cell lysates (S20) were isolated from the supernatants after centrifugation of crude cell or tissue lysates at 20,000 g for 20 min. To prepare nuclear cell lysates containing core histones, cells were lysed in a hypotonic buffer [10 mmol/L KH2PO4, 1 mmol/L MgCl2, 50 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 2 mmol/L DTT (pH 7.2)] with 30 strokes of a tight-fitting Dounce homogenizer (18). After centrifugation of crude lysates at 10,000 g for 10 min, cell pellets containing nuclear histones were resuspended in lysis buffer and sonicated for 10 min as nuclear lysates (P10). Protein concentrations in cell lysates (S20) and nuclear lysates (P10) were measured using the bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of cellular proteins were resolved by electrophoresis in either 10% or 14% SDS-polyacrylamide gels and transferred to nitrocellulose filters for Western immunoblotting as described previously (12–14). Antibodies specific to Akt, Raf-1, MEK1/2, p38, c-Jun NH2-terminal kinase (JNK), acetylated H2A on Lys5, acetylated H2B on Lys12, H2B protein, acetylated H3 on Lys9, H3 protein, acetylated H4 on Lys8, procaspase-3, active caspase-3, active caspase-7, active caspase-8, full-length and cleaved poly(ADP-ribose) polymerase (PARP; 89-kDa cleaved PARP and 116-kDa full-length and cleaved PARP), and β-actin were purchased from Cell Signaling Technology. Specific antibodies to phosphorylated forms of Akt, Raf-1, B-Raf, MEK1/2, ERK1/2, p38, and JNK were also purchased from Cell Signaling Technology. Specific antibodies to H-Ras, B-Raf, ERK1/2, p53 (FL-393), p27kip1, and p21Cip1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antigen-antibody complexes on filters were detected by the SuperSignal chemiluminescence kit (Pierce).

**Statistical Analysis**

Statistical significance was analyzed by the Student’s t test. A P value of ≤0.05 was considered significant.

**Results**

**Tumorigenicity of J82 Cells Was Induced by Oncogenic H-Ras**

To determine the effects of oncogenic H-Ras on tumorigenic potential of human urinary bladder J82 cancer cells,
cell clones were stably transfected with the expression plasmid vector pcDNA4/TO-E-H-ras. As shown in Fig. 1A, ectopic expression of oncogenic H-Ras in J82 cells resulted in a distinctive morphologic change from a round, ruffled edge morphology (Fig. 1A, a) to an elongated, transformed morphology (Fig. 1A, b). Based on this change, the H-Ras expression level, and an enhanced cancerous ability of anchorage-independent growth in soft agar (19), we developed a single clonal J82-Ras cell line from selected cell clones. As shown in Fig. 1B-1, J82-Ras cells (Fig. 1B, lane 2) expressed a level of oncogenic H-Ras comparable with the counterpart level in the human bladder cancer T24 cell line (Fig. 1B, lane 3), which hosts the oncogenic H-ras mutant gene (20). Colony formation in soft agar showed an average of 45 colonies developed from 3,000 J82 cells and an average of 90 colonies developed from 3,000 J82-Ras cells (Fig. 1C). The cancerous ability of anchorage-independent growth in J82 cells was increased by oncogenic H-Ras. To conclude if J82-Ras cells had acquired tumorigenic ability, we inoculated J82-Ras and parental J82 cells into immunodeficient mice. Consistent with others’ report (21), animals inoculated with parental J82 cells did not develop any xenograft tumors in 8 weeks. In contrast, nude mice inoculated with J82-Ras cells developed visible xenograft tumors in 15 days; tumors developed to an average size of 0.3 cm in diameter by 20 days and 1 cm by 30 days. Histopathologic evaluation of J82-Ras–derived xenograft tumor tissues revealed neoplastic cells that invaded the adjacent skeletal muscle (Fig. 1D, a) and a cohesive sheet of neoplastic cells characterized by ill-defined cytoplasmic borders, abundant slightly vacuolated eosinophilic cytoplasm, round to elongate nuclei, and multiple prominent nucleoli (Fig. 1D, b). Additionally, marked anisokaryosis (Fig. 1D, b) and anisocytosis as well as numerous binucleate and occasional multinucleate tumor giant cells were noted. The histopathologic diagnosis confirmed that expression of oncogenic H-Ras induced the tumorigenic ability of J82 cells to produce poorly differentiated, xenograft adenocarcinoma in vivo.

Increased Cell Susceptibility to HDACIs by Oncogenic H-Ras

To determine if expression of oncogenic H-Ras increased cell susceptibility to FR901228 for inducing cell death, we compared cell growth and survival rates of J82-Ras cells with parental J82 cells responding to FR901228 treatment. We detected that expression of oncogenic H-Ras did not result in any significant difference in the growth rate of J82-Ras cells versus parental J82 cells (Fig. 2A, 0 nmol/L control lines). Treatment with 1 nmol/L FR901228 reduced cell growth of both parental J82 and J82-Ras cells. Treatment with 5 nmol/L FR901228 resulted in growth inhibition of both parental J82 and J82-Ras cells in 24 h. In contrast to a modestly reduced cell survival rate in parental J82 cultures, 5 nmol/L FR901228 treatment significantly reduced cell survival in J82-Ras cultures by 48 h, indicating significantly higher cell death in J82-Ras cultures than in parental J82 cultures. Prolonged incubation with FR901228 at either 1 or 5 nmol/L induced increased cell death in both
parental J82 and J82-Ras cultures. We observed a higher extent of apoptotic-like cell morphology with cell shrinkage and loss of cell contact induced by FR901228 treatment in J82-Ras cultures than in parental J82 cultures (data not shown). Although induction of apoptotic-like cell death by FR901228 was in a dose- and time-dependent manner, FR901228 at 5 nmol/L induced a distinguishable, higher level of apoptosis in J82-Ras cultures than in parental J82 cells. Cell death by apoptosis in these cultures was verified by flow cytometry. As shown in Table 1, apoptotic cell population in attached cells was higher in J82-Ras cultures (increased from 2% to 7% and 24%) contrasted to parental J82 cultures (increased from 2% to 4% and 13%) after FR901228 treatment.

We used a clonogenic assay (22) to further determine whether the increased cell susceptibility to FR901228 by oncogenic H-Ras was attributed to a reduced resistance of oncogenic H-Ras–expressed J82 cells. As shown in Fig. 2B, ~600 clones (>30 cells per colony) developed from 1 × 10^4 untreated J82 and J82-Ras cells in 7 days (control). After 48 h of FR901228 treatment, we detected that ~25 clones had grown slowly and survived in J82 cultures (reached 30 cells per colony) by 14 days and none survived in J82-Ras cultures (Fig. 2B, FR). Evidently, expression of oncogenic H-Ras in J82 cells increased susceptibility and reduced cell resistance to FR901228-induced apoptosis.

To expand our investigation of the proapoptotic role of oncogenic H-Ras in increasing cell susceptibility to HDACs, we compared the susceptibility of J82 and J82-Ras to the induction of apoptosis by FR901228 and TSA, with the susceptibility to the human bladder cancer T24 cell line, which hosts the endogenous, oncogenic H-ras mutant gene (20). As shown in Fig. 2C, FR901228 treatment at either 5 or 25 nmol/L reduced cell viability of J82-Ras and T24 cells to higher degrees than in J82 cells. Both J82-Ras and T24 cells also showed a higher susceptibility than J82 cells to TSA-reduced cell viability in a dose-dependent manner (Fig. 2D). Interestingly, T24 cells showed a susceptibility similar to J82-Ras cells in response to either FR901228 or TSA treatment. Evidently, both FR901228 and TSA showed selectivity to induce cell death of oncogenic H-Ras–expressed human urinary bladder cancer J82 and T24 cells.

### Differential Regulation of Caspase Pathways by FR901228

To detect the apoptotic pathway induced by FR901228 in selective apoptosis of J82-Ras cells, we initially studied activation of executor caspase-3 by FR901228 in parental J82 and J82-Ras cells. We observed a higher level of procaspase-3 in J82-Ras cells than in parental J82 cells (Fig. 3A-1, lane 3 versus lane 1); FR901228 treatment increased the procaspase-3 protein level in parental J82 cells (Fig. 3A-1, lane 2 versus lane 1) but decreased procaspase-3 in J82-Ras cells (Fig. 3A-1, lane 4 versus lane 3). FR901228 treatment resulted in a higher level of active caspase-3 in J82-Ras cells (Fig. 3A-2, lane 4) than in parental J82 cells (Fig. 3A-2, lane 2). Studying the activation course of caspase-3 in FR901228-induced apoptosis revealed that FR901228 treatment did not induce detectable levels of active caspase-3 in J82 cells until 48 h (Fig. 3B-1, lane 3); in contrast, it induced an early caspase-3 activation in J82-Ras cells in 24 h (Fig. 3B-1, lane 5). Accelerated caspase-7 activation by FR901228 was also detected in J82-Ras cells (Fig. 3B-2, lanes 5 and 6) compared with parental J82 cells (Fig. 3B-2, lanes 2 and 3). Concomitantly, PARP, a downstream substrate of caspase-3 and caspase-7 (23), was accordingly cleaved in parental J82 cells (Fig. 3B-3, lane 3) and J82-Ras cells (Fig. 3B-3, lanes 5 and 6). Expression of oncogenic H-Ras in J82 cells seems to accelerate FR901228-induced activation of caspase-3 and caspase-7 and proteolysis of PARP.

To determine the upstream initiator caspses, which may contribute to the activation of caspase-3 and caspase-7, we detected that active caspase-8 was induced by FR901228 in parental J82 cells by 48 h (Fig. 3B-4, lane 4), and FR901228 treatment induced an early activation of caspase-8 in J82-Ras cells (Fig. 3B-4, lanes 5 and 6). Interestingly, caspase-9 was induced by FR901228 in J82-Ras cells (Fig. 3B-5, lanes 5 and 6) but was not induced in parental cells (Fig. 3B-5, lanes 1–3). Accordingly, the caspase-8 to caspase-3 and caspase-7 pathway was induced by FR901228 in parental J82 cells. Both caspase-8 and caspase-9 to caspase-7 pathways were induced by FR901228 in J82-Ras cells. The caspase-9 pathway seems to be potentiated by oncogenic H-Ras for activation in FR901228-induced selective apoptosis of J82-Ras cells.

### Table 1. Flow cytometric analysis of FR901228-treated cells

<table>
<thead>
<tr>
<th>Treatment (h)</th>
<th>J82</th>
<th>J82-Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Apoptotic (%)</td>
<td>2 ± 1</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Live (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G0-G1</td>
<td>52 ± 2</td>
<td>51 ± 6</td>
</tr>
<tr>
<td>S</td>
<td>22 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>G2-M</td>
<td>26 ± 3</td>
<td>44 ± 3</td>
</tr>
</tbody>
</table>

NOTE: J82 and J82-Ras cultures were treated with 5 nmol/L FR901228 for 0, 24, and 48 h. Anchored cells were trypsinized from cultures, and cell populations were analyzed by flow cytometry to detect apoptotic cell population and live cells in each phase of the cell cycle. Each value represents a mean of results from two independent experiments ± SD. The Student’s t test was used to analyze statistical significance of b versus a (P < 0.05).
To verify the role of caspase-3 and caspase-7 in FR901228-induced cell death, J82 and J82-Ras cells were pretreated with the potent inhibitor Ac-DEVD-CHO to specifically inhibit caspase-3 and caspase-7 (24) followed by FR901228 treatment. Pretreating cells with Ac-DEVD-CHO reduced FR901228-induced cleavage of PARP in both parental J82 (Fig. 3C-1, lane 4 versus lane 3) and J82-Ras (Fig. 3C-1, lane 8 versus lane 7) cells. Although treatment of cells with Ac-DEVD-CHO alone did not induce any detectable cleaved PARP (Fig. 3C-1, lanes 2 and 6), the treatment resulted in a modest reduction of cell viability (Fig. 3C-2). Pretreatment with Ac-DEVD-CHO did not significantly reverse the low degree of FR901228-reduced viability of J82 cells, but it significantly attenuated the FR901228-reduced viability of J82-Ras cells (Fig. 3C-2). We also used AEBSF-HCl (25), a serine protease inhibitor, in a similar study, but pretreatment with AEBSF-HCl did not enhance or suppress any FR901228-reduced cell viability (data not shown). These results indicate that caspase-3 and caspase-7 played important roles in the FR901228-induced selective apoptosis of oncogenic H-Ras–expressed J82 cells. In addition, we treated xenograft tumor tissues of J82-Ras cells with FR901228 in vitro and detected active caspase-3, caspase-7, and cleaved PARP (Fig. 3D-1 to D-3, lanes 2 and 3); this result verified that the FR901228-induced caspase activation also occurred in a three-dimensional tumor tissue structural environment.

Regulation of Cyclin-Dependent Kinase Inhibitors, p53, and Histones by FR901228

FR901228 treatment induces growth arrest of human breast cancer MCF7 and MDA-MB231 cells in the G2-M phase of the cell cycle (11). However, FR901228 treatment growth arrests mouse 10T1/2 cells and Ras-expressed 10T1/2 cells in the G0-G1 phase of the cell cycle (12, 13). To study if growth arrest contributed to FR901228-induced growth inhibition of J82 and J82-Ras cells, we initially verified that cell proliferation in both parental J82 and J82-Ras cultures was suppressed by FR901228 treatment in 24 h (Fig. 4A). Subsequently, flow cytometric analysis revealed that FR901228 treatment of either J82 or J82-Ras cultures resulted in decreased cell populations in the G0-G1 phase, substantially decreased cell populations in the S phase, and increased cell populations in the G2-M phase (Fig. 4B). These results indicate that FR901228 inhibited cell proliferation in both parental J82 and J82-Ras cultures in vitro.

![Figure 3](https://example.com/figure3.png)
detected that p21Cip1 was increasingly induced by FR901228 in J82 (Fig. 4B-2, lanes 2 and 3) and J82-Ras (Fig. 4B-2, lanes 5 and 6) cells. Induction of p21Cip1 seems to correlate with induction of growth inhibition, and p27Kip1 induction seems to correlate with completion of cell growth arrest by FR901228 in the G2-M phase of the cell cycle (Table 1). In addition, we detected that the mutant p53 was progressively reduced by FR901228 in J82 (Fig. 4B-3, lanes 2 and 3) and J82-Ras (Fig. 4B-3, lanes 5 and 6) cells. The reduction of the mutant p53 was correlated with FR901228-induced apoptosis.

Modification of core histones through acetylation has been reported to play a critical role in the growth arrest of cell proliferation (26, 27). We detected that acetylation of core histones H2A on Lys 5 (Fig. 4C-1), H2B on Lys 12 (Fig. 4C-2), H3 on Lys 9 (Fig. 4C-3), and H4 on Lys 8 (Fig. 4C-4) was profoundly increased by FR901228 treatment in both parental J82 (Fig. 4C-4, lane 2) and J82-Ras (Fig. 4C-4, lane 5) cells by 24 h; however, acetylation was substantially reduced in cells treated with FR901228 for 48 h (Fig. 4C-4, lanes 3 and 6). The H2B protein level was reduced by FR901228 treatment in both parental J82 and J82-Ras cells (Fig. 4C-5, lanes 3 and 6). FR901228 progressively reduced the H3 protein level in J82-Ras cells undergoing apoptosis (Fig. 4C-6, lanes 5 and 6) than in parental J82 cells mainly undergoing growth arrest (Fig. 4C-6, lanes 2 and 3). However, we were unable to detect H2A and H4 protein with two commercially available specific antibodies. These results indicate a novel effect of oncogenic H-Ras on the reduction of histone contents induced by extended treatment with FR901228.

Roles of the ERK, PI3K, and p38/SAPK Pathways in FR901228-Induced Apoptosis

The ERK, PI3K, and p38/SAPK pathways are modulated by FR901228 to play different roles in apoptosis of H-Ras–transformed and nontransformed mouse 10T1/2 cells (12, 13). To investigate the role the ERK pathways may play in J82-Ras and parental J82 cells undergoing FR901228-induced selective apoptosis, we used U0126 to specifically inhibit MEK1/2 activity (13, 14, 28). As shown in Fig. 5A-1, treatments with U0126 alone for 48 h modestly reduced cell viability in cultures of J82 and J82-Ras. Although U0126 treatment failed to affect FR901228-reduced viability in parental J82 cells, it enhanced FR901228-reduced viability of J82-Ras cells. Thus, expression of oncogenic H-Ras induced the survival role of the ERK pathway in J82-Ras cells responding to the FR901228-induced apoptosis. To identify targets in the ERK pathway for reducing cell viability, we studied FR901228-induced effects on activation-related phosphorylation and protein levels of ERK pathway kinases B-Raf, Raf-1, MEK1/2, and ERK1/2. We detected that protein levels of oncogenic H-Ras were not affected by FR901228 treatment (Fig. 5A-2). The overall phosphorylation level of B-Raf was elevated in J82-Ras cells (Fig. 5A-3, lane 4) compared with the counterpart phosphorylated kinase in parental J82 cells (Fig. 5A-3, lane 1). FR901228 treatment increased the overall phosphorylation of B-Raf in parental J82 cells (Fig. 5A-3, lanes 2 and 3) but reduced both the overall phosphorylation and cognate

Figure 4. Regulation of cyclin-dependent kinase inhibitors, mutant p53, and histones in FR901228-treated cells. A, J82 and J82-Ras cells were treated with 5 nM FR901228 for 24 h. Inhibition of cell growth by FR901228 was determined by the blockage of BrdUrd (BrdU) incorporation into cellular DNA. Quantification of BrdUrd-labeled cells was determined with an ELISA reader. Relative cell growth rate was normalized by the value of BrdUrd detected in untreated cells, set as 100%. Columns, mean of triplicates; bars, SD. B-1 to B-4 and C-1 to C-6, J82 and J82-Ras cultures were treated with 5 nM/L FR901228 for 0 h (lanes 1 and 4), 24 h (lanes 2 and 5), and 48 h (lanes 3 and 6). Western immunoblotting with specific antibodies was used to detect p21Cip1, p27Kip1, p53, and β-actin in cell lysates (B-1 to B-4) and acetylated H2A, acetylated H2B, acetylated H3, acetylated H4, total H2B protein, and total H3 protein in nuclear lysates (C-1 to C-6).
protein levels of B-Raf in J82-Ras cells (Fig. 5A-3 and A-5A-3 and A-4, lanes 5 and 6). Although B-Raf protein levels may vary slightly between experiments, in general, they were unchanged in parental J82 cells treated with FR901228. Adjusted by the cognate protein level of B-Raf, the specific phosphorylation level of B-Raf was elevated by FR901228 treatment in both parental J82 and J82-Ras cells. Adjusted with β-actin levels (Fig. 5A-11), FR901228
treatment resulted in significant reduction of B-Raf protein in J82-Ras cells. B-Raf protein seems to be a distinct target for FR901228-induced reduction in J82-Ras cells but not in parental J82 cells.

Expression of oncogenic H-Ras also elevated the overall activation-related phosphorylation level of Raf-1 in J82-Ras compared with parental J82 cells (Fig. 5A-5 and A-6, lanes 2, 3, 5, and 6); based on the cognate Raf-1 protein level, the specific phosphorylation level of Raf-1 was slightly increased in parental J82 cells and reduced in J82-Ras cells. Adjusted with β-actin levels, FR901228 treatment resulted in significant reduction of Raf-1 protein (Raf-1/actin) in both parental J82 and J82-Ras cells. Thus, Raf-1 protein seems to be a common target for FR901228-induced reduction in J82 and J82-Ras cells.

To further investigate the ERK downstream pathway from Raf regulated in FR901128-treated cells, we detected that phosphorylation levels of MEK1/2 (Fig. 5A-7) and ERK1/2 (Fig. 5A-9) were not noticeably increased in J82-Ras cells (Fig. 5A-9, lane 4 versus lane 1). Although treatment of J82-Ras cells with FR901228 suppressed the overall phosphorylation and protein levels of B-Raf (Fig. 5A-5 and A-5A-3 and A-4, lanes 5 and 6) and Raf-1 (Fig. 5A-5 and A-6), it increased phosphorylation of MEK1/2 (Fig. 5A-7) and ERK1/2 (Fig. 5A-9). In contrast, FR901228 treatment did not induce any noticeable change in phosphorylation of MEK1/2 and ERK1/2 in parental J82 cells (Fig. 5A-7 and A-9, lanes 2 and 3). Increased phosphorylation/activation of MEK1/2 and ERK1/2 seems to correlate with increased specific phosphorylation of B-Raf in J82-Ras cells. Expression of oncogenic H-Ras in J82 cells seems to potentiate the ERK downstream kinases for activation in response to FR901228 treatment.

While investigating the PI3K, the p38/SAPK, and the JNK/SAPK pathways involved in FR901228-induced cell death, we detected that expression of oncogenic H-Ras in J82 cells (lane 4 versus lane 1) did not increase the phosphorylation of Akt (Fig. 5B-1) and p38 (Fig. 5B-3) but changed the phosphorylation profiles of JNK (Fig. 5B-5). FR901228 treatment did not result in any significant change in the overall phosphorylation of Akt in either J82 (Fig. 5B-1 and B-5B-1 and B-2, lanes 2 and 3) or J82-Ras (Fig. 5B-1 and B-2, lanes 5 and 6) cells. Interestingly, FR901228 treatment profoundly reduced phosphorylation of p38 in parental J82 cells (Fig. 5B-3, lanes 2 and 3), but it did not result in any change in phosphorylation of p38 in J82-Ras cells (Fig. 5B-3, lanes 5 and 6). Thus, expression of oncogenic H-Ras prevented p38 from dephosphorylation induced by FR901228. On the other hand, FR901228 treatment profoundly reduced phosphorylation of JNK but not cognate protein in parental J82 cells (Fig. 5B-5 and B-6, lanes 2 and 3) and in J82-Ras cells (Fig. 5B-5 and B-6, lane 6). In pursuing the role the PI3K, the p38/SAPK, and the JNK/SAPK pathways may play in FR901228-induced cell death, we detected that treatments with LY294002, which specifically inhibits PI3K activity (13, 29), alone modestly reduced cell viability in cultures of J82 and J82-Ras, but it failed to result in any significant change in the FR901228-reduced viability (Fig. 5C-1). The PI3K pathway was unlikely to play a role in the FR901228-induced growth inhibition or apoptosis of J82 and J82-Ras cells. After using SB203580, which specifically inhibits p38 activity (13, 30), to block the p38/SAPK pathway, we detected that SB203580 treatment attenuated FR901228-reduced viability in J82-Ras cells but not in parental J82 cells (Fig. 5C-2). Therefore, the p38/SAPK pathway seems to be differentially maintained by oncogenic H-Ras and to play a proapoptotic role in FR901228-induced apoptosis of J82-Ras cells. Using SP600125, which specifically inhibits JNK activity (31, 32), to block the JNK/SAPK pathway, we detected that J82-Ras cells were less susceptible to SP600125 treatment than parental J82 cells, and SP600125 treatment additionally enhanced FR901228-reduced viability in parental J82 cells but not in J82-Ras cells (Fig. 5C-3). The JNK/SAPK pathway seems to play a survival role in parental J82 cells.

Although expression of oncogenic H-Ras induced phosphorylation of both p54 and p48 JNK and induced resistance to SP600125, suppression of the JNK/SAPK pathway by FR901228 treatment may still contribute to the induction of cell death in J82-Ras cells.

Discussion

Understanding the proapoptotic ability of oncogenic H-Ras to enhance the cytotoxicity of anticancer agents to induce selective apoptosis provides cellular and molecular bases for developing therapeutic strategies to target Ras-related human cancers. In our previous studies (12–14), we showed that expression of oncogenic H-Ras in mouse 10T1/2 cells increases susceptibility to FR901228 but not the potent apoptosis inducer staurosporine for inducing selective apoptosis. In this report, we present evidence to verify the selectivity of HDACIs to target human bladder cancer cells associated with oncogenic H-Ras expression as summarized in Fig. 6.

The human urinary bladder carcinoma J82 cell line hosts wild-type ras genes and is not tumorigenic to immune-deficient mice (15, 16, 21). In our studies, introduction of the oncogenic H-ras gene into J82 cells promoted cells to acquire the tumorigenic ability to develop xenograft tumors in immune-deficient mice; however, it was also accompanied by the novel proapoptotic property for FR901228 and TSA to induce selective apoptosis. The human urinary bladder carcinoma T24 cell line, in which oncogenic H-Ras is endogenously expressed (20), showed a comparable level of susceptibility with J82-Ras cells to both FR901228 and TSA for inducing cell death. However, expression of oncogenic H-Ras in J82 cells did not increase susceptibility to other agents, including U0126, LY294002, SB203580, SP600125, and Ac-DEVD-CHO, which is consistent with our results from studying mouse fibroblasts (13). The proapoptotic ability of oncogenic H-Ras that facilitates agents to induce cell death is HDACI-specific but is not a...
Scheme of targets in FR901228-treated J82-Ras cells

- Destabilization of anti-apoptotic Raf and mutant p53
- Accelerated induction of extrinsic caspase-8 and intrinsic caspase-9 pathways
- Resistance of pro-apoptotic p38 to deactivation
- Nucleosomal alterations via constant acetylation of core histones

Selective apoptosis

Figure 6. Proposed targets by FR901228 to induce selective apoptosis of H-Ras–expressed cells.

cell type-specific property. HDACIs may activate the proapoptotic potential of oncogenic H-Ras. In addition, expression of the oncogenic v-src gene in 10T1/2 cells resulted in cellular transformation but did not increase cell susceptibility to FR901228 for inducing apoptosis (result not shown). Whether the selectivity of HDACIs to induce cell death of oncogenic H-Ras–expressed cells is applicable to other oncogene-expressed cells needs to be broadly investigated.

Caspase pathways were differentially induced by FR901228 between oncogenic H-Ras–expressed and parental J82 cells. Previously, we reported that caspase-3 plays an important role in FR901228-induced selective apoptosis of Ras-transformed 10T1/2 cells (12–14). Here, we detected that FR901228 induced not only accelerated caspase-3 activity but also caspase-7 in the selective apoptosis of oncogenic H-Ras–expressed J82 cells compared with parental J82 cells. Inhibition of caspase-3 and caspase-7 alleviated FR901228-reduced cell viability, which clearly verified the contributing role of these caspases in the induction of apoptosis by FR901228. Investigating their upstream caspase pathways, we detected that both the caspase-8 and caspase-9 pathways were activated in oncogenic H-Ras–expressed J82 cells, but only the caspase-8 pathway was induced in parental counterpart J82 cells after FR901228 treatment. Other studies have shown that FR901228 treatment induces the extrinsic Fas pathway-dependent activation of caspase-8 and caspase-3 in human osteosarcoma and leukemia cells (33, 34) and induces the intrinsic mitochondrial pathway-dependent activation of caspase-9 and caspase-3, but not caspase-8, in lung cancer cells (35). Either the extrinsic caspase-8 or the intrinsic caspase-9 initiator pathway is reportedly sufficient to activate its downstream executioners, caspase-3 and caspase-7 (36). Whether the additional activation of caspase-9 in J82-Ras cells, but not in parental J82 cells, plays an enhancing role in the accelerated induction of selective apoptosis in J82-Ras needs to be clarified.

Expression of oncogenic H-Ras in J82 cells resulted in an increased cell population in the G2-M phase of the cell cycle in response to FR901228-induced cell growth arrest. Studies of cyclin-dependent kinase inhibitors revealed distinct regulation of p21Cip1 and p27Kip1 in the course of FR901228-induced cell growth arrest of J82 cells. High levels of p21Cip1 were initially induced in 24 h and subsequently reduced by 48 h in both parental J82 and J82-Ras cells, whereas the p27Kip1 level was continuously increased in the course of FR901228 treatment. Increased expression of p27Kip1 has been postulated to play a role in complete suppression of human cancer cell growth (37). In fact, FR901228 induced growth inhibition of both parental J82 and J82-Ras cells in 24 h. Accordingly, an early induction of p21Cip1 plus a progressive induction of p27Kip1 may be required to growth arrest J82 and J82-Ras cells. Both p21Cip1 and p27Kip1 have been postulated to play multiple roles in the regulation of apoptosis, protein assembly, and gene transcription in addition to inhibitors of the cell cycle (38). The significance of the distinct, sequential regulation of p21Cip1 and p27Kip1 in FR901228-induced cell growth arrest and apoptosis remains to be determined. On the other hand, it is interesting that, in J82-Ras cells, a lower level of p21Cip1 and a higher level of p27Kip1 were induced compared with parental J82 cells undergoing FR901228-induced growth arrest. We reported previously that FR901228 treatment induces p21Cip1 expression in nontransformed 10T1/2 cultures in which cells are mainly arrested in the G0-G1 phase of the cell cycle; in contrast, FR901228 treatment reduces the basal level of p21Cip1 in Ras-transformed 10T1/2 cultures in which significant apoptosis is induced (12). We did not detect any induction of p27Kip1 in either nontransformed or Ras-transformed 10T1/2 cultures after FR901228 treatment (data not shown). Sandor et al. (39) observed that human colon cancer HCT116-derived cell clones lacking p21Cip1 are not arrested in the G1 phase but are arrested in the G2-M phase of the cell cycle following FR901228 treatment; they suggested that p21Cip1 is required for FR901228-induced G1 arrest and G2-M arrest in the absence of p21Cip1. J82 cells host the mutant p53 gene (15), and the mutant p53 protein was progressively and substantially reduced in J82 and J82-Ras cells undergoing FR901228-induced growth arrest followed by apoptosis. It has been suggested that FR901228 induces a novel p53-related feedback activity that results in depletion of mutant p53 protein in association with increased cytotoxicity of FR901228 to tumor cells (40, 41). Accordingly, enhanced reduction of p21Cip1 and depletion of mutant p53 conceivably contribute to FR901228-induced selective apoptosis of oncogenic H-Ras–expressed J82 cells.
It was expected that FR901228 treatment induced profound acetylation of core histones H2A, H2B, H3, and H4 in both J82 and J82-Ras cells. However, it was unexpected that acetylated histones and their protein contents were subsequently reduced in cells undergoing FR901228-induced growth inhibition or apoptosis. Particularly, reduction of H3 content by FR901228 seems to be accelerated by expression of oncogenic H-Ras in J82 cells in association with enhanced cell death. Modification of core histones through acetylation has been reported to play a critical role in the growth arrest of cell proliferation (26, 27). It is possible that oncogenic H-Ras–induced modification of histones in conjunction with apoptosis-related chromosomal DNA fragmentation contributes to the enhanced histone reduction in J82-Ras cells undergoing FR901228-induced cell death. However, the mechanism for reducing histone content and the role of reduced histones in FR901228-induced apoptosis remain to be clarified.

The ERK pathway was targeted by FR901228 treatment in both parental and oncogenic H-Ras–expressed J82 cells. FR901228 treatment resulted in substantial reduction of Raf-1 protein in both parental J82 and J82-Ras cells, a result consistent to the outcome of substantially reduced Raf-1 protein in FR901228-treated mouse 10T1/2 cells (12). It has been suggested that FR901228 treatment results in destabilization of Raf-1 protein due to dissociation from Hsp90 (42, 43). Possibly, the association between Raf-1 and Hsp90 is a target for FR901228 to reduce Raf-1 protein in J82 and 10T1/2 cells, regardless of Ras activation. On the other hand, expression of oncogenic H-Ras increased phosphorylation/activation of B-Raf. It has been suggested that Ras-activated B-Raf, but not inactive B-Raf, is subjected to regulation by Hsp90, and inhibition of Hsp90 activity destabilizes activated B-Raf for degradation (44). Therefore, activated B-Raf may become susceptible to the Hsp90-involved degradation induced by FR901228 treatment in J82-Ras cells. Raf family members have been reportedly involved in cell survival (45). In contrast to a maintained, unchanged B-Raf level and a reduced Raf-1 level in parental J82 cells, reduction of both Raf-1 and B-Raf conceivably contributed to the increased susceptibility of J82-Ras cells to FR901228-induced apoptosis.

FR901228 treatment stimulated the survival role of the ERK pathway in J82-Ras cells but not in parental J82 cells. Although expression of oncogenic H-Ras in J82 cells increased phosphorylation of both B-Raf and Raf-1, it did not lead to significant phosphorylation of downstream kinases MEK1/2 and ERK1/2 until FR901228 treatment. FR901228 treatment resulted in reduction of both overall phosphorylated B-Raf and B-Raf protein in J82-Ras cells; however, the specific phosphorylation of B-Raf was highly increased. Possibly, the FR901228-increased specific phosphorylation contributed to the increased specific kinase activity of B-Raf, leading to the induction of downstream kinases MEK1/2 and ERK1/2 in J82-Ras cells. In contrast, MEK1/2 and ERK1/2 were not activated by FR901228 in parental J82 cells. Consequently, blockade of the ERK pathway by inhibition of MEK1/2 activation resulted in enhanced cell death in FR901228-treated J82-Ras cells but not in parental J82 cells. Thus, the downstream ERK pathway plays an antiapoptotic role in FR901228-induced apoptosis of J82-Ras cells. Accordingly, suppression of the downstream ERK pathway enhanced the FR901228-induced selective apoptosis of J82-Ras cells.

The PI3K pathway, in addition to the ERK pathway, was also unconventionally regulated in J82 cells. Akt is downstream from PI3K and pTEN, and its activation is positively regulated by PI3K and negatively regulated by pTEN (46). Previously, we showed that expression of oncogenic H-Ras in 10T1/2 cells induces the PI3K pathway to activate Akt, and blockage of PI3K activity enhances FR901228-induced apoptosis in both nontransformed and Ras-transformed 10T1/2 cells (13). Clearly, the PI3K pathway plays an antiapoptotic role in FR901228-induced cell death of mouse cells. Here, expression of oncogenic H-Ras in J82 cells, in which the pTEN gene is deleted (16), did not increase phosphorylation of Akt. Suppression of PI3K activity did not change FR901228-reduced cell viability. Thus, the PI3K pathway is unlikely to play a role in FR901228-induced cell death of either J82 or J82-Ras cells. On the other hand, the p38/SAPK pathway was also not induced by oncogenic H-Ras in J82 cells. However, in contrast to a suppression of the p38/SAPK pathway induced by FR901228 in parental J82 cells, FR901228 did not suppress the p38/SAPK pathway in J82-Ras cells. Inhibition of p38 activity attenuated the FR901228-reduced cell viability in J82-Ras cells, but not in parental J82 cells, indicating that a proapoptotic role of the p38/SAPK pathway was induced in J82-Ras cells responding to FR901228-induced apoptosis. On the other hand, inhibition of JNK activity enhanced the FR901228-reduced cell viability in parental J82 cells, but not in J82-Ras cells, indicating a survival role of the JNK/SAPK pathway in parental J82 cells responding to FR901228-induced apoptosis. Possibly, expression of oncogenic H-Ras in J82 cells had suppressed the survival JNK activity; FR901228 treatment further reduced JNK phosphorylation as a contributing factor to the resulting cell death. These unconventionally regulated pathways may indicate an outcome from multiple mutations occurring in human cancers.

Current Ras-targeted anticancer approaches mainly focus on inhibition of Ras protein synthesis, interference with Ras processing to functional sites, or blockage of downstream Ras effectors, approaches based on understanding the roles of oncogenic Ras in tumorigenesis (5). Growing evidence indicates that transformed cells are much more sensitive than normal cells to growth-inhibitory and apoptotic effects of HDACIs (6, 47). Other evidence has shown that anticancer agents, including 5-fluorouracil (48), etoposide VP16 (49), cisplatin (50), lovastatin (51), and arsenic (52), selectively induce apoptosis of oncogenic H-Ras–expressed cells. Our studies verified the proapoptotic activity of oncogenic H-Ras that facilitated HDACIs to induce cell death of human urinary bladder cancer J82 cells. Our results suggest a potential value of HDACIs in treating human cancers involving activation of H-Ras. However,
whether human cancers involving H-Ras overexpression are potential targets for HDACI therapy needs to be studied. Although the precise role of H-Ras activation in urinary bladder tumorigenesis remains controversial, clinical and basic studies have suggested that activation of H-Ras accompanied by inactivation of tumor suppressor p53 plays important roles in tumorigenesis to high-grade invasive urothelial tumors (53, 54). Our previous studies showed that expression of oncogenic H-Ras alone in mouse embryo fibroblasts induces cellular transformation and tumorigenicity in immune-deficient mice and increases cell susceptibility to FR901228 for inducing apoptosis (12–14). Our current report indicates that expression of oncogenic H-Ras in bladder tumor J82 cells, which host mutant p53, Rb, and pTEN genes, promotes tumorigenesis and increases cell susceptibility to HDACIs for inducing apoptosis. Possibly, HDACI therapy may be applicable to human cancers that acquire oncogenic H-Ras mutation at various stages of tumorigenesis but that possibility needs to be verified clinically. FR901228 has been shown to exhibit strong activities inhibiting class I HDAC-1 and HDAC-2, mild activity inhibiting class II HDAC-4, and weak activity inhibiting class II HDAC-6, assayed in vitro (55). Some studies have suggested that class I HDACs are important in the regulation of proliferation and survival in cancer cells (7, 56, 57). Selective inhibition of class I HDACs by FR901228 conceivably contributes to its selectivity in control of cancer cells. In addition, a recent report showed that ectopic expression of oncogenic H-Ras elevates reactive oxygen species in ovarian epithelial cells (58). Two HDACIs, SAHA and MS-275, have been shown to cause an accumulation of reactive oxygen species in transformed cells but not in normal cells (59). Whether expression of oncogenic H-Ras and FR901228 treatment additively increased reactive oxygen species in the selective induction of apoptosis of J82-Ras cells needs to be clarified. Multiple mutations occur within human cancer cells; consequently, affected signaling pathways are unconventionally regulated. It is important that the values of individual molecular targets need to be considered in individual cases for designing therapeutic protocols using HDACIs in combination with other agents to treat human urinary bladder cancers involving H-Ras activation.

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