A novel combination: ranpirnase and rosiglitazone induce a synergistic apoptotic effect by down-regulating Fra-1 and Survivin in cancer cells

Maria E. Ramos-Nino¹ and Benjamin Littenberg²

Departments of ¹Pathology and ²Medicine and Nursing, University of Vermont, Burlington, Vermont

Abstract
Accumulating evidence supports the idea that two known phosphatidylinositol 3’-kinase (PI3K) downstream proteins, Fra-1 and Survivin, are potential targets for cancer therapy. Increased expression of Fra-1, a Fos family member of the transcription factor activator protein-1, has been implicated in both the maintenance and the progression of the transformed state of several cancer cells. In addition, high Survivin expression in tumors correlates with more aggressive behavior, lower response to chemotherapeutic drugs, and shortened survival time. Previously, we reported that, in malignant mesothelioma cells with increased PI3K activity, small-molecule inhibitors of the PI3K/AKT pathway acted cooperatively with the amphibian RNase chemotherapy drug ranpirnase to inhibit cell growth. Because the thiazolidinedione antidiabetic drug rosiglitazone targets the PI3K/AKT pathway, we investigated the effect of the combination of these two drugs in cell survival in several cancer cell lines. We show here that the combination of ranpirnase and rosiglitazone synergistically decreases cell viability and increases cell apoptosis in several cancer cell lines. Cell killing is associated with decreased Fra-1 and Survivin expression and knockdown of Fra-1 increases cell killing by ranpirnase in a dose-dependent manner but not by rosiglitazone. The drug combination does not have a synergistic effect on killing in Fra-1 knockout cells, showing that Fra-1 modulation accounts in part for the synergism. The novel drug combination of ranpirnase and rosiglitazone is a promising combination to treat cancers with increased PI3K-dependent Fra-1 expression or Survivin. [Mol Cancer Ther 2008; 7(7):1871–9]

Introduction
Fra-1 and Survivin are key signaling proteins in the development and progression of various cancers. Fos family member Fra-1 accumulates in transcription factor activator protein-1 complexes during the malignant progression of several tumors and has been implicated in both the maintenance and the progression of the transformed state. The causal role of Fra-1 in cellular transformation has been documented in several systems, including esophagus (1), breast (2, 3), and thyroid (4, 5). Ectopic expression of Fra-1 in vitro increases the cell motility and metastatic behavior of mammary adenocarcinoma cells (6) and lung epithelial cells (7). Lastly, Fra-1 is induced by lung carcinogens, such as cigarette smoke and asbestos (7). Fra-1 is a predominant component of the activator protein-1 complex in asbestos-induced mesothelioma and proliferating rat mesothelioma cells, whereas overexpression of a dominant-negative Fra-1 mutant inhibits the growth of these cells in soft agar (8). Survivin is an IAP protein abundantly expressed in fetal tissues (9) and neoplasms (10) but undetectable in most normal, terminally differentiated adult tissues (10). High Survivin expression by tumors correlates with more aggressive behavior, decreased response to chemotherapy agents, and shortened survival times compared with Survivin-negative cancers (reviewed in ref. 11). These findings support Fra-1 and Survivin as potential targets for cancer therapy.

Here, we present a novel drug combination (ranpirnase and rosiglitazone) capable of down-regulating both Fra-1 and Survivin in several cancer cell lines. The anticancer effect of ranpirnase (reviewed in ref. 12) has been documented both in vitro (13–19) and in vivo (14, 16, 18, 20). This amphibian RNase drug holds promise as a chemotherapeutic tool, thanks to its low toxicity to normal cells and effectiveness against cancer cells. Previously, we reported that, in malignant mesothelioma cells with increased kinase activity levels of AKT, both LY294002 and wortmannin [two inhibitors of the phosphatidylinositol 3’-kinase (PI3K)/AKT pathway] act cooperatively with ranpirnase to inhibit cell growth (19). Because the antidiabetic thiazolidinedione, peroxisome proliferator-activated receptor (PPARγ) agonist, rosiglitazone down-regulates the PI3K/AKT pathway, and because thiazolidinediones are potentially useful in treating several cancers (21, 22) through both PPARγ-dependent and PPARγ-independent mechanisms, we investigated the combination of the thiazolidinediones rosiglitazone and ranpirnase. Specifically,
we tested the combination of ranpirnase and rosiglitazone in several cancer cell lines, showing that two PI3K down-stream targets, Fra-1 (23) and Survivin (24), are down-regulated by the combination. Furthermore, we showed a synergistic, apoptotic effect of ranpirnase and rosiglitazone in some cancer cell lines directly associated with the expression of Fra-1. This drug combination could be an important chemotherapeutic alternative in some cancers.

**Materials and Methods**

**Cell Lines**

The breast cancer cell lines MDA-MB-231 (231), T47D, and MCF7, the ovarian cancer cell lines SKOV-3 and CAOV-3, the prostate cancer cell lines PC3, DU-145, and LnCaP, the lung carcinoma cell lines NCI-H292 (H292) and N1792 (all from the American Type Culture Collection), and the mesothelioma cell lines MP5 and MP2 (kindly donated by Dr. Harvey Pass, New York University) were maintained in frozen stocks. Cells were incubated at 37°C in 5% CO2 until ~80% to 90% confluency in DMEM (Life Technologies) containing 5% fetal bovine serum (FBS) and 1 g/L glucose. Cells were then starved overnight in DMEM containing 0.5% FBS before treatments with ranpirnase, rosiglitazone, or in combination for 2 or 6 days and then collected. When indicated, medium was changed again after treatment to DMEM/F-12 containing 0.5% FBS and 1 A mol/L insulin to activate the PI3K pathway.

**Small-Molecule Inhibitors and Chemicals**

Stock solution of the PI3K small-molecule inhibitor LY294002 was diluted in DMSO and used at effective nontoxic concentrations (20 A mol/L; ref. 25; Calbiochem). Ranpirnase (Onconase, kindly provided by Dr. Kuslima Shogen, AlfaCell) was used at three concentrations (0.1, 1, and 10 A g/mL medium) and prepared as aliquots from a lyophilized stock solution subsequently frozen at −20°C. Rosiglitazone (Cayman Chemical) was dissolved in DMSO and used at concentrations of 10 and 20 A mol/L (<IC50 for all cells tested). GW9662 (Cayman Chemical), an irreversible PPARγ antagonist, was prepared in the same manner and used at a concentration of 2 A mol/L. All untreated control cells received DMSO in medium.
MTS Assay for Cell Viability

Assays were done on 96-well microtiter plates after plating of $7.7 \times 10^4$ per well. Cells were then cultured for 24 h in complete medium before changing to medium containing 0.5% FBS with ranpirnase at 0.1, 1, or 10 µg/mL, rosiglitazone at 10 or 20 µmol/L, or their six combinations or DMSO-containing medium (solvent control). Cell viability was measured by the colorimetric MTS Assay, CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), per the manufacturer’s recommendations. MTS is a tetrazolium salt that undergoes a color change caused by its bioreduction of MTS into a water-soluble formazan. The conversion of MTS into the aqueous-soluble formazan is accomplished by dehydrogenase enzymes found in active mitochondria, with reaction occurring only in living cells. The quantity of formazan product measured by the amount of 490 nm light absorbance is directly proportional to the number of living cells in culture. Briefly, 20 µL MTS reagent was added per well, and plates were incubated at 37°C for 2 to 3 h. Finally, the absorbance of each well was read at 490 and 650 nm. ΔA from these two wavelengths were reported as the corrected viability. Fold changes were calculated with respect to the control as a measure of cell viability.

Flow Cytometry

Near-confluent cells were maintained in complete medium containing 0.5% FBS overnight before addition of ranpirnase at 1 µg/mL, rosiglitazone 20 µmol/L, or their combination or DMSO-containing medium (solvent control). At 48 h, medium was removed and adherent cells were harvested by trypsinization. Combined cells were resuspended at $10^6$/mL in staining solution (50 µg/mL propidium iodide, 0.1% Triton X-100, and 32 µg/mL RNase A) in PBS and incubated for 30 min at 37°C before analysis of 10,000 cells per group per time point in triplicate. The distribution of cells, including cells with a hypodiploid DNA content indicative of apoptosis or necrosis, was determined using a Coulter Epics Elite flow cytometer and appropriate software as described previously (26). To determine number of apoptotic cells, cells were stained with Annexin V and propidium iodide in the dark for 15 min and 5,000 events per sample and analyzed by flow cytometry.

Figure 2. Treatment of cancer cells with the combination of ranpirnase and rosiglitazone for 48 h increased the proportion of sub-G0-G1 cells in 5 of 12 human cancer cell lines as determined by flow cytometry using propidium iodide. A, T47D; B, MCF7; C, 231; D, LNCaP; E, DU-145; F, PC3; G, SKOV3; H, CAOV3; I, MP5; J, MP2; K, H292; L, H1792. Columns, mean of two samples per group; bars, SE. Experiments were repeated twice. *, P < 0.05 in comparison with control; †, P < 0.05 in comparison with both single treatments.
cytometry as described above. For staining, cell pellets were suspended in 93 μL of 1× binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂], 5 μL propidium iodide at a final concentration of 2.5 μg/mL (Sigma), and 2 μL FITC-labeled Annexin V (BD Bioscience). Cells with Annexin V–positive staining were scored as apoptotic.

Growth Curves

Cells (n = 2-3 plates per group per time point) were plated at ~1 × 10⁵ per six-well plate in complete medium, allowed to attach for 24 h, and then treated with inhibitors at different time points. Cells were removed by trypsinization, and aliquots were counted using a hemocytometer to determine total cell number.

Western Blot Analyses

Near-confluent MM cells were washed three times with cold PBS before centrifugation at 14,000 rpm for 1 min. The pellet was resuspended in lysis buffer [20 mmol/L Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mmol/L NaCl, 2 mmol/L EDTA, 25 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 2 mmol/L pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 1 mmol/L DTT, 10 mmol/L NaF, and 1% aprotinin], incubated at 4°C for 15 min, and centrifuged at 14,000 rpm for 20 min. The amount of protein in each supernatant was determined using the Bio-Rad protein assay (Bio-Rad). Protein (30 μg) in sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L DTT, and 0.1% (w/v) bromophenol blue] was electrophoresed in 10% SDS-polyacrylamide gel and transferred to nitrocellulose using a semidry transfer apparatus (Ellard Instrumentation). Blots were blocked in buffer [TBS containing 5% nonfat dry milk + 0.1% Tween 20 (Sigma)] for 1 h, washed three times for 5 min each in TBS/0.1% Tween 20, and incubated at 4°C with an antibody specific to Fra-1 (R-20) at a 1:500 dilution or Survivin (D8) at 1:100 dilution (Santa Cruz Biotechnology). Blots were then washed three times with TBS/0.1% Tween 20 and incubated with a specific peroxidase-conjugated secondary antibody for 1 h. After washing blots three times in TBS/0.1% Tween 20, protein bands were visualized with the LumiGlo enhanced chemiluminescence detection system (Kirkgaard and Perry Laboratories) and quantitated by densitometry (27). Blots were reprobed with an antibody to α-Tubulin (Santa Cruz Biotechnology) to validate equal loading between lanes.

Constructs and Transfection Techniques

siFra-1 RNA interference duplexes were constructed from sequence information on mature mRNA extracted from the EST database using the open frame region from the cDNA sequence of exon 2 of the Fra-1 gene. The small

Figure 3. Cancer cells resistant to synergistic killing by the combination of ranpirnase and rosiglitazone were tested for cell growth (by cell count) and apoptosis (by count of Annexin V–positive cells by flow cytometry) after 6 d of treatment. A and B, PC3. C and D, H1792. E and F, 231. Mean ± SE of two samples per group. Experiments were repeated twice. *, P < 0.05 in comparison with control; †, P < 0.05 in comparison with both single treatments.

interfering RNA pool sequences targeting Fra-1 corresponded to the 107 to 126, 124 to 143, and 230 to 249 coding regions relative to the first nucleotide of the start codon. The sequences were BLAST searched (National Center for Biotechnology Information database) against EST libraries to ensure the specificity of the small interfering RNA molecule. The small interfering RNA duplexes or a scramble control were transfected into cancer lines using LipofectAMINE 2000 (Invitrogen) as recommended by the manufacturer. Cells were incubated with complexes overnight, and the medium was replaced the next day. Cells were allowed to recover for 48 h before treatments.

**SYBR Green Real-time Quantitative PCR**

Total RNA (1 µg) was reverse transcribed with random primers using the Promega AMV Reverse Transcriptase kit according to the recommendations of the manufacturer. PCR amplifications were done using the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Reactions were done in a 50 µL reaction mixture that included 25 µL SYBR Green JumpStart Taq ReadyMix (Sigma), distilled H₂O, DNA template, and 0.2 µmol/L each primer from QuantiteTect primer assays (Qiagen). Amplification was done by initial denaturation at 94°C for 2 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension for 1 min at 72°C. This was followed by a dissociation cycle of 95°C for 15 s, 60°C for 15 s, and 95°C for 15s. Threshold cycles (Ct) for both Fra-1 mRNA and the 18S rRNA control were determined. Original input RNA amounts were calculated using the comparative Ct method (2^(-ΔΔCt)) to analyze changes in gene expression in the samples relative to the untreated control sample. Duplicate assays were done with RNA samples isolated from at least two independent experiments. The values obtained from cDNA and 18S controls provided relative gene expression levels for the gene loci investigated (25).

**Immunofluorescence**

Dual confocal fluorescence approaches were used to determine if colocalization of Fra-1 was specific to nuclear proliferating cell nuclear antigen (PCNA)–positive cells. Cells grown on coverslips were fixed in 100% methanol for 1 h on ice, washed in PBS, and incubated in 0.1% Tween 20 in PBS for 30 min at room temperature. After incubation in blocking solution (2% dry milk, 0.1% Tween 20 in PBS) for 30 min at room temperature, cells were incubated with a cocktail of primary antibodies [mouse anti-PCNA (PharMingen; 1:1,000) and rabbit polyclonal anti-Fra-1 antibody (R-20; Santa Cruz Biotechnology; 1:100)] for 1 h at room temperature. Cells were washed twice for 20 min in blocking solution and once for 10 min in PBS. PCNA was detected using Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes) and diluted 1:400 in 10 µg/mL bovine serum albumin/PBS. Fra-1 was detected using Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes) and diluted 1:200 in 10 µg/mL bovine serum albumin/PBS. Controls were run using only primary or secondary antibodies. Following a final wash in PBS, sections were counterstained with SYTOX Green (1:1,000 in PBS; Molecular Probes), washed once in PBS, mounted on glass slides using AquaPoly/Mount (Polysciences), and examined using
for each sample, confocal images were collected in fluorescence modes followed by electronic merging of the images.

**Statistical Analyses**

All experiments used multiple replicate determinations \((n = 2, 3, \text{or } 8)\) per group, per time point. Experiments were done in duplicate. Results were evaluated by one-way ANOVA using the Student’s-Newman-Keuls’ procedure for adjustment of multiple pairwise comparisons between treatment groups. Differences with \(P\) values <0.05 were considered statistically significant.

**Results**

**Combination of Ranpirnase and Rosiglitazone Synergistically Reduced Viability and Increased Death in Several Cancer Cell Lines**

To test the hypothesis that the combination of the two drugs is synergistically antineoplastic, all 12 cancer cell lines were treated with ranpirnase (1 \(\mu\)g/mL), rosiglitazone (20 \(\mu\)mol/L), or their combination for 48 h. Cell viability measured by MTS assay showed that the combination of ranpirnase and rosiglitazone had a significant synergistic effect on the reduction of cell viability in 7 of the 12 cell lines tested (Fig. 1).

**Combination of Ranpirnase and Rosiglitazone Increased the Proportions of sub-G0-G1 Cells**

To prove the hypothesis that ranpirnase increased cell killing cooperatively with rosiglitazone, we determined the effects of ranpirnase (1 \(\mu\)g/mL), rosiglitazone (20 \(\mu\)mol/L), and their combination on the cell cycle kinetics of the 12 cancer cell lines. As early as 48 h after treatment, 5 of the 12 cell lines showed a significant increases \((P < 0.05)\) in the proportions of cells in sub-G0-G1 (apoptotic and necrotic cells; Fig. 2). These changes occurred with concomitant decreases in cells in G0-G1 and increases in the numbers of cells in G2-M, suggesting cell cycle arrest (data not shown).

**Combination of Ranpirnase and Rosiglitazone Triggered a Reduction in Proliferation and Increased Apoptosis in Several Cancer Cell Lines**

To determine the mechanism of cell death from the combination of ranpirnase and rosiglitazone, PC3, H1792,
and 231 cell lines were examined using the Annexin V assay to detect apoptotic cell death after 6 days of treatment (Fig. 3B, D, and F). These cell lines were originally selected because of their resistance to the drug combination after 48 h of treatment in previous tests (Fig. 2). Compared with control cells, significantly higher concentrations of Annexin V–positive cells were observed. Longer-term treatment showed that even these relatively resistant cell lines underwent apoptosis when exposed to the combination for 6 days. Growth curves (Fig. 3A, C, and E) also showed a synergistic decrease in cell growth at 6 days with the combination of ranpirnase and rosiglitazone.

Combination of Ranpirnase and Rosiglitazone Decreased Expression of Fra-1 and Survivin

Fra-1 expression in mesothelioma cell lines is regulated by the ERK1/2 or PI3K pathways in a cell-dependent manner (25). To determine which of these two pathways is affected by the combination, we selected two mesothelioma cell lines. The MP5 line has been shown previously to have a PI3K-dependent Fra-1 expression, whereas the MP2 line has been shown to have an ERK1/2-dependent Fra-1 expression (25). Ranpirnase and rosiglitazone in combination down-regulated Fra-1 only in the cell line with a PI3K-dependent Fra-1 expression but not in the other cell line (Fig. 4). The use of the PI3K inhibitor LY294002 further confirmed the PI3K effect on Fra-1 expression.

Survivin, another PI3K-dependent protein, was also down-regulated by the PI3K inhibitor and by the combination of ranpirnase and rosiglitazone in the MP5 cell line but only by the drug combination in the MP2 cell line. A second set of cancer lines (231 and DU-145) was treated for 48 h with ranpirnase, rosiglitazone alone, or in combination and then washed and incubated for 3 h with fresh DMEM/F-12 complete medium containing 0.5% FBS and 1 μmol/L insulin. These conditions were shown previously to result in a peak Fra-1 expression in these cell lines (data not shown). Results under these favorable conditions for Fra-1 expression still showed a synergistic down-regulation of Fra-1 in both cell lines (Fig. 5). Down-regulation of Survivin was observed only in the DU-145 cell line.

Decreased Expression of Fra-1-Induced Apoptosis

To determine if the down-regulation of Fra-1 accounted for the apoptotic effect produced by the drug combination, all cell lines were transfected with siFra-1 or scramble control and tested for Fra-1 knockout by real-time quantitative PCR. Only the cells showing >50% reduction in Fra-1 expression were used in this experiment (Fig. 6A). After cell transfection with the RNA interference, cells were left for 5 days and tested for apoptosis using the Annexin V assay. As shown in Fig. 6B, significantly increased apoptosis was observed in cell lines MP5, 231, and H292. The use of immunofluorescence showed that proliferating cells expressed nuclear Fra-1; dying cells did not (Fig. 6B).
Fra-1 Expression Increased Drug Resistance to Ranpirnase

To directly observe if the knockdown of Fra-1 increased the efficacy of ranpirnase, rosiglitazone, or their combination, cell line H292 (a cell line with high transfection efficiency) was studied. Cells were transfected with siFra-1 or scramble control and left to rest for 48 h before treatment. Cells were then treated with different concentrations of ranpirnase (0.1, 1, or 10 μg/mL), rosiglitazone (10 or 20 μmol/L), or their combination for 48 h. The PPARγ inhibitor GW9662 (2 μmol/L) was added to observe the influence of PPARγ. Figure 7 shows that ranpirnase reduced viability in a dose-dependent manner. The knockdown of Fra-1 significantly increased the efficacy of ranpirnase alone at all concentrations but not rosiglitazone alone. The use of the drug combination produced a synergistic effect on the scramble control transfected cells but not in Fra-1 knockdown cells. The synergistic effect of rosiglitazone and ranpirnase was not changed by the modulation of PPARγ.

Discussion

In human mesothelioma cell lines, we have shown previously that the increased expression of survival pathways, frequently activated in cancer as the PI3K pathway (28), predicts the efficacy of chemotherapeutic drugs such as cisplatin and ranpirnase (19, 29). The data presented here show that the combination of ranpirnase with rosiglitazone, an antidiabetic drug targeting the PI3K pathway (30), results in synergistic killing of several cancer cell lines. The mechanism of cell death was determined to involve cell apoptosis. We also investigated whether two recognized PI3K-regulated proteins [Fra-1 (25) and Survivin (24)], key to the development and maintenance of several cancers, were modulated by the drug combination. Furthermore, we investigated the effect of knocking down Fra-1 on the cell killing and apoptosis found with combinations of ranpirnase with rosiglitazone.

Under the experimental conditions of our studies, the combination of ranpirnase and rosiglitazone down-regulates Fra-1 in a cell-dependent manner. We have shown previously that the regulation of Fra-1 in mesothelioma could be dependent on the ERK1/ERK2 pathway (8, 25) or on the PI3K pathway (25). The down-regulating effect on Fra-1 produced by the combination of ranpirnase and rosiglitazone suggests that the combination acts through PI3K.

Results presented here also show that the knockdown of Fra-1 can induce apoptosis. This finding could partially explain the killing effect of the drug combination in these cell lines. The knockdown of Fra-1 in the cell line H292 further shows that the synergistic effect of the two drugs is partially related to the modulation of Fra-1. To test whether the synergism between these two drugs was related to the ability of rosiglitazone to activate PPARγ, we used a PPARγ antagonist together with the combination. The synergistic effect was independent of PPARγ.

Clinical trials of ranpirnase alone (Onconase) showed heterogeneity in therapeutic efficacy (19). These differential responses might reflect varying survival signaling mechanisms. Our results suggest that combined therapeutic use of ranpirnase and rosiglitazone may overcome the resistance produced in some cancer cells by the activation of survival pathways and their targets. Further in vitro studies are warranted.

Conclusions

Antineoplastic drug efficacy depends, in part, on the survival signaling pathways activated in specific tumor cells. The novel drug combination of ranpirnase and rosiglitazone is a promising combination to treat cancers with increased PI3K-dependent Fra-1 expression or Survivin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Molecular Cancer Therapeutics

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Maria E. Ramos-Nino and Benjamin Littenberg


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