Reexpression of Tumor Suppressor, sFRP1, Leads to Antitumor Synergy of Combined HDAC and Methyltransferase Inhibitors in Chemoresistant Cancers

Simon J. Cooper1, Christina A. von Roemeling1, Kylie H. Kang1, Laura A. Marlow1, Stefan K. Grebe2, Michael E. Menefee3, Han W. Tun3, Gerardo Colon-Otero3, Edith A. Perez3, and John A. Copland1

Abstract

Metastatic solid tumors are aggressive and mostly drug resistant, leading to few treatment options and poor prognosis as seen with clear cell renal cell carcinoma (ccRCC) and triple-negative breast cancer (TNBC). Therefore, the identification of new therapeutic regimes for the treatment of metastatic disease is desirable. ccRCC and TNBC cell lines were treated with the HDAC inhibitor romidepsin and the methyltransferase inhibitor decitabine, two epigenetic modifying drugs approved by the U.S. Food and Drug Administration for the treatment of various hematologic malignancies. Cell proliferation analysis, flow cytometry, quantitative PCR, and immunoblotting techniques were used to evaluate the antitumor synergy of this drug combination and identify the reexpression of epigenetically silenced tumor suppressor genes. Combinatorial treatment of metastatic TNBC and stage IV ccRCC cell lines with romidepsin/decitabine leads to synergistic inhibition of cell growth and induction of apoptosis above levels of individual drug treatments alone. Synergistic reexpression of the tumor suppressor gene secreted frizzled-related protein one (sFRP1) was observed in combinatorial drug-treated groups. Silencing sFRP1 (short hairpin RNA) before combinatorial drug treatment showed that sFRP1 mediates the growth inhibitory and apoptotic activity of combined romidepsin/decitabine. Furthermore, addition of recombinant sFRP1 to ccRCC or TNBC cells inhibits cell growth in a dose-dependent manner through the induction of apoptosis, identifying that epigenetic silencing of sFRP1 contributes to renal and breast cancer cell survival. Combinatorial treatment with romidepsin and decitabine in drug resistant tumors is a promising treatment strategy. Moreover, recombinant sFRP1 may be a novel therapeutic strategy for cancers with suppressed sFRP1 expression. Mol Cancer Ther; 11(10); 2105–15. ©2012 AACR.

Introduction

Cancer is a multistep process facilitated by the accumulation of genetic abnormalities resulting in genomic instability and the mutation of tumor suppressor and oncogenic genes. Furthermore, epigenetic changes in cancer lead to modulations of gene expression through mechanisms of DNA methylation and histone deacetylation. The hypermethylation of cytosines in areas of rich CpG islands and deacetylation of histones that facilitate a tighter formation of chromatin contribute to the inappropriate silencing of gene expression. Histone deactylase (HDAC) inhibitors such as trichostatin A and romidepsin (FK228), and methyltransferase inhibitors such as decitabine (DAC; 5-aza-2’-deoxycytidine) are capable of reversing these epigenetic events and suppressing the cancer phenotype. The HDAC inhibitor romidepsin exhibits antitumor properties in human cell lines both in vitro and in vivo (1). Studies have identified that romidepsin treatment of tumor cells leads to inhibition of angiogenesis and cell growth, while inducing apoptosis, cell death, and cell differentiation (2–6). Romidepsin was approved by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma in 2009, and for peripheral T-cell lymphoma (PTCL) in 2011. It continues to be actively investigated as an anticancer therapeutic for both hematological and solid malignancies.

Methyltransferase inhibitors are analogues of cytosine that incorporate into the DNA during replication before covalently linking with DNA methyltransferases leading to global loss of gene methylation (7). Treatment of cancer cell models with the methyltransferase inhibitor decitabine leads to suppression of growth and apoptosis through reexpression of silenced genes and the activation

Authors’ Affiliations: 1Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center; 2Departments of Medicine and Laboratory Medicine & Pathology, Mayo Clinic, Rochester, Minnesota; and 3Division of Hematology/Oncology, Internal Medicine Department, Mayo Clinic, Jacksonville, Florida

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S.J. Cooper and C.A. von Roemeling contributed equally to the work.

Corresponding Author: John A. Copland, Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center, 4500 San Pablo Road, Jacksonville, FL 32224; Phone: 904-953-6120; Fax: 904-953-0277; E-mail: copland.john@mayo.edu

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of p53 and p21Waf1/Cip1 (8–10). Studies have identified that decitabine causes G2 arrest, reduces clonogenic survival, and inhibits growth while causing DNA fragmentation and activating the ATM and ATR DNA repair pathways (11). In 2006, decitabine was FDA approved for the treatment of myelodysplastic syndromes (MDS).

Constitutive activation of the Wnt signaling pathway as a mechanism for cancer development was first identified in colon cancer (12). The binding of secreted Wnt family members to frizzled receptor complexes on the cell surface leads to activation of downstream gene targets through either the canonical/β-catenin pathway or one of the noncanonical/β-catenin–independent pathways (13). Composition of the Wnt/frizzled complex governs which of these pathways are activated. Canonical Wnt signaling influences genes associated with cell proliferation, survival, and invasion (14), whereas noncanonical pathways regulate those involved in cell adhesion, migration, and cytoskeletal reorganization (15). sFRP1, secreted frizzled-related protein 1, functions as a negative regulator of Wnt signaling by sequestering Wnt proteins and heterodimerizing with frizzled to form nonfunctional receptor complexes. However, in colorectal, ovarian, lung, hepatocellular, kidney and breast cancer, hypermethylation of the sFRP1 promoter, and subsequent loss of expression has been identified allowing aberrant Wnt signaling (14, 16–20).

Renal cell carcinoma (RCC) is the third most prevalent urological cancer, and is the tenth most common cause of cancer death in men and ninth in women (21). Clear cell RCC (ccRCC) is the largest subtype of RCC and accounts for approximately 80% of renal cancers. Breast cancer is the most common cancer in women with triple-negative breast cancer (TNBC), accounting for approximately 15% of newly diagnosed cases. TNBCs are associated with poor prognosis, a higher mitotic index, and younger age (22). In ccRCC and breast cancer, early diagnosis and treatment dramatically increase median survival rates as when metastatic, these cancers are mostly aggressive and drug resistant. Development of metastatic disease in ccRCC patients reduces the 5-year survival rate to less than 10% (23) and in TNBC reduces survival to around 18 months (24). Therefore, there is a dire need for new chemotherapeutic drug therapies in these drug resistant cancers.

Materials and Methods

Reagents

Romidepsin was generously provided by Celgene Corporation and the National Cancer Institute (NCI). Decitabine (5-aza-2'-deoxycytidine) was purchased from Sigma-Aldrich and recombinant human sFRP1 from R&D Systems.

Cell line verification

Genomic DNA was used for short tandem repeat (STR) analysis of all cell lines at John Hopkins University Fragment Analysis Facility using the StemElite service in July 2010. Furthermore, KIJ265T cells were verified by AmpF/STR Identifier analysis (Applied Biosystems). The renal origin of KIJ265T cells was validated by Immunohistochemistry (Supplementary Fig. S1). Cells were fixed using 2% paraformaldehyde (Sigma), permeabilized using 1% Triton X-100 (Sigma), and blocked with diluent containing background reducing components (Dakocytomation) before staining with primary antibody. Control slides were prepared by excluding primary antibody during staining. Primary antibodies included RCC-Ma (Cell Marque Corporation), podocin (ABCAM), gamma glutamyl transpeptidase (Lifespan Biosciences), Pax2 (Lifespan), and aquaporin2 (Santa Cruz). The KIJ265T cells were identified to be VHL mutant (Exon 2 c.407T>C; protein modification of p.F136S) by DNA sequencing.

Cell culture

The A498, MDA-231, and BT20 cell lines (American Type Culture Collection) and KIJ265T cells (derived from primary tumor site stage IV human ccRCC patient tissue) were maintained in Dulbeccos’ Modified Eagles’ Medium (DMEM; Cellgro) supplemented with 10% FBS (Hyclone) and 1% glutamyl transpeptidase (Lifespan Biosciences), Pax2 (Lifespan), and aquaporin2 (Santa Cruz). The KIJ265T cells were identified to be VHL mutant (Exon 2 c.407T>C; protein modification of p.F136S) by DNA sequencing.

Drug treatments and proliferation assays

Cells were plated (1 × 10^5 per well) in 12-well plates (Midwest Scientific) and treatments carried out in triplicate. For monotherapeutic treatments, cells were treated for 72 hours with a dose range of 0.01 to 10 μmol/L decitabine or 0.01 to 100 nmol/L romidepsin. dimethyl sulfoxide (DMSO) was used for vehicle control. Cells were trypsinized and counted using a Coulter Particle Counter (Beckman). For combination doseouts, cells were treated with a dose range of 0.1, 1, or 10 μmol/L decitabine. After 48 hours, romidepsin was added in combination to the decitabine at a dose range of 0.5 to 7.5 nmol/L for a further 24 hours. Appropriate monotherapeutic and DMSO treatments were included and administered in accordance with combinatorial time points. An optimal combinatorial dose of 1 μmol/L decitabine for 72 hours with the addition of 5 nmol/L romidepsin for the last 24 hours was used in further treatments.

For treatment of cells with recombinant human sFRP1, MDA-231, and KIJ265T cells were seeded in 96-well culture plates at 5,000 cells per well in 100 μL supplemented
Samples were normalized to GAPDH and dehydrogenase (GAPDH; Hs99999905_m1) were used. (Hs00231122_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) were used. Samples were normalized to GAPDH and ΔΔCT methods used to calculate fold expression changes (25).

**Protein expression analysis**

Cells were seeded (1 × 10^5) on 100 mm plates (Midwest Scientific) and treated with the optimal combinatorial dose. Cells were lysed using M-PER reagent containing phosphatase inhibitors (Pierce) and protease inhibitor cocktail (Roche). Quantification and transfer to 0.2 μmol/L Immobilon Psq membranes were carried out as previously described (26). Membranes were incubated overnight in primary antibody [β-actin (Sigma-Aldrich), caspase-3, sFRP1, or PARP (Cell Signaling)] before treatment with secondary species-specific horseradish peroxidase-labeled antibodies (Jackson ImmunoResearch) for 45 minutes at room temperature. Supersignal chemiluminescent kit (Pierce) was used for detection.

**Cell death analysis via flow cytometry**

Cells were treated with the optimal combinatorial therapy with appropriate control groups. After 72-hour treatment, adhered and floating cells were collected and resuspended in ×1 cold binding buffer (BD Pharmingen) at 1 × 10^6 cells/mL. Cells were propidium iodide (BD Pharminogene) stained for 10 minutes and fluorescence-activated cell-sorting analysis conducted using Accuri C6 flow cytometer (Accuri). Unstained cells were used as controls for setting the cell population parameters.

**Methylation-specific PCR**

Genomic DNA was isolated from the 4 cell lines and treated with sodium bisulfite using the EZ DNA Methylation-Startup Kit (Zymo Research) according to manufacturer’s instructions. Purified bisulfite-treated DNA was amplified with primers specific for methylated or unmethylated sFRP1 at an annealing temperature of 58°C as previously described (14, 27). Positive control templates were bisulfite-treated Universal Methylated Human DNA. Sequence analysis of the methylation patterns of 10 clones for each cell line, treated with DMSO or combinatorial drug treatments, using previously published primers (14) were conducted at ATCG, Inc.

**Luciferase reporter assay**

KIJ265T and MDA231 cells were transfected with 0.5 μg per well TOPflash luciferase reporter construct (kind gift from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD), 0.5 μg/well sFRP1 expression vector (kind gift from Dr. Jeffrey Rubin, NIH/NCI) or pcDNA3.1 empty vector control (Invitrogen) and 10 ng/well control Renilla luciferase reporter using Fugene 6 transfection reagent (Roche). Cells were collected 48 hours posttransfection and reporter activity measured using the Dual Luciferase Kit (Promega). Data are normalized to Renilla luciferase and reported as mean ± SD of replicates of 3.

**Lentivirus and infections**

MISSION shRNA pLKO.1 constructs (Sigma-Aldrich) were used to make self-inactivating shRNA lentiviruses for sFRP1 [target sequence 5’-CGAGATGCTTAAGTGT-GACAA-3’ (clone NM_003012.3-758s1c1)], and a nontarget random scrambled sequence control (SHC002). For virus transduction, 2 × 10^5 MDA-231 or KIJ265T cells were incubated with lentivirus plus 5 μg/mL polybrene (American Bioanalytical) for 24 hours. Clones were identified by puromycin (Fisher Scientific) selection.

**Statistical analysis**

Data are presented as the mean ± SD and comparisons of treatment groups were analyzed by 2-tailed paired Student t test. Data for comparison of multiple groups are presented as mean ± SD and were analyzed by ANOVA. P < 0.05 was considered statistically significant.

**Results**

**Single drug therapy**

Individual 72-hour drug treatments of romidepsin (chemical structure Fig. 1A) or decitabine (chemical structure Fig. 1B) were evaluated in 2 ccRCC stage IV cell lines (A498 and KIJ265T) and 2 TNBC cell lines (MDA231 and BT20) for their ability to inhibit cell proliferation. Romidepsin was identified as a potent inhibitor of cell proliferation in all cell lines producing significant inhibition in the 2.5 to 100 nmol/L range (Fig. 1C). Treatment with decitabine had minimal effect on cell proliferation and was unable to provide an IC50 in the 0.01 to 10 μmol/L drug range (Fig. 1D). These data identify romidepsin as a potent inhibitor of cell proliferation in ccRCC and TNBC cell lines.

**Combination therapy induces synergistic responses in ccRCC and TNBC cell lines**

Combinatorial treatments of romidepsin and decitabine were evaluated for their ability to inhibit cell proliferation (Fig. 2). Drug doses identified in the single drug exposure...
experiments were evaluated in these investigations. Romidepsin doses were standardized to 0.1, 0.5, 2.5, and 5 nmol/L, with 5 nmol/L being a dose that induced approximately 50% cell death 72 hours posttreatment. Because decitabine had little effect upon cell proliferation, doses of 0.1, 1, and 10 μmol/L were chosen for combinatorial treatments. Treatment protocols for these studies evaluated the response of the cells to either a 24-hour treatment of romidepsin alone, a 72-hour treatment of decitabine alone or treatment for 72 hours of decitabine with the final 24 hours in combination with romidepsin. Please note, in these combinatorial therapy treatments, the cells were exposed to romidepsin for 24 hours and not 72 hours as in the single drug exposures (Fig. 1). In all cell lines combinational drug treatment induced greater inhibition of cell proliferation than single drug treatments alone.

Experimental cell lines were further evaluated for drug-induced cell death. Propidium iodide staining of cell lines treated with 5 nmol/L romidepsin or 1 μmol/L decitabine alone or in combination-identified synergistic induction of cell death in the combination drug therapy group (Fig. 3). In ccRCC KIJ265T cells, combinatory treatment induced cell death 21.1% above DMSO controls (Fig. 3B). For the other cell lines, A498, MDA231, and BT20, cell death with combination treatment was induced 13.6%, 10.7%, and 10.8%, respectively, compared with DMSO controls. Single drug exposures did not consistently induce cell death in the tested cell lines.

**Combinational treatment therapy induces apoptosis**

Drug-treated cells were analyzed for markers of apoptosis to elucidate mechanisms of cell death. Total proteins from cells treated under the optimal dosing regimen, with single or combinatorial therapy showed cleavage of caspase-3 and PARP only in the combinatorial drug-treated groups (Fig. 4A). These events were absent in single drug exposure and control groups indicating that in combination these drugs are potent inducers of apoptosis.

**sFRP1 expression is induced by combinational therapy**

To elucidate the molecular events taking place in cells treated in combination with romidepsin and decitabine, we analyzed molecular targets we have previously identified to be directly or indirectly affected by epigenetic silencing in cancer. Expression levels of protein and RNA from the cell lines were examined for RhoB (28, 29), p21Waf1/Cip1 (29), TβRIII (30), GATA3 (30), and sFRP1 (14) after single or combinational treatments (data not shown). Of these molecular targets, sFRP1 showed consistent synergistic upregulation of RNA and protein expression across all cell lines treated with combinatorial therapy (Fig. 4A and Table 1). Methylation-specific PCR established that the sFRP1 promoter was hypermethylated in all experimental cell lines. Treatment with decitabine alone or in combination with romidepsin led to demethylation of the sFRP1 promoter region (Fig. 4B) verifying that reexpression of sFRP1 at the RNA and protein level is because of the reversal of epigenetic silencing. Bisulfite DNA sequencing confirmed a global decrease of sFRP1 promoter methylation in all cell lines after combinatorial treatment compared with DMSO-treated controls (Supplementary Fig. S2).
Silencing of dual treatment-induced sFRP1 leads to gain of cell survival

Because sFRP1 is a tumor suppressor gene, we hypothesize that reexpression of sFRP1 in dual therapy-treated cancer cells is sufficient to modulate cell survival. Therefore, endogenous levels of sFRP1 were shRNA silenced in MDA231 and KIJ265T cell lines. Cells were treated with 5 nmol/L romidepsin and 1 μmol/L decitabine in combination and the expression of sFRP1 RNA message evaluated (Fig. 5A). Combinatorial treatment induced expression of sFRP1 in the KIJ265T and MDA231 nontarget cell lines approximately 600- and 1,300-fold, respectively, when compared with nontreated, nontarget controls. shRNA silencing of sFRP1 before combinatorial treatment reduced this expression by 6-fold or greater in KIJ265T and MDA231 cells. Loss of inducible sFRP1 was observed to reduce the effects of combinatorial treatment on KIJ265T and MDA231 cells. Following combinatorial treatment, the growth of KIJ265T sFRP1 shRNA silenced cells was unchanged compared with shRNA nontarget DMSO controls (Fig. 5B). Thus, the loss of inducible sFRP1 completely abolished the growth inhibitory effects of combinatorial treatment in KIJ265T cells. Combinatorial drug responses (romidepsin 5 nmol/L, decitabine 1 μmol/L) in sFRP1 shRNA silenced cells were 35% and 50% less responsive in KIJ265T and MDA231 cells than shRNA nontarget-treated controls (Fig. 5B). Total cell protein analysis showed that sFRP1 shRNA silenced cells had reduced levels of PARP cleavage identifying that cell survival after combinatorial treatment was because of an inhibited apoptotic response (Fig. 5C). Therefore, sFRP1 is a target of romidepsin/decitabine treatment and its reexpression plays a significant and critical role in the inhibition of cell growth/induction of cell death when these drugs are administered in combination.

Human recombinant sFRP1 inhibits cell proliferation

To verify that epigenetic silencing of sFRP1 is vital for TNBC and ccRCC cell survival, we reintroduced recombinant human sFRP1 to the cells and examined effects on cell proliferation and apoptotic cell death. Escalating doses of recombinant sFRP1 (142.8 pmol/L–1.4 nmol/L)
Figure 3. Synergistic induction of cell death in decitabine (5A2D) and romidepsin (Rom) combinatorial treated ccRCC and TNBC cell lines. A498 (A), KIJ265T (B), MDA231 (C), and BT20 (D) cell lines treated with monotherapeutic doses of decitabine or romidepsin were analyzed versus combination treatment for drug effects leading to cell death, analyzed via flow cytometry. Only combinatorial treatment was sufficient to induce cell death.
led to a dose-dependent decrease in KIJ265T and MDA231 cell proliferation (Fig. 5D) identifying that low nanomolar doses of sFRP1 are capable of inhibiting cancer cell growth. This inhibition was verified to be through induction of apoptosis 28 hours after a single dose of 1.4 nmol/L recombinant sFRP1 (Fig. 5D). Reexpression of sFRP1 by transient transfection in KIJ265T and MDA231 cells inhibited Wnt activated β-catenin/T-cell factor–mediated transcription as measured by the TOPflash reporter assay (Supplementary Fig. S3A) indicative that sFRP1 attenuates oncogenic Wnt signaling in ccRCC and TNBC cells. These data show that romidepsin and decitabine in combination are a potential drug therapy option for the treatment of ccRCC and TNBC, and that this mechanism of

![Figure 4. Analysis of sFRP1 expression and markers of apoptosis in ccRCC and TNBC cell lines treated with 1 μmol/L decitabine (5A2D) and 5 nmol/L romidepsin (Rom). A, protein lysates from treated A498, KIJ265T, MDA-231, and BT-20 cells were probed for PARP and caspase-3 cleavage identifying synergistic apoptotic escalation with combinatorial treatment. Accumulation of sFRP1 expression was observed with combination treatment. B, cell lines were examined for methylation status of the sFRP1 promoter and the ability of single and combinatorial drug treatments to modulate these methylation events via methylation specific PCR utilizing defined primers for the amplification of methylated or unmethylated sequences of sFRP1 (M, methylated, U, unmethylated).](image)

**Table 1.** Induction of sFRP1 RNA expression observed with combinatorial treatment

<table>
<thead>
<tr>
<th>sFRP1</th>
<th>Ctrl CT value</th>
<th>Ctrl fold change</th>
<th>5A2D fold change</th>
<th>Rom fold change</th>
<th>Rom:5A2D fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A498</td>
<td>38.5</td>
<td>1.0 ± 0.1</td>
<td>11.1 ± 2.9</td>
<td>1.4 ± 0.9</td>
<td>35.3 ± 2.5*</td>
</tr>
<tr>
<td>KIJ265T</td>
<td>34.3</td>
<td>1.0 ± 0.5</td>
<td>2.5 ± 0.7</td>
<td>20.6 ± 1.1</td>
<td>62.4 ± 6.6*</td>
</tr>
<tr>
<td>MDA231</td>
<td>39.2</td>
<td>1.0 ± 0.2</td>
<td>95.4 ± 15.9</td>
<td>4.8 ± 3.4</td>
<td>1,302.6 ± 49.5*</td>
</tr>
<tr>
<td>BT20</td>
<td>27.7</td>
<td>1.0 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>3.6 ± 0.4*</td>
</tr>
</tbody>
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NOTE: Statistically significant increases in RNA expression (*, P < 0.05 Student t test) are bold faced, with the highest reexpression values observed in combinatorial treatment groups designated with an asterisk.
activity is predominantly through synergistic reexpression of sFRP1.

Discussion

Despite recent progress made in therapeutics for ccRCC and TNBC, there is an urgent need for more effective therapies (31, 32). Our preclinical studies identify that combinatorial treatment of ccRCC and TNBC cell lines with decitabine and romidepsin leads to inhibition of cell proliferation and the induction of apoptosis as measured by caspase-3 and PARP cleavage. Combinatorial epigenetic treatment induces the reexpression of the Wnt signaling pathway antagonist sFRP1, an event that is not seen with single drug treatments alone, identifying that the epigenetic silencing of sFRP1 plays a prominent role in the onset and progression of clear cell RCC and TNBC. Furthermore, treatment of ccRCC and TNBC cells with low doses of exogenous sFRP1 produced dose-dependent inhibition of cancer cell growth (142.8 pmol/L–1.4 nmol/L) through induction of apoptosis. It is our belief that these findings reveal potential treatment options for those patients diagnosed with advanced ccRCC and TNBC. Because sFRP1 is a secreted protein, these data also suggest that sFRP1 reexpression could be used as a molecular biomarker of response to this combinatorial therapeutic regimen.

We and others have published that the promoter of sFRP1 is epigenetically silenced in all stages of ccRCC suggesting that silencing of sFRP1 is an early event in ccRCC carcinogenesis (14, 33, 34). In our earlier publication, reexpression of sFRP1 within a ccRCC cell line with no endogenous sFRP1 expression led to reduced tumor growth in vitro and in vivo. Recently, this was confirmed

Figure 5. Loss of sFRP1 facilitates cell survival in ccRCC and TNBC cell lines treated in combination with decitabine (5A2D) and romidepsin (Rom). A, real-time PCR of KIJ265T and MDA-231 cells infected with sFRP1 shRNA shows a significant decrease in sFRP1 reexpression with combinatorial drug treatment versus control (*, P < 0.05 Student t test). B, resulting loss of sFRP1 reexpression yielded an increase in cell survival of KIJ265T and MDA-231 cells when treated with combinatorial dose of 1 μmol/L 5A2D and 5 nmol/L Rem. C, attenuation of apoptosis in KIJ265T and MDA-231 sFRP1 knockdown cells was shown via reduction in PARP cleavage versus nontarget controls with combination treatment. D, decreased proliferation of KIJ265T and MDA-231 parent cell lines was observed in a dose-dependent manner when treated with recombinant human sFRP1 and verified to be through the induction of apoptosis as seen by PARP cleavage, after a single dose of 1.4 nmol/L sFRP1.
by others who also identified that loss of sFRP1 in RCC cells increases their tumorigenic potential and that methylation of sFRP1 in RCC was associated with reduced patient survival although this was not independent of tumor stage, size, and grade (35). In breast cancer, methylation silencing of sFRP1 is also associated with poor patient survival (27), whereas studies in breast cancer cells examining the role of sFRP1 on carcinogenesis have identified similar findings to those observed in ccRCC. Expression of sFRP1 in many breast cancer cell lines leads to decreased proliferation potential, decreased ERK1/2 activity, induced apoptosis, and suppressed colony formation (36, 37), whereas loss of sFRP1 enhances breast cancer growth via increases in cell viability (37). More importantly, xenograft models using the breast cancer cell line MDA-MB-231 identified that reexpression of sFRP1 led to inhibited xenograft outgrowth and lung metastasis in vivo (38). Taken together, these findings identify sFRP1’s critical role as a tumor suppressor in breast and renal cancer.

Furthermore, we have previously identified that a number of downstream targets of the Wnt signaling pathway are overexpressed in patient ccRCC tissue samples and that reexpression of sFRP1 in ccRCC cell lines inhibits their expression (14). There is also evidence that in both RCC and breast cancer epigenetic silencing of other Wnt antagonists occurs, implicating a role for aberrant Wnt signaling in tumorigenesis. In primary breast tumors, methylation silencing of sFRP1, 2, 5, and DKK1 was identified to be present in 40%, 77%, 71%, and 17% of all tissues, respectively (37). Loss of sFRP5 in breast cancer is associated with reduced overall survival (39) although loss of sFRP2 showed no correlation with patient outcome (40). In ccRCC, sFRP1, sFRP2, sFRP4, sFRP5, and DKK3 are frequently silenced in tumor tissues (14, 41–43). Reexpression of sFRP5 in RCC cells inhibits anchorage independent colony formation, cell invasion, and induces apoptosis suggesting that like sFRP1, sFRP5 is a tumor suppressor gene (41). Further analysis is required so that we can evaluate the role of these antagonists of Wnt signaling within our experimental renal and breast cancer cell lines. More importantly, it needs to be ascertained whether combination treatment with romidepsin and decitabine leads to the reexpression of these epigenetically silenced Wnt antagonists and the roles that they play on cell proliferation and apoptosis. Care must also be taken to identify that reexpression of these soluble Wnt antagonists does not lead to the activation of Wnt signaling as previously observed (44, 45). Clearly, multiple soluble Wnt antagonists are silenced in ccRCC and TNBC delineating the importance of Wnt upregulation in tumor promotion. Thus, it makes sense that reexpression of sFRP1 has a profound effect upon cell proliferation and cell survival as sFRP1 is known to bind and antagonize multiple Wnts as well as the Wnt membrane binding partners, frizzleds leading to Wnt/β-catenin signaling repression (18).

Decitabine and romidepsin have been approved by the FDA for treatment of MDS and cutaneous T-cell and PTCL, respectively. Very few clinical trials exist that examine the combinatorial effects of these 2 agents on human cancers. One clinical trial (NCT00037817) used a 72-hour IV infusion of decitabine followed by a 4-hour IV infusion of romidepsin for the treatment of patients with pulmonary and pleural malignancies while a recently completed phase II clinical trial assessed decitabine/romidepsin combinatorial treatments in patients with relapsed or refractory leukemia, MDSs, or myeloproliferative disorders (NCT00114257). Findings from both of these studies have yet to be published. Phase 1 clinical trials examining individual treatment of romidepsin in RCC patients at a dose of 9.1 mg/m² led to a partial response in one patient after two 21-day cycles of treatment and was maintained for a further 6 cycles (4). Treatment with decitabine at a dose of 75 mg/m² resulted in tumor stabilization in one RCC patient (46) and at dose levels of 0.15 and 0.25 mg/kg stable disease was observed in 3 patients (47). Although no clinical trials have been published with these individual agents in breast cancer, recent research identifies that poor prognosis breast cancers express high levels of deoxycytidine kinase (DCK) whose phosphorylation is needed for the pharmacological activity of decitabine. Screening for expression of DCK would allow for the selection of breast cancer patients that are more likely to respond to nucleoside analog treatments (48). From our findings, we identify that combinatorial treatment with decitabine and romidepsin is effective at inducing cell death in ccRCC and TNBC cell lines and therefore, clinical trials of this combination in patients with these solid tumors would be of interest.

We have identified in these studies, one epigenetically silenced tumor suppressor gene whose loss of expression plays a significant role in cancer cell survival. We understand that there are many more genes modulated by this drug treatment that we have not investigated. To truly understand the actions of the combinatorial treatment of romidepsin and decitabine in ccRCC and TNBC cell lines, genome wide analysis of gene regulation after treatment must be undertaken (49). We predict that there are a number of drug targetable genes that are reexpressed after combinatorial treatment that possess antitumor activity. We further suggest that the administration of chemotherapeutic or immunotherapeutic agents at the end of epigenetic combinatorial treatment could further increase the inhibition of cell growth or induction of cell death within drug resistant cancer cells. In single drug exposure studies, treatment of the ACHN ccRCC cell line with decitabine reversed resistance to interferon and in combination-induced 85% cell apoptosis (50). Thus, the analysis of decitabine/romidepsin modulated genes and the identification of novel signaling pathways could be of clinical relevance for the targeted treatment of ccRCC and TNBC.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S.J. Cooper, L.A. Marlow, H.W. Tun, E.A. Perez, J.A. Copland


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.J. Cooper, K.H. Kang, S.K. Grebe, J.A. Copland

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.J. Cooper, C.A. von Roemeling, L.A. Marlow, H.W. Tun, E.A. Perez, J.A. Copland

Writing, review, and/or revision of the manuscript: S.J. Cooper, C.A. von Roemeling, L.A. Marlow, S.K. Grebe, M.E. Menetere, H.W. Tun, C. Colon-Otero, E.A. Perez, J.A. Copland

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.A. von Roemeling, K.H. Kang, L.A. Marlow, J.A. Copland

Study supervision: J.A. Copland

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