SIRT1-Activating Compounds (STAC) Negatively Regulate Pancreatic Cancer Cell Growth and Viability Through a SIRT1 Lysosomal-Dependent Pathway

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Abstract

Purpose: Recent studies suggest that SIRT1-activating compounds (STAC) are a promising class of anticancer drugs, although their mechanism of action remains elusive. The main goal of this study is to determine the role of STACs as a potential therapy for pancreatic cancer. In addition, we also explored the mechanism by which these compounds affect pancreatic cancer.

Experimental design: Using in vitro (cell culture experiments) and in vivo (xenograft experiments) approaches, we studied the role of SIRT1 agonists (STAC) in human pancreatic cancer cell viability and growth.

Results: We show that SIRT1 is highly expressed in pancreatic cancer cells and that the STACs SRT1720, SRT1460, and SRT3025 inhibited cell growth and survival of pancreatic cancer cells. STACs enhanced the sensitivity of pancreatic cells to gemcitabine and paclitaxel, indicating that these drugs could be used in combination with other chemotherapy drugs. We also show that STACs were very effective in inhibiting tumor xenograft growth. In mechanistic studies, we observed that STACs activated a SIRT1 lysosomal-dependent cell death. Furthermore, the effect of STACs on cell viability was also dependent on the expression of the endogenous SIRT1 inhibitor DBC1.

Conclusions: Taken together, our results reveal an essential role for SIRT1 and lysosomes in the death pathway regulated by STACs in pancreatic cancer cells. Clin Cancer Res; 1–12. ©2015 AACR.

Introduction

Pancreatic cancer is a leading cause of cancer-related death. The prognosis of pancreatic cancer patients is very poor. Chemotherapy is the primary treatment for most patients with pancreatic cancer; however, it has had limited impact on patient survival (1–2). Therefore, new therapeutic options are highly needed.

Sirtuins are NAD-dependent deacetylases that are involved in physiologic processes such as aging and cancer (3–6). To date, seven mammalian sirtuins have been identified (SIRT1–SIRT7; refs. 5, 6). The sirtuin SIRT1 deacetylates histones and nonhistone proteins that are involved in metabolism, cell growth, apoptosis, and senescence (4). The role of SIRT1 in tumorigenesis and cancer progression is not clear, and data suggest that its role is tissue-type and context specific (7). On one hand, SIRT1 inhibition leads to growth arrest and apoptosis in lymphoma, breast, lung, and epithelial cancer (8–10). On the other hand, studies support the role of SIRT1 as a tumor suppressor (11, 12). SIRT1 has been shown to be significantly upregulated in acute myeloid leukemia (AML), prostate, gastric, and colon cancer, but downregulated in glioma and ovarian cancers (12–16). Thus, SIRT1 may modulate a delicate balance between suppression and promotion of oncogenesis, depending on its level of activity, spatial and temporal distribution, and the stage of tumorigenesis (17).

Recent studies provided evidence for the potential clinical use of SIRT1-activating compounds (STAC; refs. 18–20). STACs are potent small-molecule activators of SIRT1 (21, 22), and their use is being evaluated for metabolic syndrome, age-related diseases, and cancer (23–26). These compounds have been shown to induce apoptosis of myeloma cells (19) and necrosis of breast cancer cells (27). In contrast, SRT1720 promoted tumor cell migration and lung metastasis in mice (28), suggesting that the effects of STACs are complex and need to be evaluated in a cancer-specific manner. Specifically, one major question is which effects of these compounds are SIRT1-dependent, as some studies did not evaluate (19, 29), or describe SIRT1-independent effects of STACs (28, 30).

In the current study, we show that STACs inhibited cell viability and growth of pancreatic cancer cell lines and also tumor growth
Pancreatic cancer incidence is on the rise. Currently, this cancer continues to have extremely poor prognosis and therapeutic options are limited. Novel therapies are urgently needed for this disease. Here, we described preclinical evidence that SIRT1-agonists (STACs) induce pancreatic cancer cell death via a SIRT1/lysosomal–dependent process. Our data are extremely novel and presents one of the few examples of well documented SIRT1-mediated effects of STACs. In addition, the effects of these specific SIRT1 agonists have never been reported in pancreatic cancer. We believe that our findings justify further studies on the role of STACs as a therapeutic option in pancreatic cancer. We propose that in the future STACs may improve the therapeutic outcome of pancreatic cancer patients.

in vivo. We also show that STACs induced apoptosis via a mechanism that involves SIRT1 and lysosomes. Our findings indicate that STACs may serve as a potential therapy for pancreatic cancer.

**Materials and Methods**

**Cell lines**

The source, characterization, authentication, and culture conditions for the cell lines PaTu8988t, Panc-1, SU86.86, and HPDE were previously described (31). Cells were provided by Dr. D. Billadeau or from ATCC. Cultures used for experiments were reinitiated every 4 to 6 months from the cryopreserved stocks. The pancreatic cancer cells lines possess K-ras and/or p53 mutations that were authenticated/validated by DNA sequence analysis using published primers flanking each mutated exon. Cells were plated and maintained in media containing 1% FBS for 24 to 48 hours before experiments. All experiments were performed in media containing 1% FBS as, in these conditions, STACs were more effective than in 10% FBS (Supplementary Fig. S1).

**Drugs, inhibitors, and antibodies**

SRT1720, SRT1460, and SRT3025 were from Sirtris Pharmaceuticals. Paclitaxel, 3-methyl-adenine (3-MA), and DBC1 were from Cayman Chemical Company. SIRT1 polyclonal antibody was from Bethyl Laboratories. PARP-1, cleaved caspase-3, -Actin antibody was from Sigma-Aldrich, and DBC1 -tubulin antibodies were from Cell Signaling Technology. Glycerol-1-phenylalanine-beta-naphthylamide (GPN) was from Cayman Chemical Company. SIRT1 polyclonal (for immunoblotting) and β-tubulin antibodies were from Abcam. B-Actin antibody was from Sigma-Aldrich, and SIRT1 antibody was from Bethyl Laboratories. PARP-1, cleaved caspase-3, LC3, p62, and SIRT1 mouse monoclonal (for immunofluorescence) antibodies were from Cell Signaling Technology. Cathepsin B antibody was from Santa Cruz Biotechnology.

**Cell viability assay**

Cells were trypsinized and cell suspensions were mixed with Trypan blue dye. Cells were scored as live or dead based on Trypan blue exclusion.

**MTT assay**

Cells were plated in 96-well plates (3–5 × 10³/well). Twenty-four hours later, cells were treated with the drugs, and incubated for 24 to 72 hours at 37°C. Then, cells were incubated with 20 μL of 5 mg/mL MTT for 2 hours and the resulting formazan crystals were dissolved in dimethyl sulfoxide (200 μL). Absorbance was measured at 560 nm. The effect of the drugs on cell viability was assessed as percentage cell viability compared with vehicle-treated control cells, which were assigned 100% viability. IC₅₀s were calculated using CalcuSyn software (Biosoft).

**Short interfering RNA**

Nontargeting siRNA (Dharmacon # D001210-03-20) was used as control. siRNAs to knockdown human SIRT1 were: ON-TARGETplus SMARTpool siRNA from Dharmacon (#1) and siRNA with the sense strand 5’-AGAGUUCCACCACACCCG (5’-AAACGGAGCCUACUGAACAUU) (#2). The siRNA duplex against DBCA1 was: BDC1 siRNA sense strand, 5’-AAACGGAGCCUACUGAACAUU. Transfections were performed with 50 nmol/L of siRNA using Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were replated and allowed to attach for 24 hours. Cells were then treated with drugs and used for specific assays.

**Western blot analysis**

Cells were lysed in 20 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40, 5 mmol/L NaF, 50 mmol/L 2-glycerophosphate, and protease inhibitors (Roche). Lysates were centrifuged at 12,000 rpm at 4°C for 10 minutes. Samples were separated through a SDS-PAGE, transferred to Immobilon P membranes, and immunoblotting was performed with specific antibodies. Blots are representative of at least three experiments.

**Soft agar colony formation assay**

Cells were seeded in 6-well plates (10,000/well) in 0.35% agar over 0.6% bottom agar layer in growth media containing 5% FBS and SRT1720 or SRT1460. Colonies measuring ≥50 μm were counted after 7 to 10 days of culture using a cell colony counter (Gelcount, Oxford Optronix).

**ATP measurements**

ATP levels were measured using ATPlite Luminescence assay system from PerkinElmer according to the manufacturer’s instructions.

**Tumor xenograft study**

Female athymic nu/nu mice were obtained from the NCI (Bethesda, MD). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Mayo Clinic (protocol A39511). Subconfluent Panc-1 cells were harvested by trypsinization. Viability of cells was verified by Trypan blue exclusion. Only suspensions with 90% cell viability were used. Panc-1 cells were injected subcutaneously in both flanks of 5- to 6-week-old female athymic nu/nu mice [4 × 10⁴ cells in 100 μL of PBS:Matrigel (1:1/site)]. After 14 days of implantation, when the tumor volume reached approximately 60 mm³, mice were randomized in two groups: (i) untreated control (vehicle only, PBS containing 1% Hydroxypropyl-β-cyclodextrine and 12% propylene glycol); (ii) SRT1720 or SRT3025 (50–200 mg/kg, daily for 10 days by oral gavage). Tumor volumes were measured weekly for an additional 10 days with a caliper and calculated using CalcuSyn software (Biosoft).
calculated using the formula \( V = \frac{4}{3}\pi(l \times w \times d) \), where \( l \) is the length, \( w \) is the width, and \( d \) is the depth.

Immunofluorescence, LysoTrackerRed staining, and confocal microscopy

For immunofluorescence analysis, nontransfected or transfected Su86.86 cells were plated on coverslips. Cells were fixed with 3% paraformaldehyde for 10 to 12 minutes, permeabilized with 0.2% Triton X-100 for 15 minutes, preincubated with blocking buffer (4% BSA in PBS) and incubated with primary antibodies (LC3- 1:1000; p62- 1: 800; SIRT1- 1:1000; Cathepsin B 1:800; LAMP-2 1:800) overnight. Coverslips were washed with PBS and incubated with fluorescence-tagged secondary antibodies (Alexa Fluor 488 and/or 568, Molecular probes, Invitrogen) in blocking buffer for 2 hours followed by counterstaining with DAPI and imaged immediately.

SIRT1 activity measurement

SIRT1 activity was measured with a fluorometric assay (Enzo Life Sciences, catalog number BML-AK555-0001) as described before (32). The proteins used were recombinant SIRT1 from bacteria (Biomol) and GST-DBC1 purified from baculovirus (33). Recombinant SIRT1 (1 μmol/L) was incubated in vitro with 5 μmol/L DBC1. SRT1720 was used at 1 μmol/L.

Quantification of mRNA

mRNAs from human biospecimens were obtained from the Mayo Clinic Tissue Registry under an approved Institutional Review Board (IRB) protocol. RNA was isolated from a set of pancreatic adenocarcinoma patient samples for which frozen, paired tumor and nontumor tissue sample was available. RNA was isolated from pancreatic cell lines using the RNeasy kit (Qiagen). mRNA abundance was analyzed by quantitative PCR using an Applied Biosystems 7900HT thermal cycler and TaqMan Gene Expression Assay probe sets (Applied Biosystems) were: 18S (Hs00700620_m1), SIRT1 (Hs01009055_m1), DBC1 (Hs00217777_m1), and GAPDH (Hs00275899_g1). Gene expression in primary pancreatic cancers was normalized to 18S. In cells, the relative expression of target genes was normalized to GAPDH. The changes in expression were calculated relative to control pancreatic cells (HPDE).

Results

STACs reduce cell viability and growth of pancreatic cancer cells

To explore the role of STACs as a therapy for pancreatic cancer, we first examined their effect on the cell viability of pancreatic cancer cell lines. Treatment of these cells with SRT1720 inhibited cell viability in a dose-dependent manner (Fig. 1A). All pancreatic cancer cells were more sensitive to this drug than control HPDE cells. There was a significant difference in the sensitivity of cells to SRT1720, with PaTu8988t and Su86.86 cells being the most sensitive. When different lengths of treatment with SRT1720 were analyzed, we observed the majority of decrease in cell viability in the first 24 hours (Fig. 1B), indicating that SRT1720 quickly decreases cell viability.

The effect of SRT1720 on cell viability and growth was confirmed by additional assays (Fig. 1C–E). In PaTu8988t cells there was a dose-dependent decrease in the number of viable cells counted by Trypan blue exclusion assay after treatment with SRT1720 (Fig. 1C). ATP can be used as a marker of cell viability, as ATP levels rapidly decline when cells undergo necrosis or apoptosis. There was a marked decrease in ATP levels in cells incubated with SRT1720 (Fig. 1D). As colony formation of human pancreatic cancer cells in soft agar is one of the best in vitro indicators of malignant behavior (34), we measured the number of colonies of PaTu8988t cells in soft agar during treatment with SRT1720. Treatment with this compound resulted in significantly fewer colonies than vehicle treatment, and this effect was dose-dependent (Fig. 1E).

We also investigated whether other STACs had similar effects on pancreatic cancer cells in vitro. Treatment with different concentrations of SRT1460 (24) inhibited cell viability in a dose-dependent manner (Fig. 1F), with all pancreatic cancer cells being more sensitive than the control HPDE cell. SRT1460 was also shown to inhibit cell growth by the Trypan blue exclusion and in the anchorage-independent cell growth assays in PaTu8988t (Supplementary Fig. S2A and S2B). In contrast, SRT3025 (35–36), a STAC that has been tested in humans (37), decreased viability mainly of Su86.86 cells in culture. PaTu8988t and Panc-1 cells were not as sensitive to the effect of the drug (Fig. 1G). Our observations strongly demonstrate that STACs inhibit cell viability and growth of pancreatic cancer cells, with SRT1720 and SRT1460 being the most effective STACs.

As conventional therapeutic agents for pancreatic carcinoma produce minimal survival benefit as single agents, numerous efforts have focused on drug combinations (2). We tested STACs in combination with gemcitabine and paclitaxel, drugs that have been used as therapies for pancreatic cancer. Our results show that, in both PaTu8988t and Su86.86 cells, combinations of STACs with gemcitabine or paclitaxel caused a greater decrease in cell survival than addition of the STACs alone (Supplementary Fig. S3). The only exception was combination of gemcitabine and SRT1460 in PaTu8988t, which was not more effective than SRT1460 alone. These results together indicate that STACs have the potential be used in combination with other drugs in pancreatic cancer.

STACs prevent pancreatic tumor growth in vivo

We next tested the in vivo effect of STACs using a xenograft animal model of pancreatic cancer. SRT1720 treatment markedly decreased tumor size in comparison with the vehicle-treated tumors (Fig. 2A). When different doses of SRT1720 were compared, there was a dose-dependent effect on tumor growth, with both 200 and 100 mg/kg being significantly different from vehicle treatment (Fig. 2B). As shown in Fig. 2C and D, tumor size and weight were greatly decreased in SRT1720-treated mice. During the treatment period, no obvious treatment-related toxicity was observed. The food intake and body weight of both groups of animals remained similar (Supplementary Fig. S4). We also performed in vivo experiments with Panc-1 xenografts using SRT3025. Similar to SRT1720, SRT3025 treatment also inhibited tumor growth in vivo (Fig. 2E), even though it was not as effective in inhibiting the viability of Panc-1 cells in culture.
lysosomes in vehicle-treated cells and showed a punctate staining. This is a distinctive sign of LMP (38, 39). Cathepsin B localized to the lysosomes, and upon changes in lysosomal acidi- fication, it diffuses out of the lysosome (38). Five hours treatment with SRT1720 markedly decreased lysosome staining with LysoTracker Red (Fig. 3A). To distinguish between changes in lysosome staining with an antibody for Lysosome-associated membrane protein 2 (LAMP-2) shows that STACs did not completely disrupt the lysosomes (Supplementary Fig. S5). It is then likely that STACs are promoting LMP.

STAC-induced cell death is lysosomal dependent, but lysosomal permeabilization is not sufficient to cause cell death

Next, we investigated the mechanism underlying the effect of STACs in pancreatic cancer cells. In breast cancer cells, it was proposed that SRT1720 promotes cell death through a SIRT1-independent induction of lysosomal membrane permeabilization (LMP; ref. 27). To determine whether STACs regulate lysosomal function in pancreatic cancer cells, SU86.86 cells were treated with SRT1720 and then incubated with LysoTracker Red. The LysoTracker Red dye concentrates in the lysosomal compartment and upon changes in lysosomal acidiﬁcation or LMP, it diffuses out of the lysosome (38). Five hours treatment with SRT1720 markedly decreased lysosome staining with LysoTracker Red (Fig. 3A). To distinguish between changes in lysosomal acidiﬁcation or LMP, we performed staining with cathepsin B antibody. Translocation of cathepsins from lysosomes to cytosol is a distinctive sign of LMP (38, 39). Cathepsin B localized to lysosomes in vehicle-treated cells and showed a punctate staining. However, upon treatment with SRT1720, cathepsin B staining decreased and became diffuse (Fig. 3A). Immunofluorescence staining with an antibody for Lysosome-associated membrane protein 2 (LAMP-2) shows that STACs did not completely disrupt the lysosomes (Supplementary Fig. S5). It is then likely that STACs are promoting LMP.

Figure 1. STACs decrease pancreatic cancer cell survival and growth. A, cells were treated with vehicle (control) or different concentrations of SRT1720 and submitted to MTT analysis 72 hours after treatment. IC50 (µmol/L) were: Patu8988t, 1.2 ± 0.07; SU86.86, 1.13 ± 0.04; Panc-1, 2.1 ± 0.04. B, SU86.86 and Patu8988t cells were treated with vehicle or 2 µmol/L SRT1720 for different time periods. Cells were submitted to MTT analysis. C, Patu8988t cells were treated with vehicle (control) or SRT1720 for 72 hours and cells were counted by Trypan blue dye exclusion assay. D, ATP levels were measured in pancreatic cancer cells after treatment with vehicle (control) or 2.5 µmol/L SRT1720 for 48 hours. E, Patu8988t cells were grown in soft agar containing vehicle or different concentrations of SRT1720 for 7 days. Graph shows quantitative analysis of colony number. F, cells were treated with vehicle or different concentrations of SRT1460 and submitted to MTT analysis 72 hours after treatment. IC50 (µmol/L) were: Patu8988t, 1.62 ± 0.13; SU86.86, 2.31 ± 0.23; Panc-1, 0.66 ± 0.02; HPDE, 2.39 ± 0.29. G, cells were treated with vehicle or different concentrations of SRT3025 for 72 hours and submitted to MTT analysis. SRT3025 IC50 (µmol/L) for SU86.86 cells was 0.98 ± 0.13. Vehicle-treated cells (control) were assigned as 100% cell viability. Values are mean ± SEM of at least three independent experiments. *, P < 0.05, compared with vehicle.

To determine whether the effect of STACs on the lysosomes was critical for regulation of pancreatic cancer cell viability, we used the lysosomotropic agents bafilomycin A1, chloroquine, and ammonium chloride to inhibit lysosomal acidification and function (40). None of the three drugs decreased SU86.86 or Patu8988t cell viability (Fig. 3B and 3C), indicating that blocking lysosome acidification for 24 hours does not inhibit pancreatic cancer cell viability. Interestingly, these agents signiﬁcantly reversed the effect of SRT1720 on cell viability (Fig. 3B and 3C). Similar results were also obtained when lysosome inhibitors were added in combination with SRT1460 and SRT3025 (Fig. 3D). To confirm that lysosome inhibitors were indeed reducing lysosome acidification, we tested the effect of bafilomycin, ammonium chloride, and chloroquine on LysoTracker Red staining. Similar to SRT1720, these drugs decreased LysoTracker staining (Supplementary Fig. S6). To determine toxicity to animals and could not be tested in vivo. These data show that STACs are also effective in inhibiting pancreatic tumor growth in vivo.
whether lysosome permeabilization was sufficient to regulate pancreatic cancer cell viability, we used GPN, a drug that osmotically permeabilize the lysosomes (41). GPN quickly decreased LysoTracker and cathepsin B staining, indicating lysosomal permeabilization, but did not inhibit pancreatic cell viability (Fig. 3E and F). We concluded that the inhibition of pancreatic cancer cell viability induced by STACs clearly depends on lysosomal function, but in contrast to breast cancer cells, permeabilization of the lysosomes alone is not sufficient to decrease pancreatic cancer cell viability.

It has been shown that pancreatic cancers require autophagy for tumor growth, and that inhibition of autophagy leads to tumor regression (42). Because lysosomes are important for autophagy (39), we investigated the expression of autophagy markers in cells treated with STACs. All three STACs increased expression of the autophagy marker LC3-II, detected by both immunofluorescence punctate staining and immunoblotting (Fig. 3G and H and Supplementary Fig. S7). As an increase in LC3-II could be an indicator of increased autophagy or inhibition of autophagy flux (43), we measured levels of p62/SQSTM1. p62 is a ubiquitin binding protein that binds LC3-II and is degraded after fusion of autophagosome and lysosome, being considered a marker for autophagy flux (44). Because we observed an increase in p62 levels with SRT1720 treatment (Fig. 3G and H and Supplementary Fig. S7), it implies that STACs inhibit the autophagy flux in pancreatic cancer cells.

To study the role of autophagy in the pathway regulated by STACs, we blocked autophagy using the PI3K inhibitor 3-MA (40). Treatment with 3-MA alone for 24 hours did not decrease cell viability (Fig. 3I). When 3-MA was added in combination with
SRT1720 in Patu8988t cells, there was an increase in cell survival compared with SRT1720 alone (Fig. 3I). However, in SU86.86 and Panc-1 cells, combination of SRT1720 and 3-MA caused an even greater inhibition of cell viability than SRT1720 alone (Fig. 3I). Because 3-MA has different effects in these cell lines, it appears that the block in autophagy induced by STACs is not the main mechanism involved in the STAC-induced cell death. This conclusion is also supported by the observation that multiple lysosomal inhibitors did not cause cell death in pancreatic cancer cells at the time course analyzed (Fig. 3B and C), but they did impair autophagy, as shown by an increase in LC3-II and p62 levels (Supplementary Fig. S8). These results also indicate that the PI3K pathway, and possibly autophagy, has different roles in the pathway regulated by SRT1720 in distinct pancreatic cancer cells. In Patu8988t, the PI3K pathway appears to regulate the effect of SRT1720. In contrast, the PI3K inhibitor 3-MA was very effective in sensitizing SU86.86 and Panc-1 cells to SRT1720, suggesting that combination of STACs...
and PI3K inhibitors could be an effective treatment for some pancreatic tumors.

**SIRT1720 induces apoptosis in pancreatic cancer cells, which is prevented by lysosomal inhibitors**

To further explore the role of STACs in pancreatic cancer cells, we analyzed other downstream signaling pathways activated by STACs. Treatment of Patu8988T and SU86.86 cells with SRT1720 and SRT1460 increased the cleavage of PARP-1 and caspase-3, indicating an induction of apoptosis (Fig. 4A and B). These increases were both time- and dose-dependent (Fig. 4A and Supplementary Fig. S9). As lysosomal acidification is required for pancreatic cancer cell death, we tested the effect of lysosomal inhibitors in the apoptosis induced by SRT1720. SU86.86 cells were treated for different time periods with 5 μmol/L SRT1720 alone and in combination with 1 mmol/L bafilomycin A1 (BafA1). Expression levels of SIRT1, LC3-II, p62, cleaved caspase-3, and cleaved PARP-1 were determined by immunoblotting.

**Figure 4.** SRT1720-induced apoptosis is prevented by lysosomal inhibitors. A, Patu8988T cells were treated with vehicle or 5 μmol/L SRT1720 for different time periods. Expression levels of SIRT1, cleaved caspase-3, and cleaved PARP-1 were determined by immunoblotting. B, SU86.86 cells were incubated with vehicle, 5 μmol/L SRT1720 or 5 μmol/L SRT1460 for 24 hours, and expression of PARP-1 and cleaved caspase-3 were determined by immunoblotting. C, SU86.86 cells were treated for different time periods with 5 μmol/L SRT1720 alone and in combination with 1 mmol/L bafilomycin A1 (BafA1). Expression levels of SIRT1, LC3-II, p62, cleaved caspase-3, and cleaved PARP-1 were determined by immunoblotting.

Among the pancreatic cancer cells, SU86.86 had the highest levels of DBC1 (45), in the pancreatic cancer cell lines and HPDE. SIRT1 protein levels were higher, while DBC1 expression was lower, in all pancreatic cancer cells than in HPDE cells (Fig. 5A). Therefore, the ratio SIRT1/DBC1 was higher in pancreatic cancer cells than in HPDE cells (Fig. 5A). This suggests that pancreatic cancer cells may have a higher amount of free SIRT1, which may be more easily activated by STACs. Indeed, our *in vitro* experiments show that SRT1720 activates SIRT1 better in the absence of DBC1 (Supplementary Fig. S12). To determine whether the mRNA expression of SIRT1 and DBC1 relate to their protein levels, we measured the mRNA levels of both proteins in the different cell lines. Similar to the protein expression, the ratio of SIRT1/DBC1 mRNA expression was higher in the pancreatic cells than in the control HPDE cell (Fig. 5B and Supplementary Fig. S13).

To evaluate whether the effect of STACs on pancreatic cancer cell viability depends on SIRT1, we tested the effect of these compounds in cells knocked down for SIRT1. A strong reduction in SIRT1 levels was observed in cells upon transfection with SIRT1 siRNA compared with control siRNA (Fig. 5B). SU86.86 and Patu8988T cells transfected with SIRT1 siRNAs were considerably less sensitive to SRT1720 than control siRNA-transfected cells (Fig. 5B). In addition, SRT1460 inhibition of cell viability was also SIRT1-dependent (Supplementary Fig. S14). These results show that the antisurvival effect of STACs in pancreatic cancer cells is SIRT1-dependent.

### SIRT1 and DBC1 regulate the effect of STACs on cell viability

It is inconclusive from the literature whether the effects of STACs are dependent on SIRT1. To establish the role of SIRT1 in the effect of STACs in pancreatic cancer cells, we first evaluated the protein expression of SIRT1 and its endogenous inhibitor, DBC1 (45), in the pancreatic cell lines and HPDE. SIRT1 protein levels were higher, while DBC1 expression was lower, in all pancreatic cancer cells than in HPDE cells (Fig. 5A). Therefore, the ratio SIRT1/DBC1 was higher in pancreatic cancer cells than in HPDE cells (Fig. 5A). This suggests that pancreatic cancer cells may have a higher amount of free SIRT1, which may be more easily activated by STACs. Indeed, our *in vitro* experiments show that SRT1720 activates SIRT1 better in the absence of DBC1 (Supplementary Fig. S12). To determine whether the mRNA expression of SIRT1 and DBC1 relate to their protein levels, we measured the mRNA levels of both proteins in the different cell lines. Similar to the protein expression, the ratio of SIRT1/DBC1 mRNA expression was higher in the pancreatic cells than in the control HPDE cell (Fig. 5B and Supplementary Fig. S13).
We further investigated the expression of SIRT1 and DBC1 in pancreatic cancer tumor samples from patients. There was a significant variability in the expression of SIRT1 and DBC1 in pancreatic cancer samples, but in general, the expression of SIRT1 was higher and the expression of DBC1 was lower in samples from pancreatic tumors than in normal tissue (Supplementary Fig. S15). Our data together suggest that the high ratio of SIRT1/DBC1 in pancreatic cancer cells could explain why these cells are more sensitive to SIRT1 activators than normal HPDE cells. The relative expression of SIRT1 and DBC1 may serve as molecular markers for the responsiveness to SIRT1 activators in vivo.

SIRT1 regulates the effect of STACs in the lysosomes and in apoptosis

Immunofluorescence of SIRT1 in SU86.86 cells showed that in vehicle-treated cells SIRT1 is present in the cell nuclei. However, upon SRT1720 treatment, a fraction of SIRT1 translocated to the cytosol, increasing the ratio of cytoplasmic to nuclear SIRT1 (Fig. 6A). This increase in cytoplasmic SIRT1 is consistent with the hypothesis that SIRT1 may be regulating lysosomes in the cytosol. Thus, we investigated whether the effects of SRT1720 on lysosomes were SIRT1-dependent. SIRT1 siRNA transfection abolished SIRT1 staining, while control siRNA cells showed nuclear localization of SIRT1 (Fig. 6A). Treatment with SRT1720 quickly decreased Lyso-Tracker Red staining by 3 hours in control siRNA-transfected cells, but was not as effective in SIRT1 siRNA–transfected cells (Fig. 6B). In addition, the cathepsin B distribution after SRT1720 treatment was also dependent on SIRT1. While cathepsin B shows a diffuse distribution after SRT1720 – transfected cells (Fig. 6C). These results collectively show that the effects of SRT1720 on LMP are SIRT1-dependent.

To further explore the role of SIRT1 in the mechanism of cell death induced by STACs, we transfected SU86.86 cells with control, SIRT1, and DBC1 siRNAs. 48 hours after transfection, cells were treated with SRT1720 for 16 hours and the expression of proteins involved in apoptosis and autophagy was analyzed.
In control siRNA-treated cells there was an increase in cleavage of caspase-3 and PARP-1 induced by SRT1720, and also an increase in levels of LC3-II and p62, confirming the induction in apoptosis and the inhibition of autophagy (Fig. 6D). In the presence of SIRT1 siRNA, there was a marked decrease in the cleavage of PARP-1 and caspase-3 induced by SRT1720 (Fig. 6D). However, the increase in expression of LC3-II and p62 induced by SRT1720 was not influenced by absence of SIRT1. In the presence of DBC1 siRNA there was still cleavage of caspase-3 and PARP-1, and an increase in LC3-II and p62 (Fig. 6D). These results together demonstrate that SIRT1 is required for regulation of lysosomal function and apoptosis induced by SRT1720, but does not appear to be involved in the inhibition of autophagy flux by this drug.

Discussion

The current study clearly shows a role for STACs as a potential therapy for pancreatic cancer. Here we describe that STACs inhibit cell survival of a panel of pancreatic cell lines, reduce anchorage-dependent and-independent pancreatic cancer cell growth, and inhibit xenograft tumor growth. STACs have been shown to differ in pharmacokinetics, potency, and toxicity (24). The most effective STACs in pancreatic cancer cell lines were SRT1720 and SRT1460. However, even though SRT3025 was less effective in vitro, it did inhibit Panc-1 tumor growth in vivo. This indicates that although all STACs inhibit survival and growth of pancreatic cancer cells, different types of pancreatic tumors may have different sensitivities to specific STACs.
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Moreover, we and others show that SRT1720 and SRT3025 are well tolerated in mice and have the potential to be used in several diseases (19, 26, 27, 35, 36).

One of the main questions in STAC research at this time is whether the biologic effects of STACs are mediated by SIRT1 activation. The original study describing the development and effects of STACs did not show a clear SIRT1 dependency for its effects (24). Furthermore, another study questioned the direct binding and activation of SIRT1 by STACs (30). In tumor biology studies, the dependency on SIRT1 was only investigated in one report which found confounding results, with the effect of STACs being SIRT1-dependent in vivo but not in vitro (27). Our study is the first to demonstrate a clear SIRT1-dependent biologic effect of multiple STACs in several parameters in cancer cells. Importantly, our findings highly suggest that the effects of STACs in pancreatic cell growth, apoptosis, and lysosomal function are SIRT1-dependent. Our data are in agreement with at least two recent studies demonstrating that STACs indeed bind and activate SIRT1 directly, at least in vitro (46, 47). Interestingly, we also observed that STACs had SIRT1-independent effects, such as the blockage of autophagy.

Our data together support the idea that SIRT1 plays a key role in the survival and proliferation of pancreatic cancer cells. Previous reports show that inhibition of SIRT1 by EX527 or SIRT1 knockdown in pancreatic cancer cells reduces cell proliferation, and induces apoptosis and senescence (48). However, SIRT1 inhibition in vitro by EX527 was shown to promote xenograft tumor growth (49), indicating that the effect of SIRT1 in pancreatic cancer is complex, and it is possible that this activation or inhibition of SIRT1 disturbs the growth of pancreatic cancer cells.

Although studies have explored the role of SIRT1 in cancer progression and the mechanisms involved in this regulation, little is known about the signaling pathways regulated by STACs in cancer. In multiple myeloma cells SRT1720 was shown to increase apoptosis, presumably through a pathway dependent on SIRT1-AMPK (19). In breast cancer cells, SRT1720 induces cell death through a lysosomal-dependent pathway, involving LMP and necrosis, and there is no induction of apoptosis (27). In addition, the SRT1720-induced cell death in breast cancer cells occurred irrespective of SIRT1. In contrast, in pancreatic cancer cells, lysosomes are required for SRT1720-induced cell death, and this pathway appears to involve induction of apoptosis and is highly dependent on SIRT1. In fact, accumulating data support the role of cathepsins as effectors of LMP-initiated cell death pathways, including LMP-induced apoptosis (39). This suggests that the lysosomal cell death pathway is connected with other death pathways, like apoptosis. Our findings indicate that STACs act by a coordinated pathway involving multiple components including SIRT1, lysosomes, and apoptotic components. A key factor in determining the type of cell death mediated by lysosomes (necrosis or apoptosis) seems to be the magnitude of lysosomal permeabilization and consequently the amount of proteolytic enzymes released into the cytosol (50). It will be important to explore in the future how STACs regulate lysosomal permeabilization and function, and ultimately the cell death process in pancreatic cancer cells. One possibility is that STACs are lysosomotropic agents that are being sequestered in the lysosomal compartment causing an increase in lysosomal permeability (51). This hypothesis is supported by the fact that STACs are basic, they displace LysoTracker Red from inside the lysosomes, and their effect is blocked by inhibitors of lysosome acidification. It is also possible that SIRT1 activation by STACs may lead to SIRT1 translocation to the cytoplasm and targeting of lysosomal proteins and function. However, at this point, except for the fact that STACs lead to increases in cytoplasmic SIRT1, we do not have mechanistic demonstration that this translocation is necessary for the cell killing induced by STACs.

The role that autophagy plays in the mechanism of cell death induced by STACs is still not clear. All STACs appeared to block autophagy. However, autophagy inhibitors did not promote cell death when added for the same duration as STACs. In addition, SIRT1 knockdown did not affect the expression of autophagy markers induced by STACs, supporting the notion that inhibition of autophagy is not the primary mechanism involved in the cell death. Nevertheless, it is possible that the autophagy block contributes to the effect of STACs in pancreatic cancer cells.

Human pancreatic tumors are hypovascular in nature and large areas of tumor survive under nutrient and oxygen starvation. However, human pancreatic tumor cells show extraordinary ability to tolerate extreme states through the modulation of energy metabolism. Therefore, agents that retard the tolerance of cancer cells to nutrient starvation represent a novel approach in anticancer drug discovery (52). Our study shows that pancreatic cancer cells are more sensitive to STACs in low serum conditions, implying that STACs may be a good therapy under nutrient-starved conditions in the pancreatic tumor microenvironment.

The STAC doses that were effective in our animal studies have been shown to lead to micromolar plasma levels (24). We observed that these concentrations were pharmacologically relevant in killing pancreatic cancer cells in vitro. Our findings also reveal that SIRT1 is a key molecular target for pancreatic cancer treatment. As new STACs, with high safety and efficacy, are developed it will be of great importance to test them as therapies for pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Acknowledgments**

The authors thank Gina Warner for editing the manuscript.
Grant Support
This work was supported by the Pancreatic Cancer SPORE project from NIH/NCI to E.N. Chini. Grant (grant: CA102701-08) and the Mayo Clinic Center for Cell Signaling in Gastroenterology (NIDDK P30DK084567). J.M. Espindola-Netto is a recipient of a grant from Coordenacão de Aperfeicoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Received July 29, 2015; revised November 18, 2015; accepted November 30, 2015; published OnlineFirst December 11, 2015.

www.aacrjournals.org Clin Cancer Res; 2016 0F11

Published OnlineFirst December 11, 2015; DOI: 10.1158/1078-0432.CCR-15-1760

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SIRT1-Activating Compounds (STAC) Negatively Regulate Pancreatic Cancer Cell Growth and Viability Through a SIRT1 Lysosomal-Dependent Pathway

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Clin Cancer Res  Published OnlineFirst December 11, 2015.

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