SRT1720 Induces Lysosomal-Dependent Cell Death of Breast Cancer Cells
Tyler J. Lahusen and Chu-Xia Deng

Abstract
SRT1720 is an activator of SIRT1, a NAD⁺-dependent protein and histone deacetylase that plays an important role in numerous biologic processes. Several studies have illustrated that SRT1720 treatment could improve metabolic conditions in mouse models and in a study in cancer SRT1720 caused increased apoptosis of myeloma cells. However, the effect of SRT1720 on cancer may be complex, as some recent studies have demonstrated that SRT1720 may not directly activate SIRT1 and another study showed that SRT1720 treatment could promote lung metastasis. To further investigate the role of SRT1720 in breast cancer, we treated SIRT1 knockdown and control breast cancer cell lines with SRT1720 both in vitro and in vivo. We showed that SRT1720 more effectively decreased the viability of basal-type MDA-MB-231 and BT20 cells as compared with luminal-type MCF-7 breast cancer cells or non-tumorigenic MCF-10A cells. We demonstrated that SRT1720 induced lysosomal membrane permeabilization and necrosis, which could be blocked by lysosomal inhibitors. In contrast, SRT1720-induced cell death occurred in vitro irrespective of SIRT1 status, whereas in nude mice, SRT1720 exhibited a more profound effect in inhibiting the growth of allograft tumors of SIRT1 proficient cells as compared with tumors of SIRT1-deficient cells. Thus, SRT1720 causes lysosomal-dependent necrosis and may be used as a therapeutic agent for breast cancer treatment.

Introduction
Breast cancer is the most common type of cancer to affect American women. It is estimated that over 200,000 women will develop breast cancer in 2014 and about 40,000 will die from the disease (1). There are many treatment options for women with ER⁺/PR⁺/HER2⁻ luminal-type breast cancers, but for basal-type ER⁺/PR⁺/HER2⁻ breast cancers (triple-negative breast cancers, TNBC), there are few options currently available (2, 3). Therefore, novel therapeutics targeting TNBCs and other breast cancers that are resistant to current therapies are desperately needed. In this study, we wanted to elucidate whether the small-molecule compound SRT1720 is a potential therapeutic agent for breast cancer. SRT1720 was initially identified as an activator of SIRT1, a NAD⁺-dependent deacetylase, which was shown to positively affect glucose and lipid homeostasis (4–7). However, more recently other researchers have demonstrated that SRT1720 has SIRT1-independent effects and does not directly activate SIRT1 (8).

Anticancer drugs may cause cell death either through the process of apoptosis or necrosis (9, 10). Apoptosis, also known as programmed cell death, results in the activation of caspases to cause membrane swelling and DNA fragmentation (11). Necrosis is another type of cell death that is not dependent on caspase activation but results from an alternative mechanism such as lysosomal membrane permeabilization (LMP), thus causing cell proteolysis and membrane disruption (12). In a previous study, it was shown that SRT1720 induced apoptosis of myeloma cells (13), while another study reported that SRT1720 could promote tumor cell migration and lung metastasis in mice (14). Neither study investigated whether such effects of SRT1720 were dependent on SIRT1. Therefore, it is inconclusive whether the effect of SRT1720 on cancer cells is dependent on SIRT1.

To determine the effect of SRT1720 in breast cancer and study its underlying mechanism, we treated SIRT1-deficient and proficient breast cancer cells with SRT1720. Our data demonstrated that SRT1720 caused lysosomal-dependent cell death in breast cancer cells in vitro irrespective of their SIRT1 status. SRT1720 could also inhibit the growth of allograft tumors in nude mice that was partially mediated by SIRT1. These data reveal that SRT1720 has both SIRT1-dependent and independent functions and may potentially be a therapeutic agent for the treatment of breast cancer cells.

Materials and Methods
Cell lines and reagents
All human breast cancer cell lines (MCF-7, T47D, SKBR3, MDA-MB-231, SUM149, HS578T, and BT-20) and the A549 lung adenocarcinoma cells were obtained from ATCC and cultured with DMEM (Invitrogen) supplemented with 10% FBS (Sigma) and 1% l-glutamine (Invitrogen). All cell lines from ATCC are authenticated. Mouse mammary tumor cells were from MMTV-neu mice (Neu) and from Brcα+/-;p53+/-;MMTV-Cre mice (69), respectively (15, 16). MCF10A immortalized mammary epithelial cells were obtained from ATCC and cultured with DMEM/F12 (1:1; Invitrogen) supplemented with 5% horse serum.
concentration of the supernatant was quantified with a Bio-Tek plate reader. For production of lentiviral particles, 293T cells (3 × 106) were seeded in 100-mm dishes and 1 mL of viral supernatant was added for another 24 hours. The medium containing lentiviral particles was then collected, centrifuged at 2,000 rpm for 5 minutes, filtered through a 0.45 μm filter, and aliquots were stored at −80 °C. For transduction of lentiviral particles, MDA-MB-231 (5 × 105) cells were seeded in 100-mm dishes and 1 mL of viral supernatant was added to 7 mL of medium after cell attachment. The cells were transduced for 24 hours in the presence of polybrene (8 μg/mL; Sigma). Cells stably expressing SIRT1 shRNA were selected for 48 hours in the presence of puromycin (2 μg/mL; Sigma) before plating for experiments.

Western blot analysis

Cells were harvested from subconfluent plates and whole-cell lysates were prepared for immunoblot analysis. Cells were washed with cold PBS and lysed with lysis buffer containing: 1% NP-40, 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10% glycerol, 50 mmol/L NaF, 2 mmol/L EGTA, 2 mmol/L EDTA, 1 μg/mL pepstatin A, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 100 μg/mL AEBSF, and 1 mmol/L sodium orthovanadate. The lysate was centrifuged at 14,000 rpm at 4 °C for 10 minutes and the protein concentration of the supernatant was quantified with the Bio-Rad protein assay reagent (Bio-Rad Laboratories). Protein lysates (25–50 μg) were prepared in SDS-loading buffer containing the reducing agent β-mercaptoethanol (Sigma), heated at 95 °C for 4 minutes, resolved by SDS-PAGE on 4% to 12% Tris-glycine gels (Invitrogen), and then transferred to polyvinylidene difluoride Immobilon-P membranes (EMD Millipore). The membranes were blocked with 4% nonfat milk in PBS for 30 minutes, incubated for 2 hours with primary antibodies diluted in 1% milk in PBS, washed three times with PBS-T (PBS, 0.1% Tween-20), and then incubated with either an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for 45 minutes. The blots were washed three times with PBS-T and the protein bands were detected on enhanced chemiluminescence film (Denville Scientific) with the Immobilon chemiluminescence detection reagent (EMD Millipore). Proteins were detected with the following antibodies: monoclonal anti-SIRT1 (1F3), LC3 (Cell Signaling Technology), and monoclonal anti-β-actin (Sigma).

Results

SIRT1720 inhibits the growth of breast cancer cells in vitro in a SIRT1-independent manner

SIRT1720 was previously shown to inhibit the proliferation of myeloma cell lines through the induction of apoptosis (13). In our study, we determined whether SRT1720 affected the viability of breast cancer cell lines. Human and mouse breast cancer cells,
2.5 MB-231 cells was decreased by 66% to 86% with a dose range of SRT1720 from 2.5 to 10 \( \text{mol/L} \) treatment. Next, we treated MDA-MB-231 cells with a dose range further experiments as they were more sensitive to SRT1720 SRT1 knockdown cells. D, viability of control cells as compared with sh-

SRT1720 treated WT as compared

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there was no signi

There was no significant difference in cell viability in SRT1720-treated sh-

SRT1 knockdown cells. D, viability of WT and Sirt1 \(^{-/}\) MEF cells treated with SRT1720 for 24 hours. There was no significant difference in cell viability in SRT1720 treated WT as compared with Sirt1 \(^{-/}\) MEFs.

SRT1720 decreases the viability of breast cancer cells independent of SIRT1 status. A, MTT viability assay of cancer cell lines treated with different doses of SRT1720 for 24 hours. B, MTT dose response of MDA-MB-231 breast cancer cells treated with SRT1720 for 24 hours. There were significant decreases in cell viability with 5 (66%), 7.5 (80%), and 10 (86%) \( \text{mol/L} \) of SRT1720 \((** P < 0.001)\), but less with 2.5 \( \text{mol/L} \) (9%; ** P < 0.01). C, viability of control and SIRT1 shRNA knockdown MDA-MB-231 cells treated with 5 \( \text{mol/L} \) of SRT1720 for 24 hours. There was no significant difference in cell viability in SRT1720-treated sh-

SRT1 knockdown cells as compared with sh-

SRT1 knockdown cells. D, viability of WT and Sirt1 \(^{-/}\) MEFs (Fig. 1C and D). Therefore, SRT1720 decreases the viability of MDA-MB-231 cells and MEF cells independently of SIRT1 expression. We also compared the effect of resveratrol, another reported activator of SIRT1 \((21, 22)\), on the viability of MDA-MB-231 cells. There was a 30% decrease in the viability of sh-control MDA-MB-231 cells treated with 80 \( \text{mol/L} \) of resveratrol as compared with a decrease of 47% for sh-SIRT1 knockdown cells, thus suggesting that SIRT1 is not required for resveratrol-induced cell death (Supplementary Fig. S2). We next determined whether the effect of SRT1720 on breast cancer cells was dependent on other members of the sirtuin family by cotreatment with the pan-sirtuin inhibitor nicotinamide. Our results showed that other sirtuin members also do not have a role in SRT1720-induced cell death (Supplementary Fig. S3).

SRT1720 decreases the viability of breast cancer cells by increased necrosis

A decrease in cell viability may result from decreased cell-cycle progression or through increased cell death. The cause of a reduction in cell viability was determined by measuring apoptosis and necrosis. Cells were treated with SRT1720 for 8 hours and apoptosis/necrosis was measured by annexin V/propidium iodide staining, respectively. After 5 \( \text{mol/L} \) of SRT1720 treatment, 1% of the cells were positive for early apoptosis/necrosis (double positive for annexin V and propidium iodide; Fig. 2A). Measurement of necrosis with propidium iodide alone showed a 13% and 24% increase in necrotic cells with 5 and 10 \( \text{mol/L} \) SRT1720, respectively, after 8 hours of SRT1720 treatment (Fig. 2B). The pan-caspase inhibitor ZVAD was used to determine whether apoptosis had any role in SRT1720-induced cell death. There was a 74% reduction in cell viability in SRT1720-treated cells; however, cotreatment...
of MDA-MB-231 cells with ZVAD did not reduce cell death caused by SRT1720 treatment (Fig. 2C). Also, SRT1720 did not cause caspase-3 cleavage as was observed with the proteosome inhibitor bortezomib (Fig. 2D).

SRT1720 caused the formation of acidic vacuoles and an increase in autophagy markers

Treatment of MDA-MB-231 breast cancer cells with SRT1720 for 8 hours resulted in the formation of acidic vacuoles (Fig. 3A) as detected by acridine orange staining. Acridine orange will emit red fluorescence in a low pH environment, which can be used to measure acidic vacuoles. Autophagy is a cellular process by which the cell catabolizes its own cytoplasmic organelles during nutritional deprivation. It is mainly a survival pathway, which may result in cell death during prolonged nutrient deprivation. One of the markers for autophagy is the autophagosome membrane marker LC3 that is converted from LC3-I to LC3-II through lipidation involving both ATG7 and ATG3, which leads to LC3 fusion with autophagosome membranes (23). We generated MDA-MB-231 cells with stable expression of LC3-GFP to measure the effect of SRT1720 on autophagy by observation of GFP puncta formation. Treatment with SRT1720 resulted in an increase in the number of cells with an average of eight GFP puncta per cell (Fig. 3B). An immunoblot analysis of LC3 will show an increase in the lower LC3-II band during autophagy. We observed that treatment with SRT1720 caused an increase in the autophagy marker LC3-II, and this was decreased in sh-SIRT1 knockdown MDA-MB-231 cells (Fig. 3C). This validates a previous study demonstrating that SIRT1 is involved in the regulation of autophagy (24). Next, we determined whether blocking the PI3K pathway responsible for initiation of autophagy would affect autophagy induction by SRT1720. Cotreatment of MDA-MB-231 cells with the type-III PI3K inhibitor, 3-MA, resulted in decreased LC3-II formation caused by SRT1720 treatment (Fig. 3D). Also, LC3-II formation was reduced in Sirt1 and Atg7 knockout MEFs (Fig. 3E). Therefore, SRT1720 increases the formation of the autophagy marker, LC3-II, through SIRT1 and ATG7. Then we determined whether blocking autophagy induction by SRT1720 with 3-MA or Atg7 knockout cells could affect cell death. MDA-MB-231 cells treated with either SRT1720 or 2.5 mmol/L 3-MA resulted in a 65% and 18% decrease in cell viability, respectively. However, cotreatment of 3-MA with SRT1720 did not reduce SRT1720-induced cell death (Fig. 3F). In addition, treatment of WT MEFs with 5 μmol/L of SRT1720 resulted in a 22% decrease in cell viability, whereas the number of Atg7 knockout cells decreased by 80% (Fig. 3G). Therefore, these results suggest that autophagy is increased during SRT1720 treatment due to cellular stress as a mechanism to promote cell survival.
SRT1720 induces lysosomal membrane permeabilization

Necrotic cell death may be caused by permeabilization of the lysosomal membrane (12). LMP causes the release of cathepsins and other hydrolases from the lysosomal lumen to the cytosol (25). To determine whether the death of MDA-MB-231 cells was caused by permeabilization of the lysosomal membrane, cells were incubated with either acridine orange or LysoTracker Red. Acridine orange and LysoTracker Red normally concentrate in the lysosomal compartment and emit red fluorescence, but upon permeabilization, these dyes will diffuse out of the lysosome (12). In the case of acridine orange, the lysosomal vacuoles become yellow as a result of an increase in the pH. After treatment of MDA-MB-231 cells with SRT1720 for 16 hours, the cells were incubated with acridine orange and observed by microscopy. SRT1720 caused an increase in yellow stained cells, therefore indicating an increase in lysosomal membrane permeability (Fig. 4A). An analysis of the cells by FACS showed that there is a dose-dependent decrease in the percentage of red fluorescent cells with SRT1720 treatment. The percentage of red cells decreased by 68% with 7.5 μmol/L and 95% with 10 μmol/L of SRT1720 (Fig. 4B). We also incubated the cells with LysoTracker Red and observed a 66% and 90% decrease in LysoTracker Red staining by FACS analysis after treatment with 7.5 and 10 μmol/L of SRT1720, respectively (Fig. 4C).

Lysosomal inhibitors block SRT1720-induced cell death

As a result of our observation that SRT1720 increases LMP, we next determined whether inhibitors of lysosomal
acidification could affect SRT1720-induced cell death. The lysosomotropic alkalinazation agents, ammonium chloride, bafilomycin A1, and chloroquine, affect lysosomal acidification through different mechanisms. Ammonium chloride directly neutralizes lysosomal pH, bafilomycin A1 is an inhibitor of the V-ATPase that pumps protons into the lysosome, and chloroquine blocks the fusion of the lysosome and autophagosome (23, 26, 27). We assessed the viability of MDA-MB-231 cells treated with SRT1720 and lysosomal inhibitors. We observed that ammonium chloride, bafilomycin A1, and chloroquine could significantly reduce SRT1720-induced cell death. Treatment with SRT1720 alone resulted in a 79% decrease in viable cells, whereas cotreatment with ammonium chloride, bafilomycin A1, and chloroquine resulted in only a 23%, 20%, and 30% decrease in cell viability, respectively (Fig. 5A). We also observed that ammonium chloride could significantly reduce the death of Hs578T breast cancer cells caused by SRT1720 treatment (Supplementary Fig. S4). There was an 84% decrease in cell viability with 10 μmol/L of SRT1720 treatment compared to controls (Fig. 5B). LMP of MDA-MB-231 cells decreases the level of p62 protein, which indicates an increase in the rate of autophagy by SRT1720, whereas ammonium chloride inhibits SRT1720-induced p62 degradation by inhibiting lysosome function (Fig. 5D). We also determined whether SRT1720 caused activation of the endoplasmic reticulum (ER) stress pathway. ER stress is caused by a malfunction in protein processing in the ER lumen, which leads to an increase in unfolded/misfolded proteins (28). This will cause a block in protein translation by stress-kinase–induced phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α) and an induction of genes necessary for the proper folding of proteins including activating transcription factor 3 (ATF3; ref. 29). As a result of SRT1720 treatment, the phosphorylation of eIF2α and protein level of ATF3 was increased (Fig. 5D). Also, the cells were cotreated with ammonium chloride to determine whether the inhibition of SRT1720-induced death could be through the
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Figure 5.
LysoTracker Red-positive cells after treatment with SRT1720 and the lysosomal inhibitor NH4Cl. Cotreatment with NH4Cl significantly reduced the percentage of necrotic cells after SRT1720 treatment (***, P < 0.001).

Discussion
SIRT1 is the initial member of the 7-member sirtuin family of protein and histone deacetylases (7, 31, 32). Numerous studies have revealed important functions of SIRT1 in many biologic processes including cell proliferation, differentiation, apoptosis, senescence, metabolism, calorie restriction, lifespan regulation, and tumorigenesis (5, 6, 33–39). Although SIRT1 was initially considered as a tumor promoter in some studies, recent work has uncovered a tumor suppressor function of SIRT1, especially from...
analyzing SIRT1-mutant mouse models (17, 30, 40). Although SIRT1 might have both tumor promoter and tumor suppressor activity depending on the tissue context (41–43), a few studies have tested the effectiveness of a SIRT1 activator, SRT1720, for cancer therapy. These studies demonstrated that SRT1720 could serve as an inhibitor of myeloma (13); however, it could also promote tumor cell migration and lung metastasis in mice (14). In addition, there have been conflicting data on the use of SRT1720 as a direct activator of SIRT1 (8).

In the present study, we sought to determine whether SRT1720 could affect breast cancer cells in vitro and in vivo in a SIRT1-dependent manner. We observed that SRT1720 caused increased cell death in multiple breast cancer cell lines and other tumor types. In the panel of breast cancer cell lines that we tested, the basal-type cells including MDA-MB-231 were more sensitive to SRT1720 than luminal-type MCF-7 or non-transformed cells. However, unexpectedly the increased cell death with SRT1720 treatment in vitro was independent of SIRT1 levels. We also treated mice with SRT1720 in which neu mouse mammary tumor cells were grown in the mammary fat pad. SRT1720 also inhibited the growth of neu tumors, but in contrast with in vitro, this effect was partially dependent on SIRT1. It is unclear why there was a difference but it may be due to the function of SIRT1 in the stromal environment. It will be important to explore the role of SIRT1 and the tumor extracellular environment. From our in vitro experiments, we determined that SRT1720 caused cell death by necrosis but not apoptosis. We observed that SRT1720 activated several cellular stress pathways including autophagy and the unfolded protein response. However, the effect of SRT1720 was independent of the activation of these pathways because inhibition of either autophagy or the unfolded protein response through either chemical or genetic means did not inhibit SRT1720-induced cell death. Interestingly, we found that MDA-MB-231 cells are more resistant to SRT1720 treatment when cultured with conditioned media from cells plated at a high density. This observation suggests that a secreted factor from MDA-MB-231 cells may attenuate the effect of SRT1720. Future studies should be directed to identify this secreted factor as it may help to further understand the mechanism of action of SRT1720.

We identified LMP as the cause of SRT170-induced cell death. This could be verified with three different chemical modifiers of lysosomal function including bafilomycin A1, chloroquine, and ammonium chloride, which could all inhibit LMP and cell death caused by SRT1720. It has been established that cancer cells exhibit structural and functional changes in their lysosomes as compared with normal cells that allow cancer cells to survive (44). Therefore, targeting lysosomes to induce cell death has emerged as a possible target for cancer therapy to overcome apoptosis-resistant cells and drug resistance to chemotherapy (31). Many therapeutic agents have been reported to effect the death of cancer cells through modulation of lysosome function (25). Therefore, SRT1720 may serve as a useful agent for studying lysosomal-dependent cell death in cancers, which may eventually lead to the development of effective therapeutic approaches for cancer prevention and treatment.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.J. Lahusen, C.-X. Deng
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