Histone/protein deacetylase SIRT1 is an anticancer therapeutic target

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Abstract: SIRT1, a member of the NAD+-dependent histone/protein deacetylase family, is involved in chromatin remodeling, DNA repair, and stress response and is a potential drug target. 5-fluorouracil (FU) and the S1-type DNA methylating agent temozolomide (TMZ) are anticancer agents. In this study, we demonstrate that sirt1 knockout mouse embryonic fibroblast cells are more sensitive to FU and DNA methylating agents than normal cells. Based on these findings, the chemotherapy efficacy of SIRT1 inhibitors in combination with FU or TMZ were tested with human breast cancer cells. We found that treatments combining SIRT1 inhibitors with FU or TMZ show synergistic reduction of cell viability and colony formation of breast cancer cells. Thus, inhibition of SIRT1 activity provides a novel anticancer strategy.

Keywords: Breast cancer, drug resistance, SIRT1 histone deacetylase, 5-fluorouracil, methylating agents

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women [1]. Currently, drug resistance remains the most perplexing problem in breast cancer therapy. The future of cancer treatment lies in tailoring regimens to individual patients by identifying response predictors and developing novel therapeutic agents. Both SIRT1 histone deacetylase and thymine DNA glycosylase (TDG, a base excision repair (BER) enzyme) are key factors controlling cell cytotoxicity by 5-fluorouracil (FU) [2-4]. In addition, SIRT1 [5-7] and TDG [8, 9] regulate estrogen receptor alpha (ERα) signaling. Thus, SIRT1 and TDG are attractive targets for breast cancer therapeutics.

SIRT1 is a NAD+-dependent histone/protein deacetylase that has been linked with gene silencing, control of the cell cycle, apoptosis, energy homeostasis, and aging (reviewed in [10, 11]). It has been observed that Sirt1 knockout mice die in early postnatal stages [12] and that SIRT1-defective or knockdown cells are more sensitive to several DNA damaging agents [2, 3, 12-16]. SIRT1 is known to deacetylate histones, thereby silencing gene transcription. SIRT1 also deacetylates many non-histone proteins including many DNA repair enzymes, DNA methyltransferase 1 (DNMT1), and tumor suppressor p53 [15-19]. We recently reported that SIRT1 deacetylates TDG and inhibits TDG expression to modulate TDG activity and substrate specificity [20]. Besides repairing T:G mismatches, TDG enhances the activity of many transcription factors [8, 9] and participates in active DNA demethylation [21, 22], thus activating gene expression.

5-Fluorouracil (FU) is an important cancer therapeutic agent that acts as a thymidylate synthase inhibitor to block dTMP synthesis [23]. Administration of FU leads to lower levels of dTMP with elevated dUMP and dFUMP concomitantly. Incorporation of dUMP and dFUMP into DNA causes rapidly dividing cancer cells undergo cell death. FU also affects RNA metabolism that contribute significantly to the toxicity of the drug [24]. FU has been used to treat several types of cancer including breast, colon, rectum, and head and neck cancers. Studies have fur-
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other shown that FU can combine with other chemotherapy drugs to treat breast cancer before or after surgery [23]. However, drug resistance remains a significant limitation to the clinical use of FU. Therefore, new strategies to overcome FU-resistance have been intensively explored. It has been shown that TDG deficiency causes resistance while SIRT1 deficiency causes increased sensitivity to FU [2-4]. Thus, modulating the expression levels or activities of SIRT1 and TDG may overcome FU-resistance.

The $S_n$-type DNA methylating agents, such as N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) and temozolomide (TMZ) cause cell cycle arrest and apoptosis mainly by generating O6-methylguanine (MeG). TMZ has been clinically used to treat astrocytoma (an aggressive brain tumor) [25-27] and melanoma [28]. MeG lesions can be repaired by the suicide enzyme MeG methyltransferase [29], however, MeG methyltransferase is inactive in most solid tumor cells [30]. When MeG is not repaired, it pairs with thymine during DNA replication [31]. This prompts DNA repair enzymes including TDG to initiate futile repair and apoptosis [9, 32-36]. Thus, TDG-deficient cells are resistant to methylating agents of $S_n$ type [9].

In this report, we focus on SIRT1 as a therapeutic target for breast cancers. As compared to normal cells, sirt1 knockout mouse embryonic fibroblast (MEF) cells are more sensitive to FU and $S_n$-type DNA methylating agents. We show that sirtinol and EX-527 (a specific SIRT1 inhibitor) can enhance the cytotoxicity of FU and TMZ to breast cancer cells. Our results provide new strategies to overcome or limit drug resistance.

Materials and methods

Cell culture

Triple negative metastatic human breast cancer cell line MDA-MB-231 (Cell Biolabs, Inc) was derived from the pleural effusion of a cancer patient [37]. Cells were maintained at 37°C in 5% CO₂ in MEM (Life Technology) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin. MCF10A cells (Michigan Cancer Foundation) were maintained in DMEM/F12 (Life Technology) supplemented with 5% horse serum with additions of 20 ng/ml epithelial growth factor, 0.5 µg/ml hydorcortione, 0.1 µg/ml cholera toxin, 1 µg/ml insulin, and penicillin/streptomycin. MCF7 cells (American Type Cell Culture) were maintained in DMEM (Cellgro) supplemented with 10% fetal bovine serum and penicillin/streptomycin. MCF7Ca cells (obtained from Dr. Angela Brodie at University of Maryland) were derived from MCF7 by stably transfection with the human aromatase (an estrogen biosynthetic enzyme) gene. MCF7Ca cells were cultured similarly as MCF7 except with an addition of 0.7 mg/ml G418. Sirt1$^{+/+}$ (wild-type) and sirt1$^{-/-}$ (knockout) MEF cells (obtained from Dr. Toren Finkel at NIH) were maintained in DMEM (Invitrogen) supplemented with 15% fetal bovine serum and 1% Penicillin-Streptomycin.

Western blotting

The antibodies used for Western blotting were: ERα (gift from Dr. Chen-Yong Lin at Georgetown University), SIRT1 (Millipore), TDG (from Primo Schar, University of Basel, Switzerland), β-actin (Sigma-Aldrich), and horseradish peroxidase-conjugated anti-mouse/anti-rabbit antibodies (BioRad). Cell extracts (about 25 µg of total protein) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for Western blotting [38].

Cell viability and colony formation assays

Cell viability was measured using the neutral red uptake assay [39]. SIRT1 wild-type and knockout MEFs were seeded in 96-well flat bottom tissue culture plates. One day post-seeding, the cells were treated with FU (Sigma-Aldrich), MNNG (VWR), TMZ (Axxora), or DMSO for 24 h. The cells were then recovered in regular media for 2-3 days. MDA-MB-231 cells were treated with sirtinol (Axxora), EX-527 (Sigma-Aldrich), FU, and/or TMZ for 3 days or left untreated, then recovered in regular media for 2-3 days. The plates were incubated for 2 h in regular medium containing 40 µg/ml of neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride, Sigma). After the cells being washed with PBS, the dye was extracted from each well with acidified ethanol solution and the absorbance at 540 nm was read by a Multiskan Spectrum microplate spectrometer (Thermo Lab systems).

For clonogenic survival assays, cells were seeded at 5000 cells per well in 6-mm dishes and
Figure 1. Sirt1 defective cells are more sensitive to FU and S1-type DNA methylating agents. A, C and E: Wild-type and sirt1 knockout MEFs were treated with increasing doses of FU, MNNG, or TMZ, respectively, followed by 2-3 day recovery. Cell viability was measured as described in Materials and Methods. Data were normalized to the mock treatment controls (as the value of 100%). B, D and F: Wild-type and sirt1 knockout MEFs were treated with FU, MNNG, or TMZ, respectively, and allowed to grow into colonies for 10 days. Colony formation was measured as described in Materials and Methods. The formed colonies were counted, and the data were normalized to the mock treatment controls (as the value of 100%). Error bars indicate SD; n ≥ 3. Two stars indicate that p-values are smaller than 0.05.
treated with drugs as described above. Regular media was replaced after treatment. After 10 days, cells were stained with 0.5% crystal violet in 20% methanol and counted.

**Apoptosis TUNEL assay**

The apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in accordance with the manufacturer’s protocol (Promega) [38]. Images were captured using a Nikon E400 fluorescent microscope with an attached CCD camera.

**Results**

Sirt1-knockout mouse cells are more sensitive to 5-fluorouracil and S\textsubscript{n}-1-type DNA methylating agents

SIRT1-defective or knockdown cells have been shown to be more sensitive to several DNA damaging agents [3, 12, 13, 15]. In addition, SIRT1 is up-regulated in FU-resistant cells and SIRT1 silencing significantly lowers the resistance to FU in FU-resistant cells [2]. Therefore, we compared wild-type and sirt1 knockdown MEF cells for sensitivity against FU. We first determined the cellular viability in response to different doses of FU. Sirt1-knockout cells showed significantly higher sensitivity to FU at all tested FU concentrations (5-15 µM) than the control cells (Figure 1A). We also determined the colony formation of sirt1 knockout cells after FU treatment. As shown in Figure 1B, Sirt1-depleted MEF cells had significantly reduced ability to form colonies following FU treatment compared to the control cells. Moreover, sirt1-knockout cells undergo apoptosis at 24 h and 72 h after FU treatment (Figure 2B). Thus, it is concluded that SIRT1 controls the cellular sensitivity to FU.

SIRT1 has never been reported to link to sensitivity to S\textsubscript{n}-1-type DNA methylating agents. Because SIRT1 interacts with TDG and TDG-defective cells are resistant to DNA methylating agents [9], we began examining the role of SIRT1 in response to methylating agents. We measured the sensitivity of Sirt1-depleted MEF cells to MNNG and TMZ. Sirt1-defective cells were only slightly more sensitive to MNNG and TMZ than control cells when recovered in regular media for 2 days in cell viability assays (Figure 1C and 1E). However, after 3 days of recovery, Sirt1-knockout cells showed significantly higher sensitivity to MNNG and TMZ than the control cells (Figure 1C and 1E). Similarly, sirt1-knockout cells formed fewer colonies than the control cells after treatment with MNNG and TMZ (Figure 1D and 1F). At 24 h after MNNG treatment, sirt1-knockout cells did not show more apoptotic cells as compared to control cells (Figure 2C, 2\textsuperscript{nd} column). However, apoptotic cells did increase in sirt1-knockout cells, but not in control cells, at 72 h after MNNG treatment (Figure 2C, 3\textsuperscript{rd} column). These results demonstrate that SIRT1 plays a significant role in modulating cytotoxic effects of FU and DNA methylating agents.

**SIRT1 is up-regulated and TDG is down-regulated in MDA-MB-231 breast cancer cell**

Before testing drug effects on breast cancer cells, we examined SIRT1 and TDG expression in several breast cell lines by Western blotting. MCF7 and MCF7Ca cell lines are ER positive (ER\textsuperscript{+}) while MDA-MB-231 and non-cancer MCF10A cells are ER negative (ER\textsuperscript{-}). We observed that SIRT1 was expressed in breast cancer cells but not in MCF10A cells (Figure 3, 2\textsuperscript{nd} panel), in particular, MDA-MB-231 cells had very high SIRT1 expression (Figure 3, 2\textsuperscript{nd} panel, lane 4). This finding is consistent with those in Alvala et al. [40]. Moreover, TDG protein was expressed in the ER\textsuperscript{+} cell lines, but was very low in ER\textsuperscript{-} cell lines (Figure 3, 3\textsuperscript{rd} panel).

We chose MDA-MB-231 breast cancer cell line as our model system to examine drug effects because this cell line is invasive and is resistant to several anti-cancer agents [41]. This cell line displays a high activity of DNA methyltransferases [42], low expression of miR-34a [43], and extensive DNA methylation of the CpG island in the promoter region of the ER\textalpha gene [42]. In addition, MDA-MB-231 cells express a mutant p53 and lack the tumor-suppressor kinase LKB1, making them very resistant to drug treatments [44].

**SIRT1 inhibition leads to increased sensitivity to 5-fluorouracil in human breast cancer cells**

To investigate the role of SIRT1 in drug resistance in human breast cancer cells, we employed two SIRT1 inhibitors (sirtinol and EX-527) and FU. Sirtinol is a pan-inhibitor of
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SIRTs, whereas EX-527 has high specificity for SIRT1 but not SIRT2 [45]. It has been shown that sirtinol induces cell death in breast cancer cells and EX-527 causes cell cycle arrest at G1 phase in MCF7 cells [45]. First, we measured the sensitivity of MDA-MB-231 breast cancer cells to various concentrations of FU in the absence and presence of 40 μM sirtinol. We observed synergistic effects in the range of 20-100 μM FU (Figure 4A). When FU concentrations are higher than 250 μM, there was no statistically significant difference between treatments with FU alone and FU plus sirtinol.

Second, we examined individual drug concentrations that slightly inhibited cell viability of MDA-MB-231 cells. Sirtinol (40 mM), EX-527 (40 mM), and FU (20 mM) reduced cell viability of MDA-MB-231 cells by about 30%, 20%, and 10%, respectively (Figure 4B, columns 2-4). Next, we determined whether SIRT1 inhibitors in combination with FU had any effect on cell viability (Figure 4B, last 2 columns). With the combination of sirtinol and FU, 39% cells were viable; while with the combination of EX-527 and FU, 29% cells were viable with combination indices (CI) of 0.62 and 0.43, respectively. CI values that are less than one indicate drug synergism. Sirtinol (40 mM), EX527 (40 mM), and FU (20 mM) reduced colony formation of MDA-MB-231 cells about 20%, 20%, and 50%, respectively (Figure 4C, columns 2-4). Combination treatments, sirtinol and FU or EX-527 and FU, led to 12% of cells forming colonies (Figure 4C, last 2 columns). There are strong synergistic effects (CI values ≈ 0.3) with SIRT1 inhibitors and FU in the colony formation assay. Thus, SIRT1 inhibitors can provide anticancer therapeutics by enhancing efficacy of FU.

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Figure 2. Sirt1-knockout cells undergo apoptosis after treatment with FU and MNNG. Wild-type and sirt1 knockout MEFs were treated with DMSO (A), 15 µM of FU (B), or 15 µM of MNNG (C) for 24 h and then grown for additional 24 h or 72 h. Cells were stained with DAPI and subjected for TUNEL assay to detect DNA (blue) and apoptotic cells (green). 0 h reflects the time after treatment.
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TMZ alone at 15 µM could reduce cell viability by 15% (Figure 5B, 4th column) and at 5 µM could reduce colony formation by 50% (Figure 5C, 4th column). With the combination of sirtinol and TMZ, 45% cells were viable (Figure 5B, 5th column) and 6% of cells formed colonies (Figure 5C, 5th column). With the combination of EX-527 and TMZ, 50% cells were viable (Figure 5B, last column) and 20% of cells formed colonies (Figure 5C, last column). Significant synergistic effects with SIRT1 inhibitors and TMZ were observed in both assays. There were strong synergistic effects (CI values < 0.5) with SIRT1 inhibitors and FU in the colony formation assay. The CI value was 0.15 when MDA-MB-231 cells were treated with sirtinol and TMZ in the colony formation assay (Figure 5C, 5th column). Sirtinol exhibits a better therapeutic effect than EX-527 by enhancing the TMZ-caused colony reduction (Figure 5C, compare 5th and 6th columns). Thus, SIRT1 inhibitors can provide anticancer therapeutics by enhancing efficacy of TMZ.

Discussion

Conventional chemotherapy often encounters drug resistant cancer cells. To overcome this problem, novel therapeutic strategies are in an urgent need to be developed, and one of the attractive strategies is the combination of new drugs in chemotherapy. Here, we show that SIRT1 inhibitors can provide anticancer therapeutics by enhancing the efficacy of FU and TMZ to human breast cancer cells. We found that the combination treatments produced synergistic inhibition of cell proliferation and colony formation, compared with single treatments. Our strategies are based on the findings that Sirt1 defective mouse cells are significantly more sensitive to FU and S1,1-type methylating agents compared to the control cells. Our results are consistent with several reports demonstrating that SIRT1 is involved in FU resistance [2, 3, 43, 46]. Downregulation of SIRT1 sensitizes colon and breast cancer cells to FU [2, 3]. Synergistic antitumor effect of tenovin-6 (an inhibitor of SIRT1 and SIRT2) has been observed in combination with FU in colon cancer cells [46]. Ectopic expression of miR-34a, one of the SIRT1 suppressors, attenuates the resistance to FU [2]. Our findings that SIRT1 plays a role in resistance to MNNG and TMZ are novel. Although TMZ has not been used to treat breast cancer patients, our finding reveals a potential novel therapeutics. A combination of SIRT1 inhibitors with TMZ may be a novel therapy for breast cancer, thus, SIRT1 is a therapeutic target for breast cancers in FU and TMZ chemotherapies.

The molecular mechanism for SIRT1's effect on protecting cells from apoptosis upon FU and TMZ treatments remains to be further investigated. There are several possible mechanisms to be explored based on SIRT1 activity and SIRT1 interacting proteins. First, because SIRT1 is a histone deacetylase, SIRT1 may regulate chromatin structure. A compact chromatin is less accessible to drugs. If this is the case, SIRT1 will protect cells from many anticancer drugs. This is supported by the findings that SIRT1-defective or knockdown cells are more sensitive to ultraviolet light, methyl methanesulfonate, H2O2, ionizing irradiation, and FU [2, 3, 12-16]. Second, because SIRT1 usually acts as gene silencers [11], SIRT1 may suppress anti-apoptotic or tumor suppressor genes such as p53 [47], thus enhancing cell survival. Third, because SIRT1 deacetylates many non-histone proteins and is involved in DNA repair and response to stress [48, 49], SIRT1 may protect cells from DNA damage induced by FU and TMZ. For example, Kabra et al. have suggested that SIRT1 expression provides a cell survival advantage under cellular stress [3].
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One of the SIRT1 target proteins is TDG [20]. We have shown that SIRT1 interacts with TDG, suppresses TDG expression, reduces TDG acetylation, and alters the DNA substrate specificity of TDG [20]. Because Kunz et al. [4] have shown that inactivation of TDG significantly increases cell’s resistance towards FU, we favor a model that SIRT1 promotes FU resistance by reducing TDG expression and deacetylating TDG and APE1 (the 2nd enzyme in BER) [15], therefore reducing FU cytotoxicity in cancer cells. In this case, an abasic (AP) site generated by TDG is converted to a nick by APE1 and is further repaired by BER. However, in the presence of SIRT1 inhibitors (Figure 6), the amount of AP-DNA is greatly increased because (i) overproduced and hyper-acetylated TDG (Ac-TDG) exhibits higher activity toward FU/G and (ii) acetylated APE1 cannot form a complex with XRCC1 [15], leading to unbalanced and inefficient repair. Spontaneous breakage at AP sites generates strand breaks. These single-stranded breaks can be converted to double-stranded breaks during DNA replication or through breakage at neighboring AP sites and thus cause FU cytotoxicity.

Figure 4. SIRT1 inhibitors and FU have synergistic effects on cytotoxicity of breast cancer cells. (A) Viability assay with various FU doses. MDA-MB-231 breast cancer cells were treated with different doses of FU in the absence or presence of 40 µM sirtinol for 2 days or left untreated, then recovered in regular media for 2 days. Percentage (%) of cell viability was normalized with untreated control. Error bars indicate SD; n = 3. Combination indices (CI) were determined with the equation: % of combined treatment/(% of treatment A x % of treatment B). CI values less than one indicate synergism. One, two and three stars represent CI (0.5-1), CI (0.25-0.5), and CI (< 0.25), respectively. (B) Viability assay with fixed drug doses. MDA-MB-231 cells were treated with 40 µM sirtinol, 40 µM EX-527, and 20 µM FU singly or in combination similar as in (A). The averages of percentage (%) of cell viability were shown above each bar. (C) Clonogenic survival assays. MDA-MB-231 cells were treated with 40 µM sirtinol, 40 µM EX-527, and 20 µM FU singly or in combination for 2 days or left untreated, then recovered in regular media for 10 days and colonies were counted. The data were calculated and presented as in (B).
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For the first time, we show that SIRT1 deficiency or inhibition causes increased sensitivity to S\textsubscript{\text{N}}1-type DNA methylating agents such as MNNG and TMZ. S\textsubscript{\text{N}}1-type methylating agents represent an important class of chemotherapeutics, but the molecular mechanisms underlying their cytotoxicity are unclear. Their toxicity appears to result from the processing of MeG-containing mispairs by DNA repair enzymes. It has been shown that the persistence of MeG induces DNA damage response by the mismatch repair system [36] and MBD4-dependent BER [32]. Similarly, TDG can remove T from T/MeG and has also been suggested to initiate futile repair cycles or DNA damage response [9]. We have shown that MNNG induces TDG foci formation and enhances TDG interaction with the checkpoint clamp Rad9-Rad1-Hus1 [50]. The interaction between SIRT1 and TDG suggests that TDG may mediate SIRT1-dependent MNNG cytotoxicity. It is also possible that SIRT1 reduces cytotoxicity of methylating agents by mediating mismatch repair [36] or MBD4-dependent BER [32]. The requirement of 72 h to observe MNNG-induced apoptosis in sirt1\textsuperscript{−/−} cells is consistent with the finding that cell cycle is arrested in the second G2 phase of mismatch defective cells after MNNG treatment [51].

Through interactions with and modification of many DNA repair enzymes, SIRT1 maintains genomic integrity and regulates the cellular response to stress. The status of SIRT1 expression in cancer patients is therefore likely to determine their response to chemotherapy. We have demonstrated the synergistic effect of SIRT1 inhibitors and conventional chemotherapies with FU and TMZ treatments to human breast cancer cells. These combined chemotherapies may be most effective on SIRT1 overproducing and/or TDG under-producing cancers. Thus, SIRT1 inhibitors could be potential therapeutic molecules for enhancing drug efficacy in treating tumors.
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Disclosure of conflict of interest

The authors declare no conflict of interest.

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