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Linda Chen April 9, 2023

Investigating the role of SAMHD1 in response to Camptothecin and Sacituzumab Govitecan in triple-negative breast cancer cell line MDA-MB-231

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Abstract

Investigating the role of SAMHD1 in response to Camptothecin and Sacituzumab Govitecan in triple-negative breast cancer cell line MDA-MB-231

By Linda Chen

Due to the lack of response to hormone therapy and therapies targeting human epidermal growth factor receptor 2 (HER2), triple-negative breast cancer (TNBC) patients need biomarkers to identify individual sensitivity to conventional treatments inducing DNA damage. Protein Sterile Alpha Motif and Histidine-Aspartic acid domain containing protein 1 (SAMHD1) was previously reported as biomarker for Camptothecin (CPT) and Poly (ADP-ribose) polymerase (PARP) inhibitor sensitivity in human osteosarcoma cell line, and it has a characterized role in facilitating homologous recombination (HR) mediated DNA double-strand break (DSB) repair. We hypothesized that loss of SAMHD1 might mediate sensitization of TNBC cell line to selected DNA damaging agents such as CPT and Sacituzumab Govitecan (SG). Interestingly, our present results show that loss of SAMHD1 does not sensitize MDA-MD-231 cells to DNA damaging agents CPT and SG as hypothesized. Additionally, we validated our findings by taking CtBP (C-terminal binding protein) interacting protein (CtIP) as a control and indeed observed a similar effect. Overall, our results reflect TNBC cell lines might mediate chemoresistance to SG and CPT upon SAMHD1 and CtIP depletion by activating other DNA repair pathways.

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Introduction

Breast cancer is one of the most prevalent and invasive cancer type among patients globally. It represents 10.4% of total cancer incidents, and causes 15% of total cancer death, being the second leading cause of death from cancer [1]. Proliferation of many breast cancer types can be stimulated by hormones, and thus those cells express surface receptors of estrogen (ER) and progesterone (PR) and overexpress human epidermal growth factor 2 (HER2) which also promotes growth [2]. Common treatments for these cancers include hormone therapies that inhibit production or binding of hormone and targeted therapy that attacks HER2 [3]. Triple-negative breast cancer (TNBC) is a type of aggressive breast cancer which lacks the ER and PR receptors and overexpression of HER2. As a result, TNBC patients respond poorly to hormone and targeted therapies [2] and have more restricted treatment options. Other conventional treatments for breast cancer, including ionizing radiation (IR), chemotherapy and second/third line treatment using poly ADP-ribose polymerase (PARP) inhibitors, promote cancer cell death by inducing more DNA damage [4]. However, resistance to DNA damaging agents impairs the effect of these treatments. Therefore, biomarkers for identification of resistance to DNA damage are important in personalization of treatments, aiming to promote therapeutic efficacy and to reduce toxicity.

Since change in DNA damage response (DDR) pathways can alter cells' response to DNA damaging agents, activity of proteins involving in DDR pathways could be potential indicators of cellular response. Sirtuins are a family of cellular signaling proteins, some of which have NAD+ dependent deacetylase activity. Previous studies from our lab have shown that depletion of Sirtuin 2 (SIRT2) results in higher sensitivity to DNA double-strand breaks (DSBs) inducing agents. Additionally, there are reports showing that SIRT2 depletion sensitizes cells to replication stress, IR, camptothecin (CPT), PARP inhibitor veliparib, cisplatin, gemcitabine, hydroxyurea (HU), and mitomycin C (MMC) [5-9].

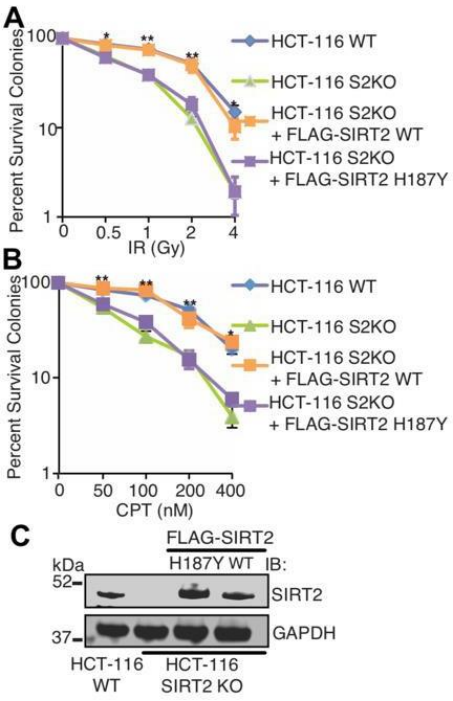

Figure 1. SIRT2 Deacetylase Activity Mediates Resistance to DSB-Inducing Agents. (A-B) HCT- 116 SIRT2 WT and KO cells were treated with IR or CPT and assayed 14 d later for colony survival. (C) Western blot showing HCT-116 SIRT2 KO cells complemented with FLAG-SIRT2 WT and H187Y. * $p<0.05$, ** $p<0.001$.

A preliminary study that used HCT-116 human colorectal carcinoma cells found increased sensitivity to IR and CPT in SIRT2 knockout (KO) cells, compared to wild type (WT) cells; further, this study showed that this hypersensitivity in KO cells were rescued by expression of WT SIRT2, but not SIRT2 H187Y which has inactive deacetylase function [10] (Figure 1). These results suggest that the deacetylase activity of SIRT2 is responsible for mediating sensitivity to DNA damaging agents. Therefore, acetylation status of substrates of Sirtuin might be potential biomarkers for cellular sensitivity.

Another Sirtuin family member Sirtuin 1 (SIRT1) deacetylates protein SAMHD1 at K354; this deacetylation promotes SAMHD1 binding to DSBs, which facilitates binding of core HR factor CtIP to DSBs and promotes HR mediated DSB repair [11]. Previous studies

show that depletion of SAMHD1 and its acetylation at K354 increase cellular sensitivity to DNA damaging agents like PARP inhibitors [11] and CPT [12] in U2OS human osteosarcoma cell line (Figure 2).

These suggests that the acetylation status of SAMHD1 might have a role in regulating cellular response to DNA damaging agents and in predicting patient outcome under related treatments. However, whether this observed regulatory role by SAMHD1 applies to TNBC cell line remains unknown.

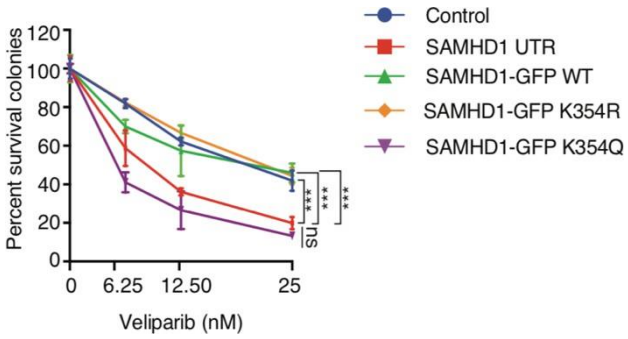

Figure 2. SAMHD1 deacetylation at K354 mediates PARP inhibitor resistance. U2OS cells silenced for endogenous SAMHD1 and expressing SAMHD1-GFP WT, deacetylation mimic K354R, or acetylation mimic K354Q were treated with indicated concentrations of veliparib and assayed for colony survival 10-12 days later. The mean +/- SEM from 3 independent replicas is shown. P values (***p<0.001) were determined by two-way ANOVA followed by a Bonferroni test.

CPT is a Topoisomerase I inhibitor which interferes with DNA religation by Topoisomerase I during DNA replication, thereby generating increased amount of DSBs and promoting cell death [13]. Many CPT analogs are common chemotherapeutic treatments against cancer. Sacituzumab govitecan (SG) is a novel antibody-drug conjugate which is used in clinics to target TNBC that remain metastatic after at least 2 breast cancer therapies, including 1 for metastatic disease [14]. In SG, SN-38 (the active metabolite of CPT analog Irinotecan) is conjugated with antibody against trophoblast cell-surface antigen 2 (Trop-2) [15], which is expressed in the majority of breast cancer cells including MDA-MB-231. Clinical studies have shown longer progression-free survival and overall survival in SG-treated patients compared to single-agent-chemotherapy-treated patients with metastatic TNBC [16]; efficacy of SG is not dependent on Breast Cancer Gene (BRCA) status, but

could be lower with low-Trop-2-expression[16]. Additional biomarkers are needed to predict treatment outcomes of SG.

Here we used MDA-MB-231 cell line, a well-established TNBC cell line that has wild type (WT) BRCA1 and mutant TP53 [17, 18]. We determined the inhibitory effect of 2 DNA damaging agents (CPT and SG) on MDA-MB-231, and we observed different involvement of SAMHD1 in regulation of cellular sensitivity in MDA-MB-231 cell line.

Results

Effect of Camptothecin (CPT) on MDA-MB-231 cell viability

The effect of CPT on MDA-MB-231 cell viability was investigated by measuring percentage survival of MDA-MB-231 cells under increasing dosage of CPT, compared to cells treated with DMSO control. During standardization process, optimal treatment time of 72 hours was selected based on the proposed doubling time of 21~36 hours of MDA-MB-231 cell line [19-22] to ensure that most cells would at least pass one S phase which allows the action of CPT during DNA replication. 96-well plates with black wall, transparent bottom and binding surface were selected to minimize luminescence signal crosstalk.

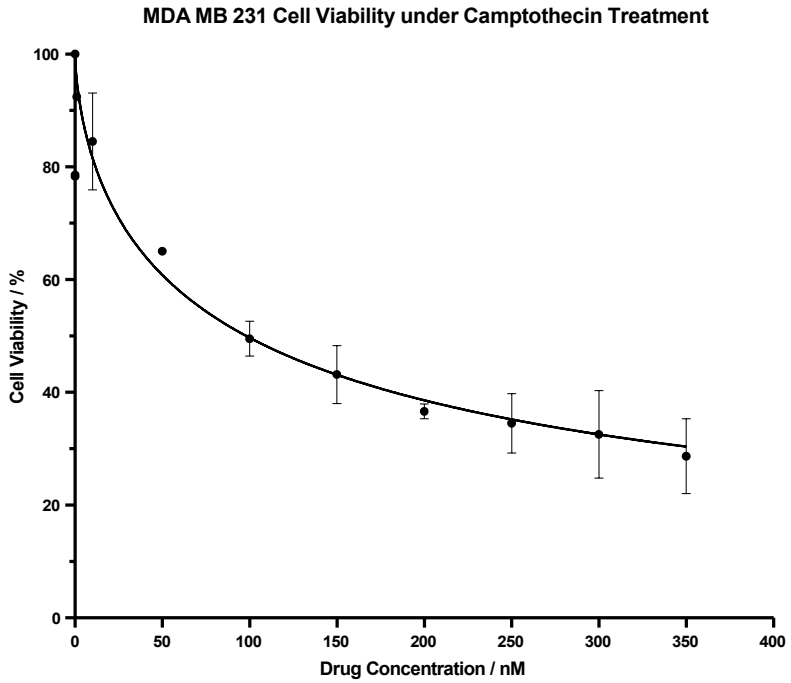

Figure 3. MDA-MB-231 cell line shows does-dependent response to CPT at lower concentrations MDA-MB 231 cells (8000 cells/well) were treated with increasing concentration of CPT. Cell viability was measured after 72 hours of treatment using CellTiter-Glo Luminescent assay. Relative cell viability was normalized against cells with no CPT treatment. Experimental triplicates were taken.

CPT showed does-dependent inhibitory effect on MDA-MB-231 cell viability (Figure 3). A working concentration that can effectively sensitize this cell line is around 98nM, determined by reduction of 50% of cell viability. However, the inhibition weakens as the concentration increases (57% decrease of relative survival from 0~150nM; 14.5% decrease of

relative survival from 150~350nM). This suggests that MDA-MB-231 cell line might show resistance to higher concentration of CPT. With experimental triplicates and biological triplicates, CPT exhibited an inhibition concentration that will cause 25% reduction of cell viability at 25nM. 50nM concentration was selected as the treatment dosage for the rest of the experiments because it effectively caused 35% reduction of cell survival, while remaining in the range of concentrations to which the cell line was still responsive.

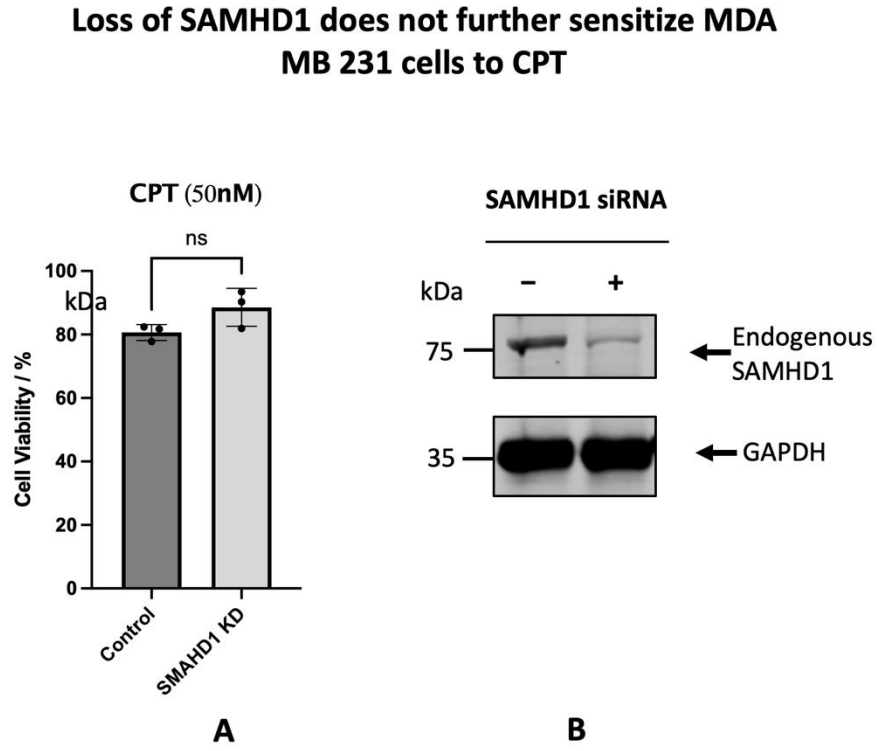

Figure 4. Knockdown of SAMHD1 does not further sensitize MDA-MB-231 cells to CPT (A) MDA-MB 231 cells show similar sensitivity to CPT with and without endogenous SAMHD1. **(B)** Western blot showing knockdown of SAMHD1 at 24hr post transfection. Cell viability was measured after 72 hours of treatment using CellTiter-Glo Luminescent assay. Relative cell viability was normalized against cells with no CPT treatment. Experimental triplicates were taken. p values ($p<0.05$) were determined using ordinary One-Way ANOVA.

Loss of SAMHD1 produces no synergistic effect with CPT in MDA-MB-231 cell line

Based on the verified CPT potency, to investigate whether SAMHD1 mediates sensitivity to CPT, effect of CPT on cell viability with and without endogenous SAMHD1 was measured.

Endogenous SAMHD1 in MDA-MB-231 cells were silenced via RNA interference. With biological triplicates and 6 experimental repeats, the relative percentage survival under 50nM CPT treatment of cells with silenced SAMHD1 was comparable to that of control cells with non-targeting siRNA (Figure 4A). This shows that under downregulation of endogenous SAMHD1 (Figure 4B), cellular response to DNA damaging effect of CPT was not significantly enhanced. While study has shown that silencing of SAMHD1 further sensitized U2OS cells to CPT, this phenotype might not be applicable to MDA-MB-231 cell line [12]. As the passage numbers increase, decreasing sensitivity to CPT treatment was observed.

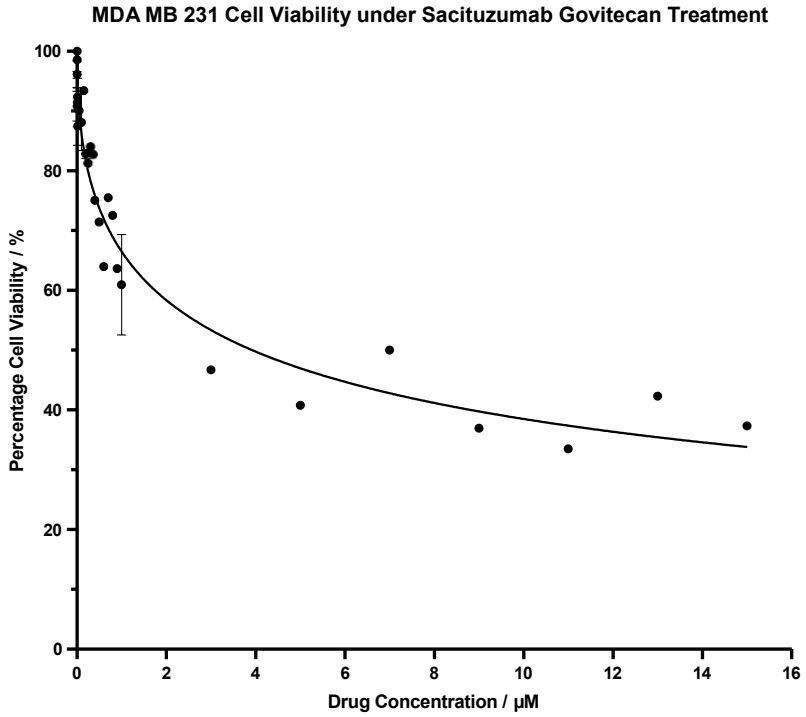

Figure 5. MDA-MB-231 cell line shows does-dependent response to Sacituzumab Govitecan at lower concentrations MDA-MB-231 cells (8000 cells/well) were treated with increasing concentration of Sacituzumab Govitecan (SG). Cell viability was measured after 72 hours of treatment using CellTiter-Glo Luminescent assay. Relative cell viability was normalized against cells with no SG treatment. Experimental triplicates were taken.

Effect of Sacituzumab Govitecan (SG) on MDA-MB-231 cell viability

The experimental setup remains the same as cell viability assays using CPT. Cells with 72-hour SG treatment showed decreased survival rate with increasing drug

concentration, while cells with 24-hour treatment showed no change in survival (data not shown). This suggests that the effect of SG is not impaired after passing its 11-to-16-hour half-life [23, 24].

SG showed does-dependent inhibitory effect on MDA-MB-231 cell viability. Likewise, the results of SG treatment show that, the reduction of cell viability weakens at the higher concentration as compared to lower concentration (53% decrease of relative survival from 0~3μM; 10% decrease of relative survival from 3~15μM). This suggests that MDA-MB-231 cell line shows resistance to SG surpassing a certain concentration threshold. With experimental triplicates and biological triplicates, SG exhibited an inhibition concentration that causes 25% reduction of cell viability at 0.4μM. 2μM concentration of SG was selected as the treatment dosage for the rest of the experiments because of two reasons. First, it caused 40% cell death; and hence was effective in inhibition. Second, the concentration chosen was not effective in causing chemoresistance in MDA-MB-231 cells.

Loss of SAMHD1 produces no synergistic effect with SG in MDA-MB-231 cell line

With biological triplicates and 6 experimental repeats, the relative survival under 2 μM SG treatment of cells with downregulated endogenous SAMHD1 (Figure 4B) was similar as that of control cells treated with non-targeting siRNA (Figure 6), suggesting that loss of SAMHD1 does not further sensitize MDA-MB-231 cells to SG. Equivalent results were seen in the case of CPT, wherein MDA-MB-231 cells showed higher survival as the passage number increases.

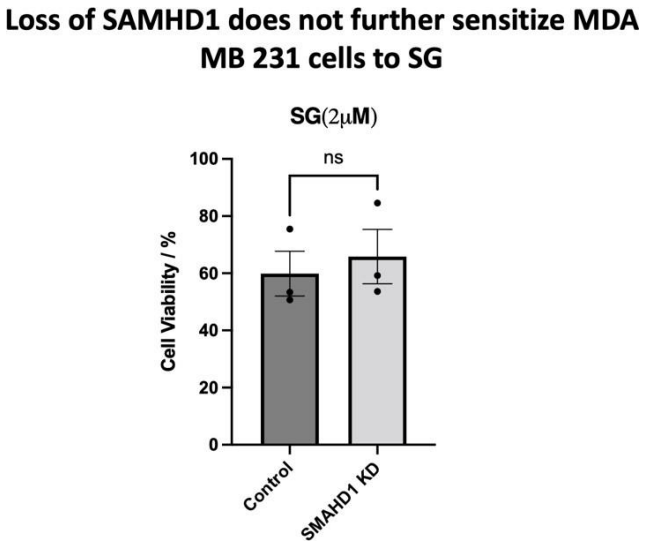

Figure 6. Knockdown of SAMHD1 does not further sensitize MDA-MB-231 cells to SG. MDA-MB-231 cells show similar sensitivity to SG with and without endogenous SAMHD1. Cell viability was measured after 72 hours of treatment using CellTiter-Glo Luminescent assay. Relative cell viability was normalized against cells with no SG treatment. Experimental triplicates were taken. p values ($p<0.05$) were determined using ordinary One-Way ANOVA.

Loss of CtIP and SAMHD1 produces no synergistic effect with CPT and SG

To verify the observation that loss of SAMHD1 does not further sensitize MDA MB 231 cells to CPT and SG, CtIP knockdown was selected as positive control. CtIP is one of the core factors in homologous recombination (HR), and its silencing leads to less effective HR mediated DSB repair. In line with this, CtIP depleted cells should show enhanced sensitivity to DNA damaging agents. We tested the viability of MDA-MB-231 cells in response to CPT and SG treatment, upon SAMHD1 knockdown and CtIP knockdown. The latter is used as a positive control, alongside with cells treated with non-targeting siRNA as negative control.

Upon depletion of both CtIP and SAMHD1, MDA-MB231 cells were not further sensitized to DNA damaging agents CPT and SG, (Figure 7A, 7B). While under same treatment condition, CtIP knockdown led to a greater reduction in cell viability than SAMHD1 knockdown. However, this reduction is not statistically significant tested with One-Way ANOVA analysis. Western blot was taken to test knockdown efficiency from

samples of the same batch of cells. The effectiveness of SAMHD1 knockdown was still intact at 120hr post transfection, same time point as the measurement of CellTiter-Glo assay (Figure 7C). This verifies that the nonsignificant change in cellular response was not caused by insufficient silencing. However, although CtIP knockdown at 24hr post transfection was effective, it was not clearly shown at 72hr and 120hr time points due to ineffective binding of primary antibody (Figure 7C). Therefore, the question that whether the nonsignificant change in sensitivity was caused by less dependence of cells on HR or by less effective CtIP knockdown still needs to be further verified.

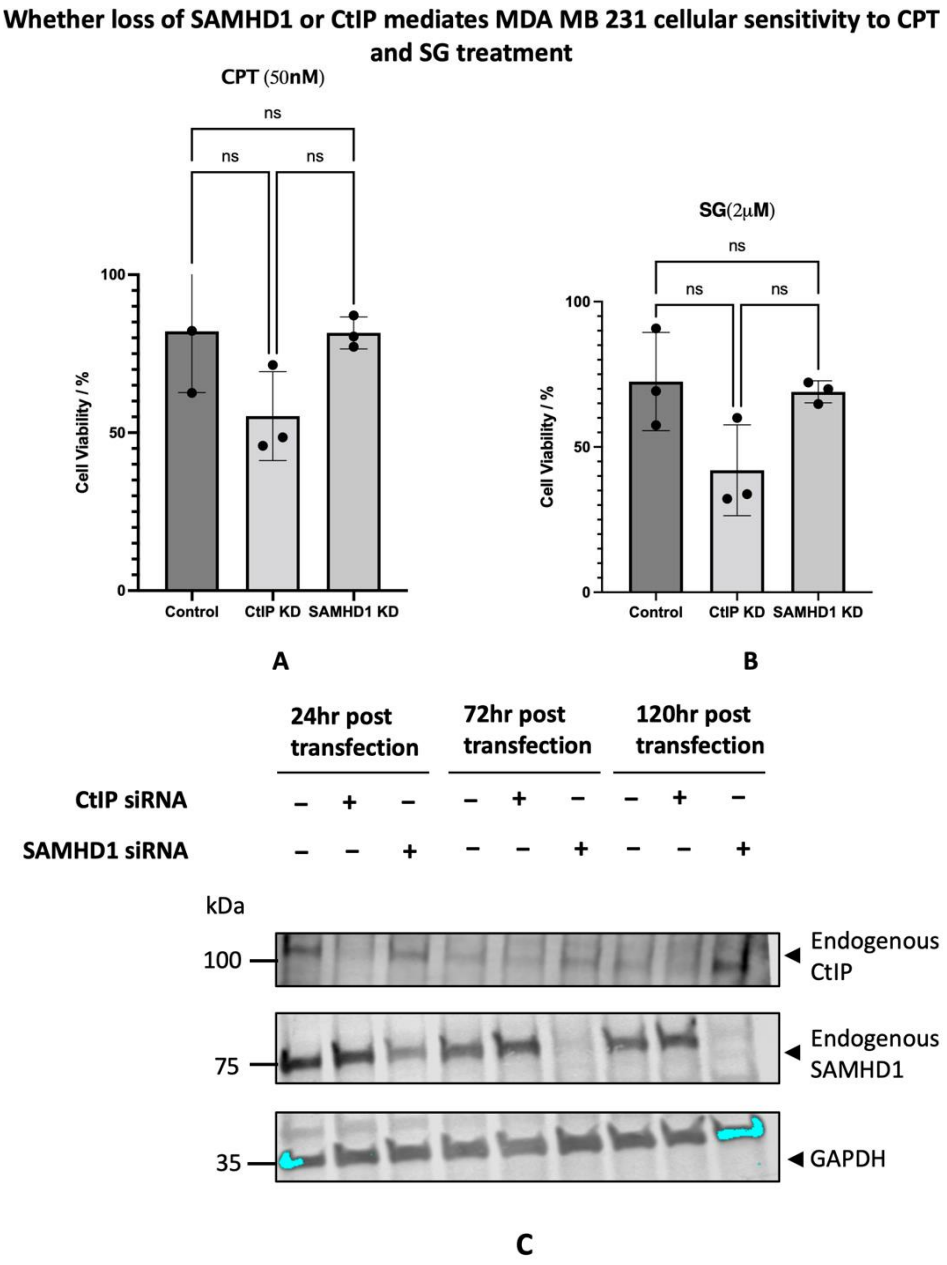

Figure 7. Knockdown of SAMHD1 and CtIP do not further sensitize MDA-MB-231 cells to CPT and SG (A, B) MDA-MB-231 cells show similar sensitivity to CPT & SG with and without endogenous SAMHD1. Cells with CtIP knockdown show higher sensitivity to both drugs, but this decrease of survival is not statistically significant. **(C)** Western blot showing knockdown of SAMHD1 and CtIP at 24hr, 72hr and 120hr post transfection. Cell viability was measured after 72 hours of treatment using CellTiter-Glo Luminescent assay. Relative cell viability was normalized against cells with no DNA damaging agent treatment. Experimental triplicates were taken. p values ($p<0.05$) were determined using ordinary One-Way ANOVA.

Discussion

Our study adds data to the dose-dependent response to CPT and the novel chemotherapeutic agent SG in MDA-MB-231 cell line. Overall, our results show that the working concentration of CPT to sensitize MDA-MB231 cells is around 98 nM. Interestingly, the concentration of CPT optimized during this study varies from the previously reported CPT concentrations [25-27].CPT and SN-38 both induce DNA DSB via Topoisomerase I inhibition, and SG promotes more targeted delivery of SN-38 via Trop-2 antibody expressed on breast cancer cells. Previous studies showed that: low dosage of SG could stimulate chemoresistance in MDA-MB-231 cells by activating HR mediated DNA repair pathway, but this resistance was overcome when surpassing a certain concentration threshold [28]. However, our results are not concurrent with previous published studies, and interestingly, we observed an even 40-folds higher working concentration of SG in sensitizing the MDA-MB231 cells, less effective as that of CPT. We are not able to understand why a higher dosage of SG is not able to sensitize cells. One of the plausible causes we hypothesize is that at higher concentrations, cells might circumventing HR and utilizing other repair pathways to gain chemoresistance and increase survival. Another alternative hypothesis is that MDA-MB-231 cell line shows increased chemoresistance due to elevated interaction with the Trop2 antibody or linker region in SG, which in turn might interfere with delivery and activity of SN-38.

Study has shown that under induction of DNA damage, SAMHD1 promotes HR by facilitating binding of CtIP onto DSB sites in U2OS cell [11]. It was expected that since MDA-MB-231 cell line has WT BRCA gene that maintains HR mediated DSB repair, loss of core HR factor CtIP and its mediator SAMHD1 might impair DNA repair efficacy and increase cellular sensitivity. However, here we demonstrated that the loss of both CtIP and

SAMHD1 did not further sensitize cells to DNA damaging agents. More biological replicates of the cell viability assay and corresponding Western Blot could be done to confirm the reproducibility of the results and knockdown efficiency of CtIP. Cell proliferation assays like clonogenic assay could be done to verify the observed phenotype: testing of whether the ability to proliferate might be impaired could also indicate cellular sensitivity to DNA damage. If the results remain consistent, due to lack of synergy, SAMHD1 might not be a biomarker for efficacy of CPT and SG in MDA-MB-231 cell line. The lack of sensitivity to CtIP knockdown in MDA-MB-231 cell line is not previously reported; this might be attributed to the specific microenvironment and mutation profile of the specific cell line, which might render the cell line less dependent on CtIP. For example, the mutation of p53 tumor suppressor.

In respect of mechanism of action, future experiments could investigate whether the HR efficiency is downregulated in CPT or SG treated MDA-MB-231 cells with SAMHD1 or CtIP knockdown, in comparison with control cells to study if the particular cell line has DNA repair mechanisms that are less dependent on CtIP or HR.

Future experiments could also test for the effect of SAMHD1 and CtIP knockdown on sensitivity to CPT and SG in nontumorigenic breast epithelial cell line like MCF10A. If cellular sensitivity of MCF10A increases as in U2OS cell line, this could support the idea that MDA-MB-231 cell line has mutations that alter the role of SAMHD1 and CtIP in mediating cellular sensitivity; if both MCF10A and MDA MB 231 show similar sensitivity upon SAMHD1 and CtIP knockdown, this suggests that the differed involvement might be due to cell type difference instead of mutations that contribute to invasiveness of MDA-MB-231.

Our study shows that the presence and absence of one of SIRT1/2 substrate SAMHD1 does not mediate MDA-MB-231 cellular sensitivity to selected DNA damaging agents: common therapeutic agent CPT and novel antibody-conjugate SG. Therefore, the acetylation status of SAMHD1 might not be an effective biomarker for predicting cellular resistance and patient outcome under treatment of SG and CPT. However, future studies could investigate whether SAMHD1 sensitizes MDA-MB-231 cells to other DSB inducing agents like IR and PARP inhibitors. Moreover, other SIRT1/2 substrate including ATRIP, BARD1 and CDK9 could also be studied for potential role in regulation of cellular sensitivity to multiple DNA damaging agents.

Materials and Methods

Cell Culture

MBA-MD 231 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM media (Gibco, catalog number 11995065) supplemented with 10% Fetal Bovine Serum (FBS).

DNA Damaging Agents

Camptothecin (CPT) was purchased from Sigma-Aldrich (product number PHL89593), diluted with DMSO to 5mM stock aliquots, stored in -20°C.

Sacituzumab Govitecan was purchased from Selleck (catalog number E2841, stock concentration 6.243mM in DMSO), stored in -20°C.

RNAi Silencing

Endogenous *SAMHD1* and *CtIP* were silenced using siRNA. 3×10^5 cells were plated in 100mm cell culture dishes or 6-well culture plates. 24 hours post seeding, cells were transfected with siRNA at a final concentration of 50nM. siRNA and Lipofectamine RNAiMAX reagent were added to 2 separate tubes of Opti-MEM Reduced Serum Media. The 2 tubes were shaken for 5 minutes for proper mixing. The transfection mixture was allowed to stand for 20 minutes, with 1-minute shaking every 5 minutes. Transfection mixture was added to cell culture plate, on top of freshly added DMEM media. Cells were grown and harvested as needed for subsequent assays.

Opti-MEM Reduced Serum Media: Gibco, catalog number 31985070

siRNA used: SAMHD1-5'UTR (ACGCAUGCUGAAGCTAAGTAA); non-targeting siRNA (Dharmacon, catalog number D-001210-02-05); CtIP (Dharmacon, catalog number D-011376-01-0020)

Lipofectamine RNAiMAX reagent: Invitrogen, catalog number 13-778-150

CellTiter-Glo Luminescent Cell Viability Assay

Cell survival was measured using CellTiter-Glo Luminescent Cell Viability Assay Kit purchased from Promega (catalog number G7572). Substrate and buffer were mixed, and mixture was thawed following protocol.

Cells were seeded in 96-well plate (8000 cells in 200μL per well), 24 hours post transfection. 24 hours post seeding, media was replaced with DNA damaging agents of desired concentrations (100μL per well). 72 hours post treatment, 100μL of CellTiter-Glo mixture reagent was added on top of media in each well. Plate was gently shaken for 2 minutes for proper mixture and cell lysis, followed by 10 minuets of standing. Luminescence produced was measured by the BioTek Synergy H1 microplate reader, using BioTek Gen5 Microplate Reader and Imager software.

96-well plate: lumox catalog number NC1913418

Western Blot

Harvested cells were resuspended in 0.75% CHAPS buffer (10% glycerol, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM DTT, 1 μM TSA, 20 μM NAM, 0.75% CHAPS, protease inhibitors). Cells were lysed for 30 min on ice, and cell debris was cleared by centrifugation. Protein concentration was determined by Bradford. 50μg of protein was loaded and resolved by 10% SDS-PAGE gel, transferred to PVDF membrane and probed with the antibodies of

interest. Signal was detected using the Li-Cor Odyssey system with the ImageStudio 5.2 software.

Primary antibodies used: SAMHD1 (Origene #TA502024, 1:1000); GAPDH (Sigma, G9545, 1:2000); CtIP (Millipore # MABE1060; 1:1000)
Secondary antibodies used: donkey anti-rabbit IR Dye 680 (Licor Biosciences # 926-68023); donkey anti-mouse IR Dye 800 (Licor Biosciences # 926-33212); donkey anti-rabbit IR Dye 800 (Licor Biosciences # 926-33213); donkey anti-mouse IR Dye 680 (Licor Biosciences # 926-68022).

Statistical Analysis

For each biological replicate of the CellTiter-Glo assay, the mean of experimental repeats of each treatment condition was calculated. Background signal (reading for pure media) was subtracted. Cell viability was calculated as percentage cell survival: within cells of the same transfection status (control, SAMHD1 KD, CtIP KD), divide mean luminescence signal of each treatment group (CPT, SG) by mean luminescence signal of the group not treated with DNA damaging agents, and multiplies by 100. p values are determined using ordinary One-Way ANOVA (threshold: $p<0.05$).

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