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Synergistic Inhibition of Hepatocellular Carcinoma Growth by Cotargeting Chromatin Modifying Enzymes and Poly (ADP-ribose) Polymerases

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Abstract

Hepatocellular carcinoma (HCC) is a particularly lethal form of cancer, yet effective therapeutic options for advanced HCC are limited. As poly(ADP-ribose) polymerases (PARPs) and histone deacetylases (HDACs) are emerging to be among the most promising targets in cancer therapy, and sensitivity to PARP inhibition depends on homologous recombination (HR) deficiency and inhibition of HDAC activity blocks the HR pathway, we tested the hypothesis that co-targeting both enzymatic activities could synergistically inhibit HCC growth and defined the molecular determinants of sensitivity to both enzyme inhibitors. We discovered that HCC cells have differential sensitivity to HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and PARP inhibitor Olaparib, and identified one pair of cell lines, termed SNU-398 and SNU-449, with sensitive *versus* resistant phenotype to both enzyme inhibitors, respectively. Co-administration of SAHA and Olaparib synergistically inhibited the growth of SNU-398 but not SNU-449 cells, which was associated with increased apoptosis and accumulated unrepaired DNA damage. Multiple lines of evidence demonstrate that the hepatic fibrosis/hepatic stellate cell activation may be an important genetic determinant of cellular sensitivity to both enzymatic inhibitors, and coordinate activation or inactivation of the aryl hydrocarbon receptor (AhR) and cAMP-mediated signaling pathways are involved in cell response to SAHA and Olaparib treatment.

Conclusion—These findings suggest that combination therapy with both enzyme inhibitors may be a strategy for therapy of sensitive HCC cells, and identification of these novel molecular determinants may eventually guide the optimal use of PARP and HDAC inhibitors in the clinic.

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Keywords

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INTRODUCTION

Poly (ADP-ribose) polymerases (PARPs) are a family of enzymes that share a catalytic PARP homology domain and the ability to poly(ADP-ribosyl)ate protein substrates(1, 2). Among them, PARP1 and PARP2 are activated by single- and double-strand breaks (SSB and DSB, respectively) and play a critical role in the base excision repair pathway by binding to DNA breaks and recruiting DNA repair proteins to the site of damage (1–3). Inhibition of PARPs induces accumulation of large numbers of unrepaired SSBs, leading to the collapse of replication forks during S phase and the consequent generation of double-strand breaks. Normally, these replication-associated DSBs would be repaired by the error-free homologous recombination (HR) repair pathway with no deleterious effect seen (4). However, in cells in which HR is defective, such as the BRCA-mutated cells, DSBs can be repaired by a more error-prone nonhomologous end-joining (NHEJ) pathway, resulting in chromosome aberrations and cell lethality (3, 5). Indeed, accumulating evidence has proposed that PARP inhibition might be a useful therapeutic strategy for the treatment of a wider range of tumors bearing a variety of deficiencies in the HR pathway or displaying properties of “BRCAness” (3, 5, 6).

DSB repair occurs within chromatin and can also be modulated by chromatin-modifying enzymes. In this context, histone deacetylases (HDACs) are critically important to enable functional HR by controlling the expression of HR-related genes and promoting the proper assembly of HR-directed subnuclear foci (7). In contrast, inhibition of HDAC enzymes down-regulates the expression of HR DNA repair proteins and impairs recruitment of these key HR proteins to the site of DNA damage, resulting in a decrease in homology-directed repair of DSBs (7–10). These findings indicate a potential therapeutic utility of HDAC inhibitors in cancer patients with tumors that have overactive HR or in combination with anti-tumor agents that induce damage repaired by HR.

Hepatocellular carcinoma (HCC) is one of the most lethal cancers worldwide, yet effective therapeutic options for advanced HCC are limited (11, 12). Emerging evidence that development of HCC is associated with perturbed DNA damage response and repair pathway (11) opens the possibility of targeting the components of DNA damage response pathway for the treatment of HCC. In this study, we investigated the hypothesis that co-targeting the enzymatic activities of PARPs and HDACs could synergistically inhibit the growth of HCC and defined the molecular determinant of sensitivity to both enzymatic inhibitors.

EXPERIMENTAL PROCEDURES

The human HCC cell lines HepG2, PLC/PRF/5, SNU-398, and SNU-449 were obtained from American Type Culture Collection, and Huh-7 cell line was purchased from Japanese Collection of Research Bioresources. The pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the PARP inhibitor Olaparib were obtained from Merck and Selleck Chemicals, respectively. All experimental assays, including cell viability assay, flow cytometric analysis of apoptosis, pulsed field gel electrophoresis assay, colony formation assay, antibodies and Western blot analysis, microarray gene expression arrays and data analysis, quantitative real-time PCR, and statistical analyses, are described in Supporting Information in detail.

RESULTS

Differential Sensitivity of HCC Cells to SAHA and Olaparib Treatment

To test whether co-targeting the enzymatic activities of PARPs and HDACs could inhibit the growth of HCC cells, five well-characterized human HCC cell lines were treated with different concentrations of SAHA or Olaparib alone for 96 hrs and their growth was subsequently assayed using the standard XTT assay. Results showed that SAHA inhibited the proliferation of HepG2, Huh7, PLC/PRF/5, and SNU-398 but not the SNU-449 cells in a dose-dependent manner (Fig. 1A). In contrast, Olaparib effectively inhibited the proliferation of HepG2, Huh7, and SNU-398 but not PLC/PRF/5 and SNU-449 cells in a dose-dependent manner (Fig. 1B). These results suggest that HCC cells have differential sensitivity to SAHA and Olaparib, probably due to the heterogeneous genetic background (Table S1). Given that the genetic background of SNU-398 and SNU-449 cell lines are relatively comparable (Table S1) and that both cell lines exhibit striking sensitive (SNU-398) *versus* resistant (SNU-449) phenotype to both enzyme inhibitors (Figs. 1A and 1B), we chose this pair of cell lines as a model to investigate the synergistic action of SAHA and Olaparib in HCC cells and to define the underlying mechanisms.

We next investigated the growth inhibitory effects of combination of SAHA and Olaparib at very low concentrations in HCC cells by XTT assays. As shown in Fig. 1C, incubation of SNU-398 cells with 0.5 μ M SAHA or 3 μ M Olaparib alone for 96 hrs did not significantly alter cell viability, whereas the simultaneous treatment with SAHA and Olaparib at the same concentrations resulted in a significant reduction of cell viability. In contrast, SAHA had no detectable effect on cell viability when combined with Olaparib at the same concentrations in resistant SNU-449 cells (Fig. 1C). Colony-forming assay further confirmed these results, showing a greater inhibition of clonogenicity in SNU-398 but not SNU-449 cells following SAHA and Olaparib treatment (Figs. 1D and S1). Together, these findings suggest that HCC cells have differential sensitivity to SAHA and Olaparib and co-administration of both inhibitors had a synergistic anti-proliferative effect in the sensitive SNU-398 but not the resistant SNU-449 cells.

Synergistic Induction of Apoptosis in the Sensitive but not Resistant Cells Following SAHA and Olaparib Treatment

To investigate the underlying mechanisms of the synergistic anti-proliferative effect of SAHA and Olaparib, cells were treated with 0.5 μ M SAHA, 3 μ M Olaparib alone or in combination for 24 hrs, and then subjected to flow cytometry analysis of DNA stained with propidium iodide, which has been widely used for the evaluation of apoptosis by determination of the percentage of events which accumulated in the sub-G1 position in different experimental models. As shown in Figs. 2A and S2A, combination of SAHA with Olaparib resulted in 12.4% apoptosis in SNU-398 cells as compared with 4.32% and 2.21% for individual agent alone, respectively. In contrast, in SNU-449 cells combination of SAHA with Olaparib resulted in 1.57 % apoptotic cells compared with 1.08% and 1.50% for each agent alone. The effects of SAHA and Olaparib treatment on apoptosis were further determined by FITC Annexin V staining, which is used to quantitatively determine the percentage of apoptotic cells within a population based on the property of cells to lose membrane asymmetry in the early phases of apoptosis. Results showed that co-administration of SAHA and Olaparib significantly increased apoptosis (12.8%) in SNU-398 cells compared to treatment with individual agent alone, whereas only a minor effect was observed in SNU-449 cells (Figs. 2B and S2B). These results suggest the synergism in inducing apoptosis by SAHA and Olaparib in the sensitive SNU-398 but not resistant SNU-449 cells.

We next analyzed the expression of apoptosis-related proteins in both cell lines by Western blot analysis. Results showed that co-administration of SAHA and Olaparib resulted in a synergistic increase in the levels of the cleaved Caspase-3 and cleaved PARP in SNU-398 cells as compared with SAHA or Olaparib treatment alone (Figs. 2C, S3A, and Table S2). In contrast, the same effect in SNU-449 cells was only modest (Figs. 2C, S3A, and Table S2), indicating that SNU-398 cells are more sensitive to the combination of both agents than SNU-449 cells do. Together, these results suggest that the anti-proliferative activity of SAHA and Olaparib in SNU-398 cells was, at least in part, attributable to apoptosis induction. In addition, it is noteworthy that treatment of cells with SAHA down-regulated the expression of HDAC1 and HDAC2 proteins in SNU-398 cells (Figs. 2C, S3A, and Table S3).

Combination of SAHA and Olaparib Leads to An Accumulation of Unrepaired DNA Damage and to A Decrease in HR DNA Repair Proteins

To test whether co-administration of SAHA and Olaparib causes a decrease in DSB repair capacity of HCC cells, we performed a pulsed field gel electrophoresis (PFGE) to assess for DNA repair ability of HCC cells upon HDAC and PARP inhibition. Results revealed that combination of SAHA and Olaparib resulted in a significant increase in the levels of unrepaired DNA damage in SNU-398 cells (Figs. 2D and 2E). In contrast, no significant increase in DSBs was detected in SNU-449 cells after treatment with SAHA and Olaparib. These results indicate a possible mechanism by which SAHA enhances cellular sensitivity to Olaparib through abrogation of DSB repair pathway.

We next investigated the levels of repair proteins in both cell lines by Western blot analysis, and found that co-treatment with SAHA and Olaparib synergistically down-regulated the protein levels of checkpoint protein 1 (Chk1), Chk2, and fanconi anemia group D2 protein (FANCD2) in SNU-398 but not SNU-449 cells (Figs. 2F, S3B, Tables S2 and S4). Interestingly, the expression levels of p53-binding protein 1 (53BP1), a mediator of DNA damage checkpoint, were significantly down-regulated in SNU-449 cells as compared with SNU-398 cells following treatment with both inhibitors (Figs. 2F and S3B). In contrast, other repair proteins such as ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR) and H2AX remain unaffected (Figs. 2F and S3B). It has been shown that silencing Chk1, Chk2, and FANCD2 proteins leads to a reduction in HR repair and an increased sensitivity to PARP inhibitor (6). In contrast, loss of 53BP1 partially restores the HR defect and decreases sensitivity of BRCA1-deleted cells to DNA-damaging agents (13). Thus, activation of diverse DNA repair pathway by SAHA- and Olaparib-induced DNA damage in SNU-398 and SNU-449 cells might contribute to the distinct sensitivity to both enzyme inhibitors.

Hepatic Fibrosis/Hepatic Stellate Cell Activation May be An Important Genetic Determinant of Sensitivity to Both Enzyme Inhibitors

To understand the molecular basis of the differential sensitivity of SNU-398 and SNU-449 cells to SAHA and Olaparib, we next performed genome-wide expression profile analysis to identify the genetic differences between both cell lines using the Affymetrix Human Exon 1.0 ST arrays. Statistical analysis of the data generated a set of 1147 differentially expressed genes with p -value <0.05 and fold change expression ± 1.5 , and the top 50 differentially expressed genes are shown in Table S5. Among them, 53.90 % were up-regulated, whereas 46.10% were down-regulated in SNU-449 cells as compared with SNU-398 cells (Fig. 3A). We next performed hierarchical clustering analysis of the differentially expressed genes and the normalized log2 ratio values were used to obtain the heatmap (Fig. 3B). The gene leaf nodes were optimized in the heat maps representing the differential expression of genes between both cell lines.

To investigate the functions of these differentially expressed genes, we next performed Gene Ontology (GO) analysis on all the sets of genes with a *p*-value cutoff set to 0.1, and found that 43.16 % of these differentially expressed genes are related to cell component, followed by 37.85 % to molecular function, and 18.89 % to biological process (Fig. 3C). The detail with GO terms for the above mentioned comparisons is shown in Table S6. We next carried out Ingenuity Pathways Analysis (IPA) on all these differential expressed genes and Fischer's exact test was applied with *p*-value < 0.05. The top 10 significant functions and canonical pathways, in which these differentially expressed genes are involved, are shown in Figs. 3D and 3E, respectively. The most likely function and significant canonical pathway of these differentially expressed genes are cancer and hepatic fibrosis/hepatic stellate cell activation, respectively.

Hepatic fibrosis represents the consequences of a sustained wound healing response to chronic liver disease, in which hepatic stellate cell (HSC) activation is an initial event. Following liver injury of any etiology, HSCs undergo a response known as "activation", which is the transition of quiescent cells into proliferative, fibrogenic and contractile myofibroblasts (14). Activated HSCs are resistant to most pro-apoptotic stimuli including serum deprivation, doxorubicin, etoposide, and oxidative stress mediators (15) as supported by an increased expression of a number of pro-survival factors (16). As shown in Table S7, we discovered that a number of pro-growth and pro-survival factors were significantly up-regulated in SNU-449 cells as compared with SNU-398 cells. Most of them are growth factors and cytokines that promote cell proliferation, angiogenesis and prevent apoptosis.

To test whether these survival factors are involved in cell sensitivity to SAHA and Olaparib for HCC, we next validated the expression of several survival factors, including chemokine (C-C motif) ligand 2 (CCL2), endothelin 1 (EDN1), fibroblast growth factor 2 (FGF2), fibroblast growth factor receptor 1 (FGFR1), and platelet-derived growth factor beta polypeptide (PDGFB) in both cell lines by qPCR. Consistent with the microarray data, we repeatedly found that all of 5 genes were up-regulated in resistant SNU-449 cells as compared with sensitive SNU-398 cells (Fig. 4A and Table S8). Given that EDN1 is most highly up-regulated gene in resistant SNU-449 cells and has been shown to promote tumor cell proliferation, survival, angiogenesis, invasion and metastasis (17), we next tested the hypothesis that induced expression of EDN1 in sensitive SNU-398 cells could convert its sensitive phenotypes to a more resistant one. To this end, SNU-398 cells were transfected with pCMV6-AC/hEDN1 expression plasmid and assessed the effect of EDN1 on cellular sensitivity to both enzyme inhibitors. As shown in Fig. 4C, induced expression of EDN1 in sensitive SNU-398 cells (Fig. 4B and Table S9) significantly increases cell viability following SAHA and Olaparib treatment as compared with empty vector control. In addition, we noticed that SAHA and Olaparib treatment still can significantly inhibit cell growth as compared with DMSO control in EDN1-overexpressed cells (Fig. 4C, right panel), raising the possibility that other pro-survival factors may also contribute to HCC sensitivity to both inhibitors. Collectively, these results suggest the hepatic fibrosis/hepatic stellate cell activation may be an important genetic determinant of sensitivity to both enzymatic inhibitors due to up-regulation of numerous survival factors.

Activation of the Aryl Hydrocarbon Receptor (AhR) Pathway Is An Important Feature of the Anti-Proliferative Effects of Both Enzyme Inhibitors in Sensitive HCC cells

To further identify markers that may predict response to SAHA and Olaparib treatment, we next performed the expression profiling analysis in both cell lines following treatment with 0.5 μ M SAHA, 3 μ M Olaparib alone or in combination for 24 hrs, and the larger number of transcript expression changes was observed in SNU-398 cells than SNU-449 cells. The normalized log2 ratio values of the differentially regulated genes in each comparison were used to obtain the heat maps (Fig. S4). In this context, 68 and 21 genes were found to be

induced by SAHA treatment in SNU-398 and SNU-449 cells, respectively (Fig. 5A, Tables S10 and S11). When cells were treated with Olaparib and compared with DMSO control, total 28 and 5 genes were specifically induced in SNU-398 and SNU-449 cells, respectively (Fig. 5B, Tables S12 and S13). Interestingly, total 165 and 45 genes were specifically induced in SNU-398 and SNU-449 cells, respectively, following co-treatment with SAHA and Olaparib (Fig. 5C, Tables S14 and S15).

To further investigate the functions of these regulated genes by SAHA and Olaparib treatment, we performed GO analysis on all the sets of genes with a *p*-value cutoff set to 0.1. As shown in Fig. 5D, 51.71 % of these induced genes in SNU-398 cells related to molecular function, followed by 32.68 % to biological process and 15.61% to cellular component. In contrast, 42.11% of differentially expressed genes induced by SAHA and Olaparib in SNU-449 cells were linked to cellular component, 31.58% to biological process, and 26.32 % to molecular function. The details with GO terms for all the above mentioned comparisons are shown in Tables S16 and S17, respectively. We next performed Ingenuity pathway analysis on all the genes that were influenced by SAHA and Olaparib treatment. Excitedly, we found that the most significant canonical pathway, in which the induced genes might be involved, is the aryl hydrocarbon receptor (AhR) signaling in SNU-398 cells (Fig. 6A and Table S18), and that the most likely functions of the genes regulated by SAHA and Olaparib in SNU-398 cells are cancer, cellular development, cell death, cell growth and proliferation (Fig. 6B).

The AhR is a ligand-activated basic helix-loop-helix transcription factor that mediates gene activation events induced by environmental contaminants. Previous studies have suggested that AhR signaling is involved in normal liver growth and development (18) and modulates the susceptibility of hepatocytes to the pro-apoptotic effects of tumor necrosis factor- α and Fas stimulation (19). To further test whether AhR signaling is involved in growth inhibitory effect of SAHA and Olaparib in sensitive SNU-398 cells, we next carried out qPCR analysis to validate the altered expression of several representative genes that are involved in the AhR signaling, including aldehyde dehydrogenase 3 family, member B1 (ALDH3B1), cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), CYP1B1, and p21 (Table S18). In agreement with the microarray data, we found that ALDH3B1 was down-regulated, whereas CYP1A1, CYP1B1, and p21 were up-regulated, in SNU-398 cells following SAHA and Olaparib treatment (Fig. 6C and Table S19).

Aldehyde dehydrogenases (ALDHs) are critical enzymes in the metabolism of endogenous and exogenous aldehydes (20), which are up-regulated in a variety of human cancer tissues and cell lines resulting in resistance to anti-cancer drugs (21). ALDH3B1 belongs to the ALDH3 family (20) and is highly expressed in liver and has an important role in the defense against oxidative stress (20). Given that ALDH3B1 was down-regulated in sensitive SNU-398 cells following SAHA and Olaparib treatment (Fig. 6C and Table S19), we hypothesized that restoration of ALDH3B1 in sensitive SNU-398 cells could reverse its sensitive phenotype to both inhibitors. Interestingly, we found that expression of ALDH3B1 (Fig. 6D and Table S9) significantly increases cell viability following inhibition of both SAHA and PARP (Fig. 6E), probably by protecting cells from the damaging effects of oxidative stress (20). Thus, we concluded that activation of the AhR signaling pathway in sensitive SNU-398 cells may be an important feature of the anti-proliferative effects of both enzyme inhibitors.

Dysregulation of the cAMP-Mediated Signaling May Contribute to Resistance to Both Enzyme Inhibitors

In contrast, the genes induced by SAHA and Olaparib in SNU-449 cells are primarily involved in the cAMP-mediated signaling (Fig. 7A). cAMP is a signaling molecule

important for a variety of cellular functions, and exerts its effects by activating the cAMP-dependent protein kinase, which transduces the signal through phosphorylation of different target proteins. These representative genes involved in the cAMP-mediated signaling are shown in Table S20. Ingenuity pathway analysis revealed that the functions of these genes are involved in genetic disorder, hematological disease, and carbohydrate metabolism (Fig. 7B). qPCR analysis further demonstrated that cAMP-dependent protein kinase type II-beta regulatory subunit (PRKAR2B) and regulator of G-protein signaling 4 (RGS4) were up-regulated, whereas calcium/calmodulin-dependent protein kinase II beta (CAMK2B) was down-regulated, in SNU-449 cells after SAHA and Olaparib treatment (Fig. 7C and Table S21). Interestingly, a recent study using a synthetic lethal small-interfering RNA screen of cancer cell lines found that silencing of PRKAR2B by siRNAs resulted in a 62% decrease in cell growth following treatment with PARP inhibitor as compared with control siRNA-transfected cells (22), indicating that PRKAR2B has a potential role in cell resistance to PARP inhibitor. In light of our results that co-treatment of SAHA and Olaparib resulted in an over 20-fold increase in the levels of PRKAR2B in resistant SNU-449 cells (Fig. 7C and Table S21) and previous report from other laboratory (22), we next tested the possibility that over-expression of PRKAR2B in sensitive SNU-398 cells could decrease sensitivity to both enzyme inhibitors. As expected, we found that exogenous expression of PRKAR2B (Fig. 7D and Table S9) in SNU-398 cells significantly increases cell viability in the presence of SAHA and Olaparib (Fig. 7E). Thus, these results suggest that up-regulation of cAMP-mediated signaling, especially, PRKAR2B, may be an important marker of cell resistance to SAHA and Olaparib for HCC. Interestingly, PRKAR2B has been documented to be down-regulated in human cancer cells and its over-expression induces growth inhibition (23). One possibility is that, like other tumor suppressor genes Chk1, Chk2, and BRCA1, PRKAR2B has a potential role in DSB repair and can enable HCC cells to survive DNA damage that is induced by SAHA and Olaparib.

DISCUSSION

The PARP expression is significantly increased in human HCC compared with adjacent non-tumor tissues (24, 25) and inhibition of PARP-1 decreases HCC growth (26). Interestingly, HDAC inhibitors have been shown to exert its antitumor activity against HCC in preclinical models (12). The unanswered questions in this field are whether co-targeting both enzymatic activities could synergistically inhibit HCC growth and what is the genetic determinant of cellular sensitivity to both enzyme inhibitor therapies. In this study, we report that co-targeting the enzyme activities of PARPs and HDACs synergistically inhibited the growth of sensitive HCC cells. The possible mechanism could be that, inhibition of PARP induces the generation of DSBs, which is normally repaired by the error-free HR repair pathway (Fig. 8A). When co-administrated with HDAC inhibitor that blocks the HR-mediated repair pathway (7–10), cells exhibit a mimic HR-deficient phenotype, resulting in PARP hyper-activation and PARP inhibitor sensitivity (27) (Fig. 8B). Our findings also highlight the notion that a deficiency in HR is a determinant of sensitivity to PARP inhibition and not BRCA1/2 deficiency per se, because both SNU-398 and SNU-449 cell lines express wild-type BRCA1 (28). In support of our findings, it has been shown that PARP inhibitors can inhibit the growth of breast cancer cells irrespective of their BRCA1 status (29), and Olaparib monotherapy resulted in responses in patients with high-grade serous ovarian carcinoma without germline *BRCA1* or *BRCA2* mutation (30).

Another novel finding is that the differentially expressed genes between the sensitive and resistant cell lines are primarily involved in the hepatic fibrosis/hepatic stellate cell activation (Fig. 3E), which is characterized by up-regulation of numerous pro-growth and pro-survival factors. One key question is whether the hepatic fibrosis/hepatic stellate cell activation signaling is connected to DNA repair pathway, which could explain the

differential sensitivity of both cell lines to SAHA and Olaparib. Indeed, most of these genes, such as Met proto-oncogene and epidermal growth factor receptor, can promote DNA repair by up-regulating proteins involved in DNA repair (31, 32), which may be one of mechanisms by which SNU449 cells are resistant to SAHA and Olaparib treatment. Indeed, we demonstrate that induced expression of EDN1 converts the sensitive phenotype of SNU-398 cells to a more resistant one (Fig. 4D), suggesting a potential role of EDN1 in cell resistance to both inhibitors. EDN1 has been shown to activate various kinases that are known to be involved in the survival of cell signaling and proliferation and to inhibit apoptosis of cancer cells by various mechanisms (17). Moreover, a recent study demonstrated that EDN1 reduces DNA damage and/or enhances DNA repair in human melanocytes following ultraviolet radiation (33). As EDN1 is up-regulated in SNU-449 cells relative to SNU-398 cells, it could be one of the genes that protect SNU-449 cells from DNA damage induced by SAHA and Olaparib treatment. Thus, we conclude that the hepatic fibrosis/hepatic stellate cell activation may be a novel determinant to the sensitivity of HCC cells to SAHA and Olaparib treatment.

In summary, the findings presented here establish that HCC cells have heterogeneous response to HDAC- and PARP- inhibitors and the hepatic fibrosis/hepatic stellate cell activation may be genetic determinant of cellular sensitivity to HDAC- and PARP- inhibitors. Combination therapy with a selective HDAC- and PARP-inhibitor may be a strategy for therapy of sensitive HCC cells, in which the coordinate activation or inactivation of the AhR and cAMP-mediated signaling pathway is an important feature of the anti-proliferative and pro-apoptotic effects of both inhibitors. Thus, treatment targeting DNA repair mechanisms seems to provide new hope for treatment of HCC and identification of these novel determinants may eventually guide the optimal use of PARP and HDAC inhibitors in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Differential sensitivity of human HCC cells to SAHA and Olaparib. (A–C) Cells were treated with SAHA (A), Olaparib (B) alone or in combination (C) at the indicated concentrations for 96 hrs and then subjected to XTT assays. (D) Cells were treated with DMSO, 0.5 μM SAHA, 3 μM Olaparib alone or in combination for 6 weeks, stained with 0.05% crystal violet. The colonies were counted under the microscope (100 $\times$). The representative images are shown in Fig. S1.

Fig. 2. SAHA and Olaparib synergistically induced apoptosis and compromised DNA repair in the sensitive HCC cells. (A–B) Cells were treated with 0.5 μ M SAHA, 3 μ M Olaparib alone or in combination for 24 (A) or 12 (B) hrs, subjected to staining with propidium iodide (A) or FITC-Annexin V (B), and then analyzed by flow cytometry. The representative FACS images are shown in Fig. S2 and the quantitation results from three independent experiments are shown in A and B, respectively. (C, F) Cells were treated with 0.5 μ M SAHA, 3 μ M Olaparib alone or in combination for 48 hrs, and protein extracts were subjected to Western blot analysis with the indicated antibodies. The overall results of quantification of Western blots using ImageJ program are shown in Table S2. *NS*, non-specific. (D–E) Cells were treated with 2.5 μ M SAHA, 15 μ M Olaparib alone or in combination for 24 hrs, and then subjected to PFGE using a CHEF-DR III Pulsed Field Electrophoresis Systems. Representative gel image is shown in D and quantitation results from three independent experiments are shown in E. Arrow in panel D indicates the released DNA fragments.

Fig. 3.

Expression profile analysis of differentially expressed genes between SNU-398 and SNU-449 cells using Affymetrix human exon 1.0 ST arrays. (A) Pie Chart shows the percentage of the up-regulated (green) or down-regulated (blue) genes. (B) Heatmap shows the hierarchical clustering of differentially expressed genes between both cell lines. The color scale represents the degree of expression of the genes. (C) GO analysis using GeneSpring GX 10.0.2 shows that differentially expressed genes between both cell lines match with three broad GO terms, including cellular components, biological process, and molecular function. (D–E) Ingenuity Pathway Analysis of differentially expressed genes between both cell lines. The top 10 possible functions and canonical pathways of these differentially expressed genes are shown in D or E, respectively.

Fig. 4.

Hepatic fibrosis/hepatic stellate cell activation signaling is involved in cellular sensitivity to SAHA and Olaparib in HCC cells. (A) qPCR validation of differentially expressed genes that are involved in hepatic fibrosis/hepatic stellate cell activation signaling in SNU-398 and SNU-449 cells. (B) SNU-398 cells were transfected with pCMV6-AC/hEDN1 expression plasmid or empty vector. After 48 hrs of transfection, total RNA was isolated and subjected to qPCR analysis of *EDN1* gene expression. (C) SNU-398 cells were transfected with pCMV6-AC/hEDN1 expression plasmid or empty vector for 48 hrs and then seeded into 96-well dishes overnight. Cells were treated DMSO or 0.5 μ M SAHA in combination with 3 μ M Olaparib for 48hrs and then subjected to XTT assays.

Fig. 5.

Identification of differentially expressed genes in SNU-398 and SNU-449 cells treated with 0.5 μ M SAHA, 3 μ M Olaparib alone or in combination for 24 hrs using Affymetrix Human Exon 1.0 ST arrays. (A–C) Venn diagrams show the number of differentially expressed genes in both cell lines treated with SAHA (A), Olaparib (B) alone or in combination (C) as compared with DMSO control. (D) GO analysis of the differentially expressed genes between SNU-398 and SNU-449 cells following treatment with SAHA and Olaparib. Pie charts show the differentially expressed genes matching with three broad GO terms, including cellular components, biological process, and molecular function.

Fig. 6.

Activation of the AhR pathway is an important feature of the anti-proliferative effects of both enzyme inhibitors in sensitive HCC cells. (A–B) Ingenuity pathway analysis reveals top 5 canonical pathways (A) and functions (B) of the altered genes in SNU-398 cells following SAHA and Olaparib treatment. (C) qPCR validation of the altered expression of genes that are involved in the AhR signaling pathway in SNU-398 cells. (D) SNU-398 cells were transfected with pcDNA3.1(-)/hALDH3B1 expression plasmid or empty vector. After 48 hrs of transfection, total RNA was isolated and subjected to qPCR analysis of *ALDH3B1* gene expression. (E) SNU-398 cells were transfected with pcDNA3.1(-)/hALDH3B1 expression plasmid or empty vector for 48 hrs and then seeded into 96-well dishes overnight. Cells were treated DMSO or 0.5 μ M SAHA in combination with 3 μ M Olaparib for 48hrs and then subjected to XTT assays.

Fig. 7.

Up-regulation of cAMP-mediated signaling may contribute to HCC cell resistance to both enzyme inhibitors. (A–B) Ingenuity pathway analysis reveals top 5 canonical pathways (A) and functions (B) of the altered genes in SNU-449 cells following SAHA and Olaparib treatment. (C) qPCR validation of the altered expression of genes that are involved in the cAMP-mediated signaling pathway in SNU-449 cells with or without SAHA and Olaparib treatment. (D) SNU-398 cells were transfected with pCMV6-XL5/hPRKAR2B expression plasmid or empty vector. After 48 hrs of transfection, total RNA was isolated and subjected to qPCR analysis of *PRKAR2B* gene expression. (E) SNU-398 cells were transfected with pCMV6-XL5/hPRKAR2B expression plasmid or empty vector for 48 hrs and then seeded into 96-well dishes overnight. Cells were treated DMSO or 0.5 μM SAHA in combination with 3 μM Olaparib for 48hrs and then subjected to XTT assays.

A. PARP inhibitor Olaparib alone **B. PARP inhibitor Olaparib in combination with HDAC inhibitor SAHA** **Fig. 8.**

The proposed working model. PARP inhibition by Olaparib results in the formation of additional DSBs owing to the conversion of unrepaired SSBs during replication. These DSBs would be repaired by the error-free HR repair pathway in HR-proficient cells, resulting in cell survival and therapeutic resistance. However, in cells in which HR is defective, DSBs can be repaired by a more error-prone NHEJ pathway, which causes large numbers of chromatid breaks and aberrations, leading to cell death (A). When Olaparib is in combination with SAHA, SAHA blocks HR-mediated repair pathway, which is needed for repairing the DSBs induced by Olaparib, resulting in a synergistic effect on cell death (B).