Antitumor Effects in Hepatocarcinoma of Isoform-Selective Inhibition of HDAC2

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Abstract

Histone deacetylase 2 (HDAC2) is a chromatin modifier involved in epigenetic regulation of cell cycle, apoptosis, and differentiation that is upregulated commonly in human hepatocellular carcinoma (HCC). In this study, we show that specific targeting of this HDAC isoform is sufficient to inhibit HCC progression. siRNA-mediated silencing of HDAC inhibited HCC cell growth by blocking cell-cycle progression and inducing apoptosis. These effects were associated with deregulation of HDAC-regulated genes that control cell cycle, apoptosis, and lipid metabolism, specifically, by upregulation of p27 and acetylated p53 and by downregulation of CDK6 and BCL2. We found that HDAC2 silencing in HCC cells also strongly inhibited PPARγ signaling and other regulators of glycolysis (ChREBPα and GLUT4) and lipogenesis (SREBPIC and FAS), eliciting a marked decrease in fat accumulation. Notably, systemic delivery of HDAC2 siRNA encapsulated in lipid nanoparticles was sufficient to blunt the growth of human HCC in a murine xenograft model. Our findings offer preclinical proof-of-concept for HDAC2 blockade as a systemic therapy for liver cancer. Cancer Res; 74(17); 1–10. © 2014 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the third most lethal neoplasm causing an estimated 700,000 deaths annually (1). In the United States, the incidence of HCC has doubled over the past two decades, with only 30% to 40% of patients being eligible for curative treatments due to the late diagnosis, underlying liver disease, and lack of effective treatment options (2–4). HCCs are phenomenologically and genetically heterogeneous tumors driven by diverse molecular mechanisms (5). However, HCCs exhibit certain common traits selected through genomic and epigenetic alterations (6, 7). Identification of both common and HCC subtype-specific genomic alterations may provide an opportunity for anticancer treatment through targeted therapy (8).

Histone deacetylases (HDAC) belong to a family of chromatin-modifying enzymes that repress gene expression by removing acetyl groups from histone substrates (9). There are 11 known human HDAC isoforms, which are subdivided into four classes based on function and DNA sequence similarity. These include class I (HDAC1, 2, 3, 8), class IIa (HDAC4, 5, 7, 9), class IIb (HDAC6, 10), and class IV (HDAC11). Within the family, HDAC class I enzymes show widespread activity throughout the body and are frequently overexpressed in many diseases, including cancer (10). Given the functional significance of HDACs for epigenetic regulation of a large number of genes and signaling cascades in human cancer (9, 11), the pharmacologic targeting of HDACs is emerging as a promising anticancer strategy (12).

Currently, two small-molecule inhibitors of HDACs (HDACi), including SAHA (vorinostat) and FK-228 (romidepsin), have been approved by the U.S. Food and Drug Administration (FDA) for treating subcutaneous T lymphoma and/or peripheral T-cell lymphoma, and many more HDACi are undergoing evaluation for clinical application (12, 13). However, despite some progress in preclinical models (14), most of the known first-generation HDACi showed limitations in clinical trials, mainly due to ineffectiveness at low concentrations in solid tumors and unspecific binding to all or several HDAC isoforms, thereby increasing the off-target effects (15). Panobinostat (LBH589), a novel cinnamic hydroxamic acid HDACi, has shown to be active on HDAC class I and II isoforms at nanomolar concentrations (16) and to overcome imatinib-resistant KIT mutation in a combinatorial trial (17). In addition to efforts for reduction in pharmacologic concentration, a number of HDAC isoform–selective or -specific inhibitors are currently in development to abolish the adverse effects in clinic (18).

In the course of our studies of gene expression signatures as predictors of survival classes of patients with HCC, we identified...
a limited number of genes with expression patterns that were significantly associated with disease prognosis (19). We then validated these findings in a preclinical model of liver cancer (20, 21) by therapeutically targeting two of the identified “survival” genes (COP1 or CSN5) using RNA interference (RNAi). RNAi is an intrinsic cellular mechanism that triggers a sequence-specific degradation of target mRNA (22, 23). Lipid nanoparticles (LNP) were used as an effective delivery vehicle for the small interfering RNA molecules (siCOP1 and the siCSN5) minimizing systemic toxicity (24–27).

Here, we extended our studies to address the therapeutic utility of the selective targeting of another survival gene, HDAC2, the overexpression of which significantly correlated with length of HCC patient survival (19). HDAC2 has been shown to prevent apoptosis and promote cell-cycle progression by inhibiting p53 tumor suppressor and increasing MYC expression (28–30). Given our past experience in several preclinical studies, we used RNAi-based LNP as a treatment strategy to allow liver tissue selective drug accumulation and effective silencing of a target RNA in HCC cells while reducing unwanted immune response (20, 31, 32). We also performed microarray profiling and a subsequent protein analysis to better understand the mechanism and therapeutic utility of HDAC inhibition in HCC. HDAC2 knockdown by siRNA was isoform selective and caused a strong suppression of cancer cell growth. The growth-inhibitory effects were driven by a subset of molecular alterations, including upregulation of p27, acetylated p53, and downregulation of CDK6, BCL-2, and peroxisome proliferator-activated receptor γ (PPARγ). In vivo delivery of HDAC2 siRNA effectively reduced liver tumor growth in mice.

Materials and Methods

siRNA

All native HDAC2 siRNA duplexes used for in vitro studies were chemically synthesized by Ambion (HDAC2-1, siRNA ID# 120208; HDAC2-2, siRNA ID# 120209; HDAC2-3, siRNA ID# 120210). For in vivo applications, HDAC2-1 siRNA was synthesized in a large scale by Integrated DNA Technologies and 2'-OMe (2'-O-Me) modifications (31). Modified target siRNAs were encapsulated into LNP as described by Jeffs and colleagues (24). Negative control siRNA molecules that do not target any endogenous transcript were used for control experiments. Silencer Negative Control #1 siRNA (Ambion) and LNP-formulated βgal 478siRNA (32) were used for in vitro and in vivo studies, respectively.

Cell culture and transfection of siRNA in vitro

Liver cancer cell lines, PLC and HepG2, were obtained from the American Type Culture Collection (ATCC). Huh7 from Riken Cell Bank Japan (deposited by Dr. Nam-Ho Huh), and Huh7 from Health Science Research Resource Bank Japan, were passaged for <6 months, and maintained as recommended by the provider. ATCC performed cell line authentication using DNA fingerprinting by short tandem repeat analysis. Riken Cell Bank and Health Science Research Resource Bank did not provide information on the method of authentication. All cell lines were karyotyped upon receipt for future reference. Cells were plated at 30% density 24 hours before transfection. Lipofectamine 2000 was mixed with siRNA molecules in a volume of 50 µL Opti-MEM I (both from Invitrogen). The medium was replaced 24 hours after transfection. The negative control siRNAs (NCsiRNA) were used in the same quantity and transfected to the cells simultaneously.

Microarray analysis

Total RNA was extracted, quality controlled, in vitro transcribed, and hybridized on Sentrix whole-genome BeadChips, human Ref-8v3 (Illumina), as previously described (33). Image analysis and data extraction were performed automatically using Illumina GenomeScan Software. All microarray data were submitted to the Gene Expression Omnibus database with the accession number GSE52232. The genomic data were analyzed using the Bootstrap t-test and ANOVA (10,000 repetitions) between treatment and control (n = 4, each). To explore the functional relationships among the significant genes with altered expression in HCC cells treated with HDAC2 siRNA compared with controls, their functional associations were validated by independent pathway analysis tools, Pathway Studio (Ariadne Genomics) and Ingenuity Pathway Analysis (Ingenuity Systems).

Systemic administration of LNP-formulated siRNA and bioluminescence imaging in vivo

All procedures were performed in accordance with the guidelines of the NIH animal care committee. Huh7-Luc+ (5 × 10⁵) cells were injected into the splenic pulp of 6-week-old male SCID/Beige mice (Charles River Laboratories) as previously described (20, 21, 33). LNP-formulated siRNAs (2 mg/kg) were injected into the lateral tail vein, every 3 days, for a total of three doses. Tumor growth was monitored by bioluminescence imaging (BLI) for 4 weeks with 3 or 4 day intervals using an IVIS Imaging System and analyzed by Living Image Software (Xenogen).

Statistical analysis

Statistical comparisons were conducted using a Bootstrap t-test with 10,000 repetitions. The Mann–Whitney U test was used when normality assumptions were not satisfied. All data are shown as means ± SEM. Analyses were conducted using R statistical software (v. 3.0.1). P values ≤ 0.05 (*) and ≤ 0.01 (**) were considered as statistically significant.

Measurement of cell proliferation and apoptotic cell death, real-time RT-PCR, Western blot analysis, and generation of HCC reporter cell line expressing luciferase

See Supplementary Materials and Methods.

Results

siRNA silencing of HDAC2 inhibits proliferation and induces apoptosis in HCC cells

To address the biologic functions of HDAC2, two HCC cell lines, Huh7 and HepG2, were transfected with three variants of HDAC2-specific siRNA (HDAC2-1, -2, or -3). To select the siRNA eliciting the highest treatment efficacy and target
Gene knockdown, we tested a 5 to 20 nmol/L dose range for each HDAC2 siRNA at optimal experimental conditions. Cells were plated at 30% confluence 24 hours before transfection and treated with siRNA mixed with the same amount of cationic lipids. HDAC2-1 siRNA at a concentration of 15 nmol/L caused a maximum growth suppression of approximately 80% in both the Huh7 and HepG2 cells after 4 days of treatment (Fig. 1A). This dose was, therefore, selected for all subsequent studies. In concordance with the phenotypic assay result, HDAC2-1 siRNA was the most effective at target gene silencing in both of the examined tumor cell lines (Fig. 1B). HDAC2 knockdown was specific as transfection with NCsiRNA did not affect target gene expression. The decrease in cell viability was confirmed by microscopic observation in 4 days of target gene silencing (Fig. 1C). The growth-suppressing effect was reproduced in two additional HCC cell lines, Huh1 and PLC/PRF/5 (Fig. 1D). The growth inhibition in HDAC2-depleted HCC cells was attributed, in part, to blockade of cell-cycle progression. Flow cytometry analysis revealed that Huh7 and HepG2 cells transfected with HDAC2-1 siRNA had increased G0–G1 and reduced G2–M fractions, suggesting cell-cycle arrest in the G1 phase (Fig. 2A). More significantly, HCC cells with silenced HDAC2 showed a strong induction...
of apoptosis as measured by the formation of denatured single-stranded DNA that formed in apoptotic cells as opposed to necrotic cells or cells with DNA breaks (Fig. 2B). This coincided with increased activation of caspase-3, one of the primary executioners of apoptosis (Fig. 2C), and a concomitant accumulation of apoptosis-specific caspase-cleaved K18 (ccK18) fragments (Fig. 2D). The subsequent Western blot analysis of cleaved caspase-9 and caspase-3 (Fig. 2E) confirmed that HDAC2 silencing mediates a caspase-dependent mechanism of apoptosis. These data show that molecular targeting of HDAC2 in human HCC cells blocks cell-cycle progression and induces apoptosis.

Figure 2. HDAC2 siRNA silencing delays cell-cycle progression and induces apoptosis in Huh7 and HepG2 cells. A, FACS analysis of cell-cycle progression 2 days after transfection with NC and HDAC2-1siRNA. B, detection of apoptosis 3 days after transfection experiments by ApoStrand ELISA Apoptosis Detection Kit. Data represent three independent experiments (**, P < 0.01; Bootstrap t test). NT, no treatment; NC, negative control siRNA; HDAC2, HDAC2-1siRNA. C, caspase-3 activation as detected by the CaspSELECT Caspase-3 Immunoassay Kit 2 days after transfection experiments. Data represent three independent experiments. ***, P < 0.01; Bootstrap t test. D, detection of caspase-cleaved keratin 18 (ccK18) fragments by M30 Apoptosense ELISA 2 days after transfection experiments. Data represent three independent experiments. ***, P < 0.01; Bootstrap t test. E, Western blot analysis of HDAC2 and the indicated proteins functionally involved in caspase cascade. Whole-cell lysates were prepared 2 days after treatment with NC or HDAC2-1siRNA. Actin was included as a loading control.
The molecular mechanisms underlying the growth inhibitory effects of HDAC2 siRNA knockdown

To study the molecular basis of growth inhibition caused by HDAC2 loss, we compared the global gene expression profiles of HDAC2-deficient Huh7 and HepG2 cells to those of control cells transfected with NCsiRNA. HDAC2 siRNA knockdown for 48 hours caused a strong (>8 fold) inhibition of HDAC2 mRNA in both Huh7 and HepG2 cells (Fig. 3A). Western blot analyses confirmed a great and specific decrease of only HDAC2 protein, albeit at a slightly different degree in the two tested cell lines (Fig. 3B). The expression levels of HDAC1, HDAC3, HDAC4, HDAC5, and HDAC8 isoforms were unaffected as compared with NCsiRNA controls on mRNA and protein levels.

The global gene expression analysis revealed that HDAC2-specific knockdown led to up- and downregulation of 1,104 genes in Huh7 and 286 genes in HepG2 cells (Fig. 3C). Comparing these two gene sets showed a statistically significant overlap of 97 genes as defined by a Bootstrap t test with 10,000 repetitions and at least a 2-fold change (P < 0.001). Among these, 88 genes showed the same directional regulation (15 up- and 73 downregulated genes) and were considered as a common HDAC2 knockdown signature (Fig. 3C and Supplementary Table S1). Besides a strong downregulation of HDAC2, the dysregulated genes were enriched for cell death and proliferation ontology terms (Fig. 3D). Consistent with phenotypic changes, PathwayStudio analysis of the common HDAC2 knockdown signature revealed that the expression levels of TP53I3, BTG2, KLF11, and TAGLN involved in proapoptotic activity and growth suppression were upregulated, whereas key regulators of cell growth TGFB1 and BPS6K43 were repressed (Fig. 3E). Furthermore, both examined HCC cell lines showed a decrease in the mRNA levels of the SKP2 gene, which encodes a substrate-targeting subunit of the ubiquitin ligase complex known to control entry into the S phase, thereby inducing the degradation of the cyclin-dependent kinase inhibitors p21 and p27 (34). Of importance, in support of the recent findings that drug-mediated histone deacetylation could modulate nuclear receptor binding to chromatin and/or to factors involved in mediating their functions (35, 36), the signature also revealed downregulation of the PPARγ (Fig. 3E, Fig. 4C), a critical transcription factor linking lipid and metabolic changes to gene expression, these results reinforce a previously unrecognized role for HDACs in control of lipid metabolism (35, 36). Thus, knockdown of a single HDAC2 gene coordinatey affects a restricted number of defined oncogenic pathways acting in concert to decrease cell proliferation, induce apoptosis, and reduce lipogenesis, providing a rationale for therapeutic targeting of HDAC2 in HCC.

Systemic silencing of HDAC2 by siRNA encapsulated into LNP suppresses orthotopic liver tumor growth

The next studies were performed to validate whether systemic siRNA silencing of HDAC2 could suppress liver tumor growth. To enhance siRNA stability in vivo and prevent unwanted immune activation, native HDAC2-1siRNA was chemically modified by selective incorporation of 2’OMe uridine or guanosine nucleosides into one strand of the siRNA duplex, and encapsulated into LNP (31, 32). The modified siRNAs were then screened for in vivo activity by evaluating the extent of growth and target gene inhibition, and cytokine induction using murine Flt3L dendrocytes isolated from mouse bone marrow. Among the variants tested, the HDAC2 3/7 sequence was the most effective in inhibiting Huh7 cell growth with more than 95% of target gene silencing (Fig. 5A, and B). In addition, this modified sequence resulted in a minimal induction of IL6 as compared with the treatment with a native HDAC2-1siRNA (Fig. 5C).

To test the therapeutic efficacy of HDAC2 gene targeting, we used an orthotopic xenograft model of human HCC in immuno-compromised NOD/SCID mice and B11 as a method of monitoring the kinetics of tumor growth. All mice received an intrasplenic injection of Huh7-luc+ reporter cells (0.5 × 10⁶), and on day 8 were randomly assigned to either the treatment (LNP-siHDAC2 3/7) or control (LNP-siG478) groups before starting siRNA therapy. The results showed that in control mice treated with LNP-siG478 targeting β-galactosidase, tumors grew very rapidly, and at the end point of the study (28 days) occupied a significant portion of liver parenchyma (Fig. 6A, and C). By this time, the majority of control mice developed ascites, reflecting impaired liver function, characteristic of end-stage...
Figure 3. Transcriptomic analysis of gene expression following HDAC2 knockdown. A, fold changes in expression levels of HDAC isoforms in Huh7 and HepG2 cells treated for 2 days with 15 nmol/L HDAC2-1siRNA as compared with NCsiRNA based on microarray. B, Western blot analysis of HDAC1, 2, 3, 4, 5, and 8. Actin was used as a loading control. 1 si., HDAC2-1siRNA. C, heatmaps of the differentially expressed genes (on the left) and 88 genes commonly deregulated (on the right) in Huh7 and HepG2 cells 2 days after transfection with 15 nmol/L of HDAC2-1siRNA after normalization to the corresponding cells were treated with NCsiRNA. \( n = 4 \) for each condition, 10,000 repetitions in Bootstrap ANOVA with contrast tests and a threshold cutoff of 2-fold change; \( P < 0.001 \); red, induced; green, repressed; log2-based scale. D, functional relation of the significant genes. The table demonstrates the key changes (cell death and antiproliferation following HDAC2 knockdown). E, common regulators in HDAC2 knockdown 88-gene signature analyzed by PathwayStudio software.
liver cancer. In contrast, three intravenous injections of LNP-siHDAC2 3/7 caused a significant suppression of Huh7-luc\(^+\)-derived tumors in liver (Fig. 6A, and B). Upon histopathologic evaluation, mice receiving LNP-siHDAC2 3/7 therapy showed an improved histology with single and much smaller tumors. As well, a decrease in liver-to-body ratios reflected the reduced tumor burden (Fig. 6C, and D). Taken together, HDAC2 is an important regulator of HCC growth and that specific targeting of HDAC2 by RNAi could be a novel therapeutic modality for HCC.

**Discussion**

HDACs are commonly dysregulated in cancer and, therefore, represent promising targets for therapies (10, 12, 13). In addition to recent reports in HCC (14, 19), increasing evidence
downregulation of HDAC2 mRNA in Huh7 cells treated with Bootstrap inhibition of Huh7.

However, the anticancer therapy by a selective inhibition of effective therapeutic strategy against various cancer types. metrial cancer (39), implying that HDAC2 targeting may be an colorectal cancer, prostate cancer, ovarian cancer, and endo-
types such as cutaneous T-cell lymphoma, gastric cancer, treated with 

Figure 5. Selection of HDAC2 3/7 for in vivo application based on the inactivation of tumor cell growth and minimal cytokine induction. A, inhibition of Huh7-luc cell growth after transfection with 30 nmol/L of SNALP-formulated HDAC2-1 (native) or its modified variants (HDAC2-3/6–8, HDAC2-4/6–8, HDAC2-5/6–8). The siRNA transfectants were examined by MTT assay at 4 days after treatment [P < 0.05 (n = 3) by Bootstrap t test]. LNP-Luc, LNP-formulated siRNA targeting luciferase. B, downregulation of HDAC2 mRNA in Huh7 cells treated with HDAC2 3/7. Total RNAs were extracted at 48 hours after treatment with 15 nmol/L of siRNA [P < 0.01, Bootstrap t test]. C, quantification of IL6 levels after HDAC2 siRNA treatment. Cytokine supernatants of FBL-derived dendrocytes were assayed for IL6 using ELISA 24 hours after siRNA treatment. The data are shown as means ± SEM of triplicate experiments [P < 0.01, Bootstrap t test].

demonstrates aberrant expression of HDAC2 in diverse cancer types such as cutaneous T-cell lymphoma, gastric cancer, colorectal cancer, prostate cancer, ovarian cancer, and endome-trial cancer (39), implying that HDAC2 targeting may be an effective therapeutic strategy against various cancer types. However, the anticancer therapy by a selective inhibition of a disease-specific HDAC is still lacking.

The present study provides evidence that siRNA targeting of HDAC2 in HCC cell lines elicits a strong target specificity and inhibits only one HDAC2 target protein among the tested class I and class IIa HDAC isoenzymes. Over the past decade, at least 21 siRNAs have been evaluated for more than a dozen diseases, including recent preclinical studies targeting polo-like kinase 1 (PLK1) and kinesin spindle protein (KSP; ref. 27). The primary obstacle for therapeutic application of RNAi is the successful in vivo delivery to the targeted cell (40, 41). In this study, we used LNP technology to protect the systemically administered siRNA from glomerular filtration and serum nucleases, thereby extending siRNA half-life and increasing its potency while suppressing unwanted immune responses (24–26, 31, 32). Indeed, treatment with a moderate (2 mg/kg) dose of 2′OMe-modified HDAC2 siRNA encapsulated in LNP was sufficient to mediate a potent silencing of the target mRNA and the effective suppression of HCC growth in vivo without cytokine induction or toxicity associated with unmodified siRNA. Although more work is needed to optimize the therapeutic dose and address potential side-effects, these findings suggest the clinical utility of HDAC2 siRNA targeting for treatment of HCC disease with high target selectivity.

In agreement with published data (28, 42, 43), inhibition of HDAC2 protein caused a block in cell-cycle progression and extensive apoptosis in all the examined HCC cell lines. On the molecular level, it was paralleled by a coordinated dysregulation of genes acting downstream of HDAC2-regulated transcription factors. Microarray profiling showed that phenotypic changes triggered by HDAC2 siRNA silencing were associated with an increased expression of proapoptotic effectors KLF11, BTG2, and TP53I3, and downregulation of key regulators of cell growth such as SKP2 and TGFβ1. This was paralleled by a strong reduction in the protein levels of CDK2, CDK4, CDK6, cyclin D1, and cyclin E, whereas the expression of cdk inhibitor p27 was increased, providing a mechanism for HDAC2 silencing-mediated inhibition of cell-cycle progression. We also show that HDAC2 siRNA knockdown increased the levels of p53 acetylation, which is known to boost the p53 recruitment to promoter-associated complexes and upregulate the expression of p53 target genes involved in cell-cycle control and apoptosis. Of note, HDAC2 knockdown enhanced p53 acetylation both in HepG2 (with wild-type p53) and Huh7 cells (with mutant p53). Furthermore, siRNA-mediated HDAC2 silencing caused a downregulation of PPARγ, which was associated with reduction in expressions of lipogenic enzymes and decreased lipid accumulation. This is in agreement with recent findings that genetic deletion of HADC1 and HADC2 in cultured mesenchymal precursor cells (35) or in vivo treatment with a novel class II-selective inhibitor MC1568 (36) interfered with PPARγ signaling pathways. There is increasing appreciation that PPARγ signaling in addition to control of lipid homeostasis also influences inflammation, hepatic fibrosis, and cancer. Given the involvement of PPARγ signaling networks in diverse physiologic and pathologic processes (37, 44, 45) and the conflicting data about the role of PPARγ as either an anti- or protumorogenic factor in HCC (46, 47), the mechanisms whereby HDAC1 interferes with the PPARγ signaling pathways may be of significance for the clinical use of HDACi and require thorough investigation.

In conclusion, this is the first study to report that targeting of HDAC2 by RNAi-based nanoparticles allowed a selective inactivation of only one disease-specific HDAC isoform and achieved consistent therapeutic benefits in a preclinical model of HCC, providing an attractive therapeutic modality for liver cancer.
Disclosure of Potential Conflicts of Interest

I. MacLachlan received commercial research support from Tekmira Pharmaceuticals Corp. No potential conflicts of interests were disclosed by the other authors.

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