The American Journal of Surgery 213 (2017) 645–651

Contents lists available at ScienceDirect

The American Journal of Surgery

journal homepage: www.americanjournalofsurgery.com

Suberoylanilide hydroxamic Acid, a histone deacetylase inhibitor, alters multiple signaling pathways in hepatocellular carcinoma cell lines

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ARTICLE INFO

Article history:
Received 30 June 2016
Received in revised form
6 October 2016
Accepted 9 December 2016

Keywords:
Histone deacetylase inhibitor
SAHA
Vorinostat
Hepatocellular carcinoma
Apoptosis
Notch
AKT
Raf-1
STAT3

ABSTRACT

Introduction: Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, has preclinical efficacy in hepatocellular carcinoma (HCC), despite an unclear molecular mechanism. We sought to further investigate the effects of SAHA on HCC. We hypothesize SAHA will inhibit HCC cellular proliferation through apoptosis and aid in further profiling SAHA's effect on HCC oncogenic pathways.
Methods: HCC cell lines were treated with various concentrations of SAHA. Cell proliferation was determined by MTT and colonogenic assays. Cell lysates were analyzed via Western blotting for apoptotic and oncogenic pathway markers. Caspase glo-3/7 was used to assess apoptosis.
Results: SAHA treatment demonstrated significant (<0.05) reduction in cell growth and colony formation through apoptosis and cell cycle arrest. Western analysis showed reduction in Notch, pAKT and pERK1/2 proteins. Interestingly, phosphorylated STAT3 was increased in all cell lines.
Conclusions: SAHA inhibits Notch, AKT, and Raf-1 pathways but not the STAT3 pathway. We believe that STAT3 may lead to cancer cell progression, reducing SAHA efficacy in HCC. Therefore, combination of SAHA and STAT or Notch inhibition may be a strategy for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) continues to be a significant burden on the health care system as it represents the 6th most common cancer worldwide.[1] Over the course of several decades, HCC has risen in occurrence and is now among the most common occurring malignancies with incidence in the United States steadily increasing. Despite improvements in early diagnostic capabilities along with improved traditional tactics and the emergence of novel treatment strategies, 5-year survival following HCC diagnosis remains dismal at less than 20%.[2] Concomitant liver disease and cirrhosis contribute to both morbidity and mortality in these patients often limiting traditional therapeutic approaches such as resection, transplantation, and TACE.[3] Consequently, advanced cases of HCC have limited treatment options and traditional systemic chemotherapy such as doxorubicin and sorafenib provide a limited survival advantage and a high toxicity profile prompting alternative approaches.

Histone deacetylases (HDACs) regulate chromatin structure through the modulation of acetyl groups. This, in turn, alters deoxyribonucleic acid (DNA) conformation and subsequent manipulation of gene expression.[4] In total, 18 HDAC enzymes have been categorized and subsequently divided into classes I–IV. HDAC classes I and II, a zinc-binding class, are unique in their many off target effects. Outside of histone targeting, HDAC classes I and II also modulate transcription factors and cell cycle regulators.[5–7] In addition, these classes have demonstrated a predilection for overexpression in various cancer types.[8,9] Therefore, targeting these two classes may be of particular interest.

Histone deacetylase inhibitors (HDACis) prevent the deacetylation of histones within the chromatin structure, consequently increasing the histone component within the DNA. This leads to a

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http://dx.doi.org/10.1016/j.amjsurg.2016.12.001
0002-9610/

relaxed, loosened chromatin structure allowing for a larger transcriptional response. HDACis are plentiful and have been extensively studied in hematological malignancies and recently have transitioned into solid tumors. One HDACi that has been studied is suberoylanilide hydroxamic acid (SAHA). SAHA, marketed as Vorinostat, is a class I and II HDACi that has had promising in vitro results. SAHA is one of a series of hydroxamic acid-based hybrid polar compounds that exhibit growth arrest through apoptotic induction and cell cycle arrest via inhibition of the HDAC system.[10,11]

Furthermore, preclinical models in prostate cancer as well as hematological cancers have demonstrated beneficial activity; thereby, providing proof of concept.[12] Preclinical models have shown a mitochondrial-mediated apoptotic induction where phase I–III clinical trials have shown translational promise.[13–15] Moreover, clinical trials have shown HDACis have the capacity to improve sensitivities of traditional chemotherapies.[16] Among the HDACis, SAHA has demonstrated efficacious preclinical and clinical success, at tolerated doses. Phase I studies demonstrated a maximum tolerated dose (MTD) of 200 mg twice daily or 250 mg three times daily. Dose-limiting toxicities (DLT) were fatigue, nausea, vomiting, and diarrhea. Common adverse effects include diarrhea, nausea, fatigue, and anorexia. Higher grade adverse events included thrombocytopenia.[17] It was the first HDACi to be approved by the Food and Drug Administration (FDA) in the treatment of T cell cutaneous lymphoma following multiple clinical trials. Despite the delineation of SAHA, its detailed molecular mechanism by which apoptosis is induced is poorly understood. Furthermore, clinical trials have stalled as SAHA has shown limited effectiveness as a single agent therapy. Given our improved understanding of molecular pathways and the desire to improve the clinical effects of SAHA, there is an urgent need to further molecularly profile SAHA administration.

In this present study, we examined the anti-proliferative effect of SAHA on HCC cell lines. Here, we provide promising evidence that SAHA, a HDACi, leads to a reduction in cellular proliferation through induction of apoptosis by altering various oncogenic signaling pathways. Interestingly, SAHA treatment activates STAT3 pathway while blocking other dominant oncogenic pathways. These findings provide a novel mechanism of action of SAHA and allows for future translational work with combination therapy.

2. Materials and methods

2.1. Cell lines and reagents

Three human hepatocellular carcinoma (HCC) cell lines were used in this study. Huh-7 (a kind gift from Dr. Chisari, The Scripps Research Institute, La Jolla, CA), Hep3B and HepG2 (American Type Culture Collection (ATCC), Rockville, MD, USA). All three cell lines represent a diverse demographic. Huh-7 cells are a derivative from an Asian male decent whereas Hep3B and HepG2 are derived from a black female and white male respectively. In addition, the p53 genetic signature is varied among the three cell lines. Huh-7 is a p53 mutant variant whereas Hep3B and HepG2 are p53 deficient and p53 wild type respectively. All three cell lines were cultured as described.[18] Briefly, Hep3B cells was cultured in Eagle's Minimum Essential Medium (EMEM) whereas Huh-7 and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with nonessential amino acids (NEAA, Life Technologies, Carlsbad, CA, USA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Life Technologies). These cell lines were additionally supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture media was replaced every 2–3 days. SAHA (Selleckchem, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and treated at various concentrations indicated.

2.2. Cellular proliferation and colony forming assays

Cellular proliferation on the HCC cell lines was measured by using colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described.[18] Cells were seeded in 96-well plates and treated with SAHA (0–10 μM) in quadruplicates for up to 96 h. At each time point, viability was determined after the addition of 0.5 mg/mL MTT and the absorbance was measured at 540 nm using a spectrophotometer (Infinite M200PRO Microplate reader; TECAN, San Jose, CA, USA). The reported cellular viability represents the average of three experiments. The ability to form colonies following SAHA administration was determined by the measurement of colonogenic cell survival as previously described.[19]

2.3. Caspase-3 and -7 activity levels

Confirmatory apoptotic studies measured the cleavage of caspase-3 and -7 utilizing the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) as previously described.[19] Briefly, 10 μg of protein lysate in 25 μL was mixed with equal volume of the Caspase-Glo reagent and incubated at room temperature for thirty minutes. Following incubation, the activity level of caspase-3 and -7 was determined by luminescence using the Infinite M200PRO Microplate reader (TECAN).

2.4. Western blot analysis

Following 48 h of SAHA treatment, cells were collected and lysed in radioimmunoprecipitation assay (RIPA, Thermo Fisher Scientific, Waltham, MA, USA) buffer as previously described.[20] Protein concentrations were quantified using the bicinchoninic acid assay method (BCA, Thermo Fisher) and 30 μg of protein were analyzed by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were then transferred to nitrocellulose membranes (Bio-Rad Laboratories) using a *Trans*-Blot Turbo (Bio-Rad Laboratories) and the membranes were blocked in 5% milk solution for one hour. The membranes were incubated overnight at 4 °C with the respective antibodies. Following day, membranes were washed with PBS-T wash buffer (1 X PBS, 0.05% Tween-20) three times for five minutes each. Membranes were then incubated in their representative secondary antibody (horse-radish peroxide conjugated anti-rabbit or anti-mouse, Abcam, Cambridge, MA, USA, 1: 10,000 dilution) for a minimum of 1.5 h. Following the secondary antibody incubation, membranes were again washed for three times for five minutes each. Detection of the immune complexes was assessed using chemiluminescence detection kit (Thermo Fisher). Images of the complexes were taken using the Molecular Imager ChemiDocXRS$^+$ imager (Bio-Rad Laboratories).

2.5. Statistical analysis

Determination of statistical significance was calculated by analysis of variance (ANOVA) using a statistical analysis software package (IBM SPSS Statistics version 22, New York, NY, USA). Statistical significance was achieved with p-values of <0.05. Data were represented as ± standard error.