Comparative Effects of HDAC Inhibitor SAHA and MDM2 Inhibitor RG7388 in LNCaP Prostate Cancer Cells

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Abstract

Alterations in gene expressions are often due to epigenetic modifications that can influence cancer development, growth, and progression. Acetylation of histone is one of the most common epigenetic modifications that is regulated largely by histone acetyltransferases (HATs). Due to their ability for imparting epigenetic alterations, HDAC (Histone Deacetylase) inhibitors such as SAHA, which is Suberoylanilide Hydroxamic Acid (also known as Vorinostat), are in clinical trials as a new class of drugs with promising impact on the control of cancer growth and metastatic process. On the other hand, RG7388 is a newly developed inhibitor that is specific for an oncogene-derived protein called MDM2, which is also in clinical trials for the treatment of cancers. One of the common characteristics for these two drugs is their ability to induce p21 expression through distinct mechanisms. This difference was expected to trigger cell cycle arrest and cell death through mechanisms that are dissimilar. In this regard, the molecular mechanism whereby SAHA can induce cell cycle arrest and trigger cell death is still evolving.

Similarly, the effect of RG7388 for producing anti-cancer effect is undergoing significant level of investigations, because of its ability to produce p53 dependent and p53 independent effects. We performed experiments to measure the cell cycle arrest and cytotoxic effects of SAHA and RG7388 using LNCaP (Prostate cancer) cells. The cell cycle arrest, cytotoxicity, and pro-apoptotic effects of the treatments were assessed by using cell viability assessment methods, fluorescence assay with DEVD-amc (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) fluorogenic substrate for Caspase 3/7 and also by immunoblotting methods. Our results from LNCaP cells confirmed that SAHA and RG7388 treatments were able to induce cell death via combination of cell cycle arrest and cytotoxic mechanisms. In LNCaP prostate cancer cells the induction of cell cycle arrest was evidenced by the elevation of p21 protein levels following SAHA and RG7388 treatments. Interestingly, SAHA was able to elevate p21 expression without elevating the p53 levels in LNCaP cells. As expected, inhibition of MDM2 with RG7388 was able to induce p21 expression by elevating p53, due to strong inhibition of MDM2. Though the mechanisms may be different, both cells were showing clear evidences of cell death after treatment with 7.5 µM of SAHA and 2.0 µM of RG7388.

It was clearly evident that RG7388 treatment was inducing cell death by elevating p21 through p53 dependent mechanisms in LNCaP cells. It has been reported in the literature that the elevated levels of acetylated histones (H2 and H3) and consequent activation of intracellular signals are responsible for the transcription of p21 gene and consequent cell cycle arrest and cell death that are typically observed when HDAC inhibitors (HDACi) are used. We are speculating that our findings could lead to the development of newer treatments for prostate cancers using HDACi or MDM2 inhibitors.

Abbreviations: HATs: Histone Acetyl Transferases; HDAC: Histone Deacetylase; SAHA: Suberyl Anilide Hydroxamic Acid

Introduction

Prostate cancer is one of the most common causes of cancer-related deaths among men worldwide. The survival rate for prostate cancer patients with distant metastasis is only 30% versus 98% survival for patients with localized disease. Therefore, the need for new drugs and efficient strategies for treating aggressive cancers continues to exist. Although the development of cancer is triggered by a complex process of tumorigenesis, the epigenetic changes are also very common during cancer development, as these changes affect gene transcription without modifying the underlying DNA sequence. In normal cells the histones are modified via acetylation, methylation and phosphorylation to meet certain needs of the cellular function [1,2]. Acetylation of histone is one of the most
common epigenetic modifications that is regulated largely by histone acetyltransferases (HATs), which transfer the hydrophobic acetyl group from acetyl coenzyme A to specific lysine residues on the N-terminal tails of histone H2A, H2B, H3 and H4 [3].

This addition leads to the neutralization of positive charge of the amino group and increases the steric interference leading to the loosening of the histone- DNA structure that is more accessible for transcription machinery and gene activation [4,5]. On the other hand, the DNA stretching, and activation of the gene expression can be turned off when histone is deacetylated [4,5]. The histone deacetylation is carried out by enzymes that belong to two families, the classical HDAC family and SIR2 family [6]. There are 11 HDAC isoenzymes that deacetylate histones within the nucleus, and the specific HDACs are differentially regulated to modulate the expression of various groups of genes [7]. The gene expression can be activated when histone deacetylase inhibitor such as SAHA is used. This will induce growth arrest, cell death or differentiation, indicating that the net effect of histone deacetylation blockade is an inhibition of cell proliferation [8]. Thus, the HDACi have been reported to stimulate much enthusiasm in the field of oncology, resulting in clinical trials and approval by the Food & Drug Administration (FDA) of SAHA and other antimtor agents with HDACi ability.

Use of HDACi effectively inhibits HDAC activity, increases histone acetylation to commensurate cancer cell with necessary transcription to induced cancer cell differentiation, arrest of cell cycle progression, and induction of cancer cell apoptosis. In addition, several studies have so far confirmed that SAHA can increase p21WAF1/CIP1 levels and its function, resulting in cell cycle arrest and induction of apoptosis in cancer cells [9,10]. In several metastatic sarcoma as MDM2 gene has been shown to encode a protein in the molecular weight range of 90 KD (p90) that can form an oligomeric complex with both mutant and wild type p53 proteins. The interaction between MDM2 and p53 is believed to occur through direct binding involving a specific binding domain of the MDM2 protein [11-14]. In addition, it has been well established that MDM2 contains ubiquitin ligase (E3) activity in its carboxy terminus which is also responsible for the poly-ubiquitination of p53 and consequent proteasomonal degradation.

One of the effective methods of blocking MDM2 mediated mechanisms is by inhibiting its interaction with p53 protein. Historically, disruption of protein-protein interaction used to be a challenging task due to the large binding interface of the protein partners. However, the well-defined, small interface of MDM2-p53 made it possible to design small-molecule inhibitors such as RG7388 to target the MDM2-p53 interaction. The RG7388 is one of the latest generations of MDM2 inhibitors with good selectivity and potency combined with improved bioavailability [15]. Various studies conducted utilizing RG7388, to rescue p53 and activate downstream cell cycle arrest or apoptotic pathway so far have yielded interesting results [16]. However, the detailed mechanisms of cell cycle arrest and cell death induced by RG7388 has not been very well established. The current study has provided some interesting insights into the mechanisms mediated by SAHA and RG7388 through p21 expression.

Materials and Methods

Reagents

Histone deacetylase (HDAC) inhibitor SAHA was purchased from Selleckchem (Houston, TX, USA) and MDM2 inhibitor RG7388 was purchased from Med Chem Express (New Jersey, USA). The primary antibodies against p53, p21, BAX and Cleaved PARP (1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). MDM2 antibody (1:500) was purchased from Santa Cruz Biotechnology. The β-actin antibody (1:2000) was purchased from Sigma-Aldrich (St. Louis, MO, USA). ECL was purchased from Millford, MA). Nitrocellulose membrane (0.45 µm) was purchased from Amersham (GE Healthcare Life Sciences USA). All other chemicals used in this experiment were of research grade.

Cell Culture and Drug Treatments

Human prostate adenocarcinoma cell line (LNCaP) was provided by Dr. Thomas Powell (Cleveland Clinic Foundation, Cleveland, OH) The LNCaP cells were cultured with RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1.5 g/L sodium bicarbonate antibodies (1% Amphotericin B and 1% penicillin G – Streptomycin). The cells were maintained in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. When LNCaP cells reached 75-80% confluency, they were treated with SAHA (7.5 µM) or RG7388 (2.0 µM) for 24 hrs. After incubation the cells were lysed, and protein was extracted for western blot analysis. Similarly, the cell viability assays and fluorescence staining were also performed after treating the cells with the above-mentioned drugs.

Cell Viability Assessment Using Trypan Blue Dye Exclusion and MTT Methods

The LNCaP cells were plated at a density of 5x10⁴ cells/well in 96-well plates, and incubated at 37°C under 95% air and 5% CO2 for 24 hrs. Once the cells reached 75-80% confluency they were treated with different concentrations of the drugs for 24 hrs. After incubation, the viability of the cells was assessed using Trypan Blue Dye Exclusion (TBDE) method and also by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In TBDE method, after removing the incubation medium, equal parts of 0.4% trypan blue dye was added to the cell suspension. The mixture was allowed to incubate for less than three minutes at room temperature. The cell viability was counted using the TC20 automated cell counter from Bio-Rad (Hercules, CA). For the MTT assay, the cells were seeded into a 96-well plate at a density of 5x10⁴ per well (200 µl) and treated with different concentrations of SAHA (0.5, 2.5, 5.0, 7.5, 10.0 µM) and RG7388 (1.0, 2.0, 2.5, 5.0, 7.5 µM). After 24 hrs of treatment, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and the cells were incubated at 37°C.
for an additional 3-4 hrs. At the end of incubation, 200 μl of DMSO was added to each well. The plate was gently rotated on an orbital shaker for few minutes to completely dissolve the precipitates. The absorbance was read at 650 nm with a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Protein Preparation and Western Blot Analysis**

After 24 hrs of treatment the cells were lysed with RIPA (Radio-Immunoprecipitation Assay) buffer, containing the protease inhibitor cocktail and sodium orthovanadate (Santa Cruz Inc., Dallas, TX, USA), for 30 min at 4°C. Cell lysates were clarified at 4°C for 20 min at 14,000 rpm and then protein concentrations were determined by using bicinchoninic acid (BCA) protein assay method (Thermo Fisher Scientific, Grand Island, NY, USA). For the western blot analysis equal concentration of proteins were separated using 7.5-12% sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane. After protein transfer the nitrocellulose membranes were blocked using solution containing 5% non-fat dry milk in TBS (Tris-buffered saline) After necessary washes the membranes were probed with antibodies specific for MDM2, p53, p21, CDK1, BAX, Cleaved PARP and β-actin. Finally, for the detection of targeted proteins, the membranes were incubated in a solution containing LumiGLO Reserve Chemiluminescent substrate from KPL (Gaithersburg, MA, USA). Densitometric analyses of the bands in the western blots were performed using the ImageJ program (NIH Image, Bethesda, MD).

**Fluorescence Imaging for Cell Death Assessment**

The DEVD-amc is a fluorogenic substrate specific for Caspase 3/7. This is a cell permeant reagent that consists of a four-amino acid peptide (DEVD) as indicated before, conjugated to a nucleic acid-binding dye. The peptide sequence is based on the PARP cleavage site Asp216 for Caspase-3. Once cleaved from DEVD the amc (7-Amino-4-methylcoumarin) can be excited around 502 nm to emit fluorescence that can be measured at 535 nm. Uncleaved DEVD substrate is intrinsically non-fluorescent. However, during apoptosis, Caspase-3 and Caspase-7 are activated, which can cleave the DEVD-amc and generate green fluorescence. For determining the effects of the drugs, the cells were treated with SAHA and RG7388 for 24 hrs. After the incubation the cells were washed and then incubated with DEVD-amc substrate for 30 minutes. The fluorescence images were captured using a DMI3000 B Leica microscope (Illinois, USA).

**Statistical Analysis**

The data are presented as mean ± SD Statistical significance between the groups was analyzed by one-way analysis of variance (ANOVA) followed by LSD (Least Significant Difference) test. P < 0.05 was considered statistically significant indicated by * P < 0.001 was indicated by **.

**Results**

**Reduction of Cell Viability by SAHA and RG7388 Treatments on LNCaP**

The cytotoxic effects of SAHA and RG7388 on LNCaP cells were determined using TBDE and MTT assay methods. The treatment effects of SAHA and RG7388 in monotherapy are shown in Figure 1. Following 24 hrs treatments the RG7388 produced significant reduction in the cell viability. As can be seen in Figure 1, the IC₅₀ for SAHA after 24 hrs of treatment was found to be 7.5 µM. On the other, RG7388 produced stronger cytotoxic effects on LNCaP cells and as a result the IC₅₀ was found to be 2.0 µM after 24 hrs of treatment.

**Effect of SAHA and RG7388 Treatments on Cell Cycle Related Gene Expressions**

Based on the results shown in Figure 2, both SAHA and RG7388 induced strong cell cycle arrest in LNCaP cells. In accordance with this conclusion, treatments with SAHA and RG7388 showed significant elevation in the levels of p21. The elevation caused by SAHA was only around 1.3 folds whereas RG7388 treatment induced 3.5 folds (P<0.001) increase in p21 levels. However,
treatment of LNCaP cells with SAHA did not elevate neither MDM2 nor p53 protein levels after 24 hrs of treatment. As expected RG7388 treatment elevated both MDM2 and p53 levels by nearly 95% and 120% (P<0.001) respectively in the LNCaP cells (Figure 2).

![Graph showing expression levels of MDM2, p53, p21, CDK1, and Beta Actin for Control, SAHA, and RG7388 treatment groups.](image)

**Figure 2:** Effect of SAHA and RG7388 on Cell Cycle related gene expressions in LNCaP cells. Figure shows upregulation of p53, p21 and MDM2 levels in LNCaP cells after treatment with 2.0 μM concentration of RG7388. The right panel shows the results of relative band intensity of the western blot bands that was measure using ImageJ software. The normalization of protein levels is depicted using the β-actin bands. Data are presented as means ± S.E.M. from minimum of three independent experiments. *, P < 0.05 vs. Control; **, P < 0.001 vs. Control.

**Apoptotic effects of SAHA and RG7388 Treatments on LNCaP cells**

![Fluorescence images showing apoptotic cell death induced by SAHA and RG7388 treatment](image)

**Figure 3:** Apoptotic cell death in LNCaP cells shown by Fluorescence image obtained using the DEVD-amc. The results of cell death induced by SAHA and RG7388 treatment for 24 hrs is shown in Figure 3A. The effects of the treatment on cell death observed in by light microscopic imaging is shown in Figure 3B. The apoptotic cell death following SAHA and RG7388 treatment was analysed using the fluorescence staining method with DEVD-amc fluorogenic substrate that is specific for Caspase 3/7 (Figure 3A). The cells that were treated with RG7388 produced high levels of green fluorescence as a result of the cleavage of DEVD-amc by the activated Caspases (Figure 3A). Interestingly, the SAHA treatment did not produce high levels of green fluorescence, which suggested very low-level cleavage of the DEVD-amc substrate in this treatment. The light microscopic imaging of the un-stained cells also showed significant reduction in the cell number after RG7388 treatment (Figure 3B). The results of the imaging experiments were supported by the increased levels of the PARP cleavage and BAX obtained using western blot after treating the cells with RG7388 (Figure 4). Interestingly, SAHA treatment was not able to elevate BAX levels in LNCaP cells, rather it produced slight decrease after 24 hrs. In addition, SAHA treatment did not produce PARP cleavage after 24 hrs of treatment with 7.5 µM concentration (Figure 4). It is interesting that in LNCaP cells SAHA was able to induce p21 expression without any changes in the expression levels of p53.
Though p53 levels were not elevated, SAHA treatment showed measurable level of cell death. However, the level of cell death induced by SAHA was slightly lower than the extend of cell death induced by RG7388 treatment in 24 hrs. The greater effects of RG7388 was supported by significant decrease in CDK1 levels that was observed in addition to the elevation of BAX and PARP deavage in LNCaP cells. Evidently, treatment of LNCaP prorate cancer cells with SAHA was not able to show elevation in the levels of BAX nor cleavage of PARP after 24 hrs treatment. In addition, the CDK1 levels were only slightly lowered compared to the controls after SAHA treatment. Thus, the effects of RG7388 towards cell cycle arrest and pro-apoptotic mechanisms were lot more significant in LNCaP cells and showing strong correlation with p21 elevation and PARP cleavage.

**Discussion**

Our results with cytotoxicity experiments and western blot analysis suggested that, following SAHA treatment, cell cycle arrest should be occurring due to elevation of p21WAF1/CIP1 through p53 independent pathway in LNCaP cells. However, our results with RG7388 dearly showed that the effects of the MDM2 inhibitor is mediated through the p53 dependent pathway subsequent to 24 hrs treatment. It has been previously reported that histone deacetylase inhibitors (HDACi) such as SAHA can effectively kill cancer cells that are growing in cultures or implanted in animals to form xenograft tumors [16]. Since these compounds also induce cell cycle arrest and differentiation, HDACi are considered as good candidates for targeted therapies. Many of the anticancer effects of SAHA are known to be mediated through transcriptional induction of p21 gene and elevating its protein levels. It has been demonstrated that SAHA induced p21 promoter activity is primarily through two Sp1 sites located at 782 and 769 relative to the transcription start site of the gene, in a p53-independent manner [17].

Two Sp1 and Sp3 are the major transcription factors that are known to bind to the Sp1 site of the p21 promoter and induce gene expression, however, it was reported earlier that SAHA did not alter their DNA binding activities. In addition, Sp3 was reported to produce suppressive effects in the absence of SAHA treatment. Since SAHA mediated induction of p21 is completely independent of p53, it was suspected to be influenced by histone acetylation status. The acetylation-dependent mechanism is accounted for the induction of p21 in cells that lack functional p53 also. It has been reported in the literature that the elevated levels of acetylated histones (H2 and H3) and the activation of intracellular signals are responsible for the transcription of p21 gene and consequent cell cycle arrest and cell death that are typically observed when HDAC inhibitors (HDACi) are used. Thus, our new findings in this study with SAHA has offered some new insights into the cell cycle arrest and cytotoxic effects that can be mediated through HDAC inhibition.

As it has been reported in many experimental models, RG7388 was expected to bind to MDM2 and prevent its interaction with p53 and cause p21 elevation. In LNCaP cells RG7388 produced effects that are very consistent with this speculation through its specific binding with MDM2. The high affinity binding of RG788 with MDM2 is very well characterized through molecular modeling approach as well as in vitro screening methods. The X-ray crystal structures of the N-terminal domain of MDM2 that complexes with the N-terminal of p53 has revealed that, the binding site of p53 in MDM2 is formed by 14 residues: Leu54, Leu57, Ile61, Met62, Tyr67, Gln72, Val75, Phe86, Phe91, Val93, His96, Ile99, Tyr100, and Ile101. MDM2 has a deep hydrophobic cleft on which the p53 protein, after adopting α-helical conformation, interacts through three hydrophobic residues: Phe19, Trp23 and Leu26 that are buried deeply in the MDM2 cleft. To disrupt this interaction MDM2 inhibitors must mimic primarily these three hydrophobic interactions.

Structurally, modelling and simulation studies have revealed that, 4-chlorophenyl and neopentyl group of RG7388 occupy the Trp23 and Phe19 pockets, while its 3-chlorophenyl group occupies the Leu26 pocket and partakes in an additional π-π interaction with the His96 residue. In addition, the pyrrolidine Cα carbonyl was shown to form a hydrogen bond with NH of His96 [18]. Although, RG7388 and some of the other MDM2 inhibitors such as Nutlin-3 have a shared some common mechanism of action, RG7388 has
superior potency and specificity for MDM2 compared to other MDM2 inhibitors, which is probably responsible for the cell cycle arrest and the cell death effects observed. From our studies it is very clear that in LNCaP prostate cancer cells SAHA and RG7388 produce p21 elevation through two different pathways. As a result, the outcomes of the treatment are distinct and deserves further exploration before these two drugs are used to complement each other’s mechanisms.

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