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Nanoparticle formulations of histone deacetylase inhibitors for effective chemoradiotherapy in solid tumors

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Histone deacetylase inhibitors (HDACIs) represent a class of promising agents that can improve radiotherapy in cancer treatment. However, the full therapeutic potential of HDACIs as radiosensitizers has been restricted by limited efficacy in solid malignancies. In this study, we report the development of nanoparticle (NP) formulations of HDACIs that overcome these limitations, illustrating their utility to improve the therapeutic ratio of the clinically established first generation HDACI vorinostat and a novel second generation HDACI quisinostat. We demonstrate that NP HDACIs are potent radiosensitizers *in vitro* and are more effective as radiosensitizers than small molecule HDACIs *in vivo* using mouse xenograft models of colorectal and prostate carcinomas. We found that NP HDACIs enhance the response of tumor cells to radiation through the prolongation of γ-H2AX foci. Our work illustrates an effective method for improving cancer radiotherapy treatment.

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1. Introduction

Histone deacetylases (HDACs) are enzymes involved in the regulation of gene expression and chromatin modification [1]. Aberrant activity of HDACs has been implicated in cancer development. Consequently, the inhibition of HDACs has emerged as a promising strategy to reverse aberrant epigenetic states associated with cancer [2]. There has been extensive development of histone deacetylase inhibitors (HDACIs) as a new class of therapeutics for both solid tumors and hematologic malignancies. Unfortunately, these efforts have only resulted in approval of HDACIs (vorinostat (suberoylanilide hydroxamic acid, SAHA) and romidepsin (depsipeptide)) for the treatment of cutaneous T-cell lymphoma [3,4]. In the clinic, HDACIs have not significantly improved outcomes in solid malignancies compared with current standard therapies [2]. One of the potential clinical applications of HDACIs is to improve radiotherapy treatment, a treatment that more than 60% of all cancer patients will receive [5]. Preclinical studies have indicated that a number of HDAC inhibitors are effective radiosensitizers [6], agents that sensitize tumor cells to radiotherapy, in a variety of solid malignancies such as colorectal cancer [7] and prostate cancer cells [8]. However, the radiosensitization effects have been associated with only mild improvements in efficacy. Given the promise of HDACIs as radiosensitizers, the identification of strategies to improve their therapeutic ratio is needed.

The specific mechanisms by which HDACIs induce radiosensitization remains unresolved, but may be due in part to the prevention of the DNA double strand (DBS) repair, the principal mechanism of action of radiotherapy, leading to subsequent tumor cell death [6,9]. HDACIs have been shown to prolong the formation of phosphorylated histone H2AX (γ-H2AX), a marker of DSBs, following radiation [9–11]. HDACIs may promote the stabilization of DNA DSBs through a variety of mechanisms including the downregulation of specific DNA repair molecules such as Ku70, Ku86, Rad50 and Rad51 [6,12]. HDAC inhibition may also lead to

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hyperacetylation of histones, leading to a more relaxed chromatin state [1]. This may enhance exposure of DNA to radiation-induced damage.

It has been postulated that the efficacy of established first-regeneration HDACIs were limited in solid tumor indications due to their suboptimal potency for specific HDAC enzymes and transient induction of histone acetylation in tumor tissue [13]. In agreement with this notion, it has been shown that prolonged exposure of HDACI vorinostat is necessary for tumor growth inhibition. Furthermore, vorinostat's inhibitory activity is rapidly reversible upon removal of the drug [14]. This may explain the limited efficacy of vorinostat in combination with radiotherapy in solid malignancies. Thus, more potent second-generation HDACIs, such as quisinostat (JNJ-26481585) have been developed with the goal to prolong pharmacodynamic response and to increase efficacy [13]. Quisinostat have been shown to exert antiproliferative activity against a wide panel of cancer cell lines at nanomolar concentrations. The potent and prolonged activity of quisinostat is found to translate into higher *in vivo* potency in preclinical colorectal cancer tumor models than vorinostat. However, more potent HDACIs can also be associated with increased toxicity to normal tissues [15].

Therefore, there are two key limitations in the current use of HDACIs as radiosensitizers. First, clinically established HDACIs may be inefficient at sustaining inhibition of DSB repair, leading to limited efficacy in improving radiotherapy. Second, more potent HDACIs may sensitize both tumor and normal cells to the effects of radiotherapy, leading to increased toxicity. Thus, there is strong interest in the development of novel strategies to further improve their therapeutic ratio in chemoradiotherapy. One approach is to utilize nanoparticle (NP) drug delivery vehicles. NPs preferentially accumulate in tumors and have low distribution in normal tissue [16,17]. They can also release HDACIs in a slow and controlled fashion to further increase synergy with radiotherapy (Fig. 1b). We hypothesized that NP formulations of HDACIs will lead to higher therapeutic ratio when combined with radiotherapy than small molecule HDACIs. In this study, we engineered biodegradable and biocompatible NP formulations of first generation HDACI vorinostat and second generation HDACI quisinostat. These NP HDACIs were evaluated as radiosensitizers *in vitro* using two prostate and three colorectal cancer cell lines. The *in vitro* data was further validated *in vivo* using mouse xenograft models of prostate and colorectal cancers.

2. Materials and methods

2.1. Materials

Vorinostat was purchased from Biotang Inc. (Boston, MA, USA). Quisinostat was obtained from Active Biochem (Maplewood, NJ, USA). Poly (D,L-lactide-coglycolide) (PLGA) with a 85:15 monomer ratio, ester terminated, and viscosity of 0.55–0.75 dL/g was purchased from Durect Corporation (Pelham, AL, USA). PLGA with a 50:50 monomer ratio, ester terminated, and viscosity of 0.72–0.92 dl/g was purchased from Durect Corporation (Pelham, AL). Soybean lecithin consisting of 90–95% (w/w) phosphatidylcholine was obtained from MP Biomedicals (Solon, OH, USA). DSPE-PEG2000-COOH [1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy (polyethylene glycol) 2000] was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Characterization of nanoparticle vorinostat and nanoparticle quisinostat

NP vorinostat and NP quisinostat size (diameter, nm) and surface charge (ζ-potential, mV) were characterized using a Zetasizer Nano Z dynamic light scattering detector (Malvern Instruments, Westborough, MA, USA). Transmission electron microscopy (TEM) images of NP vorinostat and NP quisinostat were obtained at the Microscopy Services Laboratory Core Facility at the UNC School of Medicine.

2.3. Synthesis and characterization of nanoparticle vorinostat and nanoparticle quisinostat

PLGA-lecithin-PEG core–shell NPs were synthesized from PLGA, soybean lecithin, and DSPE-PEG-COOH using a modified nanoprecipitation technique [18]. Vorinostat and quisinostat was dissolved at a dosage of 10% (w/w) of the polymer into the PLGA/acetonitrile solution before nanoprecipitation. The NP solution was washed twice using an Amicon Ultra-4 centrifugal filter (Millipore, MA, USA) with a molecular weight cutoff of 30 kDa and then resuspended in PBS to obtain the final concentration. NP vorinostat and NP quisinostat size (diameter, nm) and surface charge (ζ-potential, mV) were characterized using a Zetasizer Nano Z dynamic light scattering detector (Malvern Instruments Ltd, Worcestershire, UK).

2.4. Nanoparticle vorinostat and nanoparticle quisinostat release

To measure the release profile of vorinostat from NP vorinostat and quisinostat from NP quisinostat, 400 μL of NP vorinostat or NP quisinostat solution at a concentration of 1 mg/mL was aliquot equally into Slide-A-Lyzer MINI dialysis microtubes with a molecular weight cutoff of 2 kDa (Pierce, Rockford, IL, USA) and subjected to dialysis against 4 L of phosphate-buffered saline (PBS) with gentle stirring at 37 °C. PBS was changed periodically during the dialysis process. At the indicated times, 0.1 mL of solution from three microtubes was removed and mixed with an equal volume of acetonitrile to dissolve the NPs. Vorinostat and quisinostat content from their respective NPs were quantitatively analyzed using an Agilent 1100 HPLC (Palo Alto, CA, USA) equipped with a C18 chromolith flash column (Merck KGaA Darmstadt, Germany). Vorinostat absorbance was measured by a UV–VIS detector at 228 nm and in 0.25 mL/min gradient (from 0:100 to 100:0) of acetonitrile/water. Quisinostat absorbance was measured by a UV–VIS detector at 228 nm and in 0.25 mL/min gradient (from 0:100 to 100:0) of methanol: water with 0.1% TFA.

2.5. Cell culture

DU145, PC3, HCT116, and SW620 cells were acquired from the Tissue Culture Facility at the Lineberger Comprehensive Cancer Center at UNC. DU145 cells were cultured in EMEM supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA, USA), nonessential amino acids (Mediatech), and penicillin/streptomycin (Mediatech), and sodium pyruvate (Gibco). PC3 cells were cultured in DMEM/F12 (Gibco) supplemented with 10% FBS and penicillin/streptomycin (Mediatech). HCT116 cells was cultured in McCoy's 5A with L-glutamine (Mediatech) supplemented with 10% FBS and penicillin/streptomycin (Mediatech). SW620 cells was cultured in DMEM-H (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Mediatech) and penicillin/streptomycin (Mediatech). SW837 (ATCC® CCL-235™) (ATCC, Manassas, VA, USA) cells were maintained in DMEM-H (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Mediatech) and penicillin/streptomycin (Mediatech).

2.6. Clonogenic survival assay

Cells were treated with 1 μM of vorinostat or 1 μM quisinostat either without NPs or encapsulated in NPs for 24 h. Cells were washed 2 times with phosphate buffer saline (PBS) after incubation. Cells were then seeded at various densities ranging from 100 to 50,000 cells in 4 mL of culture medium in 50 mL flasks following treatment. The cells were then irradiated at 0, 2, 4, 6 or 8 Gy. Radiotherapy was given using a Precision X-RAD 320 (Precision X-Ray, Inc., North Branford, CT) machine operating at 320 kvp and 12.5 mA. The dose rate at a source-subject distance of 50 cm was 2.07 Gy/min. The cells were incubated for 10 days following irradiation. After 10 days, the cells were fixed in 1:1 acetone/methanol and were stained with trypan blue. Colonies with over 50 cells were counted. The relative survival fraction was calculated by dividing the number of colonies of irradiated cells by the number of cells plated, with correction for the plating efficiency. The average plating efficiency (%) for PC3, DU145, HCT116, SW620, and SW837 cells were 52, 40, 66, 66, and 29 respectively. Survival fractions significantly lower than 0.001 were excluded from analysis.

2.7. Immunofluorescent staining for γ-H2AX

1×10^5 PC3 cells were grown in a 24-well plate and treated with 1 μM small molecule vorinostat equivalent of NP vorinostat or vorinostat. Cells were incubated for 24 h and then washed in PBS thrice and incubated with fresh medium. The cells were then treated with 2 Gy using a Precision XRAD 320. At specified times, medium was aspirated and cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Paraformaldehyde was aspirated and the cells were then washed in PBS thrice, permeabilized with 0.5% Triton X-100 followed by PBS wash thrice. Cells were then blocked with 5% bovine serum albumin in PBS for 1 h, following which mouse monoclonal anti-γ-H2AX antibody (Millipore) was added at a dilution of 1:200 in 1% bovine serum albumin in PBS and incubated for 1 h at room temperature. Cells were then washed thrice in PBS before incubating in the dark with donkey anti-mouse Alexa Fluor 594 (Invitrogen) at a dilution of 1:1000 in 1% bovine serum albumin in PBS for 1 h. The secondary antibody solution was then aspirated and the cells were washed three times in PBS. Cells were then examined using a Leica confocal microscope.

2.8. Tumor efficacy

PC3 or SW620 cells (1×10^6 cells in 200uL 1:1 RPMI-1640 and matrigel) were injected s.c. into the left flank of 6–8 week-old male Nu/Nu mice to develop xenograft tumors. Ten days after inoculation, the mice were randomly distributed into different groups for subsequent treatment. Saline, vorinostat, NP vorinostat, was tail

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