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Histone Deacetylase Inhibitor AR-42 Enhances E7-Specific CD8+ T Cell-Mediated Antitumor Immunity Induced by Therapeutic HPV DNA Vaccination

Sung Yong Lee^{1,5}, Zhuomin Huang^{1,6}, Tae Heung Kang¹, Ruey-Shyang Soong^{1,7}, Jayne Knoff¹, Ellen Axenfeld¹, Chenguang Wang⁸, Ronald D. Alvarez⁹, Ching-Shih Chen¹⁰, Chien-Fu Hung^{1,4}, and T.-C. Wu^{1,2,3,4}

¹Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

²Department of Obstetrics and Gynecology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

³Department of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

⁴Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

⁵Department of Internal Medicine, Korea University Medical Center, Seoul, South Korea

⁶Department of Gynecology, Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University, Shenzhen, China

⁷Department of General Surgery, Chang Gung Memorial Hospital at Keelung, Taiwan

⁸Department of Biostatistics and Bioinformatics, Sidney Kimmel Cancer Comprehensive Cancer Center Johns Hopkins University, Baltimore, MD, USA

⁹Department of Obstetrics and Gynecology, University of Alabama at Birmingham

¹⁰Division of Medical Chemistry, College of Pharmacy and Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA

Abstract

We have previously created a potent DNA vaccine encoding calreticulin linked to the HPV oncogenic protein E7 (CRT/E7). While treatment of the CRT/E7 DNA vaccine generates significant tumor-specific immune responses in vaccinated mice, the potency of the DNA vaccine could potentially be improved by co-administration of a histone deacetylase inhibitor (HDACi) as HDACi have been shown to increase the expression of MHC class I and II molecules. Thus, we aimed to determine whether co-administration of a novel HDACi, AR-42, with therapeutic HPV DNA vaccines could improve activation of HPV antigen-specific CD8+ T cells resulting in potent therapeutic antitumor effects. To do so, HPV-16 E7-expressing murine TC-1 tumor-bearing mice were treated orally with AR-42 and/or CRT/E7 DNA vaccine via gene gun. Mice were monitored for E7-specific CD8+ T cell immune responses and antitumor effects. TC-1 tumor-bearing mice treated with AR-42 and CRT/E7 DNA vaccine experienced longer survival, decreased tumor growth, and enhanced E7-specific immune response compared to mice treated with AR-42 or CRT/E7 DNA vaccine alone. Additionally, treatment of TC-1 cells with AR-42 increased surface expression of MHC class I molecules and increased the susceptibility of tumor cells to the

Correspondence to: Dr. T.-C. Wu, Department of Pathology, The Johns Hopkins School of Medicine, Cancer Research Building II, Room 309, 1550 Orleans Street, Baltimore, MD 21231, USA. wutc@jhmi.edu.

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cytotoxicity of E7-specific T cells. This study indicates the ability of AR-42 to significantly enhance the potency of the CRT/E7 DNA vaccine by improving tumor-specific immune responses and antitumor effects. Both AR-42 and CRT/E7 DNA vaccine have been used in independent clinical trials and the current study serves as foundation for future clinical trials combining both treatments in cervical cancer therapy.

Keywords

Cancer vaccine; human papillomavirus; cervical cancer; histone deacetylase inhibitor

Introduction

Cervical cancer is one of the leading causes of death in women worldwide. A recent estimate indicates that there are approximately 529,800 cases of cervical cancer and 275,100 mortalities annually [1]. While therapeutic techniques such as chemotherapy, radiotherapy, and surgery are currently used to treat cervical cancer, five-year survival rates for advanced cervical cancer remain low [2]. These alarming statistics validate the demand for novel therapeutic techniques to battle cervical cancer.

DNA vaccines against human papillomavirus (HPV)-associated cervical cancer represent a unique opportunity for cancer vaccine development. It is now clear that two HPV-encoded oncogenic proteins, E6 and E7, are responsible for the malignant transformation of cervical cancer and are constantly expressed in HPV-associated cancer cells. Therefore, HPV E6 and E7 represent true tumor-specific antigens and are ideal targets for developing cancer immunotherapy to control cervical cancer. Furthermore, because E6 and E7 represent foreign proteins, their use in therapeutic vaccines does not have issues related to immune tolerance. In the past, we have created a potent DNA vaccine encoding HPV-16 E7 linked to calreticulin (CRT/E7), which enhances antigen processing associated with MHC class I antigen presentation [3, 4]. Vaccination with the chimeric CRT/E7 DNA vaccine generates a significant E7-specific CD8⁺ T cell mediated immune response as well as a strong antitumor effect against an E7-expressing murine tumor model, TC-1, demonstrating the successful application of the HPV oncoprotein in generating a tumor-specific immune response [5]. The encouraging results from preclinical models have lead to several ongoing clinical trials using clinical grade CRT/E7 DNA vaccine [6, 7]. Although the CRT/E7 DNA vaccine is a promising therapeutic cancer vaccine, DNA vaccine efficacy tends to be limited by low expression of the DNA-encoded protein. To resolve this issue, treatments using multiple therapeutic modalities may be used.

Attractive supplementary therapeutic techniques may target and inhibit biological functions that promote tumor development. One such target is histone deacetylase (HDAC), an enzyme responsible for the deacetylation of histones, whose inhibition causes hyperacetylation of core histones, leading to the expression of suppressed genes and regulation of abnormal cell growth [8]. Currently, eighteen types of HDACi are known, which fall into three different classes based on their homology to yeast [9]. In addition to promoting the expression of suppressed genes and regulating abnormal cell growth, HDACi may contribute to cancer control by histone-independent mechanisms by modifying the acetylation of non-histone proteins such as p53 [10] and heat shock protein-90 [11]. These mechanisms produce antitumor effects including induced differentiation, cell growth arrest, and an increase in apoptosis [12, 13].

AR-42, the novel HDACi used in the current study, is a phenylbutyrate-based, hydroxamate-tethered short-chain fatty acid [14]. The efficacy of AR-42 proves to be dependent on

contact with the zinc cation in the active site of the HDAC enzyme. The presence of a Zn^{2+} -chelating motif, hydroxamic acid, in AR-42 permits its contact with the HDAC active site [15]. Furthermore, AR-42 has a half maximal inhibitory concentration in the nanomolar range and a consequently high inhibition efficacy [15]. As such, the optimized structure of AR-42 causes it to be a potent inhibitor of HDACs. AR-42 is similar in structure to suberoylanilide hydroxamic acid (SAHA), also known as Vorinostat, which is an FDA approved HDACi used in the treatment of cutaneous T-cell lymphoma [16]. Nevertheless, AR-42 was shown to have greater potency and antitumor effects against multiple myeloma [17], lung cancer [18], and prostate cancer [14], in cell culture systems, as well as in hepatocellular carcinoma [19] than clinically available SAHA. Thus, AR-42 is an ideal candidate to supplement anticancer therapies.

HDACi represent a great opportunity for boosting the potency of DNA vaccines. Previous studies have found that HDACi enhance the antitumor effects of DNA vaccines in preclinical models by enhancing the expression of the protein/antigen encoded by the DNA vaccine [20, 21]. Furthermore, it has been shown that cells treated with HDACi can lead to the upregulation of MHC class I and II molecules [22]. This suggests that tumor cells may become more susceptible to tumor-specific CD8⁺ T cell immunotherapy following treatment with an HDACi. Taken together, HDACi may enhance DNA vaccine potency through improved expression of the DNA transfect cell as well as increase the susceptibility of tumor cells to killing by tumor-specific CD8⁺ T cells generated by the DNA vaccine.

In the current study, we intended to demonstrate whether AR-42 could further enhance CRT/E7 DNA vaccine potency. We tested the potency of the synergistic effect of AR-42 administered orally and CRT/E7 DNA vaccine administered intradermally via gene gun in TC-1 tumor-bearing mice. Furthermore, we examined the ability of AR-42 to enhance the DNA vaccine compared to the two clinically available HDACi, SAHA and romidepsin. We showed that treatment with AR-42 could significantly increase the E7-specific CD8⁺ T cell immune responses and the antitumor effects generated by CRT/E7 DNA vaccination. Because CRT/E7 DNA vaccine and AR-42 have been separately used clinically, our data serves as an important basis for future clinical translation.

Materials and methods

Mice

Five- to eight-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All of the animal procedures were done according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Cell lines

Primary C57BL/6 mouse lung epithelial cells were cotransformed with HPV-16 E6 and E7 and an activated *ras* oncogene, as previously described [23], to generate the TC-1 cells. The generation and characteristics of the MHC class I down-regulated tumor cell line TC-1 P3 (A15) have been previously described [24] as well as the E7-specific CD8⁺ T cell line [25]. DC cell line DC2.4 was a gift from Dr. K.L. Rock [26]. TC-1, TC-1 P3 (A15) tumor cells and DC2.4 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids and 2-mercaptoethanol (2-ME, 50 μ M) at 37°C in a 5% CO₂ atmosphere.

DNA constructs

The generation of the DNA vaccine encoding CRT and E7 was described previously [5]. Briefly, for the generation of pcDNA3-CRT, CRT was first amplified by PCR using rabbit CRT cDNA as the template [27] and the primers, 5'-CCGGTCTAGAATGCTGCTCCCTGTGCCGCT-3' and 5'-CCGGGAATTCCAGCTCGTCCTTGGCCTGGC-3'. The amplified product was then cloned into the XbaI/EcoRI sites of pcDNA3 vector (Invitrogen Corp.). For the generation of pcDNA3-CRT/E7, E7 was first amplified with the set of primers, 5'-GGGGAATTCATGGAGATACACCTA-3' and 5'-GGTGGATCCTTGAGAACAGATGG-3', and cloned into the EcoRI/BamHI site of pcDNA3-CRT. For the generation of pcDNA3-GFP, the DNA fragment encoding the GFP was amplified with the primers, 5'-ATCGGATCCATGGTGAGCAAGGGCGAGGAG-3' and 5'-GGGAAGCTTTACTTGTACAGCTCGTCCATG-3 and then cloned into the BamHI/HindIII cloning sites of pcDNA3 [28]. The accuracy of the DNA constructs was confirmed by DNA sequencing. The CRT/E7 DNA vaccine has previously been tested against a mock-DNA vaccine control [5].

DNA vaccination by gene gun

DNA-coated gold particles were prepared, and gene gun particle-mediated DNA vaccination was performed according to a previously described protocol [29]. The coated gold particles (1 µg DNA/bullet) were delivered to the shaved abdominal region of the mice with a helium-driven gene gun (Bio-Rad Laboratories, Inc.) at a discharge pressure of 400 lb/in². C57BL/6 mice (five per group) were immunized with 2 µg of the CRT/E7 or CRTE6E7L2 DNA vaccine and received four boosters with the same dose at 3-day intervals. Splenocytes were harvested on day 21 after the tumor challenge.

In vivo tumor treatment experiment

For *in vivo* tumor treatment, 1×10^5 TC-1 tumor cells per mouse were subcutaneously injected into the left flank area of 5- to 8-wk-old C57BL/6 mice on day 0. After 5 days, the mice were divided into four groups (five per group), each receiving a different treatment regimen: group 1 received no treatment after the TC-1 tumor challenge, group 2 was treated daily with AR-42 by oral gavage (30 mg/kg body weight) for 10 days, group 3 was immunized with the DNA vaccine schedule as described above, group 4 was both immunized and treated with AR-42. AR-42 dosage was based on previous reports [14, 30-32]. Group 5 was immunized and treated with romidepsin intraperitoneally (ip) twice a week at 1.5 mg/kg per dose, similarly to what has been described previously [33-35]. Group 6 was immunized and treated with SAHA (IC₅₀ = 26 nM) per oral at 30 mg/kg per dose, similarly to what has been described previously [36-38]. Mice were monitored twice a week by inspection and palpation. AR-42 (NSC 736012) was a gift from Arno Therapeutics, and it is a novel hydroxamate-tethered phenylbutyrate derivative that has received IND approval from the FDA [14]. AR-42 was prepared as suspensions in vehicle (0.5% methylcellulose, 0.1% Tween 80, in sterile water). CRTE6E7L2 DNA vaccine, which has been described previously [39], was administered via gene gun in the amount of 2 µg/mouse beginning on day 5 at 3 day intervals for a total of 4 vaccinations.

Tetramer analysis of E7-specific CD8⁺ T cells

Peripheral blood samples (collected from the tail vein on day 21 after the tumor challenge) were depleted of red blood cells (RBC) by ACK lysis and were stained with PE-conjugated HPV16 H-2D^b-RAHYNIVTF tetramer reagent (NCI). Tetramer staining was combined with surface staining using FITC-conjugated anti-CD8 (BD PharMingen). Cells were analyzed on a BD FACSCalibur collecting 30,000 events.

Intracellular cytokine staining and flow cytometry analysis

Pooled splenocytes from tumor-bearing mice that were treated with the various treatment regimens were harvested 7 days after the last treatment and incubated for 24 hours with 1 µg/mL of E7 peptide containing an MHC class I epitope (amino acids 49-57, RAHYNIVTF) [40] in the presence of GolgiPlug (BD Pharmingen). The stimulated splenocytes were then washed once with FACScan buffer and stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8a (clone 53.6.7). Cells were subjected to intracellular cytokine staining using the Cytotfix/Cytoperm kit according to the manufacture's instruction (BD Pharmingen). Intracellular IFN- γ was stained with FITC-conjugated rat anti-mouse IFN- γ . All antibodies were purchased from BD Pharmingen. Flow cytometry analysis was done using FACSCalibur with CellQuest software (BD Bioscience).

MHC class I surface expression—In order to determine the in vitro expression of MHC class I molecules, A15 cells were incubated in a 6-well plate (2×10^5 cells per well) for 12 hours, and then the media were replaced with PBS or AR-42 (0.5 µM) and incubated for 24 hours. A15 cells were trypsinized, washed, and stained with florescent-conjugated H-2K^b, 2D^b antibodies (BD pPharmingen, CA, USA). The expression of MHC class I molecules on A15 cells was analyzed on a FACSCalibur cytometer.

Characterization of GFP-expression in DNA transfected dendritic cells

DC2.4 cells [26] were transfected with pcDNA3-GFP using Lipofectamine™ 2000 (Invitrogen) in a master tissue culture plate. 24 hours later, pcDNA3-GFP transfected DC2.4 cells were seeded into a 24-well round-bottomed plate (1×10^5 per well), and then treated with 2 mL of fresh medium containing 0, 0.1, 0.5, or 2.5 µM AR-42. The cells were incubated in 5% CO₂ for 24 hours at 37°C. FACSCalibur flow cytometry was used to determine the percentage of GFP-positive dendritic cells (DCs) in each DC population.

In vitro cytotoxic T lymphocyte assays after AR-42 treatment

For the characterization of the E7 peptide presentation through the MHC class I molecules on TC-1 tumor cells treated with AR-42, intracellular cytokine staining for IFN- γ and flow cytometric analysis were performed to determine the activation of E7-specific CD8⁺ T cell line [25] induced by TC-1 cells treated with or without 2.5 µM AR-42. TC-1 cells were incubated with E7-specific CD8⁺ T cells at 1:1 ratio for 15 hours. After incubation, cells were stained for CD8 and IFN- γ and analyzed by flow cytometry analysis. Luciferase-expressing TC-1 cells [41] in medium were seeded into 24-well round-bottomed plates (5×10^4 cells per well). The following day, the medium was replaced with 2 mL of fresh medium containing 0.5 µM AR-42 and incubated in a 5% CO₂ atmosphere for 24 hours at 37°C. **The media was then replaced, removing AR-42**, and 1×10^6 E7-specific cytotoxic T lymphocytes from the T cell line described previously [25] were added to each well to serve as effector cells. TC-1 cells expressing luciferase were used as target cells. After incubation, D-luciferin (potassium salt; Xenogen Corp.) was added to each well at a concentration of 15 µg/mL in medium. After 10 minutes the cells were imaged with the Xenogen IVIS 200 system.

Statistical analysis

Exploratory statistical analyses based on data from multiple independent experiments **or assays** were conducted. Summary statistics such as mean and SD were reported. Comparisons between groups were made using Student's t-test. Survival rates were estimated using Kaplan-Meier method. Survival functions among the groups were compared using log-rank test. Statistical analysis was performed using SPSS, version 17.0.

Results

AR-42 administered in combination with DNA vaccine results in reduced tumor growth and prolonged survival of TC-1 tumor-bearing mice

Previous studies have shown that tumor-bearing mice given subcutaneous (sc) administration of CRT/E7 DNA vaccine via gene gun generate improved E7-specific CD8⁺ T-cell immune responses and have reduced tumor growth rates [5]. In this study, we examined whether AR-42 administered via oral gavage could enhance these effects. Mice were sc challenged with E7-expressing TC-1 tumor cells and began treatment five days later with AR-42 (daily for 10 days) and/or the CRT/E7 DNA vaccine (4 times with 3-day intervals) as outlined in Figure 1A. As shown in Figure 1B, the tumors of the mice receiving both the DNA vaccine and AR-42 grew at a consistently reduced rate compared to the mice in the other treatment groups. Furthermore, this same group of mice survived significantly longer than the other groups of mice (Fig. 1C). This data suggests that AR-42 is able to enhance the antitumor effects generated by the CRT/E7 DNA vaccine.

TC-1 tumor-bearing mice receiving both AR-42 and CRT/E7 DNA generated greater numbers of E7-specific CD8⁺ T cells

On day 21 after tumor challenge, peripheral blood samples were collected from the tail vein of tumor-bearing mice in each treatment group, stained to identify E7-specific CD8⁺ T cells, and analyzed by flow cytometry. Mice receiving both AR-42 and CRT/E7 DNA vaccine had more than twice the percentage of E7-specific CD8⁺ T cells among PBMCs than the group receiving the DNA vaccine alone (17.78% vs 7.89%) and more than 20 times the group receiving AR-42 alone (17.78% vs 0.83%) (Fig. 2A). Additionally, splenocytes from the treated tumor-bearing mice were harvested on day 21 and incubated with E7 peptide overnight. Splenocytes were stained for intracellular IFN- γ and then analyzed by flow cytometry. Mice receiving both CRT/E7 DNA vaccine and AR-42 generated significantly more E7-specific, IFN- γ -producing CD8⁺ T cells (Fig. 2B). Collectively, these data show that AR-42 treatment enhances the generation of E7-specific CD8⁺ T cells generated by CRT/E7 DNA vaccine.

TC-1 tumor cells treated with AR-42 show increased expression levels MHC class I on the cell surface

To determine whether administration of AR-42 could increase cell surface expression of MHC class I molecules, we used a TC-1 cell clone, which was previously selected for the down-regulation of MHC, class I (A15 cells) [24]. The A15 cells were incubated with AR-42 and cell surface expression of MHC class I was analyzed by flow cytometry. As shown in Figure 3, A15 cells treated with AR-42 led to increased cell surface expression of MHC class I *in vitro* compared to untreated A15 cells.

AR-42 enhances expression of DNA-encoded proteins in DNA transfected dendritic cells

To understand whether AR-42 effects the expression level of the protein encoded by the DNA construct, which may play an important role in increasing antigen presentation in DCs, we transfected DC2.4 cells with mammalian DNA expression vector encoding a marker protein, GFP (pcDNA-GFP). One day later, DNA transfected DC2.4 cells were incubated with AR-42 at different concentrations for 24 hours. The level of GFP expression in each group was measured using flow cytometry analysis. As shown in Figure 4, GFP expression levels increased with increasing AR-42 concentration, suggesting that treatment with AR-42 can lead to increased expression of DNA-encoded proteins.

Treatment of TC-1 cells with AR-42 induces significant *in vitro* cytotoxicity

To demonstrate whether TC-1 tumor cells treated with AR-42 can lead to increased HPV E7-specific cytotoxicity, we incubated cells from an HPV-16 E7-specific CD8⁺ T cell line with TC-1 tumor cells that had been treated with or without AR-42. As shown in Figure 5A, AR-42-treated TC-1 cells induced the activation of significantly more E7-specific CD8⁺ T cells compared to untreated TC-1 cells. This data suggests that TC-1 cells treated with AR-42 can lead to enhanced presentation of E7 through the MHC class I pathway. However, the further characterization of AR-42 treated TC-1 tumors did not reveal upregulation of MHC class I expression (data not shown). This data implies that other potential mechanisms, such as increased E7 expression and/or enhanced E7 antigen processing may account for the observed phenomenon.

In order to determine if the enhanced presentation of E7 through the MHC class I pathway on TC-1 tumor cells could result in increased susceptibility of TC-1 tumor cells to killing by E7-specific CD8⁺ T cells, we incubated E7-specific CD8⁺ T cells with luciferase-expressing TC-1 tumor cells with or without pretreatment with AR-42. Untreated luciferase-expressing TC-1 tumor cells were included as a control. As shown in Figure 5B, luciferase-expressing TC-1 cells treated with AR-42 and incubated with E7-specific CD8⁺ T cells had a much greater decrease in luminescence, indicating greater cytotoxicity, than the luciferase-expressing TC-1 cells treated with either AR-42 or incubated with E7-specific CD8⁺ T cells alone. Taken together, this data suggests that TC-1 tumor cells treated with AR-42 are able to enhance MHC class I presentation, resulting in their increased susceptibility to killing by E7-specific CD8⁺ T cells.

AR-42 treatment in combination with CRT/E7 DNA vaccine generates a greater antitumor effect compared to various clinically available HDACi

Because there are two commercially available HDACi, romidepsin and SAHA, it is important to compare AR-42 with these HDACi for their ability to enhance the therapeutic antitumor effect generated by a therapeutic HPV DNA vaccine. Mice were sc challenged with TC-1 tumor cells and then were treated with different HDACi five days later, either romidepsin, SAHA or AR-42, as indicated in Figure 6A. Concurrent with HDACi administration, mice were treated with CRT/E7 DNA vaccine. As shown in Figure 6B, treatment with CRT/E7 DNA vaccine and AR-42 generated the best therapeutic antitumor effect in TC-1 tumor-bearing mice, as measured by tumor volume, among all treatment groups. This data indicates that AR-42 is the ideal HDACi to be used in combination with a therapeutic HPV DNA vaccine in order to generate a potent therapeutic antitumor effect against E6/E7-expressing tumors.

We observed similar results using the same treatment schedule with a different therapeutic HPV DNA vaccine encoding calreticulin linked to HPV-16 E6, E7 and L2 proteins (CRTE6E7L2) administered by gene gun (Supplemental Figure 1A). CRTE6E7L2 DNA vaccine has been shown to generate a potent therapeutic antitumor effect against HPV-16 E6/E7-expressing tumors [39]. Again, AR-42 in combination with CRTE6E7L2 DNA vaccine generated the most potent antitumor effect (Supplemental Figure 1B). Thus, this data has significant implications on the future clinical translation of AR-42 as a potent anti-cancer drug.

Discussion

We demonstrated the ability of AR-42, an innovative HDACi, to enhance the potency of a DNA vaccine encoding CRT linked to the HPV oncogenic protein E7. Mice treated with AR-42 and CRT/E7 DNA vaccine had reduced tumor growth and improved survival

compared to mice treated with AR-42 alone or vaccine alone. Furthermore, combination treatment generated increased E7-specific CD8⁺ T cells and increased T-cell mediated cytotoxicity compared to treatment with AR-42 or CRT/E7 DNA vaccine alone. More importantly, AR-42 was shown to be the ideal HDACi, compared to other commercially available HDACi, to be used in conjunction with a therapeutic HPV DNA vaccine to generate potent therapeutic antitumor effects. Taken together, AR-42 represents a particularly promising HDACi to be used in combination with clinical grade therapeutic HPV vaccines for the control of HPV-associated malignancies.

In the current study, treatment of TC-1 tumor cells with AR-42 alone appears to have more obvious cytotoxic effects *in vitro* (Figure 5B) compared to that *in vivo* (Figure 1B). This observation may be explained by multiple factors. For example, multiple aspects of the tumor microenvironment may inhibit the antitumor effects of treatment with AR-42 *in vivo*. In addition, the dose of AR-42 used *in vivo* may not be comparable to the dose used *in vitro*. These factors may account for the observed difference in the antitumor effect generated *in vitro* versus *in vivo*.

In the current study we have observed that AR-42 most effectively enhances the potency of therapeutic HPV DNA vaccines compared to SAHA and romidepsin. However, it is important to note that there are some limitations to comparing these HDACi, given that they were administered by different routes and regimens. The reported route of administration for romidepsin in a preclinical model is intraperitoneal injection [34, 35], while that of AR-42 [14, 30-32] and SAHA [19] is oral gavage. While the dosages used for each HDACi were based on previous publications [14, 30-38], the optimal dosages and regimens reported previously may not necessarily be applicable in the context of combinatorial treatment with DNA vaccines. Thus, it will be important to further optimize the doses and regimens of these HDACi, especially in studies involving combination treatments.

Encouraging preclinical data serve as an important foundation for future clinical trials for therapeutic HPV-associated cancer vaccines. Multiple HDACi and therapeutic HPV DNA vaccines have been tested independently in clinical trials [6, 42-44]. In addition, the HDACi Vorinostat (SAHA) is FDA approved for the treatment of cutaneous manifestations of advanced primary cutaneous T cell lymphoma [16]. Additionally, a Phase 1 trial of AR-42 treatment for multiple myeloma, chronic lymphocytic leukemia and lymphoma is currently recruiting participants [45]. Thus, in order to identify the best HDACi for future clinical trials, it will be important to perform a head to head comparison for their ability to enhance the therapeutic effect of the HPV DNA vaccine. In addition, it will be important to determine an appropriate dosage and regimen for this treatment.

Several underlying mechanisms may account for the observed enhancement, by AR-42, of HPV antigen-specific CD8⁺ T cells and antitumor effects generated by the CRT/E7 DNA vaccine administered intradermally via gene gun. It has been shown that DNA vaccines administered by gene gun can directly deliver the DNA to DCs in the skin [46]. Furthermore, previous studies have also demonstrated that AR-42 is able to enhance the expression of the protein encoded by the DNA vaccine [21]. As we show in Figure 4, treatment with AR-42 is able to enhance protein expression in DNA transfected DCs. Taken together, the DNA-transfected DCs may become more capable of priming antigen-specific CD8⁺ T cells following treatment with AR-42. Additionally, we observed that tumor cells treated with AR-42 were more susceptible to antigen-specific CD8⁺ T cell-mediated killing (Figure 5). Consequently, treatment of tumor-bearing mice with AR-42 may make DNA-transfected DCs more effective in priming HPV antigen-specific CD8⁺ T cells as well as make tumor cells killed more easily by CD8⁺ T cells, resulting in a potent antitumor effect.

In summary, we found that HDACi, AR-42, could be used in conjunction with therapeutic HPV DNA vaccine to improve the therapeutic effects against HPV-associated cancer. It will be important to further determine the suitable HDACi and therapeutic HPV vaccine as well as the optimal regimen in preparation for clinical applications of this promising cervical cancer therapeutic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *In vivo* tumor treatment experiments

C57BL/6 mice (five per group) were subcutaneously (sc) challenged with 1×10^5 TC-1 cells per mouse on Day 0. Tumor-bearing mice were treated with AR-42 and/or DNA vaccine (DNA encoding CRT/E7) beginning on day 5 as indicated in the time line. AR-42 was given daily at a dose of 30 mg/kg body weight via oral gavage. The DNA vaccine (2 µg/mouse) was given at 3-day intervals via gene gun. A. Schematic representation of the AR-42 and DNA vaccine treatment regimens. B. Graph depicting tumor volumes in the treated TC-1 tumor-bearing mice. Points, mean; bars, SD ($P < 0.01$). C. Kaplan-Meier survival analysis of the treated TC-1 tumor bearing mice ($P < 0.05$).

Figure 2. Characterization of E7-specific CD8+ T cells

A. Peripheral blood samples (collected from the tail vein on day 21) were stained with PE-conjugated HPV16 H-2D^b-RAHYNIVTF tetramer reagent and FITC-conjugated CD8 monoclonal antibody. CD8+ T cells were gated from PBMCs. B. Intracellular cytokine staining followed by flow cytometry analysis to determine the number of E7-specific CD8+ T cells in tumor-bearing mice treated with AR-42 and/or CRT/E7 DNA vaccine. On day 21 splenocytes from the treated tumor-bearing mice were harvested and incubated with E7 peptide overnight. Among complete splenocytes, E7-specific CD8+ T cells were quantified using intracellular staining for IFN- γ followed by flow cytometry analysis (top right quadrant). Representative data shown. Bar graph depicting the numbers of E7-specific IFN- γ -producing CD8+ T cells per 3×10^4 pooled complete splenocytes. Columns, mean; bars, SD (* $P < 0.01$).

Figure 3. Cell surface expression of MHC class I analyzed by flow cytometry

A. Expression levels of unstained control cells are shown as shaded peaks, A15 cells without treatment by the green line, and cells treated with AR-42 by the pink line. H-2K^b expression was assessed by staining with FITC-conjugated anti-H-2K^b mouse monoclonal antibody and H-2D^b expression was assessed by staining with PE-conjugated anti-H-2D^b mouse monoclonal antibody (mAb; BD Pharmingen), followed by analysis on a **FACSCalibur**. B. Bar graph depicts the mean fluorescence intensity (MFI) of H-2K^b or H-2D^b positive cells treated with or without AR-42. Columns, mean; bars, SD (**P*<0.05).

Figure 4. Characterization of the expression of the DNA-encoded protein in transfected DC2.4 cells treated with AR-42

DC2.4 cells were transfected with the expression vector for pcDNA3-GFP. The following day, the pcDNA3-GFP transfected DC2.4 cells were seeded into a 24-well round-bottomed plate (1×10^5 per well) and treated for 24 hours with 0, 0.1, 0.5, and 2.5 μ M AR-42. FACSCalibur flow cytometry was used to determine the percentage of increased GFP-expression in each DC population. Bar graph representing the expression of GFP in DC2.4 populations treated with AR-42. Data shown are the means of two assays performed. Columns, mean; bars, SD (* $P < 0.05$).

Figure 5. Characterization of cytotoxicity

A. Intracellular cytokine staining for IFN- γ and flow cytometric analysis to determine the activation of E7-specific CD8⁺ T cells induced by TC-1 cells treated with or without 2.5 μ M AR-42. TC-1 cells were incubated with E7-specific CD8⁺ T cells at 1:1 ratio for 15 hours. After incubation, cells were stained for CD8 and IFN- γ and analyzed by flow cytometry analysis. Left panel shows representative flow cytometry analysis. Right panel shows a bar graph representing the percentage of IFN- γ positive E7-specific CD8⁺ T-cells per 3 $\times$ 10⁴ CD8 T cells. Data shown are the means of two assays performed. Columns, mean; bars, SD (* P <0.05). B. *In vitro* cytotoxicity assay. Luciferase-expressing TC-1 tumor cells were seeded in 24-well plates (5 $\times$ 10⁴ per well). The following day, the medium was replaced with 2 mL of fresh medium containing 0.5 μ M AR-42. 24 hours later, the media was replaced again, removing AR-42, and 1 $\times$ 10⁶ E7-specific CTLs were added to the wells as indicated. Untreated luciferase-expressing TC-1 cells were included as a control. The degree of CTL-mediated killing of the tumor cells is indicated by the decrease of luminescence activity and measured with the IVIS Luminescence Imaging System Series 200 (bioluminescence signals were acquired for 3 minutes). Top panel shows representative luminescence images of the 24 well plates. Bottom panel shows a bar graph depicting the quantification of luminescence intensity in **untreated tumor cells (control)** and tumor cells treated with AR-42 and/or E7-specific CTLs. Columns, mean; bars, SD (* P <0.05). RLU, relative luciferase unit.

Figure 6. In vivo tumor treatment experiments with CRT/E7 DNA vaccine and various HDACi Groups of C57BL/6 mice (five per group) were sc challenged with 1×10^5 TC-1 tumor cells per mouse on day 0. Tumor-bearing mice were treated with romidepsin (1.5mg/kg, intraperitoneal, twice a week), SAHA (30mg/kg, per oral, IC₅₀=26 nM) or AR-42 (30mg/kg, per oral, IC₅₀=23 nM). CRT/E7 DNA vaccine was administered as indicated in the time line. DNA vaccine was given via gene gun in the amount of 2 μ g/mouse for a total of 4 vaccinations at 3 day intervals. A. Diagrammatic representation of the treatment regimens with CRT/E7 DNA vaccine and various HDACi. B. Line graph depicting the tumor volume in TC-1 tumor-bearing mice treated with the different treatment regimens. Points, mean; bars, SE ($P < 0.01$).