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Class IIb HDAC Inhibition Enhances the Inhibitory Effect of Am80, a Synthetic Retinoid, in Prostate Cancer

Mari Ishigami-Yuasa,a Hisao Ekimoto,b and Hiroyuki Kagechika*a

a Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University (TMDU); 2–3–10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–0062, Japan: and b TMRC Co., Ltd.; 1–12–12 Kitashinjuku, Shinjuku-ku, Tokyo 169–0074, Japan.

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Combination therapy is often an effective strategy to treat cancer. In this study, we examined the growth-inhibitory effects of Am80 (tamibarotene), a specific retinoic acid receptor (RAR) α/β agonist, in combination with a histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA), or a DNA methyl transferase (DNMT) inhibitor, 5-aza-2'-deoxycytidine, on androgen receptor (AR)-positive and AR-negative prostate cancer cell lines (LNCaP and PC-3, respectively). We found that the combination therapy of SAHA and Am80 showed an enhanced growth-inhibitory effect on LNCaP cells. Further studies with various HDAC isotype-selective inhibitors showed that SAHA and KDS170 (a selective class I and II HDAC inhibitor) each increased the RARα protein level in LNCaP cells. Our results indicate that the target of the enhancing effect belongs to the Class IIb HDACs, especially HDAC6. Dual targeting of Class IIb HDAC and RARα may be a candidate therapeutic strategy for prostate cancer.

Key words retinoid; Am80; histone deacetylase (HDAC) inhibitor; prostate cancer; androgen receptor-positive prostate cancer (LNCaP); retinoic acid receptor (RAR) α

INTRODUCTION

More than 10000 clinical trials of combination therapies are currently registered worldwide for the treatment of cancer, infectious diseases, metabolic diseases, cardiovascular diseases, autoimmune diseases, and neurological diseases. To determine the efficacy of combination therapy, it is important not only to evaluate the therapeutic effects when the drugs are used individually as single agents and in combination, but also to compare them with previously reported combination therapies. One potential target for combination therapy is prostate cancer. The current strategies employed for prostate cancer treatment are surgery to remove the tumor, radiation therapy to kill cancer cells or control their proliferation, and hormone therapy to inhibit progression. However, these treatment methods have significant side effects, such as impaired sexual function, weakened bones, diarrhea, nausea, and itching.1–3 Therefore, more effective treatments are needed.

Retinoids have been reported to induce apoptosis in androgen-dependent and independent prostate cancer cells,4,7 and the combination of retinoids with an organic arsenical or a histone deacetylase (HDAC) inhibitor induced apoptosis in several human and rodent prostate carcinoma cell lines in vitro and in vivo.8,9 The reported molecular mechanisms of induction of apoptosis by retinoids in prostate cancer cells include down-regulation of Bcl-2 expression,8,10,11 induction of insulin-like growth factor-binding protein-3 (IGFBP-3),12 and accumulation of tissue transglutaminase in cells.6

Epigenetic modifications of chromatin play an essential role in normal development, growth, and differentiation of cells by transcriptionally silencing specific control regions. Two major epigenetic modifications, histone deacetylation and DNA methylation, contribute to the progression and drug resistance of many cancers.13 Recent studies have shown that HDAC and DNA methyl transferase (DNMT) inhibitors can restore the expression of numerous silenced nuclear receptors (NRs), including estrogen receptor α and retinoic acid receptor (RAR) β.14,15 Therefore, combinations of NR-binding drugs with HDAC and/or DNMT inhibitors may be promising strategy for cancer treatment. Indeed, this has been tried for some cancers,16,17 but little work has been done with prostate cancer.18 Besides, these studies lead to better understanding of the biological action mechanisms of drugs and help in the discovery of new drug targets.

In this study, therefore, we examined the growth-inhibitory effects of a synthetic retinoid, Am80 (tamibarotene), in combination with HDAC and DNMT inhibitors on androgen receptor (AR)-positive prostate cancer (LNCaP) and AR-negative prostate cancer (PC-3) cells. Am80 is a specific RARα/β agonist that exhibits anticancer activity against acute promyelocytic leukemia (APL) cells and was approved for the treatment of intractable and relapsed APL in Japan in 2005.19 It also exhibits antiproliferative effects against various tumor cell lines.20 As a DNMT inhibitor, we chose 5-aza-2'-deoxycytidine (5-AzadCyd), which is a pyrimidine nucleoside analog of cytidine that is incorporated into DNA, where it blocks the action of DNMT; it was approved by the United States Food and Drug Administration (FDA) in 2006 for the treatment of myelodysplastic syndromes.21 As an HDAC inhibitor, we chose suberoylanilide hydroxamic acid (SAHA), which is a pan-HDAC inhibitor that was approved by the FDA, also in 2006, for the treatment of cutaneous T cell lymphoma (CTCL).22 As target cell lines, we used AR-positive prostate cancer cell line LNCaP and AR-negative prostate cancer cell line PC-3, which are considered representative of the early androgen-dependent stage and the advanced androgen-independent stage of the disease, respectively.

We found that the combination therapy of Am80 with
SAHA showed an enhanced growth-inhibitory effect on LNCaP cells, and we examined the mechanism involved. Our results suggest that dual targeting of Class I and II HDAC and RARα might be a candidate therapeutic strategy for prostate cancer.

MATERIALS AND METHODS

**Chemicals** 5-AzadCyd and SAHA were purchased from Tokyo Chemical Industry (Japan). Selective HDAC inhibitors were purchased from the indicated sources: MGCD0103 (Adooq Bioscience, CA, U.S.A.), R306465 (Adooq Bioscience), KD570 (Santa Cruz Biotechnology, CA, U.S.A.). These drugs were dissolved in dimethyl sulfoxide (DMSO); in all cases, the final concentration of DMSO was 0.1% or less.

**Cell Lines and Culture Conditions** LNCaP and PC-3 cell lines were purchased from RIKEN BRC (Japan) and cultured in RPMI 1640 medium (Gibco, Life Technologies, CA, U.S.A.) supplemented with 10% fetal bovine serum (Sigma-Aldrich, MO, U.S.A.) at 37°C in an atmosphere of 5% CO₂ in humidified air. Cells were harvested using trypsin–ethylenediaminetetraacetic acid (EDTA) at 70–80% confluence and collected by centrifugation (300×g for 5 min at room temperature (r.t.)). The cell pellets were resuspended in fresh medium and subcultured in T75 flasks or 96-well plates, as required.

**Measurement of Growth-Inhibitory Potency** The concentrations of single agents resulting in IC₅₀ were determined by dose–effect analysis. The cells were plated at a density of 0.5×10⁴ cells per well in 96-well plates and incubated for 24 h. Then, the cells were further incubated with drugs in the concentration range of 0.1–100 µM (final concentration) for 72 h. Cell proliferation was evaluated using the WST-8 Cell Proliferation Kit (R&D Systems, MN, U.S.A.) and the data were analyzed using GraphPad Prism. Significance was confirmed by the t-test.

**Apoptosis Assay** For staining of apoptotic cells, an annexin V-PI Assay Kit (Nacalai Tesque, Japan) was used according to the manufacturer’s protocol. Cell viability was determined by annexin V binding and propidium iodide (PI) staining using a FACSCalibur (BD Biosciences, CA, U.S.A.) flow cytometer. The samples were analyzed according to the procedure described in our previous report.²⁰

**Quantification of Prostate-Specific Antigen (PSA) in Cell Culture Supernatant** PSA levels were measured according to the procedure described in our previous report.³¹ Briefly, after drug treatment for 72 h, the supernatant was collected and the amount of PSA in the supernatant was quantified using the f-PSA enzyme immunoassay test kit (Immunospec Corp., CA, U.S.A.), according to the manufacturer’s protocol. Significance was confirmed by the t-test.

**RESULTS**

**Determination of IC₅₀ Values for Growth Inhibition of LNCaP and PC-3 Cells** To determine the appropriate concentrations of Am80 and epigenetic inhibitors for combination experiments, we first determined the IC₅₀ values for the individual compounds, Am80, SAHA, and 5-AzadCyd, in cell viability assays (Table S1). These compounds all dose-dependently inhibited the proliferation of LNCaP and PC-3 cells, and the IC₅₀ values were consistent with previously reported values. In the combination studies, all compounds were used at concentrations equal to their IC₅₀ and IC₃₀ values.

**Enhanced Effects of Am80 in Combination with Epigenetic Inhibitor** The enhanced effects of epigenetic inhibitors at various concentrations in the presence of a fixed concentration of Am80 were examined in LNCaP and PC-3 cells. As shown in Fig. 1, enhanced growth-inhibitory effects were observed in LNCaP cells treated with 25 µM Am80 and 1.6, 2.1 or 2.6 µM SAHA, whereas treatment with the individual agents did not significantly inhibit cell growth. In the case of the PC-3 cell line, the effect of the combination was not remarkable (Fig. S1). PSA has been used as a serum marker for diagnosis and prognosis of prostate cancer, and its decrease indicates attenuation of LNCaP cell activity.²⁴ The concentration of PSA was decreased by treatment of each drug or the combination (Fig. S2). Therefore, we focused on the LNCaP cell line for further experiments.

**Profile of HDAC Isotype Selectivity** SAHA is a pan-HDAC (Class I, IIb, and IV) inhibitor.²² Therefore, to identify the class of HDAC that mediates the growth-inhibitory effect observed in our study, we tested selective HDAC inhibitors. Results similar to those observed with SAHA were obtained.
when the isotype-selective HDAC inhibitor KD5170 (Class I, IIa, and IIb) was used in combination with Am80. The IC50 value of KD5170 growth inhibition in LNCaP cells changed from 0.51 µM in the absence of Am80 to 0.11 µM in combination with 25 µM Am80 (Table S2). Other isotype-selective HDAC inhibitors MGCD0103 (Class I and IV)25) and R306465 (Class I)26) did not show an enhanced growth-inhibitory effect (Table S2).

**Effect of HDAC Inhibitors on Apoptosis** Since enhancement in growth inhibition of LNCaP cells was observed with Am80 and HDAC inhibitor, we quantified the percentage of apoptotic cells after 72 h by means of annexin V staining. As expected, there was an increase in the percentage of apoptotic cells in cells exposed to the combination of Am80 and SAHA (38.1%), compared to those in cells exposed to SAHA (26.4%) or Am80 (35.5%) alone (Fig. 2). The combination of Am80 with KD5170 also showed an increase in apoptosis compared with the individual agents.

**Effect of HDAC Inhibitors on HDAC Activity** Almost all HDACs are distributed in the nucleus; however, Class IIb HDACs, which may be inhibited by SAHA and KD5170, are distributed in cytosol. To confirm the inhibition of cellular HDAC activity, we determined the HDAC activity in whole-cell lysates. SAHA and KD5170 inhibited HDAC activity by 67.0 and 42.0%, respectively, compared to the vehicle control. Interestingly, SAHA showed higher inhibitory activity than KD5170, even though KD5170 showed more potent cell growth inhibition (Fig. 3).

**Effect of HDAC Inhibitors on Protein Levels of RARα** Expression of numerous NRs, including ERα and RAR β, is silenced in several cancer cell lines,14,15) and RARα is activated by agonists, such as all-trans-retinoic acid (ATRA) and Am80, following degradation.27) That is, the existing NR level tends to be insufficient. Thus, clarifying the existence of RARα provides important information to elucidate this

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**Fig. 1. The Enhanced Growth-Inhibitory Effect of Am80 with HDAC or DNMT Inhibitor on LNCaP Cells**

LNCaP cells were treated singly or in combination as indicated for 72 h, and cell viability was determined by MTT assay. The absorbance of the vehicle control wells was taken as unity. Data are expressed as means ± standard deviation (S.D.). Experiments were performed in triplicate. n.s., Not significant, *p < 0.05; versus the vehicle; Student’s t-test.

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**Fig. 2. Apoptosis-Inducing Effect of Am80 in Combination with HDAC Inhibitors on LNCaP Cells**

LNCaP cells were treated with the indicated drugs for 72 h. The proportion of apoptotic cells (expressed as a percentage in each plot) was evaluated by flow cytometric analysis using annexin V-FITC staining.
HDAC activity was evaluated in whole-cell lysates after treatment as indicated for 72 h. HDAC activity is calculated as follows: \([\text{OD}_{450}\text{ (control - blank)} - \text{OD}_{450}\text{ (sample - blank})/\text{OD}_{450}\text{ (control - blank)}] \times 100\), where OD_{450} is absorbance at 450 nm. Data are expressed as means ± S.D. Experiments were performed in triplicate.

Here we focused on the potential value of approved epigenetic inhibitors for combination therapy with Am80. We were particularly interested in HDAC inhibitors, because they cause the accumulation of acetylated histones and activate the transcription of genes whose expression induces apoptosis. Also, HDACs are upregulated in various human prostate cancer cell lines in comparison between human prostate cancer cell lines and non-malignant prostate tissues same as in most cancers.\(^{29,30}\) Indeed, we found an enhanced growth-inhibitory effect of the pan-HDAC inhibitor SAHA with Am80 in the AR-positive prostate cancer cell line LNCaP. Further studies with various isotype-selective HDAC inhibitors revealed that KD5170 showed a similar effect, and overall, the results suggested the involvement of Class IIb HDACs. KD5170 has the highest specificity for HDAC6,\(^{31}\) and therefore we speculate that inhibition of HDAC6 may be a major target of the effect. Thus, Class IIb HDACs (HDAC6, and/or HDAC10) may be potential targets for treatment of prostate cancer.

The expression of NRs is often silenced in cancer cells, and moreover, RARα is degraded after activation through agonist binding.\(^{27}\) That is, the existing NR level tends to be insufficient, and the RARα levels in acute myelogenous leukemia cells decline further after treatment with Am80 (unpublished data). Here, we found that either KD5170 or SAHA alone increased the RARα protein level in LNCaP whole-cell lysate, though the combination treatment with KD5170 and SAHA decreased RARα protein (Fig. 4). It is well known that RARα translocates to the nucleus after binding with its agonist, retinoic acid.\(^{32}\) In this context, deacetylation of HSP90 by HDAC6 results in the retention of glucocorticoid receptor (GR) in the cytoplasm, failure of GR maturation, and decreased transcription of target genes.\(^{33,34}\) Thus, it seems plausible that the HDAC inhibitors KD5170 and SAHA promote RARα maturation in LNCaP cells. We are currently investigating whether this is indeed the case.

**CONCLUSION**

Combination treatment of Am80 with HDAC inhibitors enhanced the growth-inhibitory effect in LNCaP cells, accompanied with increased apoptosis and reduced PSA secretion. Studies with isotype-selective HDAC inhibitors suggested that Class IIb HDACs are involved in the effect. Dual targeting of Class IIb HDAC and RARα may be a promising therapeutic strategy for prostate cancer.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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