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14. ABSTRACT We hypothesized that dehydroepiandrosterone metabolites or their synthetic derivatives are able to bind to the androgen receptor with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We previously identified three potential compounds with marginal androgenic activity. Using different prostate cancer cell lines, we showed that these compounds could inhibit androgen-induced growth of androgen receptor-positive tumors <i>in vitro</i> . We have then assessed the anti-tumor activities of these compounds in mouse xenograft models for prostate cancer. Inconsistent with our <i>in vitro</i> data, treatment with the dehydroepiandrosterone derivatives even at a relatively high dose resulted in modest decreases in the growth of inoculated tumors as well as the expression of angiogenesis- and metastasis-related genes in the tumors. In addition, the compounds did not show significant chemopreventive effects on prostate carcinogenesis in the TRAMP transgenic mouse model. Competitive androgen binding assays revealed that all these compounds were able to compete significantly with androgens for androgen receptor binding. The compounds were also found to inhibit androgen-induced androgen receptor protein expression yet had little influence on the protein stability in prostate cancer cells.					
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Introduction

Although antiandrogens that can block androgen action through the androgen receptor (AR) have been widely used for the treatment of prostate cancer, the majority of available ones possess agonist activity, resulting in increases in serum prostate-specific antigen (PSA) levels, known as the antiandrogen withdrawal syndrome [1,2]. In addition, we previously found that androstenediol (Adiol), a physiological metabolite from dehydroepiandrosterone (DHEA) and a precursor of testosterone, has an intrinsic androgenic activity which was not completely antagonized by two antiandrogens clinically used, flutamide and bicalutamide (BC) [3]. Therefore, new and more effective antiandrogenic compounds with marginal androgenic activities need to be identified. Our hypothesis in the current project was that DHEA metabolites or their synthetic derivatives are able to bind to the AR with low, if any, agonist activity and thus function as better antiandrogens than currently available ones. We previously screened DHEA derivatives/metabolites for their androgenic and antiandrogenic activities and found that three compounds, 3 β -acetoxyandrost-1,5-diene-17-ethylene-ketal (ADEK), 3 β -hydroxyandrost-5,16-diene (HAD), and 3-oxo-androst-1,4-diene-17-ketal (OADK), show only marginal agonist effects and suppress significantly 5 α -dihydrotestosterone (DHT)- and Adiol-induced AR transactivations [4-6]. Thus, ADEK, HAD, and OADK have the potential to function as potent antiandrogens that carry fewer risks of withdrawal response if used for therapy in patients with prostate cancer.

We have previously assessed the effects of ADEK, HAD, and OADK on prostate cancer cell growth *in vitro*. The tasks in the approved Statement of Work in this period (months 37-48) would be to evaluate the effects of these DHEA derivatives *in vivo* (Task 2 for months 19-48), including Task 2-b (to test the anti-tumor effects of the compounds in mouse xenograft models for prostate cancer) and Task 2-c (to analyze xenograft tumors by immunohistochemistry, reverse transcription (RT)-polymerase chain reaction (PCR), and western blotting), and to determine the mechanisms of how the compounds inhibit prostate cancer growth (Task 3 for months 37-60; Tasks 3-a and 3-d).

Body

Anti-tumor effects of ADEK, HAD, and OADK in mouse xenograft models for prostate cancer

Inhibitory effects of DHEA derivatives on tumor growth have been assessed in mouse xenograft models for AR-positive prostate cancer. We used LNCaP and CWR22Rv1 because the compounds were found to significantly suppress androgen-mediated cell proliferation *in vitro*. These lines were implanted subcutaneously into the flanks of 7-8-week-old male SCID mice. After 2-4 weeks when the estimated volumes of all tumors reached 40 mm³, we started daily injection of ADEK, HAD, or OADK into mice. Because daily injections of each compound at 100 mg/Kg resulted in marginal decreases in tumor size (detailed in the previous report, July 2012), we used a higher dose (200 mg/Kg) that remained tolerable in animals. As shown in Figure 1, inoculated LNCaP/CWR22Rv1 tumors in mice treated with BC (without castration) were

significantly smaller (79%/51% reductions at 10/8 weeks, respectively) than those in the control mice. Treatment of ADEK (39%/29% at 10/8 weeks), HAD (29%/20% at 10/8 weeks), or OADK (29%/24% at 10/8 weeks) reduced the size of the LNCaP/CWR22Rv1 tumors, compared with control treatment, but the differences were not statistically significant ($P>0.05$). In addition, castration (bilateral orchietomy) significantly retarded the growth of the LNCaP tumors, and no significant additive effects of BC or each DHEA derivative were seen.

Figure 1

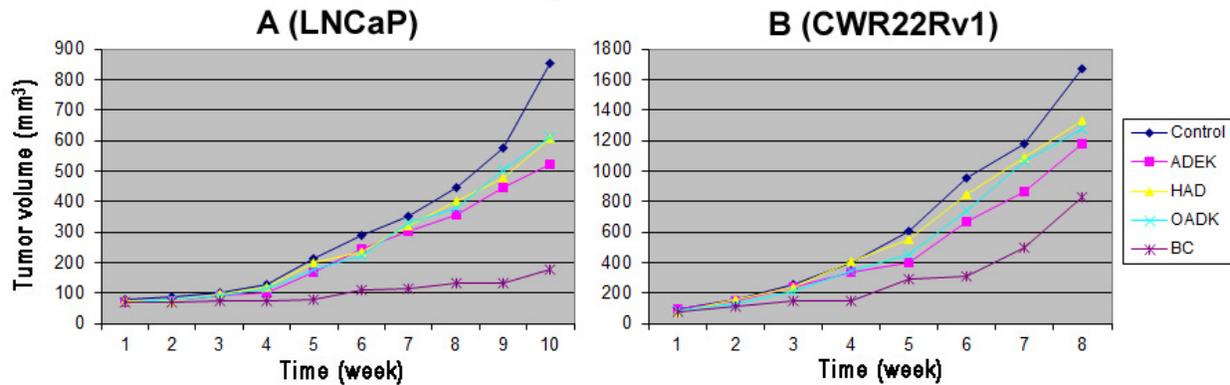
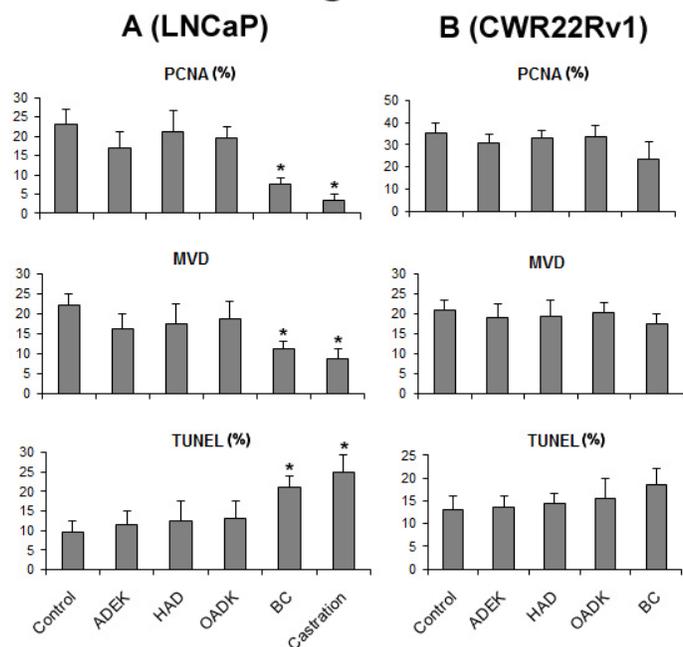


Figure 1. The effects of ADEK, HAD, and OADK on tumor progression in mouse xenograft models for prostate cancer. LNCaP (A) or CWR22Rv1 (B) cells resuspended in Matrigel (2×10^6 cells in 200 μ l per site) were implanted subcutaneously into the flanks of SCID mice, and treatment [daily intra-peritoneal injection of 200 mg/Kg each compound) began when estimated tumor volume reached 40 mm^3 calculated by the following formula: tumor weight = tumor length (mm) \times [tumor width (mm)]² \times 0.5 [7]. Tumor volume (n = 6 tumors in each group) was then monitored twice a week for 8-10 weeks.

Figure 2. The effects of ADEK, HAD, and OADK on cell proliferation, angiogenesis, and apoptosis in mouse xenograft models for prostate cancer. The LNCaP (A) and CWR22Rv1 (B) xenograft tumors described in Figure 1 were harvested for immunohistochemical and TUNEL analyses. Mean values + SDs of the percentage of PCNA-positive cells, micro-vessel density (MVD; number of vessels highlighted by CD31 staining per high-power field), and the percentage of TUNEL-positive cells in each group of tumors are shown. * $P<0.05$ (vs. control by Student's *t*-test).

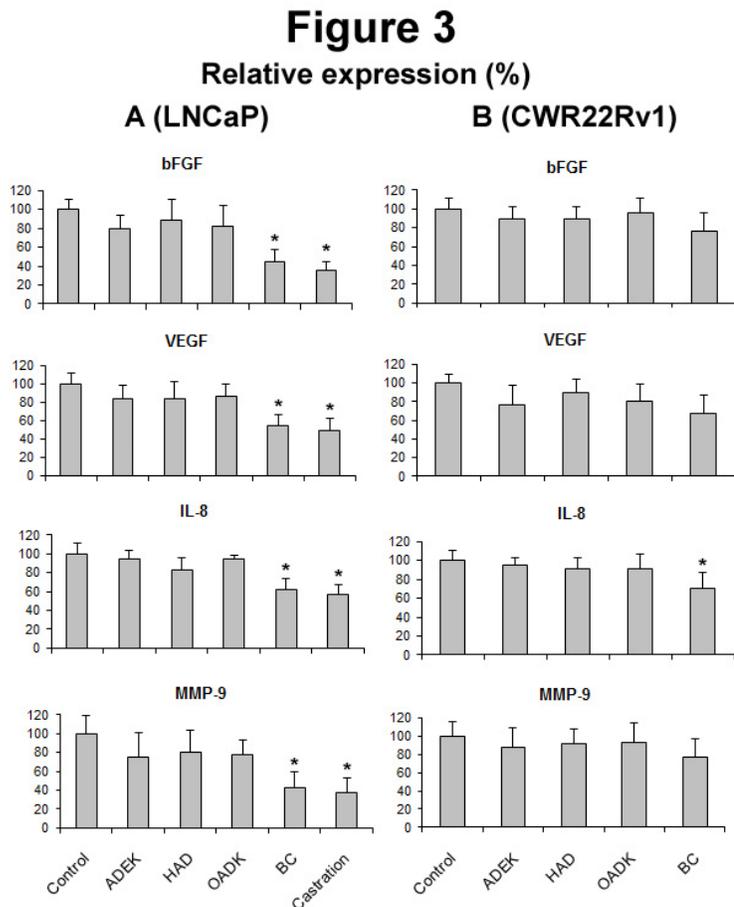
Figure 2



Some of the harvested tumor specimens were also assessed for cell

proliferation [by proliferating cell nuclear antigen (PCNA) immunostaining; Figure 2], apoptosis (by TUNEL assay; Figure 2), and angiogenesis or metastatic ability [MVD by CD31 immunostaining; Figure 2] as well as the expression of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin (IL)-8, and matrix metalloproteinase (MMP)-9 by quantitative RT-PCR; Figure 3]. Correlating with the sizes of xenograft tumors, BC or castration, but not ADEK, HAD, or OADK, significantly change these parameters. There were also no noticeable differences in the expression of VEGF, MMP-9, and E-cadherin detected by immunohistochemistry, as well as that of VEGF, MMP-2, and MMP-9 detected by Western blotting, between the tumors from the control versus ADEK/HAD/OADK groups.

Figure 3. The effects of ADEK, HAD, and OADK on angiogenesis- and/or cell invasion-related genes in mouse xenograft models for prostate cancer. The LNCaP (A) and CWR22Rv1 (B) xenograft tumors described in Figure 1 were harvested for RNA extraction. mRNA expression of bFGF, VEGF, IL-8, and MMP-9 in the tumors was analyzed by real-time RT-PCR. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of control tumors in each cell line (first lanes; set as 100%). Each value represents the mean + SD from at least three independent experiments. * $P < 0.05$ (vs. control by Student's *t*-test).



Thus, in contrast to our *in vitro* data, ADEK, HAD, and OADK did not show significant suppressive effects on AR-positive tumor growth *in vivo*. We repeated mouse xenograft experiments, using another prostate cancer cell line, VCaP, harboring a wild-type AR. However, the tumors grew very slowly in mice, and inhibitory effects of the DHEA derivatives as well as BC on tumor growth were not apparent.

Anti-carcinogenic effects of ADEK, HAD, and OADK in the TRAMP model

Alternatively, as proposed in Specific Aim 2 (Alternative approach 3), chemopreventive effects of DHEA derivatives were assessed in the TRAMP transgenic mouse model in which a premalignant lesion [prostatic intraepithelial neoplasia (PIN)], invasive prostatic adenocarcinoma, and metastasis are sequentially developed [8]. At the age of 5 weeks

prior to tumor development in the prostate, each compound at 100 mg/Kg was injected daily into the mice (n = 8/group at each time point). The mice were then sacrificed at 12 and 24 weeks. As summarized in Table 1, ADEK, OADK, or HAD did not significantly prevent the development of PIN and invasive cancer. At 24 weeks ADEK reduced the incidence of prostate cancer from 88% to 50% (p=0.282).

Table 1. The incidence of prostatic adenocarcinoma in the TRAMP mice.

Group	PIN at 12 weeks	Cancer at 12 weeks	Cancer at 24 weeks
Control	7 (88%)	3 (38%)	7 (88%)
ADEK	5 (63%)	2 (25%)	4 (50%)
HAD	6 (75%)	3 (25%)	6 (75%)
OADK	7 (88%)	3 (38%)	5 (63%)

Binding affinity of ADEK, HAD, and OADK for the AR

To determine whether the DHEA derivatives have an affinity for the AR, allowing a competition with androgens for binding, competitive androgen binding assay was performed in LNCaP with endogenous mutant AR and DU145 with transfected wild-type AR. As described [5, 6], the relative binding affinity (RBA) values were calculated. Competitive RBAs both in LNCaP and DU145 with AR were: DHT > BC > OADK > ADEK > HAD. These results confirm that the DHEA derivatives, particularly ADEK and OADK, are able to compete significantly with androgens for AR binding.

Table 2. AR ligand binding affinity.

Ligand	RBA in LNCaP	RBA in DU145 with AR
DHT	100.0	100.0
BC	48.1	28.8
ADEK	10.5	5.5
HAD	2.1	1.2
OADK	14.5	12.4

Effects of ADEK, HAD, and OADK on AR protein expression and stability

We previously reported that ADEK, HAD, and OADK inhibited androgen-induced protein expression of AR in LNCaP and CWR22Rv1 cells while they did not reduce AR expression in the absence of androgens [annual report, July 2011 & Clin Immunol Endocr Metab Drugs, 2013 (in press)]. To further determine whether the DHEA derivatives affect the stability of AR protein, Western blotting was performed in LNCaP cells pretreated with cycloheximide blocking the neosynthesis of protein. The degradation of AR protein was determined at different time points. In these pilot experiments, there were no significant differences in the ratios of AR degradation between the control versus ADEK/HAD/OADK groups in the presence and absence of DHT. These findings suggest that the DHEA derivatives have little influence on the stability of AR protein in prostate cancer cells. We will further test the effects of these compounds on AR stability in different cell lines.

Key Research Accomplishments

1. (for Tasks 2-b & 2-c) ADEK, HAD, and OADK at a high dose slightly inhibited tumor growth in mouse xenograft models for prostate cancer.
2. (for Task 2-b; *Alternative approaches for Specific Aim 2*) ADEK, HAD, and OADK marginally retarded or prevented the development of PIN and invasive prostate cancer in the TRAMP transgenic mouse model.
3. (for Task 3-a) ADEK, HAD, and OADK competed significantly with androgens for the binding to the AR in prostate cancer cells.
4. (for Task 3-d) ADEK, HAD, and OADK inhibited androgen-induced expression of AR in prostate cancer cells whereas they had little influence on the stability of AR protein.

Reportable Outcomes

Peer-reviewed Publications

1. Kawahara T, **Miyamoto H*** (corresponding author): Androgen receptor antagonists in the treatment of prostate cancer. *Clin Immunol Endocr Metab Drugs*, 2013 (accepted for publication).

Additional Peer-reviewed Publications (Underlined articles acknowledge the current award).

2. Li Y, Izumi K, **Miyamoto H*** (corresponding author): (Review) The role of the androgen receptor in the development and progression of bladder cancer. *Jpn J Clin Oncol* 42: 569-577, 2012 (July).
3. Choy B, Gordetsky J, **Miyamoto H*** (corresponding author): Clinicopathologic features of prostate cancer in patients diagnosed by age 45 who underwent radical prostatectomy. *Eur Urol* 62: 354-355, 2012 (August).
4. Subik MK, Gordetsky J, Yao JL, di Sant'Agnese PA, **Miyamoto H** (corresponding author): Frozen section assessment in testicular and paratesticular lesions suspicious for malignancy: its role in preventing unnecessary orchiectomy. *Hum Pathol* 43: 1514-1519, 2012 (September).
5. Izumi K, Li Y, Zheng Y, Gordetsky J, Yao JL, **Miyamoto H*** (corresponding author): Seminal plasma proteins in prostatic carcinoma: Increased nuclear semenogelin I expression is a predictor of biochemical recurrence after radical prostatectomy. *Hum Pathol* 43: 1991-2000, 2012 (November).

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7. Izumi K, Zheng Y, Li Y, Zaengle J, **Miyamoto H*** (corresponding author): Epidermal growth factor induces bladder cancer cell proliferation through activation of the androgen receptor. *Int J Oncol* 41: 1587-1592, 2012 (November).
8. Zhao C, Venigalla S, **Miyamoto H*** (corresponding author): Chronic inflammation on initial benign prostate biopsy is a negative predictor of subsequent cancer detection. *Pathol Int* 62: 774-776, 2012 (November).
9. **Miyamoto H**: "Differentiation between muscularis mucosae and muscularis propria" in Atlas Series of Differential Diagnosis of Tumor Histopathology, 12. Renal Pelvis/Ureter/Bladder Cancer (Tsuzuki T/Morinaga S Eds.), pp.138-146, Bunkodo, Tokyo, 2012 (ISBN 978-4-8306-2237-3).
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13. Ishiguro Y, Ishiguro H, **Miyamoto H**: Epidermal growth factor receptor tyrosine kinase inhibition up-regulates interleukin-6 in cancer cells and induces subsequent development of interstitial pneumonia. *Oncotarget* 4: 550-559, 2013 (April).
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15. Hsu J-W, Hsu I-W, Xu D, **Miyamoto H**, Liang L, Wu X-R, Shyr C-R, Chang C: Decreased tumorigenesis and mortality from bladder cancer in mice lacking urothelial androgen receptor. *Am J Pathol* 182: 1811-1820, 2013 (May).
16. Yang D-R, Lin S-J, Ding X-F, **Miyamoto H**, Messing E, Li L-Q, Wang N, Chang C:

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 22. Ishiguro H, Kawahara T, Li Y, **Miyamoto H**: "Anti-tumor activities of dexamethasone" in *Dexamethasone: Therapeutic Uses, Mechanism of Action and Potential Side Effects* (Sauvage A/Levy M Eds.), Nova Science Publishers, Hauppauge, New York, in press (ISBN 978-1-62808-406-1).
 23. Venigalla S, Wu G, **Miyamoto H*** (corresponding author): The impact of routine frozen section analysis during partial nephrectomy on surgical margin status and tumor recurrence: A clinicopathologic study of 433 cases. *Clin Genitourin Cancer* (accepted for publication).
 24. Feng L-Y, Izumi K, Lai K-P, Liang L, Li L, **Miyamoto H**, Lin W-J, Chang C: Infiltrating macrophages promote prostate tumorigenesis via modulating androgen receptor-mediated CCL4-STAT3 signaling. *Cancer Res* (accepted for publication).

Invited Speakers

1. National Cancer Institute, Urologic Oncology Branch, Bethesda, Maryland (March 2013)

2. Association of Japanese Life Scientists (Kinyokai) in the National Institutes of Health, Bethesda, Maryland (March 2013)
3. Special Lecture at the 22nd Annual Meeting of the Japanese Society for Molecular and Cellular Urology, Kochi, Japan (March 2013)
4. Special Lecture at the 15th Urological Genome Seminar, Kochi, Japan (March 2013)
5. National Taiwan University Hospital (Department of Urology), Taipei, Taiwan (March 2013)
6. Chang Gung Memorial Hospital (Genitourinary Oncology Tumor Board), Linkou, Taiwan (March 2013)
7. National Taiwan University Hospital (Department of Pathology), Taipei, Taiwan (March 2013)
8. Lecture for the Japanese Society of Urological Pathology at the 102nd Annual Meeting of the Japanese Society of Pathology, Sapporo, Japan (June 2013)

Conclusion

Using mouse xenograft models for prostate cancer (LNCaP and CWR22Rv1), we have assessed the effects of ADEK, HAD, and OADK, in comparison with that of BC, on tumor progression *in vivo*. All these DHEA derivatives, as well as BC, were previously shown to have antiandrogenic properties and significantly inhibited cell proliferation and invasion of prostate cancer lines with different AR statuses *in vitro*. Compared to these *in vitro* analyses, inhibitory effects of the three compounds at doses of 100-200 mg/Kg given subcutaneously or intraperitoneally on tumor growth were found to be less significant in animal models. To determine the mechanisms through which DHEA derivatives inhibit prostate cancer progression, we also showed that ADEK, HAD, and OADK competed significantly with androgens for the binding to the AR and that they did not significantly affect AR protein stability in prostate cancer cells. As reported [annual report, July 2010; Clin Immunol Endocr Metab Drugs, 2013 (in press)] and described in the original Project Narrative; Specific Aim 1, Alternative approach 1), new DHEA derivatives showed significant inhibitory effects on prostate cancer cell proliferation and PSA expression *in vitro*. Some of these effects were found to be even stronger than the effects of ADEK/HAD/OADK. We will further investigate these new compounds. Concurrently, we will explore the mechanisms responsible for the inhibition of prostate cancer growth by the original three compounds (*Task 3*).

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