Corrections

**NEUROSCIENCE.** For the article “Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1-dependent pathway,” by Hoon Ryu, Junghee Lee, Beatrix A. Olofsson, Aziza Mwidau, Alpaslan Deodoglu, Maria Escudero, Erik Flemington, Jane Azizkhan-Clifford, Robert J. Ferrante, and Rajiv R. Ratan, which appeared in issue 7, April 1, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 4281–4286; First Published March 14, 2003; 10.1073/pnas.0737363100), the author name Alpaslan Deodoglu should have appeared as Alpaslan Dedeoglu. The corrected author line appears below. The online version has been corrected.

Hoon Ryu, Junghee Lee, Beatrix A. Olofsson, Aziza Mwidau, Alpaslan Dedeoglu, Maria Escudero, Erik Flemington, Jane Azizkhan-Clifford, Robert J. Ferrante, and Rajiv R. Ratan

www.pnas.org/cgi/doi/10.1073/pnas.1231735100

**COMMENTARY.** For the article “What happens to genes in duplicated genomes,” by Elizabeth A. Kellogg, which appeared in issue 8, April 15, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 4369–4371; First Published April 7, 2003; 10.1073/pnas.0831050100), the pull quote in the second column on page 4370 read “Subfunctionalization occurs in some genes and is not an immediate product of polyploidization.” It should have read “Subfunctionalization occurs in some genes and is an immediate product of polyploidization.” This error occurred during the editorial process and is not the fault of the author. PNAS regrets this error.

www.pnas.org/cgi/doi/10.1073/pnas.1232296100

**CELL BIOLOGY.** For the article “Prospective identification of tumorigenic breast cancer cells,” by Muhammad Al-Hajj, Max S. Wicha, Adalberto Benito-Hernandez, Sean J. Morrison, and Michael F. Clarke, which appeared in issue 7, April 1, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 3983–3988; First Published March 10, 2003; 10.1073/pnas.0530291100), the authors note that the following statement was inadvertently omitted from the acknowledgements: “The results of this study support a patent-pending technology that is exclusively licensed to Cancer Stem Cell Genomics (CSCG) in which the authors and the University of Michigan have a financial interest.”

www.pnas.org/cgi/doi/10.1073/pnas.1131491100

**IMMUNOLOGY.** For the article “Antigen presentation by keratinocytes directs autoimmune skin disease,” by Lian Fan, Brian W. Busser, Traci Q. Lifsted, David Lo, and Terri M. Laufer, which appeared in issue 6, March 18, 2003, of *Proc. Natl. Acad. Sci. USA* (100, 3386–3391; First Published March 10, 2003; 10.1073/pnas.0437899100), the authors request that Mohamed Oukka, Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, be added to the list of authors between Traci Q. Lifsted and David Lo. The revised author line appears below.

Lian Fan, Brian W. Busser, Traci Q. Lifsted, Mohamed Oukka, David Lo, and Terri M. Laufer

www.pnas.org/cgi/doi/10.1073/pnas.1232295100
For the article “3β-Acetoxyandrost-1,5-diene-17-ethylene ketal functions as a potent antiandrogen with marginal agonist activity,” by Hiroshi Miyamoto, Padma Marwah, Ashok Marwah, Henry Lardy, and Chawnshang Chang, which appeared in issue 8, April 15, 2003, of Proc. Natl. Acad. Sci. USA (100, 4440–4444; First Published April 2, 2003, 10.1073/pnas.0831001100), in Fig. 1A the hydroxyl group in position 17 for Δ4-androstenediol (Adiol), testosterone, and dihydrotestosterone (DHT) should be connected with a single, rather than a double, bond. The conclusions are unchanged by this typographical error. The corrected figure and its legend appear below.

**Fig. 1.** The structures of DHEA derivatives and effects on AR transcriptional activity. (A) The structures of compounds nos. 5, 10, 14, 16, and 17, DHEA, Adiol, testosterone, and DHT. (B) PC-3 cells were transfected with the WT AR expression plasmid pSG5-AR and MMTV-Luc. After transfection, cells were cultured for 24 h with 1 nM DHT or 1,000 nM of various DHEA derivatives. The Luc activity is presented relative to that of EtOH treatment (white bar; set as 1-fold). Values represent the mean ± SD of at least three determinations. (C) PC-3 cells were transfected with the pSG5-AR and MMTV-Luc. After transfection, cells were cultured for 24 h with various concentrations of compounds nos. 5, 10 (ADEK), 14, or 16 in the presence of 1 nM DHT. The Luc activity is presented relative to that in the presence of DHT (black bar; set as 100%). Values represent the mean ± SD of at least three determinations.

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3β-Acetoxyandrost-1,5-diene-17-ethylene ketal functions as a potent antiandrogen with marginal agonist activity

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Contributed by Henry Lardy, February 19, 2003

The majority of available antiandrogens have been reported to possess agonist activity to induce prostate-specific antigen, which might result in antiandrogen withdrawal syndrome. Here we report the identification of 3β-acetoxyandrost-1,5-diene-17-ethylene ketal (ADEK) from dehydroepiandrosterone metabolites and derivatives as a potent antiandrogen. We found ADEK could interrupt androgen binding to the androgen receptor (AR) and suppress androgen-induced transactivations of WT AR and a mutant AR in prostate cancer cells. ADEK inhibited prostate-specific antigen expression as well as growth in LNCaP prostate cancer cells stimulated by androgen. Importantly, ADEK had only marginal agonist effects, as compared with commonly used antiandrogens such as hydroxyflutamide and bicalutamide, leading to a lower possibility of inducing withdrawal response. Moreover, ADEK could block an adrenal androgen androstenediol-induced AR transactivation that hydroxyflutamide and bicalutamide failed to block. These unique antiandrogenic activities make ADEK a potential therapeutic compound that might be able to inhibit AR-mediated prostate cancer progression. Further in vivo studies might facilitate the development of a better antiandrogen for the treatment of prostate cancer.

Antiandrogens play a major role in promoting the development and progression of prostate cancer. Consequently, since the first observation by Huggins and Hodges in 1941 (1), endocrine therapy remains the critical therapeutic option for advanced forms of prostate cancer. This therapy consists of androgen ablation by medical or surgical castration and/or inhibiting the receptor level action of androgens from both the testes and adrenal glands by antiandrogens. Thus, antiandrogens are generally used in conjunction with castration as combined androgen blockade (CAB). However, a debate is ongoing over whether the survival benefit achieved with CAB over monotherapy (castration alone or antiandrogen alone) is statistically significant (2). After a brief clinical response to the hormonal therapy in most patients, the majority eventually develop symptomatic recurrences, which have been termed androgen-independent or hormone-refractory prostate cancer, within a few years. Indeed, in males, prostate cancer is the most common malignancy and is the second-leading cause of cancer-related death (3).

Antiandrogens include a number of compounds that are able to compete with androgens, such as dihydrotestosterone (DHT), an active metabolite of testosterone in the prostate, for binding to the androgen receptor (AR). There are three nonsteroidal antiandrogens available in the United States: flutamide, bicalutamide (casodex), and nilutamide. Monotherapy of these antiandrogens does not decrease androgen concentrations, offering potential quality-of-life benefits over castration-based approaches, but there are some specific side effects, such as gynecomastia and breast pain, hepatotoxicity, visual and respiratory disturbances, and alcohol intolerance (4). In addition, antiandrogens have been reported to raise the amount of prostate-specific antigen (PSA), a tumor marker of prostate cancer and also an AR-responsive gene, during hormonal therapy. This phenomenon is known as antiandrogen withdrawal syndrome, and a subset of patients may benefit temporarily from the withdrawal of the majority of antiandrogens clinically used, including the above three drugs, as well as some steroid hormones, such as diethylstilbestrol and magesstrrol (5–7). The mechanisms responsible for antiandrogen withdrawal syndrome are not completely understood, although it is likely that AR gene mutations and/or AR coregulators, such as AR.A70, are involved in the change of antiandrogens from antagonists to agonists (6, 8–12). Thus, new and more effective antiandrogenic compounds with lower androgenic activities need to be identified.

Dehydroepiandrosterone (DHEA) is classified as belonging to the “adrenal androgens” group and has been shown to have weak androgenic activity (13). Previously, we found that some DHEA metabolites, which have very low androgenic activity, could block a precursor of testosterone Δ5-androstenediol (Adiol)-induced AR transactivation in prostate cancer cells (13, 14). However, these compounds failed to block completely the DHT-induced AR transactivation. In the present study, we have screened other DHEA derivatives/metabolites as potential antiandrogenic compounds to see whether these compounds compete with DHT and block its action on the AR. We found that one of them, compound no. 10: 3β-acetoxyandrost-1,5-diene-17-ethylene ketal (ADEK) (Fig. 1A), inhibited both DHT- and Adiol-induced AR transcription, PSA expression, and growth in prostate cancer cells.

Materials and Methods

Chemicals and Plasmids. DHT, Adiol, 17β-estradiol, progesterone, and dexamethasone were obtained from Sigma. Hydroxyflutamide (HF) was from Schering, and casodex was from ICI. Other steroid compounds, derivatives of DHEA, were synthesized. pSG5-AR and pSG5-ARA70 were used in our previous studies (11–15).

Cell Culture, Transfection, and Reporter Gene Assay. The human prostate cancer cell lines (LNCaP, PC-3, and DU145) and nonprostate cancer cell line COS-1 were maintained in RPMI or DMEM (Life Technologies, Rockville, MD) supplemented with 10% FBS. Transfections and luciferase (Luc) assays were performed as described (12, 16). Briefly, cells seeded to reach a density of 50–60% confluence in 12-well tissue culture plates were transfected with 1.5 μg of DNA according to SuperFect transfection instructions (Qiagen, Chatsworth, CA). After 2–3 h of incubation, cells were treated with medium supplemented with charcoal-stripped FBS containing either ethanol (EtOH) or

Abbreviations: DHT, dihydrotestosterone; AR, androgen receptor; PSA, prostate-specific antigen; DHEA, dehydroepiandrosterone; Adiol, Δ5-androstenediol; ADEK, 3β-acetoxyandrost-1,5-diene-17-ethylene ketal; HF, hydroxyflutamide; Luc, luciferase; R1881, synthetic androgen methyltrienolone; MMTV, mouse mammary tumor virus; EtOH, ethanol; RBA, relative binding affinity.

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ligands for 24 h. The cells were then harvested, and whole-cell extracts were used for Luc assay. The Luc activity was determined by using a Dual-Luciferase Reporter Assay System (Promega) and luminometer.

Western Blot. Western blotting assay was performed in LNCaP cells, using monoclonal PSA antibody (DAKO), as described (16). An antibody for β-actin (Santa Cruz Biotechnology) was used as the internal control. Blots were quantitated by COLLAGE software (Fotodyne, New Berlin, WI).

Ligand Binding Assay. Whole-cell extracts from COS-1 with transient transfection of pSG5-AR, or LNCaP without transfection, were incubated for 2 h at 37°C with 1 nM [3H]synthetic androgen methyltrienolone (R1881) in the presence and absence of increasing concentrations (1–10,000 nM) of unlabeled ligands. Then, hydroxyapatite (Bio-Rad) was added and stirred for 15 min at 4°C. After centrifugation and washing, radioactivity was determined by scintillation counting.

Results and Discussion
Screening of Anti-DHT Effect of DHEA Derivatives with Low Androgenic Activity on AR Transcription. For the screening of DHEA derivatives as antiandrogenic compounds, we first investigated their ability to induce AR transcriptional activity in the AR-negative PC-3 cell line. The Luc activity was determined in the cell extracts with transient transfection of WT AR plasmid and androgen response element-reporter plasmid [mouse mammary...
tumor virus (MMTV)-Luc). After transfection, the cells were treated with various DHEA derivatives at 0.1–1,000 nM. Of 17 compounds tested, only four (no. 5: 3β,7α,17β-trihydroxyandrost-5-ene; no. 10: ADEK; no. 14: 3β-acetoxyandrost-1,5-diene-17-one; and no. 16: 3β-hydroxyandrost-1,5-diene-17-one, see Fig. 1A) at 1,000 nM showed marginal induction on AR transcription, as compared with mock treatment (Fig. 1B). These four compounds were then investigated for their anti-DHT activity on AR transcription in PC-3 cells. These transfections were transfected with AR plasmid and MMTV-Luc reporter in the presence of 1 nM DHT and each of these compounds at 0.01, 0.1, or 1 μM. Whereas compounds nos. 5, 14, and 16 showed marginal suppression on DHT-induced AR transcription, ADEK suppressed it to 30% in a dose-dependent manner (Fig. 1C). ADEK was further investigated, using different cell lines and different reporters, and was also compared with nonsteroidal antiandrogens, HF and casodex. As shown in Fig. 2A, ADEK has lower androgenic activity on WT AR transcription than HF and casodex in COS-1 cells. ADEK at 1 μM suppresses DHT-induced WT AR transcription to 21%, similar to the suppression by HF and casodex. In LNCaP cell line, 10 μM HF acts as a full agonist and therefore shows no suppression of DHT-induced mutant AR transcription (Fig. 2B), consistent with the previous findings (17, 18). However, casodex and ADEK still exhibit dose-dependent suppression to 22% and 17%, respectively, and androgenic activity of ADEK is lower than that of casodex. Similar results were obtained when MMTV-Luc was replaced with PSA-Luc (data not shown). In addition, one of the AR coactivators, ARA70, which has been shown to enhance significantly the agonist activity of antiandrogens (5- to 12-fold) (11, 12), marginally enhanced AR transactivation in the presence of ADEK (~2-fold) in DU145 cells (Fig. 2C). These results indicate that ADEK acts as a potent antagonist on DHT-enhanced transactivation of both WT AR and a mutant AR. Interestingly, some compounds closely related to ADEK [nos. 14, 15 (androst-1,4-diene-3,17-dione), 16, and 17 (3β-acetoxy-17β-hydroxyandrost-1,5-diene); ADEK without ethylene ketal and acetyl groups, see Fig. 1A] do not show significant antagonistic effects. In addition, the agonist effect of ADEK is marginal and lower than that of nonsteroidal antiandrogens, suggesting that there is less possibility of inducing withdrawal response in prostate cancer patients.

Anti-DHT Effect of ADEK on PSA Expression and Cell Proliferation. The PSA is an AR-responsive gene and presently the most useful tumor marker to monitor prostate cancer progression. Therefore, it is of interest to determine whether ADEK suppresses PSA expression in prostate cancer cells. The Western blotting assay shows that DHT increases endogenous PSA expression in LNCaP cells to 4.3-fold over mock treatment (Fig. 3A, lane 1 vs. 2) and that ADEK and casodex decrease DHT-induced PSA expression by 49% (Fig. 3A, lane 8) and 58% (Fig. 3A, lane 6), respectively. HF induces PSA expression to 3.5-fold (Fig. 3A, lane 3), whereas ADEK (Fig. 3A, lane 7) and casodex (Fig. 3A, lane 5) increase it to <2-fold. We next tested the effect of ADEK on cell growth of LNCaP. As shown in Fig. 3B, DHT significantly increases cell growth, and ADEK and casodex antagonize the DHT effect. ADEK and casodex marginally increase growth in the absence of androgen. These results confirm our data of AR transcription and suggest that ADEK can inhibit androgen/AR-mediated prostate cancer progression.

Interruption of Androgen Binding to the AR by ADEK. Clinically available antiandrogens have an affinity for the AR, allowing a competition with androgens for binding. To determine whether ADEK has this common feature of AR antagonists, the competitive androgen binding assay was performed. The affinity of ligands for the AR was assessed by incubating whole-cell extracts of LNCaP or COS-1 with transfected WT AR with 1 nM [3H]R1881 in the presence of various concentrations (1–10,000 nM) of unlabeled DHT, HF, casodex, or ADEK. As described (19), the relative binding affinity (RBA) values were calculated from the constructed competitive binding curves as the ratio of concentration of unlabeled ligand and concentration of DHT required to inhibit [3H]R1881 binding by 50% (Table 1). Competitive RBAs in LNCaP cells were DHT > casodex > HF > ADEK. Similar results were obtained in WT AR-transfected COS-1 cells, although the RBAs are lower and binding of all of the compounds in competition with [3H]R1881 was weaker.
These results confirm that ADEK also competes significantly with androgen for AR binding.

Anti-Adiol Effect of ADEK on AR Transcription. In a previous report (13), we found that Adiol, which is produced from DHEA and can be converted to testosterone, also possesses intrinsic androgen activity. Among androgens it is unique in that both HF and casodex failed to block significantly Adiol-induced AR transcription in prostate cancer cells. Because castration with or without combination therapy with antiandrogen decreases the serum concentration of Adiol by only 40–50% (20, 21), our previous findings suggested that current combined androgen blockade treatment might be insufficient to block Adiol’s action in AR-positive prostate cancer. Therefore, we determined whether ADEK inhibited Adiol-induced AR transcription by measuring MMTV-Luc activity. As shown in Fig. 4, Adiol at 2.5 nM increases AR transcriptional activity in PC-3 and LNCaP to 4.5- and 2.8-fold, respectively, over mock treatment (lanes 1 vs. 2). ADEK represses Adiol-induced AR transcription up to 43%.

Table 1. Summary of AR ligand binding affinity

<table>
<thead>
<tr>
<th>Ligand</th>
<th>RBA value in LNCaP</th>
<th>RBA value in COS-1 with AR</th>
</tr>
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<tbody>
<tr>
<td>DHT</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>HF</td>
<td>23.0</td>
<td>17.1</td>
</tr>
<tr>
<td>Casodex</td>
<td>36.4</td>
<td>25.5</td>
</tr>
<tr>
<td>ADEK</td>
<td>11.1</td>
<td>6.0</td>
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</table>

RBA values were calculated from the constructed competitive binding curves as the ratio of concentration of unlabeled ligand and concentration of DHT required to inhibit [3H]R1881 binding by 50%. The RBA of DHT was set as 100.

Fig. 3. The effects of ADEK on PSA expression and cell proliferation. (A) Cell extracts from LNCaP cells cultured for 48 h with 1 μM HF, casodex, or ADEK, in the presence or absence of 1 nM DHT as indicated, were analyzed on Western blots by using an antibody to the PSA. The 33-kDa protein was detected as indicated and quantitated. β-Actin expression was used as an internal control. The normalized expression level in the second lane (DHT treatment) was set as 100%. Values represent the mean ± SD of three separate experiments. (B) LNCaP cells were cultured with 1 μM HF, casodex, or ADEK in the presence or absence of 1 nM DHT as indicated. Total cell number was counted by hemocytometer. Values represent the mean of at least three determinations.

Fig. 4. The effects of ADEK on the Adiol-induced transcriptional activity of AR. PC-3 or LNCaP cells were transfected with MMTV-Luc. The WT AR expression plasmid pSG5-AR was cotransfected in PC-3 cells. After transfection, cells were cultured for 24 h in the presence or absence of 2.5 nM Adiol and 1 μM HF, casodex, or ADEK as indicated. The Luc activity is presented relative to that in the presence of Adiol (black bars; set as 100%). Values represent the mean ± SD of at least three determinations.

Fig. 5. The effects of ADEK on the transcriptional activity of AR, progesterone receptor (PR), glucocorticoid receptor (GR), and estrogen receptor (ER). PC-3 cells were transfected with steroid receptor and its reporter (AR/MMTV-Luc, PR/MMTV-Luc, GR/MMTV-Luc, or ER/ERE-Luc). After transfection, cells were cultured for 24 h in the presence or absence of ligand [10 nM DHT, progesterone (P), dexamethasone (Dex), or 17β-estradiol (E2)] or increasing concentrations of ADEK. The Luc activity is presented relative to that of EtOH treatment (white bars; set as 1-fold). Values represent the mean ± SD of at least three determinations.
and 58% in PC-3 and LNCaP, respectively (Fig. 4, lanes 2 vs. 5), whereas HF and casodex fail to block it (Fig. 4, lanes 3 and 4). These results suggest that ADEK can suppress AR transactivation induced by classic androgens as well as adrenal androgen.

**Steroid Hormone Specificity of ADEK.** To determine whether ADEK possesses any steroid hormone activity, PC-3 cells were transfected with steroid receptor/reporter (progesterone receptor/MMTV-Luc, glucocorticoid receptor/MMTV-Luc, or estrogen receptor/ERE-Luc). As shown in Fig. 5, ADEK has some estrogenic activity, but this compound has no progesterone or glucocorticoid activity as well as no androgenic activity. This result is consistent with our previous findings that many DHEA metabolites may have some estrogen activity (14).

**Conclusion**

We have tested whether DHEA metabolites and some related steroids can block DHT-induced AR transactivation and have found a compound, ADEK, as a potential antiandrogenic drug to compete with androgens and block their action on both WT AR and a mutant AR derived from LNCaP. The compound inhibited PSA expression and growth in prostate cancer cells. Its binding affinity to the AR was sufficient for the competition with androgen. These results suggest that ADEK can control androgen-dependent prostate cancer progression. Moreover, because the androgenic activity of ADEK was very low and could not be induced by an AR coactivator, this compound might carry fewer risks of withdrawal response if used for therapy in prostate cancer patients. Further studies may allow us to develop a new and better antiandrogen to block AR-mediated prostate cancer growth.

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