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, Junghhee 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, Junghhee 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
BIOCHEMISTRY. 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 Δ5-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, 15, 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.
3β-Acetoxyandrost-1,5-diene-17-ethylene ketal functions as a potent antiandrogen with marginal agonist activity

Hiroshi Miyamoto*, Padma Marwah†, Ashok Marwah†, Henry Lardy‡, and Chawshang Chang*†

*George Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Radiation Oncology, and The Cancer Center, University of Rochester, Rochester, NY 14642; and †Institute for Enzyme Research, Department of Biochemistry, University of Wisconsin, Madison, WI 53726

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-ethylen 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.

Androgens 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 recurrence, 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, of-
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 H9252-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-en-17-one; 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 compounds 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 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, AR-A70, 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. 3A, 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 transactivation 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>
</thead>
<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>
</tr>
</tbody>
</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.
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.

This work was supported by the George Whipple Professor Endowment, National Institutes of Health Grants 60905 and 60948, and Hollis Eden Pharmaceuticals.