Suppressive effects of dehydroepiandrosterone and the nuclear factor-κB inhibitor parthenolide on corticotroph tumor cell growth and function *in vitro* and *in vivo*

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Abstract

Dehydroepiandrosterone (DHEA) is believed to have an anti-tumor effect, as well as anti-inflammatory, antioxidant, and anti-aging effects. To clarify the possible inhibitory action of DHEA on pituitary tumor cells, we tested the effects of DHEA, alone or in combination with the nuclear factor-κB (NF-κB) inhibitor parthenolide (PRT), on AtT20 corticotroph cell growth and function both *in vitro* and *in vivo*. We found that, *in vitro*, DHEA and PRT had potent inhibitory effects on pro-opiomelanocortin and NF-κB-dependent gene expression. They also suppressed the transcription activity of survivin, a representative anti-apoptotic factor, and induced apoptosis in this cell line. Furthermore, using BALB/C nude mice with xenografts of AtT20 cells *in vivo*, we found that the combined administration of DHEA and PRT significantly attenuated tumor growth and survivin expression. The treatment also decreased the elevated plasma corticosterone levels and ameliorated the malnutrition induced by tumor growth. Altogether, these results suggested that combined treatments of DHEA and PRT potently inhibit the growth and function of corticotroph tumor cells both *in vitro* and *in vivo*. This effect may, at least partly, be caused by the suppressive effects of these compounds, such as survivin and other inhibitor of apoptosis proteins, on NF-κB-mediated gene transcription.

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Introduction

Adrenocorticotropin (ACTH)-dependent Cushing's syndrome, induced either by a corticotroph tumor in the pituitary or by an ectopic ACTH-producing tumor, is a life-threatening disease, causing metabolic derangements such as diabetes mellitus, hypertension, atherosclerosis, and immune dysfunction (Arnaldi *et al.* 2003). Although surgical treatments are essential for the disorder, medical treatment is sometimes necessary in cases with incomplete removal of the tumor, recurrence, or in cases unsuitable for surgical procedure. Paez-Perada *et al.* (2001) showed that retinoic acid, an inducer of cell differentiation, has potent inhibitory effects on Cushing's adenoma using AtT20 mouse corticotroph tumor cells as a model system. More recently, Heaney *et al.* (2002) reported the effectiveness of thiazolidinedione (TZD), a peroxisome proliferator-activated receptor (PPAR)-γ agonist, on this disorder, again using the AtT20 cell xenograft. Although the precise mechanism of the effect of TZD on the pituitary tumor cell has not yet been clarified, recent studies suggest that TZD acts as an inducer of apoptosis in some other tissues (Lu *et al.* 2005, Shiau *et al.* 2005).

Dehydroepiandrosterone (DHEA) is an adrenal steroid which acts as a precursor of sex steroids such as androgen and estrogen (Miller 2002). Besides its effects as a prohormone, DHEA itself is suggested to have a variety of beneficial effects such as anti-tumor, anti-diabetic, anti-atherosclerotic, and anti-aging effects (Aoki *et al.* 2003). Some of these effects are thought to be mediated by its antioxidant properties via inhibition of nuclear factor-κB (NF-κB) (Aragno *et al.* 2002, Iwasaki *et al.* 2004), which is a transcription factor known to exert anti-apoptotic effects in neoplasia. DHEA is indeed reported to elicit cellular apoptosis in both tumor and non-tumor cells *in vitro* (Yang *et al.* 2000, Liang *et al.* 2004). Thus, it is possible that, like TZD, DHEA may exert negative effects on corticotroph tumor cell growth and function by inhibiting the NF-κB signaling pathway.

The fate of normal cells is determined by the regulation of the apoptotic mechanism, a system of programmed cell death controlling cellular survival. In contrast, this mechanism is known to be inhibited in tumor cells, by the expression of anti-apoptotic factors such as inhibitors of apoptosis proteins (IAPs) (Ambrosini *et al.* 1997, Salvesen & Duckett 2002, Schimmer 2004). Thus, induction of

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apoptosis by targeting these proteins is expected to be applicable for clinical cancer therapy (LaCasse *et al.* 1998). Although various IAPs are known to be expressed in tumor cells, some studies suggest that many of these factors, including survivin (Ambrosini *et al.* 1997, Swana *et al.* 1999), a representative IAP, are induced by the activation of the NF-κB pathway (LaCasse *et al.* 1998, Kawakami *et al.* 2005). Specific inhibition of this transcription factor is shown to induce apoptosis (Umezawa & Chaicharoenpong 2002, Horiguchi *et al.* 2003). In this regard, a sesquiterpene lactone, parthenolide (PRT), may be an appropriate candidate, because it exerts a potent inhibitory effect on the NF-κB pathway with low toxicity. The effectiveness of this compound has already been shown for a variety of human tumor cells (Nakshatri *et al.* 2004). Furthermore, it is more effective when used in combination with conventional chemotherapy and radiotherapy (Patel *et al.* 2000, Riggins *et al.* 2005).

In this study, we have examined the effects of DHEA and PRT on ACTH-secreting pituitary tumors applying an *in vitro* and *in vivo* experimental model system previously used for the examination of medical therapy for Cushing's disease (Heaney *et al.* 2002). Because DHEA and PRT are not toxic *in vivo*, and both are known to have apoptosis-inducing as well as anti-NF-κB properties, we expected the combination of these reagents to have a more significant effect. Indeed, we found in this study that DHEA and PRT, either alone or in combination, inhibited corticotroph cell function *in vitro*, and that administration of both DHEA and PRT significantly attenuated the tumor growth *in vivo*. We also found that the treatment significantly reduced the expression of IAPs such as survivin and NF-κB-dependent transcription.

Materials and Methods

Materials

NF-κB–luciferase reporter plasmid was purchased from Stratagene (La Jolla, CA, USA). DHEA was obtained from Wako Pure Chemicals (Osaka, Japan) and 17β-estradiol (E_2), testosterone, and PRT were from Sigma Chemical Co.

Cultivation and transfection

AtT20/D16 v mouse corticotroph tumor cells, or a stable transformant of the cell (AtT20PL) in which the rat pro-opiomelanocortin (POMC) gene 5′-promoter (−708 to +64; +1 indicates the transcription start site)–luciferase fusion gene was stably incorporated, was used in this study. Cells were maintained in a T_{75} culture flask with Dulbecco's modified Eagles' medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) under a 5% CO_2–95% air atmosphere at 37 °C. For each experiment, cells were plated in 24-well culture dishes with approximately 70% confluence. For transient transfection experiments, AtT20 cells were transfected with a variety of reporter plasmids using the lipofection method.

Cell culture experiments

On the day of the experiment, stock solutions of test substances (DHEA, E_2, PRT, and testosterone) were added directly to the serum-free culture medium of each dish, and the cells were incubated for 24 h. At the end of each experiment, the culture media were removed, and the cells were harvested using lysis buffer. Luciferase assay for determining the promoter activity of POMC, survivin, and NF-κB–luciferase was performed using a luciferase assay system (Promega) according to the manufacturer's instructions. ACTH secreted into the culture medium was measured by immunoradiometric assay (ACTH immunoradiometric assay kit; Mitsubishi Chemical, Tokyo, Japan).

RT-PCR

RNA was isolated using TRIZOL reagent (Invitrogen), and 2 μg of the total RNA was used for the RT reaction by Superscript III reverse transcriptase (Invitrogen). The cDNAs obtained were then amplified by PCR with Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). The sequences of primer sets for amplifying mouse IAPs were as follows: survivin forward, 5′-GCGGAGGCTG GCTTCA-3′, reverse, 5′-CTTGGCTCTCTGTCTGT CCA-3′; mouse neuronal apoptosis inhibitory protein (mNAIP) forward, 5′-ACATCACCACGTGTACTC TCA-3′, reverse, 5′-GGCTTCTGGAAGTGCACA GTG-3′; Bruce forward, 5′-GATCTTGTTGCTAGAC ACTGC-3′, reverse, 5′-GGTCTCCCTTCTGTTAGC TTC-3′; X-linked inhibitor of apoptosis (XIAP) forward, 5′-GCAATGTTTCAGTTGTCATGCG-3′, reverse, 5′-CCAGCACTAGCTAACTCTCTG-3′; inhibitor of apoptosis protein 1 (IAP1) forward, 5′-GACAAGGTCA AGTGCTTCTGC-3′, reverse, 5′-CTTACGTTCCCA GTTGCTCAG-3′; inhibitor of apoptosis protein 2 (IAP2) forward, 5′-GTGTGAACTCTACCGAATGTC-3′, reverse, 5′-CTGCGGTGCTCTGACATAGC-3′.

Quantitative real-time RT-PCR

RNA was isolated from liver and tumor tissues using Sepasol-RNA1 Super (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's instructions. cDNA was synthesized from 2 μg RNA with Superscript III reverse transcriptase in the presence of 1 μg random hexamer primers and 0·5 mM dNTPs (Promega). In survivin cDNA-specific quantitative RT-PCR assay, the reaction

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mixture contained 10 μM survivin-forward and 10 μM survivin-reverse primers, 10 μM TaqMan probe, 12·5 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), H_2O, and 2 μl cDNA, in a total volume of 25 μl. After enzyme activation (2 min at 50 °C and 10 min at 95 °C), 40 two-step cycles were performed (15 s at 95 °C and 60 s at 60 °C) by ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Mouse survivin primers and TaqMan probe were purchased from Applied Biosystems and designed as follows (Aziz *et al.* 2004): forward mouse survivin primer, 5′-GCGGAGGC TGGCTTCA-3′, reverse mouse survivin primer, 5′-CTTGGCTCTCTGTCTGTCCA-3′; TaqMan mouse survivin probe, 5′-CCACTGCCCTACCGAGAACGA GCC-3′. These yielded a 216 bp product. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers as an internal control were also purchased from Applied Biosystems. A standard curve was generated on the basis of a linear relationship between the first cycle number at which the fluorescence signal significantly increased (Ct value) and the logarithm of the starting quantity. The quantity of target mRNA in an unknown sample was determined from the Ct value using the standard curve. A control without template was included in each experiment. Non-template controls, standard dilutions, and samples were assayed in duplicate. After the relative gene expression levels for survivin value were established for each sample, these values of survivin were used for statistical analyses.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out using a Light Shift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, USA), following the manufacturer's instructions. Briefly, the extracted NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGG-3′) were labeled with ^{32}P-ATP using T_4 polynucleotide kinase and purified on QIAquick nucleotide removal kit (Qiagen). Cells were treated with DHEA (100 μM) or PRT (10 μM) for 4 h. For the EMSA, 50 μg nuclear proteins were used. Briefly, the samples were incubated with 100 000 c.p.m. ^{32}P-labeled NF-κB oligonucleotide probe for 30 min at room temperature in binding buffer containing 35 mm HEPES–KOH, pH 7·8, 0·5 mm EDTA, 0·5 mm dithiothreitol, 10% glycerol, 0·25 mm Spermidine, and 0·1 μg/μl poly (deoxyinosine-deoxycytidine) in a final volume of 40 μl. Protein–DNA complexes were resolved by electrophoresis through 5% native polyacrylamide gels containing 10% glycerol and 1 × Tris–glycine buffer. Gels were dried under vacuum and exposed for 48–72 h to Amersham Hyperfilms (Amersham Pharmacia Biotech) at 80 °C with intensifying screens. Specificity of binding was ascertained by competition with a 25-fold molar excess of unlabeled oligonucleotides.

Immunocytochemistry

AtT20 cells (5×10^3 cells/well) were plated on two-well chamber culture slides and treated with DHEA (100 μM) for 24 h. The cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7·4, for 10 min at room temperature, and incubated overnight at 4 °C with anti-NF-κB (p65) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Goat F(ab′)2 anti-mouse IgG1 fluorescein isothiocyanate (FITC) conjugate was used at a dilution of 1:200. For all fluorescent immunostaining, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 1:10 000 dilution) in 1 × PBS. The coverslips mounted with DAKO fluorescent mounting medium (DAKO Corp., Carpinteria, CA, USA) were then examined with a fluorescent microscope (Olympus, Tokyo, Japan).

Flow cytometry

Flow cytometry was performed as described in the MEBCYTO apoptosis kit (Medical and Biological Laboratories Co. Ltd, Nagoya, Japan). The Ca^{2+}-dependent phospholipid-binding protein annexin V was used as a probe for identifying cells early in apoptosis. Unfixed AtT20 cells (2×10^5 cells) which had been treated with DHEA (100 μM) and E_2 (1 μM) were washed twice with PBS and resuspended in a binding buffer containing 10 mM HEPES–NaOH (pH 7·5), 140 mM NaCl, and 2·5 mM $CaCl_2$. Cells were then incubated for 15 min with annexin V-FITC (PharMingen, San Diego, CA, USA) and propidium iodide (PI; 100 μg/ml), and were subsequently analyzed by flow cytometry with a fluorescence-activated cell sorter (FACStar; Becton Dickinson, San Jose, CA, USA).

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed using an MTT assay kit (Sigma) according to the manufacturer's instructions. Briefly, 15 mg MTT were reconstituted in 3 ml Hank's balanced solution (Invitrogen). After AtT20 cells were treated with DHEA (100 μM) and E_2 (1 μM) for 24 h, the medium was removed, and 20 μl solubilized MTT was added to each well. The plates were then incubated for 3 h at 37 °C to obtain blue formazan crystals, at which time the crystals were treated with 180 μl MTT solubilization solution. Plates were read immediately on a microplate reader.

Xenograft experiments

All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by Kochi Medical School.

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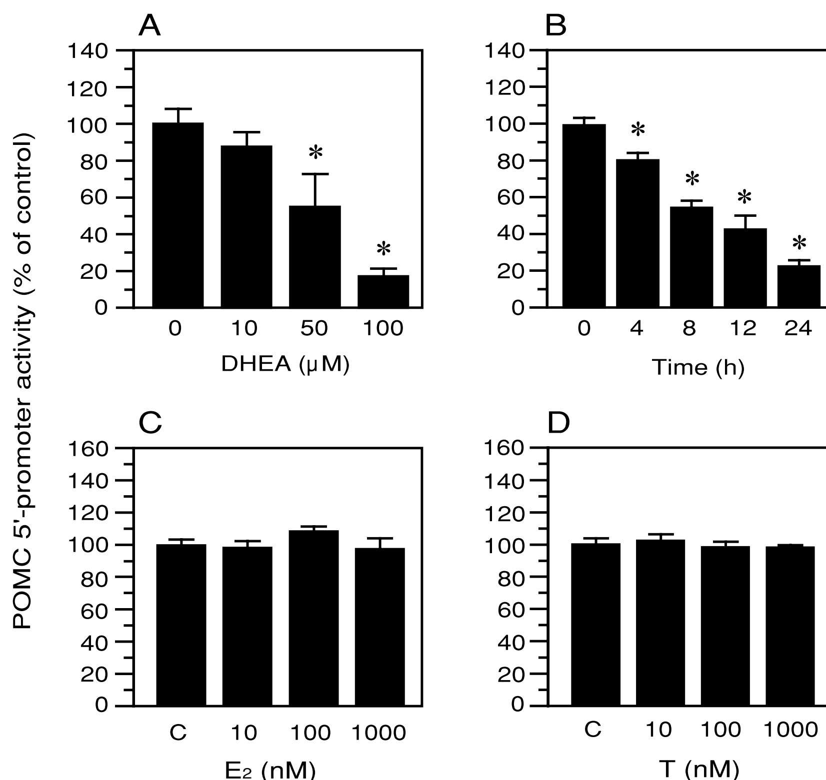

Figure 1 Effects of DHEA, E_2 and testosterone (T) on POMC gene 5′-promoter activity in AtT20PL cells. (A and B) Cells were treated with various concentration of DHEA (10–100 μM for 24 h), or with DHEA (100 μM) for defined time-intervals (4–24 h), and transcription activity of the POMC gene was determined by luciferase assay. *P<0·05 vs control [C]. (C and D) Similar experiments were performed with the two major metabolites of DHEA, (C) E_2 (10–1000 nM for 24 h) or (D) testosterone (10–1000 nM for 24 h).

Sixteen male BALB/C nu/nu mice at 8 weeks of age (approximate 22–24 g) were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained in pathogen-free conditions with irradiated chow.

Mice were subcutaneously injected with AtT20 cells (5×10^6/mouse) in matrigel (200 μl) and divided randomly into four groups (four mice each): control (vehicle only) group, DHEA group, PRT group, and DHEA+PRT group. DHEA (8 mg/mouse per day) and/or PRT (200 μg/mouse per day) dissolved in vehicle (1 vol. 95% ethanol mixed with 8 vol. mineral oil) (Aragno *et al.* 2002) were administered subcutaneously for 21 days from 1 week after AtT20 cell injection. At the end of the experiment, mice were killed, and tumor weights were measured after their careful resection and the tumors and livers were used for quantitative RT-PCR assay. Trunk blood was also collected for the corticosterone assay at the same time of day in all experiments to avoid diurnal variation. The plasma corticosterone levels were measured using commercially available RIA kits (ICN Biomedicals Inc., Costa Mesa, CA, USA).

Data analysis

All data are expressed as the means ± S.E.M. When the statistical analyses were performed, data were compared by one-way ANOVA with Fisher's protected least squares difference test, and P<0·05 was considered significant.

Results

DHEA, but not E_2 or testosterone, inhibits POMC gene transcription in AtT20 cells

DHEA inhibited the 5′-promoter activity of the POMC gene in dose- and time-dependent manners. Significant inhibition was observed at 50 μM or above, with the maximal suppressive effects at 24 h (Fig. 1A and B). This effect appeared not to be mediated by its metabolites E_2 or testosterone, because effective concentration of both hormones (10 nM–1 μM) did not show similar effects (Fig. 1C and D). These data suggest that a pharmacological concentration of DHEA inhibits corticotroph cell function *in vitro*.

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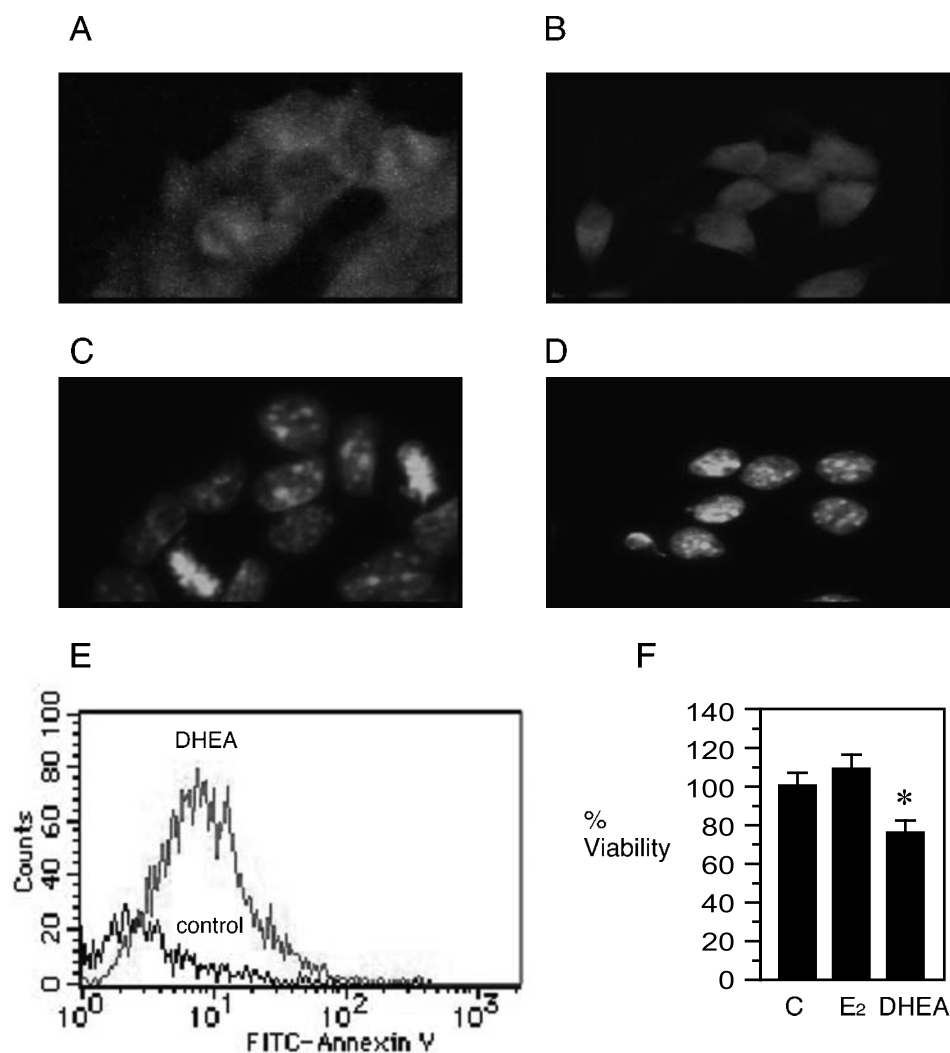

Figure 2 (A and B) Effects of DHEA on AtT20PL cells using immunocytochemical staining of NF-κB. AtT20PL cells were treated with (A) vehicle or (B) DHEA (100 μM) for 24 h, then stained using an antibody against NF-κB p65 subunit, and analyzed by fluorescent microscopy (×20). (C and D) Effect of DHEA on nuclear structure. AtT20PL cells were treated with (C) vehicle or (D) DHEA (100 μM), and stained with DAPI as described in the Materials and Methods. (E and F) Cellular apoptotic change following DHEA treatment. (E) AtT20 cells were incubated with medium (control) or DHEA (100 μM for 24 h), and then the expression pattern of annexin V was analyzed by a flow cytometric assay. (F) Alternatively, changes in cell viability were also examined using an MTT assay. E_2 was used as the active control. * $P<0{\cdot}05$ vs control (C).

DHEA induces apoptosis in AtT20 cells

To clarify the mechanism responsible for the suppressive effect of DHEA on corticotroph tumor cell function, we then examined the morphological effect of DHEA on AtT20 cells by the immunofluorescence technique using NF-κB-specific antibody. In control cells, NF-κB p65 protein was distributed in both cytoplasm and nuclei (Fig. 2A). When cells were treated with DHEA (100 μM for 24 h), the staining pattern was not changed, but most of the cells shrank in size (Fig. 2B). Furthermore, nucleus-specific staining by immunofluorescent cytochemistry revealed marked condensation of the nuclear structure, suggesting the occurrence of apoptosis in DHEA-treated cells (Fig. 2C and D). To confirm the induction of apoptosis, we examined the effect of DHEA on AtT20 cells using a flow cytometric assay. We found that annexin V-positive and PI-negative cells, suggestive of apoptotic changes, were increased by DHEA compared with control (Fig. 2E), whereas E_2 had no effect (data not shown).

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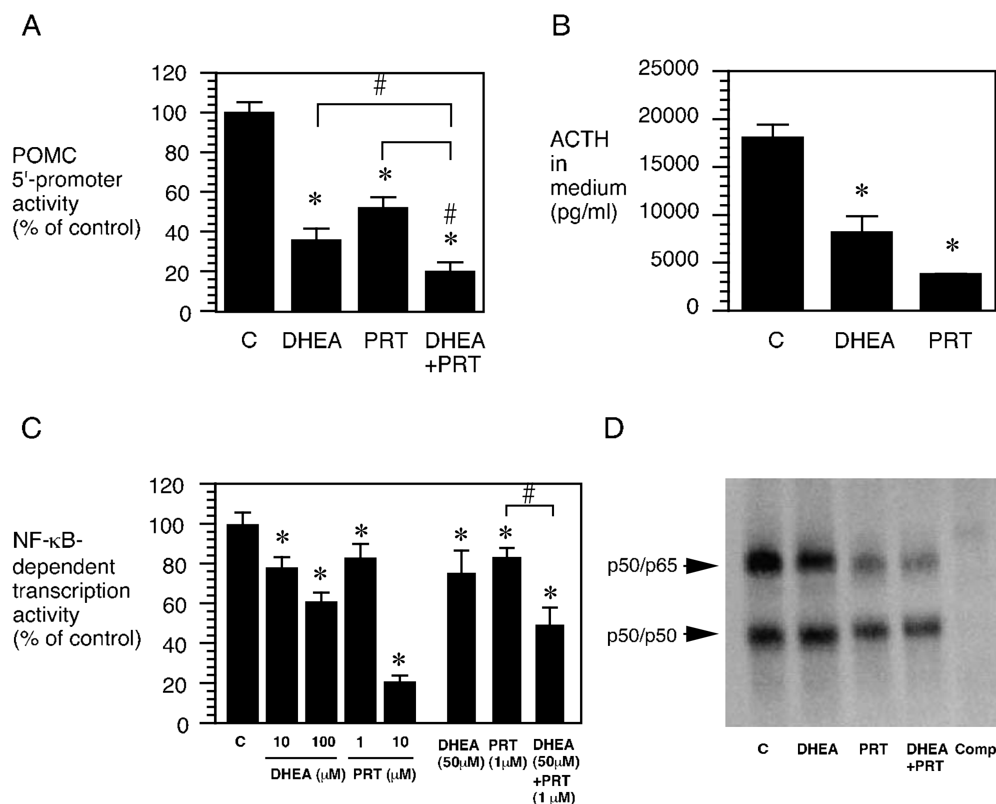

Figure 3 Effects of DHEA and PRT on corticotroph cell function. (A) AtT20PL cells were treated with DHEA (100 μM) and/or PRT (7 μM), and transcription activity of the POMC gene was determined by luciferase assay. *$P<0{\cdot}05$ vs control [C], #$P<0{\cdot}05$ vs DHEA+PRT. (B) Cells were treated as above, and ACTH secreted into culture medium during the incubation (24 h) was estimated by immunoradiometric assay. *$P<0{\cdot}05$ vs control [C]. (C) AtT20NF cells, in which NF-κB-responsive element (5 ×)-luciferase reporter gene was stably incorporated, were treated with DHEA (10–100 μM) and/or PRT (1, 10 μM), and NF-κB-dependent transcription activity was determined by luciferase assay. *$P<0{\cdot}05$ vs control [C], #$P<0{\cdot}05$ vs PRT alone. (D) Effects of DHEA and PRT on the DNA binding of NF-κB. AtT20PL cells were treated with DHEA (100 μM) and/or PRT (10 μM) for 4 h. Nuclear protein was extracted, and then applied to EMSA analysis using NF-κB-specific DNA probe. Comp, competition.

Significant reduction of cell viability by DHEA but not by E_2 was also shown by MTT assay (Fig. 2F). Thus, the above results strongly suggest that DHEA suppresses corticotroph tumor cell function by inducing apoptosis.

DHEA inhibits NF-κB-mediated transcription

Recent studies suggest that the transcription factor NF-κB is activated in a variety of tumor cells and exerts an anti-apoptotic effect (Pikarsky *et al.* 2004). Since DHEA is shown to have inhibitory effects on NF-κB-dependent transcription in other types of cell lines (Iwasaki *et al.* 2004), we speculated that DHEA induces apoptosis in AtT20 cells by inhibiting NF-κB. To confirm this hypothesis, we examined the NF-κB inhibitor PRT on POMC expression, with the result that PRT as well as DHEA significantly inhibited both POMC gene transcription and ACTH secretion (Fig. 3A and B). Furthermore, the combination of DHEA and PRT showed an additive, more suppressive effect on POMC gene expression. A similar effect was observed on NF-κB-mediated gene transcription (Fig. 3C). The inhibitory effects of these compounds were confirmed by EMSA analysis, although the effect of DHEA alone was less marked than that of PRT (Fig. 3D). Altogether, these results suggest that the inhibitory effect of DHEA on corticotroph cell function is, at least in part, mediated by suppression of NF-κB-mediated gene expression, with resultant induction of cellular apoptosis, although the additive effect with PRT suggests an NF-κB-independent mechanism as well.

DHEA inhibits the expression of survivin, a representative inhibitor of apoptosis protein

Recent studies suggest a pivotal role of the IAPs in maintaining the immortality of tumor cells (Yang *et al.*

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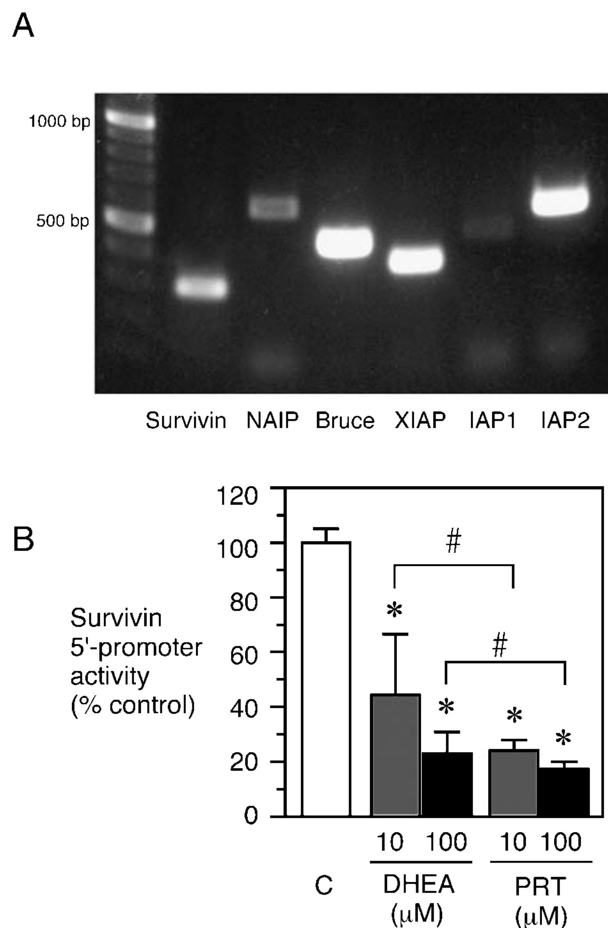

Figure 4 (A) Expression of IAPs mRNA in AtT20 cells. Total RNA was extracted from the cells, and the expression of IAPs (survivin, mNAIP, Bruce, XIAP, IAP1, IAP2) was analyzed by RT-PCR using primer sets specific to the mRNA sequence of each gene. (B) Effects of DHEA and PRT on the 5′-promoter activity of survivin gene in AtT20 cells. Cells were transfected transiently with survivin 5′-promoter–luciferase reporter plasmid, and treated with DHEA (10, 100 μM) or PRT (10, 100 μM) for 24 h. Then the transcription activity of survivin gene was determined by luciferase assay. *$P<0{\cdot}05$ vs control [C]. #$P<0{\cdot}05$ vs values with 10 μM test substance.

2003). Since the expressions of many of these IAP genes are known to be stimulated by an NF-κB-dependent mechanism, we examined the expression of IAPs in AtT20 cells by RT-PCR. The results showed that a variety of IAPs such as survivin, mNAIP, Bruce, XIAP, IAP1, and IAP2 were expressed in AtT20 cells (Fig. 4A). We then focused on the expression of survivin, a representative IAP expressed in the AtT20 cell line. When cells were treated with DHEA or PRT, the transcription activity of survivin was significantly attenuated in a dose-dependent manner (Fig. 4B). Since the promoter activity of survivin has been shown to be regulated by NF-κB (Kawakami *et al.* 2005), our results raise the possibility that DHEA and PRT inhibit the expression of IAPs like survivin and induce apoptosis through inhibition on the NF-κB pathway.

DHEA/PRT inhibits tumor growth and decreases plasma corticosterone in vivo *in nude mice with AtT20 cell xenografts*

We then examined the effects of DHEA and PRT on corticotroph tumor growth and function *in vivo* using xenografts of AtT20 cells in nude mice, an animal model of ACTH-dependent Cushing's disease used in previous studies (Heaney *et al.* 2002). In control (vehicle) mice transplanted with AtT20 cells, significant tumor growth with body weight loss was observed during the time of the examination (21 days) (Figs 5 and 6A and B). The decrease in body weight was significantly attenuated by the administration of DHEA but not by PRT (Fig. 6A), and neither treatment inhibited the tumor growth (Fig. 6B). Interestingly, however, the combined administration of both DHEA and PRT significantly suppressed both body weight loss and tumor growth (Fig. 6A and B). Indeed, mice receiving the combined treatment had a markedly smaller tumor size and looked much less cachexic, compared with control mice treated with vehicle alone (Fig. 5).

To examine the possible deleterious effects of the combined administration of DHEA and PRT on non-tumor cells, we examined the expression level of survivin mRNA in xenografts and in liver cells as a control, by quantitative RT-PCR. We found that survivin mRNA was detected in normal hepatocytes as well as AtT20 xenograft cells by conventional RT-PCR (Fig. 6D). When the expression level of survivin using quantitative RT-PCR was examined, we found that the combined treatment of the mice with DHEA and PRT significantly decreased the expression level of survivin mRNA in xenograft cells *in vivo* (Fig. 6C), whereas the same treatment did not influence survivin expression in normal hepatocytes (Fig. 6D).

Finally, we examined the plasma corticosterone levels in the nude mice xenograft model. We found that the plasma corticosterone level was significantly lower in the mice treated with DHEA alone or both DHEA and PRT, and that there was no statistically significant difference between the two groups.

Discussion

Although the first choice in the treatment of ACTH-dependent Cushing's disease, either of pituitary origin or ectopic, is removal of the tumor by surgical procedure, medical therapy is sometimes needed for patients with unresectable tumor and/or recurrence/metastasis (Hammer *et al.* 2004). Recent studies suggest that retinoic acid and TZD such as rosiglitazone are the possible

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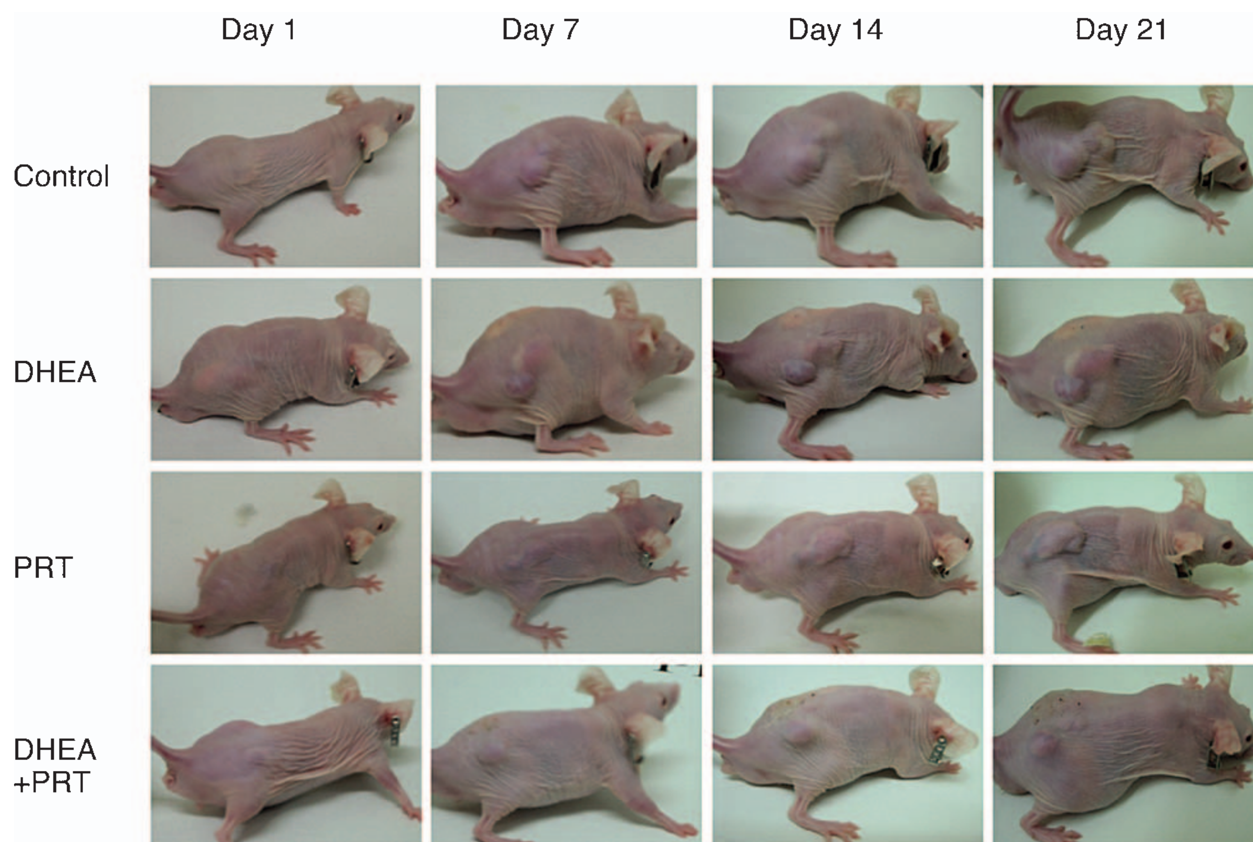

Figure 5 Effects of DHEA and/or PRT on tumor growth in the nude mouse/AtT20 xenograft model *in vivo*. Mice were divided into four groups (control, DHEA, PRT, and DHEA+PRT; $n=4$ in each group). All the mice received a subcutaneous xenograft of AtT20 cells (5×10^6/mouse), and 7 days later they started to receive treatment with vehicle (control) or with DHEA (8 mg/mouse per day) and/or PRT (200 μg/mouse per day) for 21 days. During the experiment, the appearance of tumor mass and the nutritional condition of each mouse with xenografts were serially recorded. Representative photographs of mice in each group are shown.

candidate drugs for clinical application, and promising preliminary data are reported in the latter case (Heaney *et al.* 2003). In this study, we have examined the possible effects of DHEA and PRT, both of which have anti-NF-κB activity with apoptosis-inducing properties on tumor cells. We found that, *in vitro*, both compounds decreased the activity of corticotroph tumor cells by inducing apoptosis. Furthermore, *in vivo*, the combination of the two drugs significantly suppressed tumor growth as well as function in mice receiving AtT20 xenografts.

DHEA is usually recognized as an adrenal sex steroid precursor, which is known to be converted to testosterone and E_2 in peripheral organs (Ebeling & Koivisto 1994). However, DHEA may also be expected to have a variety of beneficial effects, and is sometimes used as a supplement in healthy and, especially, aged people (Baulieu 1996), although the receptor for DHEA has not been identified and the molecular mechanism remains to be clarified (Widstrom & Dillon 2004). In the present study, we found an inhibitory effect of DHEA on POMC gene transcription in AtT20 cells *in vitro*. This effect seems not to be mediated by E_2 or testosterone, because no direct effects of these metabolites were observed. However, the effective concentration of DHEA needed was much higher than that in the peripheral blood of healthy subjects (10–30 nM) (Iwasaki *et al.* 2004). This suggests that a pharmacological concentration of DHEA is needed to exert an anti-tumor effect. Recently, Iwasaki *et al.* (2004) reported that a high dose of DHEA and/or DHEA-sulfate exerts anti-NF-κB effects in hepatocytes. Furthermore, a pharmacological dose of DHEA is reported to have anti-tumor effects by inducing apoptosis (Yang *et al.* 2000). Since NF-κB is an anti-apoptotic transcription factor, it is possible that DHEA exerts its anti-tumor effect by inhibiting NF-κB and subsequently inducing apoptosis. Indeed, in our study, we found that treatment of the cells with DHEA caused morphological changes such as nuclear condensation, which could be compatible with the occurrence of apoptosis. Furthermore, flow cytometric analysis showed the increase in annexin

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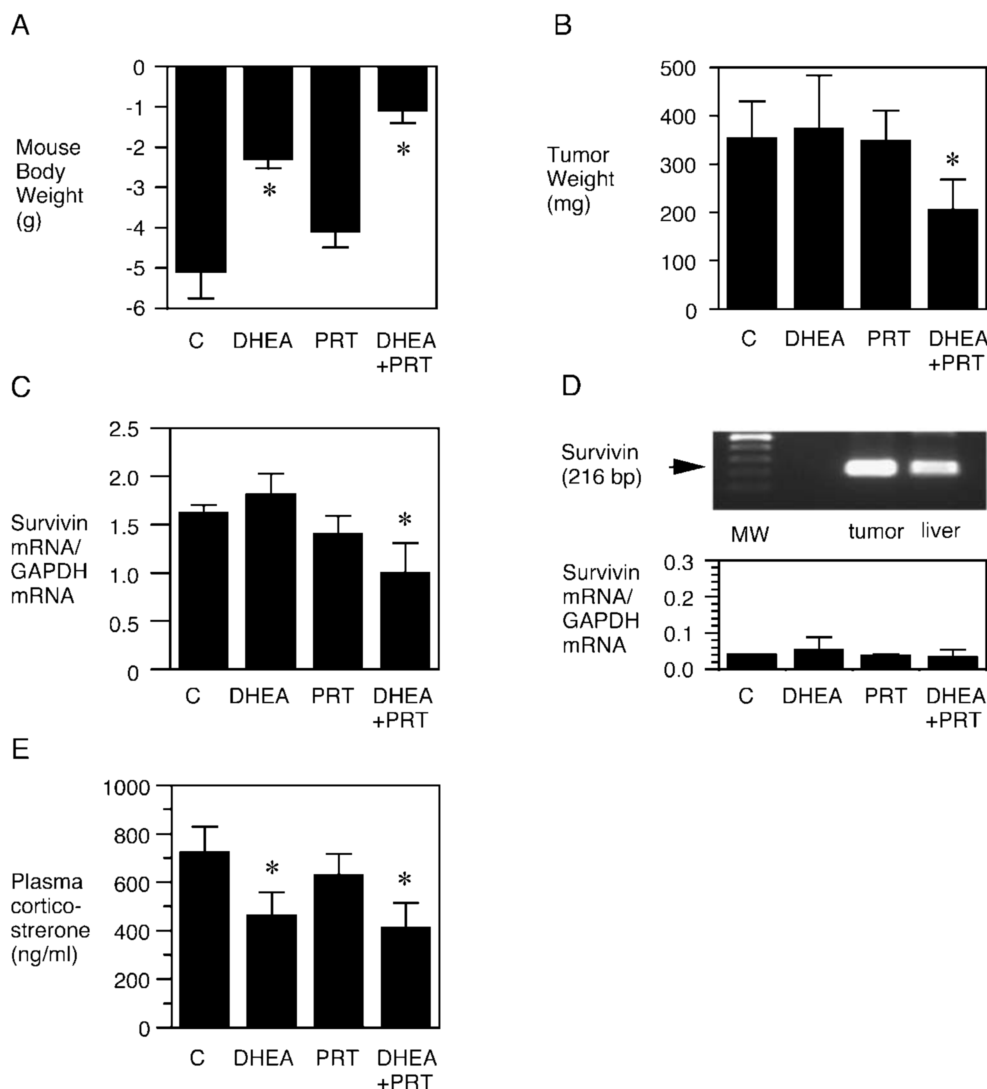

Figure 6 Effects of DHEA and/or PRT on various parameters in mouse xenograft model. (A and B) Mouse body weight and tumor weight. (A) Body weight in each mouse was determined at the end of the experiment (day 21), and compared with that at day 1. (B) At the same time, the subcutaneous tumor was resected and weighed. *P<0·05 vs control [C]. (C) Survivin mRNA in the xenograft. Total RNA was isolated from the grafted tumor cell at the end of experiment (day 21), and the expression level of survivin was determined by quantitative RT-PCR. GAPDH mRNA level was also estimated and used as an internal control. *P<0·05 vs control [C]. (D) Survivin mRNA in normal tissue (liver). Total RNA was isolated from hepatic tissues, and analyzed by conventional RT-PCR (upper panel) or by quantitative real-time RT-PCR as control. (E) Plasma corticosterone levels. Peripheral blood of each mouse was obtained at the end of experiment (day 21), and applied for the RIA of corticosterone. * P<0·05 vs control [C].

V-positive and PI-negative cells following DHEA treatment, further confirming the apoptosis-inducing effect of the hormone.

NF-κB is a transcription factor which is involved in inflammation/cytokine-mediated gene transcription. However, the factor is frequently activated in tumor cells, and possibly makes the cells anti-apoptotic and immortalized. In AtT20 cells, NF-κB is suggested to be involved in POMC gene transcription following immunological stimuli (Kovalovsky *et al.* 2004). Furthermore, it is well known that NF-κB induces a variety of IAPs such as survivin, mNAIP, Bruce, XIAP, IAP1, and IAP2, all of which we confirmed to be expressed in AtT20 cells as well. Thus, it is reasonable that inhibition of NF-κB causes apoptosis by decreasing survivin and other IAPs, as well as by decreasing the POMC expression.

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DHEA and PRT showed an additive effect on POMC as well as NF-κB-dependent gene transcription *in vitro*, suggesting that the two compounds have differing effects on NF-κB function. PRT is known to exert an anti-NF-κB effect by inactivating inhibitory kB (IkB) kinase (Kwok *et al.* 2001). DHEA, in contrast, may inhibit NF-κB through its antioxidant effect by altering the intracellular redox state (Gao *et al.* 2005, Kabe *et al.* 2005), through inhibition of IκB ubiquitination is shown in diabetic rats (Aragno *et al.* 2002), or through activation of PPARα (Kochan & Karbowska 2004). Thus, we assume that the combined effects of the two drugs are caused, at least partly, by their inhibitory effects on NF-κB function, although the effects on other signaling pathway(s) are not ruled out.

The additive effects of DHEA and PRT were also observed *in vivo*, especially in anti-tumor growth and anti-survivin effects. In these cases, administration of DHEA or PRT alone was not effective, and this may simply be because the blood level of each drug was less than that which we tested *in vitro*. Interestingly, DHEA alone had a significant inhibitory effect both on plasma corticosterone levels and on the cachexic effect; this also suggests that DHEA and PRT are acting partly through different molecular mechanisms. In the former case, Chang *et al.* (2003) reported the direct effect of DHEA on adrenal cells to inhibit corticosterone secretion by inhibiting steroidogenic enzymes downstream from cytochrome P450 side-chain cleavage enzyme (scc) and by inhibiting steroidogenic acute regulatory protein (StAR) expression. Thus, we assume that the combined use of DHEA and PRT is more efficient for inhibiting both growth and function of corticotroph tumor cells.

Because NF-κB is known to play an important role in normal cell function (Barnes & Karin 1997), inhibition of NF-κB may cause adverse effects in non-tumor cells. However, mice treated with both DHEA and PRT had much less weight loss and cachexia compared with control xenograft mice, and no tendency to immunodeficiency was observed. We also confirmed *in vivo* that the combined use of DHEA and PRT did not affect the expression of survivin mRNA in a normal organ like the liver. Thus, we assume that inhibition of NF-κB by these two drugs does not cause deleterious effect(s) on non-tumor tissues/cells, as far as we observed in our animal model.

DHEA is a endogenous hormone, has virtually no toxicity, and indeed is used frequently as a supplement in healthy people (Villareal & Holloszy 2004). PRT is also already used for the treatment of malignant lymphoma and other tumors in clinical practice (Ross *et al.* 1999, Curry *et al.* 2004). Furthermore, each drug has properties to inhibit corticotroph cell function as well as growth, and simultaneous administration shows significant additive effects both *in vitro* and *in vivo*. Therefore, the combination of these two compounds might be suitable in functional endocrine neoplasia like corticotroph tumor although further basic studies are absolutely necessary.

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