

Effects of tubastatin A on adrenocorticotrophic hormone synthesis and proliferation of AtT-20 corticotroph tumor cells

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Abstract. Cushing's disease is an endocrine disorder characterized by hypercortisolism, mainly caused by autonomous production of ACTH from pituitary adenomas. Autonomous ACTH secretion results in excess cortisol production from the adrenal glands, and corticotroph adenoma cells disrupt the normal cortisol feedback mechanism. Pan-histone deacetylase (HDAC) inhibitors inhibit cell proliferation and ACTH production in AtT-20 corticotroph tumor cells. A selective HDAC6 inhibitor has been known to exert antitumor effects and reduce adverse effects related to the inhibition of other HDACs. The current study demonstrated that the potent and selective HDAC6 inhibitor tubastatin A has inhibitory effects on proopiomelanocortin (*Pomc*) and pituitary tumor-transforming gene 1 (*Pttg1*) mRNA expression, involved in cell proliferation. The phosphorylated Akt/Akt protein levels were increased after treatment with tubastatin A. Therefore, the proliferation of corticotroph cells may be regulated through the Akt-Pttg1 pathway. Dexamethasone treatment also decreased the *Pomc* mRNA level. Combined tubastatin A and dexamethasone treatment showed additive effects on the *Pomc* mRNA level. Thus, tubastatin A may have applications in the treatment of Cushing's disease.

Key words: Cushing's disease, Adrenocorticotrophic hormone, Proopiomelanocortin, Corticotroph tumor, Histone deacetylase

CUSHING'S DISEASE is an endocrine disorder characterized by hypercortisolism, mainly caused by pituitary ACTH-producing adenomas [1, 2]. ACTH then stimulates the production of glucocorticoids, such as cortisol in humans and corticosterone in rodents, by the adrenal glands. An increase in glucocorticoids in turn inhibits both corticotropin-releasing factor production in the hypothalamus and ACTH production in the pituitary as a negative feedback mechanism. Indeed, autonomous ACTH secretion results in excess cortisol production from the adrenal glands, and corticotroph adenoma cells disrupt the normal cortisol feedback mechanism [3]. Histone deacetylase (HDAC) can modulate ACTH and glucocorticoid production as well as cell proliferation in corticotrophs [4, 5].

HDACs regulate genes related to cell proliferation or apoptosis, and the modification of histone acetylation affects tumor growth [6]. In cancers, unregulated or aberrantly expressed HDACs repress the expression of tumor-suppressor genes, leading to tumor growth [7]. Therefore, HDAC inhibitors block tumor growth and cell proliferation [8-10]. Eighteen different types of HDACs, categorized into four different classes, have been identified [11]. Among these HDACs, class I HDACs (HDAC1–HDAC3, HDAC8, and HDAC11) and class IIb HDACs (HDAC6 and HDAC10) can be modulated by hydroxamic acid HDAC inhibitors. In corticotroph tumor cells, the pan-HDAC inhibitor suberoylanilide hydroxamic acid inhibits cell proliferation [12]. Additionally, trichostatin A also blocks cell proliferation and ACTH synthesis by modulating its precursor proopiomelanocortin (*Pomc*) and alters ACTH secretion. However, even though trichostatin A exerts potent antiproliferative effects in cancer cells, adverse effects have limited its further development [13].

Among HDACs, HDAC1, HDAC2, and HDAC6 are key targets to reduce the cytotoxic effects of pan-HDAC inhibition [14]. Romidepsin, a selective inhibitor of HDAC1/HDAC2, also decreases *Pomc* mRNA level and blocks cell proliferation [15]. Moreover, *Hdac1* knock-down decreases cell proliferation, demonstrating that

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HDAC1 is involved in the proliferation of corticotroph cells. On the contrary, HDAC6 is a class IIb HDAC that is uniquely localized in the cytoplasm [16]. HDAC6 controls heat shock protein 90 (HSP90) acetylation and modulates HSP90/glucocorticoid receptor protein–protein interactions. Human HSP90 β is cloned based on homology to HSP90 α , as they share 60% overall homology. HSP90 α and HSP90 β are also localized in the cytoplasm. Recent studies have used selective HDAC6 inhibitors, such as tubastatin A [17], and small interfering RNA (siRNA)-mediated knockdown to determine the roles of HDAC6 [18]. Tubastatin A decreases cell migration and proliferation, and tumor growth [19]. Thus, the use of this selective HDAC6 inhibitor has been known to not only result in antitumor effects but also reduce adverse effects related to the inhibition of other HDACs.

Pituitary tumor-transforming gene 1 (*Pttg1*) is a biomarker for pituitary tumor cell proliferation, in AtT-20 cells. *Pttg1* knockdown inhibits cell proliferation, and *Pttg1* is associated with cell proliferation in corticotroph tumor cells [5].

It has not been determined yet whether the selective inhibition of HDAC6 decreases ACTH synthesis and cell proliferation in corticotroph tumor cells. It is also interesting to determine whether the effects of the inhibitor tubastatin A would be modulated under glucocorticoid treatment. Therefore, in the current study, we examined the effects of selective inhibition of HDAC6 with tubastatin A on *Pomc* and *Pttg1* expression and cell proliferation under excess glucocorticoid in AtT-20 murine corticotroph tumor cells.

Materials and Methods

Materials

Tubastatin A was purchased from ApexBio Technology (Houston, TX, USA). Dexamethasone was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

Cell culture

Mouse pituitary corticotroph tumor AtT-20 cells, purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 100 μ g/mL streptomycin, and 100 U/mL penicillin under a humidified atmosphere (5% CO₂ and 95% air) at 37°C, as reported previously [20]. The cells were cultured in six-well plates at 1.5×10^5 cells/cm² for 2 days before each experiment. On day 3, the medium was changed to DMEM supplemented with 0.2% bovine serum albumin and then cultured for 12 h.

RNA extraction

The cells were treated with the medium alone (control) or medium containing 0.1–10 μ M tubastatin A for the indicated times. The total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol, and stored at –80°C until analysis. The total RNA (0.5 μ g) was used as a template for synthesizing cDNA using random hexamers as primers with the SuperScript first-strand synthesis system for reverse transcription quantitative polymerase chain reaction (RT-qPCR; Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions.

RT-qPCR

The cDNA was synthesized as described previously [20], and then subjected to real-time (RT)-qPCR as follows. Transcript-specific primer and probe sets (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA, USA) were used to perform RT-qPCR to determine the mRNA levels of the following mouse transcripts: *Pomc* (NM_001278581.1), *Pttg1* (NM_001131054.1), *Hsp90 α* (NM_010480.5), *Hsp90 β* (NM_008302.3), and *Hdac6* (NM_001130416.1). To standardize gene expression levels, β 2-microglobulin (*B2m*) was selected as a reference gene, because the *B2m* mRNA level did not differ significantly from those of the controls across all treated samples. RT-qPCR was performed using 25- μ L reaction mixtures containing 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems), 1 \times Assays-on-Demand Gene Expression Products for each of the transcripts (Mm00437762_m1 for *B2m*, Mm00435874_m1 for *Pomc*, Mm00479224_m1 for *Pttg1*, Mm00658568_gH for *Hsp90 α* , Mm00833431_g1 for *Hsp90 β* , and Mm00515945_m1 for *Hdac6*), and 500 ng cDNA. StepOnePlus Real-Time PCR System (Applied Biosystems) was used for amplification under the following thermal cycling conditions: 95°C for 20 sec, followed by 40 cycles at 95°C for 1 sec and 60°C for 20 sec.

All expression data are expressed as a function of the threshold cycle (C_T) for quantitative analyses using StepOne software v2.3 (Applied Biosystems). Analyses of diluted samples of genes of interest (*Pomc*, *Pttg1*, and *Hdac6*) and the reference gene (*B2m*) revealed identical amplification efficiencies. Relative quantitative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method.

Cell proliferation assay

The cells were treated with 0.1–10 μ M tubastatin A for 48 h. Viable cells were enumerated using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Western blotting

In order to examine the effects of tubastatin A and dexamethasone on the protein expression of phosphorylated Akt (p-Akt) and Akt, western blotting was performed. The cells were washed twice with phosphate-buffered saline (PBS) and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation and the supernatant was recovered. The boiled samples were subjected to electrophoresis on a 4%–20% gradient polyacrylamide gel, and proteins were transferred on to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block[®] buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the membrane was incubated with each antibody, anti-p-Akt (1:500) or anti-Akt (1:15,000) (Cell Signaling Technology, Beverly, MA) for 1 h, washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (1:25,000 or 1:100,000 dilution, respectively; Daiichi Kagaku). For detection, the chemiluminescent substrate SuperSignal West Pico (Pierce Chemical Co., Rockford, IL, USA) was used, and the membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY).

Statistical analyses

Each experiment was performed three times. The samples were analyzed in triplicate for each group of experiments. The experimental results are expressed as mean \pm standard error of the mean. The data were subjected to analysis of variance, followed by Scheffe's multiple comparison tests. Significance was set at $p < 0.05$.

Results

Effects of tubastatin A on the *Pomc*, *Pttg1*, and *Hdac6* mRNA levels

To determine the concentration- and time-dependent effects of tubastatin A on *Pomc* mRNA levels, AtT-20 cells were incubated with various concentrations of tubastatin A for different periods. The time-course study also showed that 10 μ M tubastatin A significantly decreased the *Pomc* mRNA level ($p < 0.0001$) after treatment for 2–24 h (Fig. 1A). The *Pomc* mRNA level was decreased in a concentration-dependent manner ($p < 0.005$), with significant effects initially occurring at 1 μ M tubastatin A (Fig. 1B). Furthermore, 10 μ M tubastatin A also significantly decreased the *Pttg1* mRNA level

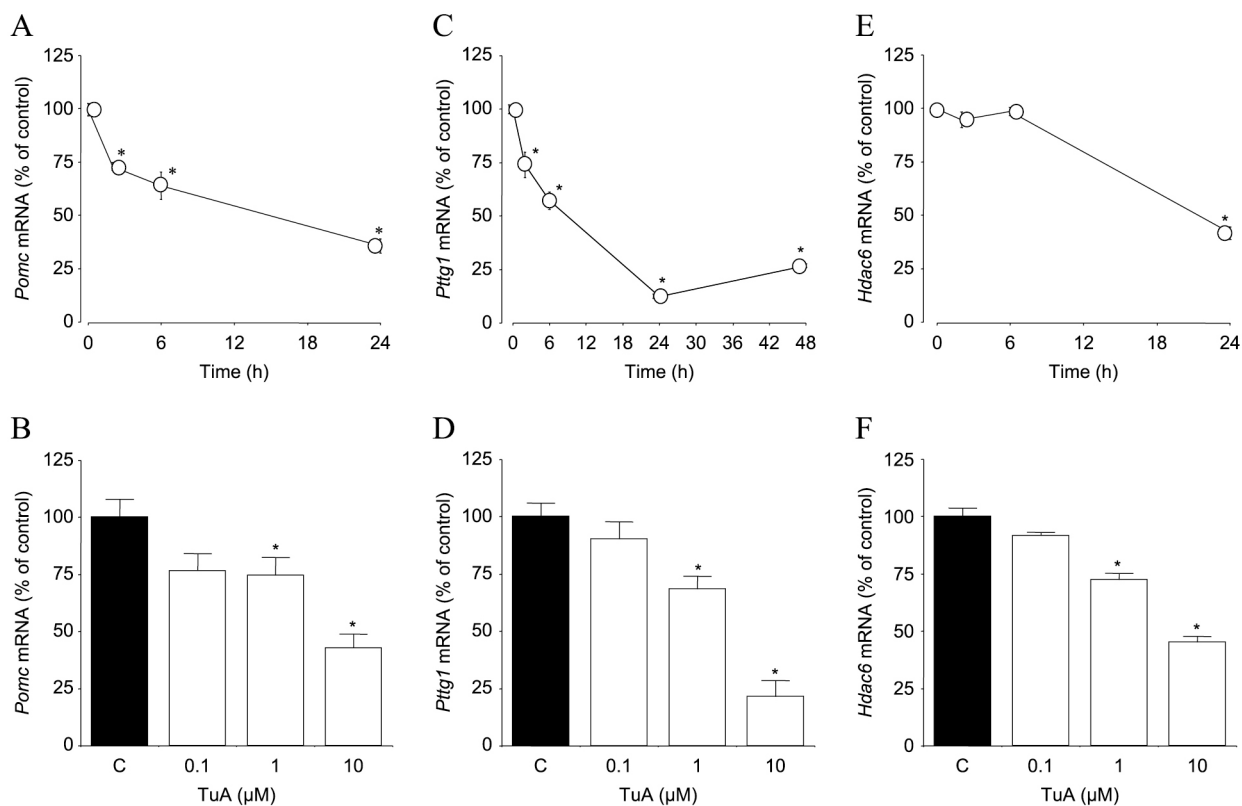


Fig. 1 Effects of tubastatin A on *Pomc*, *Pttg1*, and *Hdac6* mRNA in AtT-20 cells. (A, C, and E) Time-dependent effects of tubastatin A on *Pomc*, *Pttg1*, and *Hdac6* mRNA levels. The cells were cultured in medium containing 10 μ M tubastatin A. (B, D, and F) Concentration-dependent effects of tubastatin A on *Pomc*, *Pttg1*, and *Hdac6* mRNA. The cells were cultured for 24 h in medium containing 0.1–10 μ M tubastatin A (TuA). Data are expressed as mean \pm standard error of the mean. * $p < 0.05$ compared with time 0 or the control (C). The cells were treated in triplicate, and the average of three independent experiments is shown ($n = 3$).

($p < 0.0001$) after treatment for 2–48 h (Fig. 1C). The *Pttg1* mRNA level was decreased in a concentration-dependent manner ($p < 0.005$), with significant effects initially occurring at 1 μ M tubastatin A (Fig. 1D). The time-course study showed that 10 μ M tubastatin A significantly decreased the *Hdac6* mRNA level ($p < 0.0001$) after treatment for 24 h (Fig. 1E). The *Hdac6* mRNA level was decreased in a concentration-dependent manner ($p < 0.0001$), with significant effects initially occurring at 1 μ M tubastatin A (Fig. 1F).

Effects of tubastatin A on cell proliferation

The proliferation of AtT-20 cells was inhibited in a concentration-dependent manner following treatment with tubastatin A ($p < 0.0001$), with significant effects first observed at 5 μ M (Fig. 2).

Effects of dexamethasone on the *Pttg1* and *Hdac6* mRNA levels

To determine the concentration- and time-dependent effects of dexamethasone on *Pttg1* and *Hdac6* mRNA levels, AtT-20 cells were incubated with various concentrations of dexamethasone for different periods. The time-course study showed 100 nM dexamethasone significantly decreased the *Pttg1* mRNA level ($p < 0.0001$), with significant effects observed at 24 and 48 h of treatment (Fig. 3A). The time-course study also showed that 100 nM dexamethasone significantly decreased the *Hdac6* mRNA level ($p < 0.0005$), with significant effects observed at 24 h of treatment (Fig. 3C). Moreover, both *Pttg1* and *Hdac6* mRNA levels were decreased in a

concentration-dependent manner ($p < 0.01$ and $p < 0.0005$, respectively), with significant effects initially occurring at 1 and 10 nM dexamethasone, respectively (Fig. 3B and 3D).

Effects of tubastatin A and dexamethasone on the *Pomc* and *Pttg1* mRNA levels

The *Pomc* and *Pttg1* mRNA levels were reduced in cells treated with either tubastatin A or dexamethasone (Fig. 4A and 4B). In cells treated with both tubastatin A and dexamethasone, the *Pomc* mRNA level was further reduced; thus, the combined treatment showed additive effects on the *Pomc* mRNA level (Fig. 4A). Such additive effects were not observed on the *Pttg1* mRNA level (Fig. 4B).

Effects of tubastatin A and dexamethasone on the p-Akt/Akt protein levels

The p-Akt/Akt protein levels increased 120 min after treatment with tubastatin A (Fig. 5A), but it was increased 360 min after dexamethasone treatment (Fig. 5B). The additive effects were not observed on the p-Akt/Akt protein levels (Fig. 5C).

Discussion

In this study, we demonstrated that tubastatin A, a potent and selective HDAC6 inhibitor, decreased the *Pomc* mRNA level in corticotroph tumor cells. Tubastatin A also decreased the *Hdac6* mRNA level. Therefore, the effects of tubastatin A on the *Pomc* mRNA level could be attributed to its inhibition of *Hdac6* expression. However, the time-course study revealed that the *Hdac6* mRNA expression level was not suppressed at 2 and 6 h, whereas the *Pomc* mRNA level was decreased at both 2 and 6 h. In fact, HDAC inhibitors have been shown to act by decreasing the HDAC activity, but not the gene expression levels [21]. Notably, tubastatin A acts *via* the increase in histone acetylation of specific substrates [22]. Indeed, HDAC6 acts as an HSP90 deacetylase [23], enhancing its chaperone function. HDAC6 inactivation by tubastatin A leads to HSP90 hyperacetylation, resulting in a loss of chaperone activity [23]. Riebold *et al.* also showed that Cushing's disease is caused by the overexpression of HSP90 protein and that it can be treated with an appropriate HSP90 inhibitor [24]. In our previous study, we showed that the HSP90 inhibitors 17-allylamino-17-demethoxygeldanamycin and CCT018159 suppress ACTH synthesis and secretion in corticotroph tumor cells [25]. The HDAC6 inhibitor tubastatin A may act *via* its suppression of HSP90 activity.

Somatic driver mutations in ubiquitin-specific protease 8 (*USP8*) have been identified in Cushing's disease

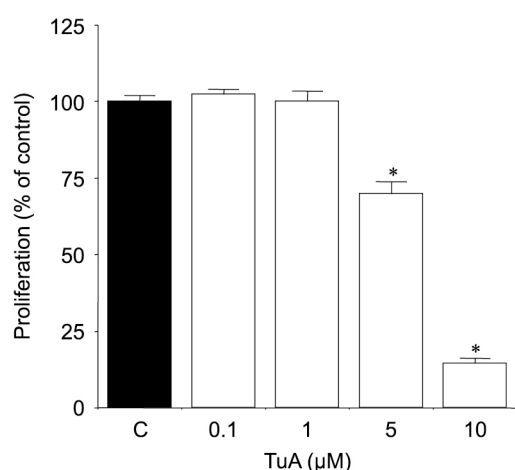


Fig. 2 Effects of tubastatin A on the proliferation of AtT-20 cells. The cells were cultured for 48 h in medium containing 0.1–10 μ M tubastatin A (TuA). Data are expressed as mean \pm standard error of the mean. * $p < 0.05$ compared with time 0 or the control (C). The cells were treated in triplicate, and the average of three independent experiments is shown ($n = 3$).

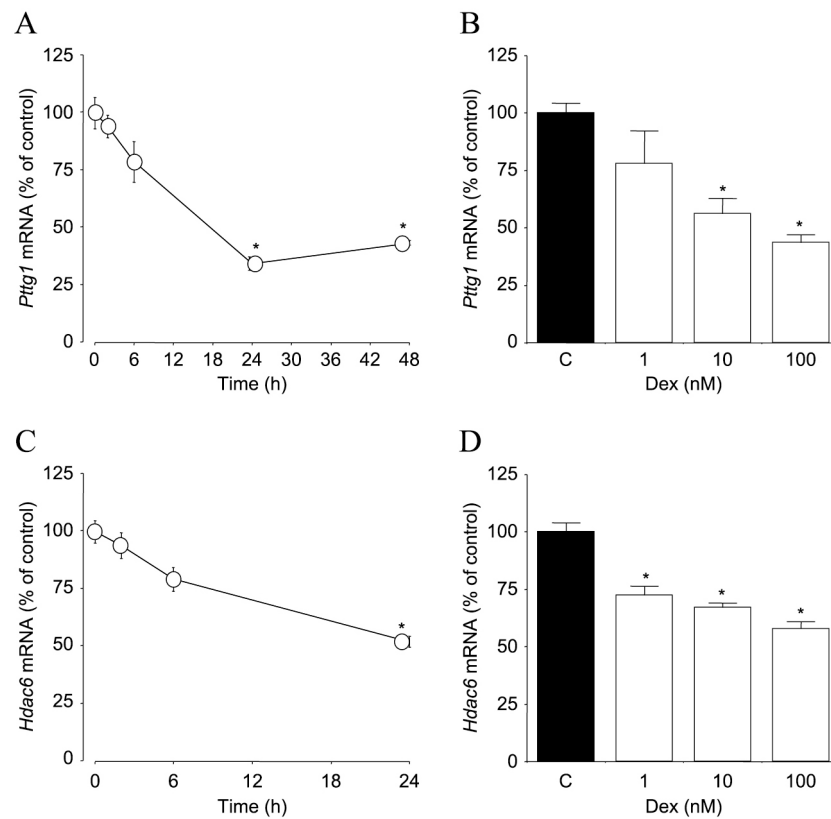


Fig. 3 Effects of dexamethasone on *Hdac6* and *Pttg1* mRNA in AtT-20 cells. (A and C) Time-dependent effects of dexamethasone on *Hdac6* and *Pttg1* mRNA levels. The cells were cultured in medium containing 100 nM dexamethasone. (B and D) Concentration-dependent effects of dexamethasone on *Hdac6* and *Pttg1* mRNA levels. The cells were cultured for 24 h in medium containing 1–100 nM dexamethasone (Dex). Data are expressed as mean \pm standard error of the mean. * $p < 0.05$ compared with time 0 or the control (C). The cells were treated in triplicate, and the average of three independent experiments is shown ($n = 3$).

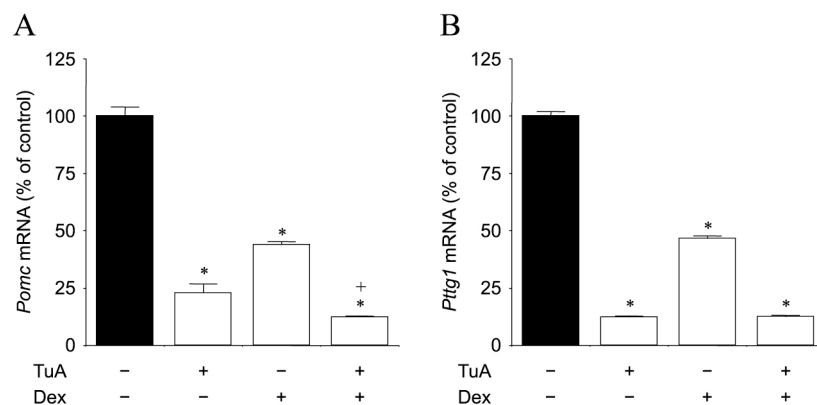


Fig. 4 Effects of tubastatin A and dexamethasone on *Pomc* and *Pttg1* mRNA levels in AtT-20 cells. The cells were incubated with medium containing 10 μ M tubastatin A (TuA) for 24 h and/or 100 nM dexamethasone (Dex) for 24 h, or with control medium. (A) Effects of tubastatin A and dexamethasone on the *Pomc* mRNA level. (B) Effects of tubastatin A and dexamethasone on the *Pttg1* mRNA level. Data are expressed as mean \pm standard error of the mean. * $p < 0.05$ compared with the control (C). ⁺ $p < 0.05$ compared with either TuA or Dex. The cells were treated in triplicate, and the average of three independent experiments is shown ($n = 3$).

[26, 27]. The mutations hyperactivate *USP8*, resulting in the inhibition of epidermal growth factor receptor (EGFR) degradation [27]. Thus, increased EGFR stability contributes to the maintenance of EGFR signaling in

Cushing's disease. Importantly, HDAC6 regulates EGFR trafficking and degradation [28], and therefore tubastatin A could affect EGFR signaling *via* cytoplasmic HDAC6. Related studies have not been performed yet; therefore,

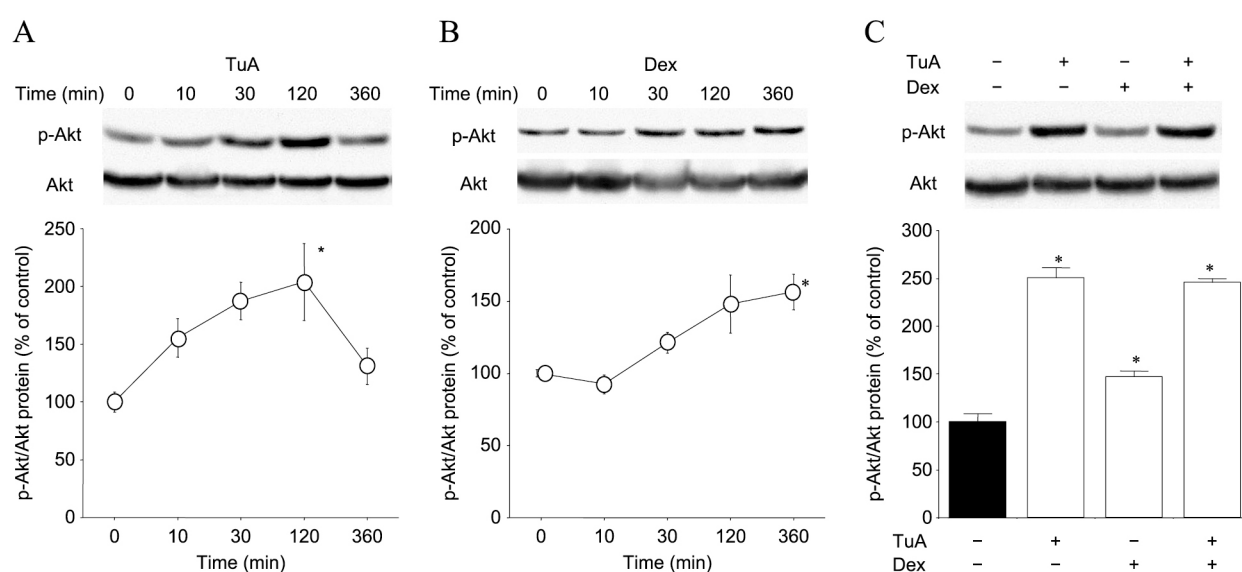


Fig. 5 Effects of tubastatin A and dexamethasone on the p-Akt/Akt protein levels in AtT-20 cells. The cells were incubated in medium containing 10 μ M tubastatin A (TuA) and/or 100 nM dexamethasone (Dex) or with control medium. (A) Effects of tubastatin A on p-Akt/Akt protein levels. (B) Effects of dexamethasone on p-Akt/Akt protein levels. (C) Effects of tubastatin A and dexamethasone on p-Akt/Akt protein levels. The cells were incubated in medium containing 10 μ M TuA for 120 min and/or 100 nM Dex for 360 min or with control medium. Data are expressed as mean \pm standard error of the mean. * p < 0.05 compared with time 0 or the control (C). The cells were treated in triplicate, and the average of three independent experiments is shown (n = 3).

further studies are needed to assess this mechanism.

In this study, dexamethasone also decreased *Pomc* mRNA level, and combined treatment with tubastatin A and dexamethasone exerted additive effects on the *Pomc* mRNA level. Glucocorticoids inhibit *Pomc* gene expression directly in the pituitary gland [29]. Negative glucocorticoid-response elements contribute to the suppression of *Pomc* expression by glucocorticoids [8, 30]. NeuroD1 is also involved in the glucocorticoid-mediated negative regulation of *Pomc* in the pituitary gland [29]. Tubastatin A may act by suppressing HSP90 activity (Fig. 6). Overexpression of HSP90 restrains the release of mature glucocorticoid receptor, leading to partial glucocorticoid resistance [24]. A C-terminal HSP90 inhibitor restores glucocorticoid sensitivity [24]. Therefore, it is possible that tubastatin A restores glucocorticoid sensitivity by modulating the HSP90 activity. Thus, tubastatin A and dexamethasone may regulate *Pomc* expression additively. In the context of hypercortisolism in Cushing's disease, the HDAC6 inhibitor tubastatin A may effectively decrease autonomous ACTH production.

Our findings showed that tubastatin A decreases *Pttg1* mRNA level and AtT-20 cell proliferation. *Pttg1* expression contributes to the proliferation of pituitary adenomas. In our previous studies [5, 25], we found that HSP90 and HDAC inhibitors decrease *Pttg1* mRNA level in AtT-20 corticotroph tumor cells. Thus, *Pttg1* may be involved in the HSP90 or HDAC inhibitor-induced suppression of proliferation of corticotroph tumor cells.

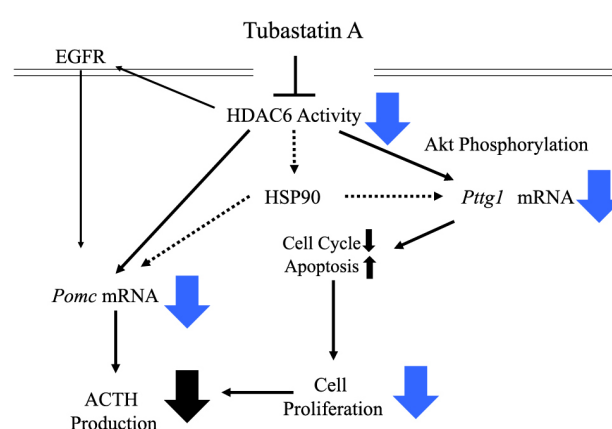


Fig. 6 Proposed signaling mechanisms of inhibitory effects of tubastatin A on ACTH synthesis and cell proliferation. The results determined in this study are indicated with a thick up or down blue arrow, whereas those not determined in this study are indicated with a thick up or down black arrow. The indirect effects presumably mediated by HSP90 are indicated with a dotted line.

Although dexamethasone decreased *Pttg1* mRNA level and AtT-20 cell proliferation, combined treatment with tubastatin A and dexamethasone showed no additive effects on the *Pttg1* mRNA level, because the potent effect of tubastatin A to downregulate *Pttg1* mRNA expression might mask the additive effect of dexamethasone.

Akt pathway activation is also involved in the proliferation of corticotroph tumors [22]. Furthermore, the additive

effects were not observed on the p-Akt/Akt protein levels. Notably, glucocorticoids inhibit cell migration *via* the inhibition of HDAC6 [31]. Akt phosphorylation has been suggested to be upstream of Pttg1 regulation (Fig. 6), and additional factors also may be involved in the regulation. Thus, through this Akt-Pttg1 pathway, the proliferation of corticotroph cells may be regulated.

In human corticotroph tumor cells, the pan-HDAC inhibitor suberoylanilide hydroxamic acid has been reported to exert antitumor effects [12]. In the current study, we used mouse corticotroph tumor cells. However, it is unclear whether these results would also be observed *in vivo* or in human corticotroph tumor cells. Thus, further studies are required to evaluate these mechanisms in human corticotroph cells.

In conclusion, we found that the selective HDAC6 inhibitor tubastatin A decreased *Pomc* and *Pttg1* mRNA levels in and blocked the proliferation of AtT-20 murine corticotroph tumor cells. The proliferation of corti-

cotroph cells may be regulated through the Akt-Pttg1 pathway. Combined tubastatin A and dexamethasone treatment showed additive effects on *Pomc* mRNA levels. Overall, these findings suggest that tubastatin A may have applications as a novel therapy for Cushing's disease.

Contributors

All authors participated in writing the manuscript and approved the final manuscript.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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