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Ubiquitin-specific protease 8 inhibitor suppresses adrenocorticotropic hormone production and corticotroph tumor cell proliferation

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Abstract. Cushing’s disease is primarily caused by autonomic hypersecretion of adrenocorticotropic hormone (ACTH) from a pituitary adenoma. In Cushing’s disease, mutations in the ubiquitin-specific protease 8 (USP8) have been detected. These mutations are associated with hyperactivation of USP8 that prevent epidermal growth factor receptor (EGFR) degradation. This leads to increased EGFR stability and results in the maintenance of EGFR signaling in Cushing’s disease. USP8 inhibitors can suppress the growth of various tumors. In this study, the effects of a potent USP8 inhibitor, DUBs-IN-2, on ACTH production and cell proliferation were examined in mouse corticotroph tumor (AtT-20) cells. Proopiomelanocortin (Pomc) mRNA levels and ACTH levels were decreased in AtT-20 cells by DUBs-IN-2. Further, cell proliferation was inhibited, and apoptosis was induced by DUBs-IN-2. Transcript levels of pituitary tumor-transforming gene 1 (Pttg1), a pituitary tumor growth marker, were increased; and transcript levels of stress response growth arrest and DNA damage-inducible 45 (Gadd45β) and Cdk5 and ABL enzyme substrate 1 (Cables1) mRNA levels were increased in response to the drug. Gadd45β or Cables1 knockdown partially inhibited the DUBs-IN-2-induced decrease in cell proliferation, but not Pomc mRNA levels. Both GADD45 β and CABLES1 may be responsible, at least in part, for the USP8-induced suppression of corticotroph tumor cell proliferation. USP-8 may be a new treatment target in Cushing’s disease.

Key words: Cushing’s disease, Adrenocorticotropic hormone, Cell proliferation

CUSHING’S DISEASE is a rare condition caused by hypercortisolism resulting from hyperproduction of adrenocorticotropic hormone (ACTH) from pituitary corticotroph adenomas [1, 2]. Cushing’s disease induces severe complications such as hypertension, hyperglycemia, osteoporosis, infections, atherosclerosis, and mental disorders [3]. The primary treatment for Cushing’s disease is surgical excision of the adenoma from the pituitary, but curative surgery is still challenging, and additional therapies are required to treat the resulting hypercortisolism [4-6]. Among the drugs to use suppress ACTH production, a somatostatin receptor type 5 analogue is a recently identified potential therapeutic for Cushing’s disease.

Somatic driver mutations in ubiquitin-specific protease 8 (USP8) have been identified in 35% to 60% cases of Cushing’s disease [7-9]. These mutations hyperactivate USP8, preventing epidermal growth factor receptor (EGFR) degradation, leading to increased EGFR stability, and resulting in the maintenance of EGFR signaling in Cushing’s disease. USP8 mutations also enhance the promoter activity of proopiomelanocortin (Pomc) by stabilizing EGFR signaling. Accordingly, phosphorylated EGFR expression has been found in most cases of Cushing’s disease [10], and an EGFR inhibitor could be an effective treatment for EGFR-related tumors. Indeed, the EGFR tyrosine kinase inhibitor gefitinib has been shown to suppress ACTH production and tumor growth in an experimental mouse model of Cushing’s disease [11].

DUBs-IN-2 is a potent deubiquitinase enzyme inhibitor of USP8 [12]. A proteasome inhibitor, bortezomib, has been used to treat multiple myeloma [13], and inhibition of USP8 might be effective in overcoming gefitinib resistance in non-small cell lung cancers [14]. In this study, we examined the effects of the potent deubiquitinase enzyme inhibitor DUBs-IN-2 on ACTH production and cell proliferation in AtT-20 corticotroph tumor cells.

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Materials and Methods

Materials
DUBs-IN-2 (9-oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile) was purchased from MedChem Express (Princeton, NJ, USA).

Cell culture
Mouse AtT-20 corticotroph tumor cells were obtained from ATCC (Manassas, VA, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C with 5% CO₂. One day before each experiment, the cells were starved with DMEM containing 0.2% bovine serum albumin.

RNA isolation
AtT-20 cells were grown to 40% confluence and incubated either with or without DUBs-IN-2 at the indicated concentrations (0.1–10 μM) for the indicated (0–24 h) times. Total cellular RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Total RNA (0.5 μg) was used as a template to synthesize cDNA with random hexamer primers using the SuperScript First-Strand Synthesis System for Reverse Transcription-Polymerase Chain Reaction (RT-PCR; Invitrogen Corp., Carlsbad, CA, USA) [15, 16].

Quantitative RT-PCR
The resulting cDNAs were subjected to quantitative RT-PCR. Expression of indicated genes was assessed by PCR using the specific primer and probe sets (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA, USA) targeting Pomp (NM_008895.3), pituitary tumor-transforming gene 1 (PtgI) (NM_001131054.1), stress response growth arrest and DNA damage-inducible 45 (Gadd45β) (NM_008655.1), and Cdk5 and ABL enzyme substrate 1 (Cables1) (NM_001146287.1) mRNA. To standardize expression levels, β2-microglobulin (B2mg) was used as a reference gene, because its levels were not significantly altered by any of the treatments. The 25-μL real-time PCR solution used in each reaction contained TaqMan universal PCR master mix (Applied Biosystems), gene expression products (Mm00435874_m1 for mouse Pomp, Mm00479224_m1 for mouse PtgI, Mm00435123_m1 for mouse Gadd45β, Mm00491531_m1 for mouse Cables1, and Mm00437762_m1 for mouse B2mg), and 500 ng cDNA. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used for amplification with the following cycle parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Cell proliferation assay
AtT-20 cells were incubated with the indicated concentrations (0.1–10 μM) of DUBs-IN-2 for 48 h. Cell viability was then determined using a Cell Counting Kit-8 (Dojin, Kumamoto, Japan).

Cell death assay
AtT-20 cells were incubated with the indicated concentrations (0.1–10 μM) of DUBs-IN-2 for 24 h. DNA fragmentation was measured using a Cell Death Detection ELISA Kit (Roche, Penzberg, Germany), and the fragmentation of each enrichment factor was then calculated according to the manufacturer’s instructions.

RNA interference experiments
All small interfering (si) RNA fragments were purchased from Qiagen. HiPerFect Transfection Reagent (Qiagen) was used to transfect AtT-20 cells with siRNA fragments according to the manufacturer’s protocol. The target mRNA levels were determined using cells that were seeded in 12-well plates at a density of 12.0 × 10⁴ cells/well. The cultures were incubated for 48 h in 1 mL of culture medium containing control siRNA (siControl) or the experimental siRNAs, that is, Gadd45β-specific siRNA (siGadd45β; Mm_Gadd45b_4) or Cables1-specific siRNA (siCables1; Mm_Cables1_4). The Gadd45β, Cables1, Pomp, and B2mg mRNA levels were then assayed via quantitative RT-PCR. Cell proliferation was measured using cells cultured in 100 μL of culture medium containing siRNA fragments in 96-well plates (1.5 × 10⁴ cells/well density) with the medium changed after 24 h of incubation. Cell viability was measured 48 h post-transfection using a Cell Counting Kit-8.

EGFR quantification
Cells were incubated with 0.1–10 μM DUBs-IN-2 for the indicated (0–24 h) times. After each treatment, cells were washed with cold phosphate-buffered saline (PBS). The cells were then lysed with Laemmli sample buffer. Lysates were centrifuged, and supernatants were collected, and western blot analyses were performed [17]. In brief, whole cell lysates were heated, and then proteins were separated on a 2–15% gradient polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane (Daichi Kagaku, Tokyo, Japan). Membranes were blocked with Detector Block buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and incubated for 1 h with an anti-EGFR antibody (1:1000 dilution) (Boster Biological Technology, Pleasanton, CA, USA) and anti-β-actin antibody (1:10000 dilution) (ab8227, Abcam, Cambridge, MA). After washes with PBS containing 0.05% Tween 20, the membrane was incubated with horseradish peroxidase-labeled anti-rabbit immuno-
globulin G (Daiichi Kagaku). Hybridization signals were detected using the chemiluminescent substrate SuperSignal West Pico (Pierce Chemical Co., Rockford, IL, USA), and the membrane was exposed to a BioMax film (Eastman Kodak Co., Rochester, NY, USA). β-actin was used as a reference gene to standardize EGFR expression levels.

**ACTH assays**

AtT-20 cells were incubated with the indicated concentrations (0.1–10 μM) of DUBs-IN-2 for 24 h, and the culture medium was then collected. ACTH levels were measured using an ACTH enzyme-linked immunosorbent assay (ELISA) kit (MD Bioproducts, Zurich, Switzerland).

**Statistical analysis**

Data are shown as a mean ± standard error of the mean. Statistical analyses were performed with ANOVA, followed by Fisher’s protected least-significant difference post hoc test. P values < 0.05 were considered statistically significant.

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**Results**

**DUBs-IN-2 suppresses Pomc mRNA and ACTH levels**

DUBs-IN-2 significantly decreased Pomc mRNA levels (ANOVA; p < 0.0001) to 37% of the basal level 24 h after treatment with DUBs-IN-2 (Fig. 1A). The effects were dose-dependent (ANOVA; p < 0.005), and significant effects were observed at a concentration of 10 μM (Fig. 1B). Treatment with 10 μM DUBs-IN-2 also significantly reduced ACTH levels to 67% in the culture medium (Fig. 1C).

**DUBs-IN-2 inhibits cell proliferation and induces cell death**

Treatment with 10 μM DUBs-IN-2 inhibited cell proliferation (Fig. 2A) and increased the level of cytoplasmic histone-associated DNA fragmentation (Fig. 2B). The increased level of cytoplasmic histone-associated DNA fragmentation indicates the promotion of cell death.
DUBs-IN-2 decreases Ptg1 mRNA levels and increases Gadd45β and Cables1 mRNA levels

DUBs-IN-2 (10 μM) significantly decreased Ptg1 mRNA levels (ANOVA; $p < 0.0001$) to 27% of the basal level 24 h after treatment with DUBs-IN-2 (Fig. 3A). Ptg1 mRNA levels were significantly decreased by DUBs-IN-2 (ANOVA; $p < 0.0001$), and significant effects were observed at a concentration of 10 μM DUBs-IN-2 (Fig. 3B). DUBs-IN-2 (10 μM) significantly increased Gadd45β and Cables1 mRNA levels (ANOVA; $p < 0.005$) to 266% and 174%, respectively, of the basal level 24 h after treatment with DUBs-IN-2 (Figs. 3C and 3E). Gadd45β and Cables1 mRNA levels were significantly decreased by DUBs-IN-2 (ANOVA; $p < 0.005$), and significant effects were observed at a concentration of 10 μM DUBs-IN-2 (Figs. 3D and 3F).

Effects of GADD45β and CABLES1 on POMC transcript levels and cell proliferation

AtT-20 cells were transfected with transcript-specific siRNA fragments. The transfected cells showed 66% and 64% reduction in Gadd45β and Cables1 mRNA levels (Table 1). Gadd45β or Cables1 knockdown did not affect basal Pome mRNA levels or cell proliferation (Figs. 4 and 5). DUBs-IN-2 significantly inhibited Pome mRNA levels and cell proliferation. Gadd45β or Cables1 knockdown also failed to alter the decrease in Pome mRNA levels (Figs. 4A and 5A), but individual knockdown of both gene partially inhibited the DUBs-IN-2-induced decreases in cell proliferation (Figs. 4B and 5B).

DUBs-IN-2 decreases EGFR protein expression

The protein levels of EGFR decreased to 43% within 24 h of treatment with DUBs-IN-2 (ANOVA; $p < 0.005$; Fig. 6A). The effects were dose-dependent (ANOVA; $p < 0.01$), and significant effects were observed at a concentration of 10 μM (Fig. 6B).

Discussion

In our previous study [18], we determined that an EGFR inhibitor suppressed autonomic ACTH production in AtT-20 corticotroph tumor cells. In the present study, we found that USP8 inhibitor DUBs-IN-2 decreased Pome mRNA levels in AtT-20 cells. DUBs-IN-2 also decreased AtT-20 cell proliferation and induced DNA fragmentation in corticotroph tumor cells, suggesting that this inhibitor induces cell death and reduces cell growth. Because the drug does not affect the cell viability of other endocrine or nonendocrine cells [19], this inhibitor shows a specific effect on pituitary tumor cells. Decreased ACTH levels might also be achieved by inhibiting tumor cell proliferation and ACTH synthesis [20].

In the present study, DUBs-IN-2 was also found to gradually decrease Ptg1 mRNA levels in AtT-20 cells. PTTG1 is associated with aggressive cell proliferation [21] and is a hallmark of tumorigenesis [22-24]. PTTG1 expression contributes to the proliferation of pituitary adenomas, including those formed from corticotroph tumor cells [25]. Our previous studies [25, 26] showed that heat shock protein 90 and histone deacetylase inhibitors decrease Ptg1 mRNA levels in AtT-20 cells. Thus,
PTTG1 expression may be related to USP8-induced corticotroph tumor cell proliferation.

Both GADD45β and CABLES1 are pituitary suppressors that inhibit cell proliferation and tumorigenesis. A loss of function mutation in GADD45β has been associated with the progression of tumorigenesis in human pituitary gonadotroph tumors [27]. CABLES1 is a critical regulator of corticotroph growth, and its expression is lost in about more than half of human pituitary ACTH-producing adenomas [28]. Thus, loss-of-function mutations in the CABLES gene are a cause of Cushing’s disease [29]. Gadd45β and Cables1 mRNA levels increased with DUBs-IN-2 treatment. Cell proliferation

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<th>Table 1</th>
<th>Effects of each specific siRNA</th>
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<tr>
<td></td>
<td>Control siRNA</td>
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<tr>
<td>Gadd45β</td>
<td>100.0 ± 6.6</td>
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<td>Cables1</td>
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Fig. 3  Effects of DUBs-IN-2 on Ptg1, Gadd45β, and Cables1 mRNA levels in AtT-20 corticotroph tumor cells. (A, C, and E) Time-dependent effects of DUBs-IN-2 on Ptg1, Gadd45β, and Cables1 mRNA levels. AtT-20 cells were incubated for 2, 6, and 24 h with 10 μM DUBs-IN-2. (B, D, and F) Dose-dependent effects of DUBs-IN-2 on Ptg1, Gadd45β, and Cables1 mRNA levels. AtT-20 cells were incubated for 24 h with 0.1–10 μM of DUBs-IN-2. *p < 0.05 (compared with basal [0] or control [C]). The cells were treated in triplicate, and the average of three independent experiments is shown (n = 3).
also decreased in response to DUBs-IN-2. Knockdown of Gadd45β and Cables1 each partially restored cell proliferation, suggesting that GADD45β and CABLES1 may be involved in the USP8 inhibitor-induced decrease in cell proliferation. Thus, both GADD45β and CABLES1 pathways are at least partially involved in the decrease in corticotroph tumor cell proliferation in response to DUBs-IN-2. However, it is unclear whether these effects are mediated by EGFR down-regulation.

Although Fukuoka’s report suggested that their AtT-20 cells did not express endogenous EGFR [10], our AtT-20 cells expressed EGFR protein. The reason for this discrepancy is unclear, but AtT-20 cells are known to have subtypes. Each AtT-20 subtype might have a differ-

Fig. 4 Effects of GADD45β on Pmc mRNA levels and AtT-20 corticotroph tumor cell proliferation. (A) Effects of GADD45β on Pmc mRNA levels. AtT-20 cells were incubated for 24 h in 1 mL of culture medium containing control or Gadd45β-specific siRNA (siGadd45β). (B) Effects of GADD45β on cell proliferation. AtT-20 cells were incubated for 48 h in 100 μL of culture medium containing control or Gadd45β-specific siRNA (siGadd45β). *p < 0.05 (compared with control). †p < 0.05 (compared with DUBs-IN-2 containing control siRNA). The cells were treated in triplicate, and the average of three independent experiments is shown (n = 3).

Fig. 5 Effects of CABLES1 on Pmc mRNA levels and AtT-20 corticotroph tumor cell proliferation. (A) Effects of CABLES1 on Pmc mRNA levels. AtT-20 cells were incubated for 24 h in 1 mL of culture medium containing control or Cables1-specific siRNA (siCables1). (B) Effects of CABLES1 on cell proliferation. AtT-20 cells were incubated for 48 h in 100 μL of culture medium containing control or Cables1-specific siRNA (siCables1). *p < 0.05 (compared with control). †p < 0.05 (compared with DUBs-IN-2 containing control siRNA). The cells were treated in triplicate, and the average of three independent experiments is shown (n = 3).
ent character. However, consistent with a previous report [8], we show that USP8 knockdown reduced EGFR protein levels. USP8 inhibitors may decrease autonomous EGFR signaling via changes in EGFR expression. In fact, EGFR signaling contributes to ACTH production and corticotroph tumor growth [11], although the mechanism underlying this effect has not been determined in AtT-20 cells. It has not been known any candidates of USP8 targets other than EGFR. However, it is known that this USP8 inhibitor affects the EGFR pathway. Our study indicates that inhibition of USP8 promotes EGFR degradation, leading to decreased EGFR stability and EGFR signaling. Thus, this receptor and downstream proteins could be promising targets for the treatment of Cushing’s disease.

Although the USP8 inhibitor had potent effects on ACTH production and cell proliferation in mouse corticotroph tumor cells, it is unclear whether this drug is able to suppress them in vivo or in human corticotroph tumor cells. Studies to determine this are required in the future. Additionally, USP8 may contribute to the pathogenesis in 35% to 60% cases of Cushing’s disease [7-9]. However, some additional factors would cause this disease. For example, corticotroph adenomas overexpress heat shock protein 90, and some of Cushings’s diseases may be caused by overexpression of the heat shock proteins [30, 31].

In conclusion, USP8 signaling contributes to ACTH production and cell proliferation in AtT-20 cells. Therefore, a USP8-targeting therapy offers a promising approach to treat Cushing’s disease.

Compliance with Ethical Standards

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Disclosure potential conflicts of interest

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

Human participants were not involved in this research.

Authors’ Contributions

All authors were involved in conducting the experiments, drafting the manuscript, and approving the final manuscript.

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