

# Inhibitory effects of a selective Jak2 inhibitor on adrenocorticotrophic hormone production and proliferation of corticotroph tumor AtT20 cells

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**Purpose:** The primary cause of Cushing's disease is adrenocorticotrophic hormone (ACTH)-producing pituitary adenomas. EGFR signaling induces *POMC* mRNA-transcript levels and ACTH secretion from corticotroph tumors. The Jak-STAT pathway is located downstream of EGFR signaling; therefore, a Jak2 inhibitor could be an effective therapy for EGFR-related tumors. In this study, we determined the effect of a potent and selective Jak2 inhibitor, SD1029, on ACTH production and proliferation in mouse AtT20 corticotroph tumor cells.

**Materials and methods:** AtT20 pituitary corticotroph tumor cells were cultured after transfection with *PTTG1*- or *GADD45β*-specific siRNA. Expression levels of mouse *POMC*, *PTTG1*, and *GADD45β* mRNAs were evaluated using quantitative real-time polymerase chain reaction. ACTH levels were measured using ACTH ELISA. Western blot analysis was performed to examine protein expression of phosphorylated STAT3/STAT3. Viable cells and DNA fragmentation were measured using a cell-proliferation assay and cell-death detection ELISA, respectively. Cellular DNA content was analyzed using fluorescence-activated cell sorting.

**Results:** SD1029 decreased *POMC* and *PTTG1* mRNA and ACTH levels, while increasing *GADD45β* levels. The drug also decreased AtT20-cell proliferation and induced apoptosis, but did not alter cell-cycle progression. SD1029 also inhibited STAT3 phosphorylation. *PTTG1* knockdown inhibited *POMC* mRNA levels and cell proliferation. However, combined treatment with *PTTG1* knockdown and SD1029 had no additive effect on *POMC* mRNA levels or cell proliferation. *GADD45β* knockdown inhibited the SD1029-induced decrease in *POMC* mRNA levels and also partially inhibited the decrease in cell proliferation.

**Conclusion:** Both *PTTG1* and *GADD45β* may be responsible, at least in part, for the Jak2-induced suppression of ACTH synthesis and cell proliferation. Accordingly, therapies that target EGFR-dependent Jak2/STAT3 may have clinical applications for treating Cushing's disease.

**Keywords:** Cushing's disease, adrenocorticotrophic hormone, *POMC*, pituitary tumor, treatment

## Introduction

The primary cause of Cushing's disease is the hypersecretion of adrenocorticotrophic hormone (ACTH) from a pituitary adenoma.<sup>1,2</sup> ACTH then in turn stimulates the release of glucocorticoids from the adrenal glands. However, in pituitary adenoma cells, the normal cortisol-feedback mechanism of the hypothalamic-pituitary-adrenal axis is disturbed, resulting in hypercortisolism, which can lead to diabetes mellitus, hypertension, atherosclerosis, immune dysfunction, and other metabolic derangements.<sup>3</sup> ACTH production may have a partial association with cell masses or pituitary corticotroph tumor-cell growth.<sup>4</sup> The primary treatment for Cushing's disease is excision of tumors

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from the pituitary, but unsuccessful excision necessitates further therapy to treat the resulting hypercortisolism.<sup>5,6</sup> Therefore, Cushing's disease requires effective medical therapy as a complementary treatment.

Tumor growth is at least partially determined by cell proliferation, the progression of the cell cycle, and an interruption of apoptosis.<sup>7</sup> The *PTTG1* oncogene was first cloned from the pituitary tumor of rat,<sup>8</sup> and *PTTG1* has since been identified as a signature gene expressed by pituitary tumors.<sup>9,10</sup> *PTTG1* is involved in several important processes, including cell-cycle progression, increased pituitary-cell proliferation, and the promotion of murine pituitary development.<sup>11,12</sup> Our previous study demonstrated that a decrease in *PTTG1* levels contributed to a decrease in AtT20 corticotroph tumor-cell proliferation.<sup>13</sup> Histone-acetylation modification has also been identified as playing an important role in the control of *PTTG1* expression.<sup>14</sup> In contrast, the stress-responsive *GADD45* gene family is involved in a range of related processes that include the maintenance of genomic stability, DNA repair, and active DNA demethylation, as well as cell-cycle control, cell survival, and apoptosis.<sup>15</sup> Additionally, *GADD45* $\beta$  is both a putative downstream target of p53 and a novel pituitary suppressor that blocks proliferation, survival, and tumorigenesis when expressed.<sup>16</sup>

Mutations in the deubiquitinase gene *USP8* have been found in ACTH-producing pituitary adenoma cells derived from humans.<sup>17</sup> A mutational hotspot hyperactivates *USP8*, contributing to the rescue of EGFR from lysosomal degradation and ensuring EGFR-stimulatory signaling in Cushing's disease. The Jak-STAT pathway is located downstream of EGFR signaling. Therefore, a Jak2 inhibitor might be an effective treatment for EGFR-related tumors.

SD1029, a compound initially used as an antifungal agent, is a potent, cell-permeable Jak2 inhibitor that blocks cell-cycle progression and both suppresses tumor cell proliferation and induces cellular differentiation.<sup>18</sup> SD1029 inhibits the nuclear translocation of STAT3, and then targets the anti-apoptotic proteins of activated STAT3.<sup>19</sup> The present study involved the application of SD1029 to AtT20 corticotroph tumor cells and revealed its effects on cell proliferation and ACTH production. To elucidate other potential mechanisms of SD1029 action, we also assessed the roles of *GADD45* $\beta$  and *PTTG1* in these effects.

## Materials and methods

### Materials

SD1029 was acquired from Merck KGaA (Darmstadt, Germany) and used throughout the study as a standardized

solution. After dissolving SD1029 in dimethyl sulfoxide, it was diluted further with cell-culture medium to between 100 nM and 10  $\mu$ M. Many of the cell-culture conditions, techniques, and protocols used by Nakada et al were adapted for use in the current study.<sup>13</sup>

### Cell-culture conditions

AtT20 pituitary corticotroph tumor cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in DMEM with 10% FBS, 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin in T75 culture flasks at 37°C under a humidified 5% CO<sub>2</sub> atmosphere. AtT20 cells were subsequently cultured in six-well plates at a density of 1.5 $\times$ 10<sup>5</sup> cells/well for 3 days prior to each experiment, and culture medium was exchanged with fresh medium every 48 hours. Exogenous factors within the FBS were minimized 1 day prior to each experiment by washing and serum-starving the AtT20 cells overnight with DMEM supplemented with 0.2% bovine serum albumin. Total cellular RNA or protein was collected at the conclusion of each experiment and then stored at -80°C until use.

### RNA extraction and quantitative real-time PCR

Both RNA extraction and quantitative real-time polymerase chain reaction (PCR) generally followed the procedures outlined by Nakada et al.<sup>13</sup> For the control and experimental treatments, AtT20 cells were incubated for the times indicated in each figure with medium alone or medium containing SD1029. Dose-dependent SD1029 effects were induced by incubating AtT20 cells for the times indicated under control or experimental SD1029 conditions, with a gradient of SD1029 concentrations ranging from 100 nM to 10  $\mu$ M. Total cellular RNA was extracted using an RNeasy minikit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol at the conclusion of each experiment. Then, 0.5  $\mu$ g of RNA was used as a template for synthesizing cDNA with random hexamer primers using the SuperScript first-strand synthesis system for reverse-transcription PCR (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

In accordance with the study by Nakada et al,<sup>13</sup> both total cellular RNA extraction and cDNA synthesis were performed.<sup>20,21</sup> Real-time PCR was performed using the resulting cDNA. Transcript-specific primer and probe sets (Assays-on-Demand gene-expression products; Thermo Fisher Scientific) were used to perform quantitative real-time PCR to assay the expression levels of three mRNA mouse transcripts: *POMC* (NM\_008895.3), *PTTG1* (NM\_001131054.1),

and *GADD45 $\beta$*  (NM\_008655.1). To standardize gene-expression levels, *B2MG* was used as a reference gene. Across all treated samples, *B2MG* mRNA levels did not significantly differ from those of controls. The 25  $\mu$ L real-time PCR reactions contained TaqMan universal PCR master mix (Thermo Fisher Scientific), gene-expression products (Assays-on-Demand) for each of the transcripts (with Mm00437762\_m1 for *B2MG*, Mm00435874\_m1 for *POMC*, Mm00479224\_m1 for *PTTG1*, and Mm00435121\_g1 for *GADD45 $\beta$* ), and 500 ng of cDNA. An ABI Prism 7000 sequence-detection system (Thermo Fisher Scientific) was used for amplification, with thermal cycling conditions in accordance with the study by Nakada et al: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.<sup>13</sup>

In order to eliminate the risk of DNA contamination, transcript-specific primer sets and a TaqMan probe spanning exon–exon junctions were used in each of the real-time PCR assays. All expression data were expressed as a function of the threshold cycle ( $C_T$ ) for quantitative analyses using the ABI Prism 7000. Analyses that used diluted samples of genes of interest (*POMC*, *PTTG1*, and *GADD45 $\beta$* ) and the reference gene (*B2MG*) revealed identical amplification efficiency.

## ACTH assay

Following the procedure outlined by Nakada et al,<sup>13</sup> AtT20 cells were incubated for 24 hours at 37°C with a gradient of SD1029 concentrations. A sample of each incubation medium was then aspirated, and ACTH levels in the supernatants were measured using an ACTH enzyme-linked immunosorbent assay kit (MD Bioproducts, Zurich, Switzerland). As determined by Nakada et al,<sup>13</sup> intra- and interassay coefficients of variation are 6.7% at 42.2 pg/mL and 7.1% at 42.3 pg/mL ACTH. The coefficients of variation for ACTH fragment (amino acids 1–24), ACTH fragment (amino acids 18–39), and  $\alpha$ -melanocyte-stimulating hormone were –3.4%, –2.4%, and –1.7%, respectively.

## Cell-proliferation, cell-death, and cell-cycle assays

Protocols again reflected those of Nakada et al for cell-proliferation, cell-death, and cell-cycle assays.<sup>13</sup> A range of SD1029 concentrations were used for AtT20-cell incubation at 37°C for 48 hours. The Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) was used to assess the number of viable cells for all samples from each treatment.

The same AtT20 cells in the same SD1029 conditions were incubated at 37°C for 24 hours for the cell-death assay. A cell death-detection enzyme-linked immunosorbent-assay

kit (Hoffman-La Roche, Basel, Switzerland) was used to measure DNA fragmentation, and each enrichment factor was calculated according to the manufacturer's protocol.

Cell-cycle assays were conducted following a 24-hour incubation period for AtT20 cells in either control medium or a 10  $\mu$ M SD1029 medium. Cells were harvested after trypsinization of the cell cultures, centrifugation, and resuspension of the cell pellets in Triton X-100. After treatment with 0.5% RNase A, the resuspended cells were incubated at 37°C for 30 minutes with 50  $\mu$ g/mL propidium iodide, a fluorescent intercalating agent. Fluorescence-activated cell sorting (FACS) was used to analyze the stained cellular DNA content. The resulting flow-cytometry images were analyzed using FACSDiva (Becton Dickinson, Franklin Lakes, NJ, USA) to determine cell-cycle profiles.

## RNA-interference experiments

Both *PTTG1*- and *GADD45 $\beta$* -specific siRNA fragments and control siRNAs were designed. All siRNA fragments were purchased from Qiagen. HiPerfect transfection reagent (Qiagen) was used to transfect AtT20 cells with siRNA fragments following the manufacturer's protocol. Target mRNA levels in samples were determined from cells that were seeded in 12-well plates at a density of  $12 \times 10^4$  cells/well; cultures were incubated for 48 hours in 1 mL of culture medium containing control siRNA (siControl) or the experimental siRNAs, that is, the *PTTG1*-specific siRNA (siPTTG1; Mm\_Pttg1\_7) or the *GADD45 $\beta$* -specific siRNA (siGADD45 $\beta$ ; Mm\_GADD45 $\beta$ \_4). *PTTG1*, *GADD45 $\beta$* , *POMC*, and *B2MG* transcript levels were then assayed via quantitative reverse-transcription PCR. Cell proliferation was measured from cells cultured in 200  $\mu$ L of culture medium containing siRNA fragments in 96-well plates ( $1.5 \times 10^4$  cells/well density); the medium was changed after 24 hours of incubation. Cell viability was measured at 48 hours post-transfection using the Cell Counting Kit 8.

## Western blot analysis

Western blot analysis was conducted following the protocol described by Yamagata et al, with minor modifications.<sup>22</sup> Protein expression of phosphorylated STAT3/STAT3 in AtT20 cells was assessed by Western blot analysis. After being washed twice with phosphate-buffered saline, cells were lysed with Laemmli sample buffer and centrifuged to remove cell debris. The supernatant was recovered from samples, which were boiled to denature the proteins, and electrophoresed on a 4%–20%-gradient polyacrylamide gel. After electrophoresis, the proteins were cross-linked

to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). Detector Block buffer (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used to block the cross-linked proteins on the membrane, followed by a 1-hour incubation with a 1/1,000 dilution of anti-STAT3 antibody (Cell Signaling Technology, Danvers, MA, USA) or a 1/1,000 dilution of anti-pSTAT3 antibody (Cell Signaling Technology). Membranes were washed with 0.05% Tween 20 phosphate-buffered saline, followed by incubation with horseradish peroxidase-labeled antirabbit immunoglobulin G (Daiichi Kagaku). SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Dallas, TX, USA) was used to detect antibodies, after which the membrane was exposed to BioMax film (Eastman Kodak, Rochester, NY, USA) for visualization.

## Statistical analyses

Each in vitro experiment was performed in triplicate. Following Nakada et al,<sup>13</sup> within each group of experiments, samples were run in duplicate or triplicate. Data were recorded, and are presented as mean  $\pm$  SE. Analysis of variance (ANOVA) and subsequent Fisher's protected least significant difference post hoc tests or unpaired Student's *t*-tests were used to analyze the data, and  $P < 0.05$  was considered significant.

## Results

### Effect of SD1029 on POMC transcript and ACTH levels

To determine the dose- and time-dependent effects of SD1029 on *POMC* mRNA levels, AtT20 cells were incubated with the compound at various concentrations and for various incubation periods. *POMC* mRNA levels were significantly decreased by 10  $\mu$ M SD1029 (ANOVA,  $P < 0.05$ ); over the course of a 24-hour SD1029 incubation, *POMC* transcript levels were reduced to 69% of levels in the control (Figure 1A). Additionally, *POMC* transcript levels decreased in a dose-dependent manner throughout SD1029 incubations (ANOVA,  $P < 0.05$ ), with significant effects occurring at a concentration of 10  $\mu$ M (Figure 1B). Similarly, ACTH levels of the cell-culture medium also decreased significantly under SD1029 treatment for an SD1029 concentration of 10  $\mu$ M ( $P < 0.0001$ , Figure 1C).

### SD1029 effects on cell proliferation and death

The effects of SD1029 on the proliferation and death of AtT20 cells were also revealed through SD1029 incubations. Cell proliferation exhibited a dose-dependent decrease under SD1029 treatments, with significant (ANOVA,  $P < 0.0005$ )

effects observed at 10  $\mu$ M (Figure 2A). Cytoplasmic histone-associated DNA fragmentation was used as a proxy for cell-death induction by SD1029; again, at 10  $\mu$ M SD1029 concentration, DNA fragmentation increased significantly (Figure 2B).

### SD1029 effects on cell-cycle profile

Flow cytometry was used to assess the cell-cycle distribution of the cultures. FACS analyses indicated that the percentages of G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phase cells were not modulated by incubation with SD1029 at 10  $\mu$ M (Figure 3).

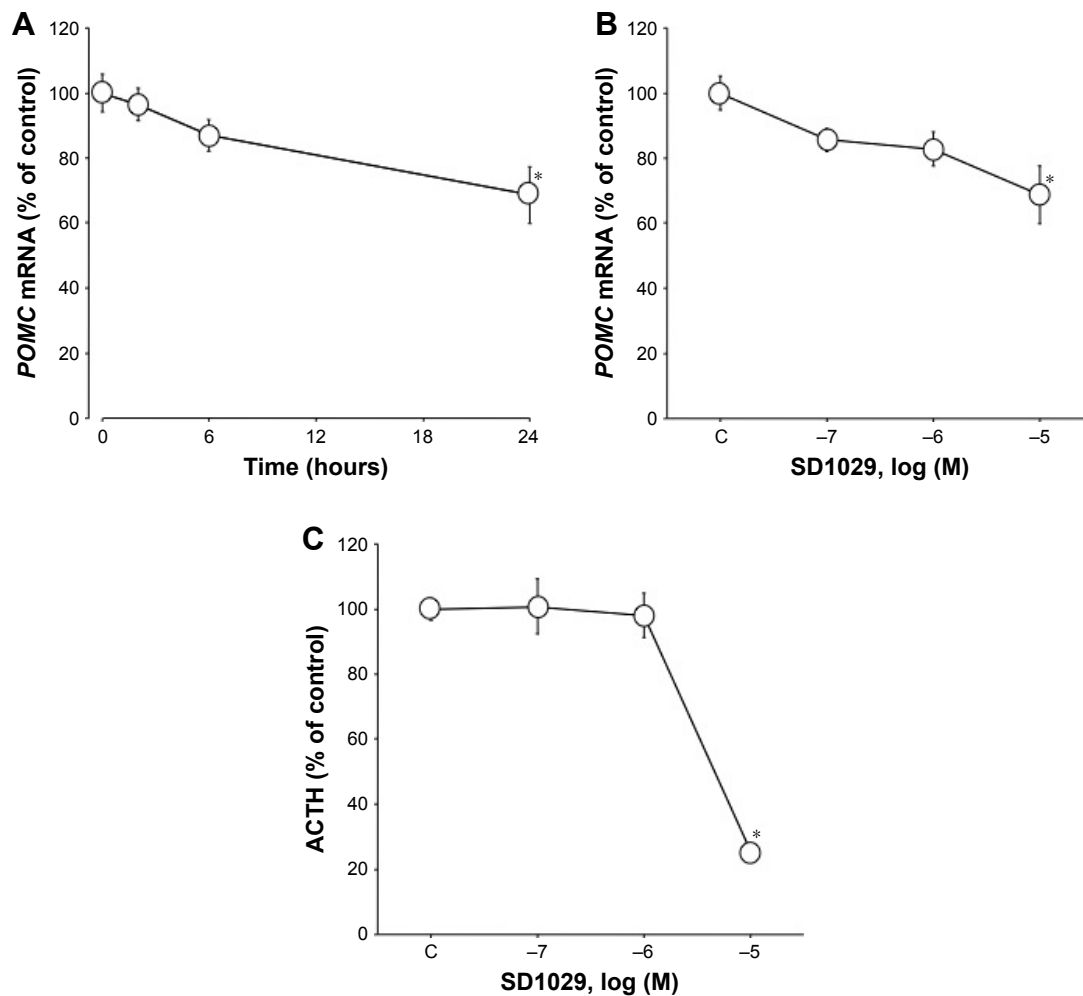
### SD1029 effects on PTTG1 and GADD45 $\beta$ transcript levels

Time-course cultures indicated that 10  $\mu$ M SD1029 significantly decreased and subsequently increased *PTTG1* (ANOVA,  $P < 0.05$ ) and *GADD45 $\beta$*  transcript levels (ANOVA,  $P < 0.0001$ ). Within the first 24 hours of SD1029 incubation, *PTTG1* transcript levels decreased to 77% of the level of the control treatment (Figure 4A); additionally, *PTTG1* levels decreased as SD1029 concentrations increased (ANOVA,  $P < 0.005$ ), with significant effects initially occurring at 10  $\mu$ M SD1029 (Figure 4B). SD1029 also significantly increased *GADD45 $\beta$*  transcript levels in a time- and dose-dependent manner (Figure 4C and D).

### Effects of PTTG1 and GADD45 $\beta$ on POMC transcript levels and cell proliferation

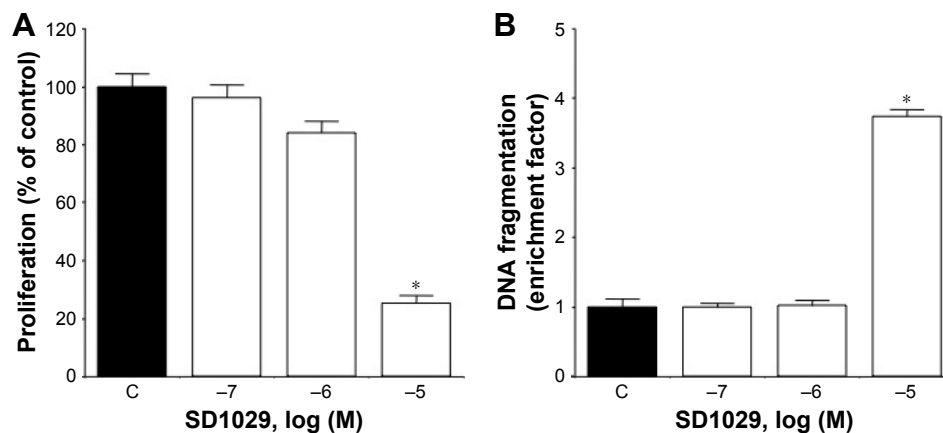
The roles of *PTTG1* and *GADD45 $\beta$* , gene candidates for cell-proliferation regulation, were also examined in AtT20 cells. After transfection with transcript-specific siRNA fragments, *PTTG1* and *GADD45 $\beta$*  mRNA levels were reduced by 42% and 67%, respectively. *PTTG1* knockdown and SD1029 each individually induced a significant decrease in *POMC* transcript levels (ANOVA,  $P < 0.001$ , Figure 5A). Combined treatment with *PTTG1* knockdown and SD1029 had no additive effect on *POMC* transcript levels ( $P < 0.001$ , Figure 5A). *PTTG1* knockdown or SD1029 alone significantly inhibited cell proliferation ( $P < 0.05$ , Figure 5B). Combined treatment with *PTTG1* knockdown and SD1029 had no additive effect on cell proliferation ( $P < 0.001$ , Figure 5B).

*GADD45 $\beta$*  knockdown did not modify basal *POMC* mRNA levels or cell proliferation (Figure 5C and D). SD1029 significantly decreased *POMC* mRNA levels and cell proliferation. *GADD45 $\beta$*  knockdown also significantly inhibited the decrease in *POMC* mRNA levels (Figure 5C), and its knockdown partially inhibited the observed decrease in cell proliferation (Figure 5D).



**Figure 1** Effect of SD1029 on *POMC* mRNA and adrenocorticotropic hormone (ACTH) levels in AtT20 cells.

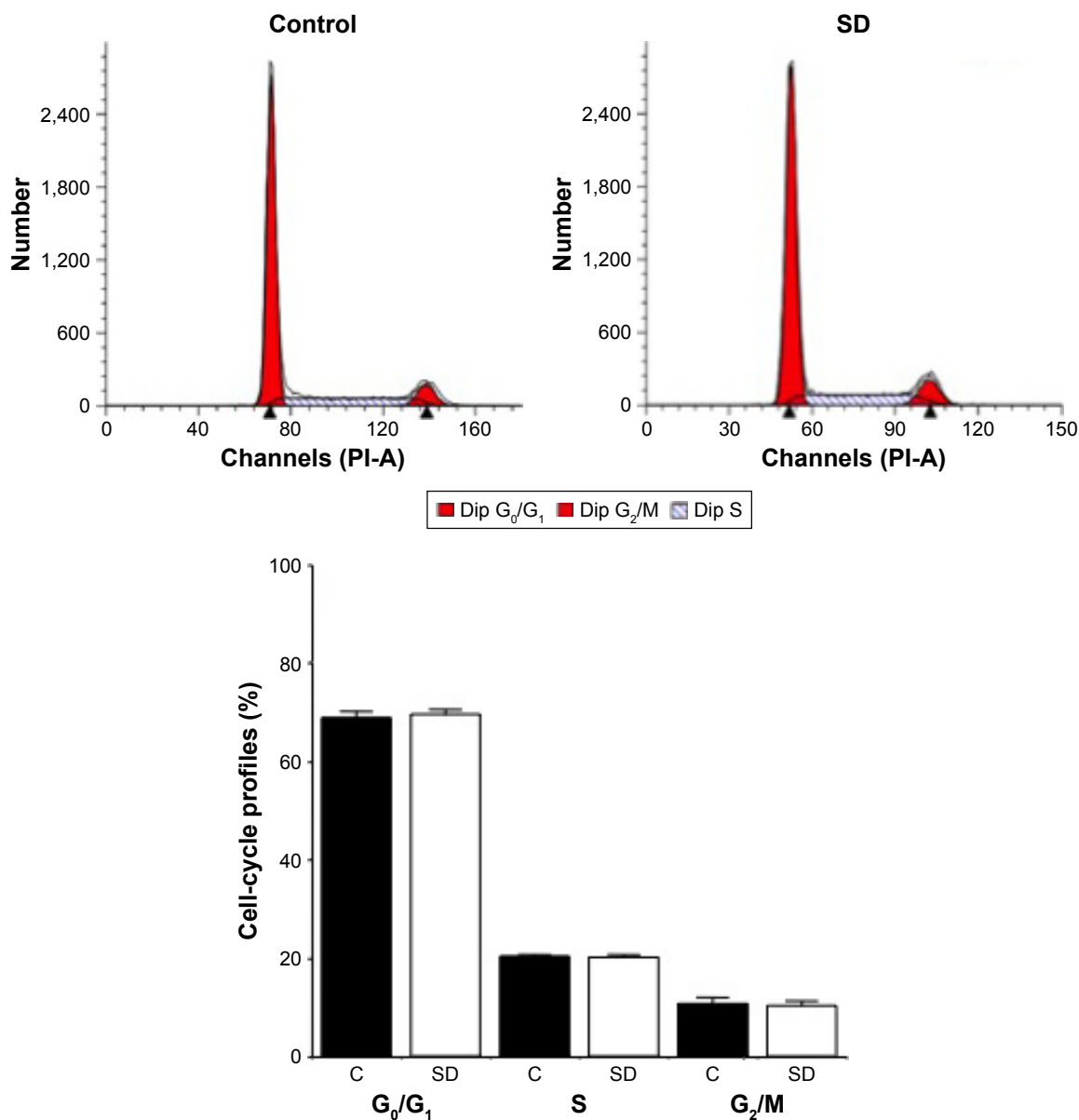
**Notes:** Cell-culture treatments were conducted in triplicate, and the averages of three independent experiments are shown (the average of triplicates was considered as  $n=1$ , in this experiment  $n=3$ ). One-way ANOVA was conducted, followed by Fisher's protected least significant difference post hoc test to determine differences between treatment means ( $*P<0.05$  compared with control [C]). **(A)** Time-dependent effect of SD1029 on *POMC* mRNA levels; cells were incubated with medium containing 10  $\mu$ M SD1029. **(B)** Dose-dependent effects of SD1029 on *POMC* mRNA levels; cells were incubated for 24 hours with medium containing between 100 nM and 10  $\mu$ M SD1029. **(C)** Dose-dependent effects of SD1029 on ACTH levels in AtT20 cells; cells were incubated for 24 hours with medium containing between 100 nM and 10  $\mu$ M SD1029.



**Figure 2** Effect of SD1029 on the proliferation and death of AtT20 cells.

**Notes:** Cell-culture treatments were conducted in triplicate, and the averages of three independent experiments are shown. One-way ANOVA was conducted, followed by Fisher's protected least significant difference post hoc test to determine differences between treatment means ( $*P<0.05$  compared with control [C]). **(A)** Effect of SD1029 on the proliferation of AtT20 cells; cells were incubated for 48 hours with medium containing between 100 nM and 10  $\mu$ M SD1029. Viable cells were measured using Cell Counting Kit 8. **(B)** Effect of SD1029 on cell death in AtT20 cells; cells were incubated for 24 hours with medium containing between 100 nM and 10  $\mu$ M SD1029. DNA fragmentation was assayed using a cell-death detection enzyme-linked immunosorbent-assay kit.





**Figure 3** Effect of SD1029 on the cell-cycle profile of AtT20 cells.

**Notes:** Control cells were treated with the medium and vehicle. Representative graphs are shown in the upper panels. Duplicate cell treatments were conducted, and the average of three independent experiments is shown in the lower panel. Unpaired Student's *t*-tests were conducted to determine differences between treatment means. Cells were incubated for 24 hours with 10  $\mu$ M SD1029 (SD) or the dimethyl sulfoxide control (C). Cellular DNA content was analyzed by flow cytometry, and cell-cycle profiles were determined using BD FACSDiva software.

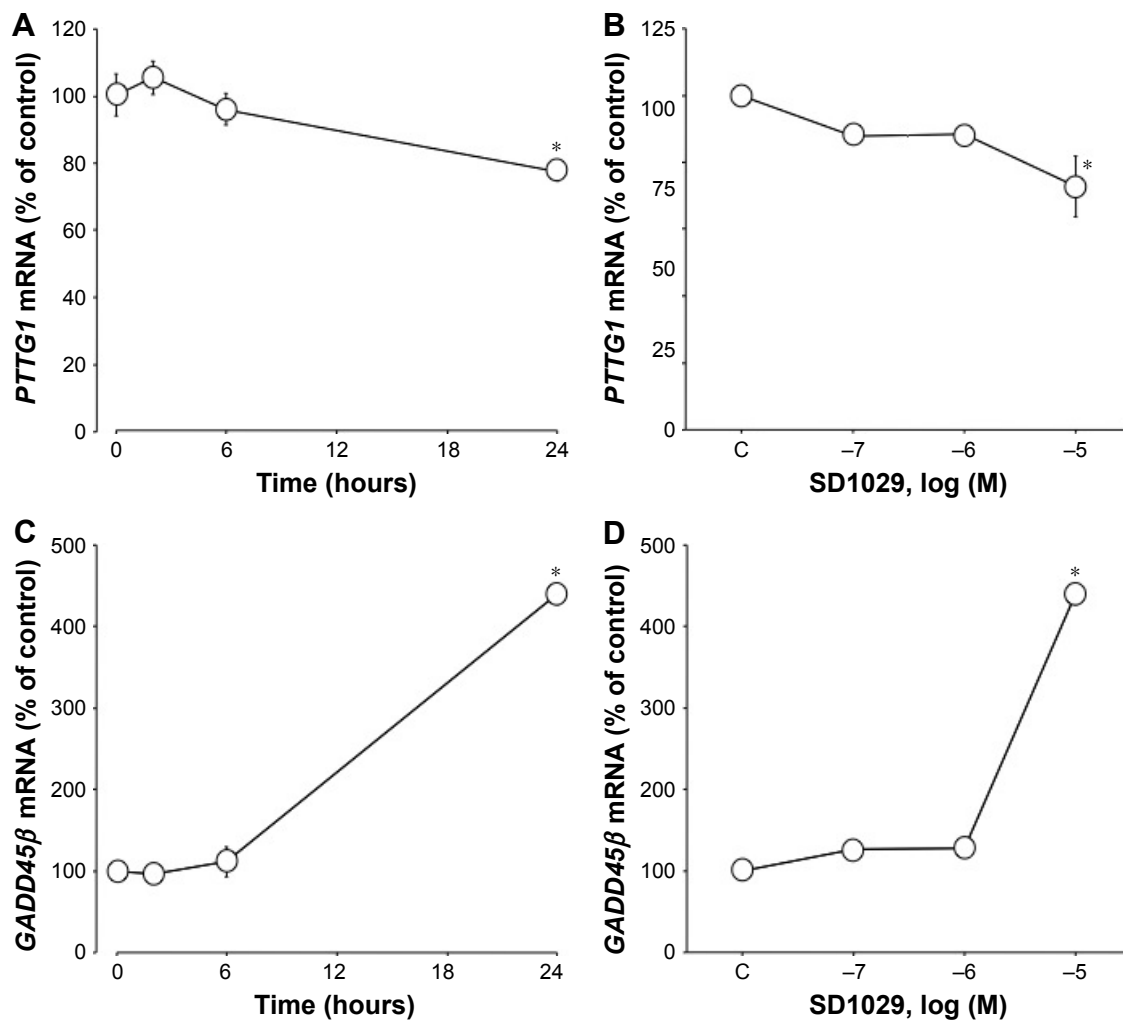
## Time-dependent changes in SD1029-induced STAT3 phosphorylation

AtT20 cells were incubated with 10  $\mu$ M SD1029 to determine their effects on STAT3 phosphorylation. Time-course incubation revealed that SD1029 significantly inhibited STAT3 phosphorylation from 15 to 120 minutes during the incubation period (ANOVA,  $P < 0.0001$ , Figure 6).

## Discussion

This study demonstrated that application of the Jak2 inhibitor SD1029 to AtT20 cell cultures decreased both *POMC*

transcript levels and basal ACTH levels. These in vitro experiments suggest the Jak2 inhibitor suppresses both the autonomic synthesis and release of ACTH in corticotroph tumor cells. SD1029 was also found to inhibit AtT20-cell proliferation. This is supported by previous research on SD1029 that showed the compound increases DNA fragmentation in AtT20 cells, implicating it in the induction of cell death in corticotroph tumor cells.<sup>23,24</sup> FACS analyses indicated that SD1029 did not modulate the percentage of AtT20 cells in any phase of the cell cycle, suggesting that this compound does not attenuate cell-cycle progression, nor does it arrest



**Figure 4** Effect of SD1029 on *PTTG1* and *GADD45β* mRNA levels in AtT20 cells.

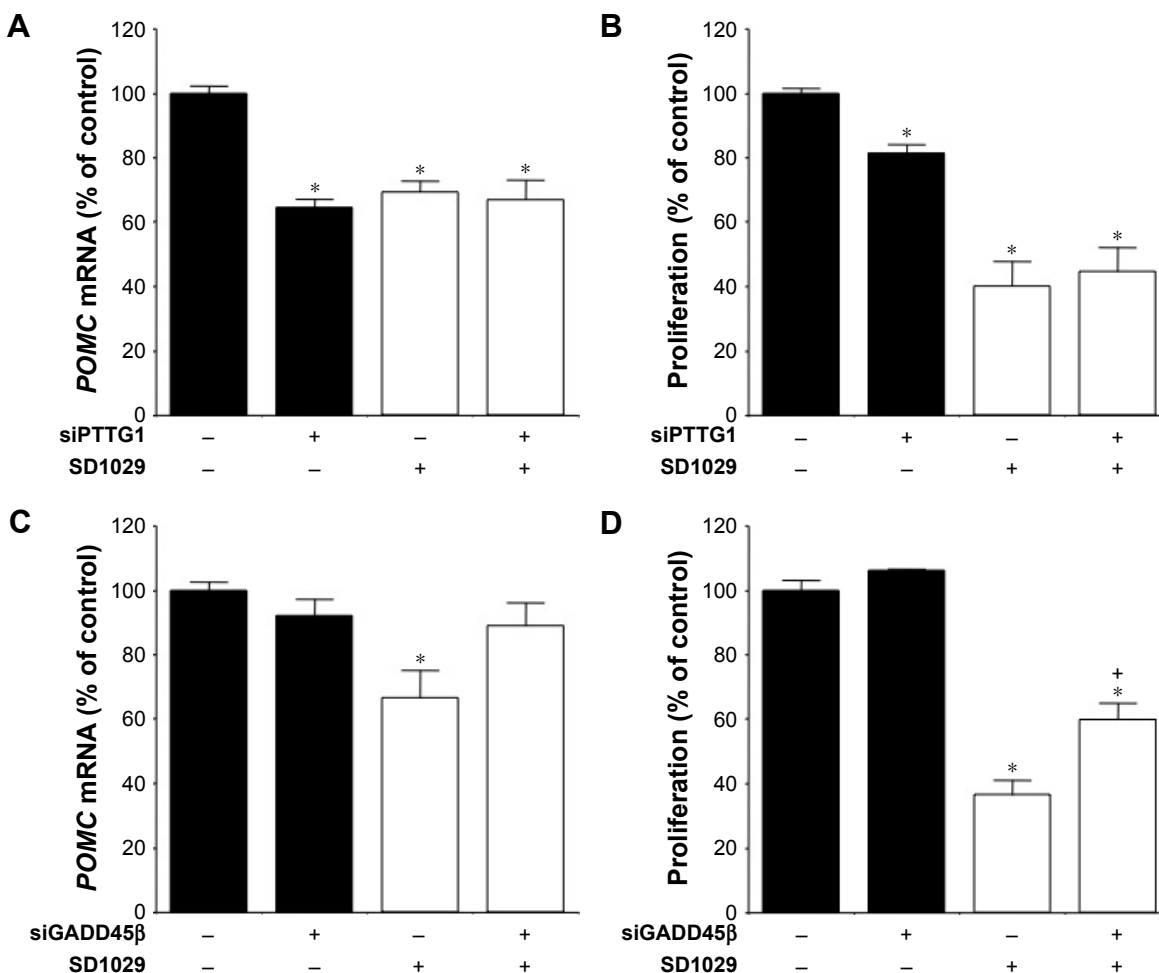
**Notes:** Triplicate cell treatments were conducted, and the average of three independent experiments is shown. One-way ANOVA was conducted, followed by Fisher's protected least significant difference post hoc test to determine differences between treatment means (\* $P < 0.05$  compared with control [C]). (A, C) Time-dependent effect of SD1029 on *PTTG1* and *GADD45β* mRNA levels; cells were incubated with medium containing 10  $\mu$ M SD1029. (B, D) Dose-dependent effects of SD1029 on *PTTG1* and *GADD45β* mRNA levels; cells were incubated for 24 hours with medium containing between 100 nM and 10  $\mu$ M SD1029.

the cell cycle.<sup>23</sup> ACTH production is dependent upon both cell proliferation and synthesis; as such, decreased overall cell growth via apoptosis may contribute to the decreased ACTH levels observed in the culture medium.

*PTTG1* is a key signaling molecule in pituitary tumors,<sup>25</sup> and is known to facilitate cell-cycle progression and increase pituitary-cell proliferation.<sup>12</sup> Accordingly, the overexpression of *PTTG1* results in the proliferation of pituitary gonadotroph cells.<sup>10</sup> Previous studies have shown that histone deacetylases or HSP90 inhibitors repress *PTTG1* mRNA levels.<sup>13,26</sup> In the present study, the application of SD1029 to AtT20 cells gradually decreased *PTTG1* transcript levels. *PTTG1* was implicated by these AtT20 cell-culture experiments in the Jak2 inhibitor-induced suppression of cell proliferation. Moreover, *PTTG1* knockdown inhibited *POMC* transcript levels and cell proliferation in the current study; however,

combined treatment with *PTTG1* knockdown and SD1029 had no additive effect on *POMC* transcript levels or cell proliferation. Combined, *PTTG1* is at least partially implicated in the Jak2-induced suppression of ACTH synthesis and cell proliferation that was observed in AtT20 cells.

*GADD45β*, a putative downstream target of the tumor-suppressing protein p53, has been shown to inhibit cell proliferation and survival in normal, healthy pituitary tissues.<sup>16</sup> Accordingly, a loss-of-function mutation in *GADD45β* may contribute to the initiation and/or progression of tumorigenesis in human gonadotroph tumors.<sup>16</sup> Overall, SD1029 increased *GADD45β* mRNA levels. SD1029 also decreased *POMC* mRNA levels and cell proliferation. *GADD45β* knockdown inhibited SD1029-induced decreases in *POMC* mRNA levels, suggesting that *GADD45β* may also be involved in the Jak2 inhibitor-induced suppression of *POMC*



**Figure 5** Effect of *PTTG1* and *GADD45β* on *POMC* mRNA levels and proliferation in AtT20 cells.

**Notes:** Cell treatments were conducted in triplicate, and the average of three independent experiments is shown. One-way ANOVA was conducted, followed by a Fisher's protected least significant difference post hoc test to determine differences between treatment means ( $^*P < 0.05$  compared with control). **(A)** Effect of *PTTG1* on *POMC* mRNA levels; cells were seeded in 12-well plates at a density of  $1.2 \times 10^4$  cells/well and incubated for 48 hours in 1 mL of culture medium containing control or *PTTG1*-specific siRNA (siPTTG1). **(B)** Effect of *PTTG1* on cell proliferation; cells were seeded in 96-well plates at a density of  $1.5 \times 10^4$  cells/well and incubated for 48 hours in 200  $\mu$ L of culture medium containing control siRNA or siPTTG1. **(C)** Effect of *GADD45β* on *POMC* mRNA levels; cells were seeded in 12-well plates at a density of  $1.2 \times 10^4$  cells/well and incubated for 48 hours in 1 mL of culture medium containing control or *GADD45β*-specific siRNA (siGADD45β). **(D)** Effect of *GADD45β* on cell proliferation; cells were seeded in 96-well plates at a density of  $1.5 \times 10^4$  cells/well and incubated for 48 hours in 200  $\mu$ L of culture medium containing control siRNA or siGADD45β. The symbols "+" and "-" mean culture medium with and without the material, respectively.

mRNA levels. Its knockdown also partially inhibited the decrease in cell proliferation. Therefore, the *GADD45β* pathway may be involved in the suppression of proliferation in corticotroph tumor cells.

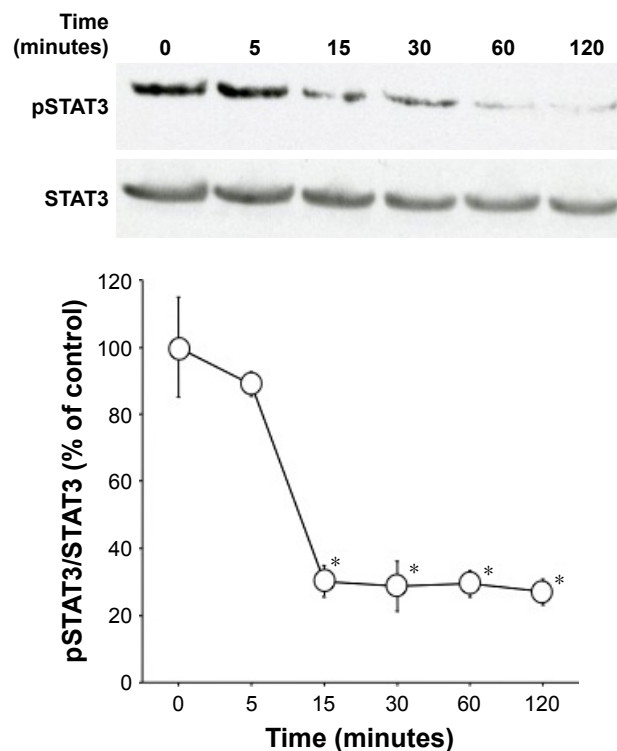
EGFR signaling induces *POMC* mRNA levels and ACTH secretion in corticotroph tumors.<sup>27</sup> Accordingly, blocking EGFR-tyrosine activity suppresses ACTH production and hence tumor growth.<sup>27</sup> Cleavage of USP8 contributes to increased deubiquitination of EGFR, impairing its downregulation and enhancing cellular EGFR signaling.<sup>17</sup> Therefore, this receptor may be a candidate target for Cushing's disease therapies. The Jak-STAT pathway is located downstream of the EGFR-signaling pathway.<sup>28</sup> Therefore, Jak2/STAT3 signaling is a critical element in EGFR-driven migration and invasion<sup>29</sup> and a potential target for cancer therapy.<sup>30</sup> STAT3 is also required for

*POMC* transcription.<sup>31</sup> Indeed, STAT3 has been shown directly to regulate *POMC* in the pituitary and hypothalamus.<sup>31,32</sup> The tolerance of normal cells to the loss of Jak2/STAT3 function has driven recent research efforts to identify molecules capable of Jak2/STAT3 inhibition,<sup>19</sup> yielding SD1029 as a novel potent Jak2 and STAT3 inhibitor. In our study, SD1029 decreased STAT3 phosphorylation in corticotroph AtT20 tumor cells. EGFR-dependent Jak2/STAT3 therapies have potential clinical implications for treating Cushing's disease.

## Conclusion

In AtT20 cell, SD1029 decreased *POMC* transcript and ACTH levels and proliferation. Additionally, SD1029 decreased and increased *PTTG1* and *GADD45β* transcript levels, respectively. Both *PTTG1* and *GADD45β* may be





**Figure 6** Time-dependent changes in SD1029-induced STAT3 phosphorylation. **Notes:** AtT20 cells were incubated with medium containing 10  $\mu$ M SD1029 for the durations shown. Cells treatments were conducted in duplicate, and the average of three independent experiments is shown. One-way ANOVA was conducted, followed by Fisher's protected least significant difference post hoc test to determine differences between treatment means (\* $P$ <0.05 compared with control [0 minutes]).

responsible, at least partially, for the Jak2-induced suppression of cell proliferation and ACTH synthesis. SD1029 decreased STAT3 phosphorylation. Accordingly, research on Cushing's disease therapies should focus on EGFR-dependent Jak2/STAT3 therapies.

## Acknowledgment

We thank the Department of Social Medicine, Hirosaki University Graduate School of Medicine for assistance with the FACS analysis.

## Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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