# Inhibitory effects of trichostatin A on adrenocorticotropic hormone production and proliferation of corticotroph tumor AtT-20 cells

Trichostatin A による AtT-20 腫瘍細胞における ACTH 産生と細胞増殖の抑制作用

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## Abstract.

Cushing's disease is primarily caused by adrenocorticotropic hormone (ACTH)-producing pituitary adenomas. Pituitary tumor-transforming gene 1 (*PTTG1*) expression, a hallmark of pituitary tumors, stimulates pituitary cell proliferation. Histone deacetylases (HDACs) play an important role in regulating gene transcription and HDAC inhibitors induce cellular differentiation and suppress tumor cell proliferation. HDAC inhibitors also repress *PTTG1* mRNA levels. Trichostatin A (TSA) is a potent cell-permeable HDAC inhibitor that blocks cell cycle progression. In the present study, we determined the effect of TSA on ACTH production and cellular proliferation in mouse AtT-20 corticotroph tumor cells. TSA decreased proopiomelanocortin (*POMC*) mRNA levels in AtT-20 cells and reduced ACTH levels in the culture medium of these cells. The TSA-induced decreases in *POMC* mRNA levels were not modulated when TSA and dexamethasone were simultaneously administered. Drug treatment also decreased AtT-20 cell proliferation, induced apoptosis, and increased the percentage of cells in G0/G1 phase using flow cytometry. TSA decreased *PTTG1* mRNA levels. Furthermore, *PTTG1* knockdown inhibited cellular proliferation. Its knockdown also inhibited *POMC* mRNA and ACTH levels. TSA inhibits ACTH production and corticotroph tumor cell proliferation. TSA may inhibit cellular proliferation, and ACTH synthesis and secretion by decreasing *PTTG1* expression.

*Key words*: Cushing's disease, Adrenocorticotropic hormone (ACTH), Proopiomelanocortin (POMC), Pituitary tumor, Trichostatin

Cushing's disease is primarily caused by adrenocorticotropic hormone (ACTH)-producing pituitary adenomas [1, 2]. In adenoma cells, the normal cortisol feedback mechanism of the hypothalamic-pituitary-adrenal axis is disturbed, resulting in hypercortisolism which results in metabolic derangements such as diabetes mellitus, hypertension, atherosclerosis, and immune dysfunction [3]. ACTH production may be associated with cell mass or pituitary corticotroph tumor cell growth [4]. Tumor growth is determined, at least in part, by cellular proliferation, cell cycle progression, and apoptosis [5].

Pituitary tumor-transforming gene 1 (*PTTG1*) is an oncogene that was first cloned from a rat pituitary tumor [6]. PTTG1 expression is a hallmark of pituitary tumors [7, 8]. PTTG facilitates cell cycle progression, increases pituitary cell proliferation, and promotes murine pituitary development [9]. Heat shock protein 90 (Hsp90) is an essential molecular chaperone involved in the folding and stabilization of client proteins which regulate the survival of cancer cells. Hsp90 inhibitors have been reported to repress PTTG1 expression in carcinoma cells [10, 11], and are thought to act in a similar way in pituitary tumor cells. Previous studies have demonstrated that Hsp90 inhibitors decreased *POMC* mRNA and ACTH levels in AtT-20 cells [12, 13]. These drugs have also been shown to decrease *PTTG1* mRNA and cellular proliferation in AtT-20 cells. The modification of histone acetylation plays an important role in controlling PTTG1 expression. Histone deacetylase (HDAC) inhibitors suppress *PTTG1* mRNA levels in a manner similar to Hsp90 inhibitors [11].

Of the numerous HDACs have been identified, the prototypical ones are HDACs 1, 2, and 3. HDACs play an important role in regulating gene transcription. HDAC inhibitors induce cellular differentiation and suppress tumor cell proliferation [14]. Trichostatin A (TSA), originally used as an antifungal drug, is a representative hydroxamic acid-based natural product that possesses an HDAC inhibitor activity. TSA is a potent, cell-permeable HDAC1 and 2 inhibitor which blocks cell cycle progression.

In the present study, we examined the effect of TSA on ACTH production and cellular proliferation in AtT-20 corticotroph tumor cells. To further elucidate the possible mechanisms of TSA action, we also examined the effect of PTTG1 on cellular proliferation.

## Materials and Methods

### *Materials*

TSA was purchased from Calbiochem (San Diego, CA, USA), dissolved in dimethyl sulfoxide (DMSO), diluted with cell culture medium, and used at concentrations between 10 nM and 10 μM.

### *Cell culture*

AtT-20 pituitary corticotroph tumor cells were cultured in T75 culture flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere of 5% CO2. The cells were seeded in 6-well plates at a density of 1.5 × 105 cells/well and grown for 3 days before each experiment. The culture medium was changed every 48 h. To minimize the effect of exogenous factors contained within FBS, one day before each experiment, cells were washed and serum starved overnight with DMEM supplemented with 0.2% bovine serum albumin. At the end of each experiment, total cellular RNA or protein was collected and stored at −80°C until the relevant assay was performed.

### *RNA extraction*

Cells were incubated with medium alone (control) or medium containing TSA for the indicated times. To examine the dose-dependent effects of TSA, cells were incubated for the indicated times with medium alone (control) or medium containing increasing concentrations of TSA (100 nM to 10 μM). At the end of each experiment, total cellular RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 0.5 μg of total RNA with random hexamer primers using the SuperScript First-Strand Synthesis System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR; Invitrogen Corp., Carlsbad, CA) in accordance with the manufacturer's instructions.

*Quantitative real-time RT-PCR*

Total cellular RNA extraction and cDNA synthesis were performed as previously described [15, 16]. The resulting cDNA was subjected to real-time PCR as described below. The expression of mouse *POMC* (NM_008895.3) and *PTTG1* (NM_001131054.1) mRNA were evaluated using quantitative real-time PCR with specific primer and probe sets (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA). ß2-microglobulin (B2MG) was used as a reference gene to standardize expression levels. *B2MG* mRNA levels were not significantly altered by any of the treatments used in this study. Each real-time PCR reaction consisted of 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 1 × Assays-on-Demand Gene Expression Products (Mm00435874_m1 for mouse *POMC*, Mm00479224 m1 for mouse *PTTG1*, Mm00435121 g1 for mouse stress response gene growth arrest and DNA damage-inducible 45ß (*GADD45ß*), and Mm00437762_m1 for mouse *B2MG*), and 500 ng of cDNA in a total volume of 25 µL. Amplification was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using 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.

Specific sets of primers and a TaqMan probe spanning the exon–exon junction were used in the real-time PCR assays and the results should not, therefore, have been affected by DNA contamination. Data were collected and recorded using the ABI PRISM 7000 SDS software (Applied Biosystems) and are expressed as a function of the threshold cycle (CT). The amplification efficiency of each gene of interest and the reference gene were found to be identical when analyzed using diluted samples.

Relative quantitation of gene expression was calculated using the 2-ΔΔCT method. In brief, for each sample assayed, the CT values for the gene of interest and reference gene were determined. The CT of the gene of interest was then corrected by subtracting the CT of the housekeeping gene (ΔCT) for each sample. Untreated control samples were used as reference samples, and the ΔCT of all

experimental samples was reduced by the average ΔCT of the control samples (ΔΔCT). Finally, the abundance of the experimental mRNA relative to the control mRNA was calculated using the formula 2-ΔΔCT.

*ACTH assay*

Cells were incubated at 37°C for 24 h with the indicated concentrations of TSA. The media was then aspirated and the ACTH level in the supernatants was measured using an ACTH enzyme-linked immunosorbent assay (ELISA) kit (MD Bioproducts, Zurich, Switzerland). The intra- and inter-assay coefficients of variation (CV) were 6.7% at 42.2 pg/mL and 7.1% at 42.3 pg/mL ACTH, respectively. Cross-reactivity with ACTH fragment (1–24), ACTH fragment (18–39), and α-melanocyte-stimulating hormone was −3.4%, −2.4%, and −1.7%, respectively.

*Cellular proliferation assay*

Cells were incubated at 37°C for 48 h with the indicated concentrations of TSA. Viable cells were measured using the Cell Counting Kit-8 (Dojin, Kumamoto, Japan). All of the samples from each experiment were determined in the same assay.

*Cell death detection assay*

Cells were incubated at 37°C for 24 h with the indicated concentrations of TSA. DNA fragmentation was measured using the Cell Death Detection ELISA Kit (Roche, Penzberg, Germany) and each enrichment factor was calculated in accordance with the manufacturer's instructions.

*Cell cycle analysis*

AtT-20 cells were incubated for 24 h with medium alone (control) or medium containing 1 μM TSA. The cells were then harvested by trypsinization, pelleted by

centrifugation, and resuspended in Triton X-100. The cells were treated with 0.5% RNase A and stained with propidium iodine (50 μg/mL) at 37°C for 30 min and the cellular DNA content was analyzed using fluorescence-activated cell sorting (FACS). The cell cycle profiles were determined using BD FACSDiva™ software (Becton Dickinson, Franklin Lakes, NJ).

### *RNA interference experiments*

*PTTG1*-specific and control small interfering (si) RNAs were designed and purchased from QIAGEN. Cells were transfected with siRNA using HiPerFect transfection reagent (QIAGEN) in accordance with the manufacturer's protocol. To determine *PTTG1* mRNA levels, cells were seeded into 12-well plates at a density of 12 × 104 cells/well and incubated in 1 mL of culture medium containing either control siRNA (siControl) or *PTTG1*-specific siRNA (siPTTG1; Mm_Pttg1_7) for 48 h. The expression of *PTTG1, POMC*, and *B2MG* mRNA was then examined using quantitative RT-PCR. The ACTH level in the medium was measured using an ACTH ELISA kit. To measure cell proliferation, cells were seeded in 96-well plates at a density of 1.5 × 104 cells/well and incubated for 24 h in 200 μL of culture medium containing siControl or siPTTG1 prior to changing the media. Viable cells were measured 48 h after transfection using the Cell Counting Kit-8.

### *Statistical analysis*

Each *in vitro* experiment was performed 3 times. Samples were run in triplicate for each group of experiments. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using analysis of variance (ANOVA) followed by Fisher's protected least-significant difference post hoc test or using the unpaired Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

## Results

### *Effect of TSA on POMC mRNA and ACTH levels*

AtT-20 cells were incubated with TSA to determine the time- and dose-dependent effects on *POMC* mRNA levels. A time course study indicated that 1 μM TSA significantly decreased *POMC* mRNA levels. *POMC* mRNA levels fell to 37% of the control value (Fig. 1A) within 24 h of TSA addition. *POMC* mRNA levels were also decreased in a TSA dose-dependent manner (ANOVA; $P < 0.0001$), with significant effects observed from 100 nM to 1 μM (Fig. 1B). Similarly, the ACTH level in the cell culture medium was also decreased in a dose-dependent manner (ANOVA; $P < 0.005$), with significant effects observed from 100 nM to 1 μM TSA (Fig. 1C). To further examine a possible interaction between TSA and glucocorticoids, the effect of TSA and dexamethasone on *POMC* mRNA levels was examined. Both TSA and dexamethasone individually decreased *POMC* mRNA levels (Fig. 1D). However, the TSA-induced decrease in *POMC* mRNA levels were not modulated when TSA and dexamethasone were simultaneously added (Fig. 1D).

### *Effect of TSA on cellular proliferation and cell death*

AtT-20 cells were incubated with TSA to determine the dose-dependent effects on cellular proliferation. Cellular proliferation decreased in a TSA dose-dependent manner (ANOVA; $P < 0.0001$), with significant effects observed from 100 nM to 10 μM TSA (Fig. 2A).

To examine whether TSA induced cell death, the level of cytoplasmic histone-associated DNA fragmentation was determined. DNA fragmentation increased in a TSA dose-dependent manner (ANOVA; $P < 0.0001$), with significant effects observed from 100 nM to 10 μM TSA (Fig. 2B).

### *Effect of TSA on the cell cycle profile*

Cell cycle distribution was assessed using flow cytometry. FACS analyses

indicated that the percentage of cells in G0/G1 phase was increased after incubation with 1 μM TSA. Reciprocally, the percentage of cells in S phase decreased after incubation with 1 μM TSA (Fig. 3).

*Effect of TSA on PTTG1 mRNA levels*

A time course study indicated that 1 μM TSA potently decreased *PTTG1* mRNA levels (ANOVA; $P < 0.005$, Fig. 4A). Within 24 h of TSA addition, *PTTG1* mRNA levels fell to 62% of the control value (Fig. 4A). *PTTG1* mRNA levels also decreased in a dose-dependent manner (ANOVA; $P < 0.0005$), with significant effects observed from 100 nM to10 μM TSA (Fig. 4B). As a positive control, GADD45ß levels were also measured in this experiment. TSA significantly increased *GADD45ß* mRNA levels in timedependent and dose-dependent manners (ANOVA; $P < 0.001$, Fig. 4C and 4D).

*Effect of PTTG1 on cellular proliferation, POMC mRNA, and ACTH levels*

We proceeded to examine the role of PTTG1 in AtT-20 cells since PTTG1 could be involved in the regulation of cell proliferation. *PTTG1* mRNA levels were reduced by 42% in cells transfected with *PTTG1*-specific siRNA ($P < 0.01$; Fig. 5A) and *PTTG1* knockdown significantly inhibited cellular proliferation ($P < 0.005$; Fig. 5B). Its knockdown also significantly inhibited POMC mRNA and ACTH levels ($P < 0.05$; Fig. 5C and 5D).

## Discussion

In this study, we showed that the HDAC inhibitor TSA decreased *POMC* mRNA levels and the basal level of ACTH in the culture medium of AtT-20 cells. These results suggest that the HDAC inhibitor suppresses the autonomic synthesis and release of ACTH in corticotroph tumor cells. We also showed that TSA decreased AtT-20 cell proliferation. In accordance with this finding, TSA was found to increase DNA fragmentation in AtT-20 cells, suggesting that it induces cell death in corticotroph tumor cells. FACS analyses indicated that TSA increased the percentage of AtT-20 cells in G0/G1 phase, suggesting that the drug may attenuate cell cycle progression or cause cell cycle arrest in G0/G1 phase. This would also result in the inhibition of cellular proliferation. Decreased cell numbers in TSA cultures could contribute to the observed decrease in ACTH levels.

Valproic acid, an HDAC2 inhibitor, has been reported to prevent the suppression of *POMC* promoter activity by glucocorticoid in AtT-20 cells [17]. In our study, TSA, an HDAC1 and 2 inhibitor, treatment resulted in decreased levels of *POMC* mNA. The effect was not modulated by glucocorticoid treatment of the AtT-20 cells. It is not clear whether this discrepancy is a result of differences between *POMC* promoter activity and mRNA levels. *HDAC2* mRNA levels have been reported to be decreased in corticotroph tumors [18], but no such data exist for the expression of other HDAC subtypes. It is possible that valproic acid and TSA exhibit different specificities for the HDAC subtypes, and this could account for the observed results. Even in the presence of glucocorticoids, TSA appeared to decrease ACTH production.

Previous studies have shown that HDAC inhibitors repress *PTTG1* mRNA levels in a manner similar to Hsp90 inhibitors [11]. In the present study, we found that TSA gradually decreased *PTTG1* mRNA levels in AtT-20 cells, similar to the Hsp90 inhibitors 17-AAG and CCT018159 [12]. PTTG1 has been shown to facilitate cell cycle progression and increase pituitary cell proliferation [9], and that the overexpression of PTTG1 results in the proliferation of pituitary gonadotroph cells

[8]. These findings suggest that PTTG1 may be involved in the HDAC inhibitor-induced suppression of cellular proliferation in AtT-20 cells. Indeed, in the present study, *PTTG1* knockdown inhibited cellular proliferation. Its knockdown also inhibited POMC mRNA and ACTH levels. Taken together, these findings indicate that PTTG1 may be responsible, at least in part, for the TSA-induced suppression of cellular proliferation, and ACTH synthesis and secretion in AtT-20 cells.

## Conclusion

TSA decreased *POMC* mRNA and ACTH levels in AtT-20 cells. The drug also decreased *PTTG1* mRNA levels and cellular proliferation, presumably due to the induction of cell cycle arrest and cell death. *PTTG1* knockdown inhibited cellular proliferation. Thus, TSA may inhibit cellular proliferation, as well as ACTH synthesis and secretion, via decreased PTTG1 expression in AtT-20 cells.

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## Conflicts of Interest

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

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## Figure Legends

Fig. 1

Effect of TSA on proopiomelanocortin (*POMC*) mRNA and adrenocorticotropic hormone (ACTH) levels in AtT-20 cells. Cells were treated in triplicate and the average of 3 independent experiments is shown (the average of triplicates was considered as n = 1, in this experiment n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference post hoc test. $*P < 0.05$ (compared with control [C]). (A) Time-dependent effect of TSA on *POMC* mRNA levels: cells were incubated with medium containing 1 μM TSA. (B) Dose-dependent effects of TSA on *POMC* mRNA levels: cells were incubated for 24 h with medium containing between 10 nM and 1 μM TSA. (C) Dosedependent effects of TSA on ACTH levels in AtT-20 cells: cells were incubated for 24 h with medium containing between 10 nM and 1 μM TSA. (D) Effect of TSA and dexamethasone on *POMC* mRNA levels: cells were incubated for 24 h with medium containing 1 μM TSA (T) and/or 100 nM dexamethasone (Dex).

Fig. 2

Effect of TSA on cellular proliferation and cell death in AtT-20 cells. Cells were treated in triplicate and the average of 3 independent experiments is shown. Statistical analysis was performed using one-way ANOVA followed by Fisher's protected least-significant difference post hoc test. $*P < 0.05$ (compared with control [C]). (A) Effect of TSA on cellular proliferation in AtT-20 cells: cells were incubated for 48 h with medium containing between 10 nM and 10 μM TSA. Viable cells were measured using Cell Counting Kit-8. (B) Effect of TSA on cell death in AtT-20 cells: cells were incubated for 24 h with medium containing between 10 nM and 10 μM TSA. DNA fragmentation was measured using Cell Death Detection ELISA Kit.

Fig. 3

Effect of TSA on the cell cycle profile of AtT-20 cells. Control cells were treated with medium and vehicle. A representative histogram is shown in the upper panels. Cells were treated in duplicate and the average of 3 independent experiments is shown in the lower panel. Statistical analysis was performed using one-way ANOVA followed by Fisher's protected least-significant difference post hoc test. $*P < 0.05$ (compared with control [C]). Cells were incubated for 24 h with 1 μM TSA or vehicle containing DMSO. Cellular DNA content was analyzed using flow cytometry and the cell cycle profiles were determined using BD FACSDiva™ software.

Fig. 4

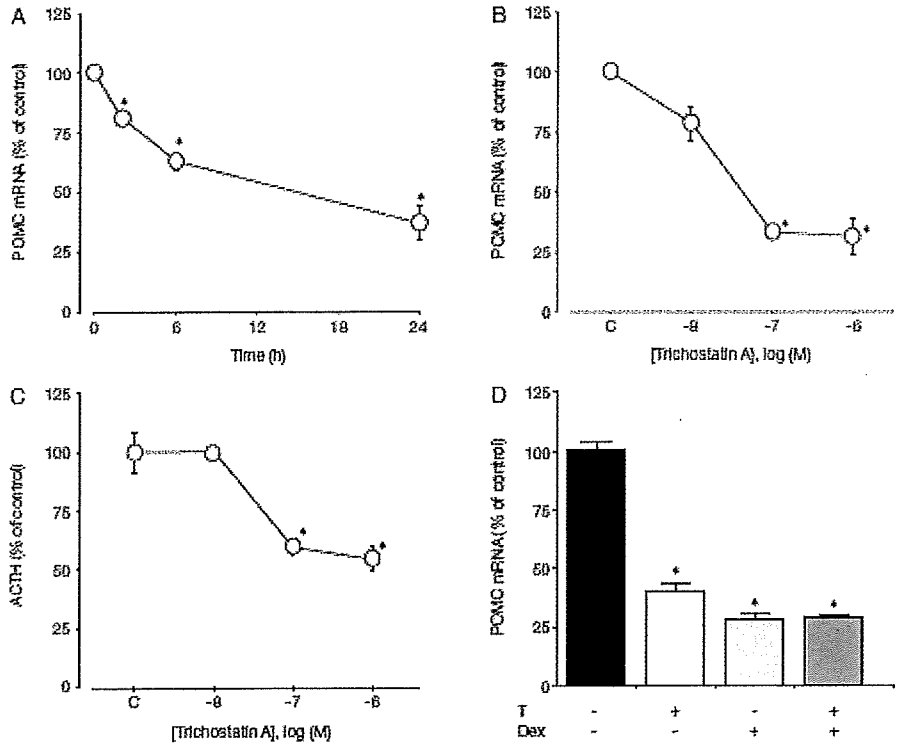
Effect of TSA on pituitary tumor transforming gene 1 (*PTTG1*) mRNA levels in AtT-20 cells. Cells were treated in triplicate and the average of 3 independent experiments is shown. Statistical analysis was performed using one-way ANOVA followed by Fisher's protected least-significant difference post hoc test. $*P < 0.05$ (compared with control [C]). (A and C) Timedependent effect of TSA on *PTTG1* and *GADD45β* mRNA levels: cells were incubated with medium containing 1 μM TSA. (B and D) Dose-dependent effects of trichostatin A on *PTTG1* and *GADD45β* mRNA levels: cells were incubated for 24 h with medium containing between 100 nM and 10 μM TSA.

Fig. 5

Effect of *PTTG1* on cellular proliferation, *POMC* mRNA, and ACTH levels in AtT-20 cells. Cells were treated in triplicate and the average of 3 independent experiments is shown. Statistical analysis was performed using the unpaired Student's *t*-test. $*P < 0.05$ (compared with control). (A, C, and D) Effect of *PTTG1* on *PTTG1* mRNA, *POMC* mRNA, and ACTH levels: cells, seeded into 12-well plates at a density of 12 × 104 cells/well, were incubated for 48 h in 1 mL of culture

medium containing control or *PTTG1*-specific small interfering (si) RNA (siControl and siPTTG1, respectively). (B) Effect of *PTTG1* on cellular proliferation: cells, seeded into 96-well plates at a density of 1.5 × 104 cells/well, were incubated for 48 h in 200 μL of culture medium containing control or *PTTG1*-specific small interfering (si)RNA (siControl and siPTTG1, respectively).

Fig. 1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0
0
6
12
18
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Time (h)
B
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C
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-7
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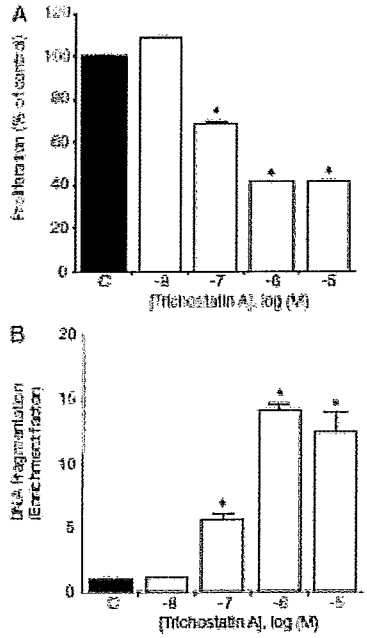
T
Dex

Fig. 2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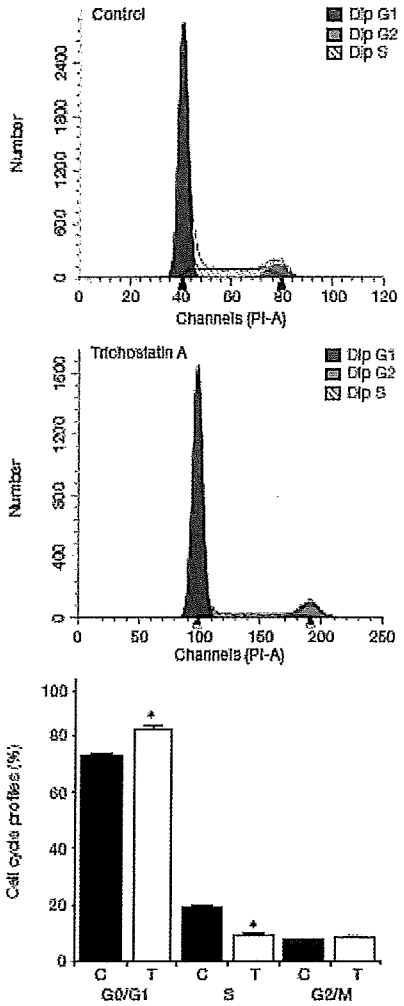
B
DNA fragmentation (Enrichment factor)
[Trichostatin A], log (M)

Fig. 3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Number
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Channels (PI-A)
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Fig. 4

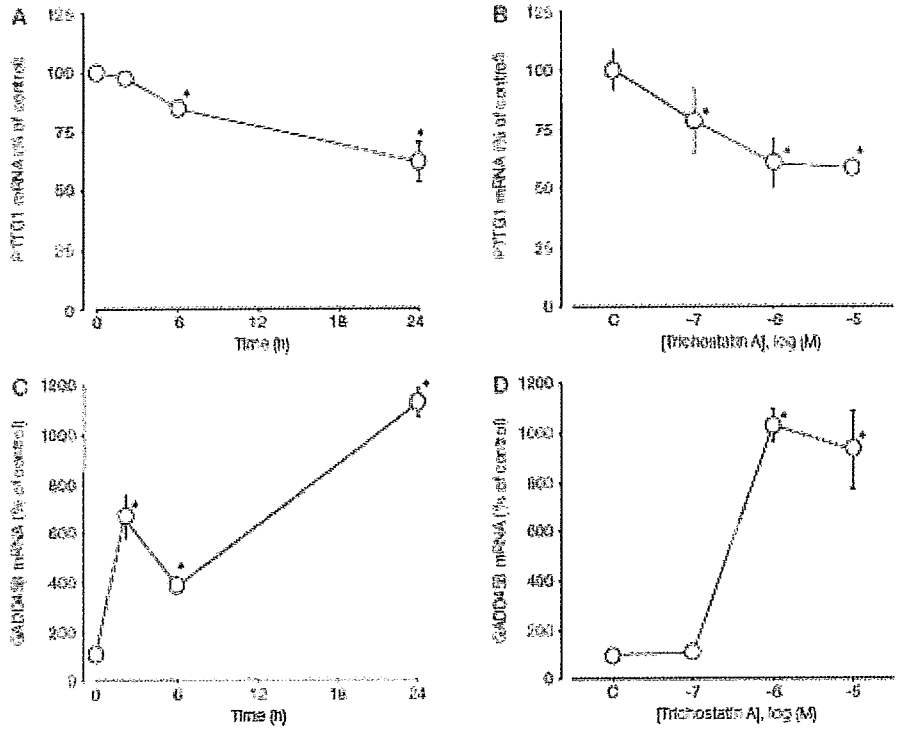


Fig. 5