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Prolyl oligopeptidase inhibition-induced growth arrest of human gastric cancer cells

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ABSTRACT

Prolyl oligopeptidase (POP) is a serine endopeptidase that hydrolyzes post-proline peptide bonds in peptides that are <30 amino acids in length. We recently reported that POP inhibition suppressed the growth of human neuroblastoma cells. The growth suppression was associated with pronounced G_0/G_1 cell cycle arrest and increased levels of the CDK inhibitor $p27^{kip1}$ and the tumor suppressor p53. In this study, we investigated the mechanism of POP inhibition-induced cell growth arrest using a human gastric cancer cell line, KATO III cells, which had a p53 gene deletion. POP specific inhibitors, 3-({4-[2-(*E*)-styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) and benzyloxycarbonyl-thioprolyl-thioprolinal, or RNAi-mediated POP knockdown inhibited the growth of KATO III cells irrespective of their p53 status. SUAM-14746-induced growth inhibition was associated with G_0/G_1 cell cycle phase arrest and increased levels of $p27^{kip1}$ in the nuclei and the pRb2/p130 protein expression. Moreover, SUAM-14746-mediated cell cycle arrest of KATO III cells was associated with an increase in the quiescent G_0 state, defined by low level staining for the proliferation marker, Ki-67. These results indicate that POP may be a positive regulator of cell cycle progression by regulating the exit from and/or reentry into the cell cycle by KATO III cells.

1. Introduction

Prolyl oligopeptidase (POP, EC 3.4.1.26) is an oligopeptidase that cleaves proline-containing peptides that are <30 amino acids in length and belongs to the POP family of serine proteases (family S9 of clan SC). In mammals, high POP activities have been detected in the brain, kidney, and testis [1]. In general, POP has been considered to be a cytosolic enzyme, but it has been detected as a membrane-bound form and in the nucleus of non-neuronal cell lines and neuronal cell cultures [2]. POP may play a role in numerous biological processes, such as learning and memory [3,4], signal transduction [5], and protein secretion [6]. However, no conclusive results have yet been reported, and the primary physiological role of POP remains to be elucidated.

POP involvement in cell division was suggested in several studies [7–10]. POP was shown to be involved in cell proliferation and DNA replication in a mouse Swiss 3T3 cell line [7]. In addition, POP activity is commonly elevated in many cancers [8,9]. More recently, POP was found in various cell types in both the cytoplasm and nuclei in mouse whole-body sections and at the cellular level in peripheral tissues by immunohistochemical study. The nuclear colocalization of the POP protein and Ki-67, a proliferation marker protein, suggested that POP is involved in cell proliferation [10].

We recently reported the effects of POP inhibitors, 3-({4-[2-(*E*)-styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) and benzyloxycarbonyl-thioprolyl-thioprolinal (Z-TT-CHO), and small interfering RNAs directed against human POP on the growth of NB-1 human neuroblastoma cells [11]. Our results indicated that POP inhibition suppressed the growth of NB-1 cells without inducing cell death. SUAM-14746-induced growth inhibition was associated with pronounced G_0/G_1 cell cycle arrest and reduced levels of phosphorylated retinoblastoma protein (pRb), cyclin E, and cyclin dependent kinase (CDK) 2, and increased levels of the CDK inhibitor $p27^{kip1}$ and the tumor suppressor p53. SUAM-14746 also induced a transient inhibition of S and G_2/M cell cycle phase progression, which was correlated with retarding the decreases in the levels of cyclins A and B. RNAi-mediated POP knockdown also resulted in inhibition of NB-1 cell growth, and this effect was accompanied by G_0/G_1 cell cycle arrest.

p53-Dependent G1 cell cycle arrest is well documented. After cells are exposed to stress, the half-life of the p53 protein significantly increases, and p53 accumulates in the nucleus of treated cells. A primary mechanism by which p53 negatively controls cell cycle progression is through the transcriptional activation of p21WAF1/CIP1 [12]. p21WAF1/CIP1 inhibits cyclin-dependent kinases, which block the phosphorylation and subsequent

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inactivation of pRb and causes the arrest of cell cycle progression in the G1/S phase. p53 has also been reported to be involved in G2/M cell cycle arrest as well as G1 arrest [13]. Because POP inhibition not only arrests the cell cycle at the G0/G1 phase but also impedes S and G2/M phase progression, we hypothesized that POP inhibition-induced growth suppression may be mediated by a p53-dependent pathway.

Thus, in the present study, we investigated the mechanism of cell growth inhibition by POP inhibition using a human gastric cancer cell line, KATO III, which had a p53 gene deletion [14]. In this cell line, the p53 pathway in POP inhibition-induced cell growth arrest was not a primary contributor to the inhibition mechanism. Our results also indicated that POP inihibitor-mediated cell cycle arrest in KATO III cells was associated with an increase in the proportion of cells in the quiescent G_0 state. This suggests that POP is a positive regulator of cell cycle progression, which regulates the exit from and/or reentry into the cell cycle by KATO III cells.

2. Materials and methods

2.1. Cells and reagents

KATO III (JCRB0611) human gastric cancer cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's Medium (DMEM) and RPMI-1640 medium (Sigma–Aldrich) supplemented with 50 μg/ml of kanamycin and 10% fetal calf serum (FCS). T98G (RCB1954) human glioblastoma cells were from the RIKEN RRC cell bank (Ibaraki, Japan) and cultured in DMEM supplemented with 50 μg/ml of kanamycin and 10% FCS. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The POP inhibitor 3-({4-[2-(*E*)-Styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) was obtained from the Peptide Institute (Osaka, Japan).

Benzyloxycarbonyl-thioprolyl-thioprolinal (Z-TT-CHO) was synthesized by a previously reported method [15]. Anti-CDK2 and anti-pRb2/p130 antibodies were purchased from BD Bioscience (San Jose, CA, USA). Anti-cyclin E and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cyclin D3, anti-CDK4, anti-CDK6, anti-p27Kip1, anti-pRb, anti-mouse IgG, and anti-rabbit IgG HRP-conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). An anti-Ki-67 antibody was purchased from Thermo Scientific (Waltham, MA, USA). An Alexa Fluor 488 anti-mouse IgG antibody was obtained from Molecular Probes (Eugene, OR, USA).

2.2. Cell growth assay and cell cycle analysis

Cell growth was assessed using a WST-1 assay (Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 3×10^3 cells/well. After incubation for 24 h, cells were treated with 0.5% DMSO (control) or with a range of doses of a POP inhibitor. After different treatment periods, the WST-1 reagent was added to cells for 4 h at 37 °C. The amount of formazan produced was determined using a spectrophotometric microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Cell viability was assessed using a trypan blue exclusion assay. Cells were seeded in 24-well plates at a concentration of 3×10^4/ml and incubated for 24 h. Cells were then treated with different concentrations of a POP inhibitor and stained with trypan blue.

Cell cycle stages were assessed by staining with propidium iodide (PI). Cells were treated with a POP inhibitor (60 μM) or 0.5% DMSO. At the indicated times, cells were harvested, washed with PBS, and gently fixed with 70% cold ethanol. Cells were then washed with PBS, treated with PBS containing 0.1 mg/ml of RNase at room temperature (RT) for 30 min, and resuspended in PBS containing 50 μg/ml of PI. Stained cells were analyzed with a FACScan (BD Bioscience) flow cytometer. Data were analyzed using ModFit software (Verity Software House, Topsham, ME, USA).

2.3. Ki-67 and PI staining for quiescent G_0 cell cycle status

Quiescent cell status was assessed using Ki-67/PI staining as previously described [16], with some modifications. Briefly, cells were gently fixed with 70% cold ethanol, washed with PBS containing 10% FCS, and then incubated with an anti-Ki-67 antibody at RT for 30 min. They were then washed, incubated with an Alexa Fluor 488 anti-mouse IgG antibody at RT for 30 min in the dark, washed, and resupended in PBS containing 10% FCS and 50 μg/ml of PI. Stained cells were analyzed with a FACScan flow cytometer.

2.4. Western blot analysis

Cells were rinsed with ice-cold PBS, suspended in CelLytic™ M lysis/extraction reagent (Sigma–Aldrich Co., St Louis, MO, USA) supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, bestatin, E-64, pepstatin A, and leupeptin. Cells were lysed using three freeze–thaw cycles. Cell extracts were centrifuged and the supernatants were analyzed by 12% SDS–PAGE. The proteins in gels were transferred to nitrocellulose membranes (Bio-Rad), and blocked for non-specific binding with 5% skim milk in Tris-buffered saline, pH 7.4, with 0.1% Tween-20 (TBST) at RT for 1 h. The membranes were incubated with a primary antibody, followed by incubation with a corresponding HRP-conjugated secondary antibody. Immune complexes were detected using LumiGLO® Reagent (Cell Signaling). Immunoblotting for actin was used to verify equivalent amounts of loaded protein. Protein concentrations were determined using a BCA™ Protein Assay Kit (Thermo Scientific).

2.5. POP silencing by siRNA

The 25-nucleotide modified synthetic RNAs (stealth RNAi) were custom synthesized (Invitrogen, Carlsbad, CA, USA). The primer sequences were: hPOP-siRNA sense, 5′-GGGUGGAGCUGAGUUAUCUGAUGAU-3′, and antisense,

5′-AUCAUCAGAUAACUCAGCUCCACCC-3′. Stealth RNAi negative control was directly ordered from Invitrogen. Cells were seeded on a 60-mm dish and incubated for 24 h. Cells were transfected with 33 nM of hPOP-siRNA or negative control using Lipofectamine 2000 (Invitrogen). After 48 h, transfected cells were used for cell growth determinations.

3. Results

3.1. POP inhibition effects on cell proliferation and cell cycle progression

To investigate the effects of POP inhibition on KATO III cells' proliferation, we used POP specific inhibitors, SUAM-14746 and Z-TT-CHO, and POP silencing with siRNA. SUAM-14746 is a POP inhibitor that was obtained by screening numerous thiazolidine derivatives [17,18]. KATO III cells were treated with different concentrations of SUAM-14746, and then assessed for proliferation every 24 h by WST-1 assay. Fig. 1A shows cell growth was inhibited after treatment with SUAM-14746 in a time- and dose-dependent manner. The viability of cells treated with SUAM-14746

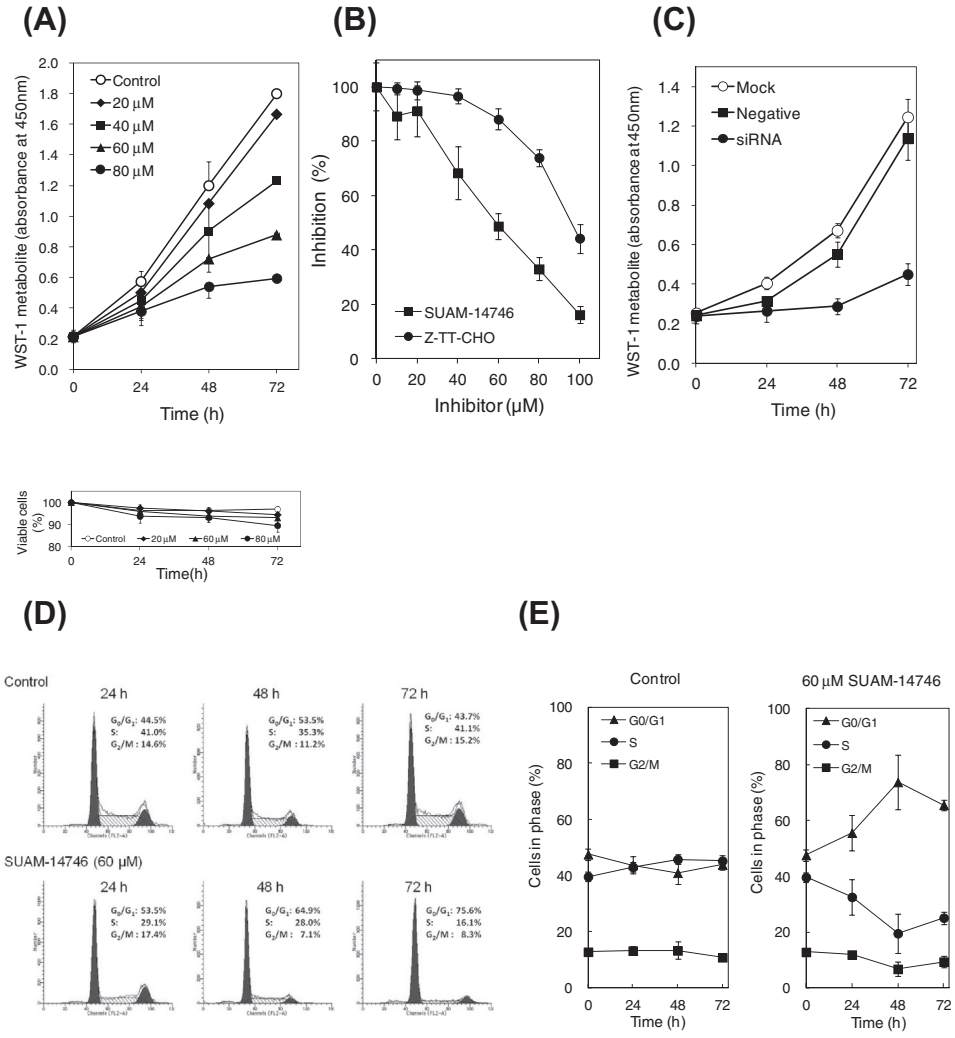


Fig. 1. Human POP-induced cell growth inhibition is associated with pronounced G_0/G_1 arrest in KATO III cells. (A–C) POP inhibition effects on KATO III cells growth. Cell growth was determined by the WST-1 method. (A) (upper panel) KATO III cells were treated with the indicated concentrations of SUAM-14746 for 24, 48, and 72 h. (lower) Cell viability after treatment with SUAM-14746 was determined using trypan blue exclusion assay with results expressed as the percentages of viable cells. Each point and error bar corresponds to the mean and SD of three experiments, respectively. (B) KATO III cells were incubated with different concentrations of SUAM-14746 or Z-TT-CHO for 72 h. Results are means ± SDs ($n = 3$). (C) KATO III cells were transfected with 33 nM siRNA duplexes. After 48 h, transfected cells were incubated for 24, 48, and 72 h. Cells were then used for a cell growth assay with the WST-1 method. (D and E) Changes in cell cycle phase distributions in SUAM-14746-treated KATO III cells. Exponentially growing cells were treated with 60 μM SUAM-14746. At the times indicated, cells were labeled with PI, and their DNA contents were determined by FACS analysis. (D) Results of representative experiments are shown. (E) Each point represents the mean ± SD of three separate experiments.

remained >90% for at least 72 h. Fig. 1B shows at 72 h, 60 μM SUAM-14746 inhibited cell growth by approximately 50%.

An anti-proliferative effect of Z-TT-CHO, a specific POP inhibitor that was designed based on its substrate specificity by Tsuru et al. [15], was also observed, although its effect was less than that of SUAM-14746. These results indicated that POP inhibitors suppressed cell proliferation rather than inducing cell death by KATO III cells for up to 72 h.

To determine whether POP knockdown affected KATO III cell growth, cell growth was assessed after POP knockdown. Transfection with hPOP-siRNA, and not the negative control siRNA, resulted in significant cell growth inhibition (Fig. 1C).

To further elucidate the mechanism of cell growth inhibition by SUAM-14746, we determined its effect on cell cycle distributions. Treating KATO III cells with 60 μM SUAM-14746 resulted in an increased G_0/G_1 fraction, which was accompanied by decreases in the proportions of both S phase and G_2/M phase cells (Fig. 1D and E). Thus, SUAM-14746-mediated growth inhibition of KATO III cells was associated with G_0/G_1 phase arrest. These results indicated that the p53 pathway during POP inhibition-induced cell growth arrest was not primarily involved in the mechanism of inhibition.

3.2. SUAM-14746 effects on the cell cycle regulator expression levels

We investigated the mechanism of SUAM-14746-induced G_0/G_1 arrest. We observed that treatment with 60 μM SUAM-14746 reduced the expression levels of G_1-S cyclins (cyclin D_3 and E) and their CDKs (CDK4/6 and CDK2) as compared with their control levels (Fig. 2A). To acquire further insights into the mechanism of SUAM-14746-induced G_0/G_1 phase arrest, we determined the expression level changes of the CDK inhibitor $p27^{kip1}$, retinoblastoma protein (pRb), and pRb2/p130. It was previously reported that quiescent cells expressed high levels of $p27^{kip1}$ [19] and pRb2/p130 [20]. Fig. 2B shows treating cells with SUAM-14746 resulted in upregulating the $p27^{kip1}$ expression in cell nuclei and pRb2/p130, and a remarkable reduction in the pRb expression levels. Thus, SUAM-14746-mediated cell cycle arrest in KATO III cells may have been associated with an increased proportion of cells in the quiescent G_0 state.

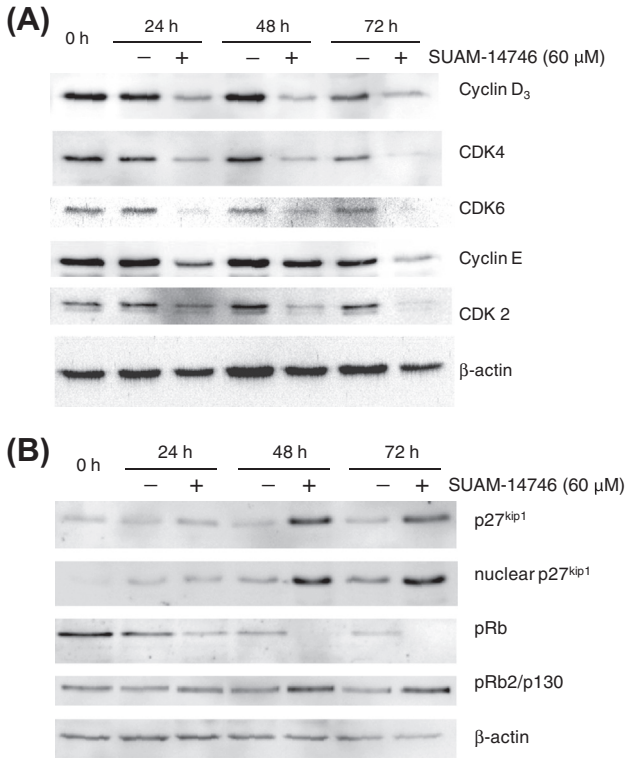


Fig. 2. SUAM-14746 treatment effects on the levels of cell cycle regulator proteins in KATO III cells. Exponentially growing cells were treated with 60 μM SUAM-14746. At the times indicated, total cell lysates or nuclear fractions were prepared and subjected to Western blotting using an antibody directed against each protein. β-Actin was used as a loading control.

3.3. SUAM-14746 induces a quiescent G_0 state in KATO III cells

To confirm that POP inhibitor-induced arrest involved a quiescent G_0 state, we also analyzed the effects of SUAM-14746 on the expression of the proliferation marker Ki-67 in KATO III cells by flow cytometry. The Ki-67 protein is present in all phases of the cell cycle (G_1, S, G_2, and M), but absent in the quiescent G_0 state. It is an excellent marker for determining the so-called growth fraction of a given cell population [21]. In Fig. 3A, the histogram shows that the Ki-67 expression level was affected by SUAM-14746. The high and low Ki-67 expression levels by cells were determined based on cells having higher and lower expression than that indicated by the dashed line, respectively. In addition, KATO III cells were simultaneously analyzed for their cell cycle distributions by PI staining.

Quiescent G_0 cells were defined as those with low Ki-67 expression among the G_0/G1 population of cells. KATO III cells that were treated with 60 μM SUAM-14746 had an increased proportion of cells in G_0 as compared with control cells (Fig. 3B). Fig. 3C shows that the control cells had a low proportion of G_0 cells (31.1% or less) in the G_0/G_1 phase up to 72 h, whereas treatment with 60 μM SUAM-14746 resulted in a majority of cells (46.4% for 24 h, approximately 60% for 48–72 h) in G_0. Thus, SUAM-14746 treatment had significantly increased the proportion of cells in the G_0 state as compared with control cells. The appropriateness of this criterion of Ki-67/PI staining was confirmed using T98G human glioma cells, which were previously shown to be arrested in the G_0 phase under serum starvation [22,23].

To investigate cellular G_0 entry and exit, T98G cells were precultured in the presence of 10% FCS for 48 h, and then serum-depleted for 72 h. They were subsequently cultured by adding serum (Fig. S1). Cells were primarily arrested at 72 h after serum starvation, and began to grow again at 24 h after serum addition. The decreases in the Ki-67 expression level under serum starvation in T98G cells were similar to the SUAM-14746-induced decreases of the Ki-67 expression.

The effects of removing SUAM-14746 on cell cycle progression were also assessed using Ki-67/PI staining. Fig. 3D shows that KATO III cells treated with 60 μM SUAM-14746 for 48 h had increased proportions of cells in the G_0 state. However, after removing SUAM-14746, this proportion significantly decreased. After 48 h, the cell cycle distribution was similar to that of control cells. These results indicated that the POP inhibitor-induced quiescent G_0 state was reversible.

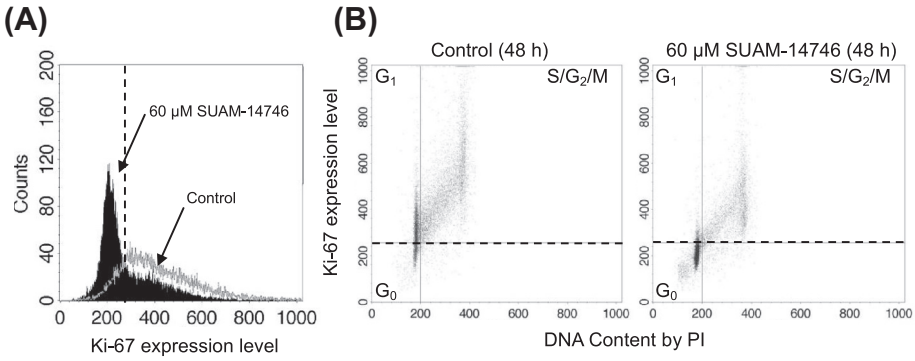
4. Discussion

Previous studies have shown that a POP inhibitor and POP knockdown suppressed the growth of NB-1 neuroblastoma cells [11]. Because POP inhibition increased the levels of p53 protein and not only arrested cells at G_0/G_1 but also impeded S and G_2/M phase progression, we hypothesized that POP inhibitor-induced growth inhibition may be mediated by a p53-dependent pathway. The present study was undertaken to acquire insights into the mechanism of cell cycle arrest by POP inhibition using p53 null KATO III cells as a model. We found that SUAM-14746 treatment caused G_0/G_1 phase cell cycle arrest in KATO III cells irrespective of their p53 status. Thus, we suggest that the p53 pathway in SUAM-14746-induced growth inhibition does not primarily contribute to this mechanism of inhibition.

To further examine the mode of cell cycle arrest induced by POP inhibition, we also analyzed cell cycle regulators' expression. This showed that POP inhibition resulted in upregulating those components necessary for maintaining KATO III cells in the G_0 phase. Treating KATO III cells with SUAM-14746 resulted in decreased levels of pRb and increased $p27^{kip1}$ and pRb2/p130 levels. The pRb2/p130 protein levels are increased in quiescent cells in which pRb2/p130 functions but are lower in proliferating cells due to turnover [24]. Thus, an increase in pRb2/p130 levels should occur to facilitate cells entry into G_0. In addition, $p27^{kip1}$ is an important component of the G_0 state that must be degraded upon cellular activation [25].

Moreover, POP inhibitor-mediated cell cycle arrest in KATO III cells was associated with an increased proportion of cells in a quiescent G_0 state, defined by low level staining with the proliferation marker Ki-67. In addition, the POP inhibitor-induced quiescent G_0 state was reversible. These results suggested that POP may be a positive regulator of cell cycle progression, which regulates the exit from and/or reentry into the cell cycle in KATO III cells. Thus, analyzing POP functions during cell proliferation may aid in elucidating a novel cell cycle regulation mechanism.

RNAi-mediated knockdown of POP also inhibited cell growth by KATO III cells as well as NB-1 cells. These results clearly demonstrated that POP was involved in cell cycle progression. However, we were unable to determine whether the peptidase activity of POP participated in this process. There appears to be some consensus that POP has a non-hydrolase activity. POP was found to physically interact with α-tubulin in a yeast two-hybrid screen [6]. POP has also been reported to bind to growth-associated protein 43 (GAP-43) and regulate synaptic plasticity without its peptidase activity [26]. Moreover, α-synuclein aggregation is associated with the pathology of Parkinson's disease and POP accelerates α-synuclein aggregation via a protein–protein interaction [27]. These results suggested that POP is involved in various intracellular processes independent of its catalytic activity through its protein–protein interactions with other cytosolic proteins.



(C)

		Percent cells in phase			
	Phase	0 h	24 h	48 h	72 h
Control	G_0/G_1	31.8 ± 1.4	41.9 ± 1.3	41.9 ± 1.9	50.3 ± 0.3
	G_1	17.0 ± 1.2	23.8 ± 1.5	28.2 ± 1.6	19.2 ± 0.7
	G_0	14.9 ± 2.6	18.1 ± 2.3	13.7 ± 1.6	31.1 ± 0.8
	S	51.1 ± 0.6	43.7 ± 1.3	45.7 ± 0.9	40.2 ± 0.9
	G_2/M	17.1 ± 0.8	14.5 ± 0.4	12.3 ± 1.0	9.5 ± 0.7
SUAM-14746 (60 μM)	G_0/G_1		58.7 ± 1.6**	70.9 ± 0.9**	63.4 ± 0.8**
	G_1		12.4 ± 1.7**	9.7 ± 2.7**	4.9 ± 0.2**
	G_0		46.4 ± 3.2**	61.2 ± 2.7**	58.5 ± 1.4**
	S		29.9 ± 1.0**	21.3 ± 0.7**	26.4 ± 0.2**
	G_2/M		11.4 ± 0.6**	7.9 ± 0.3**	10.2 ± 0.9

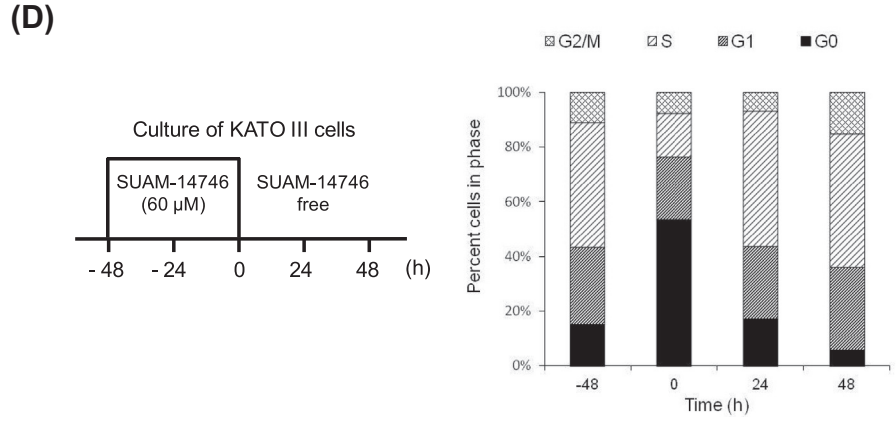


Fig. 3. POP inhibition increases the percentage of KATO III cells in the quiescent G_0 phase of the cell cycle. (A and B) Determination of the quiescent G_0 state in KATO III cells by FACS analysis. KATO III cells were stained for the proliferation marker Ki-67. The high and low Ki-67 expressions levels were determined for cells with expression higher and lower than the dashed line, respectively. Cells were also simultaneously assessed for their cell cycle distributions by PI staining. Quiescent G_0 cells were defined as those with low Ki-67 expression among the G_0/G_1 population of cells. Results of one representative experiment are shown. (C) Percentages of cells in different cell cycle phases among KATO III cells treated for the indicated periods with 0.5% DMSO (control) or 60 μM SUAM-14746. Results are means ± SDs ($n = 6$). $^{**}P < 0.01$ significantly different compared with control by Student's *t-test*. (D) Reversible arrest of the quiescent G_0 state induced by SUAM-14746. KATO III cells were precultured in the presence of 60 μM SUAM-14746 for 48 h. Then SUAM-14746 was removed for 48 h, and cells subsequently grew. Percentages of cells in different cell cycle phases among KATO III cells. Results are the means of three experiments.

We recently identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a POP binding protein in NB-1 cells. The interaction between POP and GAPDH was required for genotoxic stress-induced GAPDH nuclear translocation and cell death [28]. It has been reported that GAPDH nuclear accumulation is a cell cycle-dependent process. In human lung carcinoma cells, nuclear GAPDH is primarily detectable in the S phase, it remains high at G_2/M, and it significantly decreases by the end of mitosis. At G_0/G_1, GAPDH was nearly completely excluded from the nucleus [29]. Reversible nuclear translocation of GAPDH upon serum depletion in NIH-3T3 cells was also reported [30]. Taken together with the results of these studies, our results indicate that a POP-GAPDH interaction may be involved in regulating cell cycle progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.051.

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