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Prolyl oligopeptidase participates in cell cycle progression in a human neuroblastoma cell line

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ABSTRACT

Prolyl oligopeptidase (POP) is a post-proline cleaving enzyme, which is widely distributed in various organs, with high levels in the brain. In this study, we investigated the effects of a selective POP inhibitor, 3-({4-[2-(*E*)-styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746), on the growth of NB-1 human neuroblastoma cells. SUAM-14746 treatment for 24–72 h suppresses the growth of NB-1 cells without cell death in a dose-dependent manner (10–60 μM). Similar suppressive effects were observed with another POP inhibitor benzyloxycarbonyl-thioprolyl-thioprolinal. The SUAM-14746-induced growth inhibition in NB-1 cells was associated with pronounced G_0/G_1 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 transient inhibition of *S* and G_2/M phase progression, which was correlated with retardation of the decrease in the levels of cyclins A and B. Moreover, RNAi-mediated knockdown of POP also led to inhibition of NB-1 cell growth and the effect was accompanied by G_0/G_1 arrest. These results indicate that POP is a part of the machinery that controls the cell cycle.

1. Introduction

Prolyl oligopeptidase (POP, also known as prolyl endopeptidase or post-proline cleaving enzyme, EC 3.4.21.26) is a serine peptidase characterized by oligopeptidase activity. POP activities are highly conserved in mammals and are known to be widely localized in different organs, with high concentrations in the brain [1,2]. POP may play roles in many biologic processes such as the maturation and degradation of peptide hormones and neuropeptides [3,4], learning and memory [5], signal transduction [6], and protein secretion [7]. However, conclusive results have not yet been reported, and the primary physiologic role of POP remains to be elucidated.

Involvement of POP in cell division has been suggested in several papers. The POP activity in rat liver during postnatal development has been found to be highly correlated with the extent of proliferation and differentiation of liver cells [8]. Studies with POP inhibitors have revealed more detailed information about the involvement of POP in cell division. Repeated administration of a POP inhibitor to partially hepatectomized rats was found to significantly suppress the rate of liver regeneration, and this suppression was reversed by termination of inhibitor treatment [9]. Addition of a POP inhibitor to cultured *Sarcophaga peregrina* imaginal disks prevented the process of disk differentiation from entering the elongation stage [10]. In addition, a POP inhibitor was found to inhibit DNA synthesis and cell proliferation in an embryonic *Sarcophaga* NIH-Sape-4 cell line [11]. Furthermore, POP has been shown to be involved in cell proliferation and DNA replication in the mouse Swiss 3T3 cell line [12]. POP activity is significantly higher in tumors of the prostate, lung, and sigmoid than in healthy tissues [2,13]. More recently, POP was found to be present in various cell types in both the cytoplasm and nucleus of the cells on immunohistochemical studies. The nuclear colocalization of POP protein and Ki-67, a proliferation marker protein, suggests that POP may be involved in cell proliferation [14]. Together, these observations indicate that POP possesses properties related to regulation of cell growth. However, the cell proliferation mechanisms involving POP are still unknown.

In the present study, we investigated the influence of POP inhibitors and small interfering RNAs directed against human POP on the growth of NB-1 human neuroblastoma cells. The results indicate that POP is a part of the machinery controlling the cell cycle in this cell line.

2. Materials and methods

2.1. Cell culture

NB-1 (IFO50295) human neuroblastoma cells obtained from the Health Science Research Resources Bank (HSRRB) were grown in

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RPMI 1640 medium supplemented with 10% fetal bovine serum, and 50 μg/ml kanamycin in a humidified atmosphere (37 °C, 5% CO_2).

2.2. Chemicals and reagents

The tissue culture media were obtained from Invitrogen. 3-({4-[2-(*E*)-Styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) was obtained from Peptide Institute. *Z*-Thioprolyl-thioprolinal (Z-TT-CHO) was synthesized using a previously reported method [15]. Antibodies were obtained from BD Biosciences (cyclins A and B, CDK2, CDK4), Santa Cruz Biotechnology (cyclins D_1 and E, actin), Cell Signaling Technology (CDK6, p53, pRb, $p27^{kip1}$). The fluorogenic enzyme substrates Suc-Gly-Pro-MCA were obtained from Peptide Institute.

2.3. Cell proliferation and viability assay

Cell proliferation was measured using the WST-8 Cell counting kit-8 (Dojindo). The cells were seeded on 96-well plates at a density of 3×10^3 cells per well and incubated for 24 h. The cells were then treated with 0.5% DMSO (control) or with a range of doses of POP inhibitors. After various treatment periods, cells were treated with 10 μl WST-8 reagent for 4 h at 37 °C. The quantity of formazan product was measured using a spectrophotometric microplate reader (Bio-Rad) at 450 nm. Cell viability was determined using a trypan blue exclusion assay. For cell growth measurements after knockdown of POP, the cells were transfected with 25 nM of synthetic siRNAs using a transfection reagent.

2.4. Cell cycle analysis

For cell cycle analysis, 5×10^5 cells were seeded in 60 mm culture dishes and incubated for 24 h followed by treatment with POP inhibitor. At the indicated times, cells were washed with PBS, harvested, and gently fixed with 80% cold ethanol. Fixed cells were washed with PBS, and resuspended in a DNA-staining solution (0.25 mg/ml RNase, 0.5 mg/ml propidium iodide). Cells were analyzed for DNA content by using a FACScan (Becton Dickinson) flow cytometer. Data were analyzed using the ModFit software (Verity Software House). To synchronize the cells for cell cycle studies, NB-1 cells were seeded at 2.5×10^5 cells in 60 mm culture dishes and cultured for 48 h, followed by addition of 0.1 mM hydroxyurea and incubation for an additional 16 h. After washing the cells with PBS, the medium was replaced and the cells were allowed to continue the cell cycle for 10 h. Hydroxyurea (0.1 mM) was added to the cells again, and the incubation was continued for 14 h. The cells were then washed, grown in a fresh medium containing the desired concentration of POP inhibitor, and harvested every 4 h in order to monitor the different cell cycle stages.

2.5. Protein extraction and Western blot analysis

Cells were rinsed with ice-cold PBS, suspended in CelLytic™-M lysis/extraction reagent (Sigma–Aldrich) supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, bestatin, E-64, pepstatin A, and leupeptin. The cells were lysed by performing three freeze/thaw cycles. The extracts were centrifuged and the clear supernatants were analyzed using 10% SDS-PAGE. The gels were then transferred to nitrocellulose membranes

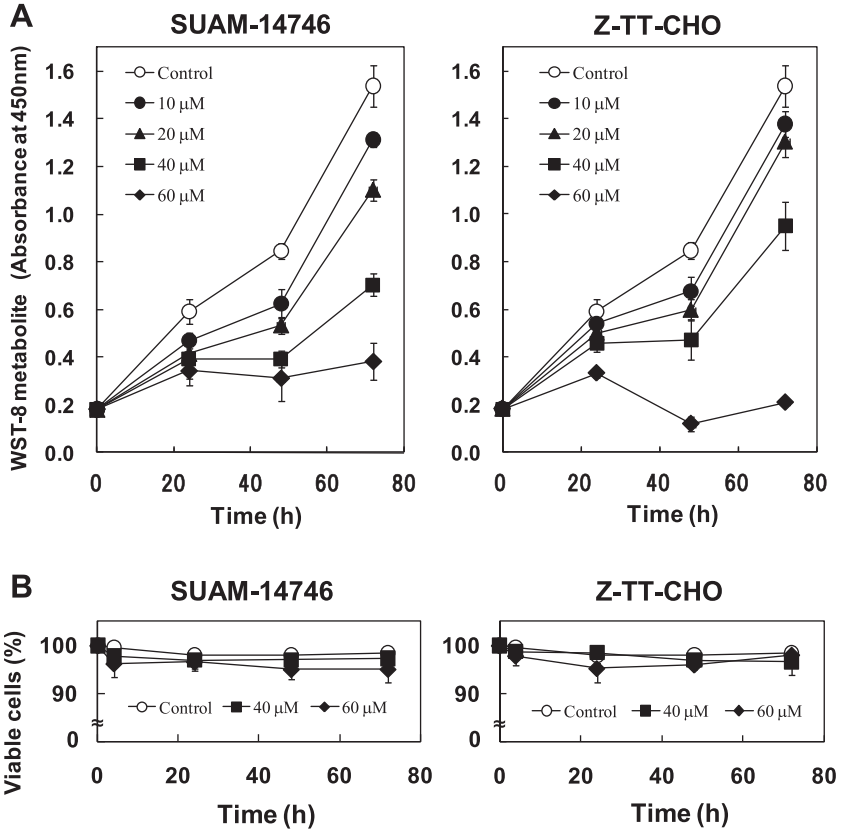

Fig. 1. Effects of POP inhibitors on the growth of NB-1 cells. NB-1 cells were treated with the indicated concentrations of SUAM-14746 and Z-TT-CHO. (A) Cell growth, determined at the times indicated, is represented as the absorbance at 450 nm. (B) The viability of cells treated with inhibitors was determined using trypan blue exclusion and is expressed as the percentage of viable cells. Each point and error bar corresponds to the mean and SD of three experiments, respectively.