Original Article

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A new method to evaluate the enzyme-suppressing activity of a leucine aminopeptidase 3 inhibitor

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Summary **The expression of leucine aminopeptidase 3 (LAP3) is associated with the prognosis for and malignant transformation of many types of tumors. Therefore, a LAP3 inhibitor may represent a new strategy for cancer therapy. Evaluating the suppression of enzyme activity by an LAP3 inhibitor is essential. Right now, leucine aminopeptidases (LAPs) purified from the porcine kidneys are the only enzymes that can be used to evaluate the suppression of enzyme activity by an LAP3 inhibitor. This approach cannot accurately reflect the suppression of human LAP3 by an inhibitor. The current study developed a new method with which to evaluate the suppression of enzyme activity by an LAP3 inhibitor. Total protein from K562 cells seldom catalyzed the LAP3 substrate. A lentivirus was used to induce K562 cells to overexpress LAP3 (K562-LAP3). After puromycin screening, flow cytometry data indicated that 98.8% of cells expressed green fluorescent protein. The expression of LAP3 in K562-LAP3 cells was also assessed using Western blotting. K562-LAP3 cells were lysed with ultrasonication. Total protein was used as an enzyme source and L-leucine p-nitroaniline hydrochloride was used as a substrate to measure enzyme activity. Total protein from K562-LAP3 cells catalyzed the substrate more than that from K562 cells did. The LAP3 inhibitor ubenimex was used as a positive control to evaluate the suppression of LAP3 enzyme activity. Results indicated that ubenimex significantly inhibited the enzyme activity of LAP3. This approach provides a convenient and accurate way to evaluate the suppression of enzyme activity by an LAP3 inhibitor.**

Keywords: Leucine aminopeptidase 3, enzyme activity, K562-LAP3 cells, inhibitor

1. Introduction

Leucine aminopeptidases (LAPs) are zinc-containing metalloproteinases and members of the aminopeptidase M1/M17 family (*1*). These enzymes can catalyze the hydrolysis of leucine residues from the N-terminus of a protein or peptide substrate (*2*). LAP3 was originally discovered by R.J. Trumbly (*3*). Since LAP3 is an LAP, its expression is closely related to the activation of peptide substrates. In breast cancer, LAP3 also enhances the invasion of breast cancer cells (*4*). Studies have found that knocking down LAP3 will make hepatocellular carcinoma cells more sensitive to cisplatin, thereby promoting cell death in those cells (*5*). In addition, the malignant transformation of human esophageal squamous cell carcinoma also results from overexpression of LAP3 (*6*). Therefore, LAP3 expression during the development of a tumor is particularly important. The synthesis of LAP3 inhibitors has attracted considerable interest because of the aforementioned findings. Thus, evaluating the suppression of enzyme activity is essential to developing an LAP3 inhibitor.

Currently, there is no method with which to evaluate the suppression of human LAP3 enzyme activity. Only Sigma (Cat. L5006) provides enzymes to evaluate

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suppression of LAP enzyme activity (*7*). However, this enzyme is derived from the porcine kidney. The amino acid sequence and structure of human and porcine LAP3 differ (Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=39*). This may cause a problem since some compounds may suppress the enzyme activity of porcine LAP3 but not human LAP3 (*8*). The current study developed a new method to evaluate the suppression of enzyme activity by an LAP3 inhibitor *in vitro*. Enzymes expressed in K562 cells did not strongly catalyze the LAP3 substrate. Therefore, a lentivirus was used to stably overexpress LAP3 in K562 cells (K562-LAP3). Enzymes obtained from K562-LAP3 cells lysed with ultrasonication were used as an enzyme source to examine the suppression of LAP3 activity by compounds. This method is convenient and easy to perform, so it can continuously and easily provide enzymes and accurately allow evaluation of the suppression of human LAP3 by an LAP3 inhibitor.

2. Materials and Methods

2.1. *Cell culture*

The human erythroleukemia cell line K562 was purchased from the China Infrastructure of Cell Line Resources (Beijing, China). K562 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and cultured in humidified air with 5% CO_2 at 37°C.

2.2. *Western blotting*

Whole-cell protein was extracted from cell homogenate and lysed in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, and 50 mM sodium fluoride) supplemented with a protease inhibitor cocktail. The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. Membranes were blocked with 10% skim milk and incubated with specific primary antibodies overnight at 4°C. The membranes were then incubated with antibodies. All antibodies were as follows: LAP3 (SANTA CRUZ, sc-376270), β-actin (SANTA CRUZ, sc-1616).

2.3. *Flow cytometry*

Cells were suspended at 5×10^5 cells/100 μL and incubated for 60 min at room temperature with an anti-LAP3 mono clonal antibody (Abcam, ab154809) (London, England). After cells were washed in PBS, the samples were resuspended in phosphate buffer saline (PBS) containing 1% FBS. Labeled cells were subsequently analyzed with flow cytometry on BD FACScan.

2.4. *Lentivirus infection*

Lentivirus particles were purchased from Genechem. Lentivirus (LV)-enhanced green fluorescent protein (EGFP) was transduced into cells. The medium was replaced with fresh culture medium after 12 h. Cells were observed under a fluorescence microscope for transduction efficiency. Cells with stable expression were screened using puromycin.

2.5. *Monoclonal cell screening*

Equal dilution was used. One hundred cells/200 μL was added to the first row of wells, 100 μL of medium was added to other wells, 100 μL of cells was removed from the first row with a Pasteur pipette and added to the second row, cells were mixed, 100 μL of cells was removed from the second row and added to the third row, and so on, until the last row. K562 cells stably overexpressing LAP3 were generated from only a single cell in a well.

2.6. *Enzymatic activity*

K562-LAP3 and K562 cells were re-suspended in PBS. Cells were disrupted with ultrasonication and seeded onto 96-well plates. Cells were combined with 1.6 mM LAP3 substrate (L-leucine p-nitroaniline hydrochloride) for 30 min at 37°C. Enzyme activity was measured at 405 nm. The analysis of data was as follows: Rate of inhibition = (expression in control group – expression in experimental group)/ expression in control group × 100%. Here, expression in the control group = wild-type expression, and expression in the experimental group = wild-type expression plus overexpression due to transduction.

2.7. *Statistical analysis*

All experiments were performed at least three times, and results are expressed as the mean ± SD. Differences between groups were determined using the Student's *t* test.

3. Results

3.1. *K562 cells were used to overexpress LAP3*

In addition to LAP3, other enzymes may also catalyze the LAP3 substrate. Therefore, the current study selected cells that minimally catalyze the substrate to reduce the influence of other enzymes. Total enzymes from several cell lines obtained *via* ultrasonication were tested for their ability to catalyze the substrate, including K562, PLC/RRF/5, and MDA-MB-231 (Figure 1). Results indicated that K562 had the least ability to catalyze the substrate. In addition, a large number of cells can easily

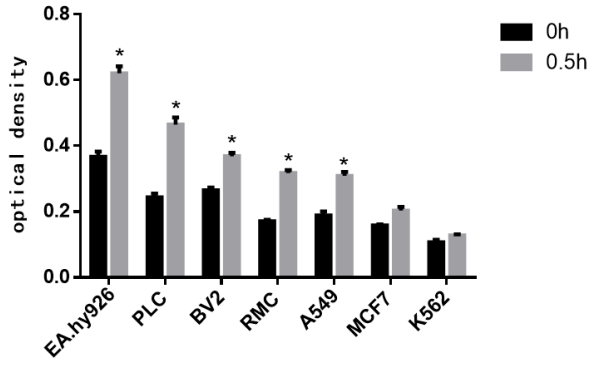

Figure 1. Catalyzing effects of different cell lines on substrates. Cells were lysed with ultrasound. Total protein was transferred to 96-well plates. One-point-six mM LAP3 substrate was added to the wells. Plates were incubated at 37°C for 30 min. Absorbance at 405 nm was measured. Each bar represents the mean ± SD. *$p < 0.05$, **$p < 0.01$.

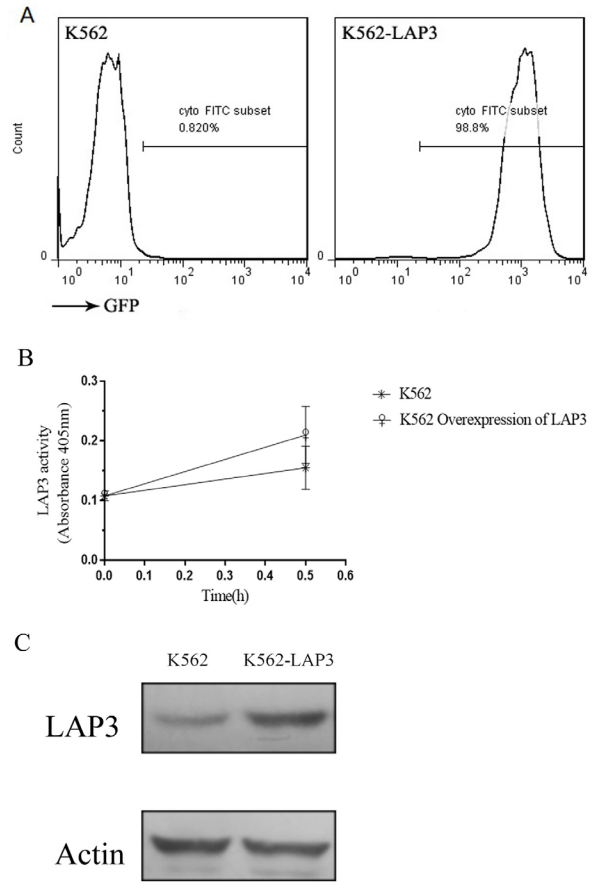

Figure 2. Infection of K562 cells with a lentivirus to overexpress LAP3. K562 cells were resuspended in complete culture medium, and 4×10^5 TU/mL of virus particles was seeded onto 96-well plates. The lentivirus LV-LAP3 was diluted with ENi.S or medium and added to each well. Simultaneously, polybrene was added to 10 μM as a viral infection adjuvant. The medium was replaced with completed culture medium after 12 h. After three days, cells with stable expression were screened using puromycin. Flow cytometry **(A)**, an enzyme activity assay **(B)**, and Western blotting **(C)** were used to test transduction efficiency.

be obtained because K562 cells are suspended cells and not adherent cells.

3.2. *LAP3 overexpression in K562 cells via lentivirus infection*

K562 cells were transduced with a lentivirus to overexpress LAP3. After puromycin screening, 98.8% of cells expressed GFP (Figure 2A). The cells were used to generate monoclonal cells. Twelve monoclonal cells were obtained, and enzyme activity was measured. No. 35 strongly catalyzed the substrate and was used in the subsequent experiment (K562-LAP3). K562-LAP3 cells exhibited higher enzyme activity than K562 cells (Figure 2B). GFP was observed under fluorescence microscopy (Figure S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=39*). Cells overexpressing LAP3 expressed a high level of LAP3 compared to parental cells (Figure 2C). In summary, K562-LAP3 cells expressed a high level of LAP3 and potently catalyzed the substrate.

3.3. *K562-LAP3 cells can be used as an enzyme source to detect the suppression of enzyme activity by an LAP3 inhibitor*

To further identify the enzyme activity of LAP3, ultrasound lysates of K562 and K562-LAP3 cells were incubated with the LAP3 inhibitor ubenimex. Results indicated that ubenimex inhibited the enzyme activity of LAP3 in a dose-dependent manner (Figure 3). This finding verified the hypothesis that K562-LAP3 cells can

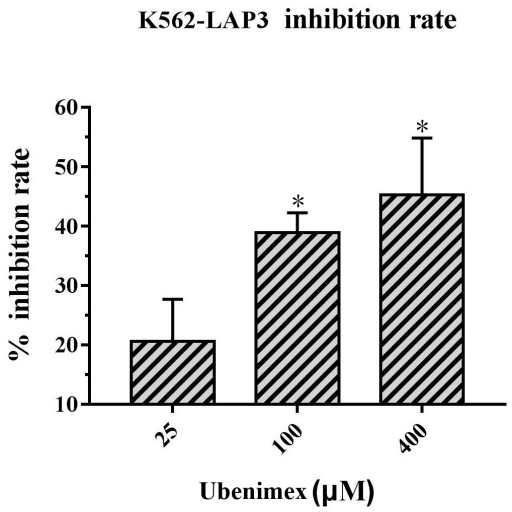

Figure 3. The LAP3 inhibitor ubenimex inhibits enzyme activity in K562-LAP3 cells. K562-LAP3 cells were lysed with ultrasound and transferred to 96-well plates. Ubenimex was added to the wells. The concentration of the drug was 25, 100, or 400 μM. After 5 min, the LAP3 substrate was added to 1.2 mM. After incubation at 37°C, absorbance was determined at 405 nm. The data were processed using the formula shown in the article. The experimental results were from three independent experiments, and similar results were obtained.

be used to evaluate the suppression of enzyme activity by an LAP3 inhibitor.

4. Discussion

LAP3 plays a key function in the tissues and cells of plants, animals, and microorganisms (*1*). LAP3 has been found to play an increasing number of roles in the development of various diseases, such as ovarian epithelial malignancy, breast cancer, endometrial cancer, human esophageal squamous cell carcinoma, and diabetes (*6,9-13*). Nonetheless, what is more interesting is the role that LAP3 inhibitors could play in treating disease. There is no method with which to evaluate the suppression of human enzyme activity by an LAP3 inhibitor. Only LAP enzyme from porcine kidney microsomes (Sigma, Cat. L5006) can be used as an enzyme source to evaluate the suppression of enzyme activity. However, the amino acid sequence and structure of human and porcine LAP differ. Therefore, there is no guarantee that an inhibitor would suppress human LAP3 in the same manner as it suppresses porcine LAP. Thus, a new method was devised to detect that suppression.

The current study used K562 cells, which weakly catalyze a substrate, as a cell source. K562 cells are suspended cells. They do not need to be digested, and they are easy to handle (*14*). There are some other enzymes that can catalyze the substrate in addition to LAP3 (*15*). The weak reaction of the enzyme from K562 cells with the substrate indicated little interference by other enzymes. All of this indicated that K562 cells were proper candidate cells.

A lentivirus was used to overexpress LAP3 in K562 cells. Lentiviral infection has several unique advantages. An exogenous gene can be stably integrated into a host chromosome in comparison to transient transfection. In addition, a lentivirus also expresses GFP in cells, and this feature can be used to evaluate infection efficiency. In addition, a lentivirus vector also carries the puromycin resistance gene, which can be used to promote infection efficiency and maintain stable expression. K562-LAP3 cells stably express LAP3 with a good reproducibility and specificity. This cell line can be cultured to obtain enough LAP3 to evaluate the suppression of LAP3 activity by a compound.

The rate of inhibition was calculated by subtracting expression by parental cells from expression by overexpressing cells. Therefore, this formula only represents the suppression of LAP3, and it eliminates the influence of other enzymes that catalyze the substrate. This guarantees that LAP3 is the only variable in this experiment, accurately reflecting the suppression of LAP3 by an inhibitor.

5. Conclusion

In this experiment, K562 cells overexpressed LAP3 as a result of lentivirus infection. The total proteins of K562-LAP3 cells served as an enzyme source, and L-leucine p-nitroanilide hydrochloride served as a substrate to measure suppression by an LAP3 inhibitor. Expression by parental cells was subtracted from expression by overexpressing cells, so the data reflected only the suppression of LAP3 enzyme activity. Therefore, this method is an accurate, economical, and convenient way to evaluate the activity of an LAP3 inhibitor.

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