Novel and highly sensitive fluorescent assay for leucine aminopeptidases

Huazhang Huang, Hiromasa Tanaka, Bruce D. Hammock, Christophe Morisseau *

Department of Entomology and UC Davis Cancer Center, University of California, Davis, CA 95616, USA

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ABSTRACT

l-Leucine aminopeptidases (LAPs) are implicated in the progress of many pathological disorders and play some regulatory roles in tumor cell proliferation, invasion, and/or angiogenesis. Thus, LAPs not only could become new diagnostic or prognostic biomarkers but also may have potential as novel molecular targets for the treatment of several cancers. Highly sensitive assays are critical for early detection of changes in LAP activity and for screening potent LAP inhibitors. In this study, we developed a novel and highly sensitive fluorescent assay for LAPs based on substituted aminopyridines as fluorescent reporters. This assay was at least 100- and 20-fold more sensitive than commercial colorimetric and fluorescent LAP substrates, respectively. We also showed that this assay was a useful tool for monitoring LAP activities in extracts from cancer cell lines, as well as for the high-throughput screening of inhibitors, which could lead to new cancer treatments.

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l-Leucine aminopeptidases (LAPs,[1] EC 3.4.11.1) are exopeptidases that catalyze the hydrolysis of leucine residues from the amino termini of protein or peptide substrates [1]. LAPs are zinc-containing enzymes of the M1 and M17 peptidase families. They play diverse biological and physiological roles in mammals, microbes, and plants by either degrading bioactive peptides or interacting with peptide-dependent signaling and DNA [2,3]. Thus, changes in LAP expression pattern and/or catalytic function result in altered peptide activation, leading to changes in tumor cell proliferation, invasion, and/or angiogenesis. For example, puromycin-insensitive leucyl-specific aminopeptidase (PI-LAP) plays a crucial role in the cell cycle progression of endothelial cells and angiogenesis via the binding and modification of phosphatidylinositol-dependent kinase [4]. Placental LAP (P-LAP) is the biomarker for the evaluation of ovarian epithelial malignancy and a target of molecular therapy [3]. P-LAP is also involved in the promotion and progression of breast cancer through oxytocin and/ or vasopressin misregulation [5] and plays a significant role in insulin regulation of Glut4 receptors in diabetic patients [6,7]. In addition, adipocyte-derived LAP (A-LAP) mediates endometrial cancer cell growth and differentiation; therefore, the assessment of A-LAP status provides clinically useful prognostic information in patients with endometrial carcinoma [8]. Thus, LAPs not only could become new diagnostic or prognostic biomarkers but also may have potential as novel molecular targets for the treatment of several cancers. Selective chemical inhibitors of LAPs may be useful probes to determine LAP functions in addition to being possible cancer therapeutics.

To develop a potent enzyme inhibitor, it is important to have reliable, sensitive, and specific assays that can be used for measuring both enzyme activity and inhibitor potency. Several assays have been developed for LAPs. Briefly, these assays are based on substrates containing an amide functionality formed with l-leucine as the acid and a common colorimetric or fluorescent reporter, such as β-naphthylamine, p-nitroaniline, or 7-amino-4-methyl-coumarin, as the amine [9–11]. Although these assays are widely used, they are not very sensitive for LAPs, with limits of detection (LODs) greater than 5 μg/ml for the colorimetric assay [11] and greater than 1 μg/ml for the fluorescent assay [9]. Furthermore, the background chemical hydrolysis of these assays is relatively high. Taken together, the currently available assays are less than suitable for high-throughput screening.

Recently, we discovered a series of novel fluorescent reporters (i.e., aminopyridine derivatives) [12]. These are small molecules with high quantum yields and large Stokes' shifts. These properties potentially make these compounds excellent reporters for aminopeptidases such as LAPs. However, these reporters have near-ultraviolet (UV) excitation (280–300 nm) and emission (380–400 nm) wavelengths. These properties suggest that their fluorescence could be quenched by high concentrations of compounds absorbing in the UV range such as those with aromatic rings and nitrogen heterocycles. Also, in some combinatorial libraries, numerous false positives could be generated given that some heterocyclic compounds, such as 2-aminoquinoline, 4-aminoquinaldine [13], and

[1] Abbreviations used: LAP, l-leucine aminopeptidase; PI-LAP, puromycin-insensitive leucyl-specific aminopeptidase; P-LAP, placental LAP; A-LAP, adipocyte-derived LAP; LOD, limit of detection; UV, ultraviolet; AMP, 5-amino-2-methoxypyridine; HL-S9, human liver S9; ADP, 5-amino-2-dimethylaminopyridine; ^{1}H NMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PTU, propylthiouracyl; DTT, dithiothreitol; BCA, bicinchoninic acid; BSA, bovine serum albumin; S/B, signal-to-background ratio; S/N, signal-to-noise ratio; PKLAP, porcine kidney LAP.

2-[4-(dimethylamino)phenyl] benzothiazole [14], can be fluorescent. Once potent leads are found, these properties are unlikely to cause serious problems. However, initial library screens will be problematic. Therefore, in this study, our aim was to obtain fluorescent reporters with both the excitation and emission wavelengths red-shifted. We used these reporters for developing novel assays for aminopeptidases. Finally, we investigated the use of such assays for inhibitor screening and measuring LAP activities from cancer cell extracts as a potential diagnostic tool.

Materials and methods

Chemicals

Porcine kidney microsomal leucine aminopeptidase, l-leucine-p-nitroanilide (**I**) (Fig. 1), 7-amino-4-methylcoumarin, and N-(tert-butoxycarbonyl)-l-leucine monohydrate were purchased from Sigma–Aldrich (St. Louis, MO, USA). l-Leucine-(4-methyl-7-coumarinyl amide (**II**) (Fig. 1) was purchased from Acros Organics (Morris Plains, NJ, USA). 3-Amino-6-methoxy-2-picoline, 5-amino-2-methoxypyridine (AMP), and 2-bromo-5-nitropyridine were purchased from Asychem (Durham, NC, USA). Pooled human liver S9 (HL-S9) was purchased from BD Biosciences (Woburn, MA, USA).

Synthesis

The fluorescent reporter 5-amino-2-dimethylaminopyridine (ADP) was prepared as described in Fig. 1 (steps a and b). This is similar to the procedure described by Heindel and Kennewell [15]. The LAP substrates **III**, **IV**, and **V** were prepared as described in Fig. 1 (steps c and d). Commercially available and optically pure l-leucine derivatives were used as starting material, and the reaction performed did not alter the chirality of the products; thus, substrates **III**, **IV**, and **V** are optically pure (S)-enantiomers. Structural identification of synthesized compounds was based on data from proton nuclear magnetic resonance (^{1}H NMR) that were acquired from a Mercury 300 spectrometer (Varian, Palo Alto, CA, USA) and by mass spectrometry. Mass spectra were obtained on a Micromass liquid chromatograph orthogonal acceleration time-of-flight mass spectrometer (Waters–Micromass, Manchester, UK) by using electrospray ionization in the positive mode, and elution was performed isocratically with a solvent mixture of acetonitrile/water/formic acid (89.9:10.0:0.1). The chemical purity of the final products was supported by the spectra described above, a single spot on thin-layer chromatography (TLC) under 254-nm wavelength, and lack of fluorescence on TLC at 254- or 360-nm wavelength. The NMR and mass spectral data suggested purities above 97% for all synthetic compounds and contamination by the fluorescent reporter to be less than 0.01% for substrates **III** to **V**.

N-(6-Methoxy-2-methylpyridin-3-yl), (S)-2-amino-4-methylpentanamide (**III**)

Reddish oil (overall 66% yield). ^{1}H NMR (CDCl$_3$): 9.38 (br, 1H, NH), 8.12 (d, J = 9.00, 1H, pyridine), 6.58 (d, J = 9.00, 1H pyridine), 3.89 (s, 3H, OCH$_3$), 3.56 (t, J = 10.5, 1H, CHNH$_2$), 2.41 (s, 3H, CH$_3$), 1.75–1.86 (3H, CH$_2$CH), 0.99 (d, J = 6.30, 3H, CH$_3$), 0.97 (d, J = 6.30, 3H. CH$_3$). Calculated mass: 251.16; found [M + H]$^+$ = 252.16.

5-Amino-2-dimethylaminopyridine (ADP)

Brownish oil (overall 55% yield). ^{1}H NMR (CDCl$_3$): δ 7.77 (d, J = 3.00, 1H, pyridine), 6.99 (dd, J_1 = 3.00, J_2 = 9.00, 1H, pyridine), 6.46 (d, J = 9.00, 1H, pyridine), 3.74 (br, 2H, NH$_2$), 3.00 (s, 6H, 2CH$_3$). Calculated mass: 137.10; found [M + H]$^+$ = 138.12.

(S)-2-Amino-N-(6-(dimethylamino)pyridin-3-yl)-4-methylpentanamide (**IV**)

Yellowish oil (overall 42% yield). ^{1}H NMR (CDCl$_3$): δ 9.26 (br, 1H, NH), 8.16 (d, J = 2.70, 1H, pyridine), 7.89 (d, J = 9.00, 1H, pyridine), 6.49 (d, J = 2.70, 1H, pyridine), 3.53 (m, 1H, CH), 3.04 (s, 6H, 2CH$_3$), 2.15 (br, 2H, NH$_2$), 1.65–1.83 (m, 3H, CHCH$_2$), 0.98 (dd, J_1 = 3.90, J_2 = 2.40, 6H, 2CH$_3$). Calculated mass: 250.18; found [M + H]$^+$ = 251.18.

N-(6-Methoxypyridin-3-yl), (S)-2-amino-4-methylpentanamide (**V**)

Reddish oil (overall 51% yield). ^{1}H NMR (CDCl$_3$): δ 9.47 (br, 1H, NH), 8.23 (d, J = 2.40, 1H, pyridine), 8.01 (dd, J_1 = 2.70, J_2 = 8.70, 1H pyridine), 6.72 (d, J = 8.70, 1H pyridine), 3.91 (s, 3H, OCH$_3$), 3.52 (t, J = 10.5, 1H, CHNH$_2$), 1.75–1.86 (3H, CH$_2$CH), 0.99 (d, J = 6.30, 3H, CH$_3$), 0.97 (d, J = 6.30, 3H. CH$_3$). Calculated mass: 237.15; found [M + H]$^+$ = 238.15.
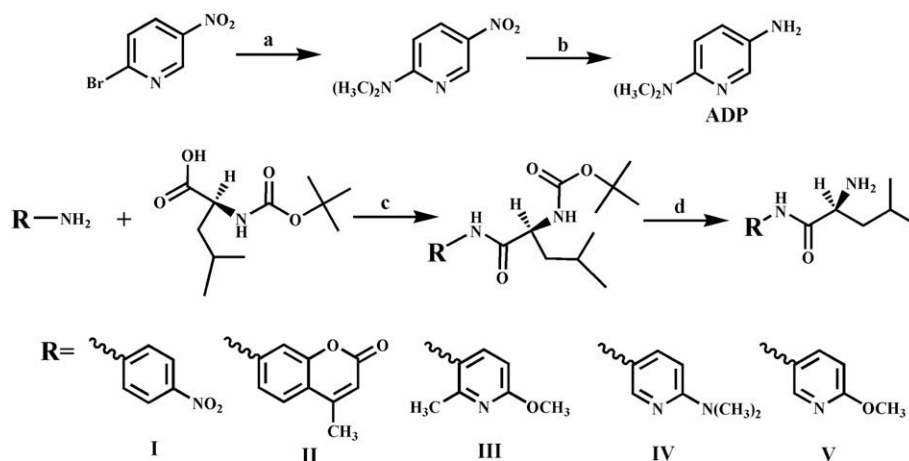

Fig. 1. Schematic preparation of a novel aminopyridine-based fluorescent reporter (ADP) and LAP substrates. Reagents and conditions: (a) dimethyl sulfoxide (DMSO), 150 to 160 °C, overnight, 60%; (b) Pt/H$_2$ in CH$_3$OH, 4 h, 90%; (c) N-(tert-butoxycarbonyl)-l-leucine monohydrate, 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU), 1,3-diisopropylcarbodiimide/CH$_2$Cl$_2$, 2 h, 96%; (d) trifluoroacetic acid (TFA), 2 h, 55%.

Optical properties determination

Optical properties (molar extinction coefficient, excitation and emission wavelengths, and quantum yield) of ADP were determined using the procedures described by Huang and coworkers [12]. The molar extinction coefficient was determined at 290 nm for final concentrations of ADP between 10 and 50 μM. To ensure that the substrates containing an aminopyridine group as a reporter (**III**, **IV**, and **V**) do not fluoresce significantly and do not interfere with the reporter fluorescent signal, their emission spectrum in phosphate buffer (0.1 M, pH 8.0) with $[S]_{final}$ = 50 μM was measured from 360 to 600 nm with the same excitation wavelength as their corresponding reporters (see Table 1).

Optimization of assay conditions

To optimize the pH of the assay buffer, the activity of porcine kidney microsomal LAP was measured in sodium phosphate buffer (0.1 M, pH 6.0–8.0) and Tris–HCl buffer (20 mM, pH 9.0). In a 96-well polystyrene flat-bottom microtiter plate, 10 μl of a diluted (0.2 μg/μl) LAP solution was added to 190 μl of buffer. The plate was then preincubated for 10 min at 27 °C. To each well was added 1 μl of a 10-mM solution of substrate **I** in ethanol ($[S]_{final}$ = 50 μM). Activity was immediately measured for 10 min at 27 °C by following the appearance of 4-nitroaniline at 382 nm every 30 s using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Assays in the absence of protein were used as a blank. Results were averaged and compared statistically. The optimal buffer was then used to examine the effect of temperature (25, 28, 31, 34, and 37 °C) on LAP activity with substrate **I**. Assays were performed in quadruplicate. The results are given as the averages ± standard deviations.

Enzymatic assays

All assays were performed in 96-well polystyrene flat-bottom microtiter plates, clear for substrate **I** and black for substrates **II** to **V**. In each well, 10 μl of diluted protein solution (or buffer for the blank) was added to 190 μl of sodium phosphate buffer (0.1 M, pH 8.0) to yield the appropriate protein concentration. The reaction was started by the addition of 1 μl of a 10-mM substrate solution in ethanol ($[S]_{final}$ = 50 μM). The reaction was followed for 10 min at 31 °C by measuring the appearance of the product every 30 s with a SpectraMax M2 spectrophotometer. The measurement parameters for the colorimetric **I** and fluorescent **II** to **V** substrates are given in Table 1. Standard curves of the various reporters (from 0 to 5 nmol) were used to quantify the measured signal (r^2 > 0.99 for all of them). Assays were performed in quadruplicate. The results are given as the averages ± standard deviations of at least three separate measurements. The LOD was defined as the amount of enzyme needed to generate activity that was three times the value of the blank. The signal-to-background ratio (S/B) was calculated by dividing the reaction average velocity in the presence of enzyme (Av_{enz}) by the average background hydrolysis rate (Av_{blank}). The signal-to-noise ratio (S/N) was calculated by dividing the reaction velocity in the presence of enzyme by the standard deviation of the background hydrolysis (SD_{blank}). The Z' factor was calculated following the method of Zhang and coworkers [16] using the following formula: $Z' = 1 - \{[3*(SD_{enz} + SD_{blank})] / (Av_{enz} - Av_{blank})\}$.

Kinetic constant determination

Kinetic constants (V_{max} and K_m) were determined for substrates **I** and **V** following the methods described above for enzyme activity. For substrate **I**, an enzyme concentration of 10 μg/ml and $[S]_{final}$ between 0.78 and 50 μM were used. For substrate **V**, an enzyme concentration of 0.12 μg/ml and $[S]_{final}$ between 1.6 and 100 μM were used. The assays were performed in quadruplicate. K_m and V_{max} were calculated by nonlinear fitting of the Michaelis–Menten equation using the enzyme kinetic module of SigmaPlot 9.01 (Systat Software). The results are given as the averages ± standard deviations of at least three separate measurements.
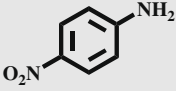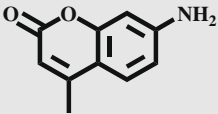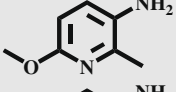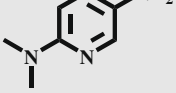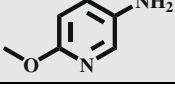
Inhibition assay

Inhibition potencies of bestatin for pig liver LAP were determined for substrates **I** and **V** following the assay methods described above. Briefly, 1 l of the inhibitor in a dimethyl sulfoxide (DMSO) solution was added to the enzyme diluted in the buffer (200 μl total, $[E]_{final}$ = 10 and 0.12 μg/ml for substrates **I** and **V**, respectively) to yield final concentrations of inhibitor between 0.78 and 50 μM. This mixture was incubated for 10 min at 31 °C prior to the addition of the substrates. IC_{50} is defined as the concentration of an inhibitor that inhibited 50% of the enzyme activity. IC_{50} values were calculated by nonlinear regression of at least five data points using SigmaPlot. The results are given as the averages ± standard deviations of at least three separate measurements.

Cell cultures

The human cancer cell lines used (293T, HepG2, HuH7, 22RV1, Du145, and T24) were obtained from the American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in RPMI 1640 medium (Mediatech) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker) under a 5% CO_2 atmosphere at 37 °C. The cells were passaged at 90 to 100% confluence by washing with phosphate-buffered saline (PBS) and trypsinization (0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid [EDTA]) for 3 to 5 min at 37 °C prior to subculture. Following trypsinization, the cell suspension was centrifuged at 400g for 5 min at 5 °C. The cell pellet was then resuspended in fresh RPM 1640 medium.

Table 1
Measurement parameters for the colorimetric and fluorescent substrates.

Substrate	Reporter structure	λ_{ex} (nm)	λ_{em} (nm)
I		382[a]	–
II		351	430
III		304	392
IV		334	450
V		302	396

[a] Absorbance wavelength.

Protein extract preparation from cell lines

Protein extracts were released from 10^6 cells by sonication (Sonic Dismembrator model 100, Fisher Scientific) for 3 s in 500 µl of cell lysis buffer (20 mM sodium phosphate [pH 7.4], 1 mM propyl-thiouracyl [PTU], 1 mM dithiothreitol [DTT], and 0.01% Tween 20). Cell debris was removed by centrifugation (10,000*g* for 15 min at 4 °C), and the supernatant was stored at −80 °C until used. For each cell line, LAP activity was measured as described above using 40 µl of extract corresponding to 8×10^4 cells. Protein concentrations were quantified by using the Pierce bicinchoninic acid (BCA) assay with fraction V bovine serum albumin (BSA) as the calibrating standard. LAP activity was measured on the extract as described above.

Results and discussion

Characterization of a novel red-shifted aminopyridine fluorescent reporter

Recently, we reported aminopyridine derivatives as novel fluorescent reporters [12]. Although these small molecules have high quantum yields and large Stokes' shifts, they have near-UV excitation (280–300 nm) and emission (380–400 nm) wavelengths that limit their usefulness. It has been shown that increasing the electron density on the electron-withdrawing group of a fluorophore yields red-shifted excitation and emission wavelengths [17–21]. Thus, to obtain a red-shifted fluorescent signal, we replaced the methoxy group of AMP, the best reporter found previously [12], with a stronger electronic donor group (e.g., dimethylamine). This new aminopyridine (ADP in Fig. 1) was synthesized following the method of Heindel and Kennewell [15] with an overall yield of 55%. As expected, compared with AMP, this replacement resulted in a substantial red shift in both excitation (∼ 30 nm) and emission (∼ 60 nm) wavelengths for ADP (Fig. 2). However, under the same conditions, the relative fluorescent intensity of ADP was lower than that of AMP (Fig. 2). This was probably due to a broader emission peak and slightly smaller quantum yield (0.89) compared with AMP (0.95) [12]. Interestingly, the molar extinction coefficient at 290 nm of ADP was found to be 5600 ± 100 M^{-1} cm^{-1}, which is approximately 2-fold higher than that of AMP and even higher than that of any other aminopyridine examined. Overall, the novel aminopyridine (ADP) seems to be a better fluorescent reporter than the ones described previously [12].

Comparison and characterization of novel LAP substrates

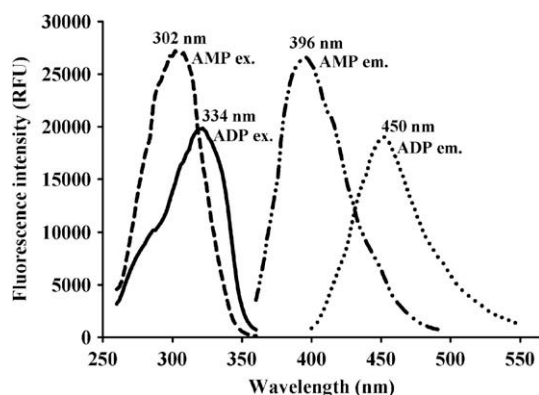The background hydrolysis in sodium phosphate buffer (0.1 M, pH 8.0) of all of the LAP substrates tested in this study (compounds I–V in Fig. 1) was determined at 31 °C. As shown in Table 2, dramatic differences in background hydrolysis were observed. Both commercial substrates (I and II) yielded the highest rates of background hydrolysis, with the absorbent substrate I being six times less stable than the fluorescent substrate II. For the aminopyridine-containing substrates, the presence of the dimethylamino group *para* in the amide function resulted in substrate IV that is roughly 10-fold less stable than the compounds (III and V) with a methoxy group at the same position. We did not observe striking differences in background hydrolysis rates over the range of pH (6.0–9.0) and temperature (25–37 °C) tested. There was a trend toward higher background hydrolysis rates with higher pH and temperature, as one would expect. Overall, although a stronger electron-donating group such as dimethylamino appeared to yield a red-shifted fluorescent reporter, it also yielded a less stable amide. One could probably optimize the electron donor effect to balance the good optical properties and chemical stability.

Using commercial porcine kidney LAP (PKLAP), we investigated the enzymatic hydrolysis of the novel LAP substrates. We first optimized the assay conditions using substrate I. At 27 °C, PKLAP activity was the highest at pH 7.0 and 8.0 (0.1 M sodium phosphate buffer), but it was dramatically lower at lower and higher pH levels. Over the time period of observation (10 min), PKLAP activity was the highest at 31 °C and decreased slowly at higher temperatures. These results are consistent with previous observations [22]. Because the fluorescence of the aminopyridines (III–V) is optimal at around pH 8.0 [12], we decided to run the enzymatic activity at pH 8.0 (0.1 M sodium phosphate buffer) at 31 °C. Using these conditions, we determined the specific activities of PKLAP for the five substrates (Table 2). The commercially available substrates (I and II) and the pyridines (IV and V) that have *para* substitution on the pyridine gave similar specific activities. In contrast, substrate III, which has a *meta* substitution on the pyridine, gave a specific activity that was roughly two orders of magnitude lower. This *meta* substitution probably interferes sterically with the optimal placement of III in the active pocket of PKLAP. No such effect was observed previously for the hydrolysis of leucine anilides by LAP from bovine lens [23].

The LOD of PKLAP was defined as the concentration of protein for a particular substrate that yields an activity signal that is at least three times the background hydrolysis rate (S/B $\geqslant$ 3) over a 10-min kinetic measurement. Because of its lower background hydrolysis and higher intensity of the reporter signal, substrate V gave the lowest LOD (Table 2). In theory, using this substrate, one can detect 100- and 20-fold lower amounts of LAP activity in comparison with the commercial colorimetric I and fluorescent II substrates, respectively. Interestingly, because of its relatively high

Fig. 2. Comparison of excitation (ex.) and emission (em.) wavelengths of AMP and ADP.

Table 2
LODs of porcine kidney LAP with colorimetric and fluorescent substrates.

Substrate	Background hydrolysis (nmol.min^{-1})	Specific activity (µmol.min^{-1}.mg^{-1})	LOD[a] (µg/ml)	S/B[b]	S/N[b]	Z'[b]
I	2.5 ± 0.1	5.1 ± 0.1	5.0	3.0	56	0.89
II	0.42 ± 0.02	9.6 ± 0.7	1.0	4.5	69	0.69
III	0.029 ± 0.007	0.044 ± 0.003	10.0	3.0	83	0.80
IV	0.34 ± 0.01	8.4 ± 0.5	1.0	4.8	93	0.79
V	0.019 ± 0.001	6.5 ± 0.4	0.05	3.4	83	0.76

[a] Minimum amount of porcine kidney LAP yielding a significantly measurable activity.
[b] Values calculated for [E] = LOD. S/B was calculated by dividing the reaction average velocity in the presence of enzyme (Av$_{enz}$) by the average background hydrolysis rate (Av$_{blank}$). S/N was calculated by dividing the reaction velocity in the presence of enzyme by the standard deviation of the background hydrolysis (SD$_{blank}$). Z' was calculated following the method of Zhang and coworkers [16] using the following formula: Z' = 1 − {[3*(SD$_{enz}$ + SD$_{blank}$)]/(Av$_{enz}$−Av$_{blank}$)}.

Table 3
Kinetic constants and substrate selectivity of PKLAP.

Substrate	V_{max} ($\mu mol.min^{-1}.mg^{-1}$)[a]	K_m (μM)	V_{max}/K_m ($\mu mol.min^{-1}.mg^{-1}.\mu M^{-1}$)	Selectivity ratio
I[b]	3.9 ± 0.2	3000 ± 200	0.0013 ± 0.0003	1
V[b]	7.7 ± 0.2	65 ± 4	0.12 ± 0.01	92

[a] V_{max} data are per milligram of protein.
[b] Assays were carried out in sodium phosphate buffer (0.1 M, pH 8.0) with 0.0236 µg/well of PKLAP and with final substrate concentrations between 1.56 and 200 µM. The reactions were followed for 10 min at 31 °C.

background hydrolysis, the substrate with the novel fluorescent reporter **IV** has an LOD similar to the aminocoumarin-based substrate **II**. For each substrate, we also calculated for [E] = LOD S/N and Z′ (Table 2). For all of the substrates, we found S/N above 50, reflecting a small variation in the reporter signal and, thus, robustness of the assays. We found Z′ values above 0.70, indicating a large separation band between the samples and blank signals. The Z′ value also indicated that these substrates could be easily used in high-throughput screening assays [16]. To test this hypothesis, we followed the enzymatic hydrolysis of substrates **I** and **V** over 90 min. Although substrate **I** was completely hydrolyzed after 20 min, only 5% of substrate **V** was hydrolyzed after 60 min, resulting in a linear response over time ($r^2 = 0.98$). This was due to the higher sensitivity of **V** that allowed using a 100-fold lower amount of enzyme. Taken together, these data show that substrate **V** will be an excellent substrate for high-throughput screening in either in a kinetic or end-point format.

To further compare substrate selectivity of porcine kidney microsomal LAP with other substrates published in the literature, we conducted a kinetic study with substrates **I** and **V** (Table 3). We found that PKLAP has a V_{max} that was 2-fold higher for **V** than for **I**, and the K_m for **V** was approximately 60-fold lower for **V** than for **I**. Taken together, these data indicate that the PKLAP has a 92-fold preference for the novel fluorescent substrate **V** than for the commercial colorimetric substrate **I**. This is consistent with the lower LOD obtained for substrate **V** (Table 2). Overall, our results clearly show that substrate **V** is an excellent substrate for LAP and is far better than the commercially available substrates.

Potential application of novel fluorescent reporters

Substituted aminopyridines used here display high fluorescence and represent a novel class of fluorescent reporters that are structurally different from aminocoumarins such as in substrate **II**.
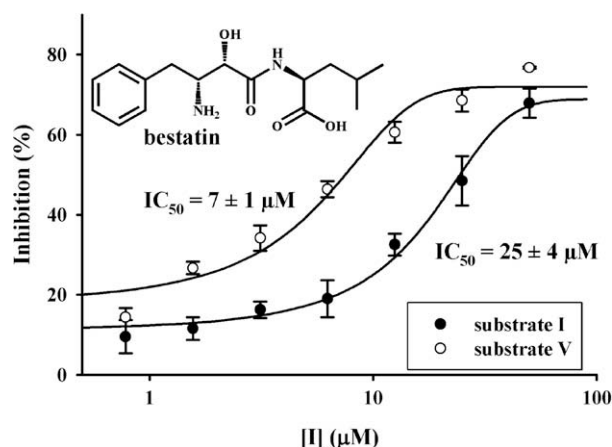
Substituted aminopyridines possess many advantages over aminocoumarins, including higher fluorescence, better aqueous solubility, and smaller size. To illustrate their potential applications and advantages over commercially available substrates, we used substrate **V** to determine inhibitor potency and detect LAP activities in cells. As shown on Fig. 3, we first looked at the inhibition of PKLAP by bestatin, a slow-binding competitive inhibitor of LAP [24–26]. The inhibition potency (IC_{50}) obtained with substrate **V** was on the same order of magnitude as that obtained with substrate **I**. Because the enzyme concentrations used were different (100-fold lower for **V**), one cannot directly compare the IC_{50} values obtained with both substrates. Nevertheless, these data suggested that **V** could be easily used to accurately determine LAP inhibition with the advantage of using much less enzyme, thereby yielding a much more sensitive assay. This is especially useful for high-throughput assay screening for potent inhibitors because the ability of an assay to distinguish among potent inhibitors is largely dependent on a low enzyme concentration.

There are many reports on the relationship between the LAPs and carcinoma [3,27–30]. These reports not only show that LAPs play regulatory roles in tumor cell proliferation, invasion, and/or angiogenesis via degradation/inactivation of target peptides (e.g., oxytocin, angiotensins, endothelin-1) that act on cancer cells as stimulatory or inhibitory factors but also suggest that LAPs possibly function to inhibit oxytocin-dependent signaling. Thus, it is important to measure LAP activity in cancer cells. However, it is difficult to measure LAP activity with commercial substrates such as **I** and **II**. As shown on Fig. 4, we were able to measure LAP activities in homogenates from six cancer cell lines with the substrate **V**. We were not able to measure LAP activity with substrate **I** on the same extracts. Like substrate **I**, substrate **V** was designed to be a general LAP activity reporter rather than to be specific for a single LAP; thus, the LAP activities observed in the cell extract could result from more than one enzyme, and caution should be taken about the interpretation of the results. Furthermore, the use of a general synthetic substrate such as **V** does not allow monitoring of the biological functions of an enzyme; rather, it is an indication of its expression/repression. We found that there was a significant increase of LAP activities ($P \leqslant 0.01$) for the T24, HepG2, and Huh7 cancer cell lines but a significant decrease ($P \leqslant 0.01$) for 22RV1 compared with LAP activity for HL-S9. There was no significant difference of LAP activity for the 293T and Du145 cancer cell lines compared with that for HL-S9. These results also indicated that LAP activity can be easily measured using a small amount of sample (the equivalent of 8×10^4 cells were used in each assay only)

Fig. 3. Inhibition of PKLAP by bestatin using colorimetric (substrate **I**) and fluorescent (substrate **V**) assays.
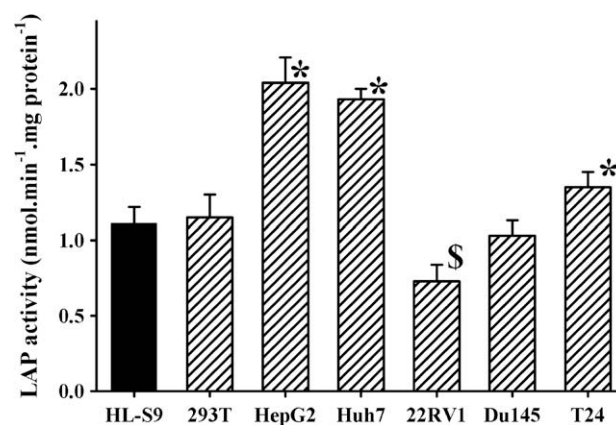

Fig. 4. LAP activities of different cancer cell lines measured with the novel fluorescent substrate **V**. LAP activity (compared with HL-S9): *Significantly increased ($P < 0.01$); $Significantly decreased ($P < 0.01$).

by using this highly sensitive fluorescent substrate. Caution is needed when using these new substrates directly on live cell cultures. Although the substrates described here are probably not too toxic to cells, the aminopyridine products could be [31]. Thus, their possible toxicity could influence the results. However, their high fluorescence permits using low concentrations of substrate (<50 µM), thereby reducing possible toxic effects.

Rapid and efficient monitoring of LAP activity is important to understand the biological functions of this enzyme as well as following the development of associated diseases [3]. The sensitivity of such assays is especially important when the sample to be tested is in limited supply. Here we have described novel fluorescent substrates for LAPs based on aminopyridines. With these new substrates, we obtained up to a 100-fold increase in sensitivity compared with currently available assays. Our assay will be highly useful for monitoring LAP activities in extracts from cancer cell lines as well as for the high-throughput screening of LAP inhibitors that could potentially lead to new cancer treatments. Finally, the technology developed here for LAPs could be easily adapted to other aminopeptidases and metalloproteases by simply changing the leucine on the substrate to a more appropriate amino acid or peptide. Furthermore, more specific substrates could be obtained by having specific amino acids at the P1′ position and the reporter group at P1 [32].

Acknowledgments

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