Bioorganic & Medicinal Chemistry Letters 21 (2011) 2403–2405

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Small-molecule probe using dual signals to monitor leucine aminopeptidase activity

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ARTICLE INFO

Article history:
Received 24 December 2010
Revised 15 February 2011
Accepted 16 February 2011
Available online 18 February 2011

Keywords:
Leucine aminopeptidases
Probe
Dual signals
2-Dicyanomethylene-3-cyano-2,5-dihydrofuran (DCDHF) unit

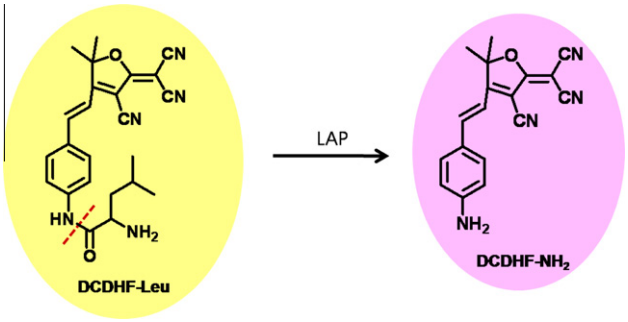
ABSTRACT

Leucine aminopeptidases (LAPs) are widely distributed in organisms from bacteria to humans, and play crucial roles in cell maintenance and cell growth. Thus, assays for LAP are necessary for measuring its activity and inhibitor potency. In this Letter, we report a small-molecule probe which exhibits colorimetric and fluorogenic changes according to LAP activity.

Aminopeptidases play important roles as transcriptional repressors, site-specific recombination factors, and viral or toxin receptors, as well as being proteolytic enzymes for the N-terminus of proteins and bioactive peptides in bacteria and animals. Leucine aminopeptidases (LAPs) are one of the exopeptidases which catalyze the hydrolysis of the N-terminal leucine residues of proteins or peptides. Some LAPs are key enzymes and are able to affect diverse biological and physiological phenomena.[1–4] They could therefore be used as diagnostic or prognostic biomarkers, and assays for their activity are necessary. Several assays for LAP activities have been developed.[4–9] Known probes for monitoring LAP activities consist of a fluorescent reporter and an amide group coupled with leucine, and they can exhibit colorimetric and fluorogenic signals on interaction with LAPs.[4] However, they have short excitation and emission wavelengths, making it hard to monitor LAP activity in living cells, and they need large amounts of LAP for high-throughput screening for potent inhibitors. To address these problems, we developed a probe, using a 2-dicyanomethylene-3-cyano-2,5-dihydrofuran (DCDHF) unit as a π-electron-accepting unit, for fluorescent measurement of LAP activity and inhibitor potency at longer wavelengths.

We designed a probe which has a DCDHF unit and a leucine-linked π-conjugated system (DCDHF–Leu). This probe has good cell permeability and can be applied in bioimaging.[10–13] The capped amine functionality of leucine could block fluorescence emission by reducing the donor ability of the NH moiety in DCDHF–Leu. However, DCDHF–Leu is expected to show color changes and fluorescence turn-on when the leucine is uncapped by treatment with LAP, forming DCDHF–NH_2 (Scheme 1).

We evaluated the optical response to LAP activity using DCDHF–Leu in vitro. When it was excited at 525 nm, DCDHF–Leu was non-fluorescent, but DCDHF–NH_2 showed significant fluorescence enhancement in HEPES buffer at pH 7.4 (Fig. 1A). The emission spectrum of DCDHF–Leu after treatment with porcine kidney microsomal LAP (PKLAP) was examined with time (Fig. 1B). The fluorescence intensity at 605 nm increased by up to 10-fold, and was saturated in 30 min. This indicates that PKLAP removes the leucine residue from DCDHF–Leu to form



Scheme 1. Probe based on the 2-dicyanomethylene-3-cyano-2,5-dihydrofuran (DCDHF) unit.

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doi:10.1016/j.bmcl.2011.02.068

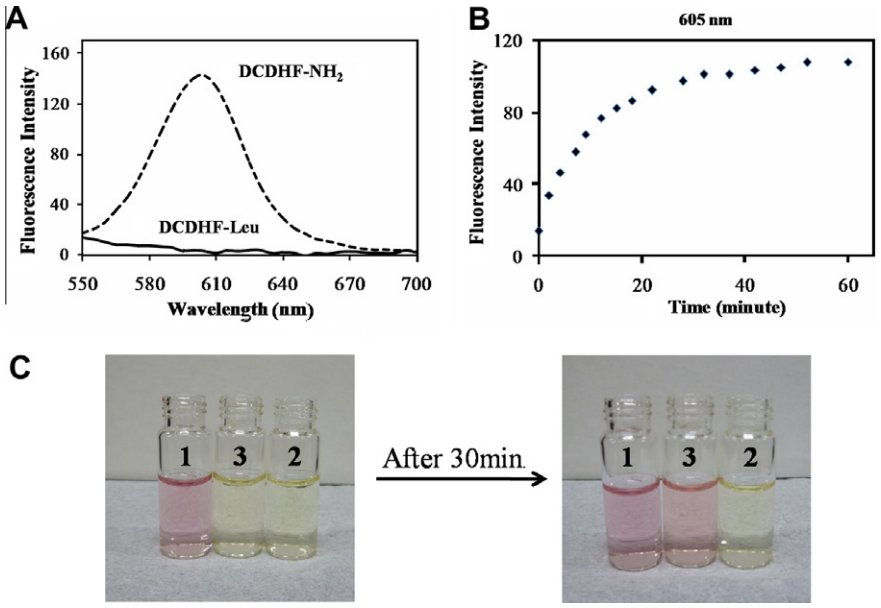

Figure 1. (A) Fluorescence spectra of DCDHF–NH_2 and DCDHF–Leu; (B) time-dependent fluorescence changes of a DCDHF–Leu probe (10 μM) with PKLAP (0.2 μg/mL) at 605 nm; (C) color change of a DCDHF–Leu probe after 30 min incubation with PKLAP: 1, 20 μM DCDHF–NH_2; 2, 20 μM DCDHF–Leu; 3, 20 μM DCDHF–Leu with 0.2 μg/mL PKLAP.

Table 1
Enzyme kinetics data

Substrate	V_{max} (μmol/min mg)	K_m (μM)	IC_{50} of bestatin[b] (μM)
l-Leucine-*p*-nitroanilide[a]	3.9 ± 0.2	3000 ± 200	25 ± 4
N-(6-Methoxypyridine-3-yl), (*S*)-2-amino-4-methylpentanamide[a]	7.7 ± 0.2	65 ± 4	7 ± 1
DCDHF–Leu	6.64 ± 0.46	8.87 ± 1.72	4.41 ± 0.61

[a] Ref. 4.
[b] Measured values using each substrate.

DCDHF–NH_2 within minutes, which means that DCDHF–Leu is a good fluorogenic substrate for PKLAP. Moreover, incubation of DCDHF–Leu with LAP for 30 min triggered a solution color change from yellow to red. It was demonstrated that PKLAP activity using the probe could also be observed by the naked eye (Fig. 1C). DCDHF–Leu is therefore an efficient probe that can measure LAP activity by fluorescent and color changes.

To determine the enzyme kinetics, the fluorescence intensity was examined with different concentrations of DCDHF–Leu and a constant amount of PKLAP. Concentrations of DCDHF–Leu from 1.2 to 50 μM, and 0.2 μg/mL PKLAP in 10 mM HEPES buffer at pH 7.4, were used (Fig. S3). The maximum velocity (V_{max}) was determined to be 6.64 ± 0.46 μmol/min mg and the Michaelis constant (K_m) was 8.87 ± 1.72 μM. Compared to previously reported data (Table 1),[4] DCDHF–Leu has a similar V_{max} and smaller K_m. These data indicate that DCDHF–Leu could bind to LAP more tightly, and that it is a better substrate for LAP than other reported or commercial substrates.

To explore whether the DCDHF–Leu probe can measure inhibitory potency, we determined the inhibition potency (IC_{50}) of bestatin, which is known to be a slow-binding competitive inhibitor of LAP.[14–16] A constant amount of PKLAP (0.2 μg/mL) was incubated with various concentrations of bestatin, from 0.6 to 50 μM, for 10 min, prior to addition of 10 μM DCDHF–Leu. The maximum

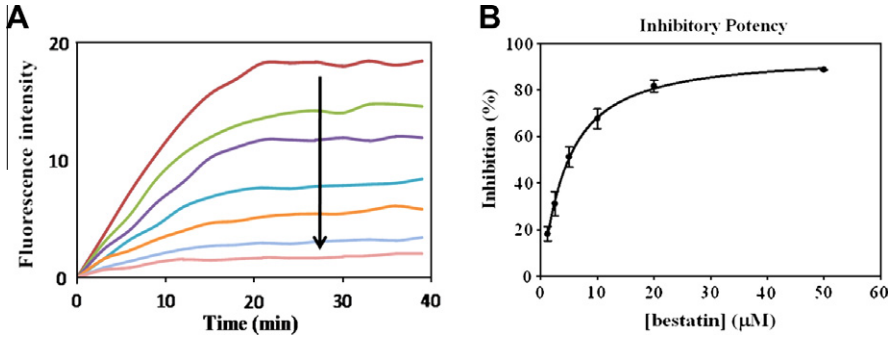

Figure 2. (A) fluorescence decrease of DCDHF–Leu with bestatin concentration (0.6, 1.2, 2.5, 5, 10, 20 and 50 μM); (B). Inhibitory potency.

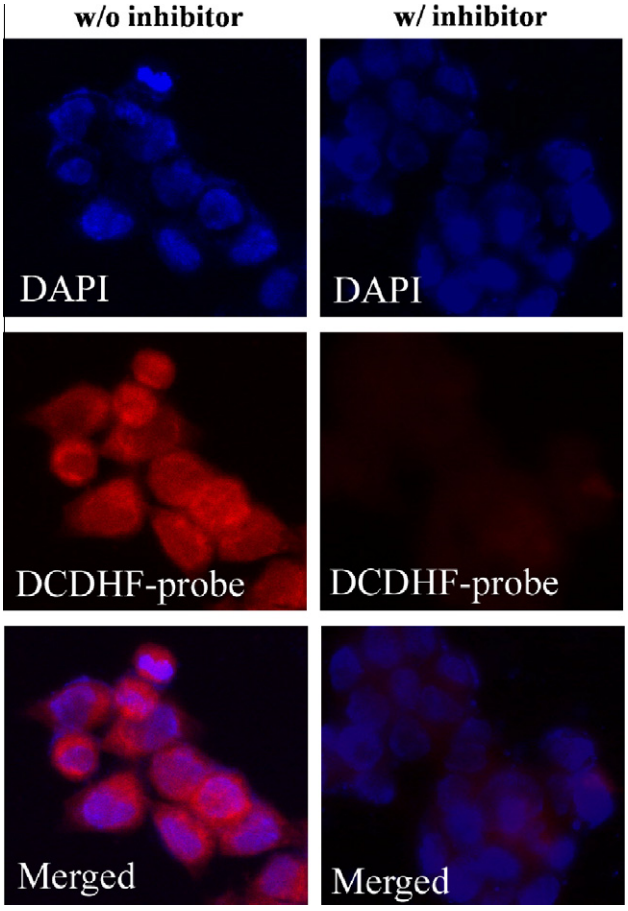

Figure 3. (A) HCT 116 cells imaging with 20 μM DCDHF–Leu incubated for 20 min (left), and with 20 μM DCDHF–Leu incubated with 100 μM bestatin for 20 min (right). Cells were fixed and stained with a DCDHF probe (red) against LAPs and with DAPI stain (blue) to identify the nucleus. Original magnification, ×400.

fluorescence intensity at 605 nm and the initial DCDHF–Leu velocity with PKLAP decreased dose-dependently (Fig. 2). The IC_{50} obtained in this experiment is 4.41 ± 0.61 μM, which is the same order of magnitude as previously reported data (Table 1).[4] Our experiment reveals that DCDHF–Leu is an excellent probe, which can detect compounds with high inhibition potency for LAP, and also enables high-throughput screening.

Finally, to demonstrate the potential practical applications of DCDHF–Leu, we applied the probe to a living cell for fluorescence imaging. DCDHF–NH_2 is a red fluorescent substrate, which has longer excitation and emission wavelengths than previously known probes.[4] Two sets of HCT 116 cells were incubated with 20 μM DCDHF–Leu, with and without bestatin, for 20 min at room temperature. The HCT 116 cells without bestatin showed red fluorescent imaging, indicating the presence of DCDHF–NH_2 from reaction with LAP; the other set, with bestatin, which could block the LAP activity, was non-fluorescent (Fig. 3).The fluorescent intensity of HCT 116 cell lysates incubated with 20 μM DCDHF–Leu and no bestatin was about six-fold higher than that of HCT 116 cell lysates incubated with the probe and bestatin (Fig. S4). The same results were obtained from the cell lysate and the living cell, indicating that DCDHF–Leu has good cell permeability. All our experiments reveal that DCDHF–Leu is an efficient probe for monitoring LAP activity in living cells.

In conclusion, we have developed a fluorescent probe (DCDHF–Leu) that can monitor LAP activity in living cells. It was demonstrated that DCDHF–Leu showed significant fluorescent enhancement and a color change on reaction with LAP. In addition, it was illustrated that DCDHF–Leu was an efficient probe for measuring the inhibitory potency of LAP inhibitors.

Acknowledgement

This study was supported by the NRF Grants funded by the MEST (No. 2009-0080734, 2010-0001842 for the Center for Next Generation Dye-sensitized Solar Cells).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.068.

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