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## *In situ* imaging of aminopeptidase N activity in hepatocellular carcinoma: a migration model for tumour using an activatable two-photon NIR fluorescent probe†

Haidong Li,<sup>a</sup> Yueqing Li,<sup>b</sup> Qichao Yao,<sup>a</sup> Jiangli Fan,<sup>a</sup> Wen Sun,<sup>ad</sup> Saran Long,<sup>ad</sup> Kun Shao,<sup>ad</sup> Jianjun Du,<sup>a</sup> Jingyun Wang<sup>cd</sup> and Xiaojun Peng<sup>ad</sup>

CD13/aminopeptidase N (APN), which is a zinc-dependent metalloproteinase, plays a vital role in the growth, migration, angiogenesis, and metastasis of tumours. Thus, *in situ* molecular imaging of endogenous APN levels is considerably significant for investigating APN and its different functions. In this study, a novel two-photon near-infrared (NIR) fluorescence probe DCM-APN was prepared to perform *in vitro* and *in vivo* tracking of APN. The *N*-terminal alanyl site of probe DCM-APN was accurately hydrolysed to the amino group, thereby liberating strong fluorescence owing to the recovery of the Intramolecular Charge Transfer (ICT) effect. By considering its outstanding selectivity, ultra-sensitivity (DL 0.25 ng mL<sup>-1</sup>) and favourable biocompatibility, the probe DCM-APN was used to distinguish between normal cells (LO2 cells) and cancer cells (HepG-2 and B16/BL6 cells). Furthermore, migration of hepatocellular carcinoma cells was apparently inhibited by ensuring that the APN catalytic cavity was occupied by bestatin. The identification of three-dimensional (3D) fluorescence in cancer tissues was completed under two-photon excitation coupled with lighting up hepatocellular carcinoma tumours *in situ*; this revealed that probe DCM-APN is an effective tool for detecting APN, thereby assisting in the early diagnosis of tumour in clinical medicine.

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## Introduction

Aminopeptidase N (APN/CD13, EC 3.4.11.2), also known as a dimeric membrane protein and a member of the zinc metalloproteinase family,<sup>1,2</sup> participates in many physiological and pathological processes, including signal transduction, neuropeptide degradation, immunological responses, and antigen processing.<sup>3</sup> APN has been extensively considered to be a marker for hematopoietic cells of myeloid origin, which facilitates the classification of human leukaemia cells through its antigenicity.<sup>4</sup> Because APN plays a vital role in tumour invasion, angiogenesis, and metastasis,<sup>5</sup> if an elevated APN is observed above a threshold, it could be employed as a cancer biomarker for assessing a patient and diagnosing clinical

cancer.<sup>6–8</sup> Therefore, developing valid methods that can be employed to track the activity of APN is considered to be of great medical significance.<sup>9–12</sup>

To date, fluorescence imaging has been extensively employed to visualize biologically significant analytes as well as to perform image-guided surgery because of its high selectivity, sensitivity, and spatial and temporal resolutions, especially in a noninvasive manner.<sup>13–23</sup> Specifically, the probes that utilize near-infrared (NIR) emission (650–900 nm) fluorescence are highly favoured for application in bio-imaging because they exhibit the ability to overcome issues such as tissue penetration and autofluorescence.<sup>24–26</sup> In addition, two-photon fluorescence microscopy (under an excitation of 700–1000 nm) provides a satisfactory cell and tissue imaging solution that can be attributed to the advantages of two-photon technology, including long wavelength excitation, minimal photo-damage, and deep tissue penetration.<sup>27–32</sup> To date, only one NIR fluorescence probe has been reported from among the few fluorescence probes employed for the detection of APN.<sup>4,33,34</sup> Unfortunately, severe cross-talk between the excitation and emission spectra<sup>4</sup> weakens its capability of gathering a valid signal.<sup>35</sup> Furthermore, to the best of our knowledge, a two-photon fluorescence probe has not been developed for tracking APN activity in both living cells and tissues.

<sup>a</sup>State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, Dalian 116024, P. R. China. E-mail: fanjl@dlut.edu.cn

<sup>b</sup>School of Pharmaceutical Science and Technology, Dalian University of Technology, 2 Linggong Road, Hi-tech Zone, Dalian 116024, P. R. China

<sup>c</sup>School of Life Science and Biotechnology, Dalian University of Technology, 2 Linggong Road, Dalian 116024, P. R. China

<sup>d</sup>Research Institute of Dalian University of Technology in Shenzhen, Gaoxin South fourth Road, Nanshan District, Shenzhen 518057, China

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Fig. 2 (a) Time response of probe **DCM-APN** versus APN. (b) Fluorescence intensity ( $F_{664\text{ nm}}$ ) changes of probe **DCM-APN** (10  $\mu\text{M}$ ) for different analytes in aqueous solution (PBS/DMSO = 7 : 3, v/v, 0.01 M, pH = 7.4). Except for special statement, the concentration of other interferents was 500  $\mu\text{M}$ , inset (1): blank; (2):  $\text{Na}^+$ ; (3):  $\text{K}^+$ ; (4):  $\text{Ca}^{2+}$ ; (5):  $\text{Ni}^{2+}$ ; (6):  $\text{Mg}^{2+}$ ; (7):  $\text{NH}_4^+$ ; (8):  $\text{F}^-$ ; (9):  $\text{Cl}^-$ ; (10):  $\text{Br}^-$ ; (11):  $\text{I}^-$ ; (12):  $\text{CH}_3\text{COO}^-$ ; (13):  $\text{HCO}_3^-$ ; (14):  $\text{CO}_3^{2-}$ ; (15):  $\text{S}^{2-}$ ; (16):  $\text{HPO}_4^-$ ; (17):  $\text{NO}_3^-$ ; (18):  $\text{SO}_4^{2-}$ ; (19):  $\text{SCN}^-$ ; (20):  $\text{NO}_2^-$ ; (21): glutathione (GSH); (22): cysteine (Cys); (23): homocysteine (Hcy); (24): ascorbic acid (AA); (25): NO; (26):  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ); (27): nitroreductase (10  $\mu\text{g mL}^{-1}$ ); (28): transglutaminase (60  $\text{mU mL}^{-1}$ ); (29):  $\gamma$ -GGT (300  $\text{ng mL}^{-1}$ ) (30): APN (100  $\text{ng mL}^{-1}$ ). (c) Inhibition tests of probe **DCM-APN** (10  $\mu\text{M}$ ) for APN. (d) Normalization merged spectra of probe **DCM-APN** pretreated with APN and **DCM-NH<sub>2</sub>** intermediate. The experiments were repeated thrice and the data are denoted as mean ( $\pm$ S.D.).  $\lambda_{\text{ex}}$  = 490 nm, slit: 10/10 nm.

$\text{Ca}^{2+}$ ;  $\text{Mg}^{2+}$ ;  $\text{Ni}^{2+}$ ;  $\text{NH}_4^+$ ;  $\text{F}^-$ ;  $\text{Cl}^-$ ;  $\text{Br}^-$ ;  $\text{I}^-$ ;  $\text{CH}_3\text{COO}^-$ ;  $\text{HCO}_3^-$ ;  $\text{CO}_3^{2-}$ ;  $\text{S}^{2-}$ ;  $\text{HPO}_4^-$ ;  $\text{NO}_3^-$ ;  $\text{SO}_4^{2-}$ ;  $\text{SCN}^-$ ;  $\text{NO}_2^-$ ), amino acids (glutathione; cysteine; homocysteine), redox species (ascorbic acid;  $\text{H}_2\text{O}_2$ ; NO) and related-enzymes (nitroreductase; transglutaminase;  $\gamma$ -glutamyltranspeptidase) was investigated. As depicted in Fig. 2b and S4,† only APN caused obvious fluorescence enhancement as it has the ability to specifically catalyze the conversion of the amide linkage of **DCM-APN** to the DCM fluorophore, thereby verifying that probe **DCM-APN** possesses excellent selectivity with respect to APN.

### Micro-environmental influence

The influence of micro-environmental conditions, including pH and temperature, was investigated. As shown in Fig. S5,† the probe **DCM-APN** (10  $\mu\text{M}$ ) exhibited good stability at different pH values ranging from 4.75 to 10.27. Temperature had little effect on the probe itself ranging from 25  $^\circ\text{C}$  to 50  $^\circ\text{C}$  (Fig. S6a†) and the fluorescence intensity of probe **DCM-APN** towards APN around 664 nm increased a maximum at 37  $^\circ\text{C}$  (Fig. S6b†). Based on the above results, the probe **DCM-APN** shows excellent performance during the recognition of APN under normal physiological conditions.

### Sensing mechanism

In order to explore the sensing mechanism of probe **DCM-APN** towards APN, the high resolution mass spectra of the resulting

catalytic product were initially recorded (Fig. S7†). Here, a distinct peak was observed at  $m/z$  = 312.1136, which was consistent with the positive ion mode of **DCM-NH<sub>2</sub>** (calcd. 312.1131 for  $[\text{M} + \text{H}]^+$ ). Additionally, normalization fluorescence spectra of **DCM-NH<sub>2</sub>** along with those of the catalytic product completely coincided with their excitation and emission spectra (Fig. 1c, 2d and S8†). This further verifies that APN exhibits the ability to catalyze substrate sites (as per Scheme 1). Furthermore, when pretreated using 100  $\mu\text{M}$  bestatin (an inhibitor of APN) in aqueous solution, the activity of APN was found to be completely suppressed (Fig. 2c and S9†) because its active sites were occupied by bestatin.

To obtain further details related to the interactions between probe **DCM-APN** and APN, molecular docking investigations based on Accelrys' Discovery Studio 2.5 platform were conducted. The X-ray crystal structure of human APN that was measured at a resolution of 1.35095 Å using amastatin (PDB: 4FYT) from the RCSB PDB database was employed. In the crystal, a zinc ion was chelated by three ligands that were provided by His388, His392 and Glu411 in the catalytic center of APN (Fig. S10†). After defining the binding sphere and molecular docking parameters (Fig. 3a), probe **DCM-APN** was docked with APN and it could easily reach the coordination center of zinc ion, which could be ascribed to the hydrophobic interaction with the *N*-terminal amino acid of peptides (Fig. S11†). Furthermore, the coordinate bond lengths in **DCM-APN** and the zinc ion were 2.117 Å and 2.256 Å (Fig. 3b), respectively, and were close to the other three bond lengths observed in APN (2.027 Å, 2.085 Å and 1.936 Å, Fig. S10†). In addition, the interactions of the existing hydrogen bonds between probe **DCM-APN** and other peptides, such as Glu355, Glu411, Ala353 and Tyr477, firmly anchor the *N*-terminal amine of probe **DCM-APN** to the active catalytic site (Fig. 3c). Therefore, using water as the catalyst, the scissile amide bond of probe **DCM-APN** was precisely hydrolyzed in the catalytic system that was centered on the zinc ions. Simultaneously, **DCM-NH<sub>2</sub>** was released with enhanced NIR fluorescence emission. To our delight, the results of the docking simulation agreed well with those of the aforementioned experimental phenomena, which completely confirmed the response of probe **DCM-APN** to APN.

### Cytotoxicity and cell imaging

To evaluate the biocompatibility of the probe, the viability of HepG-2 cells (cancer cells) and LO2 cells (normal cells) towards

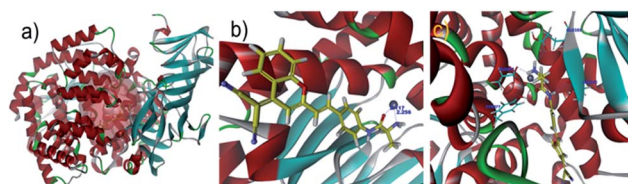


Fig. 3 (a) Stereo image of human APN with a binding location (red transparent sphere). (b) Coordination mode of probe **DCM-APN** with a zinc ion in the hydrophobic cavity. (c) Hydrogen bonds between probe **DCM-APN** and APN. The zinc ion is identified by the grey ball. All carbon atoms of probe **DCM-APN** are indicated in yellow.





various doses of probe **DCM-APN** (0–10  $\mu\text{M}$ ) was measured using standard 5-diphenyltetrazolium bromide (MTT) assays for 24 h. The experimental results are demonstrated in Fig. S12 and S13,<sup>†</sup> which reveal the considerably low cytotoxicity of the probe.

Subsequently, hepatoma carcinoma cells (HepG-2 cells, APN+) and mouse melanoma (B16/BL6 cells, APN+) were selected to monitor the endogenous APN activity on the surfaces of cell membranes.<sup>5,10</sup> Compared to the LO2 cells with a low APN expression (Fig. 4q),<sup>34</sup> the NIR fluorescence signal was obtained (Fig. 4e) after the HepG-2 cells were treated with 2.5  $\mu\text{M}$  probe **DCM-APN** for 10 min. The same features were present in B16/BL6 cells (Fig. S14<sup>†</sup>), which were ascribed to the enzymatic hydrolysis of the probe in cells exhibiting abnormal APN activity. With a prolonged incubation time, the fluorescence intensity of the cell continued to increase (Fig. S15 and S16<sup>†</sup>). Then, when incubated with 50  $\mu\text{M}$  bestatin (the inhibitor of APN) for 2 h, a significant reduction in the fluorescence intensity of HepG-2 cells could be observed (Fig. 4h). To clearly quantify the results, the fluorescence intensity values were measured using an average of nine regions of interest (ROIs) obtained from various treated cells (Fig. 4v). As observed in Fig. 4j–l, high resolution two-photon imaging of probe **DCM-APN** in living HepG-2 cells was also obtained and compared with that in Fig. S17,<sup>†</sup> which further established that probe **DCM-APN** could be employed to track endogenous APN activity *in situ* using a two-photon platform. Additionally, the cell morphology and fluorescence intensity of ROIs (Fig. S18a and b<sup>†</sup>) were observed to remain



**Fig. 4** Fluorescence imaging of probe **DCM-APN** in living cells. (a–c) As-received HepG-2 cells; (d–f) HepG-2 cells treated with 2.5  $\mu\text{M}$  of probe **DCM-APN**; (g–i) HepG-2 cells pre-incubated with 50  $\mu\text{M}$  bestatin with the addition of 2.5  $\mu\text{M}$  of probe **DCM-APN**; (j–l) two-photon fluorescence images of the HepG-2 cells treated with 2.5  $\mu\text{M}$  of probe **DCM-APN**; (m–o) as-received LO2 cells; (p–r) LO2 cells incubated with 2.5  $\mu\text{M}$  of probe **DCM-APN**; (s–u) LO2 cells pre-treated with 50  $\mu\text{M}$  bestatin with the addition of 2.5  $\mu\text{M}$  of probe **DCM-APN**; (v) the NIR fluorescence intensities were recorded by averaging the nine regions of interest (ROIs) from various treated cells (b, e, h, k, n, q and t). Error bar = RSD ( $n = 9$ ). One-photon mode:  $\lambda_{\text{ex}} = 488 \text{ nm}$  and  $\lambda_{\text{em}} = 610\text{--}660 \text{ nm}$ ; two-photon mode:  $\lambda_{\text{ex}} = 800 \text{ nm}$  and  $\lambda_{\text{em}} = 575\text{--}630 \text{ nm}$ . Scale bar = 20  $\mu\text{m}$ .



**Fig. 5** Cell motility assays of probe **DCM-APN** in HepG-2 cells. The cellular morphology of HepG-2 (a, c, e, g, i, and k) before and after a 24 h injury (control: b, f, and j; with 100  $\mu\text{M}$  bestatin: d, h, and l). (m) The distance ( $\Delta L$ ) of wounds obtained from (e–h). Error bar = RSD ( $n = 11$ ).  $\lambda_{\text{ex}} = 488 \text{ nm}$  and  $\lambda_{\text{em}} = 610\text{--}660 \text{ nm}$ . Scale bar = 20  $\mu\text{m}$ .

unchanged (Fig. S18d<sup>†</sup>) after continuous two-photon irradiation at 800 nm for 1000 s, which confirmed that probe **DCM-APN** exhibited high photo-stability. Therefore, probe **DCM-APN** might be employed to distinguish cancer cells from normal cells *via* imaging techniques.



**Fig. 6** Subcellular localization experiments in living HepG-2 cells. (a, e and i) Commercial Hoechst 33342 ( $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 410\text{--}480 \text{ nm}$ ), Mito-Tracker dye ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 500\text{--}560 \text{ nm}$ ), and Lyso-Tracker dye ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 500\text{--}560 \text{ nm}$ ) channels, respectively; (b, f and g) the NIR channel of probe **DCM-APN** ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ,  $\lambda_{\text{em}} = 610\text{--}660 \text{ nm}$ ); (c, g and k) the merged channel; (d, h and l) Pearson's correlation. Scale bar = 20  $\mu\text{m}$ .





signal using 800 nm of excitation. Based on the aforementioned results, we have verified the suitability of **DCM-APN** as the first NIR fluorescence probe for tracking APN endogenous activity in the tissue, which is useful for the early diagnosis and treatment of hepatocellular carcinoma in clinical practice.

### Fluorescence imaging of APN activity *in vivo*

Encouraged by the outstanding performance in living cells, thereby, we explored the capability of probe **DCM-APN** for tracking the APN activity in mice bearing HepG-2 xenograft tumours. As depicted in Fig. 8, the bare BABL/c mice were intratumorally injected with the probe **DCM-APN** (100  $\mu$ M, 150  $\mu$ L). Using the NightOWL II LB983 small animal *in vivo* imaging system, an obvious fluorescence signal appeared at the tumour site *in situ* (Fig. 8b), with the fluorescence intensity gradually increasing as a function of time (Fig. 8b–k). Furthermore, the respective average fluorescence statistics (ph/s) are displayed in Fig. 8l, which were consistent with those of the fluorescence images. In contrast, a considerably weak fluorescence was obtained *via* subcutaneous injection without the HepG-2 xenograft tumour (Fig. S20†), which further confirmed that the probe could selectively light-up hepatocellular carcinoma with a high signal-to-noise ratio. By considering the above results, it was revealed that the probe **DCM-APN** exhibited potential to be employed for the early diagnosis and surgical imaging of hepatocellular carcinoma in clinical practice.

## Conclusions

In summary, we have developed a novel two-photon NIR fluorescence probe **DCM-APN** for tracking endogenous APN activity in virtue of the regulation of the ICT mechanism. By specifically identifying the substrates (*i.e.*, a docking simulation), probe **DCM-APN** displayed high selectivity and ultra-sensitivity (DL 0.25 ng mL<sup>−1</sup>) for APN. It was observed to be biocompatible, and could be employed to monitor the APN activity for distinguishing between cancer cells (*e.g.*, HepG-2 and B16/BL6 cells) and normal cells (LO2 cells). Notably, it was also used to investigate the APN activity at the hepatocellular carcinoma tissue using a one/two-photon confocal fluorescence microscope. Furthermore, a wound healing assay of tumour cells has demonstrated that APN plays an important role in cell migration. More importantly, HepG-2 xenograft tumour of mice is observed to be lit up through *in situ* injection with probe **DCM-APN**, thereby demonstrating that the probe shows potential to be used as an effective tool for the early diagnosis and evaluation of synergistic cancer therapies in clinical practice.

## Conflicts of interest

The authors declare no competing financial interests.

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