ChemComm

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard <u>Terms & Conditions</u> and the <u>Ethical guidelines</u> still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.



www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxx

COMMUNICATION

Fluorometric assay of integrin activity with a small-molecular probe that senses the binding site microenvironment

Toru Komatsu,^{a,d} Aoi Takeda,^a Kenjiro Hanaoka,^a Takuya Terai,^a Tasuku Ueno,^a Yukio Tada,^c Tetsuo Nagano,^c and Yasuteru Urano^{a,b}*

s Received (in XXX, XXX) Xth XXXXXXXX 20XX, Accepted Xth XXXXXXXX 20XX DOI: 10.1039/b000000x

We have developed a small-molecular probe, consisting of cyclic RGD pentapeptide bearing a nitrobenzoxadiazole fluorophore at the 4'-residue that detects integrin $\alpha_V \beta_3$ activity in terms of fluorescence intensity decrease due to quenching of the fluorophore by interaction with tyrosine-122 at the binding site of the protein. This probe appears to be suitable for a practical, high-throughput fluorescence assay to screen small-molecular modulators of integrin activity.

- ¹⁵ Integrins are a family of proteins that transfer environmental signals into cells^[1]. So far, 24 integrin heterodimers have been identified in mammalian cells ^[2], and most of them function by binding to extracellular matrix proteins such as fibronectin or collagens to mediate proliferative and migratory signals.
- ²⁰ Downstream signaling is partly common with that of growth factor receptors^[3], and mis-regulated activation of integrins usually leads to uncontrollable cell growth and migration. For example, increased activity of integrins is often observed in metastatic cancer cell lines^[1,3]. Therefore, small molecules that
- ²⁵ modulate integrin functions would be useful both for basic studies of cell adhesion and as anticancer drug candidates^[2,4]. Integrins have active and inactive forms that cycle in response to intracellular signalling, so the existence of integrin protein does not necessarily imply activity^[3,5]. Therefore, a method for
- ³⁰ selective evaluation of integrin activity is required. One possible approach would be to use small-molecular probes that mimic the structure of extracellular proteins that binds only to the active form of integrins^[6]. However, currently available probes require special detection methods such as fluorescence polarization assay ³⁵ or surface plasmon resonance measurement.

In this study, we have developed a small-molecular fluorescent probe that binds to active integrins and shows a fluorescence signal change in response to the binding, thereby enabling rapid and reliable detection of the target protein (**Figure 1a**). A small-

- ⁴⁰ molecular fluorescent probe for integrin activity based on the aggregation-induced emission enhancement (AIEE) mechanism was recently reported ^[7]. However, although it could successfully visualize active integrins, the mechanism of fluorescence activation is based on the detection of a hydrophobic
- ⁴⁵ protein surface, so that the signal change is not specific to the binding site of integrins, and non-specific protein binding is an issue. In contrast, we set out to design a probe that would

specifically sense the microenvironment of the integrin binding site through interaction of the fluorophore with an amino acid ⁵⁰ residue at the binding site. It has been reported that electron-rich amino acid side chains can quench fluorescence via the photoinduced electron transfer (PeT) mechanism^[8]. Here, we aimed to utilize quenching of the fluorescence of nitrobenzoxadiazole (NBD) fluorophore by the hydroxyphenyl ⁵⁵ group of tyrosine-122 at the integrin $\alpha_V\beta_3$ binding site. Integrin $\alpha_V\beta_3$ has functions in angiogenesis and metastasis, so our fluorescent probe would be a useful tool to study mechanisms of integrin action and in screening assays for integrin modulators.



Figure 1. (a) Design strategy for integrin activity-based probe. (b) Possible fluorophore attachment site on c(RGDfV) peptide to ⁸⁰ sense the active site microenvironment.

Cyclic RGD peptides, such as cyclic-Arg^{1'}-Gly^{2'}-Asp^{3'}-*D*-Phe^{4'}-Val^{5'} (c(RGDfV)) and cyclic-Arg^{1'}-Gly^{2'}-Asp^{3'}-*D*-Tyr^{4'}-Lys^{5'} (c(RGDyK)) peptides, selectively bind to active integrin $\alpha_{V}\beta_{3}$ ^[9] via interaction of the side chain of the amino acid residue ss at the 4' position with Tyr¹²² of integrin β_{3} ^[10]. Since the hydroxyphenyl group of tyrosine is expected to act as a fluorescence quencher via the PeT mechanism^[11], we designed a fluorescent probe for integrin $\alpha_{V}\beta_{3}$ based on cyclic RGD peptide bearing a fluorophore at the 4' position (**Figure 1b**), anticipating ⁹⁰ that the fluorescence would be quenched upon binding to integrin.

The side chain of the 5'-residue of cyclic RGD peptide is directed outside the active site in the complex with integrin $\alpha_V \beta_3$, and many peptidyl probes based on modification at the 5'-residue have been developed^[6-7]. On the other hand, a bulky substituent s at the 4'-residue may affect binding of RGD, and there has been no report so far of a fluorescent probe with 4' modification

- no report so far of a fluorescent probe with 4' modification. Therefore, we focused on the NBD fluorophore, which is small compared to other commonly used protein-labelling fluorophores, such as fluorescein, BODIPY, rhodamine, and cyanine dyes
- ¹⁰ (**Figure S1**)^[12]. Further, the electron density of NBD fluorophore is low due to the nitro group, so that efficient fluorescence quenching by PeT could be expected (**Figure S2**)^[11]. Indeed, a Stern-Volmer plot for mixtures of the fluorophore with Tyr analogue *p*-cresol suggested that the fluorophore would be ¹⁵ quenched in close proximity to tyrosine (**Figure S3**).

The designed probe bearing N_{β} -NBD-modified D-2,3diaminopropionic acid (Dap) at the 4' position (cyclic-Arg^{1'}-Gly^{2'}-Asp^{3'}-D-Dap(NBD)^{4'}-Lys^{5'}) was synthesized by the Fmoc solid-phase method and cyclized with HATU (**Scheme S1**). For

- ²⁰ comparison, we also synthesized an RGD probe with Alexa594 at the 5'-position. 5'-Modification with a fluorophore via a lysine linker is known to have little effect on the K_d value of cyclic RGD peptides^[13]. We then compared the 4'-modified probe cRGD-NBD with the 5'-modified probe (**Figure 2a, 2c**).
- ²⁵ Surprisingly, the two probes showed quite similar K_d values (10 nM for cRGD-Alexa 594, 34 nM for cRGD-NBD; **Figure S4**). This was likely due to the small size of the NBD fluorophore. A dramatic fluorescence decrease was observed only with the NBD probe (monitored in terms of fluorescence intensity (FI)), though
- $_{30}$ both probes bound to the protein (monitored in terms of fluorescence polarization (FP)) (**Figure 2b**, **2d**). The signal decrease of cRGD-NBD in the presence of integrin $\alpha_V\beta_3$ was blocked by addition of active-site-binding cyclic RGD peptide or depletion of secondary ions (**Figure S5**), which suggested that the
- ³⁵ change occurred at the integrin binding site. Therefore, this dramatic fluorescence intensity change enables detection of integrin based on its activity.

Then, we examined whether the quenching indeed occurs via PeT from Tyr^{122} to NBD. We observed only a slight change of

- ⁴⁰ NBD absorbance upon binding with integrins (**Figure 3a**, **3b**). PeT occurs in the excited state of the fluorophore, while other possible quenching mechanisms such as stacking or aggregation operate in the ground state, and would be accompanied with a change in absorbance^{[11][14]}. Therefore, the observation of a slight
- ⁴⁵ absorbance change may suggest some contribution of a mechanism(s) other than PeT. One possibility would be π - π stacking between the fluorophore and hydroxyphenyl group of Tyr¹²². A docking study by means of molecular mechanic calculation (Merck molecular force field; MMFF94x) based on
- $_{\rm 50}$ the co-crystal structure of c(RGDfV) and integrin $\alpha_V\beta_3$ (PDB: $1L5G)^{[15]}$ confirmed that NBD and Tyr^{122} should be in sufficiently close proximity (less than 10 angstroms apart) for intramolecular electron transfer or for stacking (**Figure 3c**).

We further tested the importance of proximity of NBD ⁵⁵ fluorophore to Tyr¹²² for the fluorescence quenching by (1) preparing cRGD peptide-based probe with NBD modification at the 5'-residue, and (2) binding assay with a control protein. Integrin-binding assay of cyclic-Arg^{1'}-Gly^{2'}-Asp^{3'}-D-Tyr^{4'}-D-

Dap(NBD)^{5'} did not show fluorescence quenching, so the ⁶⁰ quenching is specific to the 4'-modified probe (**Figure S6**). Binding assay with bovine serum albumin (BSA), a control protein, did not cause fluorescence decrease (**Figure S7**).



Figure 2. Fluorescence change of cRGD-NBD on binding with integrin α_Vβ₃. (a) Expected binding mode of conventional 5'-modified probe. (b) Result of binding assay of cRGD-Alexa594 (10 nM) with soluble integrin α_Vβ₃ (100 nM) in PBS (pH 7.4) containing 1 mM MgCl₂, 2 mM CaCl₂ and 1% CHAPS. *P*_{bound}
(expected binding % of probe calculated from *K*_d; see Methods) = 90%. c(RGDfV) concentration: 1000 nM. Fluorescence intensity (FI) and fluorescence polarization (FP) are shown. n = 4. Error bar represents S.D. (c) Expected binding mode of 4'-modified cRGD-NBD (d) Result of binding assay of cRGD-NBD (10 nM)
with soluble integrin α_Vβ₃ (100 nM). *P*_{bound} = 73%. n = 4.

Thus, the 4'-NBD-modified probe, cRGD-NBD, should be suitable for specific, rapid (mix and read), high-throughput screening assay for of integrin modulators. Indeed, we could ¹⁰⁰ detect the concentration-dependent inhibitory effect of c(RGDfV), a model inhibitor, simply by monitoring the change of fluorescence intensity (**Figure 4**).

Conclusion

We have developed an integrin activity-reporting small-¹⁰⁵ molecular fluorescent probe that selectively shows a fluorescence decrease upon binding to integrin $\alpha_V\beta_3$, owing to interaction of the NBD fluorophore with the hydroxyphenyl group of tyrosine-122. Fluorometric assay with this probe should be useful for screening small-molecular modulators of integrin activity. A ¹¹⁰ similar design strategy might be applicable to other protein-small molecule interactions, since data on protein binding with small molecules are available in protein structure databases^[16].

115



- **Figure 3.** (a) Absorption spectra of cRGD-NBD (20 μ M) in PBS ²⁵ (pH 7.4) containing 1 mM MgCl₂, 2 mM CaCl₂ after addition of soluble integrin $\alpha_V\beta_3$ (20 μ M) with or without c(RGDfV) (2.5 μ M). $P_{\text{bound}} = 96\%$. Background absorbance of integrin $\alpha_V\beta_3$ was subtracted from the absorbance of samples including integrins. (b) Fluorescence spectra of cRGD-NBD (2 μ M) in PDF (2 μ
- ³⁰ PBS (pH 7.4) containing 1 mM MgCl₂, 2 mM CaCl₂ and 1% CHAPS after addition of soluble integrin $\alpha_V\beta_3$ (2 μ M) with or without c(RGDfV) (250 μ M). $P_{bound} = 88\%$. Excitation wavelength: 470 nm. (c) Molecular-mechanical calculation of lowest energy binding of cRGD-NBD at the active site of ³⁵ integrins, based on PDB1L5G.



Figure 4. Fluorescence intensity of cRGD-NBD (10 nM) with soluble integrin $\alpha_V\beta_3$ (100 nM) in PBS (pH 7.4) containing 1 mM MgCl₂, 2 mM CaCl₂, 1% CHAPS, and indicated concentrations of c(RGDfV). $P_{\text{bound}} = 73\%$. $\lambda_{\text{ex.}} = 485 \text{ nm}$, $\lambda_{\text{em.}} = 500 \text{ nm}$. n = 6. ⁵⁵ Error bar represents S. D.. Z' value was calculated to be 0.51: Z' = 1 - 3 × (S. D.₁ + S. D.₂) / (Mean₁ - Mean₂).

Notes

- ^a Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.
- ^b School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.
- ^cOpen Innovation Center for Drug Discovery, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan. Fax: 03-5841-4855; Tel: 03-65 5841-4850; E-mail: tlong@mol.f.u-tokyo.ac.jp.
- ^d Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan
- † Electronic Supplementary Information (ESI) available: Details of 70 characterization of the system. See DOI: 10.1039/b000000x/

Acknowledgements

The authors thank Akihiro Nakada for preparation of compounds, and Shimpei Iwaki for helpful discussions. This work was supported in part by MEXT (Specially Promoted Research Grants 22000006 to T.N.,

75 24689003 and 24659042 to K.H., and 24655147 to T.K.), JST (K.H., and T.K.) and Mochida Memorial Foundation for Medical and Pharmaceutical Research (T.K.).

References

- [1] J. D. Hood, D. A. Cheresh, Nat. Rev. Cancer 2002, 2, 91-100.
- 80 [2] M. Shimaoka, T. A. Springer, Nat. Rev. Drug Discov. 2003, 2, 703-716.
- [3] J. Takagi, B. M. Petre, T. Walz, T. A. Springer, Cell 2002, 110, 599-511.
- [4] (a) J. S. Desgrosellier, D. A. Cheresh, Nat. Rev. Cancer 2010, 10, 9-22; (b) R. Rathinam, S. K. Alahari, Cancer Metastasis Reviews 2010, 29, 223-237.
- [5] C. Kim, T. Schmidt, E. G. Cho, F. Ye, T. S. Ulmer, M. H. Ginsberg, *Nature* 2012, 481, 209-.
- [6] (a) K. Cheng, S. R. Kothapalli, H. Liu, A. L. Koh, J. V. Jokerst, H.
 ⁹⁰ Jiang, M. Yang, J. Li, J. Levi, J. C. Wu, S. S. Gambhir, Z. Cheng, J.
 Am. Chem. Soc. 2014, 136, 3560-3571; (b) M. Bai, D. J. Bornhop, Current Medicinal Chemistry 2012, 19, 4742-4758.
- [7] H. Shi, J. Liu, J. Geng, B. Z. Tang, B. Liu, J. Am. Chem. Soc. 2012, 134, 9569-9572.
- 95 [8] (a) H. Chen, S. S. Ahsan, M. B. Santiago-Berrios, H. D. Abru ña, W. W. Webb, *J. Am. Chem. Soc.* **2010**, *132*, 7244-7245. (b) N. Marme, J. P. Knemeyer, M. Sauer, J. Wolfrum, *Bioconjug. Chem.* **2003**, *14*, 1133-1139.
- [9] (a) A. Meyer, J. Auernheimer, A. Modlinger, H. Kessler, Current
- Pharmaceutical Design 2006, 12, 2723-2747; (b) S. L. Goodman, G. Holzemann, G. A. Sulyok, H. Kessler, J. Med. Chem. 2002, 45, 1045-1051; (c) E. Ruoslahti, Annual Review of Cell and Developmental Biology 1996, 12, 697-715.
- [10] R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk,
 H. Kessler, J. Am. Chem. Soc. 1996, 118, 7461-7472.
 - [11]T. Ueno, Y. Urano, H. Kojima , T. Nagano, J. Am. Chem. Soc., 2006, 128, 10640-10641.
 - [12] (a) K. Yoshioka, H. Takahashi, T. Homma, M. Saito, K. B. Oh, Y. Nemoto, H. Matsuoka, *Biochim. Biophys. Acta* **1996**, *1289*, 5-9; (b)
- T. Yamamoto, S. Tanaka, S. Suga, S. Watanabe, K. Nagatomo, A. Sasaki, Y. Nishiuchi, T. Teshima, K. Yamada, *Bioorg. Med. Chem. Lett.* 2011, 21, 4088-4096.
- [13]H. K. Choi, S. L. Gibbs, J. H. Lee, S. H. Kim, Y. Ashitate, F. Liu, H. Hyn, G. L. Park, Y. Xie, S. Bae, M. Henary, J.V. Frangioni, *Nat. Biotechnol.* 2013, *31*, 148-154.
 - [14] (a) Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose, T. Nagano, J. Am. Chem. Soc. 2005, 127, 4888-4894; (b) Y. Gabe, T. Ueno, Y. Urano, H. Kojima, T. Nagano, Anal. Bioanal. Chem. 2006, 386, 621-626.
- 120 [15] J. P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, M. A. Arnaout, *Science* 2002, 296, 151-155.
 - [16] R. M. Wachter, M. A. Elsliger, K. Kallio, G. T. Hanson, S. J. Remington, *Structure* 1998, 6, 1267-1277.