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A matrix metalloproteinase activation probe for painting human tumours†

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A probe that allows specific ‘painting’ of human tumours is described. Probe activation was mediated by specific matrix metalloproteinases, resulting not only in disruption of a FRET pair, but in the generation of a fragment that “fluorescently paints” human tumours. This probe demonstrated rapid and effective human tumour labelling with the potential to allow margin detection during surgical resection.

The concept of tissue-based fluorescent labelling has gained attention as a method for identification of diseased tissue margins during intra-operative cancer surgery,^{1–6} made possible with key advances in imaging instrumentation.^{7,8} Pivotal to this has been the development of fluorescent probes which provide disease-mediated contrast.⁹ Approved fluorescent contrast agents in this area include compounds such as 5-aminolevulinic acid which is selectively up-taken and metabolized by cancerous tissues to generate protoporphyrin IX.¹⁰ Other optical agents in clinical studies¹¹ include labelled nanobodies,¹² peptides, such as chlorotoxin (tozuleristide)¹³ which is currently in phase II/III studies for pediatric CNS tumours, and the bis-cyclic peptide GE-137, which targets the human hepatocyte growth factor receptor (c-MET).¹⁴ There are also a wide variety of protease based probes,^{15–21} with cathepsins being a key target where signals are generated by either covalent modification of the enzyme²² or by FRET dequenching.^{23,24} Such probes have been able to detect margins in nonmelanoma skin cancer.²⁵ Other examples of probes explored for cancer imaging include those targeting DPP-IV for esophageal cancer,^{26,27} folate receptor targeted probes,²⁸ PSMA for prostate cancer²⁹ or those developed by Tsien,³⁰ whereby MMP-2 activation liberated a cell penetrating peptide that locally tagged proximal tissue. Matrix metalloproteinase 13 (MMP-13 or

collagenase 3) is an endopeptidase overexpressed in the micro-environment around both lung tumours and fibrotic tissue, and has been shown to play a role in early invasive pulmonary adenocarcinoma.³¹ MMP-2 and -9 (gelatinase A and B) are cancer-associated endopeptidases overexpressed in a variety of malignant tumors and often associated with aggressiveness and poor prognosis.^{32,33} Here we report on the rational design, synthesis and evaluation on human cancerous tissue of a novel MMP-imaging agent (3) which allowed MMP-mediated ‘painting’ of resected human tumour tissue.

The designed probe (3) contained both a FRET pair between 5-carboxyfluorescein (FAM) and the quencher methyl red reporting on enzyme activity, as well as the incorporation of an ‘always-on’ far-red fluorophore (an in-house synthesized Cy5.5, ex/em 670/693 nm) with a spectral window distinct from FAM and away from tissue autofluorescence. The Cy5.5-fragment released enzymatically was responsible for the “tissue-painting” ability of the activated probe, which is attributable to the hydrophobicity of this fragment compared to the parent compound (Fig. 1a).

The peptide sequence (Pro-Phe-Gly-Nle-Lys-βAla, previously reported as MMP-2,9,13 substrate^{34,35}) was synthesized by Fmoc solid-phase peptide synthesis on ChemMatrix resin using Oxyma/DIC as the coupling combination (Scheme 1). At the carboxy-terminus of the peptide, three replicates of bis-ethyleneglycol and D-lysine were added to ensure both solubility and stability against proteases.³⁴ As part of this strategy we developed a novel Cy5.5 red dye, that was readily prepared on large scale in 4 steps, and contained a (5-carboxypyridin-2-yl) group to allow ready incorporation *via* an amide bond to the peptide by solid phase methods (ESI† for details). The Cy5.5, 5-carboxyfluorescein and methyl red were sequentially incorporated at the amino terminus of the peptide, on the Lys side chain (after Dde deprotection) and conjugated to the Lys(N₃) residue *via* azide/alkyne cycloaddition respectively (Scheme 1 and ESI†). Probe 3 was purified and characterized by RP-HPLC and MALDI TOF MS and was fully aqueous soluble (log *P* –1.5) (ESI† for details).

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Fig. 1 Cleavage of compound **3** by target MMPs and generation of the tissue painting fragment. (a) Mode of action of **3** showing the quenched and non-quenched fluorophores and resultant tissue staining with the Cy5.5 fragment released following MMP cleavage; (b) cleavage of **3** ($5 \mu\text{M}$) measured as fluorescence increase (compared to enzyme-free control) at 15 min; ex/em 485/528 nm (FAM, green bars) and 680/710 nm (Cy5.5, red bars) (M: marimastat, MMP inhibitor). Data is the mean of three independent replicates performed in duplicate. Error bars represent s.e.m. Statistical analysis was performed with a one-way ANOVA test compared to an enzyme-free control. * $P = 0.0147$; **** $P < 0.0001$; (c) RP-HPLC analysis revealed the decreased polarity of the Cy5.5-fragment after cleavage compared to the parent peak; (d) visual change in the octanol–water distribution before and after enzymatic cleavage of **3** showing the generation of the hydrophobic Cy5.5 fragment; (e) structure of **3** showing the fragments generated following cleavage at the Gly–Nle site by MMPs.

Target and off-target proteases were incubated with **3**. Within 15 min, as anticipated, an increase in green fluorescence (FAM signal) was measurable from the probe incubated with the active domains of MMP-2, -9 and -13 (Fig. 1b and Fig. S1, ESI[†]), which was blockable in the presence of pan-MMP inhibitor marimastat.

Probe **3** was stable against the off-target proteases (Fig. 1b), with cleavage by MMPs specifically at Gly↑Nle, as confirmed by MALDI TOF MS (Fig. S2, ESI[†]). Importantly the Cy5.5 residue did not interfere with fluorescence in the green channel following MMP activation, with data similar to the control compound **4** that contained FAM & methyl red only (see Fig. S3 and S4, ESI[†]). In solution the Cy5.5 fluorescence intensity remained constant before and after cleavage (Fig. 1b), demonstrating that its

intensity was independent of the FRET pairing. HPLC analysis confirmed that the Cy5.5-fragment obtained after cleavage was much more hydrophobic than the other two components, with its retention factor shifting from $k = 4.2$ for parent compound **3**, to $k = 8.0$ for the Cy5.5 fragment (Fig. 1c and e), while MMP-13 treatment of the peptide **3** in biphasic buffer/octanol led to migration of the cleaved Cy5.5-labelled peptide into the octanol phase (Fig. 1d and Fig. S5, ESI[†]) demonstrating the hydrophobicity “switch-on” upon cleavage.

3 ($5 \mu\text{M}$) was applied onto human lung tumour tissue, from three individual patients: two with squamous cell carcinoma and one with adenocarcinoma (MMPs presence within these tissues was confirmed by gelatin zymography,³⁶ Fig. S6, ESI[†]) and imaged over 30 min (Fig. 2 and ESI[†] videos) with a fibre-based imaging





Fig. 3 Macroscopic imaging of **3** delineating tumour margins. (a) Bright field microscopy image and fluorescence image of freshly excised lung slice, with pathologically identified adenocarcinoma (**Ad**), transition zone (**Tr**) and normal (**N**) tissue, following incubation with compound **3**. (b) Control tissue from the same patient sample without the addition of compound **3** was used as a measure of tissue autofluorescence within this spectral window. Scale bar is 1 mm.

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Conflicts of interest

There are no conflicts to declare.

References

- Q. T. Nguyen and R. Y. Tsien, *Nat. Rev. Cancer*, 2013, **13**, 653–662.
- Y. Zheng, H. Yang, H. Wang, K. Kang, W. Zhang, G. Ma and S. Du, *Ann. Transl. Med.*, 2019, **7**(Suppl 1), S6.
- M. J. Landau, D. J. Gould and K. M. Patel, *Ann. Transl. Med.*, 2016, **4**, 392.
- H. Orbay, J. Bean, Y. Zhang and W. Cai, *Curr. Pharm. Biotechnol.*, 2014, **14**, 733–742.
- T. Nagaya, Y. A. Nakamura, P. L. Choyke and H. Kobayashi, *Front. Oncol.*, 2017, **7**, 314.
- K. R. Tringale, J. Pang and Q. T. Nguyen, *Wiley Interdiscip. Rev.: Syst. Biol. Med.*, 2018, **10**, e1412.
- A. V. Dsouza, H. Lin, E. R. Henderson, K. S. Samkoe and B. W. Pogue, *J. Biomed. Opt.*, 2016, **21**, 80901.
- G. Rossi, A. Tarasconi, G. Baiocchi, G. L. De' Angelis, F. Gaiani, F. Di Mario, F. Catena and R. Dalla Valle, *Acta Biomed.*, 2018, **89**, 135–140.
- M. Koch and V. Ntziachristos, *Annu. Rev. Med.*, 2016, **67**, 153–164.
- M. O. Chohan and M. S. Berger, *J. Neuro-Oncol.*, 2019, **141**, 517–522.
- M. Gao, F. Yu, C. Lv, J. Choo and L. Chen, *Chem. Soc. Rev.*, 2017, **46**, 2237–2271.
- P. Debie, N. Devoogdt and S. Hernot, *Antibodies*, 2019, **8**, 12.
- C. G. Patil, D. G. Walker, D. M. Miller, P. Butte, B. Morrison, D. S. Kittle, S. J. Hansen, K. L. Nufer, K. A. Byrnes-Blake, M. Yamada, L. L. Lin, K. Pham, J. Perry, J. Parrish-Novak, L. Ishak, T. Prow, K. Black and A. N. Mamelak, *Neurosurgery*, 2019, **85**, 641–649.

- J. Burggraaf, I. M. C. Kamerling, P. B. Gordon, L. Schrier, M. L. de Kam, A. J. Kales, R. Bendiksen, B. Indrevoll, R. M. Bjerke, S. A. Moestue, S. Yazdanfar, A. M. J. Langers, M. Swaerd-Nordmo, G. Torheim, M. V. Warren, H. Morreau, P. W. Voorneveld, T. Buckle, F. W. B. van Leeuwen, L.-I. Odegardstuen, G. T. Dalsgaard, A. Healey and J. C. H. Hardwick, *Nat. Med.*, 2015, **21**, 955–961.
- M. Garland, J. J. Yim and M. Bogoyo, *Cell Chem. Biol.*, 2016, **23**, 122–136.
- C. Wang, Z. Wang, T. Zhao, Y. Li, G. Huang, B. D. Sumer and J. Gao, *Biomaterials*, 2018, **157**, 62–75.
- H.-Y. Hu, S. Gehrig, G. Reither, D. Subramanian, M. A. Mall, O. Plettenburg and C. Schultz, *Biotechnol. J.*, 2014, **9**, 266–281.
- E. A. Lemke and C. Schultz, *Nat. Chem. Biol.*, 2011, **7**, 480.
- M. Staderini, A. Megia-Fernandez, K. Dhaliwal and M. Bradley, *Bioorg. Med. Chem.*, 2018, **26**, 2816–2826.
- Y. Urano, in *Development of Novel Fluorogenic Probes for Realizing Rapid Intraoperative Multi-color Imaging of Tiny Tumors*, ed. Y. Toyama, A. Miyawaki, M. Nakamura and M. Jinzaki, Make Life Visible, Springer, Singapore, 2020.
- A. Mochida, F. Ogata, T. Nagaya, P. L. Choyke and H. Kobayashi, *Bioorg. Med. Chem.*, 2018, **26**, 925–930.
- E. Segal, T. R. Prestwood, W. A. van der Linden, Y. Carmi, N. Bhattacharya, N. Withana, M. Verdoes, A. Habtezion, E. G. Engleman and M. Bogoyo, *Chem. Biol.*, 2015, **22**, 148–158.
- M. J. Whitley, D. M. Cardona, A. L. Lazarides, I. Spasojevic, J. M. Ferrer, J. Cahill, C.-L. Lee, M. Snuderl, D. G. Blazer, III, E. S. Hwang, R. A. Greenup, P. J. Mosca, J. K. Mito, K. C. Cuneo, N. A. Larrier, E. K. O'Reilly, R. F. Riedel, W. C. Eward, D. B. Strasfeld, D. Fukumura, R. K. Jain, W. D. Lee, L. G. Griffith, M. G. Bawendi, D. G. Kirsch and B. E. Brigman, *Sci. Transl. Med.*, 2016, **8**, 320–324.
- J. J. Yim, M. Tholen, A. Klaassen, J. Sorger and M. Bogoyo, *Mol. Pharmacol.*, 2018, **15**, 750–758.
- Y. Liu, E. Walker, S. R. Iyer, M. Biro, I. Kim, B. Zhou, B. Straight, M. Bogoyo, J. P. Basilion, D. L. Popkin and D. L. Wilson, *J. Med. Imaging*, 2019, **6**, 016001.
- H. Onoyama, M. Kamiya, Y. Kuriki, T. Komatsu, H. Abe, Y. Tsuji, K. Yagi, Y. Yamagata, S. Aikou, M. Nishida, K. Mori, H. Yamashita, M. Fujishiro, S. Nomura, N. Shimizu, M. Fukayama, K. Koike, Y. Urano and Y. Seto, *Sci. Rep.*, 2016, **6**, 26399.
- Y. Kitagawa, S. Tanaka, Y. Kuriki, K. Yamamoto, A. Ogasawara, T. Nejo, R. Matsuura, T. Koike, T. Hana, S. Takahashi, M. Nomura, S. Takayanagi, A. Mukasa, M. Kamiya, Y. Urano and N. Saito, *Front. Oncol.*, 2019, **9**, 727.
- S. M. Mahalingam, S. A. Kularatne, C. H. Myers, P. Gagare, M. Norshi, X. Liu, S. Singhal and P. S. Low, *J. Med. Chem.*, 2018, **61**, 9637–9646.
- M. Kawatani, K. Yamamoto, D. Yamada, M. Kamiya, J. Miyakawa, Y. Miyama, R. Kojima, T. Morikawa, H. Kume and Y. Urano, *J. Am. Chem. Soc.*, 2019, **141**, 10409–10416.
- Q. T. Nguyen, E. S. Olson, T. A. Aguilera, T. Jiang, M. Scadeng, L. G. Ellies and R. Y. Tsien, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 4317–4322.
- M. Salaün, J. Peng, H. H. Hensley, N. Roder, D. B. Flieder, S. Houille-Crépin, O. Abramovici-Roels, J.-C. Sabourin, L. Thiberville and M. L. Clapper, *PLoS One*, 2015, **10**, e0132960.
- B. Bauvois, *Biochim. Biophys. Acta, Rev. Cancer*, 2012, **1825**, 29–36.
- S. Chakrabarti and K. D. Patel, *Exp. Lung Res.*, 2005, **31**, 599–621.
- A. Megia-Fernandez, B. Mills, C. Michels, S. V. Chankeshwara, N. Krstajić, C. Haslett, K. Dhaliwal and M. Bradley, *Org. Biomol. Chem.*, 2018, **16**, 8056–8063.
- M. Bradley, S. V. Chankeshwara and A. Megia-Fernandez, *WO Pat.* 2016151299A1, 2016.
- M. Toth and R. Fridman, in *Metastasis Research Protocols: Volume I: Analysis of Cells and Tissues*, ed. S. A. Brooks and U. Schumacher, Humana Press, Totowa, New Jersey, 2001.
- N. Krstajić, B. Mills, I. Murray, A. Marshall, D. Norberg, T. H. Craven, P. Emanuel, T. R. Choudhary, G. O. S. Williams, E. Scholefield, A. R. Akram, A. Davie, N. Hirani, A. Bruce, A. Moore, M. Bradley and K. Dhaliwal, *J. Biomed. Opt.*, 2018, **23**, 1–12.
- Y. Urano, M. Sakabe, N. Kosaka, M. Ogawa, M. Mitsunaga, D. Asanuma, M. Kamiya, M. R. Young, T. Nagano, P. L. Choyke and H. Kobayashi, *Sci. Transl. Med.*, 2011, **3**, 110–119.

