



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 μ 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 \uparrow 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 μ 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.

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