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NOVEL MACROCYCLIC PEPTIDE-BASED UPAR-TARGETING FLUORESCENT PROBES FOR INTRAOPERATIVE FLUORESCENCE IMAGING

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INTRODUCTION

- Intraoperative fluorescence imaging is a promising approach for real-time visualization of malignant tissue during surgery through the use of targeted fluorescent probes[1].
- The urokinase-type plasminogen activator receptor (uPAR) exhibits a desirable expression pattern on the surface of several tumor types and tumor-associated tissue, making it an attractive target for intraoperative fluorescence imaging[2].
- The development of uPAR-targeting peptides with high potency towards the receptor could facilitate the construction of peptide-based fluorescent probes and expand the imaging toolbox.
- Aim: Develop novel macrocyclic peptide-based uPAR-targeting fluorescent probes based on a known uPAR-targeting peptide AE344[3].

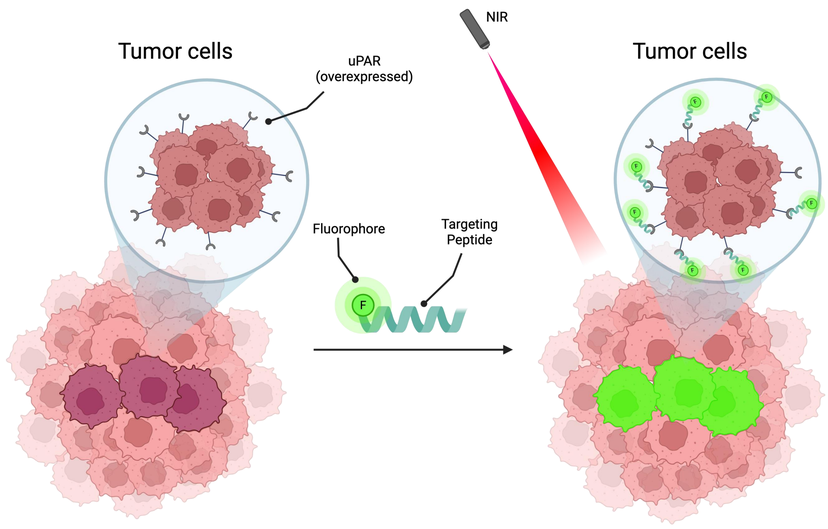


Figure 1. Illustration of intraoperative fluorescence imaging.

RESULTS

- We designed and synthesized a series of bis-thioether-linked macrocyclic uPAR-targeting peptides using cysteine cross-linking. Systematic variation of amino acid composition of AE344 and linker structures yielded 36 new uPAR-targeting peptides.
- The used linker structures were bis-electrophiles, like dibromoxylenes and dibromoketones.
- The direct binding of the peptides towards uPAR was thereafter evaluated in real time using surface plasmon resonance (SPR). The data revealed a favorable position for the macrocyclisations, and the most suitable linker structure.

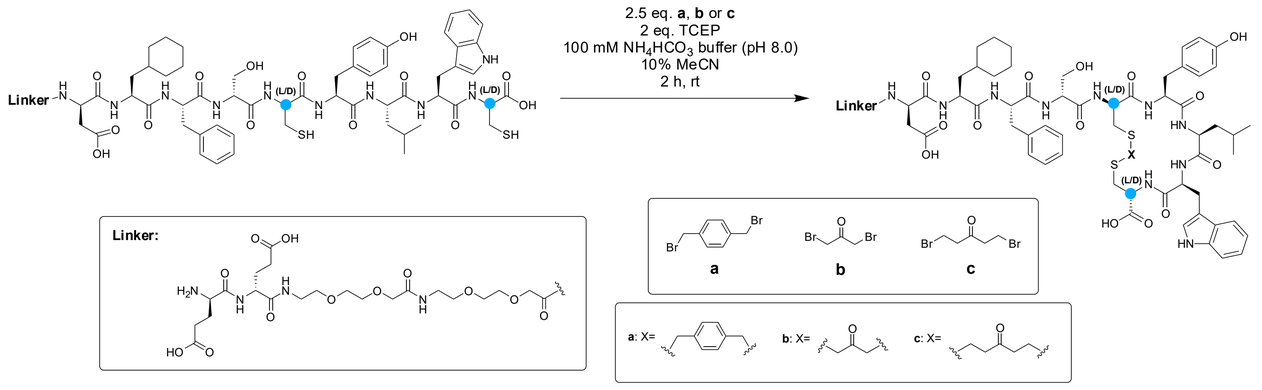


Figure 2. Reaction scheme of cysteine bis-alkylation of AE344 derivatives in solution. The blue dot represents the position of varying cysteine chirality. The left box represents a solubility linker, the upper right box the used dibromo reagents a, b and c. The lower right box represents the introduced linkage corresponding to the used reagent.

Table 1. Representation of a selection of macrocyclic uPAR-targeting peptides with a bis-thioether-linkage between positions 9 and 13[a] and kinetic parameters of peptide-uPAR interactions[b]

Peptide	Sequence	K_D (nM)	k_a (±SE) (10^5 $M^{-1}s^{-1}$)	k_d (±SE) (10^{-3} s^{-1})
AE344	E-E-O2Oc-O2Oc-D-Cha-F-s-r-Y-L-W-S	132	2.22±0.15	29.3±1.9
5b	E-E-O2Oc-O2Oc-D-Cha-F-s-C(O)-Y-L-W-C	70	3.04±0.06	21.2±0.22
6b	E-E-O2Oc-O2Oc-D-Cha-F-s-C(O)-Y-L-W-c	61	2.27±0.09	13.9±0.26
7b	E-E-O2Oc-O2Oc-D-Cha-F-s-C(O)-Y-L-W-C	75	3.70±0.09	27.9±0.36
8b	E-E-O2Oc-O2Oc-D-Cha-F-s-C(O)-Y-L-W-c	97	2.33±0.08	22.6±0.39

[a] All peptides are C-terminal acids. [b] Direct binding kinetics of all peptides were determined by SPR. The equilibrium dissociation constant (K_D) and the association (k_a) and dissociation (k_d) rates were determined from sensorgram fitting to a 1:1 interaction model. Standard errors (SE) are obtained from the fitting procedure.

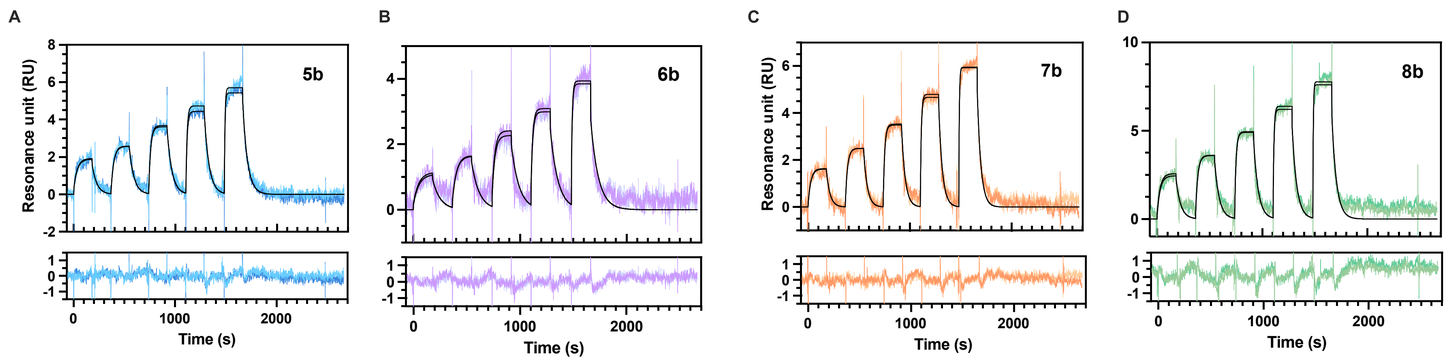


Figure 3. Direct binding kinetics of peptide-uPAR interactions analyzed by SPR. The single-cycle injections represent five consecutive injections of each analyte 5b-8b in varying concentrations. The analysis is performed in duplicates, the curve fitting to a simple 1:1 interaction model is marked with a black line. The residuals are shown in the graphs below each sensorgram.

- Selected high affinity peptides **5b** and **7b** were fluorescently labelled with a near-infrared dye IRDye800CW, yielding two novel fluorescent probes.

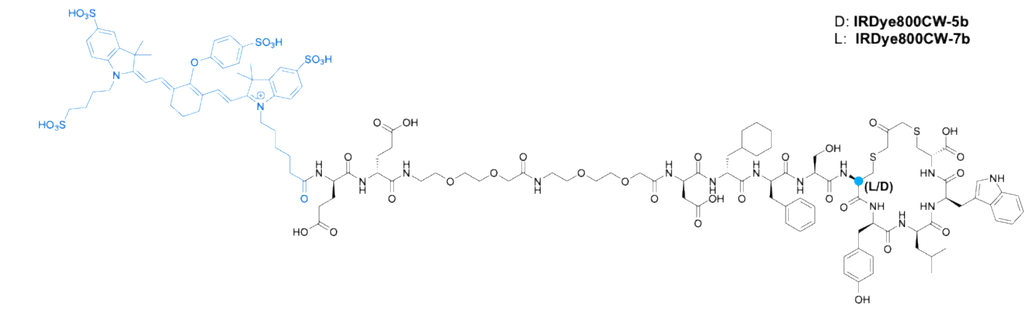


Figure 4. Structures of novel fluorescent probes IRDye800CW-5b and IRDye800CW-7b. IRDye800CW is indicated in blue.

- Exposure of the novel probes to chymotrypsin demonstrated enhanced proteolytic stability compared to a linear control probe.

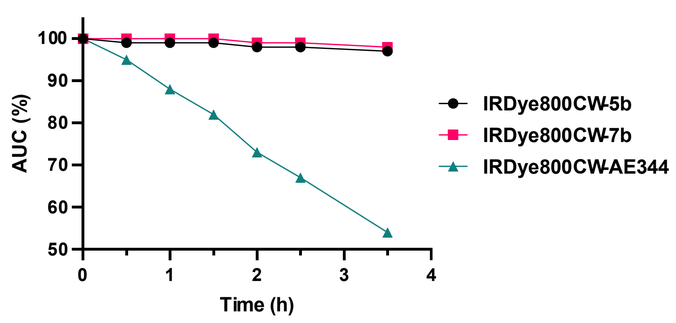


Figure 5. Fluorescent probe stability against chymotrypsin over time.

- Evaluation of the efficacy of the novel fluorescent probes in an *in vivo* fluorescence imaging study in a subcutaneous human glioblastoma xenograft tumor mouse model showed evident accumulation of the probes in uPAR-expressing tumors *in vivo* and *ex vivo*.

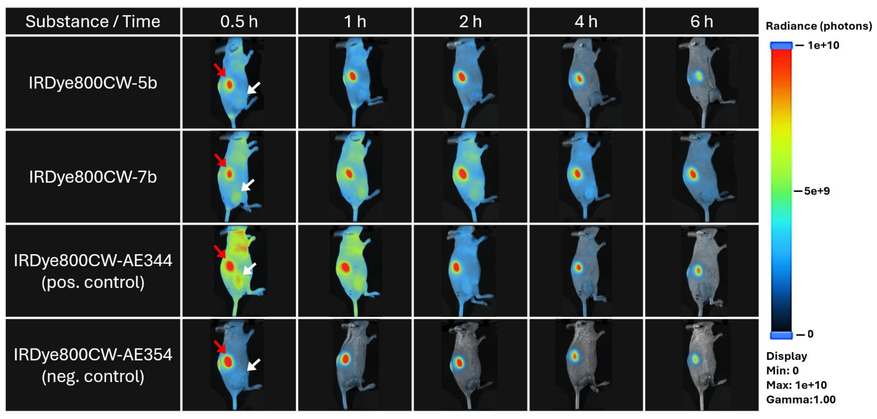


Figure 6. Dynamic *in vivo* images of tumor-bearing mice. Uptake in kidneys is indicated by a red arrow, while uptake in the flank tumor is indicated by a white arrow.

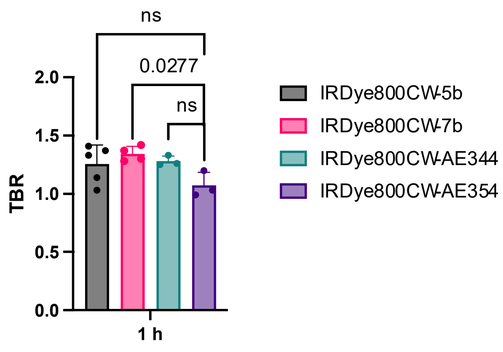


Figure 7. Tumor-to-background ratios (TBR) derived from *in vivo* fluorescent images. TBR calculations are based on the mean (ph/s/cm²/sr) for the signal and background.

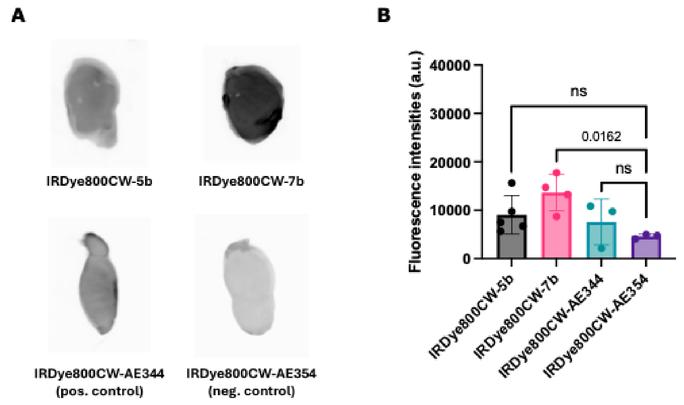


Figure 8. *Ex vivo* images of dissected tumors (A) and comparison of their mean fluorescence intensities (B).

CONCLUSION

- We designed and synthesized a series of macrocyclic uPAR-targeting peptides. Evaluation of peptide-uPAR interactions revealed a favorable position in the peptide sequence for macrocyclisations, and the most suitable linker structure.
- Two macrocyclic peptides with improved binding affinities were labelled with a near-infrared fluorophore IRDye800CW.
- Exposure of the novel probes to chymotrypsin demonstrated enhanced proteolytic stability compared to a linear control probe.
- Both developed probes showed evident accumulation in uPAR-expressing tumors *in vivo* and *ex vivo*.
- We anticipate that the developed fluorescent probes represent promising candidates for clinical translation.
- Further studies investigating probe pharmacokinetics and biodistribution in a more complex tumor model could substantiate their translational potential.

References:
1. Stewart, H. L. & Birch, D. J. S. Fluorescence Guided Surgery. *Methods Appl Fluoresc* **9**, (2021).
2. Zhai, B. T. *et al.* Urokinase-type plasminogen activator receptor (uPAR) as a therapeutic target in cancer. *J Transl Med* **20**, (2022).
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