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Near-infrared-labeled peptide multimer functions as phage-mimic for high affinity, specific targeting of colonic adenomas in vivo

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Abstract

Background—Fluorescent-labeled peptides are being developed to improve the endoscopic detection of colonic dysplasia.

Objective—To demonstrate a near-infrared peptide multimer that functions as a phage mimic for in vivo detection of colonic adenomas.

Design—A peptide multimer was synthesized using tryllysine as a dendritic wedge to mimic the presentation of peptides on phage and all peptides, including the multimer, were fluorescent-labeled with Cy5.5.

Setting—Small animal imaging facility

Subjects—Genetically engineered *CPC;Apc* mouse that spontaneously develop colonic adenomas.

Methods—NIR-labeled AKPGYLS peptide multimer was administered topically into distal colon of mice, and endoscopic images of adenomas were captured. Fluorescence intensities were quantified by target-to-background (T/B) ratios, and adenoma dimensions were measured with calipers after imaging. Validation of specific peptide binding was performed on cryosectioned specimens and cells using confocal microscopy and flow cytometry.

Main Outcome Measurements—Fluorescence T/B ratios from colonic adenomas and adjacent normal-appearing mucosa.

Results—AKP-multimer, monomer, tryllysine core and Cy5.5 resulted in mean T/B ratios of 3.85 ± 0.25 , 2.21 ± 0.13 , 1.56 ± 0.12 , and 1.19 ± 0.11 , respectively, $p < 0.01$ on in vivo imaging. Peptide multimer showed higher contrast and greater specificity for dysplastic crypts as compared to other

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Conflict of interest statement: The University of Michigan has filed a provisional patent on behalf of authors BPJ and TDW on the peptide presented in this study.

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probes. Peptide multimer demonstrated significantly greater binding to HT29 cells on flow cytometry and fluorescence microscopy in comparison to monomer and trilycine core. A binding affinity of 6.4 nM/L and time constant of 0.1136 min^{-1} (8.8 min) was measured for multimer.

Limitations—Only distal colonic adenomas were imaged.

Conclusion—Peptide multimers combine strengths of multiple individual peptides to enhance binding interactions and demonstrate significantly higher specificity and affinity for tumor targets.

Keywords

peptide; multimer; colorectal cancer; early detection; endoscopy

Introduction

Colorectal cancer is a common malignancy in western countries, and is dormant in a pre-malignant state (dysplasia) that allows for early detection.¹ Effective visualization and removal of adenomas on colonoscopy reduces risk for progression to colorectal cancer.² Colonoscopy captures white light reflected from the mucosal surface, and has significant limitations. Small adenomas (<1 mm), nonpolypoid lesions, and right sided (proximal) disease are particularly challenging.^{3–4} Clinical studies have shown a significant miss rate of >20% for adenoma.⁵ Furthermore, flat lesions are endoscopically less visible, and may represent >25% of all dysplastic lesions.⁶ Thus, novel methods that improve detection accuracy are greatly needed.

Molecular imaging techniques can visualize changes in cellular target expression that occur with cancer transformation.⁷ These methods are being developed to improve accuracy for early cancer detection in the digestive tract. By comparison to other molecular probes such as antibodies, nanoparticles, and affibodies, peptides have a low molecular weight and offer considerable advantages for in vivo imaging, including short amino acid sequences, high affinity to binding sites, minimal immunogenicity, rapid accumulation at the target, fast circulatory clearance, well-defined labeling chemistry, capacity for topical administration in high concentrations, and optimal image contrast with little concern for toxicity.⁸

Phage display is an efficient method for rapidly screening large peptide libraries and selecting ligands that bind specifically to disease targets.⁹ However, the monomer form may exhibit significantly lower specificity and less binding affinity than the original phage. The widely used M13 library presents 4–5 copies of each peptide fused to the pIII coat protein at the N-terminus, allowing for multivalent target interactions, resulting in many advantages for the multimer form.^{10–12} The use of near-infrared (NIR) fluorescence (spectral range 665–900 nm) results in greater tissue penetration, less autofluorescence background, and reduced hemoglobin absorption.¹³

We have previously selected the peptide sequence AKPGYLS, hereafter AKP using phage display technology in a genetically-engineered *CPC;Apc* mouse model of spontaneous colorectal cancer based on somatic inactivation of the *Apc* gene.¹⁴ Here, we describe the design, construction, and validation of a near-infrared-labeled peptide multimer that can be used as a phage mimic for in vivo endoscopic imaging in real time. This novel methodology can be used to evaluate molecular expression in small animal models longitudinally, and can also be directly translated into the clinic for early detection of colorectal cancer.

Materials and Methods

Cells, Chemicals and Materials

Human colorectal adenocarcinoma (HT29) and non-malignant intestinal (CCD-841Con) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC guidelines at 37°C in 5% CO₂. All peptide synthesis reagents (Anaspec, Fremont, CA or AAPTEC, Louisville, KY) were of the highest grade available (>99% purity) and used without further purification. Solvents and other chemical reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Mouse Model of Colorectal Cancer

Mice were cared for under the approval of the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan. The *CPC;Apc* mouse is genetically engineered with a Cre-recombinase driven by a Cdx2 promoter (CDX2P-9.5NLS-Cre) to target a floxed allele of the *Apc* gene.¹⁵ A somatic mutation in *Apc* results in adenomas that develop spontaneously in the distal colon beginning at ~2.5 months of age. All mice were housed in specific pathogen-free conditions and supplied water ad libitum under controlled conditions of humidity (50±10%), light (12/12 hour light/dark cycle) and temperature (25°C).

Peptide Synthesis

The peptides were prepared using standard Fmoc-mediated solid-phase synthesis. Fmoc [Fluorenylmethyloxycarbonyl] and Boc [Butyloxycarbonyl] protected *L*-amino acids were used, and synthesis was performed on rink amide MBHA [Methylbenzhydrylamine] resin.¹⁶ We synthesized three constructs: A) tetrameric form of AKPGYLS anchored on a tryllysine core (AKP-multimer); B) AKPGYLSGGGSK-CONH₂ (AKP-monomer); and C) tryllysine core (control). The structure of each construct is shown in Fig. 1. The peptides were conjugated with Cy5.5 for in vivo imaging and with FITC for flow cytometry (no Cy5.5 channel available). The unlabeled peptide (no fluorescent dye) was also synthesized for the competition study. The crude peptides were dissolved in 1:1 Acetonitrile/H₂O (v/v) and purified by prep-HPLC with a C₁₈ column (Waters Inc. Milford, MA) using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient. The final purity of the peptides was confirmed by analytical C₁₈-column. The absorbance was monitored at 214 nm for the amide bond during the purification in semi-preparative column and after purification in an analytical column. Further characterization was performed with either electrospray ionization (Waters, Milford, MA) or Q-TOF [Quadrupole time-of-flight] (Agilent Technologies, Santa Clara CA) mass spectrometry. Peptides were lyophilized (Freezone, Labconco Corp MO) before storage at -20°C.

In Vivo Imaging

For in vivo imaging, anesthesia was induced and maintained in the animals at a dose of 4% and 2%, respectively, with inhaled isoflurane mixed with oxygen via a nose cone at a flow rate 0.5 liter/min. The colon was rinsed with tap water through the instrument channel of the endoscope (Karl Storz Veterinary Endoscopy, Goleta, CA) to remove stool and mucous. The Cy5.5-labeled multimer, monomer, tryllysine core and Cy5.5 alone were then delivered into separate mice at a concentration of 100 µM in PBS containing 2.5% DMSO (dimethyl sulfoxide) to increase the solubility of the Cy5.5 fluorophore in a volume of 1.5 ml. The contrast agents were allowed to incubate for 5 min, and then the colon was rinsed 3X with a tap water to remove the unbound agents. The colon was insufflated with air, and the NIR endoscope was used to collect fluorescence with 671 nm excitation at 10 frames/second.¹³ The distal end of the NIR endoscope is ~3 mm in diameter and has a 3 Fr (~1 mm)

instrument channel. The proximal end is contained within a custom polymer housing that has a filter wheel to provide both reflectance and fluorescence images. Fluorescence images are collected through a band pass filter ($\lambda_{em} = 696$ to 736 nm, FF01–716/40–25, Semrock, Inc, Rochester, NY) in 100 ms (10 frames/sec). Endoscopic imaging of the FITC-labeled peptides was described previously.¹⁷ The videos were transferred to the computer by a firewire connection using Axiovision 4.8.1 (Carl Zeiss Microscopy, Thornwood, NY, USA) software. The distal end of this endoscope was marked at one millimeter intervals, and the distance of each lesion from the anus was recorded. The same set of mice was imaged with each of the 4 contrast agents with after a period of 72 hours for recovery in between imaging sessions. In addition, a set of FITC-labeled contrast agents was evaluated in vivo.

Image analysis

Fluorescence images collected endoscopically were exported in zvi format with 16 bit digital resolution. Sequentially-collected white light and fluorescence images for each lesion were analyzed. Separate regions-of-interest (ROI) were defined around the lesion and adjacent normal-appearing colonic mucosa on white light. These ROI's were overlaid onto the corresponding fluorescence image.¹⁷ The T/B ratio was determined by ratioing the mean fluorescence intensities from the ROI's of the lesion and normal mucosa using Labview software (National Instruments Corporation, Austin, TX, USA).

After imaging, the mice were euthanized using CO₂ overdose. The colon was harvested and divided along the length of the organ, exposing the mucosal surface. After washing 3X with 1X PBS, the specimen was placed on black paper (no fluorescence background), and fluorescence images were collected with a Xenogen IVIS spectrum (Caliper Life Sciences; Hopkinton, MA). A Cy5.5 filter set and 0.5 sec exposure time was used, and the images were exported using Living Image 2.5 software (Caliper Life Sciences; Hopkinton, MA). The dimensions of the lesions seen on fluorescence were measured with vernier calipers. The lesions found ex vivo were registered with those seen on the endoscopic image using the relative distance measured from the anus to each adenoma imaged in the distal colon. The lesions were fixed in 10% formalin buffer overnight, and submitted for histopathology (H&E).

Statistical Analysis

Differences in the T/B ratios measured from each set of lesions identified on fluorescence endoscopy using AKP-multimer-Cy5.5, AKP-monomer-Cy5.5, trilisine core-Cy5.5, and Cy5.5, were first compared using a one-way analysis of variance (ANOVA).¹⁸ Then, differences in the mean values between each pair of contrast agents were evaluated using Tukey's multiple comparisons of the means. Statistical significance was assessed at the 0.05 level. For all other methods of validation, a comparison of the difference in the mean findings between 2 groups was performed using a 2-sided Student t-test with unequal variance, and statistical significance was assessed at the 0.05 level. All results are presented as mean $\pm$ SD unless otherwise noted.

Detailed methods for peptide immunohistochemistry, competitive binding assay, fluorescence microscopy, flow cytometry, and measurement of binding affinity and kinetics are discussed in the supplementary methods.

Results

Peptide Multimer and Monomer

We developed a peptide multimer by assembling four copies of the peptide monomer AKPGYLS on a trilisine core, Fig. 1A. The C-terminus of each peptide was incorporated

into the core, exposing the N-terminus for binding with the same orientation as that on the phage. An additional lysine residue was added to the core for attaching the fluorophore, either Cy5.5 or FITC, designated by R in Fig. 1. This scaffold is designed to retain peptide binding activity outside of the context of the phage.¹⁹ Each branch of the construct contains the complete 7-mer sequence as displayed on the pIII protein of the phage. We further synthesized the peptide monomer AKPGYLS with a GGGS linker to the fluorophore to achieve the same orientation as that found on the phage, Fig. 1B. The trilycine core was also synthesized with a fluorophore for use as a control, Fig. 1C. A scrambled version of the peptide was not used as a control due to the unknown structural and binding site information for the target peptide. The chemical structures for Cy5.5 and FITC are shown in Fig. 1D and 1E, respectively.

The fluorescence emission spectra for the AKP-multimer-Cy5.5, AKP-monomer-Cy5.5, and trilycine core-Cy5.5 at a concentration of 10 μ M in 1X PBS with 671 nm excitation is shown in Fig. 1F. The peak emission occurs at ~705 nm, and the spectral range is approximately 680–950 nm. The chromatograms obtained from analytical RP-HPLC indicated a purity >95% for all peptides, Table S1. On mass spectra, the experimental m/z (mass units) measured for AKP-multimer-Cy5.5, AKP-monomer-Cy5.5, trilycine core-Cy5.5, AKP-multimer-FITC, AKP-monomer-FITC, and trilycine core-FITC, were 4003.3, 1727.97, 1136.7, 3786.0, 1510.0, and 919.5, respectively. These values agree with the expected molecular mass of 4002.3, 1726.97, and 1136.7, 3785.9, 1509.8, and 919.8, respectively.

In Vivo Imaging

The specific binding activity of the synthetic peptides to colonic adenomas was assessed in vivo in four groups of mice ranging in age from 3–6 months: 1) AKP-multimer-Cy5.5 (n=15 mice, n=34 adenomas), 2) AKP-monomer-Cy5.5 (n=14 mice, n=27 adenomas), 3) trilycine core-Cy5.5 (n=8 mice, n=17 adenomas), and 4) Cy5.5 (n=2 mice, n=5 adenomas). No cancers were found in mice in this age range. First, white light endoscopy was performed to confirm that the colonic mucosa was free of stool and mucus and to identify any grossly visible adenomas. The peptides were administered as described above, and fluorescence imaging was performed.

A white light endoscopy image of a representative adenoma (arrow) is shown, Fig. 2A. The fluorescence image of this lesion collected with AKP-multimer-Cy5.5 shows increased NIR intensity over the surface of the adenoma (arrow) with a T/B ratio of 4.69 ± 0.36 , Fig. 2B. By comparison, the AKP-monomer-Cy5.5 image shows much less fluorescence intensity from the adenoma (arrow), Fig. 2C. The fluorescence images of trilycine core-Cy5.5, autofluorescence (no contrast agent), and Cy5.5 alone shows negligible signal, Fig. 2D–F, respectively. Real-time videos of this grossly visible adenoma on white light and with AKP-multimer-Cy5.5 and AKP-monomer-Cy5.5 on fluorescence are shown in videos S1, S2, and S3, respectively.

Use of AKP-multimer-Cy5.5 achieved specific contrast enhancement that enabled detection of flat lesions that were not seen on white light endoscopy. In Fig. 3A, no adenomas are visible on white light. In Fig. 3B, the fluorescence image using the AKP-multimer-Cy5.5 reveals the presence of a flat adenoma (arrow) with T/B ratio of 4.0 ± 0.1 . This lesion was less appreciated with the AKP-monomer-Cy5.5, Fig. 3C. Out of 34 adenomas imaged, 5 were flat in architecture, and all were visualized with AKP-multimer-Cy5.5 but not with AKP-monomer-Cy5.5 and trilycine core-Cy5.5. In Fig. 4A–C, white light endoscopy images from spontaneous adenomas (arrows) are shown with corresponding fluorescence images with AKP-multimer-FITC, Fig. 4D, that reveal much greater intensity from the adenoma

compared to AKP-monomer-FITC, Fig. 4E, and trilysine core-FITC, Fig. 4F. These results suggest that binding is not fluorophore-dependent.

Image analysis

After imaging, the animals were euthanized and the colon was excised. The mucosal surface of the colon was exposed, and the size of each lesion was measured with calipers under fluorescence imaging, Fig. S1. The dimensions ranged from <1 to 6 mm. The flat lesion in Fig. 3B was found to have a dimension of 0.8 mm (black arrow), Fig. 3D (upper panel), scale bar 2.5 mm. The corresponding histology (H&E) shows features of dysplastic crypts (arrow), including elongated nuclei, disorganized structure, hyperchromaticity, and crowded lamina propria, similar to that found in human disease, Fig. 3E, scale bar 20 μm . The smallest adenoma (white arrow) on fluorescence with AKP-monomer-Cy5.5 was found to have a T/B ratio of 2.24 ± 0.04 , Fig. 3F. The size of this lesion (black arrow) measured ex vivo was 1.7 mm, Fig. 3D (lower panel). An adenoma with dimension of 5.8 mm (white arrow) can be seen nearby for comparison. The fluorescence intensities from all adenomas were quantified by calculating the mean values from the ROI's of the lesion and neighboring normal-appearing colonic mucosa and taking a ratio. The average T/B ratios for the AKP-multimer-Cy5.5, AKP-monomer-Cy5.5, trilysine core-Cy5.5, and Cy5.5 were 3.85 ± 0.25 , 2.21 ± 0.13 , 1.56 ± 0.12 , 1.19 ± 0.11 , respectively. A one way analysis of variance (ANOVA) of this data set resulted in an F-value of 24.8. Tukey's multiple comparisons showed a statistically significant difference between the mean value for AKP-multimer-Cy5.5 and that for AKP-monomer-Cy5.5, trilysine core-Cy5.5, and Cy5.5, $p < 0.01$ for all.

Peptide immunohistochemistry and competitive binding assay

We validated specific binding of the AKP-multimer-Cy5.5 and AKP-monomer-Cy5.5 to dysplastic crypts on adenoma sections ex vivo. In Fig. 5A, staining with AKP-multimer-Cy5.5 demonstrates bright NIR fluorescence from dysplastic crypts (arrow) on microscopy, scale bar 50 μm . By comparison, AKP-monomer-Cy5.5 and trilysine core-Cy5.5 showed much less fluorescence intensity, Fig. 5B and 5C. Representative histology (H&E) for adenomas and normal colonic mucosa is shown in Fig. 5D and 5H, scale bar 50 μm . Minimal NIR fluorescence intensity is seen from normal crypts on staining with all contrast agents, Fig. 5E–G.

On competitive binding, unlabeled AKP-multimer blocked AKP-multimer-Cy5.5 binding in a dose dependent manner at concentrations of 0, 100, 500, 750 and 1000 μM on fluorescence microscopy, Fig. S2. The blocking effect saturates (mean fluorescence intensity <93% of maximum) at 500 μM . No significant reduction of fluorescence intensity was observed with addition of unlabeled trilysine core (control) at concentrations up to 1000 μM , Fig. S2. The mean intensities were 34.1 ± 7.1 , 18.47 ± 1.07 , 2.55 ± 0.21 , 1.72 ± 0.04 , 1.6 ± 0.58 , and 29.68 ± 5.48 AU, respectively.

Fluorescence Microscopy

We validated specific peptide binding to the plasma membrane of cells in vitro. On confocal microscopy, we found that AKP-multimer-FITC at 10 μM bound greater than AKP-monomer-FITC to the surface of HT29 cells, Fig. S3A and S3B, but the trilysine core-FITC did not, Fig. S3C, scale bar 50 μm . By comparison, all contrast agents did not stain the (control) cells, Fig. S3D–F.

Flow Cytometry

Specific binding of the contrast agents to HT29 (target) and CCD-841Con (control) cells on flow cytometry is shown in Fig. 6. The mean result for AKP-multimer-FITC binding to

HT29 is 8644.5 ± 330.2 versus 2066 ± 89.1 , 885 ± 127.2 , and 846 ± 72.1 for AKP-monomer-FITC, trilycine core-FITC and cells only, respectively, Fig. 6A. Furthermore, the mean result for AKP-multimer-FITC binding to CCD-841Con is 3216.5 ± 28.9 versus 1572.5 ± 34.6 , 1693.5 ± 12.2 , and 1467.5 ± 43.1 for AKP-monomer-FITC, trilycine core-FITC and cells only, respectively, Fig. 6B.

Measurement of Binding Affinity

The relative fluorescence intensity at 525 nm as a function of the concentration (0–10 μ M) for AKP-multimer-FITC and AKP-monomer-FITC binding to HT29 cells is shown, Fig. 6C. A non-linear increase in intensity is observed until saturation (>99% of maximum) is reached at 750 nM for AKP-multimer-FITC and 7 μ M for AKP-monomer-FITC. Data regression yields a $K_d = 6.4$ nM/L, $R^2 = 0.973$, for AKP-multimer-FITC and $K_d = 310$ nM/L, $R^2 = 0.876$, for AKP-monomer-FITC, a factor of ~48-fold improvement.

Measurement of Binding Kinetics

The binding kinetics of AKP-multimer-FITC and AKP-monomer-FITC to HT29 cells was also evaluated on flow cytometry over time intervals ranging between 0–60 min. In Fig. 6D, the exponential increase in median fluorescence intensity as a factor of time is shown. About 50% of the maximum was observed at ~5 min and no further increase was seen after ~30 min for both contrast agents. A time constant of $k = 0.1136 \text{ min}^{-1}$ (8.8 min), $R^2 = 0.972$, and $k = 0.0663 \text{ min}^{-1}$ (15.1 min), $R^2 = 0.984$, was found for AKP-multimer-FITC and AKP-monomer-FITC, respectively. Thus, the multimer binds to HT29 cells within minutes and with a slightly faster onset by a factor of ~2-fold.

Discussion

Here, we demonstrate the construction and validation of a synthetic multimer that has been designed to mimic the binding property of multiple peptides expressed by phage. This multimer was combined with a NIR fluorophore for use as a specific contrast agent for detection of colonic adenomas on endoscopy in vivo. The multimer binding affinity (K_d) of 6.4 nM/L represents a ~48-fold improvement over the monomer alone, a result comparable to that of a good antibody. The multimer achieves binding onset in 8.8 versus 15.1 minutes (monomer) to HT29 cells, reflecting fast binding kinetics. This ~2-fold improvement was unexpected because of the multimer's higher molecular weight (4.0 versus 1.7 kD). Other molecular probe platforms, such as antibodies and activatable agents, have a much slower onset of reporter activity, requiring hours (or even days) to reach peak effect, increasing background and limiting clinical usefulness.^{20,21}

The multimer demonstrated a significant improvement in imaging performance over the monomer. On average, the T/B ratio was ~2-fold greater. With the multimer, adenomas >0.8 mm were visualized compared to >1.7 mm for the monomer. While this mouse model is not known to develop flat adenomas, we began imaging these mice at an age (3 months) when they first begin to develop adenomas. These lesions likely appear flat in architecture because they have not yet developed a growth component. The ability to detect small adenomas on endoscopic imaging with high contrast using the peptide multimer has clinical significance in their morphology being similar to flat adenomas which are believed to have significant malignant potential. The decrease in fluorescence intensity on the competition study suggests that binding occurs with the peptide rather than the fluorophore or linker.

We have previously selected a peptide (monomer) QPIHPNNM using a T7 phage display library for specific detection of grossly visible colonic adenomas using a FITC label, and measured a T/B ratio of ~2 but were unable to visualize flat and smaller sized lesions.¹⁷ The

T7 library has a low copy number of between 0.1–1 peptide per phage; limiting the likelihood for multivalent interactions with the target.²² Other peptides have been used in vivo use for cancer diagnosis and therapy.^{23–25} However, most phage-derived peptides, when used outside of the context of the phage, have limited performance, including binding affinity, specificity, T/B ratio, and tumor uptake. Previously, several groups have developed branched peptides to achieve multivalency for nuclear imaging, and also found improvements on in vivo imaging.^{26–29} Because selection of the peptide using phage display is performed in an unbiased manner, we do not know the specific cell surface target. The multimer is likely to be safe for human use in future clinical applications. The peptide and backbone were synthesized using all natural amino acids, and the Cy5.5 label belongs to the carbocyanine family, which includes indocyanine green, a FDA-approved fluorophore. Moreover, an immunogenic response is unlikely to develop as the colon will absorb little of this topically administered imaging agent.

Multimers have potential to widely expand the applicability of peptides by achieving a binding affinity that rivals that of antibodies while maintaining rapid kinetics. This novel approach is promising for in vivo diagnosis and targeted therapy in the clinic. This method can be performed as an adjunct to the current practice of endoscopic surveillance for improving the yield of detection for early disease and decreasing the risk for missed lesions in a high risk population, such as patients with FAP (familial adenomatous polyposis), HNPCC (Lynch Syndrome), and first-degree relatives with colorectal cancer.³⁰ Also, the use of multimers results in sharp lesion borders on endoscopic imaging that can be used to assess lesion size, potentially improving accuracy for monitoring conventional therapies, such as surgery, radiation and chemotherapy, and minimizing side effects. A future direction for this strategy is to visualize flat and depressed lesions that occur in the setting of ulcerative colitis.³¹ Limitations of this study include imaging of adenomas in the distal colon only. Moreover, the future clinical use of multimers presents a few challenges that will need to be addressed, including higher production costs, a stable linker (backbone), and overall long term stability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acronyms

AU	arbitrary units
FOV	field-of-view
NIR	near-infrared
PFA	paraformaldehyde
ROI	regions of interest
RT	room temperature
T/B	target-to-background
RP-HPLC	reverse phase-high performance liquid chromatography

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Fig. 1. Biochemical structure of contrast agents

A) AKP-multimer, B) AKP-monomer, C) trilysine core, D) Cy5.5, and E) FITC. R indicates either Cy5.5 or FITC fluorophore. Different components include peptide(s) binding motif (black), linker (blue), and fluorophore (red). F) Fluorescence emission spectra of AKP-multimer-Cy5.5, AKP-monomer-Cy5.5, and trilysine core-Cy5.5 using $\lambda_{\text{ex}} = 671 \text{ nm}$ excitation reveal emission peaks near 705 nm.

Fig. 2. In vivo imaging of a grossly visible colonic adenoma

A) White light and NIR fluorescence images are shown with B) AKP-multimer-Cy5.5; C) AKP-monomer-Cy5.5; D) tritylsine core-Cy5.5; E) autofluorescence; and F) Cy5.5 alone as topical contrast agent from adenoma (arrow) in distal colon of *CPC;Apc* mouse. AKP-multimer-Cy5.5 produces significantly enhanced image contrast (T/B ratio 4.69 ± 0.36) with minimal background from neighboring normal-appearing mucosa.

Fig. 3. In vivo detection of colonic adenoma with flat morphology

A) White light and NIR fluorescence images are shown with B) AKP-multimer-Cy5.5 and C) AKP-monomer-Cy5.5 as topical contrast agent from flat adenoma (arrow). AKP-multimer-Cy5.5 significantly enhances in vivo image contrast (T/B ratio 4.1 ± 0.1). D) Flat lesion (black arrow, upper panel) is found to have dimension of 0.8 mm on excised colonic specimen. Flat lesion (black arrow, lower panel) is found to have dimensions of 1.7 mm. A 5.8 mm grossly visible adenoma (white arrow) is seen nearby, scale bar 2.5 mm. E) Histology (H&E) of 0.8 mm flat lesion shows features of dysplasia (arrow), scale bar 20 μm . F) AKP-monomer-Cy5.5 is used as topical contrast agent to visualize 1.7 mm flat adenoma (arrow), T/B ratio 2.24 ± 0.04 .

Fig. 4. In vivo imaging with FITC-labeled peptides

A–C) White light endoscopy images of spontaneous adenomas (arrows) in distal colon of *CPC;Apc* mouse. Fluorescence images with D) AKP-multimer-FITC show much greater intensity compared to E) AKP-monomer-FITC and F) trilysine core-FITC, suggesting the peptide binding is fluorophore-independent.

Fig. 5. Specific binding on peptide immunohistochemistry

On fluorescence microscopy, greater NIR intensity (red) is observed from dysplastic crypt (arrow) after staining adenoma section with A) AKP-multimer-Cy5.5 versus B) AKP-monomer-Cy5.5 and C) trilylsine-core-Cy5.5, scale bar 50 μ M. D) Histology (H&E) shows crypts with dysplastic features, scale bar 100 μ m. E–G) Minimal NIR intensity is seen from staining of normal colonic mucosa with all contrast agents. H) Histology (H&E) shows normal crypts, scale bar 100 μ m.

Fig. 6. Characterization of peptide binding on flow cytometry

A) The mean value for the AKP-multimer-FITC (gray) binding to HT29 cells is 8645 compared to 2066, 885, and 846 for AKP-monomer-FITC (dashed), trilylsine core-FITC (dotted) and cells (solid), respectively. B) The mean value for the AKP-multimer-FITC (gray) binding to CCD-841Con (control) cells is 3217 compared to 1573, 1694, and 1468 for AKP-monomer-FITC (dashed), trilylsine core-FITC (dotted) and cells (solid), respectively. C) A non-linear increase in fluorescence intensity is observed with greater concentration of peptide on binding to HT29 cells until saturation is reached. Data regression yields a binding affinity $K_d=6.4$ nM/L, $R^2=0.973$, for AKP-multimer-FITC and $K_d=310$ nM/L, $R^2=0.876$, for AKP-monomer-FITC, representing a ~48-fold improvement with multimer. D) An increase in fluorescence intensity as a factor of incubation time with HT29 cells is observed. For the AKP-multimer-FITC and AKP-monomer-FITC, a time constant $k=0.1136$ min $^{-1}$ (8.8 min), $R^2=0.972$, and $k=0.0663$ min $^{-1}$ (15.1 min), $R^2=0.984$, respectively, was measured.