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# Gastroresistant oral peptide for fluorescence imaging of colonic inflammation

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ABSTRACT

The use of molecular markers for inflammation in the gastrointestinal tract could empower optical imaging modalities for early diagnosis and eventually personalized timely treatments. A major hurdle to the widespread use of functional fluorescence imaging is the absence of suitable contrast agents, in particular to be administered *via* the oral route due to the usual proteolytic susceptibility of the biomarkers. By designing a retro-inverso peptide, starting from a previously described sequence specific for N-cadherin, we achieved resistance to gastrointestinal degradation and even slightly improved specificity towards the target, both in *ex vivo* and *in vivo* experimental colitis. Simulations at fundamental molecular level suggested that the introduced retro-inverso modifications did not affect the folding of the peptide, leaving its ability to interact with the binding pocket of the monomeric N-cadherin unaltered, even when fluorescently labeled. Possible further derivatization of this sequence could be envisaged to further extend the potential of the designed retro-inverso peptide as diagnostic or theranostic agent for the oral route.

## 1. Introduction

The first, crucial tile in the multifaceted process of chasing and defeating a disease is an accurate and specific diagnosis. The majority of the diagnostic modalities enables to gather mainly anatomical and macrofunctional information. However, with the aid of biomarkers binding specifically to therapeutic targets and thanks to a restless technological improvement, a deeper understanding of molecular functions increases the possibility of timely therapeutic treatments and personalized therapies [1].

The most common route of administration of contrast media is either intravenous or topical [2,3]. Though, for some pathologies such as inflammatory diseases in the gastrointestinal (GI) tract enteral administration would be more convenient. Albeit topical application to the GI mucosa reduces the risks connected to the systemic administration of a diagnostic agent [4], it seldom enables an exhaustive screening of the disease area. An *a priori* knowledge of the region to be imaged becomes in this case a requirement [5]. Compared to topical application, the oral route represents a simpler approach to stain the full intestinal tube in a minimally invasive way. A GI contrast agent should be not (i) absorbable to minimize the risk of systemic side effects; (ii) resist digestion; (iii) bind with high affinity and specificity to the affected tissue; (iv) be retained for some time at the binding site; and (v) be easily manufactured.

The main reason underlying the scarce success of oral targeted contrast media relies, in part, on their poor ability of biomarkers in

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resisting the harsh environment of the GI tract [6]. Small ligands are generally more resistant to acid and enzymatic degradation than biologics (*e.g.* antibodies) but often lack specificity and affinity; better target-to-background ratios are generally achieved with biologics, but their permeability through the mucus and stability in the GI tract is limited [7]. Peptides have attracted increasing attention as possible molecular imaging probe owing to their intrinsic advantages over larger biomacromolecules [8,9]. Basic and sophisticated synthetic strategies allow to tune the chemical properties of the amino acid sequences while preserving the binding ability of the peptide to the target [10,11]. An elegant modification strategy to tackle the peptide susceptibility to proteolysis has been introduced in the late 70's by Goodman and Chorev [12]. While retaining the spatial arrangement of the side chains, the amino acid sequence can be made resistant to enzymatic degradation by inverting the orientation of the peptide bond. So, while by simply reverting the sequence (*i.e.* generating the retro *all*-L sequence) the structural equivalence is not necessarily retained, by also inverting the chirality (retro *all*-D or retro-inverso, RI, sequence) the side-chain topology is maintained [13,14].

In the present study, we aimed to develop an oral contrast agent for fluorescence-based detection of inflammation of the GI tract. Starting from three sequences previously identified for luminal targets [15–17], we investigated their susceptibility to pH and to proteolytic degradation in simulated GI fluids following and modified their structure to improve stability. The *ex vivo* binding affinity of the original and modified peptide to colon tissues harvested from mice with colitis was assessed. The most stable sequence showing the highest *ex vivo* target-to-control fluorescence intensity ratio was found to be the one selected against N-cadherin and modified to be a retro-inverso sequence. The peptide was then orally administered to validate the *in vitro* gastroresistance and the specificity towards inflamed tissue resulted from the *ex vivo* investigations. Computational simulations shed light on the impact of the chemical modifications on the peptide folding and on the energy profiles of the interaction between ligand and target.

In view of the function that N-cadherin has been revealed to hold in chronic conditions in the GI tract, such as Crohn's disease [18], the identification of a gastroresistant disease-specific biomarker could empower the diagnostic potential of emerging high-definition fluorescence imaging modalities, such as confocal endomicroscopy and image-guided surgery, of the inflamed, fibrotic or dysplastic GI lumen [4].

## 2. Materials and methods

### 2.1. Materials

Peptides were purchased from Peptide 2.0 Inc. (Chantilly, VA). Capital letters stand for L-amino acids, while lower-case letters stand for D-amino acids. Peptides of at least 95% purity were conjugated to FITC at their C-terminus for their further detection in the animal experiments (Table 1). The quality of the peptides was controlled by HPLC analysis, using an autosampler and pump system (Ultimate 3000, Dionex, Thermo Fisher Scientific, Reinach, Switzerland) equipped with a reversed-phase column (XBridge™ $C_{18}$ reverse-phase column (5 μm, 4.6 × 250 mm), Waters, Milford, MA) held in a column oven at 30 °C and a diode array detector (Thermo Fisher Scientific). The gradient was from 80% water to 50% acetonitrile (both with 0.1% trifluoroacetic acid) in 10 min. The peptides were detected at 220 and 440 nm. Chemicals of the highest purity were obtained from Sigma Aldrich, unless otherwise specified.

### 2.2. Stability in the GI tract

Peptides (50 μL, 1 mM) were incubated at 37 °C in 100 μL of both simulated gastric fluid USP pH 1.2 with pepsin (3.2 mg/mL) and simulated intestinal fluid USP pH 6.8 with pancreatin (10 mg/mL). After specific time points (0, 5, 15, 30, 60, 90 and 120 min) all enzymes were heat inactivated (95 °C, 10 min), the samples centrifuged (14,000 × *g*, 10 min) and the supernatants were filtered with 0.2-μm regenerated cellulose filters (BGB Analitik AG, Boeckten, Switzerland). 100 μL of the sample were mixed with 20 μL of the internal standard (GGGAGGGAGGGA peptide, GAGA, 300 μM) and after vortex mixing, peptide samples were analyzed by high performance liquid chromatography-mass spectrometry (HPLC-MS). The HPLC-MS was composed of a Rheos Allegro quaternary pump (Thermo Fisher Scientific), C18-column (XBridge, 4.6 × 250 mm, 5 μm, Waters Corp.) and XCalibur control software (Thermo Fisher Scientific). Samples were injected at an injection volume of 10 μL, a flow rate of 1 mL/min and column temperature of 35 °C. The gradient was from 80% water to 50% acetonitrile (both with 0.1% trifluoroacetic acid) in 10 min. Ionization was conducted *via* an electrospray ion source in positive mode (LTQ XL linear quadrupole ion trap, Thermo Fisher Scientific). Data were acquired by full MS and UV channel at 280 nm. Stability was assessed by the quantification of unaltered peptide. Abundance of the unaltered peptides was compared to samples at time 0. The generated peptide fragments were identified using the software tool PeptideCutter from the ExPASy Proteomics database (Swiss Institute of Bioinformatics).

### 2.3. Induction of dextran sodium sulfate (DSS) colitis

All animal experiments were approved by the Cantonal Veterinary Office Zurich (ZH215/2014), in compliance with the EU Directive 2010/63/EU for animal experiments. Female C57BL/6 mice (5–18 weeks old, 20–25 g) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice received an alfalfa-free diet Experimental diet #2222, Provimi Kliba, Penthalaz, Switzerland, throughout the experiment. Mice were housed in a specific pathogen-free facility in individually ventilated cages. Food and water were available *ad libitum*. Mice were randomly divided into 2 DSS and 2 water control groups. Acute colitis was induced by administering 3% DSS (36–50 kDa, MP Biomedicals, Illkirch, France) dissolved in drinking water for 7 days. Body weight and disease activity were determined daily. At the end of the experiment, colonoscopy was performed in a blinded manner and mice were subsequently sacrificed. Colons were removed, and colon length was measured. Colon samples were collected for hematoxylin

**Table 1**
List of peptides initially used in this study. Amino acids and fluorophore added to the original sequences are marked in bold font.

| Peptide sequence | Abbreviation | Comments | Validation[a] | Reference |
|---|---|---|---|---|
| GGGAGGGAGGGAK(FITC) | GAGA | Linear 12-mer with no functional groups on the amino acid side-chains chosen as a control for unspecific binding | *In vivo*, qualitative assessment | [19] |
| LTHPQDSPPASAK(FITC) | LTH | Linear 12-mer identified by *in vivo* phage display following intraluminal injection of phages in a murine model of gut injury | *Ex vivo*, qualitative assessment | [15] |
| SWTLYTPSGQSK(FITC) | SWT | Linear 12-mer identified by *in vitro* phage display against a human N-cadherin/Fc chimeric protein | *In vitro* ($K_d$ from surface plasma resonance) and on cells | [16] |
| ACSQSHPRHCGGGSK(FITC) | SQS | Cyclic 7-mer identified by *ex vivo* phage display on isolated bowel in a murine model of intestinal ischemia | *Ex vivo* for phage binding. No validation for the peptide | [17] |

[a] Conditions of validation of the peptide binding after phage display.

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