De novo evolution of target-specific peptides that survive oral administration

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The oral administration of peptide drugs is hampered by their metabolic instability and limited intestinal uptake, posing one of the greatest current challenges in the pharmaceutical industry. Herein, we *de novo* generated small-sized (< 1600 Da), target-specific peptide ligands that resist gastrointestinal (GI) proteases using a method that could be applied to any target. We achieved this by combining large phage libraries of a new format of conformationally constrained peptides with a built-in proteolytic-resistance selection step in the evolution process. A peptide with a nanomolar affinity for its target was orally applied to mice and efficiently resisted proteases in all regions of the GI tract; more than 30% of the peptide remained intact, and small quantities reached the blood stream. This is the first strategy for generating target-tailored and GI-stable peptides from scratch, presenting a promising path to developing orally applicable peptides that act on targets locally in the GI tract and a good starting point for future work towards the generation of systemically delivered peptides.

Orally available peptides are a major goal in the pharmaceutical industry that has not been reached for most targets; almost none of the more than 60 approved peptides are orally available[1,2]. One of the exceptions is the GLP-1 receptor antagonist semaglutide that was recently approved for oral application[3]. Oral delivery is preferred for drug administration because of its convenient self-administration, wide range of available dosage adjustments, and quick termination if adverse effects occur. The limited oral availability of peptides is mainly due to degradation by proteases in the gastrointestinal (GI) tract, poor crossing of the epithelial layer due to the large size and polar surface, and first-pass metabolism in the liver[4]. Fortunately, orally available peptide drugs derived from exceptionally stable natural peptides do exist and show that this is in principle possible[4,5,6]. For example, cyclosporine is a cyclic undecapeptide that is passively permeable due to N-methylated amide bonds and intra-molecular H-bonds[7] (oral bioavailability of around 30%), and linaclotide is a tetradecapeptide constrained by three disulfide bridges that acts locally in the intestine and thus needs

to only resist proteases and not to be absorbed.

Through powerful *in vitro* display techniques, such as phage display and mRNA display, peptide ligands for virtually any disease can be generated, but so far, no peptide isolated with such techniques was sufficiently stable to survive oral administration. Peptides can be engineered for stability by altering the amino acid sequence to eliminate vulnerable sites, cyclizing[8] or stapling[9] the backbone to impose conformational constrains that hinder protease cleavage, grafting binding sequences onto highly stable protein scaffolds such as cysteine-knots[10], or by formulating the peptides[11]. While these approaches can yield substantial improvements, the engineered peptides typically are not sufficiently stable for GI conditions. Instead of engineering peptide stability after the fact, a possible approach for discovering stable peptides is based on proteolytic phage display in which genetically encoded libraries are exposed to proteases, and only intact clones are identified by affinity selection[12–14]. In all studies that applied this principle, the proteolytic pressure used was much lower than that in the GI tract, and the stabilities of the isolated peptides were far too low to survive oral administration. For example, we previously applied proteolytic phage display to isolate peptides constrained by a chemical linker that had a substantially enhanced stability[15] though were still not able to survive GI conditions, with a half-life of only 30 min in 500-fold diluted mouse intestinal fluid. There are two likely reasons for this: i.) the chemically modified peptides were only semi-constrained, with inadequate numbers of protease-resistant library members to generate a sufficient sequence space from which to sample target-binding ligands, and ii.) the phage particles themselves were rapidly degraded in relevant concentrations of intestinal fluid[15]; this instability was due to a mutant p3 coat protein lacking six cysteines — mutated to accommodate the chemical modification technology[16].

In a recent study developing double-bridged peptides, conformationally constrained by two chemical linkers instead of only one, we discovered a peptide with a particularly

high stability in human plasma[17]. In this peptide (sequence: R$\underline{C_2}\underline{C_3}QG\underline{C_6}RVL\underline{C_{10}}$Y), Cys2 and 6 and Cys3 and 10 are bridged by chemical linkers, forming a tightly knotted structure that can explain the unusually high protease resistance. We speculated that screening full libraries of these doubled-bridged peptides would confer the additional constraints necessary to resist protease degradation during selection while still retaining sufficient sampling space to identify target binders — placing a library of peptides that could be administered orally within reach. Towards this end, we first developed a procedure to encode the chemical modifications required for double-bridged peptides without the need for the six-cysteine mutant coat protein to ensure proteolytically stable phage. Using these phage as the library backbone, we isolated target-specific peptides with nanomolar affinities that resisted proteases in undiluted simulated intestinal fluid (SIF). We also showed that they remain active in the GI tract of mice upon oral administration and were able to somewhat penetrate the epithelial layer to mark the beginnings of orally available peptides identified from *in vitro* display. Analysis of the structure and study of the chemical bridges revealed that both the particular peptide format and the selected amino acid sequences are essential for the high stability, indicating the need for these constrained, protease-resistant libraries in target-binding ligand identification.

Genetically encoding double-bridged peptides by protease-resistant phage

The *in vitro* evolution of proteolytically stable peptides (Fig. 1a) requires phage particles that resist GI-relevant protease concentrations. The highest proteolytic pressure for peptides is faced in the upper region of the small intestine, which contains high concentrations of trypsin, chymotrypsin, and smaller quantities of other proteases, including exopeptidases[18]; this pressure is mimicked using simulated intestinal fluid (SIF) made up of 10 mg ml^{-1} of porcine pancreatin in phosphate buffer. As indicated above, the disulfide-free phage previously applied for the selection of chemically constrained peptides function in 500-fold diluted SIF (20 μg ml^{-1} pancreatin)[15] but are rapidly degraded in undiluted SIF. Wild-type fd phage resist undiluted SIF for at least

30 minutes at 37°C but contain six cysteines in the p3 phage coat protein (three disulfide bridges) that can interfere with the chemical cyclization reaction applied to displayed peptides[19]. Toward the use of wild-type phage, we searched for thiol-reduction conditions that could reduce the cysteines in the displayed peptides to correctly cyclize them while leaving the six disulfide-forming cysteines in the wild-type p3 coat protein untouched. To follow the cyclization efficiency, we cloned a peptide onto the wild-type p3 that would function only when the correct reduction took place. To do this, we used a previously developed constrained peptide PK15 (^{N}AC<u></u>SDRFRNC<u></u>PADEALC<u></u>G^{C}) that binds to plasma kallikrein only when cyclized via the three cysteines with 1,3,5-tris(bromomethyl)benzene (TBMB) (K_i = 1.5 nM) but not in its linear or disulfide-cyclized form (K_i > 100 μM, Fig. 1b and Supplementary Fig. 1)[19]. Wild-type phage displaying PK15 were first incubated with different TCEP concentrations (0 to 10 mM) to reduce the three peptide-cysteines and then cyclized with TBMB using known conditions[19]. The reduction reaction was performed at 25°C and thus at a lower temperature than previously (42°C)[19] to disfavor p3 unfolding and exposing the coat protein's disulfide bridges. The number of phage that remained functional were measured by quantifying the transducing units (t.u.), and the number of phage displaying a correctly cyclized PK15 peptide were assessed by measuring the number that could be captured on immobilized plasma kallikrein (Fig. 1b). The TCEP reduction and TBMB cyclization reduced the number of infective phage by around 10-fold for most conditions. The percentage of phage captured relative to the input increased with higher reducing agent concentrations and plateaued at 0.33 mM of TCEP. The percentage of phage captured maximally was around 0.4% of the input and thus lower than that achieved with the disulfide-free phage (around 6%). Fortunately, the absolute number of wild-type phage was nevertheless higher because nearly all wild-type phage particles can infect bacterial cells, while less than 1% of disulfide-free phage particles are infective[16]. In a final test, we verified that wild-type phage treated with the optimized TCEP concentration (1 mM) and cyclization linker (20 μM) fully resisted the physiological pancreatin concentration (100% SIF; Supplementary Fig. 2a).

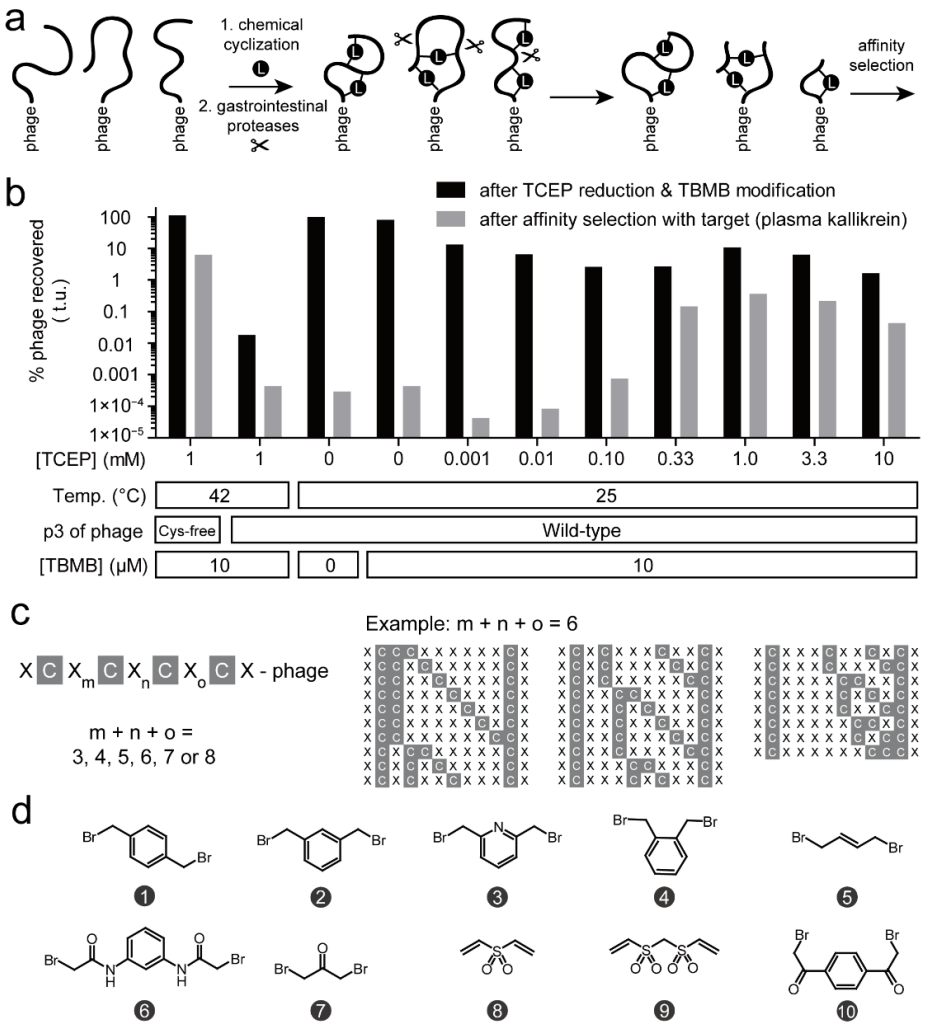

Fig. 1 Genetic encoding of double-bridged peptides by protease-resistant phage. a, Phage display selection strategy. “L” indicates chemical linkers that cross-link pairs of cysteines. Scissors represent GI proteases. **b**, Test of different reaction conditions for cyclizing the peptide PK15 displayed on protease-resistant wild-type phage. The percent of intact phage was estimated based on the residual transducing units (t.u.). Correct peptide cyclization was assessed by quantifying the number of phage captured on immobilized plasma kallikrein to which PK15 cyclized by the alkylating agent TBMB binds. **c**, Format of phage peptide library. **d**, Bis-electrophilic cyclization reagents for the generation of double-bridged peptides.

We expected to need a large diversity to find target-binding peptides that resist GI conditions, so we generated the following particularly large phage display library. We displayed random peptides of the form $XCX_mCX_nCX_oCX$ (m + n + o = 3, 4, 5, 6, 7, or 8; X = random amino acids) on phage, which gave a total of 155 different cysteine-

spacing formats (Fig. 1c) and 2×10^{10} different peptide sequences. This library was cyclized with the bi-functional reagents **1** to **10** (Fig. 1d) using suitable concentrations (Supplementary Fig. 2b), and the four cysteines could pair in three different ways per peptide sequence to generate as many as 6×10^{11} double-bridged peptides.

Enrichment of target-specific, protease-resistant peptides

The sub-libraries cyclized with the 10 linkers were separately incubated with 0.1 mg ml^{-1} of porcine pancreatin (1% SIF) for 30 min at 37ºC, which was 100-fold lower than the protease pressure in the intestine and thus rather mild for the initial rounds of panning. The protease-digested library was subsequently panned against 0.5 μg of immobilized coagulation factor XIa (FXIa) as a target (Fig. 2a). FXIa is an attractive therapeutic target because inhibition of the protease in animals prevents thrombosis without causing bleeding, which is a limitation of current anticoagulants[20]. After two and three rounds of phage selection, the DNA of 0.5 to 2 million phage from each selection was sequenced, and the peptide sequences were compared using an alignment tool[21]. Consensus groups, indicative of the isolation of target-specific peptides, were identified in selections with nine of the ten linkers (Supplementary Fig. 3). The selection with linker **7** yielded particularly strong consensus groups defined by a large number of similar but different peptides per group (Fig. 2b). The most abundant peptides of two groups, F1 and F2, were synthesized, and both inhibited FXIa, showing that the affinity selections yielded target-specific ligands (K_is of 270 ± 30 and 900 ± 100 nM; Supplementary Fig. 4).

After the first three rounds of panning, the peptide diversity remained high, as seen by hundreds of different peptides that had remained in the pool and shared similar but different sequences (Fig. 2b and 2c). In order to enrich those peptides with the highest binding affinity, we performed a fourth round, reducing the amount of immobilized target protein (100, 20, or 4 ng) and holding the pancreatin pressure at 0.1 mg ml^{-1}

(Fig. 2a). The number of phage captured with the smallest amount, 4 ng, was higher than in the control selection without target, indicating that the selection stringency could be further increased, and we therefore reduced the amount of immobilized target to 1 ng in the 5th and 6th selection rounds. In these latter two rounds, we performed selections using either 0, 0.1 or 1 mg ml^{-1} pancreatin in parallel, the highest of these concentrations being 10-fold higher than in rounds 1-4 and representing 10% SIF (Fig. 2a). DNA sequencing of clones isolated in rounds 5 and 6 showed lower diversity and a marked change in the consensus groups (Supplementary Fig. 5), as illustrated in Fig. 2b for peptides isolated in round 6 with linker **7** under high proteolytic pressure. An analysis of the most abundant peptides showed that they were enriched to different extents at the three protease pressure conditions, as shown clearly for peptide F3 that was more enriched at 1 mg ml^{-1} pancreatin concentration (10% SIF) than at a 10-fold lower concentration or no pancreatin (Fig. 2c; F3 is shown in red).

We synthesized several double-bridged peptides isolated in round 6 under the highest protease pressure (peptides F3-F12) along with two peptides selected in round 6 without pancreatin (F13, F14) and two peptides from round 2 (F1, F2), and we compared their binding affinities and stabilities (Fig 2d). The linear peptides were cyclized with an excess of **7**, the three isomers were separated by HPLC, and the binding affinity was measured in FXIa inhibition assays (Supplementary Fig. 6 and 7). For all peptides, one of the isomers was far more active than the other two, as expected for phage-selected double-bridged peptides[17]. The stability of the most active isomer of each peptide was assessed by incubation in 10 mg ml^{-1} pancreatin (100% SIF) for different time periods at 37°C and quantification of the intact peptide by LC-MS. A clear correlation between the selection pressure applied in the phage selections and the affinity as well as stability was found, as shown in Fig. 2d. Peptides isolated under high proteolytic pressure (blue) were more stable than those isolated in round 6 without pancreatin (light blue) or in round 2 (grey). In addition, peptides from round 6 had higher binding affinities than the two that were most abundant in round 2, showing that

the increased pressure for binding affinity in rounds 4 to 6 enriched for high-affinity binders.

The efficient proteolytic selection became even more evident when the abundance of peptides displaying different levels of stability were retrospectively analyzed for the different selection conditions of round 6 (Supplementary Fig. 8). The most stable peptide F3 became more abundant in the selections with higher pancreatin concentration. Conversely, peptide F4 with a medium stability was equally abundant under all three conditions, and the labile peptide F13 was diminished at higher pancreatin concentrations (Supplementary Fig. 8).

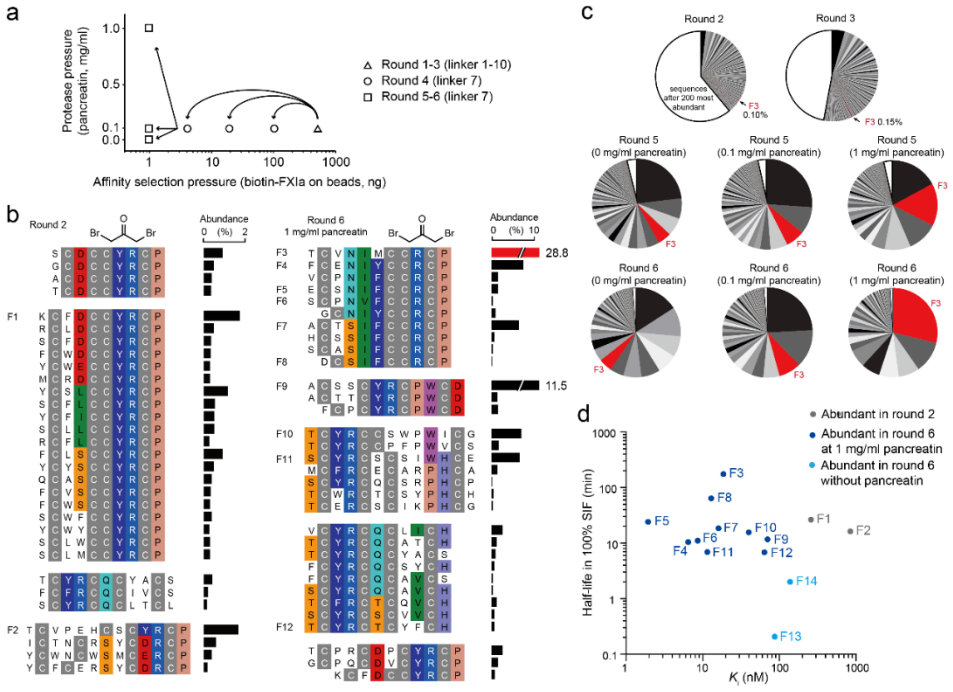

Fig. 2 Panning of double-bridged peptide libraries under protease pressure. a, Conditions applied to control the selection stringency for binding affinity (amount of target protein FXIa) and proteolytic stability (concentration of porcine pancreatin). In rounds 4-6, three selections with different target concentrations (round 4) or pancreatin concentrations (rounds 5 and 6) were performed in parallel. **b**, Sequences of peptides isolated in round 2 and round 6 (1 mg ml^{-1} pancreatin condition) using cyclization reagent **7**. Sequences isolated with other chemical linkers, in other rounds, or under other protease pressure are shown in Supplementary Figs. 3 and 5. Sequence similarities are highlighted in color, and the abundance of each peptide is indicated. **c**, Relative abundance of the 200 most frequently found sequences from rounds 2, 3, 5, and 6 in pie charts. The abundance of the protease-resistant clone F3 is shown in red. **d**, Activity and stability of peptides identified in different rounds of selection and under different protease pressure. Data is shown for the most active isomers of each peptide sequence. Mean values of the K_is are based on three measurements. The purity, K_is, and $t_{1/2}$ values of the peptides are shown in Supplementary Fig. 7.

Characterization, size reduction, and affinity enhancement of lead peptide

To determine the cysteine connectivity of the best peptides, we individually synthesized the three possible bridged isomers using orthogonal cysteine protecting groups Trt and Dpm and measured their affinity and stability. This is exemplified for F3 in Fig. 3a-c, where it was determined that isomer 2 bridged at Cys2/Cys8 and Cys7/Cys10 inhibited FXIa with a K_i of 19.2 ± 0.1 nM, while the other two isomers showed 30 and 90-fold weaker inhibition, respectively (Fig. 3b). The three isomers had very different proteolytic stabilities (Fig. 3c), with the active isomer 2 demonstrating resistance to pancreatin concentrations of 10 mg ml^{-1} (100% SIF; $t_{1/2}$ = 170 ± 30 min), which was magnitudes better than the other two isomers with 40 and 9-fold shorter half-lives in 100-fold diluted pancreatin (0.1 mg ml^{-1} of pancreatin, 1% SIF). An LC-MS analysis of the two unstable isomers revealed cleavage at peptide bonds Cys8-Arg9 and Arg9-Cys10 (isomer 1) and Arg9-Cys10 and Met6-Cys7 (isomer 3; Supplementary Fig. 9). A comparison with two clinically used oral peptide drugs, desmopressin ($t_{1/2}$ = 29.9 ± 0.3 min) and linaclotide ($t_{1/2}$ = 16 ± 4 min), showed a 6- and 10-fold higher stability for F3 isomer 2 in 100% SIF (Fig. 3d).

To facilitate epithelial-layer crossing and increase the chances for oral availability, we reduced the size of the most stable peptide, F3 isomer 2. Based on the consensus group, the N-terminal ring was less important and truncation without much activity loss was more feasible from this end. Removing the N-terminal exocyclic amino acid (Thr1; F15) reduced the binding affinity 10-fold, but this loss was reverted when the N-terminus was acetylated (F16), suggesting that the positive charge could have caused the affinity loss (Fig. 3e). Complete elimination of the N-terminal amino group on Cys2 by replacing the cysteine with mercapto-propionic acid (Cys*), eliminating the charge, yielded peptide F17 with an activity comparable to F3 (K_i [F17] = 21 ± 3 nM vs. K_i [F3] = 19.2 ± 0.1 nM). Introducing a Pro11→D-Pro mutation, identified in a parallel screen (F18; Supplementary Fig. 10), yielded F19 (K_i of 0.9 ± 0.2 nM), and further elimination of Val3 led to the truncated F20 (K_i of 3.7 ± 0.4 nM). The resulting peptide F20

contained 9-amino acids, counting the N-terminal amine-deficient cysteine (Cys*) and the three other cysteines, and had a molecular mass as low as 1134.4 Da.

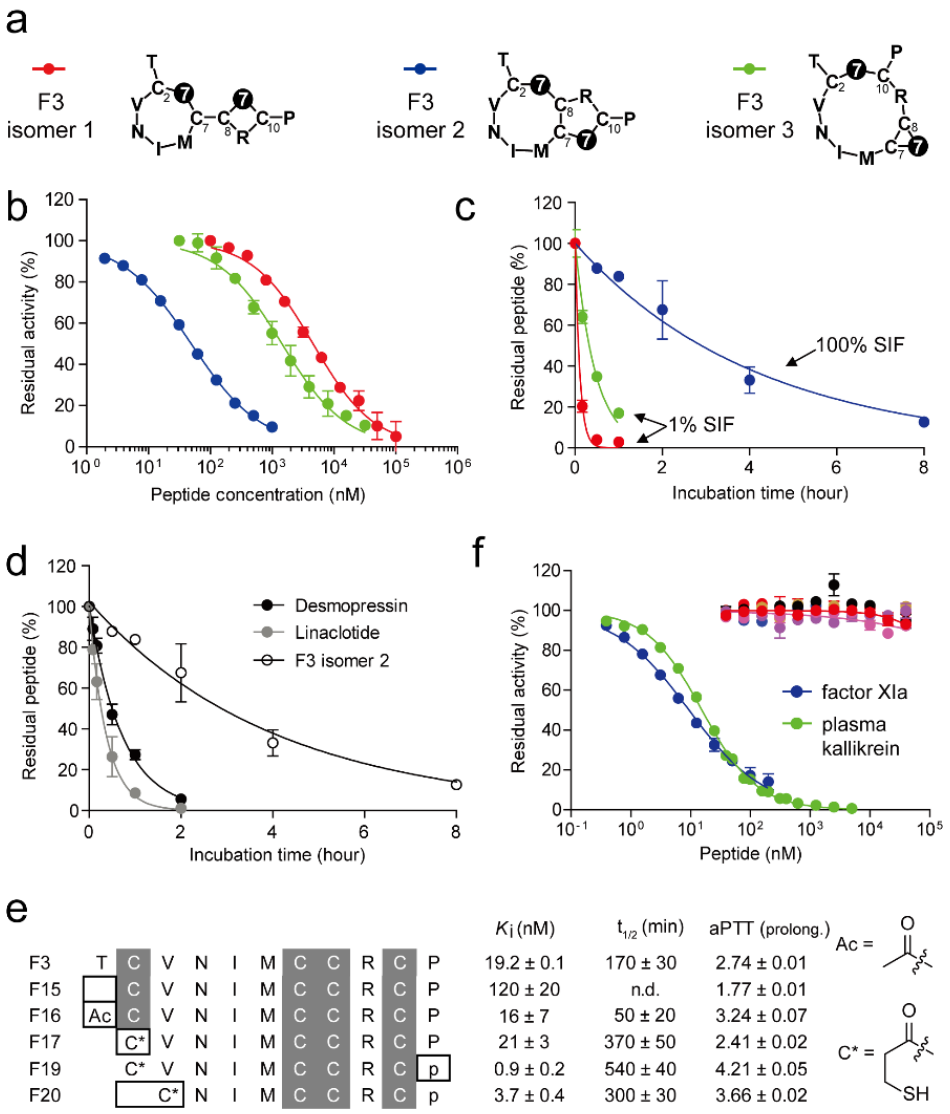

Fig. 3 Characterization and size reduction of the most stable peptide F3. a, Chemical structures of the three isomers of F3. **b**, Inhibition of FXIa by the three isomers. Means and standard deviations (SD) of two measurements are shown. **c**, Stability of F3 isomers in SIF (100% SIF used for isomer 2 and 1% SIF used for isomers 1 and 3). Means and SD of two measurements are shown. **d**, Stability of oral peptide drugs desmopressin and linaclotide in 100% SIF. The stability of F3 isomer 2 is shown for comparison (data from panel **c**). Means and SD of two measurements are shown. **e**, Activity and stability of size-reduced derivatives of F3. The amino acids modified from peptide to peptide are highlighted with a black frame. Average values and SD for FXIa inhibition (K_i), half-life in 100% SIF, and inhibition of the intrinsic coagulation pathway aPTT (fold prolongation at 30 μM peptide) are based on at least two measurements. **f**, Specificity of F20 assessed in inhibition assays with nine trypsin-like serine proteases. The curves showing no inhibition correspond to thrombin, plasmin, tPA, trypsin, FXa, uPA, and FXIIa. Means and SD of two measurements are shown.

The various size-reduced peptides displayed half-lives in 100% SIF of between 50 min and several hours and efficiently blocked activation of the intrinsic coagulation pathway, tested in human plasma *ex vivo* by assessing the prolongation of the activated partial thromboplastin time (aPTT) (Fig. 3e). A specificity profiling of proteases sharing a high sequence identity (56–84% in active site; Supplementary Fig. 11) revealed a high target selectivity for all peptides (Fig. 3f and Supplementary Fig. 12). Only plasma kallikrein, which shares the highest sequence and structure similarity with FXIa, was inhibited, though ironically this may be an advantage in antithrombotic applications as the protease is also a coagulation factor in the intrinsic pathway[20].

Helical structure and molecular basis of proteolytic stability

The structure of one of the size-reduced peptides, F19, bound to the FXIa catalytic domain was solved by X-ray crystallography and revealed a helical conformation (Fig. 4a; 2.9 Å resolution, PDB entry 6QEZ; Supplementary Fig. 13 and Supplementary Tables 1 and 2). The amino acids Asn3 to Cys9 form three i → i + 3 H-bonds and had φ and ψ dihedral angles characteristic for a 3_{10}-helix (Supplementary Fig. 13c). The helix points with its C-terminal ring towards the active site of FXIa, sticking the Arg8 side chain into the S1 sub-site, and it covers a total surface area of 649 $Å^2$. Crystallization of a second peptide that was not reduced in size, F21 (Supplementary Fig. 14) showed the same helical structure (2.9 Å resolution, PDB entry 6QF0; details of the structure are described in the supplementary results).

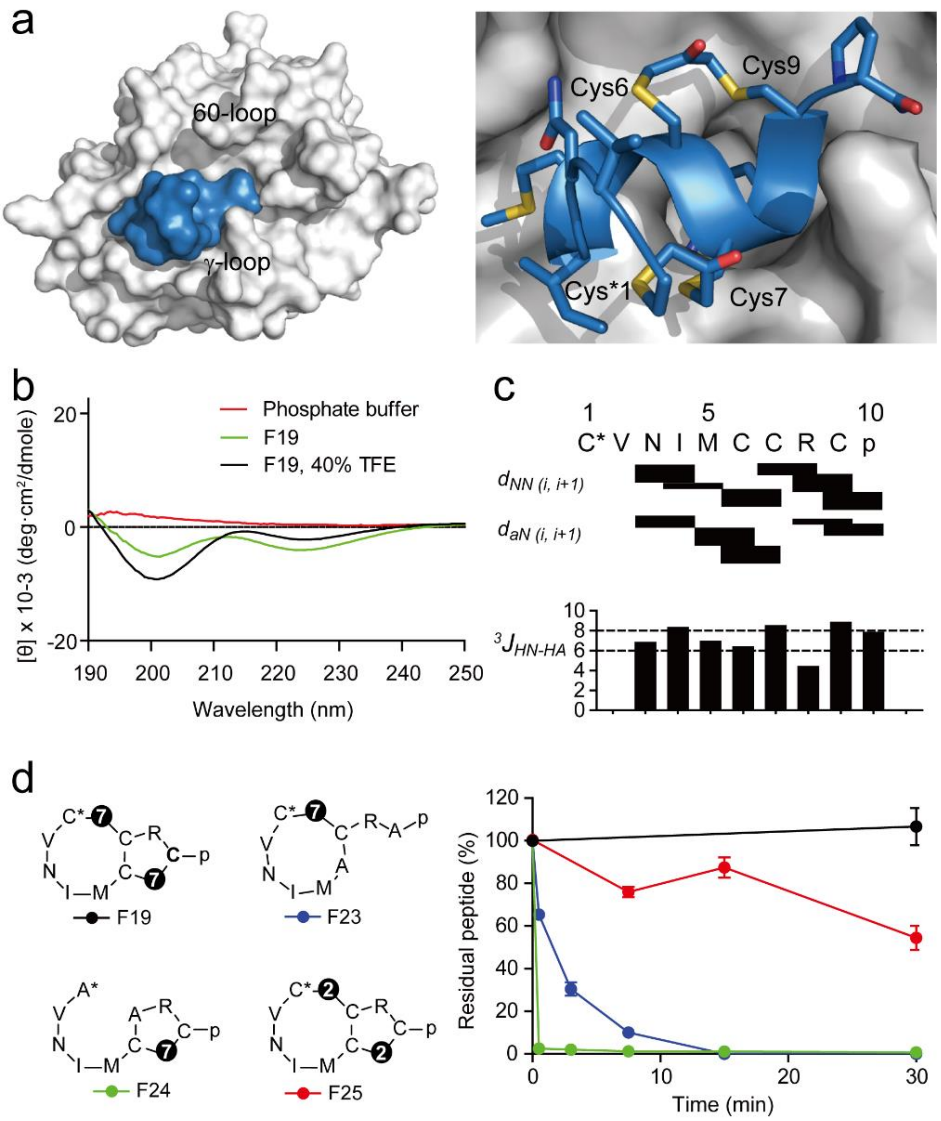

Fig. 4 Structure analysis and impact of chemical bridges on stability. a, Crystal structure of double-bridged peptide F19 bound to the active site of FXIa (PDB ID 6QEZ). Left: Surface representation of the peptide (blue) and target (grey). Right: Cartoon representation of the peptide. The four bridged cysteines are indicated. **b**, Circular dichroism (CD) spectra of F19 at a concentration of 200 µM recorded at 25°C in phosphate buffer or buffer containing 40% v/v of the helix-promoting solvent trifluoroethanol. **c**, The upper panel shows the strengths of H-H NOEs in F19. Medium- or long-range signals were not observed. The lower panel shows ^{3}J HN-HA coupling in F19. For helical and beta-sheet structures, values are typically < 6 Hz and > 8 Hz, respectively. **d**, Stability of F19 with one or two chemical bridges formed using **7** or with the two chemical bridges formed by **2** in 100% SIF. The fraction of intact peptide is shown in %. Means and SD of two measurements are shown.

We speculated that helix formation could hinder the access of proteases and explain the high proteolytic stability, as has been found for other peptides[9], though analysis of unbound F19 by CD spectroscopy (Fig. 4b) and NMR (Fig. 4c Supplementary Fig. 15)

indicated that the peptide did not form a helical structure in solution. We subsequently studied to what extent the two chemical bridges contributed to the proteolytic stability by comparing the stability of linear, single-bridged, and double-bridged peptide F19 (C*VNIMCCRCp) in 100% SIF (Fig. 4d, Supplementary Fig. 16). As expected, the linear peptide (with thiol groups removed to prevent oxidative cyclization; F22) was rapidly cleaved ($t_{1/2}$ < 1 min). Peptides containing a single bridge were also much less stable than the double-bridged F19, showing a half-life of 1.80 ± 0.07 min for Cys*1-Cys7 bridge (first ring; F23) and < 1 min for the Cys6-Cys9 bridge peptide (second ring; F24). Interestingly, the double-bridged peptide cyclized with a different linker (**2** instead of **7**; F25) had an around 10-fold shorter half-life, suggesting that the linker length and dimension are important for imposing the proper conformation for resisting proteases. Taken together, the peptide does not have a defined fold in solution, though the presence of the two linkers impose conformational constraints that hinder rapid proteolytic degradation.

Peptides remain intact in gastrointestinal tract of mice

The most stable peptides showed half-lives of more than an hour in undiluted SIF (100% SIF), which suggested that many of them could survive in the GI tract. We next tested the stability of the smallest peptide, F20, in the GI tract of mice upon oral administration and whether it could be detected in blood — indicating an ability to traverse the epithelial layer. In *ex vivo* experiments, F20 had a half-life of 41 ± 3 hrs in simulated stomach fluid (SGF), 5.0 ± 0.5 hrs in 100% SIF, and 140 ± 40 hrs in mouse plasma, respectively (Fig. 5a-c). We administrated 100 mg/kg F20 to 20 mice by gavage into the stomach, sacrificed 5 mice after each time point (15, 30, 60, and 120 min), and analyzed samples taken from the stomach, upper small intestine (duodenum and jejunum), lower small intestine (ileum), colon, and blood (Fig. 5d) for the peptide by LC-MS. The relatively high dose was applied to reliably detect and quantify the peptide in case only a small fraction of it remained in the GI tract or was absorbed into the blood stream. As a control, we performed the same experiment with the orally available

peptide drug desmopressin (Fig. 5d). At all four time points analyzed, between 32 and 45% of the administrated F20 could be recovered from the GI tract as fully intact peptide. At 15 and 30 min, most of the peptide was found in the upper part of the small intestine, at 60 min in the lower part, and at 120 min in the large intestine. In contrast, the control peptide desmopressin was found intact in only small quantities and mainly in the stomach, not the intestines. An analysis of blood samples showed that small quantities of the peptide F20 reached the blood stream with a C_{max} of 1.4 µM at 30 min and a calculated oral availability (%F) of 0.26%, which is likely too low for therapeutic use against FXIa (Fig. 5e and 5f).

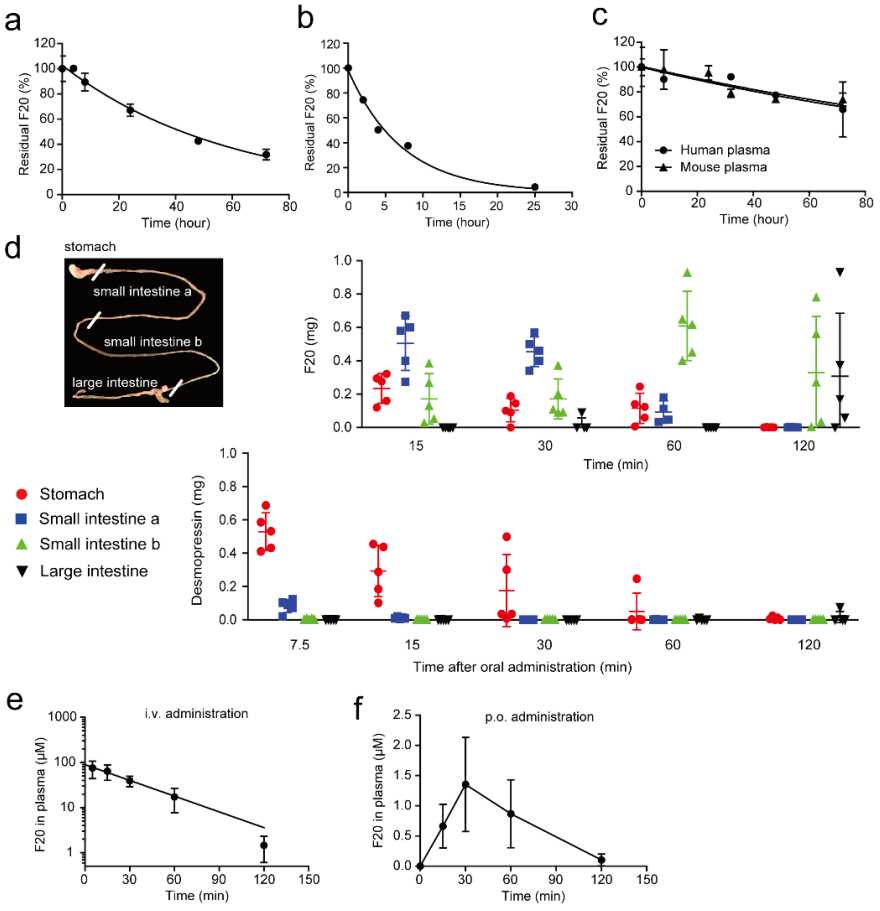

Fig. 5 Stability and oral administration to mice. Stability of F20 in (**a**) simulated gastric fluid (SGF), (**b**) 100% SIF, and (**c**) human and mouse plasma. Mean values and SD of two measurements are indicated. **d**, Quantity of intact F20 or desmopressin recovered from the GI tract organs of mice administered orally with 100 mg/kg F20 or desmopressin. n = 5 per time point. The amount of peptide indicated was normalized to a mouse weighing 20 g. The small intestine was sliced into two equal pieces labeled small intestine a (includes duodenum and jejunum) and small intestine b (ileum), as indicted. **e**, Concentration of F20 in mouse plasma following i.v. administration. Dose: 10 mg/kg, n = 5. **f**, Concentration of F20 in mouse plasma following p.o. administration. Dose: 100 mg/kg, n = 5.

The result with the FXIa inhibitor, showing long peptide survival in all regions of the GI tract and a relatively low uptake into the blood stream, suggested that our new method is particularly suited to address disease targets located in the GI tract, such as proteins in tissues of the intestinal mucosa or submucosa. One such target is the interleukin-23 receptor (IL-23R), which plays a key role in Crohn's disease and ulcerative colitis, two inflammatory conditions of the digestive tract[22]. The target has been validated by IL-23- and IL-23R-specific monoclonal antibodies, but no oral drugs are yet available. Protagonist Therapeutics and Janssen Biotech have recently disclosed a peptide-based IL-23R antagonist for oral administration, PTG-200 (compound C)[23,24]. The molecule was generated based on linear peptide precursors that were degraded within seconds, even in strongly diluted SIF. They followed a classical peptide engineering approach in which thousands of peptide variants containing unnatural amino acids were synthesized and activity- or stability-enhancing modifications were accumulated in iterative rounds of engineering to reach a nanomolar affinity and high proteolytic stability[23]. Oral administration of PTG-200 to rats showed pharmacological activity, indicating that peptides can cross the colon epithelium to reach and antagonize IL-23R. Following promising results of a Phase 1 clinical trial, the two companies announced in 2019 that they would expand the PTG-200 collaboration and co-develop second-generation antagonists[25].

To demonstrate the ability of our technology to develop peptide drug candidates with higher initial proteolytic stability (before introducing unnatural amino acids), we panned our peptide phage display library cyclized with linkers **1** to **8** against human IL-23R (Supplementary Fig. 17). After three rounds without proteolytic pressure, phage were best enriched into clear consensus groups in the library cyclized with linker **7** (Fig. 6a), and we thus panned those isolated phage in a 4th and 5th round containing proteolytic pressure. Phage were enriched at 1% SIF over background, but not at 10%, indicating that some binders survived the lower protease pressure but not the higher one.

Sequencing showed that the peptides selected under protease pressure in round 5 converged to two consensus groups (Fig. 6a). We synthesized two peptides of this round, I1 and I2, and two peptides from round 3, I3 and I4. We measured their binding to IL-23R by surface plasmon resonance (SPR) and their stability in 1% and 10% SIF. As found with the FXIa inhibitors above, peptides enriched under proteolytic pressure displayed a higher stability (Fig. 6b). The two peptides I1 and I2 had half-lives of 23 ± 1 and 7 ± 1 min in 1% SIF, and thus a much higher stability than peptides that served as a starting point for the engineering of the Protagonist/Janssen compound. Synthesis of the three regioisomers of I1 and characterization revealed that the peptide bridged at Cys2/Cys11 and Cys4/Cys7 was the active isomer (isomer 3, termed I1; Fig. 6c). A competition experiment by SPR revealed that I1 competes with IL-23 for binding to IL-23R, thus indicating that the peptide is binding to a region that is suited for therapeutic intervention (Fig. 6d).

Analysis of the degradation pathway of I1 showed that a peptide bond in the Trp-Trp-Leu loop connecting Cys7 and Cys11 was cleaved first followed by a cascade of cleavage events (Supplementary Fig. 18). Replacing Leu10 with α-methyl-Leu hindered this ring opening and rendered the double-bridged peptide highly stable ($t_{1/2}$ in 100% SIF = 4.6 ± 0.5 hrs) while conserving the binding affinity (K_d = 210 ± 10 nM; Supplementary Fig. 19). This result indicated that peptide I1 had one major vulnerable site, and protecting it in the context of the double-bridge backbone topology was sufficient to generate a peptide, I5, that resists 100% SIF with a half-life of several hours. This is in contrast to the monocyclic Protagonist/Janssen peptide that required synthesis of more than one thousand peptide variants[23], inserting a total of three unnatural amino acids, an N-terminal acetyl group, and a C-terminal amide group to reach a stability sufficiently high to attempt clinical trials. With its high binding affinity and good stability, I5 provides a new, attractive peptide starting point for developing an oral treatment of inflammatory disorders such as Crohns' disease based on IL-23R inhibition.

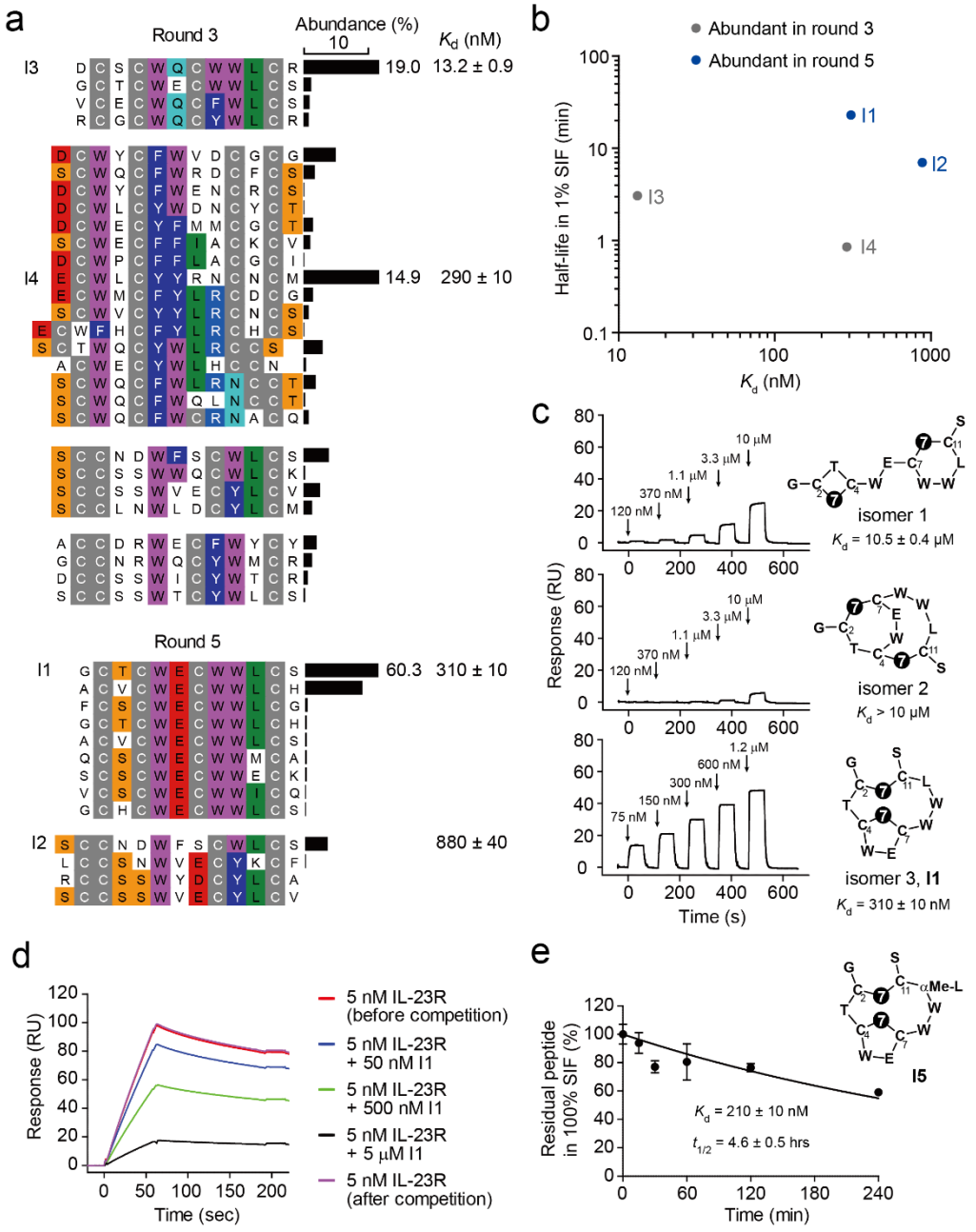

Fig. 6 Stable IL-23R antagonists. a, Peptides enriched after three phage selection rounds without protease pressure (Round 3) and after two additional rounds with 1% SIF exposure (Round 5). Mean values and SD of the K_ds are shown for the active isomers and are based on three measurements. **b**, Binding affinity and half-life in 1% SIF of the two most abundant peptides identified in rounds 3 (selection without protease pressure in round 1-3) and round 5 (protease pressure in rounds 4 and 5). **c**, Single cycle SPR sonograms of the three isomers of peptide I1. Mean values and SD of the K_ds are based on data from three measurements. **d**, Competition of immobilized IL-23 and peptide I1 for binding to IL-23R. **e**, Residual intact peptide I5 after incubation in 100% SIF, determined by LC-MS.

Despite decades of effort, the oral delivery of peptide-based drugs remains a major pharmaceutical challenge. At the moment, all biological drugs are generally delivered by intravenous or subcutaneous injection, which is effective but not desirable for patients, particularly for those with chronic conditions. Drugs based on peptides, being smaller than proteins and antibodies, have been the most amenable to oral delivery, but translating an existing bioactive peptide into an oral drug is extremely difficult. While the stability of peptides can be improved, typically by the step-wise introduction of stability-enhancing modifications, the process is cumbersome. For many peptides, the sufficiently high stability required for the GI tract still cannot be reached. Herein, we report a strategy and a first example of a *de novo* generated peptide that binds a therapeutic target with high affinity and that resists proteases in all regions of the GI tract of a mouse.

Conclusions

We generated GI-stable peptides by combining the conformationally constrained double-bridged peptides with a phage–display-based selection procedure in the hopes of fishing protease-resistant peptides out of large combinatorial libraries. The combination of the double-bridged peptide format and proteolytic phage display was technically tricky, as chemically modifiable peptide libraries had to be encoded on protease-resistant phage, which was managed using conditions that specifically reduced and alkylated the cysteines in displayed peptides without touching the three disulfide bridges in the wild-type phage p3 coat protein. This new procedure did in fact enrich for target-specific peptides with a high protease stability, indicating the new library format still provided sufficient sequence space for discovering target binders after protease digestion.

Analysis of the vast sequence data and the enrichment factors during the extensive biopanning rounds revealed that some peptide formats, characterized by specific

cysteine spacing patterns and consensus sequences, are better suited for generating protease-stable peptides. This observation is in line with the general finding that some cyclic peptides derived from nature or *in vitro* selections can more easily be improved in terms of stability while others represent evolutionary "dead ends" that seem intrinsically unstable. This suggests that peptides for oral administration may be best developed by simultaneously selecting for stability and target affinity rather than first selecting binders that are subsequently modified to improve stability.

The double-bridged peptide that best resisted pancreatin proteolysis *in vitro* also remained largely intact in all segments of the GI tract of mice, including the highly acidic stomach and the small intestine containing the enormous proteolytic pressure. While many studies with orally applied peptides or proteins use specific formulations to prevent exposure to the acidic stomach fluid or co-apply protease inhibitors to counter the activity of pancreatic proteases, we gavaged the "naked" peptide dissolved in PBS, allowing assessment of the stability independent of formulations. The oral peptide drug desmopressin, used as a positive control, was degraded rapidly in the intestine, underscoring the high stability achieved with the new approach. An analysis of blood samples showed that only a small fraction of the double-bridged peptide, below 1%, reached the blood stream, which was not surprising given the large polar surface of the peptide that even contains a positive charge. The fact remains, though, that some of the peptide did remain intact long enough to reach the blood stream and be detected, meaning that a better oral availability could be reached in the future for other peptides with a smaller polar surface, which could be achieved by removing the charges and/or N-methylating some of the peptide bonds.

For the first time, we have established a general approach for generating peptides from scratch to virtually any desired target that could simultaneously bind the target of interest and resist proteolytic degradation in the GI tract. While further work is required to achieve oral peptides that more efficiently reach the blood stream, the herein

presented approach can readily be used to develop oral peptide drugs that act locally on targets in the lumen of the GI tract, in the same fashion as the recently approved, nature-derived peptide drug linaclotide that acts on a target in the lumen of the GI tract.

Experimental Section

Quantifying peptide cyclization efficiency on phage

Phage particles that display the peptide sequence of the plasma kallikrein-specific bicyclic peptide PK15 (ACSDRFRNCPADEALCG)[19] as fusion of wild-type p3 coat protein were used to assess the reaction conditions that efficiently cyclize the peptide in presence of the p3 disulfide bridges. A phage vector named fd-tet-PK15 for producing these phage in *E. Coli* cells was cloned as described below. Disulfide-free phage displaying the peptide of PK15 were used as a positive control in this experiment. Phage were produced in 0.5 liter 2YT cultures containing 10 μg ml^{-1} tetracycline (for fd-tet-PK15) or 30 μg ml^{-1} chloramphenicol (for fdg3p0ss21-PK15) that were inoculated with glycerol stocks of TG1 *E. Coli* cells, carrying either of the two plasmids, and grown at 30°C overnight. The phage were purified by PEG precipitation and dissolved in reaction buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0). From a 0.5 liter culture, 1 × 10^{11} t.u. fdg3p0ss21-PK15 and 1 × 10^{13} t.u. fd-tet-PK15 were typically obtained. Phage were diluted with reaction buffer and incubated with various TCEP concentrations at 25 or 42°C for 30 min in 1 ml reactions to reach final concentrations of 2 × 10^9 t.u. disulfide-free phage or 2 × 10^{10} t.u. wild-type phage. Phage were then precipitated by addition of ¼ volume of PEG solution (20% [w/v] PEG 6000 and 2.5 M NaCl), the pellet dissolved in 0.9 ml reaction buffer and a small sample removed for phage titer determination. 1,3,5-Tris-(bromomethyl)benzene (TBMB) in 0.1 ml of acetonitrile was added to reach a final concentration of 10 μM TBMB and 10% acetonitrile, and the phage reaction was incubated at 30°C for 1 hr. The phage were purified by PEG precipitation and dissolved in 0.5 ml of washing buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) containing 1% (w/v) BSA and 0.1% (v/v) Tween-20. A sample was kept for titer determination and the remaining phage incubated with 0.3 μg biotinylated plasma kallikrein (3 pmoles) on 5 μl streptavidin beads (6.5-9.0 pmoles/ μl biotin binding sites) for 2.5 hr. The beads were washed three times with 0.5 ml of washing buffer and 3 times with 0.5 ml of washing buffer containing 0.1% (v/v) Tween-20. The phage were eluted by incubation for 5 min in 0.2 ml of glycine buffer (20 mM,

pH 2.2), the solution transferred to a new tube, 0.2 ml of Tris-Cl buffer (1 M, pH 8.0) was added to neutralize the pH, and the titer of all samples was determined by *E. coli* infection and plating of dilutions on 2YT agar plates containing chloramphenicol (for fdg3p0ss21-PK15; 30 μg ml^{-1}) or tetracycline (for fd-tet-PK15; 10 μg ml^{-1}).

Displaying peptide PK15 on wild-type phage

The phage vector fd-tet-PK15 was cloned for producing phage that display the peptide PK15 (ACSDRFRNCPADEALCG) as fusion of the wild-type p3. The vector was constructed by inserting DNA coding for PK15 into the vector fd-tet using a whole-plasmid PCR strategy. The following primers were used wherein PK15_forward anneals in the p3 gene (italic) and appends the PK15 DNA (bold), and fd_reverse anneals in the leader sequence. In a 50 μl PCR reaction, PK15_forward (200 nM, final conc.), fd_reverse (200 nM, final conc.), dNTP mix (200 μM each, final conc.), 20 ng of phage vector fd-tet as template, 10 μl of 5 × HF Phusion buffer and two units of Phusion polymerase (Thermo Fisher) were used. The reaction was mixed by pipetting and then immediately incubated in a thermocycler with the following program: initial denaturation for 2 min at 95°C, 25 cycles of 30 s at 95°C, 45 s at 40°C and 9 min at 72°C, and final elongation for 7 min at 72°C.

PK15_forward:

5'-CCTTTCTATGCGGCCCAGCCGGCCATGGCA**GCATGTAGCGATCGTTTTCGTA ATTGTCCGGCAGATGAAGCACTGTGTGGT**GGTTCTGGC*GCTGAAACTGTTGAA AGTTG*-3'

fd_reverse:

5'-AGGCCGGCTGGGCCGCATAGAAAGGAAC-3'

The PCR product was electrophoresed on an agarose gel, the DNA of the correct band extracted, digested with *Sfi*I and ligated with T4 ligase. The ligation mixture was incubated at 70°C for 10 min to inactivate the T4 ligase and transformed in *E. coli*

DH5α by electroporation. The sequence of constructed phage vector fd-tet-PK15 was validated by Sanger sequencing.

Assessing maximal tolerated concentrations of cyclization reagent

Potential detrimental effects of the thiol-reactive cyclization reagents on phage were tested by incubating 10^9 t.u. of phage with the chemical reagents and measuring the number of infective phage. All chemical reagents (linker **1** to **10**) used in this study were commercially available. Phage were reduced by incubation with 1 mM of TCEP at 25°C for 30 min in reaction buffer followed by precipitation of the phage with ¼ volume of 20% (w/v) PEG6000, 2.5 M NaCl to remove the excess of TCEP. Ten μl of thiol-reactive reagents at concentrations ranging from 0.1 to 6.4 mM in acetonitrile were added to 90 μl of 10^9 t.u. phage in reaction buffer, and incubated for one hr at 30°C. The number of infective phage was quantified by mixing 180 μl of exponentially growing *E. coli* TG1 cells with 20 μl of 10-fold dilutions of the phage and plating the cells on tetracycline 2YT agar plates (10 μg ml^{-1}).

Assessing maximal tolerated concentrations of pancreatin

The resistance of phage particles (with or without chemical modification) to protease degradation was tested by incubating phage with porcine pancreatin solution and measuring the number of infective phage. Disulfide-free phage or wild-type phage displaying peptide PK15 (5×10^9 t.u.) were treated with 0 or 1 mM of TCEP at 25°C for 30 min in reaction buffer followed by precipitation of the phage with ¼ volume of 20% (w/v) PEG6000 containing 2.5 M of NaCl to remove the excess of TCEP. Next, 50 μl of linker **1** at concentration of 0 or 200 μM in acetonitrile were added to 450 μl of phage in reaction buffer, and incubated for one hr at 30°C. Phage were precipitated again by adding 125 μl of 20% (w/v) PEG6000 containing 2.5 M of NaCl to remove the excess linker and dissolved in 500 μl of PBS buffer (pH 7.4). Then, 50 μl of this phage solution were added to 50 μl of porcine pancreatin in PBS buffer (pH 7.4) to reach final

pancreatin concentrations of 0, 0.001, 0.01, 0.1, 1, and 10 mg/ml, and thus SIFs of 0, 0.01, 0.1, 1, 10, and 100%. The solutions were incubated at 37°C for 30 min and the number of infective phage was quantified by mixing 180 μl of exponentially growing *E. coli* TG1 cells with 20 μl of 10-fold dilutions of the phage and plating the cells on chloramphenicol (for disulfide-free phage) or tetracycline (for wild-type phage) 2YT agar plates.

Cloning of phage peptide libraries

DNA encoding the displayed random four-cysteine peptides of the form $XCX_mCX_nCX_oCX$ (X = any amino acid, C = cysteine, m, n, o = number of random amino acids) were cloned into the phage vector fd-tet upstream of wild-type p3 by PCR amplification of the entire plasmid using degenerate primers and subsequent connection of the PCR product ends. The numbers of amino acids m, n, o are equal or larger than 0, wherein m + n + o are between 3 and 8 for the libraries XD9 to XD14. The phage vector fd-tet-PK15 was used as template for the PCR reaction. DNA encoding the peptides was appended to the plasmid DNA through forward primers that annealed in the N-terminal region of p3. The reverse primer annealed in the region coding for the p3 leader sequence. All primers contained a *Sfi*I cleavage site at the 5' end which allowed for the generation of complementary sticky ends for efficient DNA circularization by T4 ligase. The ligated DNA was purified and transformed into electrocompetent TG1 cells, plated on 2YT/tetracycline plates (10 μg ml^{-1}), and incubated over night at 37°C. The cells on the plates were recovered in 2YT media containing 10% (v/v) glycerol and stored at −80°C. The number of transformed cells was estimated based on dilutions and the total number for all libraries exceeded 2×10^{10}.

Cyclization of peptide phage display libraries

For the first round of selection, the six phage sub-libraries XD9 to XD14 were produced separately in 0.5 L of 2YT media containing tetracycline (10 μg ml^{-1}), purified by PEG

precipitation using ¼ volume of 20% (w/v) PEG6000, 2.5 M NaCl as previously described, and pooled together. PEG-purified phage (typically around 10^{14} t.u.) were reduced in 45 ml of reaction buffer with 1 mM TCEP at 25°C for 30 min. The phage solution was chilled on ice, precipitated by adding ¼ volume of ice-cold 20% (w/v) PEG6000, 2.5 M NaCl, and centrifuged at 5,000 × *g* for 30 min at 4°C. The supernatant was discarded and the phage pellets resuspended in 50 ml of reaction buffer and distributed into 10 aliquots of 4.5 ml. The peptides in each aliquot were chemically modified by adding 500 μl of the chemical reagents **1** to **10** in acetonitrile (to reach final concentrations of 20 μM for **1**, **3** and **4**, and 40 μM for **2**, **5**, **6**, **7**, **8**, **9**, **10**) and incubation at 30°C for 1 hr. The phage particles were precipitated by adding 1.25 ml of ice-cold 20% (w/v) PEG6000, 2.5 M NaCl and centrifuged at 5,000 × *g* for 30 min at 4°C. The supernatant was discarded and the phage pellets were resuspended in 4.5 ml of PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl). For the selection rounds 2 to 6, phage were produced, purified and cyclized following the same procedure but with the following modifications. Phage of each selection were produced in 25 ml 2YT culture, yielding 10^{11}-10^{12} t.u. for each selection. Phage were cyclized in volumes of one ml using the same solvent and reagent concentrations, and resuspended in 0.45 ml PBS.

Proteolytic phage selection

For the first round of selection against FXIa, phage in 4.5 ml PBS were mixed with 500 μl of 1 mg ml^{-1} porcine pancreatin (Sigma-Aldrich, CAT# P3292) in PBS (pH 7.4) to reach a condition of 1% SIF, and incubated at 37°C for 30 min. For the following rounds, phage in 0.45 ml PBS were incubated with 50 μl of 1 mg ml^{-1} pancreatin to reach 1% SIF. In round 5-6, phage in 0.45 ml PBS were alternatively also incubated without pancreatin, or with 50 μl of 10 mg ml^{-1} pancreatin to reach 10% SIF. The proteases were removed by precipitating the phage with ice-cold 20% (w/v) PEG6000, 2.5 M NaCl, centrifugation at 5,000 × *g* for 30 min at 4°C and discarding the supernatant. The phage pellets were washed twice with ice-cold 4% (w/v) PEG6000, 0.5 M NaCl to

remove the residual protease and resuspended in washing buffer containing 1% (w/v) BSA and 0.1% (v/v) Tween-20. The phage were incubated for 30 min at room temperature to allow blocking of the phage with BSA and Tween-20. For the phage selection against IL-23R, no protease pressure was used in the first three rounds and 1% SIF was used in round 4 and round 5.

Target immobilization

Human FXIa and IL-23R were immobilized on magnetic beads by random biotinylation of amino groups and addition to streptavidin or neutravidin beads. The two types of beads were used alternatingly in the six selection rounds of phage selection to disfavor enrichment of streptavidin- or neutravidin-specific peptides. Human FXIa (Cat # HFXIA; Molecular innovations) was biotinylated by incubation of 200 μg protein (2.5 nmol) in 0.8 ml PBS (pH 7.4) with a 20-fold molar excess of EZ-Link™ Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) in 5 μl DMSO (10 mM) for 1 hr at room temperature. IL-23R (purified as shown below) was biotinylated by incubating 79 μg of protein (2.1 nmol) in 0.2 ml of 20 mM HEPES (pH 7.4) and 150 mM NaCl with a 6-fold molar excess of EZ-Link™ Sulfo-NHS-LC-Biotin in 1.2 μl of DMSO (10 mM) for 2 hr on ice. Excess biotin was removed by chromatography using a PD-10 or Superdex75 10/300 GL column and PBS. The percentage of biotinylated FXIa and IL-23R was assessed by capturing 2 μg of biotinylated protein on 20 μl magnetic streptavidin beads (Dynal, M-280 from Life Technologies) and subsequent analysis of immobilized protein by SDS-PAGE as previously described[26]. Neutravidin beads were prepared by reacting 6 mg of neutravidin (Pierce) with 10 ml of tosyl-activated magnetic beads (Dynal, M-280 from Invitrogen) according to the supplier's instructions. For the first to third round of phage selection against FXIa, 0.5 μg of target protein immobilized on 20 μl strepatividin beads (1st and 3rd round) or on 10 μl neutravidin beads (2nd round) were used for each selection performed with one of the 10 different cyclization reagents. For the 4th round of selection, either 0.1 μg, 20 ng, or 4 ng of biotinylated FXIa were immobilized on 10 μl neutravidin beads. For the 5th and 6th selection round, 1 ng of biotinylated FXIa were

immobilized on 10 μl strepatividin beads (5th round) or on 5 μl neutravidin beads (6th round). For the phage selection against IL-23R, 2.5, 1.25, 0.625, 0.3, and 0.1 μg of target protein were immobilized on 20 μl of streptavidin beads (1st, 3rd, and 5th round) or on 10 μl of neutravidin beads (2nd, 4th round), respectively, and were used for the selection performed with linker 7. Biotinylated protein was immobilized on magnetic beads by incubation of protein and pre-washed beads in 200 μl PBS for 10 min at room temperature on a rotating wheel (10 rpm). The beads were washed twice with 1 ml of washing buffer and resuspended in 0.5 ml washing buffer.

Phage display panning

For the phage panning experiments, magnetic beads with immobilized FXIa or IL-23R were blocked by incubation with washing buffer containing 1% (w/v) BSA and 0.1% (v/v) Tween-20 for 30 min at room temperature on a rotating wheel (10 rpm). In parallel, phage displaying double-bridged peptides were blocked in the same buffer. Blocked beads with target protein were mixed with blocked phage and incubated for 30 min on a rotating wheel (10 rpm) at room temperature. Beads were washed eight times with washing buffer containing 0.1% (v/v) Tween-20 and twice with washing buffer. The phage were eluted by incubating the beads with 100 μl glycine buffer (50 mM, pH 2.2) for five minutes and transferred to 50 μl of 1 M Tris-Cl buffer, pH 8. Phage were incubated with 5 ml of exponentially growing *E. coli* TG1 cells (OD_{600} = 0.4−0.8) for 30 min at 37°C and the cells were plated on a 14 cm 2YT plates containing tetracycline (10 μg ml^{-1}). The next day, bacterial cells were recovered from the plates in four ml of 2YT media containing 10% (v/v) glycerol, and stored at −80°C.

Determination of peptide stability in simulated gastric and intestinal fluid

Simulated Gastric Fluid (SGF) was prepared according to USP specifications (Test Solutions, United States Pharmacopeia 35, NF 30, 2012). Sodium chloride (0.2 g) was added to a 100 ml flask and dissolved in 50 ml of water. A volume of 0.7 ml of 10 M

HCl was added to adjust the pH of the solution to 1.2. To this, 0.32 g of pepsin (Sigma-Aldrich, CAT# P7125) was added and dissolved with gentle shaking and the volume made up to 100 ml with water. Pepsin was added only after the pH was adjusted to 1.2. Simulated Intestinal Fluid (SIF) was prepared according to USP specifications (Test Solutions, United States Pharmacopeia 35, NF 30, 2012). Monobasic potassium phosphate (0.68 g) was dissolved in 25 ml of water. A volume of 7.7 ml of 0.2 M NaOH was added to adjust the pH to 6.8. To this, 1 g of pancreatin (Sigma-Aldrich, CAT# P3292) was added and shaken gently until dissolved and the volume adjusted to 100 ml with water. Pancreatin was added after adjusting the pH to 6.8 to avoid precipitation of the enzyme. The stability of peptides in SGF and SIF was tested as follows. Two μl of peptide stock solution (2 mM in DMSO) was added to 98 μl of SGF or SIF and incubated at 37°C. Samples (10 μl) were withdrawn at different intervals and added to ice-cold stop reagent (15 μl, methanol for gastric fluid and 0.1 M HCl for intestinal fluid) containing 10 μM of internal standard peptide, to inactivate the enzymes and allow quantitative determination of intact peptide remaining. All the samples were centrifuged at 10,000 × g for 10 min, and the supernatant was analyzed quantitatively by LC-MS. Linaclotide used in stability assay was purchased from Medchem (CAT# HY-17584). Desmopressin was synthesized by standard solid-phase peptide synthesis.

Determination of plasma stability

Two μl of peptide stock solution (2 mM in DMSO) was added to 98 μl of plasma (human or mouse, Innovative Research) at 37°C. Probes of 10 μl were taken at different time points (0 min, 4, 8, 24, 48, and 72 hrs), incubated at 65°C for 10 min to inactivate the plasma proteases. A volume of 5 μl of 7 M guanidine hydrochloride (pH 2.0) and 5 μl of internal standard peptide (200 μM in MilliQ H_2O) were added. The plasma proteins were precipitated by addition of cold ethanol (400 μl) and incubation on ice for 60 min. The samples were centrifuged at 10,000 × *g* for 20 min, the supernatant was transferred to new tubes and the solvent was evaporated under vacuum (speed vac) at 50°C. The samples were dissolved in 20 μl of H_2O and analyzed by LC-MS.

Animal experiments

All experiments in mice were conducted in accordance with the terms of the Swiss animal protection law and were approved by the animal experimentation committee of the cantonal veterinary service (Canton of Vaud, Switzerland).

Determination of pharmacokinetics in mice

Female BALB/cByJ mice were injected with 10 mg kg^{-1} F20 in 100 μl PBS (pH 7.4) via the tail vein (five animals per group). Blood samples (40 μl) were collected at different time points via the tail vein into tubes containing EDTA (K3 EDTA; Sarstedt, CAT# 20.1278), centrifuged at 4,000 × *g* for 10 min at 4°C and the plasma stored at −80°C. The samples were processed as described above and analyzed by LC-MS. Peptides were quantified by extracting ion chromatograms based on the peptide mass, integrating the area under the peaks of the, and normalization was based on calibration curves generated with the same peptides. Calibration curves were generated by analyzing by LC-MS mouse plasma to which defined quantities of peptide were added.

Bioavailability and quantification of peptide in GI tract

Peptide F20 dissolved in 200 μl PBS (10 mg ml^{-1}) was applied by gavage (p.o.; 100 mg kg^{-1}) to five mice (20 g weight; fasted for 15 hrs before administration) for each time point. Female BALB/cByJ mice were anesthetized with ketamine/xylazine (100/10 mg kg^{-1}) administered i.p. Blood (0.5 ml) was collected by cardiac puncture and transferred in plasma tubes containing EDTA (K3 EDTA; Sarstedt, CAT# 20.1341) and centrifuged at 4,000 × *g* for 10 min. Plasma samples (200 μl) were mixed with 50 μl of 7 M guanidine hydrochloride (pH 2.0) and 5 μl of internal standard peptide (200 μM in MilliQ H_2O). The samples were processed as described above and analyzed by LC-MS. The absolute oral bioavailability (F) was calculated with the dose-corrected area under curve (AUC) of the oral route (p.o.) divided by the AUC of the intravenous route

(i.v.):

$$F = 100 \times \frac{\text{AUC(p.o.)/Dose(p.o.)}}{\text{AUC(i.v.)/Dose(i.v.)}}$$

Stomach, small intestine and colon of mice were removed, the small intestine divided into two parts (upper part a, lower part b) and homogenized in 1 ml of organ lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.01% Triton X-100) containing 0.5% (v/v) protease inhibitor cocktail (Sigma, CAT# P8340). After centrifugation at 10,000 × *g* for 30 min at 4°C, 15 µl of supernatant was mixed with 5 µl of 7 M guanidine hydrochloride (pH 2.0) and 5 µl of internal standard peptide (200 µM in MQ H_2O). The samples were analyzed by LC-MS and the peptide quantified as described above.

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Data availability

The data supporting the results of this study are available within the paper and its Supplementary Information files. Data of the two X-ray structures are provided in the PBD database (6QEZ, 6QF0).

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Author Contributions

X.K. and C.H. conceived the strategy for proteolytic phage display with double-bridged peptides. X.K. established the phage selection procedure, cloned the libraries, performed the phage selections against FXIa, synthesized and characterized the peptides, expressed and purified FXIa, and determined the X-ray structures. J.M. expressed and purified IL-23R, performed the phage selections against IL-23R and characterized the peptides. V.C. identified the D-proline mutant. F.P. collected X-ray data and analyzed the structures. L.A.A. performed the NMR study. X.K., K.D., and C.H. wrote the manuscript with help from all authors.

Competing Interests

J.M., X.K., and C.H. are inventors of a patent protecting the IL-23R antagonists.