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Phage-encoded combinatorial chemical libraries based on bicyclic peptides

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Here we describe a phage strategy for the selection of ligands based on bicyclic or linear peptides attached covalently to an organic core. We designed peptide repertoires with three reactive cysteine residues, each spaced apart by several random amino acid residues, and we fused the repertoires to the phage gene-3-protein. Conjugation with tris-(bromomethyl)benzene via the reactive cysteines generated repertoires of peptide conjugates with two peptide loops anchored to a mesitylene core. Iterative affinity selections yielded several enzyme inhibitors; after further mutagenesis and selection, we were able to chemically synthesize a lead inhibitor (PK15; $K_i = 1.5$ nM) specific to human plasma kallikrein that efficiently interrupted the intrinsic coagulation pathway in human plasma tested *ex vivo*. This approach offers a powerful means of generating and selecting bicyclic macrocycles (or if cleaved, linear derivatives thereof) as ligands poised at the interface of small-molecule drugs and biologics.

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The discovery of new ligands to receptor, enzyme and nucleic acid targets represents the first stage in the development of therapeutic drugs. For drugs based on small organic ligands, high-throughput screening (HTS) is a popular strategy; large libraries of compounds are synthesized (or purchased), and each compound is assayed for binding to the targets. With the use of robots it is possible to screen 10^5–10^6 compounds per day, but the hits usually require further chemistry to improve their binding affinity and target specificity[1,2].

For drugs based on nucleic acids, peptides or proteins, biological selection methods offer an alternative strategy. These methods (such as phage display, ribosome display, mRNA display or RNA/DNA aptamer technologies) rely on (i) creating a diverse genetic library in which the phenotype (binding to target) of each member of the library is linked to its genotype (the encoding DNA or RNA)[3,4], and (ii) an iterative cycle in which library members are selected for binding to a target, and then amplified (by replication in a host cell, or by copying of the encoded nucleic acid *in vitro*)[5,6]. At each round of selection the binders are thereby enriched over the nonbinders. Very large libraries (10^9–10^{13} members) can be efficiently screened by a few rounds of selection, and lead hits can be refined by mutation and further selection[7–9]. The approach is very powerful and has been used to fashion ligand binding sites in antibodies[10–12] and other protein scaffolds[13], leading to the first human therapeutic antibody (adalimumab) to be approved by the US Food and Drug Administration.

Several attempts have been made to develop selection methods for the isolation of small organic ligands. Typically DNA is used as a tag that can be readily synthesized, sequenced, amplified and/or hybridized. For example, small molecules can each be conjugated to a unique DNA[14–16] (or bacteriophage[17]) tag, and the conjugates can be mixed together to create a tagged small-molecule library. After selection of the library against the target, the small-molecule 'hits' can be identified by the sequences of their (amplified) tags. Alternatively the DNA tags can be introduced during the synthesis of combinatorial chemical libraries. For example, small molecules and a corresponding tag are synthesized in parallel on the same bead[18], or hybridization of the tag is used to govern the route of chemical synthesis[19–21]. From such libraries, the synthetic route (and thereby structure) of the selected hits can be deduced from the sequence of the tag. Not withstanding their ingenuity, these methods suffer from common disadvantages; either the small molecule is linked to the DNA tag only during the first round of selection, or the method requires a high efficiency of chemical translation[20], rendering iterative cycles either impossible (and limiting application to small libraries) or technically demanding.

Methods have been described for tethering peptides through reactive cysteine side chains to the functional groups of an organic scaffold[22]. More recently others have envisaged that these methods could be used for the generation of variant peptide conjugates that mimic discontinuous epitopes of folded proteins, with binding activities assayed by HTS[23–25]. As an alternative, we sought to chemically modify the cysteine residues of the peptides displayed on phage while maintaining phage infectivity[26,27], and by harnessing the power of iterative selection have access to much greater chemical diversity than possible by HTS (**Fig. 1a**). In a first embodiment, these conjugates are reminiscent of the peptide macrocyclic drugs[28,29] (**Fig. 1b**), but whereas many peptide macrocycles (from fungus, yeast and bacteria) are made *in vivo* by nonribosomal peptide synthases, our strategy uses ribosomal synthesis. In a second embodiment, cleavage of the cyclic peptide repertoires generates an organic core decorated with highly diverse linear peptide side chains (**Fig. 1a**).

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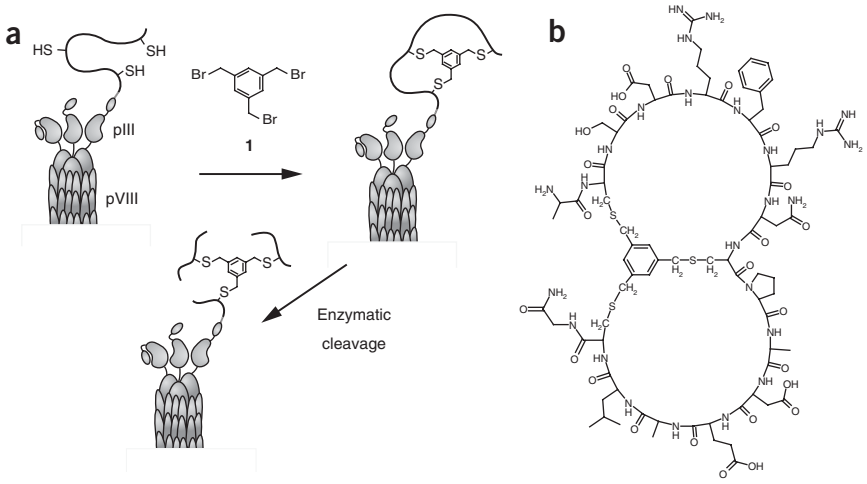

Figure 1 Generation of phage-encoded combinatorial chemical libraries and an isolated molecule. (**a**) A phage-encoded peptide with three cysteine residues is tethered to the trifunctional compound TBMB in a nucleophilic substitution reaction (see refs. 18–20 for the chemical reaction). The resulting chemical entities could optionally be further modified through enzymatic reactions such as proteolysis. (**b**) Chemical structure of a macrocyclic plasma kallikrein inhibitor isolated by phage display (PK15).

RESULTS

Conjugation of an organic scaffold to peptides on phage

We used the small organic compound tris-(bromomethyl)benzene (TBMB; **1**) as a scaffold to anchor peptides containing three cysteine residues[22,24] (**Fig. 1a**). The reaction occurs in aqueous solvents at room temperature (20–25 °C), and the threefold rotational symmetry of the TBMB molecule ensures the formation of a unique structural and spatial isomer.

We first elaborated the reaction conditions for conjugation of the peptide ^{N}GCGSGCGSGCG^{C} fused to the soluble D1-D2 domains of the phage pIII by analyzing the molecular weight of the products by mass spectrometry. However, we were unable to selectively conjugate the three cysteine residues of the peptide with TBMB while sparing the disulfide bridges of D1 and D2 (C7-C36, C46-C53 and C188-C201). This prompted us to take advantage of a recently developed disulfide-free gene-3-protein[30]. The peptide–D1-D2 (disulfide-free) fusion protein was reduced with tris-(carboxyethyl)phosphine (TCEP), the TCEP was removed and TBMB was added. A concentration of 10 μM TBMB was sufficient for quantitative reaction with the peptide fusion protein at 30 °C in 1 h, giving predominantly one product with the expected molecular mass (Δ mass expected = 114 Da; **Fig. 2a**). No product was detected with the (disulfide-free) D1-D2 protein. Reaction of TBMB with peptide–D1-D2 (disulfide-free) fusions containing only two cysteine residues (^{N}AGSGCGSGCG^{C}–D1-D2) yielded a product with a molecular mass consistent with reaction of both cysteines and the α-amino group at the peptide N terminus (**Supplementary Fig. 1a**,**b** online). Similarly, the reaction of TBMB with a peptide–D1-D2 (disulfide-free) fusion having one cysteine and a lysine (^{N}AGSGKGSGCG^{C}–D1-D2) yielded a molecular mass consistent with the reaction of the cysteine, the α-amino group of the N terminus and the ε-amino group of the lysine (**Supplementary Fig. 1c**,**d**). Thus the functional groups of TBMB preferentially react with the thiol groups of cysteines but can also react intramolecularly with primary amines in the absence of cysteine. Having identified suitable conditions, we reacted TBMB with (disulfide-free p3) phage bearing the peptide ^{N}GCGSGCGSGCG^{C}. This led to a small loss (fivefold) of phage infectivity at 10 μM TBMB, but much greater losses at higher concentrations (**Fig. 2b**); we suggest that this may be due to cross-linking of the phage coat proteins through lysine residues.

Creation of polycyclic peptide library and affinity selection

We designed a library of peptides comprising two sequences of six random amino acids flanked by three cysteines (Cys-(Xxx)$_6$-Cys-(Xxx)$_6$-Cys; **Fig. 3a**) for display on the (disulfide-free p3) phage. An alanine residue was added to the N terminus of the peptide to ensure a correct processing of the signal sequence. A Gly-Gly-Ser-Gly linker was placed between the third cysteine and the gene-3-protein. As the (disulfide-free p3) phage had a 100-fold reduced infectivity compared to wild-type phage, we grew up a 1-l volume of culture (incubated overnight at 30 °C; this typically yielded 10^{11}–10^{12} infective particles).

We tested the library (estimated 4.4 × 10^9 variants) of polycyclic peptides for binding and inhibition of the human proteases plasma kallikrein and cathepsin G. About 10^{12} purified infective phage particles were chemically modified with TBMB and then incubated with the biotinylated target proteins. After capture on magnetic streptavidin or avidin beads, the enriched phage were treated to two further rounds of selection, each round comprising amplification (by infection of bacteria), chemical conjugation and capture with the biotinylated targets. The phage titer increased after the second and third rounds, which suggests enrichment of specific binders. DNA encoding the peptides was PCR-amplified from the selected population of phage in the third round, recloned for periplasmic expression as peptide–D1-D2 (disulfide-free) fusion proteins and sequenced. This

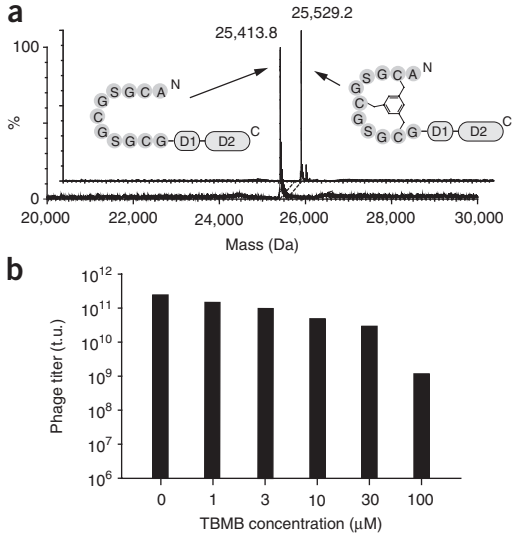

Figure 2 Conjugation of peptide fusions with TBMB. (**a**) Molecular spectrometry of the GCGSGCGSGCG–D1-D2 (disulfide-free) fusion protein before and after reaction with 10 μM TBMB, as in the Methods. The mass difference corresponds to the mesitylene core. (**b**) Titers (transducing units) of phage treated with various concentrations of TBMB as in the Methods.

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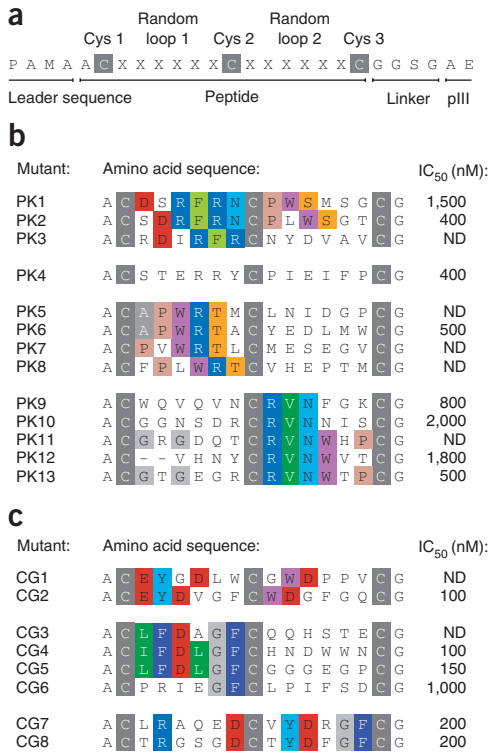

Figure 3 Sequences of selected conjugates. (**a**) Designed sequence of peptides attached to fusion proteins as expressed by phage library 1. Processing of the leader sequence upon secretion of the protein is expected to give a peptide with an N-terminal alanine, two random six-amino-acid sequences flanked by three cysteines and a Gly-Gly-Ser-Gly linker that connects the peptide to the gene-3-protein. (**b**,**c**) Amino acid sequences of conjugates selected with human plasma kallikrein (**b**) and cathepsin G (**c**) with corresponding inhibitory activity. Only those clones with sequence similarities or that were isolated multiple times are displayed. Residues of similar character are highlighted in color. ND, not determined.

revealed consensus sequences in one or both of the peptide loops (**Fig. 3b**,**c**), and several of the fusion proteins were expressed, purified, conjugated with TBMB and tested for their inhibitory activity towards protease. The best plasma kallikrein and cathepsin G inhibitors had half-maximal inhibitory concentrations (IC_{50}s) of 400 nM (PK2 and PK4) and 100 nM (CG2 and CG4), respectively, when tested as D1-D2 fusions. Because we screened the phage-selected clones for inhibition (rather than binding), we do not know whether peptide conjugates were selected that bind but do not inhibit the proteases. However, the finding that the vast majority of clones tested after the three rounds of phage selection displayed inhibitory activities suggests that inhibitors were predominantly selected.

Affinity maturation of human plasma kallikrein inhibitors

Most of the sequences of the kallikrein binders revealed consensus sequences in one or the other of the peptide loops. Three new libraries were created with each one of the three consensus regions in one loop and six random amino acids in the other loop (**Fig. 4a**). The libraries were mixed and phage panned under stringent conditions (1 nM to 200 pM biotinylated kallikrein). The random sequence converged to a new consensus, yielding clones with consensus sequences in both loops (**Fig. 4b**). Inhibition assays revealed that the IC_{50} of the best inhibitor (PK15) was 20 nM when tested as a D1-D2 fusion protein.

Activity and specificity of chemically synthesized inhibitors

Peptides corresponding to four kallikrein inhibitors from the primary selection (PK2, PK4, PK6 and PK13) and the best inhibitor from the affinity maturation selection (PK15) were chemically synthesized on a solid phase. The peptides had an alanine residue at the N terminus and an amidated glycine at the C terminus to represent the charge and chemical environment of the phage-displayed peptides. The TBMB-conjugated synthetic peptides inhibited kallikrein activity at least 250-fold more potently than the unconjugated peptides (**Table 1**). They were also more potent than the D1-D2 conjugates by a factor of more than ten (**Fig. 5a** and **Table 1**); this may be due to steric blocking of the conjugated peptide moiety by the D1-D2 moiety. The apparent inhibition constant (K_i) of the peptide conjugate PK15 (**Fig. 1b**) was calculated to be 1.5 nM using a previously described equation[31].

Incubation of the conjugate PK15 with kallikrein led to hydrolysis of a peptide bond after prolonged incubation (90% cleavage after 24 h at 37 °C), as shown by a mass gain of 18 Da, but the inhibitory activities of cleaved and uncleaved samples were similar (IC_{50} = 2.2 nM and 1.6 nM, respectively). Investigation of the cleavage site through incubation of the cleaved PK15 with leucyl aminopeptidase from *Aeromonas proteolytica* and mass spectrometric analysis of the formed products revealed that PK15 is cleaved by plasma kallikrein between Arg7 and Asn8 (**Supplementary Fig. 2** online). The amino-peptidase also cleaved off the N-terminal alanine of PK15, which confirmed that the N-terminal amine had not reacted with TBMB.

The five inhibitors were also tested against mouse plasma kallikrein (79% sequence identity) or the homologous human serine proteases factor XIa (63% sequence identity) and thrombin (36% sequence identity). None inhibited these enzymes at the highest concentration tested (10 μM).

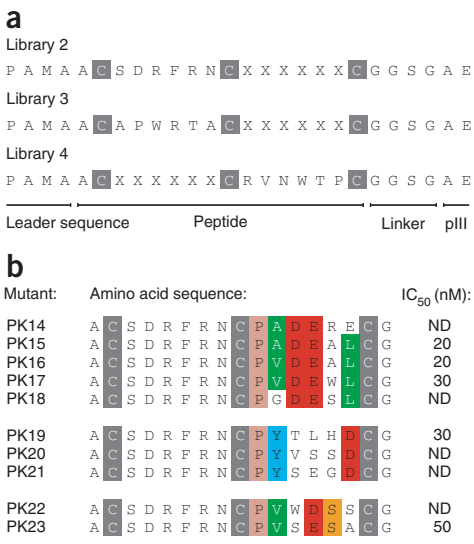

Figure 4 Affinity maturation of human plasma kallikrein inhibitors. (**a**) Design of libraries 2, 3 and 4. In each library, one of the peptide loops has the sequence of a consensus motif identified in the first selections, and the other contains six random amino acids. (**b**) Amino acid sequences of clones selected with human plasma kallikrein. All clones derive from library 2. The inhibitory activities of TBMB-modified peptide–D1-D2 fusion proteins are indicated. Only clones with sequence similarities or that were isolated multiple times are displayed. The colors highlight sequence similarities in the second binding loop. ND, not determined.

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Table 1 Chemically synthesized peptide inhibitors

Parental clones	Amino acid sequence	Mass (Da)		IC_{50} (nM)	
		Linear peptide	Bicyclic conjugate	Linear peptide	Bicyclic conjugate
PK2	H-ACSDRFRNCPLWSGTCG-NH_2	1,871.2	1,985.3	>10,000	28.6
PK4	H-ACSTERRYCPIEIFPCG-NH_2	1,942.9	2,055.9	7,181	33
PK6	H-ACAPWRTACYEDLMWCG-NH_2	1,974.8	2,088.7	5,707	21.2
PK13	H-ACGTGEGRCRVNWTPCG-NH_2	1,764.8	1,879.1	>10,000	39.1
PK15	H-ACSDRFRNCPADEALCG-NH_2	1,825	1,939.4	>10,000	1.7

The amino acid sequences of five plasma kallikrein inhibitors (17-mers) are shown. The sequences of the synthetic peptides derive from the clones PK2, PK4, PK6 and PK13 (isolated in phage selections using library 1) and from clone PK15 (an affinity-matured clone isolated from library 2). Indicated are the molecular masses and the inhibitory activities before and after the modification of the peptides with TBMB. The reduced linear peptides were incubated with plasma kallikrein, and the inhibitory activity was measured immediately to minimize the risk of peptide oxidation.

Interruption of the intrinsic coagulation pathway

Human plasma kallikrein plays a key role in the first events of the intrinsic coagulation pathway by converting factor XII to factor XIIa, which then acts on the next protease in the pathway. We tested whether conjugate PK15 could inhibit the activation of factor XIIa in human plasma samples. The pathway was triggered with caolin, and the activity of factor XIIa was measured with a colorimetric substrate. The activity of XIIa was halved in the presence of 160 nM conjugate PK15 (**Supplementary Fig. 3** online). By comparison, 5 μM aprotinin, a 6-kDa bovine serine protease inhibitor also used clinically as a plasma kallikrein inhibitor (K_i = 30 nM), was required for the same effect.

Structure determination of TBMB-modified peptide PK15

We recorded two-dimensional ^{1}H NMR spectra of the conjugate PK15 (**Supplementary Fig. 4** online) and obtained a sequence-specific assignment of the chemical shifts of the total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) spectra. From the NOE data, there was no evidence of interactions between the loops, no NOEs across the loops, and no evidence of short segments with regular secondary structure. Only one NOE (a 'weak' NOE between Arg5 HB and Cys2 HN) was between protons separated by more than two residues in the sequence. The absence of long-range and medium-range NOEs precluded a restraint-driven minimization and hinders the definition of a single defined structure with confidence.

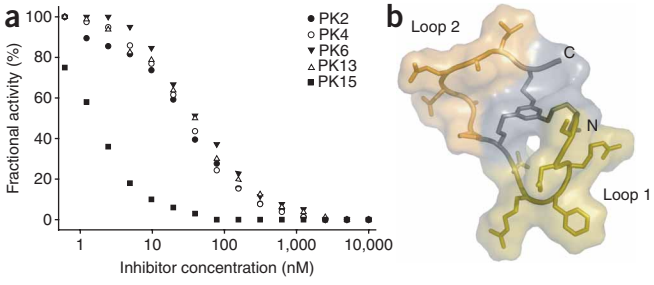

Figure 5 Inhibition of human plasma kallikrein by conjugates and NMR solution structure of conjugate PK15. (**a**) The inhibitory activity is expressed as the fractional activity (inhibited rate/uninhibited rate) at varying inhibitor concentrations (one measurement per inhibitor concentration). Clones PK2, PK4, PK6 and PK13 were isolated in phage selections using library 1. PK15 derives from library 2 and is an affinity-matured inhibitor. (**b**) The peptide loops of conjugate PK15 are shown in yellow (loop 1) and orange (loop 2). The mesitylene core, the three cysteine residues and the terminal alanine (N terminus) and glycine (C terminus) are shown in gray. The backbone atoms of the peptide are represented as a sausage, and the side chains of the amino acids are represented as sticks.

We generated a structural model of PK15 using a standard restrained simulated annealing protocol in version 1.1 of CNS (http://cns.csb.yale.edu/v1.1/) (**Fig. 5b**). Of the 50 accepted structures, none violated the NOE restraints, and the closest H-H contact in the model that did not have a corresponding NOE correlation was 3.7 Å (**Supplementary Table 1** online). The model was therefore consistent with the available experimental data. However, we have no evidence of the extent of flexibility in the loops, or of the range of conformers that may contribute to the conformational ensemble that is present in solution. In the model, the two peptide loops do not interact with each other and are arranged (but not closely packed) around the mesitylene core to which they are covalently tethered.

DISCUSSION

We have shown how the reaction of TBMB[22,24] with libraries of cysteine-rich peptides displayed on filamentous bacteriophage generates conjugates amenable to iterative selection. It was a challenge to conjugate the displayed peptide while sparing the phage, and we had to vary reagent concentrations, solvent composition and reaction temperature and use phage lacking disulfides in the gene-3-protein. From a library of $>10^9$ members and iterative selections, we succeeded in isolating potent human plasma kallikrein inhibitors (K_i = 1.5 nM) efficiently interrupted the intrinsic coagulation pathway in human plasma tested *ex vivo*, and was highly specific: it did not inhibit mouse plasma kallikrein or the homologous human plasma proteases factor XIa and thrombin. Inhibitors of human plasma kallikrein are being developed clinically for treatment of hereditary angioedema and coronary bypass surgery, but generation of small molecules that are kallikrein-specific has proven difficult (reviewed in refs. 32,33). It is therefore promising that our strategy yielded a high-affinity, specific inhibitor.

Our repertoire was built from 17-residue peptides with three cysteines, each spaced apart by six random amino acids. These conjugates should have advantages over other peptide forms. For example, compared to monocyclic peptides[34,35], the bicyclic conjugates should be more capable of extensive interactions with globular proteins and perhaps thereby more able to block protein-protein interactions; compared to disulfide-bonded cyclic peptides, the cross-links should be inert to exchange and stable in reducing environments[36]; and compared to linear peptides, the conjugates should be constrained and bind more tightly to targets (due to the smaller loss of conformational entropy on binding). Indeed, our literature review of the most potent peptide inhibitors isolated by phage display (**Supplementary Table 2** online) shows that the majority are constrained by disulfide bonds. Constrained peptides are also expected to be more resistant to protease cleavage and/or inactivation than linear peptides, as is consistent with our observations that PK15 was cleaved in one of

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the loops only after prolonged incubation with human plasma kallikrein (and even then remained active).

After conjugation with TBMB, the peptides are expected to form two six-residue loops covalently attached to a mesitylene core, as confirmed by the structure of the PK15 kallikrein inhibitor solved by NMR (**Fig. 5b**). The N-terminal peptide loop of the PK15 conjugate has a tight turn that is similar to those found in other peptide macrocycles, including the hormones oxytocin (**2**)[37,38] and octreotide (**3**)[39,40] (they both contain hexapeptides that are cyclized by disulfide bonds). However, in contrast to most peptide macrocycles, the PK15 conjugate has more polar side chains and is more soluble in aqueous solution. As hydrophobic side chains appear to be important for the interaction of antibacterial or antifungal peptide macrocyclic drugs with membranes, it may be necessary to increase the hydrophobic character of the side chains or core in our macrocycles to create drug leads.

The peptide macrocycles have some structural similarities with zinc finger proteins. In both types of molecules, polypeptide chains are coordinated via cysteine residues (in zinc finger proteins also via histidine residues) to a central molecule to gain a tertiary structure. Whereas in zinc finger proteins, cysteine and histidine residues form noncovalent bonds to Zn^{2+}, the macrocycles isolated in this work form covalent bonds to the mesitylene core. The zinc finger proteins have a slightly larger molecular mass (around 30 amino acids), have obligate secondary structure elements (an antiparallel β-sheet and an α-helix) and are likely to be more tightly folded due to these elements.

The molecular weight of PK15 (1,939.4 Da; both ring sizes are 31 atoms) is higher than that of several peptide macrocycles with biological activity, such as oxytocin, octreotide, polymyxin B (**4**), daptomycin (**5**), cyclosporine (**6**) and caspofungin (**7**) (with molecular weights of 1,000 to 1,600 and 20 to 32 atoms in the ring), but it would be possible to use shorter loops. For example, by altering the spacing of the cysteines, the loop length is readily varied, and extra segments can even be added to the peptide termini. Further variations could include mutagenesis of the loops (as with the affinity maturation of PK15), proteolytic cleavage in one or both loops to generate a mesitylene core with three peptide side chains each linked to the core through cysteines, chemical conjugation to the nascent peptide N or C termini after loop cleavage[41], use of other reactive amino acids (for example, selenocysteine[42,43]) to conjugate the peptide to the core, or use of variant organic cores. In particular, a larger organic core, or one with more functional groups, could help stabilize the peptide loops or interact directly with the target. In this respect we note that the two most potent peptide inhibitors in **Supplementary Table 2** (with inhibition constants comparable to that of PK15) both comprise at least two tryptophan residues[44,45], which may act in a similar manner in these peptides. New functionalities such as fluorescence might also be introduced via the chemical core. The ability to isolate and engineer variant conjugates by both genetic and chemical engineering on the phage, and to make further variations by routine chemical synthesis of free conjugates, makes this strategy a flexible and attractive means for generating and developing drug leads. The small size and simplicity of the chemistry may also facilitate the manufacture of products by total synthesis.

METHODS

Chemical modification of peptide repertoires with TBMB on phage. We cloned and produced phage peptide libraries that are based on the plasmid fdg3p0ss21 (ref. 30) as described in the **Supplementary Methods** online. Typically 10^{11}–10^{12} t.u. (transducing units) of polyethylene glycol (PEG)-purified phage were reduced in 20 ml of 20 mM NH_4HCO_3, pH 8 with 1 mM TCEP at 42 °C for 1 h. The phage were concentrated at 4,000 r.p.m. in a Vivaspin-20 filter (molecular weight cut off of 10,000) to 1 ml, washed twice with 10 ml ice-cold reaction buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8) and taken up in 32 ml of reaction buffer, and 8 ml of 50 μM TBMB in acetonitrile (ACN) were added to obtain a final TBMB concentration of 10 μM. The reaction was incubated at 30 °C for 1 h before nonreacted TBMB was removed by precipitation of the phage with 0.2 volumes of 20% (w/v) PEG, 2.5 M NaCl on ice and centrifugation at 4,000 r.p.m. for 30 min.

Phage selections with human plasma kallikrein and cathepsin G. We incubated biotinylated human plasma kallikrein and cathepsin G (5 to 20 μg; biotinylation as in the **Supplementary Methods**) in 0.5 ml washing buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1mM $CaCl_2$) containing 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 for 30 min. To the chemically modified phage (typically 10^{10}–10^{11} t.u. dissolved in 2 ml washing buffer), we added 1 ml of washing buffer containing 3% (w/v) BSA and 0.3% (v/v) Tween 20, and after 30 min 0.5 ml antigen was added and incubated for 30 min on a rotating wheel at room temperature. 50 μl of magnetic streptavidin beads (Dynal, M-280 from Invitrogen) pre-incubated for 30 min in 0.5 ml of washing buffer containing 1% (w/v) BSA and 0.1% (v/v) Tween 20 were added to the phage/antigen mixture and incubated for 5 min at room temperature with rotation. The beads were washed eight times with washing buffer containing 0.1% (v/v) Tween 20 and twice with washing buffer before incubation with 100 μl of 50 mM glycine, pH 2.2 for 5 min. Eluted phage were transferred to 50 μl of 1 M Tris-Cl, pH 8 for neutralization and incubated with 50 ml of TG1 cells at optical density at 600 nm (OD_{600}) = 0.4 for 90 min at 37 °C, and the cells were plated on large 2YT/chloramphenicol plates. We performed two additional rounds of panning using the same procedures. In the second round of selection, neutravidin-coated magnetic beads were used to prevent the enrichment of streptavidin-specific peptides. The neutravidin beads were prepared by reacting 0.8 mg neutravidin (Pierce) with 0.5 ml tosyl-activated magnetic beads (Dynal, M-280 from Invitrogen) according to the supplier's instructions.

Screening selected clones for inhibitory activity. We cloned the genes that encode the peptides selected in the second and third rounds of biopanning into a pUC119-based vector for expression of the peptide–D1-D2 fusion proteins (disulfide-free D1-D2 protein; the cloning and expression procedures are described in the **Supplementary Methods**). Oxidized sulfhydryl groups of the peptides were reduced by incubation of the protein (1–10 μM) with 1 mM TCEP in 20 mM NH_4HCO_3, pH 8 at 42 °C for 1 h. The reducing agent was removed by size exclusion chromatography with a PD-10 column (Amersham Pharmacia) using 20 mM NH_4HCO_3, 5 mM EDTA, pH 8 buffer. The thiol groups of the proteins were reacted by incubation with 10 μM TBMB in reaction buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8, 20% (v/v) ACN) at 30 °C for 1 h. For removal of nonreacted TBMB and concentration, the protein was filtered with a Microcon YM-30 (Millipore). The concentrations of the products were determined by measuring the optical absorption at 280 nm. The IC_{50} was measured by incubating various concentrations of the modified peptide fusion proteins (twofold dilutions) with human plasma kallikrein (0.1 nM) or cathepsin G (20 nM) and determining the residual activity in 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% (w/v) BSA, 0.01% (v/v) Triton-X100. Human plasma kallikrein activity was measured with the fluorogenic substrate Z-Phe-Arg-AMC (Bachem) at a concentration of 100 μM on a Spectramax Gemini fluorescence plate reader (excitation at 355 nm, emission recording at 460 nm; Molecular Devices). Human cathepsin G activity was measured with the colorimetric substrate N-Suc-Ala-Ala-Phe-Pro-pNA (Bachem) at a concentration of 1 mM with a Spectramax absorption plate reader (recording at 410 nm; Molecular Devices).

Chemical synthesis of bicyclic peptides. Peptides with a free amine at the N terminus and an amide at the C terminus were chemically synthesized on a 25 mg scale by solid-phase chemistry (JPT Peptide Technologies). The crude peptides (1 mM) in 1 ml 70% (v/v) 20 mM NH_4HCO_3 pH 8 and 30% (v/v) ACN were reacted with TBMB (1.2 mM) for 1 h at room temperature. The reaction product was purified by reversed-phase HPLC using a C18 column and gradient elution with a mobile phase composed of ACN and 0.1% (v/v) aqueous trifluoroacetic acid (TFA) solution at a flow rate of 2 ml min^{-1}.

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The purified peptides were freeze-dried and dissolved in DMSO or a buffer of 50 mM Tris-Cl pH 7.8, 150 mM NaCl for activity measurements.

Other methods. The protocols for the cloning of the phage libraries, the cloning and expression of peptide fusion proteins, the activity measurement of the inhibitors and the structure determination can be found in the **Supplementary Methods**.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

C.H. and G.W. conceived the experiments, analyzed the data and wrote the article; C.H. performed the experiments; T.R. and S.F. solved the NMR structure.

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