Peptide inhibitor of pancreatic lipase selected by phage display using different elution strategies

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Abstract Interference with fat hydrolysis results in the reduced use of ingested lipids. Inhibition of pancreatic lipase reduces the efficiency of fat absorption in the small intestine and thereby initiates modest long-term reduction in body weight. In an attempt to select peptides with affinity for the surface of pancreatic lipase and potential inhibitory activity, a random, cyclic heptapeptide phage-displayed library was used. Five independent selections, differing in elution step, were performed. In three selection protocols, a sequential elution strategy was applied in anticipation of improving the selection of high-affinity clones. Four heptapeptides with the highest affinity, seemingly for pancreatic lipase, were selected, synthesized, and characterized for their capacity to inhibit enzyme function. Although no clear consensus among the sequenced peptides was found, one of the selected peptides inhibited pancreatic lipase with an apparent inhibition constant of 16 μM.—Lunder, M., T. Bratkovič, S. Kreft, and B. Štrukelj. **Peptide inhibitor of pancreatic lipase selected by phage display using different elution strategies. *J. Lipid Res.* 2005.** 46: **1512–1516.**

Supplementary key words sequential elution • obesity • peptide drug leads

Obesity is a severe chronic disease that can lead to multiple long-term complications such as type 2 diabetes mellitus, hypertension, and osteoarthritis. Drugs that support the conventional strategies for decreasing excess body weight are helpful. However, centrally acting drugs may involve unpredictable risks. To date, orlistat is the only drug that reduces food intake by a peripheral mechanism of action. Orlistat, or tetrahydrolipstatin (THL), is a selective and potent inhibitor of gastric and pancreatic lipases. This inhibition is based on an almost irreversible reaction between the β-lactone secondary ester, which is the reactive part of orlistat, and the serine residue 152 at the catalytic site of the enzyme (1).

Inhibition of the digestion of dietary lipids is a logical target for pharmacological intervention because it does not involve a central mechanism of action. To obtain peptides as potential drug leads that inhibit pancreatic lipase, phage display technology was used.

Selection of peptides from phage-displayed, random combinatorial peptide libraries has proved a successful technique for discovering new ligands of enzymes and other protein targets (2–5). Results indicate that peptides isolated through phage display act as "surrogate ligands" and target only a few sites on a given protein. In many cases, biological activity can be associated with these sites. These peptides are modulators of protein function and are the starting point for identifying and synthesizing compounds with peptide characteristics but nonpeptide structures, the peptidomimetics (drug leads) (6). Phage-displayed peptide libraries have been used successfully to isolate peptide ligands directed to a functional site for which the natural ligand is not a protein or peptide (7), which is also the case with pancreatic lipase.

A random phage-displayed peptide library comprises a vast population of bacteriophages, each of which expresses a unique peptide sequence on its surface. This is accomplished by introducing a synthetic partially randomized oligonucleotide sequence into the gene for one of the phage coat proteins and results in a hybrid fusion protein. Affinity selection of phage clones displaying a specific peptide is based on immobilizing a target molecule to a solid support and incubating the phage suspension over the immobilized molecule of interest. Clones that display peptides that are sterically and electrostatically complementary to binding sites on the target molecule bind to it, whereas others are washed away. Bound clones are subsequently eluted and multiplied by infection of bacterial hosts for further rounds of selection. Finally, individual phage clones are isolated and the sequence of the inserted nucleotide fragment of the fusion coat protein is determined to give the deduced primary structure of the inserted peptide (8).

Tightly bound phage clones can be released from im-

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Abbreviations: $K_{i(app)}$, apparent inhibition constant; PBST, PBS containing Tween 20; pfu, plaque-forming units; THL, tetrahydrolipstatin.

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mobilized targets by different elution conditions. We performed five independent selections varying the elution step in an attempt to select peptides with affinity for the surface of pancreatic lipase and, possibly, inhibitory activity that could be used for the molecular design of potential antiobesity agents. Two selection protocols were basic, using only the irreversible inhibitor THL or free target. In three selection protocols, a sequential elution strategy was applied in anticipation of improving the selection of high-affinity clones. Four heptapeptides with the highest affinity for immobilized targets were selected, synthesized, and characterized for their capacity to inhibit enzyme function. Only one of these peptides (D23) inhibited pancreatic lipase {the apparent inhibition constant [$K_{i(app)}$] was 16 μM}, although the corresponding phage clone did not show a higher affinity for the immobilized target than the other tested clones. Based on the elution protocol that yielded this clone and micropanning results, the inhibitory effect is the result of binding to the active site of the enzyme and thus preventing the binding of substrate.

MATERIALS AND METHODS

Immobilization of the target molecule

Pancreatic lipase from lyophilized porcine pancreas with 70% protein content (L-0382; Sigma-Aldrich, Steinheim, Germany) was dissolved in 50 mM $NaHCO_3$ and 0.1% deoxycholic acid sodium salt (Sigma-Aldrich), pH 8.5, to a final concentration of 250 μg/ml. Maxisorp surface microtiter plate (Nalge Nunc International, Roskilde, Denmark) wells were filled with 200 μl of pancreatic lipase solution and incubated for 2 h at room temperature with gentle agitation. Pancreatic lipase solution was discarded, and microtiter plates were blocked using 250 μl of 2% BSA in PBS buffer (135 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) for 1 h at room temperature and rinsed four times with PBS containing 0.1% Tween 20 (PBST).

Affinity selection of the phage display library

Selection of peptides from a Ph.D.-C7C™ random cyclic heptapeptide phage-displayed library was carried out according to the manufacturer's instructions (New England Biolabs, Beverly, Massachusetts). An aliquot of 2×10^{11} plaque-forming units (pfu) was diluted to 100 μl with PBST and incubated in lipase-coated wells for 1 h at room temperature with gentle agitation. Nonbinding phages were then discarded by washing the wells 10 times with PBST. Bound clones were eluted with different elution strategies (**Table 1**).

In selection protocols A, B, and C, bound phages were first nonspecifically released with 100 μl of 50 mM glycine-HCl, pH 2.2, for 10 min. This eluate fraction was immediately neutralized with 100 μl of 200 mM phosphate buffer, pH 7.5. Bound phages were then sequentially eluted with THL solution, glycine-HCl (pH 2.2), and porcine pancreatic lipase solution. In all three selection protocols, THL solution was used in the last step of sequential elution. Selection protocols D and E were basic, using only THL and pancreatic lipase solution, respectively. Four rounds of biopanning were performed for each selection protocol (A–E). With the exception of the first round of each selection protocol, only the phages collected in the last elution step were amplified for the next round of biopanning.

Eluates were amplified by infecting *Escherichia coli* ER2738 host cells. After 5 h of growth at 37°C, bacteria were removed by centrifugation and phages in the supernatant were precipitated by adding one-sixth volume of polyethylene glycol/NaCl solution (20% polyethylene glycol-8000 and 2.5 M NaCl) and overnight incubation at 4°C. The precipitate was resuspended in a small volume of PBS, and amplified eluates were titered to determine phage concentration. This selection procedure was repeated three more times, increasing the Tween concentration to 0.5% in the washing steps. Finally, eluates from the last round of selection were used to infect plated bacterial host cells, and 20–40 resulting plaques were randomly selected. Individual phage clones were then grown and purified for further analysis.

Phage ELISA

Microtiter plate wells were coated with 100 μl of pancreatic lipase solution (100 μg/ml) in 50 mM $NaHCO_3$ and 0.1% deoxycholic acid sodium salt, pH 8.5, for 2 h at room temperature and blocked with 200 μl of blocking buffer (2% BSA in PBS) for 1 h. A separate set of wells was blocked with blocking buffer without previous lipase immobilization as negative controls. One hundred microliters of each selected amplified phage clone was diluted to 200 μl with blocking buffer and transferred to coated wells. Plates were incubated for 1.5 h at room temperature. Wells were then washed three times with 0.075% PBST. Horseradish peroxidase-labeled mouse anti-M13 monoclonal antibody (Amersham Biosciences, Little Chalfont, UK) in blocking buffer (1:5,000), 200 μl per well, was added and incubated for 1 h at room temperature. Finally, wells were washed four times with 0.075% PBST. Two hundred microliters of substrate solution [0.22 mg/ml diammonium 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) in 50 mM citric acid and 1.7 μl of 30% H_2O_2/ml, pH 4.0] was added and incubated for 30 min at 37°C. Absorbance at 405 nm was then determined using a microtiter plate reader. Clones giving rise to an absorbance of >0.3 (the absorbance of negative controls was typically in the range of 0.06–0.12) were selected for DNA sequencing.

DNA sequencing

Single-stranded DNA from amplified selected phage clones was isolated by denaturing coat proteins with iodide buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 4 M NaI] and precipitation with ethanol. Purified DNA was sequenced by MWG Biotech sequencing service (Munich, Germany).

TABLE 1. Elution strategies used in the selection of lipase binding peptides

Eluent Solution	Elution Step	Selection Protocol				
		A	B	C	D	E
100 μl of 0.05 M glycine-HCl (pH 2.2)	1	10 min	10 min	10 min	—	—
200 μl of 1 mM tetrahydrolipstatin (PBS)	2	60 min	—	—	—	—
200 μl of 0.05 M glycine-HCl (pH 2.2)	3	10 min	—	—	—	—
200 μl of 250 μg/ml porcine pancreatic lipase	4	—	—	60 min	—	60 min
200 μl of 1 mM tetrahydrolipstatin (PBS)	5	60 min	120 min	60 min	60 min	—

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Peptide synthesis

Selected peptides were synthesized by Jerini Peptide Technologies. The product was crude and supplied as a trifluoroacetate salt. Identity was determined by mass spectrometry. The spacer sequence Gly-Gly-Gly-Ser was added to the C terminus, and the C-terminal carboxylate was amidated to block the negative charge. The purity of the peptides was assumed to be 50%.

Inhibitory activity determination of synthetic peptides

Enzyme, peptides, and substrate were dissolved in buffer composed of solution I and solution II in volume ratio 8.5:1.5. Solution I was 41 mM Tris buffer, pH 8.4, with 1.8 mM sodium deoxycholate (Sigma-Aldrich) and 7.2 mM sodium taurodeoxycholate (Sigma-Aldrich). Solution II was 1.6 mM tartrate buffer, pH 4.0, with 0.1 mM calcium chloride. A constant amount of the enzyme (20 μg/ml) was preincubated with various amounts of a peptide on microtiter plates (TPP 96fb; Tissue Culture Plates) for 20 min at 37°C. One hundred microliters of 0.25 mM substrate *p*-nitrophenyl palmitate was then added to a final volume of 200 μl, and absorbance was measured at 405 nm at 30 s intervals for 25 min at 37°C. $Ki_{(app)}$ was determined from the rate of substrate hydrolysis with (V_i) and without (V_0) inhibitor. The slope of a plot of $V_i/V_0 - 1$ against the concentration of inhibitor [I] gives $1/Ki_{(app)}$.

Phage affinity titrations

The relative affinities of a selected phage to immobilized proteins were determined using serial dilutions of phage of known titer in phage ELISA as described above. Background signals of target protein exposed to antibody but no phage were subtracted from each value.

Micropanning

Coated and blocked (as described above) microtiter plate wells were washed four times with 0.1% PBST. Next, test wells were incubated for 30 min with agitation at 50 rpm with 1 mM THL or 1 mM peptide D23 (in buffer composed of solutions I and II as described above) to occupy binding sites on the immobilized target, and control wells were incubated with corresponding buffer. One hundred microliters of 0.5% PBST containing 10^8 phages was transferred into each well and incubated for 1 h. Next, wells were washed 12 times with 0.7% PBST. The remaining phages were eluted with glycine-HCl (pH 2.2) and their titers were determined.

RESULTS AND DISCUSSION

Assurance of quality of the immobilized target protein is one of the most important preconditions for successful selection of ligands from phage display libraries. Ideally, the activity of the immobilized protein should be retained (this is easily checked when one is dealing with enzyme targets) and the coating solution should contain no contaminants (especially proteins other than the target, because they act as "decoys" and lead to selections of ligands unrelated to the primary target of interest) (9). However, numerous reports of successful panning experiments with polyclonal antibodies (10–12) or even whole IgG from sera (13–15) encouraged us to use an isolate of pancreatic proteins enriched for triacylglycerol lipase as the source of our target protein.

Elution conditions are the second key step of affinity selection. Tightly bound phage clones can be released from immobilized target by different elution conditions. Two of the applied selection protocols (D and E) were basic. We used specific elution with a solution of the irreversible inhibitor THL or a solution of the free target (pancreatic lipase) to compete the bound phage away from the immobilized target on the plate. Specific elution seeks to release phages that are bound to the target protein binding site without releasing phages that are bound nonspecifically, for example, to the plastic support, to BSA used to block unoccupied adsorption sites, or to other proteins present in the lyophilized extract from porcine pancreas (8).

In three selection protocols (A, B, and C), a sequential elution strategy was applied in anticipation of improving the selection of high-affinity clones. Other efforts that improve the selection of high-affinity clones, such as extensive washing to dissociate low-affinity phage and increasing the concentration of Tween 20 to decrease nonspecific binding, were also incorporated in all protocols. The aim was to select clones that have strong interactions with the active site of pancreatic lipase.

In the first round, all eluates were collected and amplified to avoid the loss of high-affinity clones at the beginning of biopanning. In the next three rounds, only the phages from the last elution step were collected, and other eluates were discarded.

In selection protocol A, an alternating elution strategy was used (16). Low-pH buffer and inhibitor solution were used in turn as the elution reagents. After the third elution step of the first round of panning, 13,000 pfu could still be eluted (**Table 2**). With nonspecific elution with acidic buffer in the first step, we intended to weaken receptor-peptide interactions without regard to their specificity. Phages eluting in the later stage should have higher affinity, because the protonation effect of the atoms near the affinity site, as a result of the use of low-pH buffer, is not enough to unbind the high-affinity phages (16).

Selection protocol B was very similar, except that two-step instead of four-step elution was used and the last elution step with THL solution was prolonged. Once the elution step with glycine buffer had removed the low-affinity clones, 31,000 pfu (one-half of all eluted phages) were obtained in the second step. This proportion was much higher than in the last step of protocol A (Table 2).

Sequential elution using glycine-HCl buffer, pancreatic lipase solution, and THL solution was performed in selection protocol C. The elution step with lipase solution is aimed at eliminating phages that do not bind precisely to

TABLE 2. Titer of joined eluates in plaque-forming units and the portion of phages eluted in the last elution step determined after the first round of panning

	Selection Protocol				
Elution Variable	A	B	C	D	E
Joined eluates	1.7×10^5	6.3×10^4	1×10^5	6×10^5	3.6×10^6
Last elution step	7.6%	50%	24%	100%	100%

Phage input titer was 2×10^{11}. In selection protocols D and E, the last elution step was the only step.

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TABLE 3. Clones from different selection protocols giving rise to an absorbance of >0.3 were selected for DNA sequencing

Selection Protocol A	Peptide	Selection Protocol B	Peptide	Selection Protocol C	Peptide	Selection Protocol D	Peptide	Selection Protocol E	Peptide
A03	CLREQPQQC	B08	CTALMSASC	C10	CTALMSASC	D04	CPDANRINC	**E06**	**CSQLQTTKC**
A05	CTALMSASC	B12	CTALMSASC	**C11**	**CTALMSASC**	D10	CSQAPTPAC		
A09	CTALMSASC			C13	CTALMSASC	D11	CPPSYNGKC		
A12	CTALMSASC					D19	CPPSYNGKC		
A18	CTALMSASC					**D21**	**CPPSYNGKC**		
A21	CTALMSASC					D22	CTPTSPAMC		
A24	CTALMSASC					**D23**	**CQPHPGQTC**		

Peptides selected for synthesis are shown in boldface.

the active site of pancreatic lipase but also to other (e.g., allosteric) sites on the enzyme. This kind of elution, however, could also release phages bound to other adsorbed pancreatic proteins. The last elution step was anticipated to collect phages bound strongly to the active site that were not desorbed during competition with binding sites on the free target or with nonspecific elution.

The results clearly show that with more elution steps, fewer phage clones are obtained in the last eluate. However, the clones that persist through all previous elutions should have the highest affinity for the target.

Rapid confirmation of binding activity by ELISA was performed for 20–40 random clones from each selection protocol after the last round of panning. Clones giving rise to an absorbance of >0.3 were sequenced, and their inserted heptapeptides are listed in **Table 3**.

Nonspecific elution, with glycine incorporated in protocols A, B, and C, radically reduced the diversity of selected clones. Surprisingly, all three independently performed sequential elution protocols produced the same peptide. Eleven heptapeptide sequences of the 12 clones were exactly the same (CTALMSASC). Interestingly, this sequence, inserted in a disulfide loop, strongly resembles ankyrin repeats that are the most common protein-protein interaction motifs in nature. They occur in a large number of functionally diverse proteins, mainly from eukaryotes (17). In protocol A, another unrelated peptide was found (A03). In protocol D, greater diversity of the clones was observed. This was probably because this was the least exhaustive elution procedure. This elution strategy also led to a peptide with inhibitory activity. Even though sequential elution greatly reduces the diversity of the selected clones, this does not ensure the identification of a protein function modulator.

In selection protocol E, only one phage clone exceeded an absorbance of 0.3. The absence of elution steps that would be discarded causes greater diversity, which could be overcome by further rounds of panning. On the other hand, introducing additional rounds of selection in protocol E would probably do little to improve the specificity of interaction of the selected clones with pancreatic lipase because phages are competed away from all of the immobilized proteins with the same probability.

Because different concentrations of phage for each amplified clone were used in phage ELISA, the test gave only a rough estimate of the affinity. Therefore, four clones were selected according to their absorbance and frequency of occurrence. They were further amplified and titered. Relative affinities were determined by conducting phage ELISA using serial dilutions of phage. Absorbance readings at 405 nm produced by 5×10^{10} pfu were found to be most informative for judging the relative affinities of the dis-

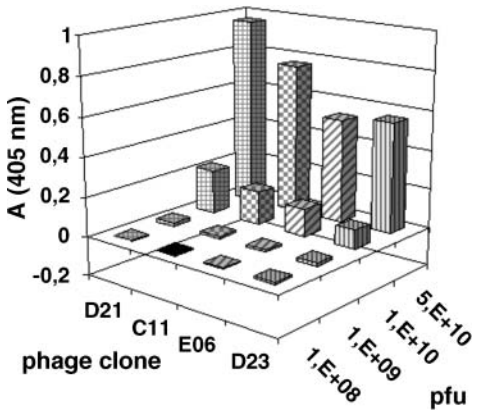

Fig. 1. Absorbance (A) at 405 nm in ELISA produced by increasing the concentrations of different phage clones. Phage clone D21 displays the highest relative affinity. pfu, plaque-forming units.

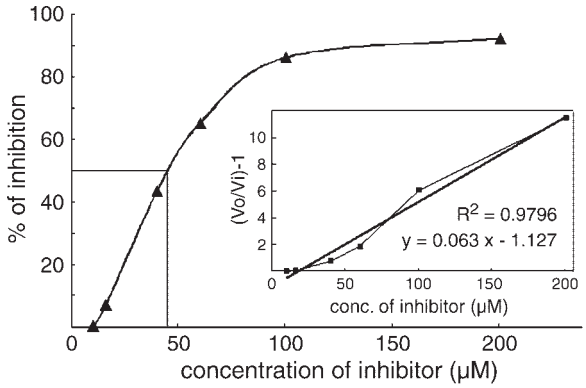

Fig. 2. Inhibitory curve for peptide D23. IC_{50} determined from the graph is <50 μM. The apparent inhibition constant [$K_{i(app)}$] was determined from the rate of substrate hydrolysis with (V_i) and without (V_0) inhibitor. The slope of a plot of $V_i/V_0 - 1$ against the concentration of inhibitor [I] gives $1/Ki_{(app)}$. Compared with tetrahydrolipstatin, which completely inhibited pancreatic lipase in this test at a concentration of <0.1 μM, peptide D23 is a much weaker inhibitor.

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played peptides. Phage D21 displayed the highest relative affinity for the immobilized target, followed by clone C11. Clones E06 and D23 exhibited the lowest affinity. At 10-fold smaller amounts of phage per well, affinity could not be detected. Results are shown in **Fig. 1**.

The presence of numerous contaminants in pancreatic lipase preparation, which increased the probability of the enrichment of irrelevant clones, required further analysis to test the specificity of interaction of selected sequences with the lipase. Therefore, the peptides that are displayed on clones C11, D21, D23, and E06 were synthesized and tested for their ability to act as inhibitors of enzyme activity. Peptide D23 inhibited pancreatic lipase with $K_{i(app)}$ of 16 μM (**Fig. 2**). Other tested peptides (D21, C11, and E06) did not inhibit enzymatic activity (data not shown), indicating that they either bind to the lipase such that they fail to block the active site or are selected on the basis of affinity to some other pancreatic protein. This is especially plausible with selection protocols E and C, comprising elution steps with a heterogeneous pancreatic isolate. However, this is possible with other selection protocols as well, because THL also inhibits other lipases (e.g., carboxyl ester lipase of pancreatic origin) (18), which could be present in the lyophilizate, or THL can bind to BSA, which contains binding sites for lipophilic molecules.

Because phage D23 was eluted from the target with THL solution, we assumed that it binds in the active site of pancreatic lipase. Phage D21 was eluted by the same elution protocol and, in spite of its strong affinity, showed no inhibitory action. Results of micropanning show that THL indeed prevents the binding of phage clones D21 and D23 and furthermore has no effect on the binding of phage clone K4 used as a negative control. Sixty-eight percent less phage D23, 60% less phage D21, and only 4% less control phage K4 (obtained in selection on streptavidin) were bound to the inhibited target. Synthetic peptide D23 binds to the same binding site as the corresponding phage, because incubation with the solution of synthetic peptide instead of THL reduced the percentage of bound phage D23 to the same extent (67% less phage). According to the micropanning experiment and elution strategy used, we conclude that peptide D23 binds to the active site of pancreatic lipase. As for peptide 21, we consider the two previously mentioned possibilities.

Results clearly indicate that the selected peptide D23 with amino acid sequence CQPHPGQTC effectively inhibits pancreatic lipase and is thus the starting drug-leading compound. Sequential elution introduced in the selection protocol drastically reduced the diversity of the selected clones, although that did not ensure the identification of a protein function modulator.

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