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An *in vivo* study of novel bioactive peptides that inhibit the growth of *Escherichia coli*

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Abstract: We have created a system in which synthetically produced novel bioactive peptides can be expressed *in vivo* in *Escherichia coli*. Twenty thousand of these peptides were screened and 21 inhibitors were found that could inhibit the growth of *E. coli* on minimal media. The inhibitors could be placed into one of two groups, 1-day inhibitors, which were partially inhibitory, and 2-day inhibitors, which were completely inhibitory. Sequence analysis showed that two of the most potent inhibitors were actually peptide–protein chimeras in which the peptides had become fused to the 63 amino acid Rop protein which was also contained in the expression vector used in this study. Given that Rop is known to form an incredibly stable structure, it could be serving as a stabilizing motif for these peptides. Sequence analysis of the predicted coding regions from the next 10 most inhibitory peptides showed that four of the 10 peptides contained one or more proline residues either at or very near the C-terminal end of the peptide which could act to prevent degradation by peptidases. Collectively, based on what we observed in our screen of synthetic bioactive peptides that could prevent the growth of *E. coli* and what has been learned from structural studies of naturally occurring bioactive peptides, the presence of a stabilizing motif seems to be important for small peptides, if they are to be biologically active.

Abbreviations: IPTG, isopropyl β-D-thiogalactoside.

Bioactive peptides are small peptides that elicit a biological activity. Over 500 of these peptides, which average 20 amino acids in size, have now been identified and characterized (1–3). They have been isolated in a variety of systems,

exhibit a wide range of actions, and have been utilized as therapeutic agents in the field of medicine, as well as diagnostic tools in both basic and applied research. Some of the better known peptides employed as therapeutic agents include calcitonin, gastrin, glucagon, luteinizing hormone releasing factor, oxytocin, secretin, somatostatin and vasopressin (4).

Where the mode of action of these peptides has been determined, it has been found to be due to the interaction of the bioactive peptide with a specific protein target (5). In most cases, the bioactive peptide acts by binding to and inactivating its protein target with incredibly high specificity. Binding constants of these peptides for their protein targets typically have been determined to be in the nM range (1,2) with binding constants as high as 10^{-12} M being reported (6,7).

There has been an increasing interest in employing synthetically derived bioactive peptides as novel pharmaceutical agents due to the inherent ability of these peptides to bind to and inhibit specific protein targets (8–10). Three obvious areas in which synthetically produced peptides could be utilized are in the development of new antibacterial, antiviral and anticancer agents. To develop antibacterial or antiviral agents, synthetically derived peptides would be isolated which could either bind to and prevent bacterial or viral surface proteins from interacting with their host cell receptors, or else prevent the action of specific toxin or protease proteins. To develop anticancer agents, synthetically derived peptides would be isolated which could bind to and prevent the action of specific oncogenic proteins.

Researchers have been engineering novel bioactive peptides through the use of two different *in vitro* approaches. The first approach involves the chemical synthesis of a randomized library of 6–10 amino acid peptides (8,9,11). In the second approach, a randomized oligonucleotide library is cloned into a Ff filamentous phage gene which allows peptides that are 6–38 amino acids in length to be expressed on the surface of the bacteriophage (10,12). The resulting peptide libraries are then usually mixed with a matrix-bound protein target. Peptides that bind are eluted and their sequences determined. From this information new peptides are synthesized and their inhibitory properties determined. Although there has been some limited success using this *in vitro* approach and a few inhibitor peptides have been developed, the use of synthetically derived peptides has not yet become a mainstay in the pharmaceutical industry.

We wanted to develop an *in vivo* approach which would allow us to isolate numerous inhibitor peptides and rationalized that anything we learned about what makes these peptides function could then be utilized in the more applied *in vitro* systems in order to develop new pharmaceutical agents. To maximize our chances of isolating inhibitor peptides, we did not want to focus our efforts on only one target and thus decided to pursue intracellularly produced peptides which could inhibit the growth of *Escherichia coli*. Any intracellular protein that was necessary for the normal cellular growth of *E. coli* would be a target in this approach.

To implement this *in vivo* approach, an oligonucleotide library capable of encoding up to 20 amino acid peptides is cloned into an expression vector which allows the peptides to either be turned off or overproduced in the cytoplasm of *E. coli*. This pool of clones is transformed into *E. coli* under repressed conditions and the resulting bacterial transformants are then screened for any that can not grow on minimal media when the peptides are overproduced. In this scheme, any transformant bacterial colony which overproduces a peptide that inhibits a protein necessary for growth on minimal media will be identified.

Experimental Procedures

Media

Rich Luria-Bertani and minimal M9 media used in this study was prepared as described by Miller (13). Ampicillin was used in rich media at a final concentration of 100 μg/mL and in minimal media at a final concentration of 50 μg/mL. Isopropyl β-D-thiogalactoside (IPTG) was added to media at a final concentration of 1 mM.

Bacterial strains and plasmids

ALS225 which is MC1061/F′*lacI*^{q1}*Z*+*Y*+*A*+ was the *E. coli* strain used in this study (14). The genotype for MC1061 is *araD139* Δ*(araABOIC-leu)7679* Δ*(lac)X74 galU galK rpsL hsr-hsm*+ (15). pLAC11 is the highly regulable expression vector that was used in this work (14).

Generating the randomized peptide library

The 93 bp oligonucleotide 5′-TACTATAGATCTATG-$(XXX)_{20}$TAATAAGAATTCTCGACA-3′,where X denotes an equimolar mixture of the nucleotides A, C, G or T, was synthesized with the trityl group on and subsequently purified using an OPC cartridge. The complementary

strand of the 93 bp oligonucleotide was generated by a fill-in reaction with Klenow using an equimolar amount of the 18 bp oligonucleotide primer 5′-TGTCGAGAATTCTTATTA-3′. After extension, the resulting ds-DNA was purified using a Promega DNA clean-up kit and restricted with *Bgl*II and *Eco*RI. The digested DNA was again purified using a Promega DNA clean-up kit and ligated to the pLAC11 vector which had been digested with the same two restriction enzymes. The resulting library was then transformed into electrocompetent ALS225 cells.

Sequencing the coding regions of the inhibitor peptide clones

The forward primer 5′-TCATTAATGCAGCTGGCACG-3′ and the reverse primer 5′-TTCATACACGGTGCCTGACT-3′ were used to sequence both strands of the inhibitor peptide clones. If an error-free consensus sequence could not be deduced from these two sequencing runs, both strands of the inhibitor peptide clones in question were resequenced using the forward primer 5′-TAGCTCACTCATTAGGCACC-3′ and the reverse primer 5′-GATGACGATGAGCGCATTGT-3′. The second set of primers were designed to anneal downstream of the first set of primers in the pLAC11 vector.

Generating antisense derivatives of the inhibitor clones

Oligonucleotides were synthesized which duplicated the DNA insert contained between the *Bgl*II and *Eco*RI restriction sites for five of the anchorless inhibitor peptides with one major nucleotide change. The 'T' of the ATG start codon was changed to a 'C' which resulted in an ACG which can not be used as a start codon. The oligonucleotides were extended using the same 18 bp oligonucleotide primer that was used to construct the original peptide library. The resulting ds-DNA was then restricted, and cloned into pLAC11 exactly as described in the section 'Generating the randomized peptide library' in Materials and Methods. The antisense oligonucleotides that were used are as follows: pPep1 (antisense), 5′-TACTATAGATCTACGGTCACTGAATTTTGTGGCTTGTTGGACCAACTGCCTTAGTAATAGTGGAAGGCTGAAATTAATAAGAATTCTCGACA-3′; pPep5 (antisense), 5′-TACTATAGATCTACGTGGCGGGACTCATGGATTAAGGGTAGGGACGTGGGGTTTATGGGTTAAAATAGTTTGATAATAAGAATTCTCGACA-3′; pPep12 (antisense), 5′-TACTATAGATCTACGAACGGCCGAACCAAACGAATCCGGGACCCACCAGCCGCCTAAACAGCTACCAGCTGTGGTAATAAGAATTCTCGACA-3′; pPep13 (antisense), 5′-TACTATAGATCTACGGACCGTGAAGTGATGTGTGCGGCAAAACAGGAATGGAAGGAACGAACGCCATAGGCCGCGTAATAAGAATTCTCGACA-3′; pPep19 (antisense), 5′-TACTATAGATCTACGAGGGGCGCCAACTAAGGGGGGGGGAAGGTATTTGTCCCGTGCATAATCTCGGGTGTTGTCTAATAAGAATTCTCGACA-3′.

Secondary structure predictions

The propensity for the inhibitor peptides to form α-helixes or β-sheets was initially considered using both Chou and Fasman (16,17) and Garnier, Osguthorpe, and Robson analysis (18,19). The peptide sequences were then analyzed using the PEPTOOL protein structure software package from BioTools Inc. The membrane spanning domain in pPep6 was analyzed using AMPHI (20), SOAP (21), SOSUI (22) and TMpredict (23) algorithms, as well as the predictive rules of Klein *et al*. (24).

Chemicals and reagents

Extension reactions were carried out using Klenow from New England Biolabs while ligation reactions were performed using T4 DNA Ligase from Life Sciences. IPTG was obtained from Diagnostic Chemicals Limited.

Results

Construction of the peptide library

In order to isolate potential inhibitor peptides, a totally randomized oligonucleotide library that encoded up to 20 amino acid peptides was cloned into the pLAC11 expression vector (14) as shown in Fig. 1 and transformed

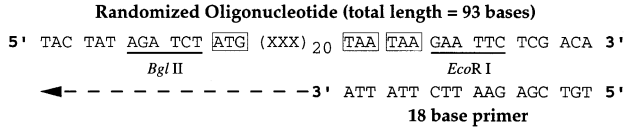

Figure 1. Scheme for generating the randomized 20 amino acid peptide library *in vivo*. The complementary strand of the 93 bp randomized oligonucleotide is generated by filling-in with Klenow using the 18 bp oligonucleotide primer. The resulting ds-DNA is digested with *Bgl*II and *Eco*RI, and then ligated into the pLAC11 expression vector which has also been digested with the same two restriction enzymes. Wherever an 'X' occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G and T, is used.

into *E. coli* under repressed conditions. Most of the routinely employed expression vectors such as pKK223-3, pKK233-2, pTrc99A and the pET series produce significant amounts of protein from cloned genes even when grown under repressed conditions (25,26). We were concerned that we might not be able to clone potent inhibitor peptides unless we were able to isolate them under completely repressed conditions. For this reason, we chose to use the highly regulable pLAC11 expression vector for our studies. Because encoded peptides that are expressed using pLAC11 are under the control of the wild-type *lac* operon, their expression can be turned on or off by the presence or absence of the gratuitous inducer, IPTG.

Researchers employing the fusion-phage technology, which also utilizes oligonucleotides to encode randomized amino acids in synthesized peptides, have used [XX(G,T)] or [XX(G,C)] codons instead of [XXX] codons to eliminate two of the three stop codons, thus increasing the amount of full-length peptides that can be synthesized without a stop codon (12). We opted to use the [XXX] codon design instead for the following reasons. First, both the [XX(G,T)] and [XX(G,C)] oligonucleotide codon schemes eliminate half of the codons and thus bias the distribution of amino acids that are generated. Second, the [XX(G,T)] and [XX(G,C)] codon schemes drastically affect the preferential codon usage of highly expressed genes and remove a number of the codons utilized by the abundant tRNAs present in *E. coli* (27,28). Interestingly, when synthesizing a 20 amino acid randomized peptide using the [XX(G,T)] or [XX(G,C)] codon design, in theory, 53% of the peptides will not contain a stop codon. This percentage is derived via the following calculation: $(31 \text{ encoding codons}/32 \text{ total codons})^{20 \text{ amino acids}}$. Using the [XXX] codon design, 38% of the peptides will not contain a stop codon: $(61 \text{ encoding codons}/64 \text{ total codons})^{20 \text{ amino acids}}$. We did not feel that the 15% increase that results from the [XX(G,T)] or [XX(G,C)] codon design was worth the possible risks of introducing codon bias and nonpreferred codons into the oligonucleotides and thus chose to utilize the [XXX] motifs in our randomized libraries.

Identifying and characterizing inhibitor peptides from the library

Using a grid-patching technique in which the clones were patched onto both rich repressing plates and minimal inducing plates, we screened 20,000 potential candidates. Minimal medium, which imposes more stringent growth demands on the cell, was chosen for the screen in order to maximize the number of inhibitors that could be found. It is well known that growth in minimal media puts more demands on a bacterial cell than growth in rich media, as evidenced by the drastically reduced growth rate, and thus a peptide that adversely affects cell growth would be more likely detected on minimal media. We isolated 21 IPTG-dependent inhibitors in this screen and found that they could be categorized into two classes based on their inhibitory properties on plates. One-day inhibitors either showed no or very little growth after 24 h, but showed significant growth after 48 h. Two-day inhibitors were completely inhibitory and showed no growth for a full 48 h. Figure 2 shows a representative plate that differentiates between a 1- and 2-day inhibitor.

To verify that all of the inhibitors were legitimate, we isolated plasmid DNA from each inhibitory clone, transformed them into a fresh background, and then checked that they were still inhibitory on plates and that their inhibition was dependent on the presence of the inducer, IPTG. This was accomplished by patching the retransformed clones onto both minimal glucose ampicillin repressing plates and minimal glycerol ampicillin IPTG inducing plates. In order to make a more accurate assessment of how inhibitory the inhibitors were, we subjected the inhibitors to growth rate analysis in liquid media. To do this, minimal cultures containing either the inhibitor to be tested or pLAC11 as a control were diluted 1 to 100 into minimal media and induced with 1 mM IPTG. Absorbance (A_{550}) readings were then taken hourly until the cultures had passed log phase. Figure 3 shows a comparison of the inhibition caused by a 1- or 2-day inhibitor using the pLAC11 vector as a control. Growth rates were determined as the spectrophotometric

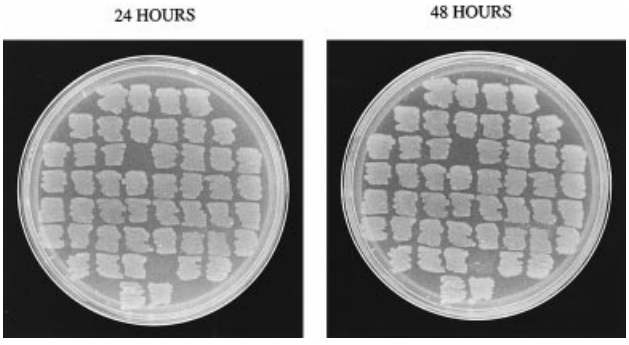

Figure 2. A minimal inducing plate showing the difference between a 1- and 2-day inhibitor. Shown here is a minimal inducing plate that contains both a 1-day and a 2-day inhibitor, photographed at 24 and 48 h. The 2-day inhibitor on row three from the top of the plate is completely inhibitory and does not grow at either 24 or 48 h. The 1-day inhibitor on row seven from the top of the plate does not grow at 24 h but does grow at 48 h.

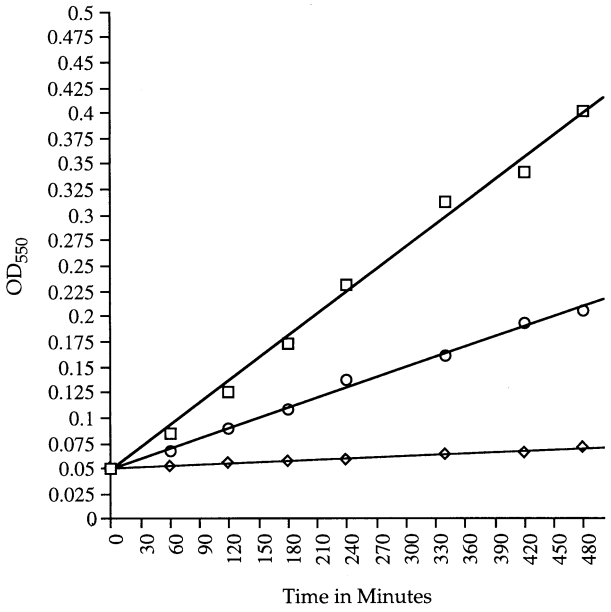

Figure 3. Growth curves showing the inhibitory effects of a 1-day vs. a 2-day inhibitor. ALS225 cells containing the pLAC11 vector (control), and the 1-day inhibitor pPep11, or the 2-day inhibitor pPep12, were grown in minimal M9 glycerol media with IPTG added to 1 mM. At ≈ 1-h intervals A_{550} readings were taken. Data points for pLAC11, pPep11, and pPep12, are indicated by squares, circles, and diamonds, respectively.

change in A_{550} per unit time within the log phase of growth. The inhibition of the growth rate was then calculated for the inhibitors using pLAC11 as a control. As indicated in Table 1, the 1-day inhibitors inhibited the bacterial growth rate at an average of 25%, whereas the 2-day inhibitors inhibited the bacterial growth rate at an average of 75%.

The hypothetical data in Table 2 show how a 25 or 75% inhibition of the growth rate affects the growth of a culture. When the control strain reaches an A_{550} of 0.640, a strain which contains a model peptide that inhibits the growth rate at exactly 50% will have only reached an A_{550} of 0.080. Thus the growth of the culture that is being inhibited by this model peptide will only be 12.5% (0.080/0.640×100) of that of the control strain at this point and the inhibitor peptide would have effectively inhibited the growth of the culture by 87.5% (100–12.5%). A 1-day inhibitor which inhibited the growth rate at 25% would have only reached an A_{550} of 0.226 when the control strain reached an A_{550} of 0.640. Thus the growth of the culture that is being inhibited by a 1-day inhibitor will only be 35.3% (0.226/0.640×100) of that of the control strain at this point and the inhibitor peptide would have effectively inhibited the growth of the culture by 64.7% (100–35.3%). A 2-day inhibitor which inhibited the growth rate at 75% would have only reached an A_{550} of 0.028 when the control strain reached an A_{550} of 0.640. Thus the growth of the culture being inhibited by a 2-day inhibitor will only be 4.4% (0.028/0.640×100) of that of the control strain at this point and the inhibitor peptide would have effectively inhibited the growth of the culture by 95.6% (100–4.4%). These calculations are consistent with the fact that 2-day inhibitors prevent the growth of bacteria on plates for a full 48 h, whereas the 1-day inhibitors only prevent the growth of bacteria on plates for 24 h.

In order to continue our analysis, we next wanted to verify that all of the candidates contained 66 bp inserts as expected (Fig. 1). Although most of them did, two of our most potent 2-day inhibitors, pPep3 and pPep14, were found to contain a huge deletion. Sequence analysis of these clones revealed that the deletion had caused the C-terminal end of the inhibitor peptides to become fused to the N-terminal end of the short 63 amino acid Rop protein (data not shown). The *rop* gene which is part of the ColE1 replicon is located downstream from where the oligonucleotide library is inserted into the pLAC11 vector (14).

Sequence analysis of the top 10 anchorless inhibitor peptides

The top 10 anchorless inhibitor peptides were sequenced and their coding sequences determined (Table 3). Eight of the 10 inhibitors are predicted to encode peptides that are terminated before the double TAATAA termination site which was incorporated into the oligonucleotide. Two of the inhibitors, pPep6 and pPep10, which contain deletions within the randomized portion of the oligonucleotide, are terminated beyond the *Eco*RI site. One of the inhibitors, pPep17, contains a termination signal just after the ATG start codon. However, just downstream is a Shine-Dalgarno site and a GTG codon which should be used as the start codon. Interestingly, the start sites of several proteins such as Rop are identical to that proposed for the pPep17 peptide (29). The average and median length for the eight peptides whose termination signals occurred before or at the double TAATAA termination site was 13 amino acids.

The characteristics of the predicted coding regions of the inhibitor peptides proved to be quite interesting. Three of the 10 peptides, pPep1, pPep13 and pPep17, contained a proline residue as their last amino acid. In addition, one of the peptides, pPep12, contained two proline residues at the $n-2$ and $n-3$ positions. Thus there appears to be a bias for the placement of proline residues at or near the end of several of the inhibitory peptides. Secondary structure analysis predicted that three of the 10 peptides contained

Table 1. **Ability of the inhibitor peptides to inhibit cell growth**

Inhibitor	Type	% Inhibition of the growth rate	Inhibitor	Type	% Inhibition of the growth rate
pLAC11 (control)	–	0	pPep11	1-day	22
pPep1	1 day	25	pPep12	2-day	82
pPep2	1 day	23	pPep13	1-day	28
pPep3	2 day	80	pPep14	2-day	71
pPep4	1 day	21	pPep15	1-day	23
pPep5	1 day	24	pPep16	1-day	24
pPep6	1 day	27	pPep17	1-day	28
pPep7	1 day	26	pPep18	1-day	24
pPep8	1 day	29	pPep19	1-day	29
pPep9	1 day	22	pPep20	1-day	19
pPep10	1 day	24	pPep21	1-day	23

Growth rates for cells containing the induced inhibitors were determined as described in the text and then the percentage inhibition was calculated by comparing these values to the growth rate of cells that contained the induced pLAC11 vector. The averaged values of three independent determinations are shown.

Table 2. **Hypothetical data from peptides that inhibit the growth rate at 25, 50 or 75%**

Time (h)	A_{550} readings on a control culture that contains pLAC11	A_{550} readings on a culture that contains a peptide that inhibits the growth rate at		
		25%	50%	75%
0	0.010	0.010	0.010	0.010
2.5	0.020	0.017	0.015	0.012
5	0.040	0.028	0.020	0.014
7.5	0.080	0.047	0.030	0.017
10	0.160	0.079	0.040	0.020
12.5	0.320	0.133	0.060	0.024
15	0.640	0.226	0.080	0.028

a known motif that could potentially form a very stable structure. pPep13, is predicted to be 72% α-helical, pPep10 is predicted to be 45% β-sheet and pPep6 is predicted to contain a hydrophobic membrane spanning domain. According to the algorithms that are commonly used to predict the presence of these motifs in proteins, a randomly generated oligonucleotide such as the one used in our studies would have had no better than a 1 in a 1000 chance of generating the motifs that occurred in these peptides (16–24,30).

Verifying that the inhibitory clones do not function as antisense

To verify that the inhibitory clones which we isolated functioned as expressed peptides and not as antisense RNA or DNA, the insert regions between the *Bgl*II and *Eco*RI sites for five of the inhibitors were recloned into the pLAC11 vector using oligonucleotides which converted the ATG start codon to an ACG codon thus abolishing the start site. In all five cases the new constructs were no longer inhibitory (Table 4), thus confirming that it is the encoded peptides that causes the inhibition and not the DNA or transcribed mRNA.

Discussion

We have developed an *in vivo* approach by which novel synthetic bioactive peptides can be isolated that inhibit the growth of *E. coli*. In our initial screening of 20,000 potential inhibitory clones, 21 inhibitors were isolated which could be grouped into two classes; 18 1-day inhibitors which were partially inhibitory and prevented the growth of bacteria on minimal plates for 24 h, and 3 2-day inhibitors which were completely inhibitory and prevented the growth of bacteria on minimal plates for 48 h. The 1-day inhibitors were found to inhibit the bacterial growth rate at an average of 25%, and thus inhibited the overall growth of the bacteria by 65%. The 2-day inhibitors were found to inhibit the bacterial growth rate at an average of 75% and thus inhibited the overall growth of the bacteria by 96%.

Two of the most potent 2-day inhibitors proved to be fusion peptides in which the C-terminus of the peptides was fused to the N-terminus of the Rop protein. Given the fact that the Rop protein is known to form an incredibly stable structure (31), Rop could be serving as a stable protein

Table 3. **Sequence analysis of the insert region from the top 10 anchorless inhibitory clones and the peptides that they are predicted to encode**

pPep1, 13 aa

CAG GAA AGA TCT ATG GTC ACT GAA TTT TGT GGC TTG TTG GAC CAA CTG CCT TAG TAA TAG TGG AAG GCT GAA ATT AAT AAG AAT TC

M V T E F C G L L D Q L P * * *

pPep5, 16 aa

CAG GAA AGA TCT ATG TGG CGG GAC TCA TGG ATT AAG GGT AGG GAC GTG GGG TTT ATG GGT TAA AAT AGT TTG ATA ATA AGA ATT C

M W R D S W I K G R D V G F M G *

pPep6, 42 aa: last 25 aa could contain a hydrophobic membrane-spanning domain

CAG GAA AGA TCT ATG TCA GGG GGA CAT GTG ACG AGG GAG TGC AAG TCG GCG ATG TCC AAT CGT TGG ATC TAC GTA ATA AGA ATT CTC

M S G G H V T R E C K S A M S N R W I Y V I R I L

ATG TTT GAC AGC TTA TCA TCG ATA AGC TTT AAT GCG GTA GTT TAT CAC AGT TAA

M F D S L S S I S F N A V V Y H S *

pPep7, 6 aa

CAG GAA AGA TCT ATG TAT TTG TTC ATC GGA TAA TAC TTA ATG GTC CGC TGG AGA ACT TCA GTT TAA TAA GAA TTC

M Y L F I G *

pPep8, 21 aa

CAG GAA AGA TCT ATG CTT CTA TTT GGG GGG GAC TGC GGG CAG AAA GCC GGA TAC TTT ACT GTG CTA CCG TCA AGG TAA TAA GAA TTC

M L L F G G D C G Q K A G Y F T V L P S R * *

pPep10, 20 aa: predicted to be 45% β-sheet, amino acids 6–14

CAG GAA AGA TCT ATG ATT GGG GGA TCG TTG AGC TTC GCC TGG GCA ATA GTT TGT AAT AAG AAT TCT CAT GTT TGA

M I G G S L S F A W A I V C N K N S H V *

pPep12, 14 aa

CAG GAA AGA TCT ATG AAC GGC CGA ACC AAA CGA ATC CGG GAC CCA CCA GCC GCC TAA ACA GCT ACC AGC TGT GGT AAT AAG AAT TC

M N G R T K R I R D P P A A *

pPep13, 18 aa: predicted to be 72% α-helical, amino acids 3–15

CAG GAA AGA TCT ATG GAC CGT GAA GTG ATG TGT GCG GCA AAA CAG GAA TGG AAG GAA CGA ACG CCA TAG GCC GCG TAA TAA GAA TTC

M D R E V M C A A K Q E W K E R T P *

pPep17, 12 aa

CAG GAA AGA TCT ATG TAG CCC AAT GCA CTG GGA GCA CGC GTG TTA GGT CTA GAA GCC ACG TAC CCA TTT AAT CCA TAA TAA GAA TTC

M * M* L G L E A T Y P F N P * *

pPep19, 5 aa

CAG GAA AGA TCT ATG AGG GGC GCC AAC TAA GGG GGG GGG AAG GTA TTT GTC CCG TGC ATA ATC TCG GGT GTT GTC TAA TAA GAA TTC

M R G A N *

The landmark *Bgl*II and *Eco*RI restriction sites for the insert region are underlined. Because the ends of the oligonucleotide from which these inhibitors were constructed contained these restriction sites, the oligonucleotide was not gel isolated when the libraries were prepared in order to maximize our oligonucleotide yields. Because of this, several of the inhibitory clones were found to contain one ($n-1$) or two ($n-2$) base deletions in the randomized portion of the oligonucleotide.

Table 4. **Antisense test of five of the anchorless inhibitory peptides**

Inhibitory peptide	% Inhibition of the growth rate	Antisense construct	% Inhibition of the growth rate
pPep1	26	pPep1-anti	0
pPep5	23	pPep5-anti	0
pPep12	80	pPep12-anti	0
pPep13	28	pPep13-anti	0
pPep19	29	pPep19-anti	0

Growth rates for cells containing the induced inhibitors or antisense constructs were determined and then the % inhibition calculated by comparing these values to the growth rate of cells that contained the induced pLAC11 vector.

anchor for these two peptides. The characteristics of the predicted coding regions of the top 10 anchorless inhibitor peptides also proved to be quite interesting. Three of the 10 peptides (30%) contained a proline residue as their last amino acid. According to the genetic code, a randomly generated oligonucleotide such as that used in our studies would only have a 6% chance of placing proline at any one position. This 5-fold bias is striking when one considers the fact that proline has been argued to prevent the ability of peptidases to degrade peptides (32,33). Additionally, one of the peptides contained a proline residue at the $n-2$ and $n-3$ positions.

It has become increasingly clear that most naturally occurring bioactive peptides whose structures have been determined contain ordered structures which should help to stabilize them. For example, dermaseptin (34), endorphin (35), glucagon (36), magainins (37), mastoparan (38), melittin (39), motilin (40), PKI 5–24 (41) and secretin (42) form α-helixes, whereas atrial natriuretic peptide (43), calcitonin (44), conotoxins (45), defensins (46), EETI II (47), oxytocin (48), somatostatin (49) and vasopressin (50) contain disulfide bonds. Collectively, both our data from synthetic bioactive peptides that can inhibit the growth of *E. coli* and data from naturally occurring bioactive peptides strongly suggest that peptide stability is of paramount concern and that the presence of structural motifs are necessary to stabilize bioactive peptides if they are to be functional. Research on developing novel synthetic inhibitor peptides for use as potential therapeutic agents over the last few years has shown that peptide stability is a major problem that must be solved if designer synthetic peptides are to become a mainstay in the pharmaceutical industry (4,51,52). Based on what has been learned in this study, it is clear that methods need to be developed which can be used to make synthetically produced bioactive peptides more stable. Our laboratory is currently investigating whether specific motifs can be incorporated into synthetic inhibitor peptides in order to make them more stable and recent experiments have shown that the deliberate incorporation of the Rop or proline motifs into synthetically engineered bioactive peptides dramatically increases the frequency at which potent 2-day inhibitors can be obtained (J.R. Walker, J.W. Warren and E. Altman, pers. commun.).

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