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Genetic selection for peptide inhibitors of angiogenin

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Abstract

The improper regulation of angiogenesis is implicit in a variety of diseases, including cancer. Angiogenin is unique among angiogenic factors in its having ribonucleolytic activity. Inhibitors of this activity could serve as chemotherapeutics. The ribonucleolytic activity of angiogenin is toxic to the Origami™ strain of *Escherichia coli*. Herein, this cytotoxicity was used to identify inhibitors from a random nonapeptide library tethered to the C-terminus of human angiogenin. The selected sequences fell into three classes: (1) extremely hydrophobic, (2) putative ClpXP substrates, and (3) slightly anionic. Two peptides corresponding to sequences in the last class were synthesized chemically and found to inhibit the ribonucleolytic activity of human angiogenin *in vitro* with micromolar values of K_i. Both peptides also inhibit bovine pancreatic ribonuclease, a homologue of angiogenin, though one exhibits selectivity for angiogenin. The affinity and selectivity of these peptides are comparable to the best known inhibitors of angiogenin. Moreover, the strategy used to identify them is general and could be applied to other cytotoxins.

Keywords

cancer; cytotoxin; genetic selection; peptide; inhibitor; ribonuclease

Introduction

In 1985, angiogenin was isolated as a human tumor-derived protein with the ability to promote angiogenesis, the growth of new blood vessels (Fett et al., 1985). When the amino-acid sequence of angiogenin was determined (Strydom et al., 1985), it was found to have 35% identity to that of the most studied enzyme of the 20th century, bovine pancreatic ribonuclease (RNase A; 3.1.27.5 (Raines, 1998)) and to retain the active-site residues that are characteristic of this family of ribonucleases (Beintema et al., 1988). Initially, there were doubts that angiogenin had ribonucleolytic activity, as it did not appear to degrade common RNA substrates (Strydom et al., 1985). It was soon shown, however, that angiogenin cleaved rRNA to a limited extent (Shapiro et al., 1986; St Clair et al., 1987). Using a hypersensitive assay, angiogenin was later found to have ribonucleolytic activity that is 10^5–10^6-fold less than that of RNase A (Leland et al., 2002). That low-level ribonucleolytic activity is, however, required for angiogenesis (Shapiro et al., 1989; Shapiro and Vallee, 1989), though the endogenous RNA substrate remains unknown (Shapiro et al., 1986; Strydom, 1998; Leland et al., 2002).

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Improperly regulated angiogenesis is involved in many diseases, most notably tumor growth (Gimbrone et al., 1972). Angiogenesis is critical for the growth and metastasis of tumors, and angiogenin levels are elevated in a variety of cancers (Katona et al., 2005). Inhibitors of angiogenic factors could lead to new cancer therapies (Folkman and Shing, 1992). The clinical success of bevacizumab (*e.g.*, Avastin™), which is a monoclonal antibody that targets the potent angiogenesis-promoter VEGF, supports the efficacy of this approach (Ferrara, 2005). Likewise, monoclonal antibodies, an antisense oligonucleotide, and small-molecule inhibitors of angiogenin have been shown to possess activity against subcutaneous tumors in athymic mice (Olson et al., 1995; Olson et al., 2001; Kao et al., 2002).

The angiogenic activity of angiogenin can be ameliorated by inhibiting its enzymatic activity (Nobile et al., 1998; Kao et al., 2002; Jenkins and Shapiro, 2003) or by interfering with its internalization pathway (Choi et al., 1997). Peptide libraries could be a source of antagonists for either purpose. Indeed, phage-display libraries have already been used to identify peptides that disrupt the interaction between angiogenin and its receptor, thereby preventing the internalization of angiogenin (Choi et al., 1997; Gho et al., 1997).

We have pursued an alternative strategy to disrupt angiogenin-induced angiogenesis. We reasoned that peptides that inhibit the ribonucleolytic activity of angiogenin could be identified by using a genetic selection system that requires the absence of enzymatic activity for cell growth. Angiogenin is a secretory protein. Its three disulfide bonds cannot form in the reducing environment of the cytosol of typical laboratory strains of *Escherichia coli*. In contrast, we found that plasmids that direct the expression of angiogenin can be toxic to Origami™ cells (Smith and Raines, 2006), which have a more oxidizing cytosol. A large decrease in the ribonucleolytic activity of angiogenin is needed to circumvent that toxicity. We have exploited this circumstance by tethering a peptide library to the solvent-exposed C-terminus of angiogenin via a glycine-rich linker. This tethering increased the effective concentration of the peptides, allowing for the isolation of inhibitors that might not be identified otherwise. From this library of over 2×10^7 random peptides, we identified many potential inhibitors of angiogenin. Two of these peptides were synthesized chemically and found to inhibit angiogenin *in vitro*, one being among the best inhibitors of angiogenin identified to date.

Materials and methods

Materials

Escherichia coli Origami™ B(DE3) cells and DH5α cells were from Novagen (Madison, WI). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Peptides were synthesized at the University of Wisconsin–Madison Peptide Synthesis Facility. The fluorogenic ribonuclease substrate, 6-FAM–dArUdAdA–6-TAMRA (where 6-FAM is a 5′ 6-carboxyfluorescein group and 6-TAMRA is a 3′ 6-carboxytetramethylrhodamine group), was from Integrated DNA Technologies (Coralville, IA). MES was purified prior to use to remove inhibitory contaminants as described previously (Smith et al., 2003). H_2O was distilled and deionized before use. All other commercial chemicals and biochemicals were of reagent grade or better, and were used without further purification.

Instruments

UV absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Palo Alto, CA). Fluorescence measurements were made with a QuantaMaster 1 Photon Counting Fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ).

Plasmids

The human angiogenin cDNA was inserted into plasmid pSH12 (Park and Raines, 2000), which was based on plasmid pGEX-4T3, at its *Nde*I and *Sal*I restriction enzyme sites. The resulting plasmid encoded a methionine residue at the start of the gene, designated Met(−1). The stop codon was replaced by three consecutive glycine codons, directly followed by a *Sal*I site. The *Sal*I site encoded two serine residues that remained after ligation of the peptide library insert. This plasmid was designated pGEX-ANG-G3S2. Three additional glycine residues were encoded by the oligonucleotides of the peptide insert, thus the full linker separating the final residue in angiogenin and the nonapeptide library was $Gly_3Ser_2Gly_3$.

Oligonucleotides for peptide library

The degenerate oligonucleotide BS9 (5′-TCGAGC$(GGT)_3(XNK)_9$TAGTAGTCGC-3′) encoded a peptide of nine random amino acid residues. The base composition of the XNK codons, X = A(32%)/G(39%)/C(21%)/T(8%), N = A(25%)/G(25%)/C(25%)/T(25%), and K = G(50%)/T(50%) was similar to that in a library designed previously to minimize the number of stop codons and to mirror closely the natural abundance of amino-acid residues in proteins (LaBean and Kauffman, 1993; Park and Raines, 2000). The gapped-duplex method was used to prepare duplex DNA for ligation while maintaining the degeneracy of the oligonucleotide (Cwirla et al., 1990; Park and Raines, 2000). Specifically, the degenerate oligonucleotide was annealed to two shorter oligonucleotides that were complementary to its nondegenerate termini (Figure 1A). The gap in the duplex DNA was filled-in after transformation of plasmid DNA into *E. coli*. The gapped-duplex DNA was prepared by annealing the degenerate oligonucleotide with oligonucleotides, BS11 (5′-ACCACCACCGC-3′) and BS10 (5′-GGCCGCGACTACTA-3′). All three oligonucleotides were phosphorylated at their 5’ ends. The oligonucleotides (1.5×10^{-11} mol for BS9 and 2.86×10^{-10} mol for BS10 and BS11) were annealed in 20 mM Tris–HCl buffer, pH 7.0, containing MgCl2 (2 mM) and NaCl (50 mM) in a total volume of 10 μL. The mixture was heated to 65 °C for 5 min and allowed to cool slowly to room temperature. The 5′ and 3′ ends of the annealed duplex are compatible with *Sal*I and *Not*I cleavage sites, respectively.

Ligation reactions

Plasmid pGEX-ANG-G3S2 (10 μg) was digested at 37 °C for 2 h with *Sal*I (15 U) and *Not*I (15 U) in 1× Promega Buffer D (which was 6 mM Tris–HCl buffer, pH 7.9, containing $MgCl_2$ (6 mM), NaCl (150 mM), and DTT (1 mM)) in a total volume of 100 μL. The restriction enzymes were inactivated by heating at 65 °C for 15 min. 0.30 M Sodium acetate buffer, pH 5.0, and three volumes of ice-cold ethanol were added to the reaction mixture, which was then incubated at −20 °C for 1 h to precipitate the DNA. The digested DNA was subjected to centrifugation at 10,000*g* for 10 min at 4 °C. The solvent was removed by aspiration with a drawn pipette, and 250 μL of ice-cold aqueous ethanol (70% v/v) was added. Again, the sample was subjected to centrifugation, and the solvent was removed by aspiration. The DNA pellet was dried for 1 min in a vacuum desiccator, and then dissolved in H_2O (20 μL). The sample was then desalted by gel-filtration chromatography with an AutoSeq G50 column (GE Healthcare, Piscataway, NJ). Solutions of the purified linear plasmid (20 μL) and annealed gapped-duplex oligonucleotides (10 μL) were incubated for 16 h at 14 °C with a ligation reaction mixture (50 μL) containing 1× ligase buffer, DNA ligase (8 U; Promega, Madison, WI), and additional ATP (1 mM). DNA was precipitated with ethanol as described above. The dried DNA pellet containing purified, ligated plasmid DNA was dissolved in H_2O (10 μL) and desalted with an AutoSeq G50 column.

Library analysis

E. coli strain DH5α was used to analyze the quality and randomness of the nonapeptide library, as plasmids encoding angiogenin are not toxic to this strain. DH5α cells were transformed by electroporation (1.80 kV, 200 Ω, 25 μF) with 1 μL of the desalted and purified ligated DNA. SOC (1.0 mL) was added immediately, and the cells were allowed to recover at 37 °C for 1 h before being grown on LB agar containing ampicillin (100 μg/mL). Electroporetic transformation of DH5a cells with ligated DNA yielded 2.4×10^7 transformants. Cultures (1 mL) were grown in LB medium containing ampicillin (100 μg/mL), and plasmid DNA was isolated with the Wizard SV Plus Miniprep kit (Promega, Madison, WI). DNA sequencing reactions (10 μL) contained Big Dye 3.1 (1.0 μL), Big Buffer (1.5 μL), ddH2O (1.5 μL), primer (1 μL from a 10 μM stock), and plasmid DNA (5.0 μL). Reaction mixtures were subjected to thermocycling (36 cycles; 96 °C for 20 s, 48 °C for 30 s, and 58 °C for 5 min). The sequencing reaction mixtures were purified with the CleanSEQ Dye-terminator Removal kit (Agencourt Bioscience, Beverly, MA). DNA sequences were obtained in the forward and reverse directions. Sequence analysis of a sample of this library indicated that >90% of clones carried inserts and that the nine XNK codons were indeed random. Of note, a fraction of the sequences (<5%) contained 1-bp inserts or deletions in the sequence encoding the nonapeptide library, even though oligonucleotide BS9 was purified by PAGE.

Genetic selection

Ligated DNA was transformed by electroporation into competent *E. coli* Origami™ cells as described above, with the following modifications. After transformation, SOC (1.0 mL) was added immediately, and the cells were allowed to recover at 37 °C for 1.5 h before being grown on LB agar containing ampicillin (100 μg/mL), kanamycin (15 μg/mL), and tetracycline (12.5 μg/mL). As Origami™ cells grow more slowly than do typical laboratory strains of *E. coli*, colonies were picked after 24–48 h of growth. Plasmid DNA was isolated and subjected to sequence analysis.

Production of ribonucleases

Plasmid pET-ANG (Leland et al., 2002) directs the production of angiogenin in *E. coli*. Angiogenin was produced and purified as described previously, except for the following modifications. To avoid trace contamination by other ribonucleases, refolded and concentrated angiogenin was purified on a new column of HiTrap SPHP resin (5 mL; GE Healthcare, Piscataway, NJ). All buffers were made with H_2O that had been treated with DEPC (0.1% v/v). The resin was equilibrated with 50 mM sodium phosphate buffer, pH 7.2. Angiogenin (15 mg) was loaded onto the column. The column was washed with 20 mL of 50 mM sodium phosphate buffer, pH 7.2, and then with 50 mL of 50 mM sodium phosphate buffer, pH 7.2, containing NaCl (0.25 M). Angiogenin was eluted from the column with 20 mL of 50 mM sodium phosphate buffer, pH 7.2, containing NaCl (0.35 M). Zymogram electrophoresis was performed to confirm that purified angiogenin was free from contaminating ribonucleolytic activity, as described previously (Leland et al., 2002). RNase A was produced and purified as described previously (Kim and Raines, 1993; Smith et al., 2003).

Inhibition of ribonucleolytic activity

Inhibition of ribonucleolytic activity was assessed at 25 °C in 2.0 mL of 50 mM MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M), 6-FAM–dArUdAdA–6-TAMRA (60 nM), and angiogenin (0.42 μM) or RNase A (2 pM), as described previously (Kelemen et al., 1999; Park et al., 2001; Smith et al., 2003). Peptide **1**, *acetyl*-LDDAEEWGG, was dissolved in assay buffer. Peptide **2**, *acetyl*-AEDYDYSWW, was dissolved in assay buffer containing CH_3CN (20% v/v). Fluorescence (F) was measured with 493 and 515 nm as the excitation and emission wavelengths, respectively. The value of $\Delta F/\Delta t$ was measured for 3 min after the addition of

enzyme. Next, an aliquot of inhibitor (*I*) was added, and $\Delta F/\Delta t$ was measured in the presence of the inhibitor for 2 min. The concentration of inhibitor in the assay mixture was doubled repeatedly in 2-min intervals. Excess RNase A was then added to the mixture to ensure that <10% of the substrate had been cleaved prior to completion of the inhibition assay. Apparent changes in ribonucleolytic activity due to dilution or other artifacts (such as protein binding to a cuvette during the course of an assay) were corrected by comparing values to an assay in which aliquots of buffer (or buffer containing CH_3CN (20% or 40% v/v)) were added to the assay. At the concentrations tested, CH_3CN had no effect on ribonucleolytic activity. Values of K_i were determined by non-linear least squares regression analysis of data fitted to the equation (Kelemen et al., 1999; Park et al., 2001):

$$\Delta F/\Delta t=(\Delta F/\Delta t)_0(K_i/(K_i+[I])) \quad (1)$$

In equation 1, $(\Delta F/\Delta t)_0$ was the ribonucleolytic activity prior to inhibitor addition.

Results and Discussion

Strategy

Ligands can be identified by scanning peptide libraries (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Landon et al., 2004; Falciani et al., 2005; Watt, 2006). Most often, such peptide libraries are generated on the surface of phage (Smith, 1985), and then screened for ligands that bind to a particular target. In contrast, we have developed a means to select (rather than screen) for peptides that not only bind to a target protein, but also inhibit its enzymatic activity.

The production of human angiogenin is not toxic to normal laboratory strains of *E. coli*, such as DH5α (Figure 1B) (Smith and Raines, 2006). Native angiogenin contains three disulfide bonds that are essential to its conformational stability and cannot form in the reducing environment of the cytosol. The Origami™ strain of *E. coli* has mutations in genes encoding glutathione reductase and thioredoxin reductase that produce a more oxidizing cytosol (Derman et al., 1993; Bessette et al., 1999). Consequently, plasmids encoding angiogenin are highly toxic to Origami™ cells (Figure 1B). Even the low level of angiogenin produced via leaky expression of the gene under control of a $\mathbf{P}_{tac}$ promoter is sufficient to kill these cells. The stringency of selection can be tuned by adding IPTG (Smith and Raines, 2006). In the absence of IPTG, a ~50-fold reduction in the ribonucleolytic activity of angiogenin is needed to allow for cell growth.

To increase the likelihood of isolating inhibitors that are capable of reducing the ribonucleolytic activity of angiogenin to this low level, a nonapeptide library was tethered to the C-terminus of angiogenin via a $Gly_3Ser_2Gly_3$ linker. The effective concentration of these peptides was much higher than if the peptide library had been fused to a second protein. This the “tethering” strategy bears some resemblence to that developed by Wells and coworkers to identify small-molecule ligands for proteins (Erlanson et al., 2000).

Peptide library

DNA encoding the nonapeptide library was ligated to a linker encoded at the 3′ end of the angiogenin gene by using the gapped-duplex method (Figure 1A) (Cwirla et al., 1990; Park and Raines, 2000). Transformation of the angiogenin/peptide library into *E. coli* DH5α cells yielded 2.4×10^7 transformants. Although the maximal size of this nonapeptide library is 5×10^{11}, the actual size is limited by the transformation efficiency of *E. coli*, among other factors. DNA sequence analysis indicated that the nonapeptide library was random and had a low occurrence of stop codons (Table 1 and Table 2).

Genetic selection

Transformation of the angiogenin/peptide library into *E. coli* Origami™ cells yielded transformants at a frequency of ~10^{-4}. This frequency of selection includes false positives. Because the selection is based on the abrogation of ribonucleolytic activity, several artifacts can lead to cell growth. False positives can be caused by random mutations that occur in the angiogenin cDNA (including insertions and deletions, stop codons, and substitutions of essential residues), recombination of plasmid DNA to excise a part or all of the angiogenin gene, or star activity by restriction enzymes during preparation of the library. For example, the restriction enzyme *Eag*I was used initially to prepare plasmid DNA for ligation. Its high level of star activity led to many false positives in which *Eag*I cut the plasmid at a site different than its consensus sequence and thereby caused a truncation or excision of the angiogenin gene in a portion of the library. Accordingly, the restriction enzyme *Not*I was used instead of *Eag*I, and yielded few false positives.

The amino-acid sequences of 246 clones were obtained that contain the intact angiogenin–peptide fusion (Table S-1). These potential positives were sorted into three classes (Table 3). First, the largest class of peptide sequences contained long stretches of hydrophobic amino acid residues. These peptides could prevent folding or cause aggregation of angiogenin in Origami™ cells. Alternatively, hydrophobic sequences at the C-terminus of proteins in *E. coli* are known to act as degradation signals (Parsell et al., 1990). Then cells could avoid angiogenin-induced toxicity by degrading the protein prior to its attack on cellular RNA. A final possibility could be that some of these hydrophobic sequences are angiogenin inhibitors, as certain largely hydrophobic peptides do bind to angiogenin with high affinity (Gho and Chae, 1997). It would be difficult, however, to resolve whether the hydrophobic peptides selected herein function as angiogenin inhibitors, not only due to the factors listed above, but also due to the difficulty of synthesizing and solubilizing these peptides.

The second class of peptide sequences occurred at a rate of ~2×10^{-6} and was distinguished by the presence of two C-terminal alanine residues (Table 3). Proteins containing an Ala–Ala dipeptide at their C-terminus are degraded by cellular proteases, including ClpXP (Parsell et al., 1990;Tu et al., 1995;Flynn et al., 2003;Levchenko et al., 2005). It is likely that cell growth in these cases is due to the degradation of angiogenin. Hence, we note that our selection system could be used to characterize the substrate specificity for bacterial protein degradation pathways.

The third class of peptides, occurring at a rate of ~1×10^{-5} was comprised of anionic sequences that did not fit into the two classes above and could be potential inhibitors of angiogenin, which has a cationic active site (Table 3). Further characterization of peptides containing cysteine residues was avoided, as they might interfere with the oxidative folding of angiogenin, thereby allowing for cell growth.

Peptide inhibitors of angiogenin

Studies involving the ribonucleolytic activity of angiogenin require special concern. The catalytic activity of angiogenin is 10^5–10^6-fold lower than that of RNase A. Thus, 1-ppm contamination of angiogenin by the common laboratory reagent RNase A would alter the results (Kao et al., 2002; Leland et al., 2002). In addition, solutions can be contaminated easily by ribonucleases that are present in abundance on human skin (Holley et al., 1961; Smith et al., 2005). Thus, angiogenin was purified in an extremely careful manner. Purified angiogenin was confirmed to be free of contaminating ribonucleases by zymogram electrophoresis (data not shown), a technique that can detect the ribonucleolytic activity of as little as 1 pg of RNase A (Blank et al., 1982; Bravo et al., 1994; delCardayré et al., 1995). In addition, the different

K_i values obtained for inhibition of RNase A and angiogenin by the peptides (*vide infra*) confirm that the angiogenin was free from contaminating RNase A.

Two peptides were tested for inhibition of angiogenin. Peptides **1** and **2** were *acetyl*-LDDAEEWGG and *acetyl*-AEDYDYSWW, respectively (Table 4). Inhibition assays were performed in 0.05 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M). The K_i values for peptides **1** and **2** were (641 ± 53) μM and (56.4 ± 4.1) μM, respectively (Figure 2; Table 4).

Selectivity of peptide inhibitors

Humans have at least 12 homologs of angiogenin involved in various biological functions (Cho et al., 2005). Accordingly, inhibitors used to prevent angiogenesis *in vivo* might need to be selective for angiogenin. The selectivity of the inhibitors was tested by determining K_i values for RNase A. Peptide **1** reduced the catalytic activity of RNase A by only ~30% at a concentration of 1.2 mM. Thus, this peptide exhibits selectivity for angiogenin. Peptide **2**, surprisingly, inhibited RNase A with a K_i value of (17.5 ± 1.1) μM, or 3-fold lower than that for angiogenin (Figure 2; Table 4).

It has been notoriously difficult to generate inhibitors that are specific for angiogenin over RNase A (Jenkins and Shapiro, 2003). Known nucleotide-based inhibitors bind angiogenin >100-fold worse than they do RNase A (Russo et al., 1996; Russo et al., 2001). Even small-molecule inhibitors identified from a large library of compounds screened solely against angiogenin had 2–3-fold greater selectivity for RNase A (Kao et al., 2002; Jenkins and Shapiro, 2003). Rationally designed derivatives of these small molecules did, however, inhibit angiogenin twofold better than RNase A (Jenkins and Shapiro, 2003), making them the most selective angiogenin inhibitors identified to date. Our findings further substantiate these results. Peptide inhibitors selected to inhibit the ribonucleolytic activity of angiogenin exhibited comparable inhibition of RNase A. Peptide **1** was twofold more selective for angiogenin, and peptide **2** was 3-fold more selective for RNase A. Thus, these peptides rank among the most selective inhibitors of angiogenin. In addition, peptide **2** ranks as among the best small-molecule inhibitors of angiogenin. Its K_i value is within ~10-fold of that for the best known inhibitor of angiogenin, benzopurpurin B, under similar assay conditions (Jenkins and Shapiro, 2003). Notably, peptide **2** exhibits a K_i value equivalent to that of a small-molecule inhibitor that exhibited antitumoral activity against subcutaneous tumors in athymic mice (Kao et al., 2002; Jenkins and Shapiro, 2003).

Previous work with peptide-based inhibitors of ribonucleases revealed that peptides corresponding to the C-terminus of these enzymes could bind and/or inhibit angiogenin (Rybak et al., 1989) or RNase A (Nakano and Sugimoto, 2003). A peptide based upon the C-terminal residues of angiogenin inhibits the enzyme with K_i = 278 μM, though at a different pH value and lower concentrations of NaCl from the assays used herein (Rybak et al., 1989). Both pH and salt concentration have large effects on inhibition assays of ribonucleases (Russo et al., 2001; Smith et al., 2003), thus direct comparison with these results is difficult.

Finally, we note that random copolymers of tyrosine and glutamate that are ~30 residues in length are known to inhibit RNase A (Sela, 1962). Intriguingly, peptide **2** contains the sequence EDYDY and inhibits both angiogenin and RNase A (Table 4). This discovery suggests that a peptide library constrained to contain several tyrosine and glutamate residues could yield even more potent inhibitors of ribonucleases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

6-FAM, 6-carboxyfluorescein; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; MES, 2-(*N*-morpholino)-ethanesulfonic acid; RNase A, bovine pancreatic ribonuclease; 6-TAMRA, 6-carboxytetramethylrhodamine.

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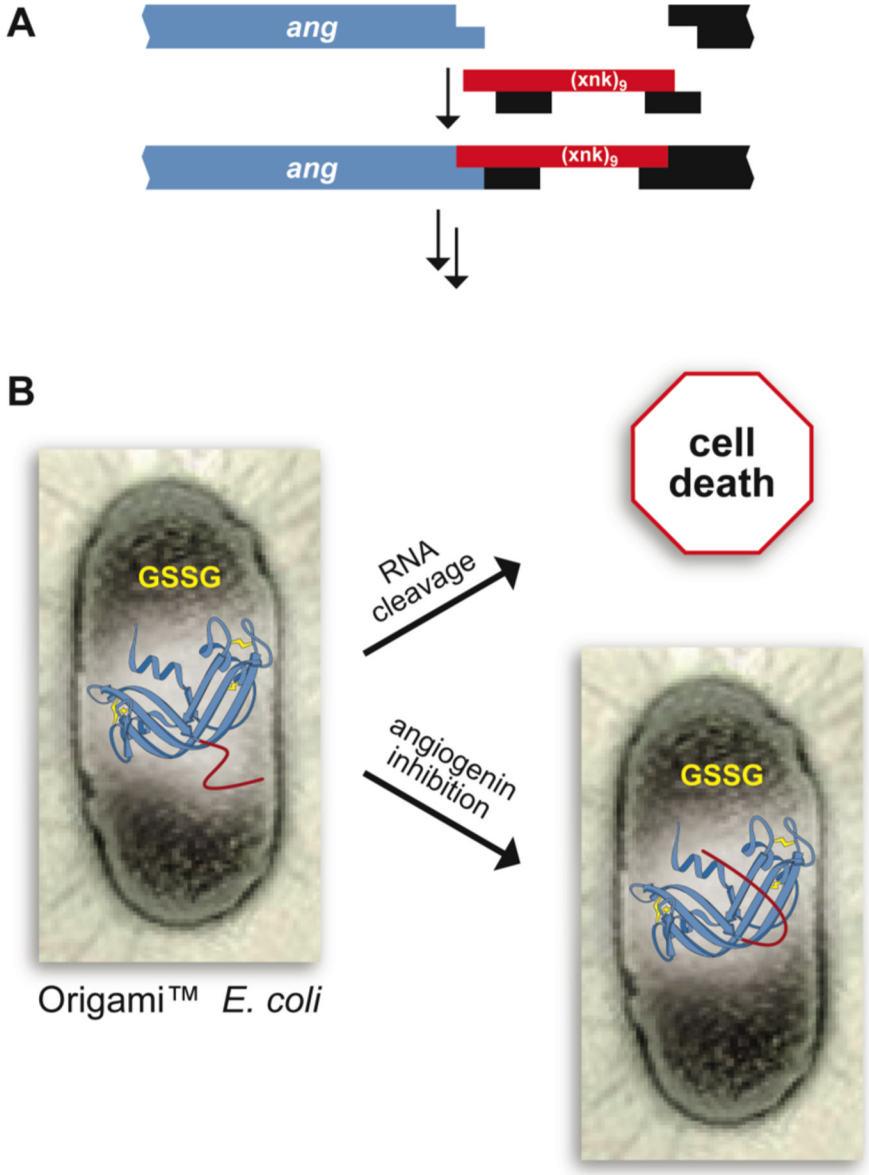

Figure 1.

Genetic selection for peptide inhibitors of angiogenin. (A) A random nonapeptide library was prepared via gapped-duplex ligation. The degenerate oligonucleotide was ligated in frame with the 3′ end of the angiogenin cDNA after a linker encoding $Gly_3Ser_2Gly_3$. Ultimately, the gap was filled-in by *E. coli*. (B) Angiogenin folds in the oxidizing environment of Origami™ cells. C-Terminal peptides (red) that do not inhibit its ribonucleolytic activity lead to cell death. Peptides that do inhibit allow for cell growth.

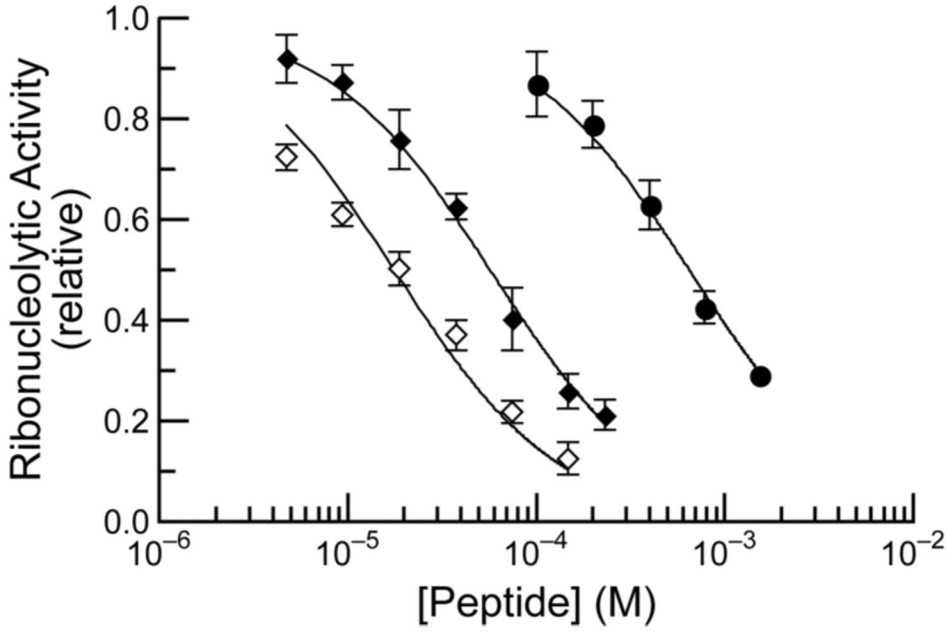

Figure 2.
Inhibition of angiogenin and RNase A by selected peptides. Assays were performed at 25 °C in 50 mM MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M). ●, Inhibition of angiogenin by peptide **1**; ◆, Inhibition of angiogenin by peptide **2**; ◇, Inhibition of RNase A by peptide **2**. Data were fitted to equation 1 to obtain values of K_i (Table 4).

Table 1

Analysis of XNK codons in peptide library

	X	N	K	%X	%N	%K
A	190	122	1	39.1	25.1	0.2
C	114	69	0	23.5	14.2	0.0
T	49	146	262	10.1	30.0	53.9
G	133	149	223	27.4	30.7	45.9
Sum	486	486	486	100.0	100.0	100.0

Table 2

Analysis of amino acid distribution in peptide library

Amino Acid	Occurrences	%
A	16	3.3
C	10	2.1
D	17	3.5
E	17	3.5
F	8	1.7
G	38	7.8
H	8	1.7
I	28	5.8
K	22	4.5
L	39	8.0
M	26	5.4
N	26	5.4
P	22	4.5
Q	17	3.5
R	63	13.0
S	40	8.2
T	25	5.1
V	45	9.3
W	4	0.8
Y	10	2.1
*	5	1.0
SUM	486	100.0

Table 3

Representative sequences selected from peptide library

Class I	Class II	Class III
AGVVIVVVS	DHGDLVPAA	VDMLLAEEM
VLEVTVVVL	VFAMDDTAA	ALAQFENMV
VTMMVYIFN	QEDMSVLAA	TTIVEDAAD
VVIDVSMIM	AERVVRRAA	YHECVSYAS
TVLIIDIRI	EDARHPRAA	AVETALMEA
VIVLILIAN	IAIHHMEAA	SYSALYEAG
VIIIVIIGE	NQVLPKRAA	LDDAEEWGG
LLVVIMSWG	NDRRAMKAA	AEDYDYSWW
SSVVVTMII	NVIMPAHAA	NCEAAEDEE
IIPIFIVTV	VDRRAA[a]	SEYLAGYGV

[a] A truncated peptide sequence was found appended to intact ANG.

Table 4

Inhibition of angiogenin and RNase A by selected peptides

Peptide	Sequence[a]	K_i (μM), Angiogenin[b]	K_i (μM), RNase A[b]
1	LDDAEEWGG	641 ± 53	>1200
2	AEDYDYSWW	56 ± 4	17.5 ± 1.1

[a] Peptides were acetylated at their N-terminus.

[b] Values of K_i were obtained in 0.05 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M).