

# Genetic selection for peptide inhibitors of angiogenesis

Bryan D. Smith<sup>1,3</sup> and Ronald T. Raines<sup>1,2,4</sup>

<sup>1</sup>Department of Biochemistry, University of Wisconsin–Madison, 433 Babcock Drive, Madison, WI 53706-1544, <sup>2</sup>Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, WI 53706-1322, USA and <sup>3</sup>Present address: Deciphera Pharmaceuticals LLC, 4950 Research Park Way, Lawrence, KS 66047, USA

<sup>4</sup>To whom correspondence should be addressed. E-mail: rtraines@wisc.edu

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

**Keywords:** cytotoxin/genetic selection/inhibitor/peptide/ribonuclease

## Introduction

In 1985, angiogenesis 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 angiogenesis 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 angiogenesis had ribonucleolytic activity, as it did not appear to degrade common RNA substrates (Strydom *et al.*, 1985). It was soon shown, however, that angiogenesis cleaved rRNA to a limited extent (Shapiro *et al.*, 1986; St. Clair *et al.*, 1987). Using a hypersensitive assay, angiogenesis 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 and Vallee, 1989; Shapiro

*et al.*, 1989), though the endogenous RNA substrate remains unknown (Shapiro *et al.*, 1986; Strydom, 1998; Leland *et al.*, 2002).

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 angiogenesis 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 (i.e. Avastin<sup>®</sup>), 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 angiogenesis 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 angiogenesis 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 angiogenesis and its receptor, thereby preventing the internalization of angiogenesis (Choi *et al.*, 1997; Gho *et al.*, 1997).

We have pursued an alternative strategy to disrupt angiogenesis-induced angiogenesis. We reasoned that peptides that inhibit the ribonucleolytic activity of angiogenesis could be identified using a genetic selection system that requires the absence of enzymatic activity for cell growth. Angiogenesis 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 angiogenesis can be toxic to Origami<sup>TM</sup> cells (Smith and Raines, 2006), which have a more oxidizing cytosol. A large decrease in the ribonucleolytic activity of angiogenesis is needed to circumvent that toxicity. We have exploited this circumstance by tethering a peptide library to the solvent-exposed C-terminus of angiogenesis 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  $2 \times 10^7$  random peptides, we identified many potential inhibitors of angiogenesis. Two of these peptides were synthesized chemically and found to inhibit angiogenesis *in vitro*, one being among the best inhibitors of angiogenesis identified to date.

## Materials and methods

### Materials

*E. coli* Origami B(DE3) cells and DH5 $\alpha$  cells were from Novagen (Madison, WI). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Peptides were synthesized at the University of Wisconsin–Madison Peptide

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

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. MES was purified prior to use to remove inhibitory contaminants as described previously (Smith *et al.*, 2003). H<sub>2</sub>O 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

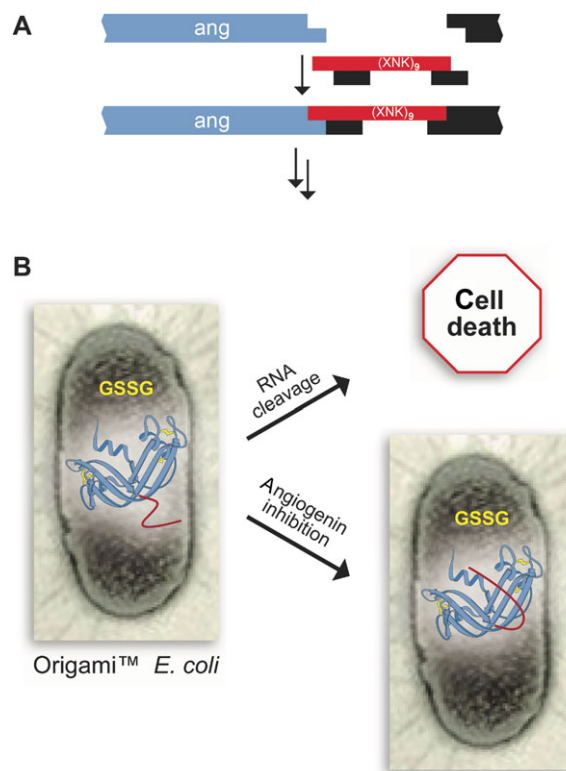
UV absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Palo Alto, CA). Fluorescence measurements were made with a QuantaMaster 1 Photon Counting Fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ).

### Plasmids

The human angiogenin cDNA was inserted into plasmid pSH12 (Park and Raines, 2000), which was based on plasmid pGEX-4T3, at its *NdeI* and *SalI* 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 *SalI* site. The *SalI* 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<sub>3</sub>Ser<sub>2</sub>Gly<sub>3</sub>.

### Oligonucleotides for peptide library

The degenerate oligonucleotide BS9 [5'-TCGAGC(GGT)<sub>3</sub>(XNK)<sub>9</sub>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 non-degenerate termini (Fig. 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'-GGCCGCGAC TACTA-3'). All three oligonucleotides were phosphorylated at their 5' ends. The oligonucleotides ( $1.5 \times 10^{-11}$  mol for BS9 and  $2.86 \times 10^{-10}$  mol for BS10 and BS11) were annealed in 20 mM Tris-HCl buffer, pH 7.0, containing MgCl<sub>2</sub> (2 mM) and NaCl (50 mM) in a total volume of 10  $\mu$ l. 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 *SalI* and *NotI* cleavage sites, respectively.



**Fig. 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<sub>3</sub>Ser<sub>2</sub>Gly<sub>3</sub>. 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.

### Ligation reactions

Plasmid pGEX-ANG-G3S2 (10  $\mu$ g) was digested at 37°C for 2 h with *SalI* (15 U) and *NotI* (15 U) in 1 $\times$  Promega Buffer D [which was 6 mM Tris-HCl buffer, pH 7.9, containing MgCl<sub>2</sub> (6 mM), NaCl (150 mM) and DTT (1 mM)] in a total volume of 100  $\mu$ l. The restriction enzymes were inactivated by heating at 65°C for 15 min. Sodium acetate buffer of 0.30 M, 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  $\mu$ l 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<sub>2</sub>O (20  $\mu$ l). 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  $\mu$ l) and annealed gapped-duplex oligonucleotides (10  $\mu$ l) were incubated for 16 h at 14°C with a ligation reaction mixture (50  $\mu$ l) containing 1 $\times$  ligase buffer, DNA ligase (8 U; Promega, Madison, WI) and additional ATP (1 mM). DNA was precipitated with ethanol as described earlier. The dried DNA pellet containing purified, ligated plasmid DNA was dissolved in H<sub>2</sub>O (10  $\mu$ l) and desalted with an AutoSeq G50 column.

### Library analysis

*E. coli* strain DH5 $\alpha$  was used to analyze the quality and randomness of the nonapeptide library, as angiogenin cannot undergo oxidative protein folding in this strain and hence is not cytotoxic. DH5 $\alpha$  cells were transformed by electroporation (1.80 kV, 200  $\Omega$ , 25  $\mu$ F) with 1  $\mu$ l 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  $\mu$ g/ml). Electroporetic transformation of DH5 $\alpha$  cells with ligated DNA yielded  $2.4 \times 10^7$  transformants. Cultures (1 ml) were grown in LB medium containing ampicillin (100  $\mu$ g/ml), and plasmid DNA was isolated with the Wizard SV Plus Miniprep kit (Promega). DNA sequencing reactions (10  $\mu$ l) contained Big Dye 3.1 (1.0  $\mu$ l), Big Buffer (1.5  $\mu$ l), ddH<sub>2</sub>O (1.5  $\mu$ l), primer (1  $\mu$ l from a 10  $\mu$ M stock) and plasmid DNA (5.0  $\mu$ l). 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 earlier, 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  $\mu$ g/ml), kanamycin (15  $\mu$ g/ml) and tetracycline (12.5  $\mu$ g/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). All buffers were made with H<sub>2</sub>O 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, and 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  $\mu$ M) 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<sub>3</sub>CN (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 with an assay in which aliquots of buffer [or buffer containing CH<sub>3</sub>CN (20% or 40% v/v)] were added to the assay. At the concentrations tested, CH<sub>3</sub>CN had no effect on ribonucleolytic activity. Values of *K<sub>i</sub>* were determined by non-linear least squares regression analysis of data fitted to the equation (Kelemen *et al.*, 1999; Park *et al.*, 2001):

$$\frac{\Delta F}{\Delta t} = \left( \frac{\Delta F}{\Delta t} \right)_0 \frac{K_i}{(K_i + [I])}. \quad (1)$$

In Eq. (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 DH5 $\alpha$  (Fig. 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 (Fig. 1B). Even the low level of angiogenin produced via leaky expression of the gene under control of a *P<sub>tac</sub>* promoter is sufficient to



**Table I.** 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

kill these cells. The stringency of selection can be tuned by adding IPTG (Smith and Raines, 2006). In the absence of IPTG, an ~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<sub>3</sub>Ser<sub>2</sub>Gly<sub>3</sub> linker. The effective concentration of these peptides was much higher than if the peptide library had been fused to a second protein. This ‘tethering’ strategy bears some resemblance to that developed by Wells and co-workers 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 (Fig. 1A) (Cwirla *et al.*, 1990; Park and Raines, 2000). Transformation of the angiogenin/peptide library into *E. coli* DH5 $\alpha$  cells yielded  $2.4 \times 10^7$  transformants. Although the maximal size of this nonapeptide library is  $5 \times 10^{11}$ , 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 (Tables I and II).

**Table II.** Analysis of amino-acid distribution in peptide library

Amino acid	Occurrences	Percentage
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
Stop codon	5	1.0
Sum	486	100.0

**Table III.** Representative sequences selected from peptide library<sup>a</sup>

Class I	Class II	Class III
AGVVVVVVS	DHGDLVPAA	VDMLLAEEM
VLEVTVVVL	VFAMDDTAA	ALAQFENMV
VTMMVYIFN	QEDMSVLAA	TTIVEDAAD
VVIDVSMIM	AERVVRRAA	YHECVSYAS
TVLIIDIRI	EDARHPRAA	AVETALMEA
VIVLILIAN	IAIHHMEAA	SYSALYEAG
VIIIIVIGE	NQVLPKRAA	LDDAEWGG
LLVVIMSWG	NDRRAMKAA	AEDYDYSWW
SSVVVTMI	NVIMPAHAA	NCEAAEED
IIPIFIVTV	VDRRAA <sup>b</sup>	SEYLAGYGV

<sup>a</sup>In *total*, 185 Class I, 10 Class II and 51 Class III sequences were selected from the library (see Supplementary material, Table S1).

<sup>b</sup>A truncated peptide sequence was found appended to intact ANG.

### Genetic selection

Transformation of the angiogenin/peptide library into *E. coli* Origami cells yielded transformants at a frequency of  $\sim 10^{-4}$ . 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 *EagI* was used initially to prepare plasmid DNA for ligation. Its high level of star activity led to many false positives in which *EagI* 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 *NotI* was used instead of *EagI*, which yielded few false positives.

The amino-acid sequences of 246 clones that contain the intact angiogenin-peptide fusion were obtained (Supplementary material, Table S1). These potential positives were sorted into three classes (Table III). 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 earlier, but also due to the difficulty of synthesizing and solubilizing these peptides.

The second class of peptide sequences occurred at a rate of  $\sim 2 \times 10^{-6}$  and was distinguished by the presence of two C-terminal alanine residues (Table III). 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  $\sim 1 \times 10^{-5}$ , comprised anionic sequences that did not fit into the two classes mentioned earlier and could be potential inhibitors of angiogenin, which has a cationic active site (Table III). Further characterization of peptides containing cysteine residues was avoided, as they might interfere with the oxidative folding of angiogenin, thereby allowing for cell growth.

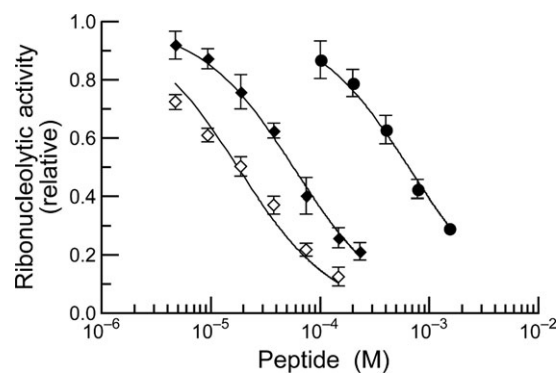
### Peptide inhibitors of angiogenin

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 IV). 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  $641 \pm 53$  and  $56.4 \pm 4.1$   $\mu\text{M}$ , respectively (Fig. 2; Table IV).

### 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  $\sim 30\%$  at a concentration of 1.2 mM. Thus, this peptide exhibits selectivity for angiogenin. Peptide 2, surprisingly, inhibited RNase A



**Fig. 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). Filled circles represent inhibition of angiogenin by Peptide 1; filled rhombi, inhibition of angiogenin by Peptide 2; open rhombi, inhibition of RNase A by Peptide 2. Data were fitted to Eq. (1) to obtain values of  $K_i$  (Table IV).

with a  $K_i$  value of  $17.5 \pm 1.1$   $\mu\text{M}$  or 3-fold lower than that for angiogenin (Fig. 2; Table IV).

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 2-fold 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 2-fold 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  $\sim 10$ -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  $K_i = 278$   $\mu\text{M}$ , 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 co-polymers of tyrosine and glutamate that are approximately 30 residues in length are known to inhibit RNase A (Sela, 1962). Intriguingly, Peptide

**Table IV.** Inhibition of angiogenin and RNase A by selected peptides

Peptide	Sequence <sup>a</sup>	$K_i$ ( $\mu\text{M}$ ), angiogenin <sup>b</sup>	$K_i$ ( $\mu\text{M}$ ), RNase A <sup>b</sup>
1	LDDAEEWGG	$641 \pm 53$	$>1200$
2	AEDYDYSWW	$56 \pm 4$	$17.5 \pm 1.1$

<sup>a</sup>Peptides were acetylated at their N-terminus.

<sup>b</sup>Values of  $K_i$  were obtained in 0.05 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M).

2 contains the sequence EDYDY and inhibits both angiogenin and RNase A (Table IV). This discovery suggests that a peptide library constrained to contain several tyrosine and glutamate residues could yield even more potent inhibitors of ribonucleases.

### Supplementary data

Supplementary data are available at *PEDS* online.

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