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The goal of this project was to identify peptides from phage display peptide libraries which bind with high affinity to the mutant EGFRvIII receptor present in breast tumors. The peptides selected were radiolabeled with technetium-99m (^{99m}Tc) and tested for their potential as agents in the detection of breast cancer. Using available phage display peptide libraries, we have identified five consensus peptides that show affinity for cells expressing the mutant EGFRvIII receptor. Characterization of these selected peptides was by ELISA and radiolabeled cell binding studies. First, the labeled phage were tested in *in vitro* assays and in mice with tumors. Specific binding of the labeled phage to the study cells was found relative to the control cells. Also, mice with tumors expressing the mutant receptor showed enhanced accumulation of the labeled phage over mice with tumors expressing the wild-type receptor. The consensus peptides were identified through analysis of the phage DNA. The peptides were synthesized, then conjugated to a chelator for radiolabeling with ^{99m}Tc. All peptides have been tested in *in vitro* assays and tested in tumor bearing mice. The *in vivo* studies show that the ^{99m}Tc-peptide clear the circulation quickly and demonstrate accumulation in breast tumor. Peptides have also been evaluated against a panel of tumor from clinical pathology. Early results suggest a distinction of peptides for various tumors.

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INTRODUCTION

The goal of this project was to identify a peptide from a phage display peptide library, which binds with high affinity to the mutant EGF receptor (EGFRvIII) on breast tumor cells. Phage display peptide libraries offer the potential of containing unique high affinity cancer diagnostic agents. In this project, the peptides selected were radiolabeled with technetium-99m (^{99m}Tc) and evaluated in *in vitro* assays and in *in vivo* mouse tumor models for their potential as agents for detection of breast cancer, through nuclear imaging. If successful this ^{99m}Tc-labeled mutant EGF-binding peptide could serve initially as an agent for the diagnosis of breast cancer, and, although not part of this proposal, as an agent in the delivery of therapeutics directly to the tumor.

For these studies we used the *in vitro* selection process referred to as "biopanning" to screen commercially available phage display peptide libraries for peptides which recognize a unique site on the mutant EGFRvIII, and do not bind to the normal EGF receptor. Phage display peptide libraries contain random sequences of peptides of equal length, with a complexity of about 2×10^9 independent sequences. These libraries contain peptides or proteins which can bind to almost any target with affinities (K_d) in the pico and micro molar range. Thus, one is no longer limited to antibodies as specific binding proteins (Ladner, 1995).

BODY

Progress on our project goals.

To review, the stated goals of this project were the following:

1. To select peptides which bind to the mutant EGF receptor (EGFRvIII) with high affinity using Phage Display Peptide Libraries which are commercially available. Potentially four peptides (high affinity binders) are to be identified for further testing.
2. The selected peptides are to be conjugated to a chelator and then radiolabeled with ^{99m}Tc for further testing.
3. These high affinity EGFRvIII binding peptides, once radiolabeled, are to be tested *in vitro* and *in vivo*. Studies *in vitro* include maximizing labeling efficiency and specific activity, testing stability of the radiolabeled peptides in serum, and characterize binding to tumor cells in culture. Studies *in vivo* are to include the biodistribution and clearance properties in the normal mouse and in a mouse tumor model.
4. Lastly, the candidate peptides will be screened using a panel of surgically resected tumors from the clinic.

If successful, a ^{99m}Tc-labeled mutant EGF-binding protein could serve as a useful agent in the diagnosis of breast cancer as well as other cancers which express the same mutant EGF receptor.

During the [rpject period we made substantial progress on our goals. To review our selection process, we are using three matched cell lines. One cell line, designated HC2 20d2/c, expresses our target,

EGFRvIII, the mutant EGF receptor, with about 2 x 10^6 receptors per cell. This cell line originated from the NIH-3T3 cell which was co-transfected with cDNA corresponding to the 801 base pair in-frame deletion. For control cells we obtained the CO12 20c2/b, which expresses the normal EGF receptor with about 10^6 copies per cell. The second control, is LTR b2 expressing the normal receptor with a low number of copies per cell, about 5-10 x 10^3 per cell.

Selection of Peptides from Phage Libraries

The phage display peptide libraries were purchased (New England BioLabs, Beverly, MA). The DNA encoding for the randomized peptides is fused to the gene which codes for the protein tips of the five coat filaments of the M13 phage. With the unique peptides readily available on the end of the filaments, they are easily available to characterize the peptide's binding to cells or receptor proteins.

In brief, for the process of selection the phage library is added to the media of a flask coated with the target cell. After an incubation period, unbound phage are removed by washing, and bound phage are eluted with a low pH buffer. The pool of bound phage is amplified, and the binding step repeated with an aliquot of the amplified phage. After repeating the binding and amplification process at least three times, individual phage clones are grown and the DNA sequenced for identification of the unique peptide clone.

Summary of First Phase (Year I)

For the first set of selection experiments a phage library kit called PhD-12 was purchased (New England Biolabs). The kit contains linear peptides of 12 amino acid in length. To increase the chances of success one incorporates a subtractive step with a control cell. In the first round of studies the selection began with the control cells, the LTR, to remove phage that bind to shared cell surface components. The LTR cells are identical to the study cells, HC2, except they lack that one unique feature, the mutant EGF receptor. After incubation with the control cells, the phage (in the supernatant) which did not bind to the control cells are transferred to the flask of HC2 cells, with the mutant receptor. After incubation, the unbound phage are discarded, and the cell-bound phage eluted with 0.2M glycine pH 2. The eluted phage are amplified and the selection cycle repeated at least three times. The purpose is to enrich the phage pool for those which bind selectively to the HC2 cells. Throughout the selection process the time of incubation, elution conditions and temperature can be varied to select for peptides with specific binding characteristics. In our case, for the first phase of this project, incubation was kept to 10 min at 37°C, and the phage were eluted with a 0.2M glycine buffer, pH 2.

Using this strategy with the PhD-12 kit, after three rounds of selection and amplification it is time to examine the phage pool for consensus, a binding peptide. Individual clones are isolated and surveyed through sequencing the DNA for the unique genetic site which codes for the filament peptide. To isolate clones samples of the amplified phage are grown on agar-agarose plates in a field of *E.coli*. The blue plaques which appear indicate a single phage clone. The clones are removed, amplified, and the DNA

isolated and prepared for sequencing according to standard procedures. From the sequence of the selected plaques, the data is evaluated for a consensus of amino acid sequences.

Of the 20 plaques selected in our first phase, nine contained an identical sequence, and another set of four shared a second common sequence. The seven remaining showed some amino acids in common, but were not complete. So we went from a pool of 10^9 independent clones to a pool where nearly half were identical.

The two consensus sequences from the PhD-12- kit are as follows:

Phage-3: H-Ser-**Pro-Trp**-Ser-**Glu**-Pro-Ala-Tyr-Thr-**Leu**-Ala-Pro-*Gly-Gly-Gly-Ser-OH*

Phage-5: H-Asn-Asn-**Pro-Trp**-Thr-**Glu**-Met-Arg-Ser-**Leu**-Leu-Ser-*Gly-Gly-Gly-Ser-OH*

The letters in bold indicate the common pattern between these two strands. The additional four carboxy terminal amino acids (in italics) were added as a leader sequence. These two peptides were commercially synthesized.

Second Phase (Year II)

A. The second phase of this project was begun with the PhD-12 kit implementing a new selection strategy. For subtraction, the LTR and the second control cell, CO12, were used in succession. Using four rounds of selection with the LTR→CO12→HC2 scheme, each selection was performed in a 25 cm^2 flask. After the rounds of selection, 30 clones were chosen, and DNA sequenced. This involved seven separate experiments. Surprisingly, the DNA sequencing results did not show any obvious consensus. The phage were then taken through a fifth round using two distinct strategies: LTR→→HC2 or CO12 →→HC2. From each of these strategies 10 clones were selected and sequenced. The consensus found was minimal. Not as we had experienced with our first set of experiments with the PhD-12 phage. The following figure lists results of the DNA sequencing of the 50 clones. The letters which are in bold text indicate a common pattern in the peptide sequence. The number indicates the clone number. The clones are arranged to best demonstrate the consensus regions.

Figure 1. P.hD-12 Kit Amino Acid Sequence of #1 ~ #30 LTR→CO12→HC2 scheme

Direction: N– terminal ⟶ C– terminal

#11.		Leu	Thr	His	**Ser**	*Ile*	**His**	**Gln**	Ala	Ser	Pro	Gly	**Leu**				
#23.						Gln	**His**	**Gln**	Leu	Asn	**Ser**	Met	**Leu**	**Pro**	***Val***	**Thr**	**Ser**
#30.					Phe	Pro	**His**	**Gln**	**Gln**	His	*Leu*	Thr	*Ser*	Asp	Leu	His	
#12.			Ser	His	Tyr	Met	Asn	Ser	Ser	*Pro*	*Leu*	Ser	*Ser*	**Pro**			
#1.				Gln	Gly	Ala	**His**	Val	Asp	*Pro*	*Leu*	**Pro**	Arg	Ile	Trp		
#26						*Ile*	**His**	*Pro*	**Gln**	Leu	Ala	Asn	**Leu**	Arg	Met	**Thr**	Gln
#13.						Ala	**His**	Lys	**Gln**	Val	Pro	His	Trp	Val	***Val***	Ser	**Ser**
#19.						Ala	**His**	Asn	Pro	Leu	Val	Tyr	Asp	*Thr*	Pro	Ile	Pro
#21.						**Thr**	**His**	**Gln**	Asn	**Phe**	Lys	Val	Pro	**Pro**	**Ser**	Tyr	Met
#16.		Tyr	Ala	Gly	Gln	Val	Thr	**Gln**	Ala	**Phe**	Phe	Gln	Thr				
#25.						**Thr**	Glu	Lys	**Gln**	**Phe**	**Ser**	Asp	**Leu**	Leu	**Ser**	Leu	Leu
#27.	Lys	Pro	**Pro**	Thr	**Ser**	**Thr**	Thr	*Pro*	Trp	**Phe**	Met	Ile					
#2.					**Ser**	Ser	Glu	Tyr	Arg	**Phe**	Gln	Ala	His	*Thr*	Lys	Asp	

#28. **Ser Thr** Asn Glu Pro Thr **Ser Pro** Gly Gln Ala Ala
#29 . **Ser** Asp Val Arg Phe ***Val*** **Ser Pro** Trp ***Thr*** Pro **Thr**
#22. Asn **Pro** Asn **Ser Thr** Trp Ser Arg ***Val*** *His* Leu Pro
#14. **Ser Thr** Ala ***Pro*** Gly Ile *His* His Pro Asn Arg **Thr**
#17. Thr **Thr** Met ***Pro*** Arg Gly Asn Phe Ala Asn Leu **Thr**
_____*_____***_____***_____***

#3. Asn Met Thr Asn **Thr Thr Leu** Pro **Pro Ala** Lys Arg
#18. Val **Pro** Thr ***Lys*** **Thr** Ala Leu **Pro Ala** Lys Val Gly
#4. ***Ser*** **Pro** Trp Leu Ile Lys **Thr Pro Ala Pro Ser Ser**
#8. **His** ***Ala*** Met **Thr Thr** Gln **Thr Pro** Trp ***Leu*** ***Pro*** Arg
#5. Asn Asp **His** Arg Phe Arg Glu Tyr **Thr** Gly His ***Leu***
#10. ***Ser*** Gln **Leu** ***Lys*** **Thr** Val **Thr** His Thr ***Leu*** ***Pro*** Pro
#9. Glu **Leu** ***Lys*** Ser **Leu** Cys Cys **Ala** Gln Thr **Ser** Arg
#7. His **Pro Ala Pro Ser** Thr Met Thr Ser Tyr Arg Ala
#20. Ala Pro ***Ala*** Trp Asn **Thr** Ser Gln Thr Arg ***Leu*** Leu
#6. Gln Ile Pro ***Lys*** **Thr** Arg Leu Ser Tyr ***Leu*** Leu **Ser**

Amino Acid Sequence of CO12 →→HC2. (#C-1~C-10) and LTR→→HC2 (#L-1~L-10)
Direction: N– terminal ⟶ C– terminal

#C-4. **Thr Leu Pro Ser Pro Leu Ala Leu Leu Thr Val His**
#C-5. Tyr **Pro** Asn Met **Pro Leu Ala Leu Leu Thr Val His**
#C-7. **Gln Asn Leu Leu Trp Leu** Thr **Ser Met** His Ala **His**
#L-5. **Thr Pro** Phe Arg **Pro Leu** Met **Leu** Gly Ala **Pro Pro**
#L-1. **Gln** Ile Ser Asp Met Asn **Arg Thr Pro Ser Pro Pro**
#C-9. Tyr **Ser Leu** Gln **Thr** Thr **Asn** Val **Pro Ser Pro Ala**
L-8. Tyr **Pro Ser** ***Thr*** **Ser** Lys ***Asn*** **Thr Pro** His Phe **Ala**

L-10. Met Val **Pro Thr** Gln Gln **Arg** Tyr Met Asp **Pro Val**
#L-9 Ala Phe Tyr **Ser Pro His Asn** Arg Ala Phe **Val** Leu
#C-8. **Gln Gly** Ile Lys Ala **His Leu** Met **Ser Ser Val Asn**

#C-10. His **Pro Gly Pro** Tyr Arg **Asn Leu Ser Ser Ser** ***His***
#L-4. Ile **Pro Ser** ***Thr*** **Ser Ser** ***Asn*** Ser His Tyr Tyr Arg
C-3. Ile Thr **Ser Ser** ***His*** **Ser Pro Thr** Gln Asp Arg Phe
#C-1. Gly **Gly Ser Leu** Val Ala Lys Ala Thr Ala **Pro Asn**
#L-3. **Thr** Ala **Leu Pro** Asp Ile Gln Asp Arg **Pro Thr** Met

B. To get a fresh new start we ordered a second phage library, this time the PhD-C7C kit which contains a 7-residue randomized peptide library in which the amino acid sequence is flanked by a pair of cysteines.

Following instructions from a technical representative at New England Bio Labs, the subtraction step was first performed with the cells as a concentrated cell pellet, to push the stochiometry in favor of low affinity phage binders. For the first three rounds the study was as described above: using an LTR→CO12→HC2 scheme. After the third round the following scheme was followed: LTR (using a packed cell pellet) →→ LTR (in a flask) →→ HC2 for an additional three rounds. After each round (round 3-6) 12-21 clones were selected for sequencing. As shown in **Figure 2,** minimal overlap appeared

through the first five rounds, but in the following scheme, two common sequences were found, as indicated in the following figure (**Figure3**).

Figure 2.

Amino Acid Sequence of C7C-1 to C7C-36

1-12 = Round III 13-24 = Round IV 25-36 = Round V

Direction: N– terminal ⟶ C– terminal

# C7C-26.				**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	Phe	**Lys**		
# C7C-36.				**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	Val	**Lys**		
# C7C-29.				**Asn**	***Ser***	**Pro**	**Leu**	***Gly***	Ser	**Lys**		
# C7C-27.				**Asn**	Met	Leu	**Leu**	***Gly***	**Arg**	Thr		
# C7C-33.	Ser	Ser	Thr	**Asn**	Asn	**Pro**	Ile					
# C7C-31.					***Ser***	Asn	**Leu**	Val	**Arg**	Tyr	Gln	
# C7C-28.				**Asn**	Asn	Ser	***Ala***	His	***Pro***	Thr		
# C7C-34.				**Asn**	Phe	Gly	Ser	Trp	***Pro***	**Lys**		
# C7C-32.				**Asn**	Met	Met	***Ala***	Met	Asn	Arg		
# C7C-25.				Pro	Arg	Val	Asp	His	**Arg**	Asn		
# C7C-12.						**Thr**	**Pro**	Thr	Trp	**Thr**	Ser	Ser
# C7C-13.			Leu	**Ser**	**Thr**	**Pro**	Asn	Arg	**Thr**			
# C7C-24.				Ile	**Thr**	**Pro**	Ser	Lys	Lys	Met		
# C7C-14.				**Ser**	**Thr**	Arg	His	Met	***Pro***	Phe		
# C7C-17.				**Ser**	His	His	Thr	Glu	***Pro***	Asp		
# C7C-15.				**Ser**	**Thr**	**Leu**	Pro	His	Ser	Arg		
# C7C-16.			Lys	Ala	**Thr**	**Leu**	Gly	**Gln**	Gln			
# C7C-18.		Tyr	Trp	Asp	**Thr**	His	**Ala**	**Gln**				
# C7C-20.				Asn	Val	**Leu**	**Ala**	Asn	His	Arg		
# C7C-23.						**Leu**	**Ala**	Ser	Ala	Ala	Ser	Arg
# C7C-21.	Trp	Gln	Met	Asn	Asn	**Leu**	**Ala**					
# C7C-5.					Pro	Val	***Leu***	**Pro**	Trp	Tyr	Ala	
# C7C-2.						Arg	***Leu***	**Pro**	Thr	Gly	**Leu**	Phe
# C7C-3.					**Ser**	**Asn**	**Ser**	**Pro**	Arg	Trp	**Leu**	
# C7C-4.			Ser	Pro	**Ser**	**Asn**	**Ser**	**Pro**	Asn			
# C7C-11.						Thr	**Ser**	**Pro**	Ile	Leu	Asn	Ser
# C7C-6.	Pro	Ala	Arg	Gln	Gln	**Asn**	**Ser**					
# C7C-7		Asp	**Gln**	**Gly**	Thr	**Asn**	Arg	***Asn***				
# C7C-1.			Leu	Ser	Leu	**Asn**	Thr	***Asn***	Tyr			
# C7C-8.	Thr	Val	**Gln**	**Gly**	Asp	Arg	**Ser**					
# C7C-9.	Gly	Pro	Lys	**Gly**	Ala	Glu	His					
# C7C-35.	Glu	Leu	Arg	Ser	Tyr	Gln	Asn					

Continuing, starting as before from round three, however, now the cells were used as a packed pellet: for example, CO12 (packed cell pellet)→→CO12 (in a flask)→→HC2 for additional rounds. After sequencing 12 clones from each round, the data revealed the same two consensus peptides as found in the previous cycle (**Figure 3**).

Figure 3.

Amino Acid Sequences of C7C- #37 to C7C- #57 —from Round VI

LTR (packed cell pellet)→→LTR (in flask)→→HC2

Direction: N– terminal ⟶ C– terminal

# C7C-37.	Asp	Pro	Ser	Lys	Leu	Gln	Met
# C7C-38.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**
# C7C-39.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-40.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-41.	*The signal too weak.*						
# C7C-42.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-43.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-44.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-45.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-46.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-47.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-48.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-49.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-50.	*The signal too weak.*						
# C7C-51.	*The signal too weak.*						
# C7C-52.	*The signal too weak.*						
# C7C-53.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-54.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-55.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**
# C7C-56.	Ser	His	Tyr	Trp	Leu	Arg	Ser
# C7C-57.	Ser	His	Tyr	Trp	Leu	Arg	Ser

A total of 21 samples from Round VI were sequenced. The sequences of 4 samples were not obtained because the signals was too weak.

1. 14 sequences share one sequence = 82.35 % (clone #C7C-39~49, 53,54,56 and 57)
2. 2 sequences share a second common sequence = 11.76% (#C7C-38 and 55), around 11.76 %. Also, #C7C-26 and 36 (from Round V) share the same sequence. It should be noted that these four sequences terminate with glycine instead of cysteine as designed in the library.

Two consensus peptides were identified in this round.

Amino Acid Sequences of Round VI C7C-1 ~ 12 ------ RoundIV

CO12 (packed cell pellet)→→CO12 (in flask)→→HC2

# R4C-1.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R4C-2.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R4C-3.	Gln	Thr	Ser	Glu	Gly	Arg	Leu	
# R4C-4.	Asn	His	Arg	Met	Ser	Thr	His	
# R4C-5.	The signals are too chaotic to be read.							
# R4C-6.	His	Ser	Lys	Ala	Ala	Ser	Ile	
# R4C-7.	Asn	Trp	Ser	Thr	His	Leu	Pro	
# R4C-8.	His	Thr	Ser	Ala	Arg	Ser	Phe	
# R4C-9.	Glu	Arg	Gly	Phe	Arg	Pro	His	
# R4C-10.	Glu	His	Ser	Leu	Lys	Pro	Ala	
# R4C-11.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R4C-12.	Asn	Thr	Pro	Gly	Gln	Lys	Gln	

Amino Acid Sequences of Round V C7C-1 ~ #12 ------ RoundV

CO12 (packed cell pellet)→→CO12 (in flask)→→HC2

# R5C-1.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R5C-2.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R5C-3.	His	Val	Gly	Ala	Ala	Thr	Asn	
# R5C-4.	Asn	Ile	Lys	Leu	Thr	Ser	Ala	
# R5C-5.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R5C-6.	***Ser***	***His***	***Tyr***	***Trp***	***Leu***	***Arg***	***Ser***	
# R5C-7.	***Ser***	***His***	***Tyr***	***Trp***	***Leu***	***Arg***	***Ser***	
# R5C-8.	***Ser***	***His***	***Tyr***	***Trp***	***Leu***	***Arg***	***Ser***	
# R5C-9.	Asn	Asn	Pro	Arg	Leu	His	Thr	
# R5C-10.	**Asn**	**Ala**	**Pro**	**Leu**	**Cys**	**Phe**	**Lys**	⇐
# R5C-11.	***Ser***	***His***	***Tyr***	***Trp***	***Leu***	***Arg***	***Ser***	
# R5C-12.	***Ser***	***His***	***Tyr***	***Trp***	***Leu***	***Arg***	***Ser***	

The two consensus peptides identified in this round are.

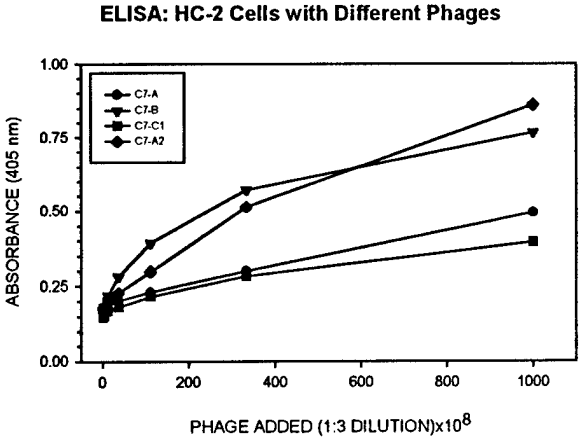
NH_2 -Cys-Asn-Ala-Pro-Leu-Cys-Phe-Lys-Gly -COOH In the report called **C7-A**

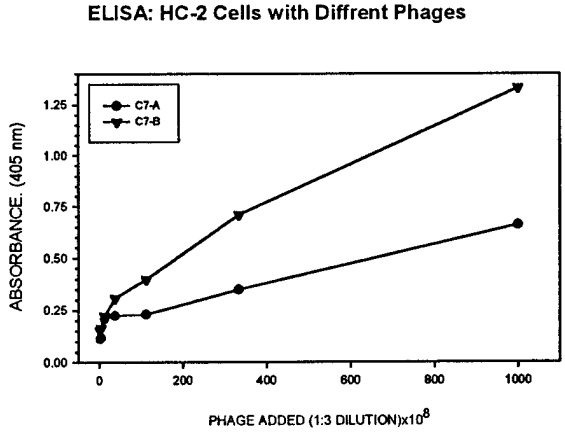
NH_2 -Cys-Ser-His-Tyr-Trp-Leu-Arg-Ser-Cys-COOH In the report called **C7-B**

ELISA: Cell Binding Assays

As before, an ELISA was used as the first step to evaluate the selected peptides. This was done with the intact phage (with the filament proteins attached) and incorporating an M-13 phage monoclonal antibody. Both the ELISA and cell binding studies with radiolabeled phage were used to evaluate the cell binding characteristics of the filament peptides. Only after showing evidence that the phage bound to the study cell were the peptides synthesized.

A number of cell assays were performed beginning with an ELISA cell binding assay (as phage filament proteins). The unlabeled phage preparations were tested against the study cell, HC2, containing the mutant EGFvIII receptor, as well as the control cells. Using a constant cell number and serial dilutions of the phage preparation, an increase was observed in phage bound to the cells. As shown in **Figure 4** the





lowest binding is observed with Phage-C7-C1, which was used as a control phage. This was a phage which survived the rounds of selection. Therefore, it was expected that it would show some degree of sticking (nonspecific binding). In this particular test peptide C7-B and C7-A2 showed the highest binding, followed by C7-A.

Figure 4

Radiolabeling of Phage with ^{99m}Tc

Conjugation of Phage with NHS-MAG3 for Radiolabeling with ^{99m}Tc

After evaluation with the ELISA the phage were radiolabeled with technetium-99m (^{99m}Tc) via the MAG3 chelator (N-hydroxysuccinimide ester of S-acetyl mercaptoacetyltriglycine, (Winnard, 1997). This approach offers a direct measure of binding characteristics rather than a sandwich type assay as is the ELISA. The standard protocol used in our laboratory for conjugation of proteins and peptides with NHS-MAG3 was followed for the phage preparations. The phage preparation in 0.1M sodium bicarbonate buffer pH 9, was incubated with the NHS-MAG3 (about 4µl of a 1mg/ml stock in DMF). After a brief incubation, the MAG3 conjugated phage was separated from free MAG3, by precipitation in polyethylene glycol. The phage pellet was then solubalized in a buffer for radiolabeling.

Figure 5a

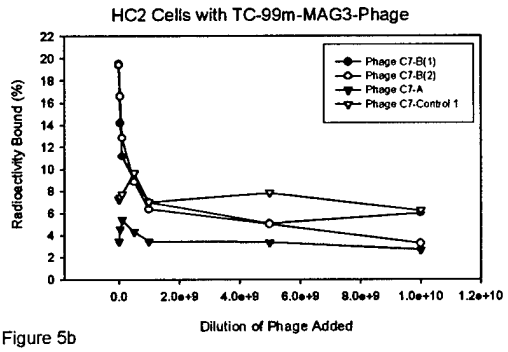
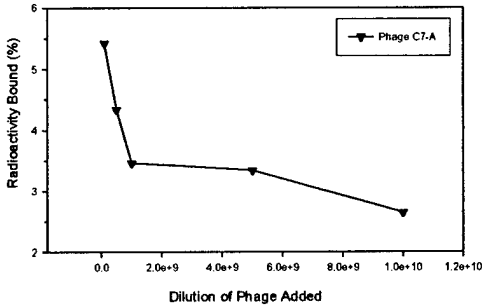


Figure 5b



For radiolabeling with ^{99m}Tc, to a solution of 10^{11} phage in 0.1M PBS was added an aliquot of sodium tartrate prepared to 50 mg/ml in 0.5 M sodium bicarbonate, 0.25 M ammonium acetate, 0.175 M ammonium hydroxide buffer, pH 9.2 for a final tartrate concentration of 7 µg/ml. After adding about 3 mCi of ^{99m}Tc-pertechnetate generator eluant, 7µl of a fresh solution of $SnCl_2 \cdot 2H_2O$ (1mg/ml in 10 mM HCl) was added. The solution was then incubated at room temperature for 30 - 60 min before purification. The labeled phage were removed by precipitation with addition of a 1:6 (v:v) dilution of polyethylene glycol, then set at 4°C for 30 min. The precipitated phage were recovered by centrifugation and dissolved in buffer. The average radiolabeling efficiency of a phage preparation was 86.6%, of which 16.5% was due to nonspecific labeling. Typically, 160µCi of ^{99m}Tc was added to 10^{11} phage.

Cell Binding Assay with ^{99m}Tc-MAG3-Phage

The radiolabeled phage were tested for binding against the specific HC2 cells using a protocol similar to the ELISA. To a constant cell number in a 96-well tissue culture plate, or eppendorf tubes, were added

serial dilutions of the ^{99m}Tc-phage, in triplicate. The cells were set on ice and incubated for 1hr, then washed and counted for incorporation of radioactivity. Eight cell binding studies were performed. Shown in **Figure 5a, b, c** are results for two studies with ^{99m}Tc-Phage C7-B, one study with ^{99m}Tc-Phage C7-A, and the control ^{99m}Tc-Phage C7-Control. The percentage of labeled phage bound versus phage dilution shows high binding for Phage C7-B, relative to the control phage. The percentage of labeled-Phage C7-A bound was low. Plotting the data for Phage C7-A on its own scale shows the expected pattern (**Figure 5b**)and **Figure 5c** shows that saturation is obtained with ^{99m}Tc-Phage C7-B.

A scatchard plot evaluation of the ^{99m}Tc-Phage C7-B data is shown **Figure 6**, indicating about 2.8 x 10^2 receptors per cell with a Kd of 162 nM. The Kd value is equal to the concentration of radioligand occupying 50% of the maximum bound, and is the inverse of the slope.

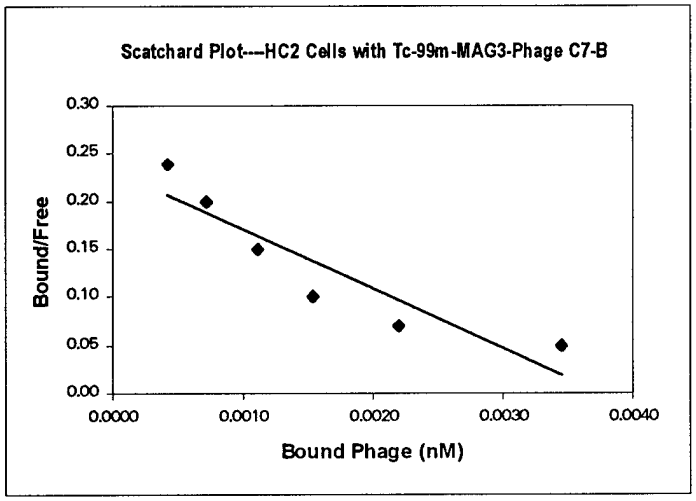


Figure 6

Synthesis of Peptides C7-A and C7-B

The two peptides C7-A and C7-B were custom synthesized commercially by Advanced ChemTech (Louisville, KY). The following sequences were obtained:

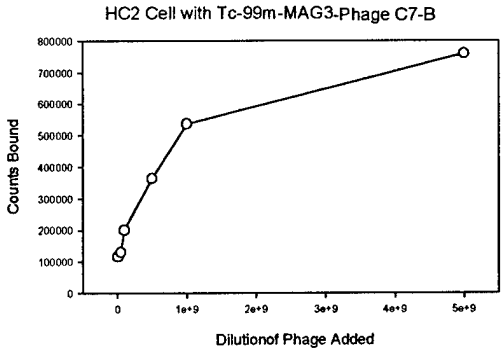
C7-A: NH_2 -Cys-Asn-Ala-Pro-Leu-Cys-Phe-Lys-Gly -COOH

C7-B: NH_2 -*Lys*-Cys-Ser-His-Tyr-Trp-Leu-Arg-Ser-Cys-COOH

So, a total of four peptides were now available.

This peptide library follows the C7C motif. Therefore, the terminal amine may be constrained by a disulfide bond. For attachment of the chelator a primary amine is needed. Therefore, into Peptide-C7-B an additional lysine was inserted near the terminal amine for conjugation purposes. Peptide C7-A already has a lysine in position #8, therefore, no additional residues were added. Most interesting is that peptide C7-A does not fit into the C7C format. This peptide is likely the result of a point mutation, with the cysteine in position 1 and 6 rather than 1 and 9. The peptide already contains a lysine plus a terminal amine.

Figure 5c



Coupling of Peptides with NHS-MAG3 and NHS-HYNIC

Two chelators were investigated for radiolabeling the peptides with ^{99m}Tc: the NHS-MAG3, as described above, and the N-hydroxysuccinimide ester of hydrazinonicotinamide (NHS-HYNIC) (Abrams, 1990).

Conjugation and Radiolabeling

NHS-Mercaptoacetyltriglycine (MAG3). The conjugation and radiolabeling of phage with ^{99m}Tc MAG3 has been described above. The following is the protocol for the conjugation and radiolabeling of these two peptides. Briefly, for conjugation with NHS-MAG3 the peptides were first prepared at a concentration of 5 mg/ml in 0.1 M HEPES buffer, pH 8.0, to which a fresh 10 mg/ml solution of NHS-MAG3 in dry dimethylformamide.(DMF) was added drop wise with agitation. The final MAG3 to peptide molar ratio was 5:1 and the volume of DMF added was always less than 10% of the total volume. The reaction mixture was then incubated at room temperature for 30-60 min before purification on a Sep Pak C-18 mini cartridge (Waters, Milford MA) as follows. The C-18 column was preconditioned with 10 ml ethanol followed with 10 ml water. Then a sample of labeled peptide was applied and the column was washed with 10 ml of H_2O to elute free pertechnetate and/or ^{99m}Tc-tartrate. The column was washed with 5ml of 8% acetonitrile (ACN), followed with 10ml of 50% ACN. The labeled peptide was eluted in the 50% ACN solution. Fractions were collected and uv absorbance at 254nm (U-2000, Hitachi Instruments, Inc, Danbury, CT) was measured. The fractions of highest peptide concentration were determined.

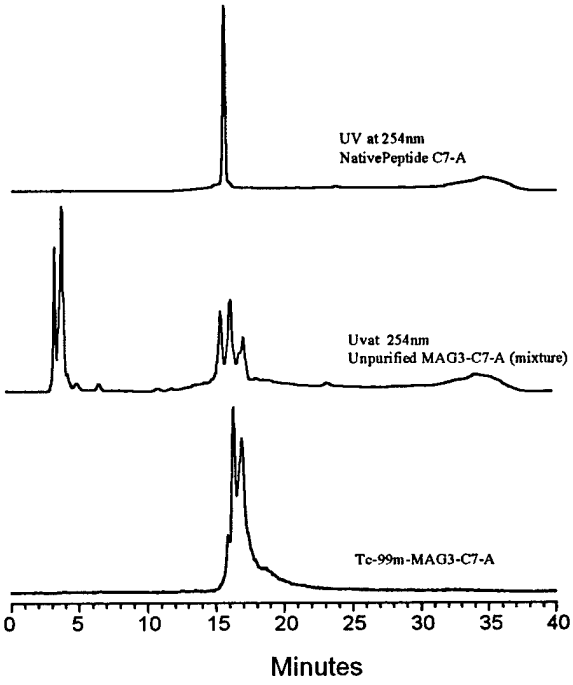
For radiolabeling, to about 20µl of the coupled peptide solution was added an aliquot of sodium tartrate prepared to 50 mg/ml in 0.5 M sodium bicarbonate, 0.25 M ammonium acetate, 0.175 M ammonium hydroxide buffer, pH 9.2 for a final tartrate concentration of 7 µg/ml. After adding about 142 µCi of ^{99m}Tc-pertechnetate generator eluant, 7µl of a fresh solution of $SnCl_2 \cdot 2H_2O$ (1mg/ml in 10 mM HCl) was added. The pH of labeling was 7.6. The solution was then incubated at room temperature for 30 - 60 min before purification over the Sep-Pak C-18 column. A purification scheme by C18 Sep-Pak was developed for each peptide with varying the percentage of acetonitrile, such that the peptide had a radiochemical purity of greater than 90%.

Fractions from the Sep-Pak column were analyzed for radiochemical purity by reverse phase HPLC on a C-18 column (YMC-pack, ODS-AMQ, S-5 µm, 25 X 0.46 cm, Waters, Milford, MA) using a Waters Millennium system with in-line UV and radioactivity detectors. The gradient system was run at a flow rate of 1 ml/min with eluant A consisting of 0.1% TFA/H_2O and eluant B was 0.1% TFA and 100% acetonitrile (ACN). For the first 5 min the system was run at 10% B, increasing over 5-8 min to 30% B, then over 8-25 min to 37% B, then over 25-30 min to 60% B, then returning to 10% B in 2 min and remaining at 10% B for 8 min. An example of the MAG3 ^{99m} Tc labeled peptide C7-A is shown in **Figure 7a**. In the figure the top panel shows the uv profile of the native peptide. The peptide has one single peak with a retention time of 15.5 min. The middle panel is the coupled unpurified peptide,

multiple peaks are found. The bottom panel is ^{99m} Tc labeled MAG3-C7-A. The labeled sample has two major peaks with retention times of 16.3 and 16.9 min. The shift in retention time signifies the binding of the chelator. When peptide C7-B was coupled with MAG3 the sample precipitated. The thiols in the MAG3 may have cross linked with the SH groups of cysteine in the peptide. Therefore, a second chelator was needed. For this we chose the NHS-HYNIC.

Figure 7a

Peptide C7-A on C18 HPLC



NHS-Hydrazinonicotinamide (HYNIC). To avoid the precipitation problem with peptide C7-B the peptides were conjugated with NHS-HYNIC using a 2:1 HYNIC to peptide molar ratio as described previously (Qu, 2001). To remove unconjugated HYNIC the sample was purified on a Sep-Pak C18 column described above (with H_2O, 8% ACN, and 50% ACN). For radiolabeling with ^{99m}Tc, about 20 µl of a 0.1 mg/ml tricine solution in water was added to about 0.1 mg of the HYNIC-peptide in 0.1 ml of 0.25 M ammonium acetate, pH 5.2. To which was added about 150µCi of ^{99m}Tc-pertechnetate generator eluant, followed by 6 µl of fresh $SnCl_2 \cdot 2H_2O$ (1mg/ml in 10 mM HCl) solution. After incubation at room temperature for 30 - 60 min, the labeled peptide was analyzed by C-18 reverse phase HPLC as described above.

^{99m}Tc-Peptide C7B showed about 25% of the radioactivity remaining on the Sep-Pak column after the 50% ACN wash. However, the labeled peptide showed a single peak on reverse phase HPLC, so the labeling was successful. ^{99m}Tc- Peptide C7A showed minimal loss on the Sep-Pak column. Reverse phase HPLC showed one major peak of the HYNIC coupled material with a retention time of 15.1 and a second minor peak with a retention time of 16 min. (shown in **Figure 7b**).

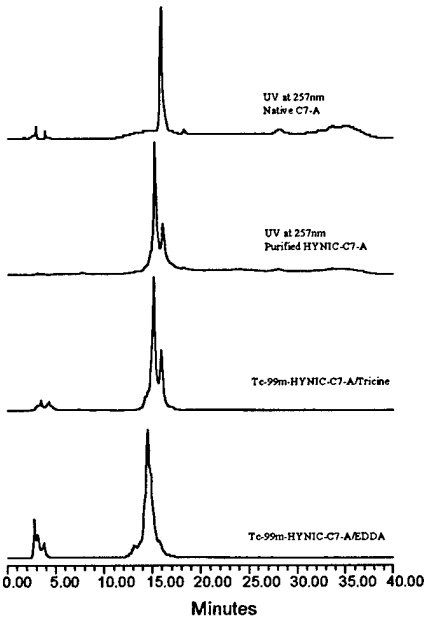
Preparation of ^{99m}Tc-labeled HYNIC-Peptides with EDDA

Others have shown and we have observed that the tricine HYNIC ^{99m}Tc complex shows binding to serum proteins. An alternative coligand in the labeling reaction is ethylenediamine triacetic acid (EDDA) (Liu G, 2001; Liu S, 1996; Decristoforo,1999a,b). However, direct labeling in the presence of EDDA results in poor labeling efficiency. To increase the labeling yield the tricine complex was first made, then the tricine was exchanged with EDDA. To prepare the ^{99m}Tc-labeled peptides with EDDA as coligand, the ^{99m}Tc-HYNIC-peptide tricine was first prepared as described above, then 0.1ml of an EDDA solution (10 mg/ml, pH7.0) was added. The solution was incubated for 30 min with heating to 70 °C. Samples were

analyzed by C-18 reverse phase HPLC. The data is shown in **Figure 7b**. The first and second panels are uv at 257nm of the native peptide C7-A and the purified HYNIC conjugated C7-A, respectively. The native peptide shows a single peak. The conjugated peptide shows a slight shift to shorter retention time, with a minor small peak which may represent the small fraction of peptide which is unconjugated. The last two panels are radioactivity traces of ^{99m}Tc-HYNIC-C7-A/tricine, and the last trace is the ligand exchange with EDDA, showing the EDDA complex. The tricine radiolabeled sample is similar the uv trace (identical retention time). The addition of EDDA and formation of the complex is verified by the slight shift to shorter retention time, 14.5 min of the ^{99m}Tc-HYNIC EDDA-peptide. The EDDA complex of peptide C7A was found to convert quantitatively to the EDDA analogue as shown in the C-18 HPLC profile (Figure 7b).

Figure 7b

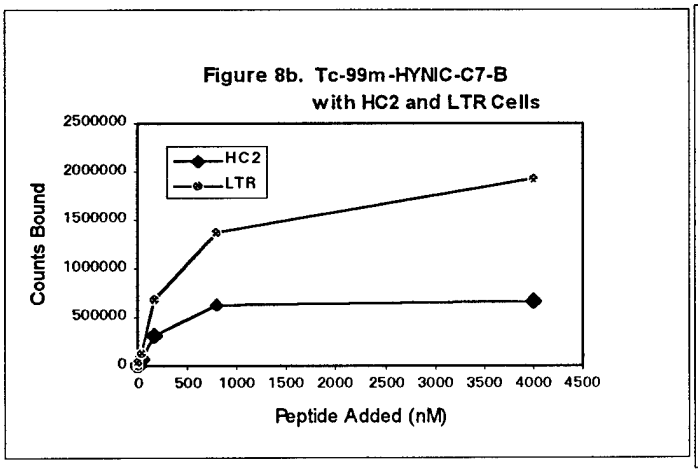
Peptide C7-A on C18 HPLC



With the C7B preparation only about 30% of the activity converted to the EDDA complex, the remainder was the tricine complex. Therefore, for further study (cell binding and mouse studies) the C7B peptide was labeled with ^{99m}Tc using tricine as the coligand. A control, labeling the peptide without the addition of a chelator showed less than 5% activity bound.

Cell Binding Studies with ^{99m}Tc-Labeled Peptides: HYNIC and MAG3

The labeled peptides were tested for binding to HC2 cells. Typically for these studies the cells, in eppendorf tubes, were used at a constant cell number (about 5 x 10^5) and serial dilutions of a labeled peptide were added. The labeled peptide ranged from about 1 ug to about 60pg per sample. **Figure 8a and 8b** show percent activity bound versus labeled peptide added and counts bound versus peptide added, respectively. Saturation is reached



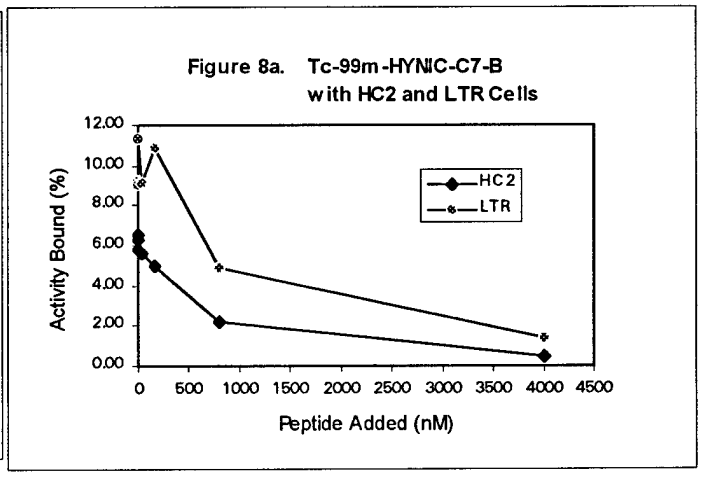
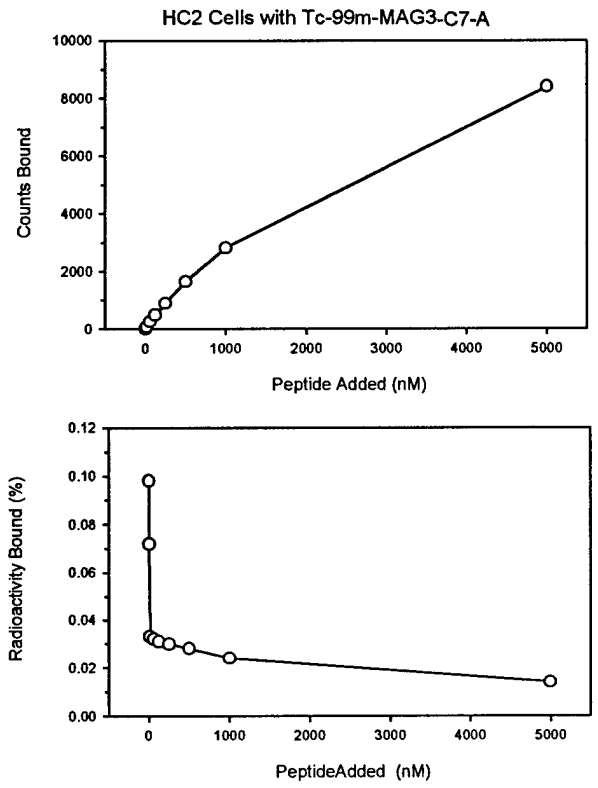


Figure 9



with peptide B on HC2 cells, and less binding is observed with the LTRs (as control cells).

Figure 9 shows a similar study with the ^{99m}Tc-MAG3-C7-A. A similar pattern is obtained as described above, although the activity bound is much lower. The same data was plotted as peptide bound (nm) versus bound peptide to free ratio, in a scatchard plot. The slope of the line = Kd, in this study the value is 4×10^{-4}M

Stability in Serum of ^{99m}Tc HYNIC and MAG3 Peptides C7-A and C7-B.

The choice of chelator is important to the in vivo stability of the radiolabel and thus to the true biodistribution and targeting of the agent in question. Size exclusion HPLC analysis was used to estimate the stability of ^{99m}Tc on each peptide preparation toward incubation at 37°C in fresh human serum. The labeled peptides were added to 37°C serum at a concentration of about 1 - 5 μg/ml, and samples were removed for analysis at various times from 5 min to 24 hrs. Recovery of radioactivity was routinely determined. All radiolabeled peptides were analyzed by size exclusion HPLC using a 1 x 30 cm Superose-12 column (Pharmacia, Piscataway, NJ), 0.1 M sodium phosphate, pH 7.0 as eluant at a flow rate 0.6 ml/min. The system was equipped with in-line radioactivity and UV detector. The HPLC system was also equipped with an in-line fraction collector (Foxy, ISCO, Lincoln, NE) and samples were counted in a NaI (Tl) gamma well counter (Cobra II, Packard Inst Co., Downers Grove, IL). A shift to higher molecular weight of the radioactivity profile could signify serum protein binding, while the presence of lower molecular weight peaks could signify a breakdown to labeled catabolites or dissociation of the radiolabel. Shown in **Figure 10** are the radio chromatograms of ^{99m}Tc-HYNIC-C7-A with tricine (left) and EDDA (right) as coligands. Top panel is the sample in saline, the middle panel is a sample removed from serum at 1hr, and the bottom panel was removed at 3hrs. These data demonstrate the strength of the EDDA complex, for only a slight shift of activity to higher molecular weight is found in the case of EDDA and more occurred with the tricine preparation. The higher molecular weight labeled species are likely to be activity binding to serum proteins.

Biodistribution of Labeled Peptides in Mice with Tumors

Both peptides were tested in mice with tumor in one thigh. To test specific binding the tumor was the HC2 which expresses the EGFRvIII receptor.

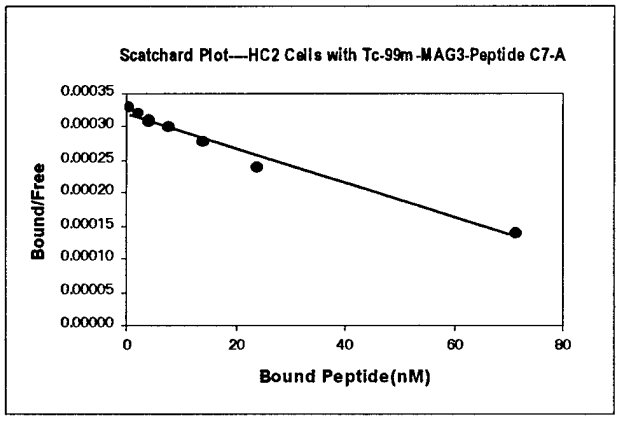
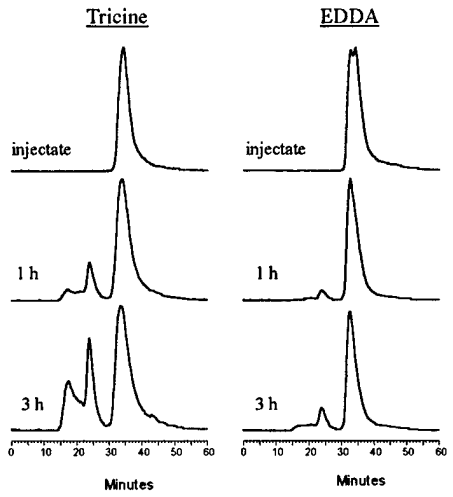
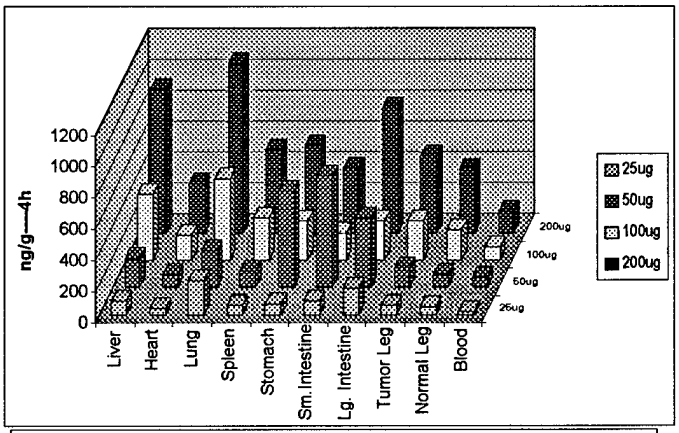
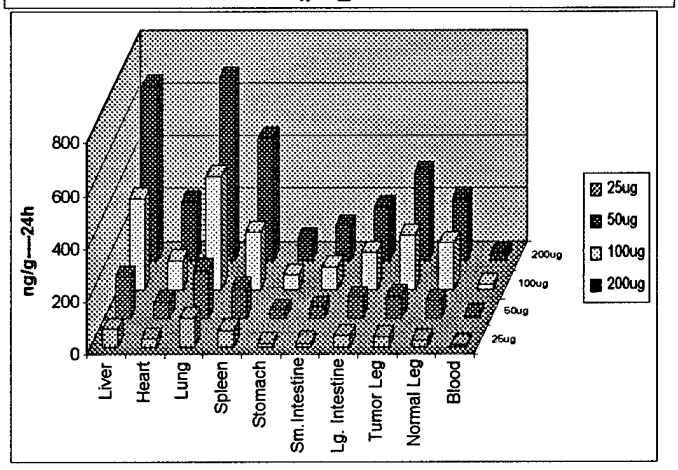


Figure 10

Incubation in Human Serum on HPLC Superose-12
Tc-99m-HYNIC-C7-A with tricine or EDDA



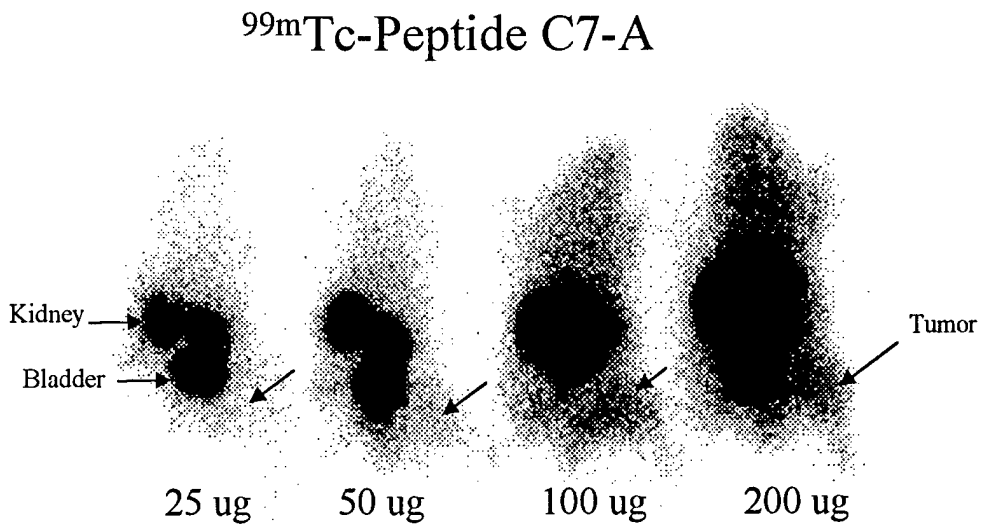




First study: Tumors were started in Swiss male nude mice (about 28 g, Taconic Labs, Germantown, NY), with $1x10^6$ cells in 0.1ml media delivered subcutaneously into the left thigh. About 10-14 days later when the tumor was about 1cm in diameter, the mice were injected via a tail vein with 0.1 ml of 50 mM PBS containing the labeled peptide. The dosage delivered was 25, 50, 100 or 200 μg, four mice per group, each with a specific activity of 10-12μCi/μg. At 3 hrs, animals were anesthetized and imaged on a gamma camera for distribution of radioactivity. After imaging, two mice from each group were sacrificed, whole blood was collected and tissues of interest were removed for counting in a NaI(Tl) well counter along with a standard of the injectate. The remaining mice were sacrificed the next morning at 24hrs post injection of labeled agents. Shown in **Figure 11** is the accumulation in tissues including tumored leg, expressed as ng per gram of tissue, at the four administered dosages. Top is at 4hrs, and bottom is 24 hrs. An increase in activity in the tumored leg over normal leg is seen, that increases with the dosage. Other than the intestinal track (likely part of the route of clearance) the liver and lung show high accumulation of activity, relative to other normal tissues.

Shown in **Figure 12** are gamma camera images of mice taken at 3hrs following administration of the labeled peptide. One animal from each dosage is shown: 25, 50, 100, 200μg, left to right. All were images simultaneously and since each received the same specific activity, the higher dosages appear overexposed. An arrow points to the tumor in the right thigh (in this view). In comparing the left to right thighs, clearly there is accumulation in the tumor. As seen in the images at the lowest dosages, the label clears from circulation primarily through the kidneys (two kidneys are seen above the bladder).

Figure 12



Another set of mice carrying the HC2 tumors received an administration of ^{99m}Tc-C7-B (labeled with HYNIC and tricine) and a set received ^{99m}Tc-C7-A (labeled with HYNIC and EDDA). For C7-A the tumor to muscle ratios were about 3:1 and tumor to blood was 6:1. Whereas with C7-B tumor accumulation increased relative to C7-A. In the case of C7-B the tumor to muscle ratio was about 5:1. However, the major organ of accumulation was the liver with about 40% of the injected dose. The C7-B peptide has shown higher binding to cells in culture. This peptide has been suspected as "sticky" in nature. Therefore, the liver accumulation may be due to its "sticky nature" as well.

Summary of Third Phase (Year III)

In vivo Selection using a PhD-12 Phage Peptide Library. The advantage with the *in vivo* selection process lies in the subtraction step. Using cells in culture for subtraction of phage, one is limited to a single cell type. By injecting the phage *in vivo* one can remove phage that stick to any organ or tissue and thus are eliminated from the pool. Therefore when a consensus is found with tumor, for example, the question of the ligand's biodistribution may be more promising.

A preliminary *in vivo* selection was performed using the PhD-12 library kit. For *in vivo* selection, nude mice (Swiss male nudes, Taconic Farms, Germantown, NY) were prepared with tumors in one thigh. When tumors were about 0.5grams one animal received via a tail vein 0.1ml of phage (PhD-12 library kit) diluted in PBS and containing approximately 2 x 10^{11} plaque forming units. Two hours following injection, the animal was killed with cervical dislocation under anesthesia (with metophane). The tumor was removed, cleaned of muscle and skin, and washed twice with 2-5ml cold PBS. The sample was diced with scissors and homogenized on ice using a hand held glass tissue homogenizer to obtain a uniform suspension. The cellular suspension was washed twice with 8ml of cold PBS. Bound phage were eluted with suspending the pellet in 0.5 ml 0.2M glycine-HCl, pH 2.2, containing 1mg/ml BSA, for 5 min. The sample was spun and the elution was repeated 2 more times to ensure recovery of all bound phage. The low pH eluant was neutralized immediately with 1M Tris-HCl, pH 9.1. The phage were amplified and titered as described above and the amplified phage were administered 3 more times for a total of 4 cycles.

The sequencing results from Round I through IV are shown. Indicated in bold type are residues forming a common pattern between sequences. Sequences from Round I of *in vivo* Phage Selection

R1-B.										Val	**Ser**	**Pro**	**Pro**	Ser	His	Ser	His	Glu	Arg	Leu	Ala		
R1-L.	Glu	Gly	Thr	Thr	Thr	Gly	Ser	Ser	Ile	**Ser**	**Pro**	**Pro**												
R1-A.				Gly	**Trp**	Ala	Thr	Ile	**Ser**	Gly	Phe	**Pro**	**Leu**	Thr	Trp									
R1-G.					**Trp**	Ser	Met	Glu	**Ser**	Pro	Arg	**Pro**	**Leu**	Ser	Gln	**Ile**								
R1-J.										His	Met	**Pro**	**Leu**	**Pro**	Val	**Ile**	Tyr	Ser	Pro	Gln	Ser			
R1-M.							Gln	Pro	**Pro**	**His**	**Leu**	Phe	**Pro**	**Leu**	**Pro**	Thr	Arg	Pro						
R1-C.	Val	Ala	Ser	Gln	Thr	Asn	Ser	**Pro**	**His**	**Leu**	Ser	Leu												

R1-N. Ala Val Asp Leu Gln **Ile** Gln **Pro** **Pro** Thr Pro Ser
R1-H. Met Pro Leu Trp Ile **Ile** Ala **Pro** **Pro** Glu Leu Tyr

R1-K. Phe Val Ser Asn Pro His Gly Leu Arg Pro Met Glu

R1-E. Asn Thr Leu Gly Phe Ser Gly Pro Val **His** **Ser** **Pro**
R1-F. Asn Asn His Asn Gly Trp Gly Ile Ala Ile Ala Val
R1-D. Ser Ser Cys Thr Lys Thr Ser Ala Cys Met **Pro** **Pro**

R1-Q. Ser Ile Arg Met His Ser Asn Thr Asp Arg Phe Gln
R1-P. Asp Met Gly Pro Ser Ser Ile His Pro His Leu Val

Sequences from Round II of *in vivo* Phage Selection

R2-Q. Ser Ser Met Met Asn **Thr** Gln Met **Arg** **Pro** Pro **Gln**
R2-F. Ala Gly Lys Leu **Thr** **Met** Pro **Arg** Phe **His** Leu Gln
R2-A. Gln Gln **Pro** **Thr** **Met** His **Arg** **Pro** **His** **Gln** Leu Ala
R2-B. His His Leu **Pro** **Thr** Tyr Leu **Arg** Thr Val His Ser

R2-J. Thr Pro Leu **Pro** **Pro** Leu Pro ***Ala*** ***Arg*** Asn Pro Leu
R2-G Val Lys **His** Ser **Pro** **Pro** **Asn** **Ala** Glu ***Ala*** ***Arg*** Ser
R2-P. Gln Ser Gln Thr Leu **His** Asn **Pro** Thr **Asn** **Ala** Asn

R2-I. His Gly **Thr** Tyr **Thr** His **Pro** **Ser** Val Pro Thr Pro
R2-M. Asp Ile His Ile ***Ser*** **Thr** ***Ile*** **Thr** Ser **Pro** **Ser** ***Pro***
R2-E. Tyr **Thr** Ser Met ***Ser*** Glu Asn **Thr** Phe Arg **Ser** ***Pro***
R2-C. **Thr** Thr **Thr** Ala Ser Asp **Thr** ***Ile*** Arg **Thr** Val **Ser**
R2-L. Asn **Thr** Ala Tyr Ser Lys Gly **Thr** Trp Pro **Thr** Gln

R2-H. **Ser** Thr Ser Tyr Asp Gly Ile **Pro** **Pro** **Thr** Val Gln
R2-D. **Ser** Pro Thr Phe Ile Gln His **Pro** Met **Thr** **Phe** Ala
R2-N. His **Ser** Lys Ile Thr Thr His Gln Gly Ala **Thr** **Phe**

Sequences from Round III of *in vivo* Phage Selection

R3-I. ***Asn*** ***Met*** Ser Lys **Leu** Ser Gly Ala Trp Glu Ile Thr
R3-L. ***Asn*** ***Met*** Pro Thr **Leu** ***Pro*** Asn Ser Arg Asn His Ala
R3-Q. Ala Ala Thr Pro Ser Gln **Ser** **Ser** **Pro** Ser **Ser** Lys
R3-D. Glu **Ser** **Ser** **Pro** **Pro** **Ser** Thr **Leu** **Ala** **Leu** ***Pro*** Leu
R3-A. Ser Cys ***Thr*** Gly **Pro** Trp Gln **Leu** **Ala** Ser Leu Thr
R3-E. Ala Val ***Thr*** Glu Ser Lys Tyr **Leu** Thr **Leu** Met Val

R3-K. Gln Ile Thr Ala Ser Phe Thr ***Lys*** Ile Thr **Asn** **Thr**
R3-P. Asn Asn Leu Ala Trp Ser Thr Ser Ala Ile ***Lys*** Ser
R3-B. Ser Leu Ser Met Tyr **Pro** **Ala** Pro Gln **Asn** **Thr** Arg
R3-F. His Asn Ile Asp Thr Trp Met Arg Thr **Pro** **Ala** Lys
R3-G. Ser Asp Arg His Asp Met Phe Lys Pro Thr Met Trp

Sequences from Round IV of *in vivo* Phage Selection

R4-B. Gly **Phe** Asn Ile Ser Phe **Ala** Ala Thr Pro Gly Gln
R4-C. **Phe** Val Glu **His** **Gly** **Ala** Trp Gln Asn Leu Pro Arg
R4-D. Ser **His** **Gly** Ser Asp Thr Ser Ala Leu Gly Ile Ala

R4-A. Ala Ser Trp Gln Gln Leu Asn Gln Arg Met Tyr Val
R4-F. Leu Leu Ser Ala Asp Leu Lys His Ser Met Arg Lys
R4-I. Ser Thr Ile Ser Met Leu Ala Ser Lys Pro Ser Arg
R4-G. Leu Pro Glu Asn Ser Val Thr Arg Ser Leu Leu His
R4-J. Ser Ser Ser Asn His Leu Met Tyr Pro Phe Leu Ser
R4-L. Ala Val Ala His Asp Phe Glu Ser His Tyr Ala Leu

R4-K. Thr Asp Asn **Thr** **Ala** Gln Thr Trp Leu Arg **Ile** Ser
R4-M. Ser His Gln Pro **Thr** **Ala** **Ile** His **Pro** **Thr** **Pro** **Ile**
R4-Q. **Thr** Phe **Ile** **Lys** **Pro** **Thr** **Pro** **Ile** Met Pro Met Gly
R4-P. Ile Thr Asn Gln Gly **Lys** Thr Phe Ala **Ile** Leu Gln
R4-R. Gly Thr Leu Gln Thr Thr Leu Asn Gly Trp Arg Ser
R4-N. Thr Thr His His Leu His Ser Lys Gln Val His Pro

From Round IV of *in vivo* selection two peptides that have close similarities were found.
R4-M. Ser His Gln Pro **Thr** Ala **Ile** His **Pro** **Thr** **Pro** **Ile**
R4-Q. **Thr** Phe **Ile** Lys **Pro** **Thr** **Pro** **Ile** Met Pro Met Gly

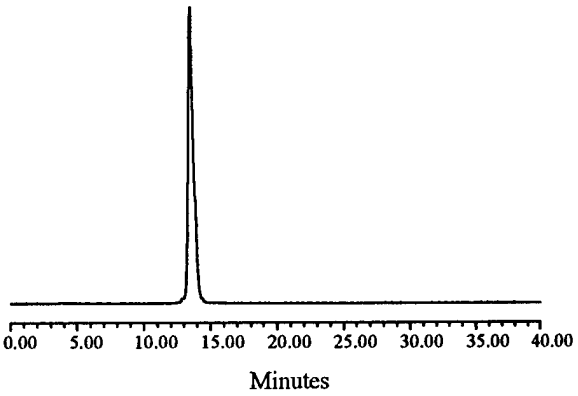
In addition, comparing this *in vivo* data to the four consensus sequences found previously with the two *in vitro* selections, other striking similarities were observed. The following comparisons are made. The R3-D is similar to Phage-3, the R4-G is similar to Phage-5, and the C7-B is similar to R4-L.

Phage #3. Ser Pro Trp **Ser** Glu **Pro** Ala Tyr **Thr** **Leu** **Ala** Pro
R3-D. Glu **Ser** Ser **Pro** Pro Ser **Thr** **Leu** **Ala** Leu Pro Leu

Phage #5. Asn Asn Pro Trp Thr Glu Met **Arg** **Ser** **Leu** **Leu** Ser
R4-G. Leu Pro Glu Asn Ser Val Thr **Arg** **Ser** **Leu** **Leu** His

C7-B. **Ser** **His**
Tyr Trp **Leu** Arg Ser
R4-L. Ala Val Ala His Asp Phe Glu **Ser** **His** Tyr
Ala **Leu**

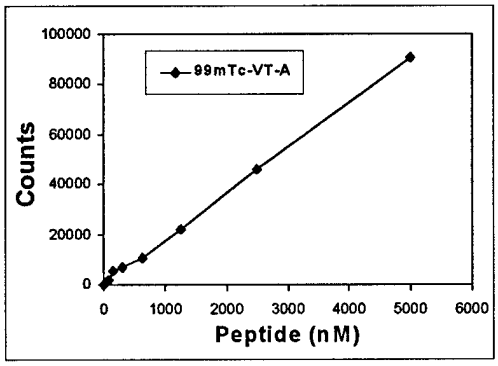
Radioactivity on C18-HPLC
Tc-99m-VT-A

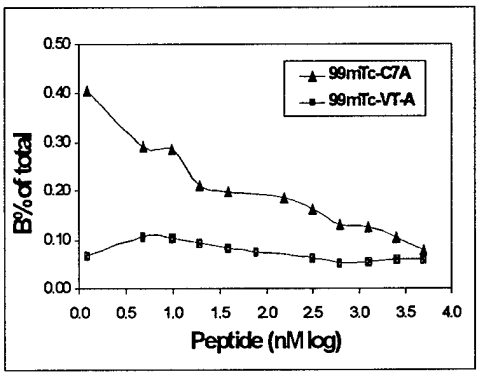


After Round Five of *In vivo* Screening. After a fifth round of selection and amplification, a consensus peptide was identified. Out of 20 clones sequenced from round five, 15 were represented by the following sequence: NH_2-Ser-Val-Ser-Val-Gly-Met-Lys-Pro-Ser-Pro-Arg-Pro-COOH. For testing, this peptide was synthesized by the in-house peptide synthesis core laboratory located at the university. On to the peptide the following additions were made. On the carboxyl end a Gly-Gly-Gly-Ser was added to serve as a linker, followed by a Gly-Gly-Gly-Cys to be used as an N3S chelator for ^{99m}Tc labeling, rather than subsequent conjugation to an NHS-MAG3 which would require a purification step. In addition, a Glu was added upon discussion with the

synthesis laboratory to adjust polarity, and a Lys was added for attachment of alternative chelators. The final molecular weight was about 2,100 Daltons. The peptide is referred to herein as VT-A.

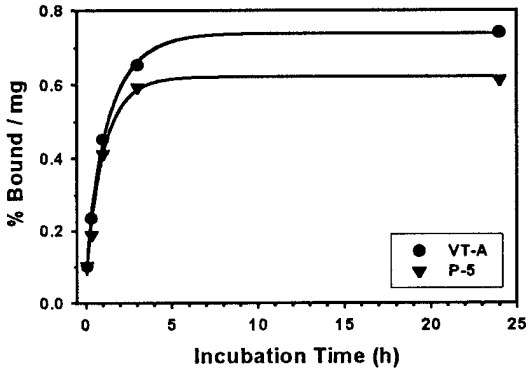
The peptide was radiolabeled with ^{99m}Tc, as typical in this laboratory for labeling of MAG3 conjugated peptides, using a tartrate solution, stannous chloride, and pertechnate. After a 45m incubation, the labeling mixture was nalyzed without purification by reverse phase HPLC using a C18 column. As shown in **Figure 13** one sharp peak was obtained. Typically a specific activity of 100μCi per μg was used with specific activity act in the range of 1mCi/μg easily achievable. The addition of the internal MAG3 like chelator into the molecule eliminated the conjugated step and subsequent need for purification. For comparison in evaluation studies, two similar consensus peptides selected by *in vitro* selection methods described above were used. The P5 a 12-mer peptide, and the C7-A, a cyclic 7-mer peptide both described previously. These control peptides were conjugated to the NHS-MAG3 and radiolabeled with ^{99m}Tc as before.

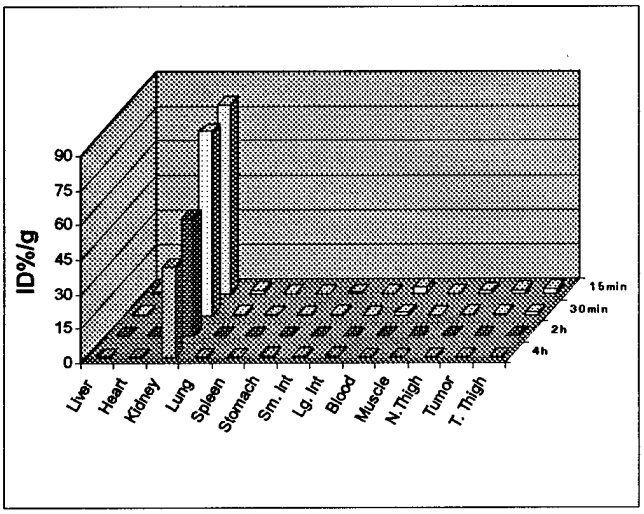


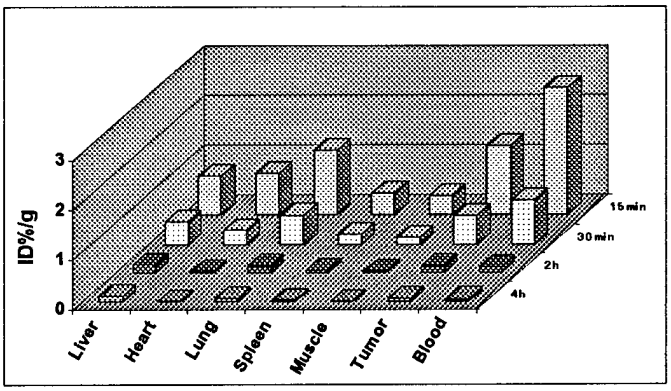


For *in vitro* evaluation cell binding tests were performed with the ^{99m}Tc-labeled VT-A peptide, concentration ranging from 1nM to 5μM and with the HC2 cells from culture (using about 5 x 10^5 cells per sample). The cells grown in a 96-well plate pretreated with 1% gelatin to enhance their adherence to the plastic. After an overnight incubation at 37°C with 5% CO_2/air a confluent monolayer was formed. In one test, a plot of counts bound versus concentration of peptide added yielded a straight line, suggesting that binding of the VT-A to the HC2 cells in culture may be non-specific **(Figure 14a)**. In another study, a comparison was made of the VT-A to the C7-A peptide selected by *in vitro* biopanning methods. As shown (**Figure 14b)** only the C7-A showed the expected response: the highest activity bound at the lowest peptide concentration, as expected. The peptide selected by *in vivo* methods, theVT-A, showed nearly no change in percentage bound over peptide concentration. Again suggesting a non specific binding of the VT-A peptide to the HC2 cells in culture.

An assay was performed using pieces of the solid HC2 tumor excised from mice, thus more representative of the *in vivo* selection situation. Cubes of about 1mm were cut and incubated with the test peptides over time in about 0.25ml of media. In this *in situ* evaluation, binding of the VT-A peptide approached saturation, as well as did the P5 peptide (**Figure 15**). Both peptides in the cube assay performed similarly. Thus, results with pieces of solid tumor differed from an assay with cells in culture with these two peptides selected by different means. Another assay was performed using solid





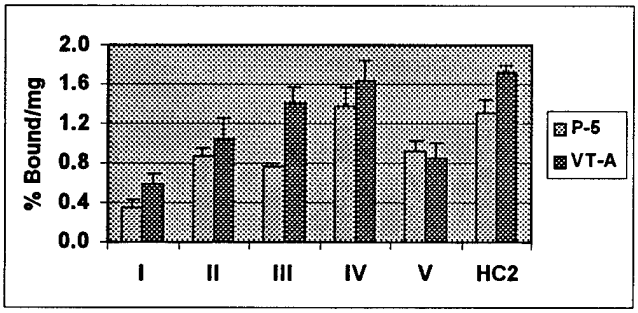


tumor cubes, comparing the HC2 xenograph from mice and five samples of human tumor from clinical pathology. The histogram (**Figure 16**) shows the percent activity bound for the two ^{99m}Tc-labeled peptides, VT-A and P5. The human tumor samples are: a human serous carcinoma (I); an adenocarcinoma of the lung (II), metastatic renal cell carcinoma (III); gastric adenocarcinoma (IV); and a metastatic ovarian tumor (V).

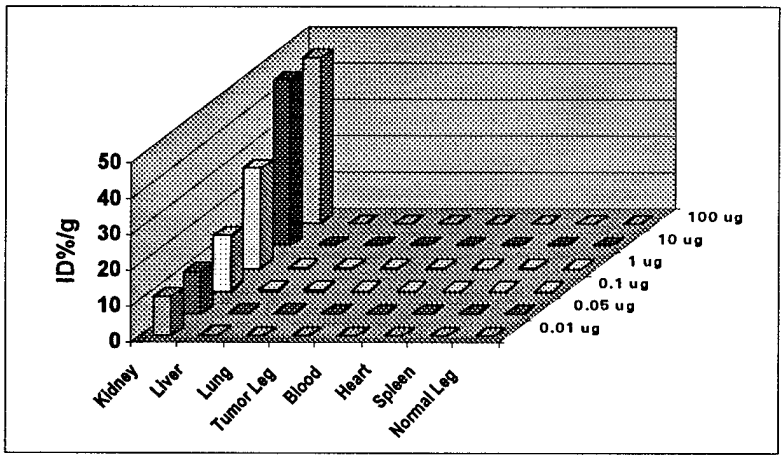
As the data indicates, a varied response was observed to the different tumor types, the VT-A showed enhanced binding to all but one of the tumors. The highest binding was observed to the mouse HC2 xenograph and the gastric adenocarcinoma. Testing these peptides against a panel of tumors of varying origin is one means by which to evaluate these peptides as potential imaging agents for detection of cancer. The VT-A peptide was evaluated in mice with HC2 tumors. In one study, the dose of peptide delivered ranged from 0.01μg to 100μg. After 4h the animals were sacrificed. As apparent in **figure 17a** independent of dosage the peptide accumulated primarily in the kidneys with minor uptake in remaining tissues. A closer look at the other tissues (**Figure 17b**) showed similar levels of activity in the liver and lung, and the tumor showed little variation with dosage. The tumor :normal leg ratio was quite variable, ranging from 2.3 t-5.2.

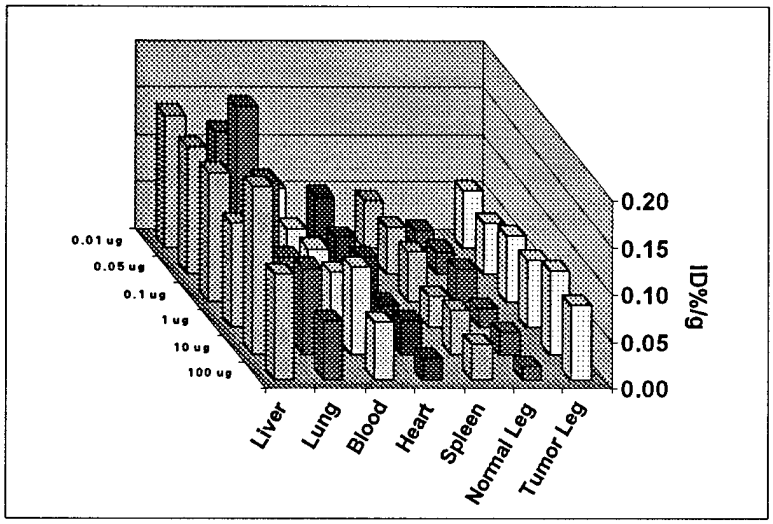
Sacrificing the animals at various times from 15m through 4h (with a dose of 40μg) showed, an immediate accumulation in the kidneys, as early as 15m about 80% of the activity was in the kidneys (**Figure 18a**). Activity in tumor and major organs with time showed minimal activity beyond 30m (**Figure 18b**). With tumor to muscle ratios improving with time to 5.2 and 5.4 at 2h and 4h respectively (**Figure 18c**). With improvements in tumor to blood ratio as well. Although we observed tumor to muscle ratios of 5:1, the low absolute uptake in tumor was attributed to rapid peptide digestion, as determined by HPLC analysis of serum and urine samples.

Shown in **figure 19** are the radioactivity profiles of the HPLC analyses using a size exclusion column (Superose-12, Pharmacia, NJ). Although the peptide appeared fairly stable in an *in vitro* incubation in human serum through 3h (left panel). Evidence of activity in urine from mice revealed a species of lower molecular weight than the starting material (right panel), appearing at the earliest time point 15m. With no intact peptide found in urine. Examination of the corresponding mouse serum at 15m showed evidence of peptide digestion (middle panel) with more than 40% of the radiolabel present as low molecular weight catabolites, but 25% still present as labeled peptide at this time. The low molecular weight species in serum was the predominant feature by 2.5h.



The following table shows a comparison of the biodistribution in mice, with HC2 tumors, of two ^{99m}Tc-labeled phage selected 12-mer peptides. One selected *in vitro*, the P-5, and the other by *in vivo* methods, VT-A. There is a marked difference in the biodistribution of these two similar 12-mer peptides. The liver is about 10-fold lower for the VT-A, which also shows very high kidney activity. The P-5 peptide shows much lower activity in kidney, and clearance appears to be through the intestinal tract. The fact that they were selected by *in vitro* and *in vivo* methods may in part account for this remarkable difference. Even though both of these peptides show low absolute tumor accumulation, the VT-A, which we know is rapidly degraded *in vivo* shows a higher tumor to muscle ratio than the P-5: a 5.4 vs 3.2, and improved tumor to blood ratio as well: 1.5 vs 0.95.





To summarize, although peptide degradation was evident, positive tumor accumulation of this *in vivo* phage selected peptide was demonstrated with tumor to muscle ratios of 5:1 attained at 2h. The peptide selected by *in vivo* methods showed improved tumor to muscle ratios over a peptide selected by *in vitro* phage library methods. Although encouraging ratios were obtained, peptide modification for *in vivo* stability for this peptide will be required.

Biodistribution at 4 h. Values shown are percent injected dose per gram of tissue. SD in parantheses.

	VT-A		P5	
Liver	0.115	(0.009)	1.27	(0.534)
Heart	0.022	(0.002)	0.008	(0.002)
Kidney	39.3	(2.335)	0.453	(0.127)
Lung	0.062	(0.010)	0.018	(0.003)
Spleen	0.032	(0.011)	0.008	(0.001)
Stomach	0.332	(0.637)	0.200	(0.146)
Sm Intest	0.581	(0.975)	12.75	(16.1)
Lg Intest	0.703	(0.254)	21.14	(13.5)
Muscle	0.015	(0.003)	0.009	(0.004)
Tumor	0.077	(0.006)	0.025	(0.003)
Blood	0.053	(0.003)	0.026	(0.003)
Tumor/muscle	5.4		3.2	
Tumor/blood	1.5		0.95	

KEY RESEARCH ACCOMPLISHMENTS:

1. With a phage display peptide library, **four** consensus peptides that show specificity for cells which carry the mutant EGFvIII receptor have been identified by *in vitro* selection methods.

2. Using *in vivo* selection methods with a phage display peptide library, **one** consensus peptide that show specificity for cells which carry the mutant EGFvIII receptor have been identified.

3. The phage carrying these peptides were radiolabeled with ^{99m}Tc after conjugation with either the NHS-MAG3 or NHS-HYNIC chelator. The radiolabeled phage carrying the specific peptides showed specificity in cells carrying the mutant EGFvIII receptor .

4. The total of **five** consensus peptides were synthesized commercially and then conjugated to NHS-MAG3 and/or NHS HYNIC for radiolabeling with ^{99m}Tc.

5. The methods of conjugation to the chelator, radiolabeling and post labeling purification were defined for each peptide with MAG3 and HYNIC. The labeled peptides showed specificity in cell binding studies.

6. The labeled peptides showed positive accumulation in tumors expressing the mutant receptor.

7. The **five** consensus radiolabeled peptides will be tested against samples of breast tumor from clinical pathology to evaluate the binding of the radiolabeled peptides in *in vitro* tissue binding assays.

8. Selection studies will continue for new peptides which show high affinity for the mutant receptor.

REPORTABLE OUTCOMES

1. Abstract and presentation: Society of Nuclear Medicine Annual Meeting June, 2000, St. Louis MO.
2. Abstract and presentation: European Association of Nuclear Medicine, Paris, France, Sept 2-6, 2000.
3. Abstract and presentation Society of Nuclear Medicine Annual Meeting, June, 2002, Los Angeles, CA
4. Paper: Labeling of Phage with ^{99m}Tc (in progress).
5. Paper: ^{99m}Tc-labeled peptides to the mutant EGF receptor. (in progress).
6. Funding applied for based upon the preliminary data obtained here, NIH RO1, July 2002.
7. Personnel funded by this project: Robin marcel, Guozheng Liu, Suresh Gupta, Ning Liu.

CONCLUSIONS

We have worked with two of the three phage peptide libraries that are available. Four consensus peptides have been identified in the investigation of these two phage display peptide libraries. The peptides appear promising based upon cell studies and studies in mice with tumors. The latest studies on samples of various tumors from clinical pathology for *in situ* testing of these radiolabeled phage peptides for clinical potential has shown promise.

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