Identification of novel peptide ligands for the cancer-specific receptor mutation EFGRvIII using a mixture-based synthetic combinatorial library

ARTICLE *in* BIOPOLYMERS · MARCH 2009

Impact Factor: 2.39 · DOI: 10.1002/bip.21117 · Source: PubMed

6 AUTHORS, INCLUDING:

Charlotte Lund Denholt
Rigshospitalet
2 PUBLICATIONS **15** CITATIONS
SEE PROFILE

Paul R Hansen
University of Copenhagen
61 PUBLICATIONS **919** CITATIONS
SEE PROFILE

Hans Skovgaard
Rigshospitalet
68 PUBLICATIONS **1,524** CITATIONS
SEE PROFILE

Nic Gillings
Rigshospitalet
60 PUBLICATIONS **620** CITATIONS
SEE PROFILE

Identification of Novel Peptide Ligands for the Cancer-Specific Receptor Mutation EFGRvIII Using a Mixture-Based Synthetic Combinatorial Library

Charlotte Lund Denholt,[1] Paul Robert Hansen,[2] Nina Pedersen,[3] Hans Skovgaard Poulsen,[3] Nic Gillings,[1] Andreas Kjær[1,4]

[1] *Department of Nuclear Medicine and PET, Copenhagen University Hospital Rigshospitalet, 2100 Copenhagen Ø, Denmark*

[2] *Department of Natural Sciences, University of Copenhagen, 1871 Frederiksberg C, Denmark*

[3] *The Department of Radiation Biology, Copenhagen University Hospital Rigshospitalet, 2100 Copenhagen Ø, Denmark*

[4] *Cluster for Molecular Imaging, University of Copenhagen, 2200 Copenhagen N, Denmark*

Received 4 July 2008; revised 30 October 2008; accepted 5 November 2008
Published online 17 November 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.21117

ABSTRACT:

We report here, the design and synthesis of a positional scanning synthetic combinatorial library for the identification of novel peptide ligands targeted against the cancer-specific epidermal growth factor tyrosine kinase receptor mutation variant III (EGFRvIII). This receptor is expressed in several kinds of cancer, in particular, ovarian, glioblastomas, and breast cancer, but not in normal tissue. *The library consisted of six individual positional sublibraries in the format, H-O_{1-6}XXXXX-NH_2, O being one of the 19 proteinogenic amino acids (cysteine omitted) and X an equimolar mixture of these. The library consisted of 114 mixtures in total. Using a biotin-streptavidin assay, the binding of each sublibrary to NR6M, NR6W-A, and NR6 cells was tested. These cells express EGFRvIII, EGFR, and neither of the receptors, respectively. The result from each sublibrary was examined to identify the most active amino acid residue at each position. On the basis of this knowledge, eight peptides were synthesized and tested for binding to EGFRvIII. We identified one peptide, H-FALGEA-NH_2, that showed more selective binding to the mutated receptor than the EGFRvIII specific peptide PEPHC1. This study demonstrates the value of using mixture-based combinatorial positional scanning libraries for the identification of novel peptide ligands targeted against the cancer-specific EGFRvIII. Our best candidate H-FALGEA-NH_2 will be radioactively labeled and evaluated as an imaging agent for positron emission tomography investigation for diagnosis, staging, and monitoring of therapy of various types of cancer.*

Keywords: cancer; combinatorial library; epidermal growth factor tyrosine kinase receptor mutation variant III (EGFRvIII)

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the Biopolymers editorial office at biopolymers@wiley.com

Correspondence to: Charlotte Lund Denholt; e-mail: charlotte.lund.denholt@rh.regionh.dk

Contract grant sponsors: Danish Cancer Society, J.S. Foundation, Aase & Ejnar Danielsen Foundation

INTRODUCTION

One of the few known cancer-specific surface markers is the epidermal growth factor tyrosine kinase receptor mutation variant III (EGFRvIII).[1] The mutated receptor lacks amino acids residues 6–273 in the extracellular domain compared with the wild-type

EGF receptor. The junction is fused by a new glycine residue, leading to a novel peptide sequence. EGFRvIII is present in number of human malignancies, in particular ovarian, breast cancer[2] and glioblastomas.[3] However, this mutation has never been identified in normal tissue.

The mutated receptor is an obvious choice as a target for development of new cancer-specific ligands.

Therapeutic approaches have included the use of unarmed MAbs[4,5] radiolabeled MAbs,[6] MAbs conjugated to immunotoxins,[7] or boronated dendrimers.[8]

Trembath et al.[9] reported a novel small molecule identified from a small-molecule library, NSC-154829, that selectively inhibited growth of different human glioblastoma cells expressing EGFRvIII but permitted normal growth of matched control cells. However, the only strategy that has made it to clinical trials to date is active immunization with peptide vaccines derived from the glycine junction of EGFRvIII.[10]

Small peptides that selectively recognize tumor cells have advantages over small molecules in terms of specificity and affinity for targets and to antibodies in terms of size.[11–14]

Firstly, the effective tissue penetration of short synthetic peptides, in combination with their selective binding and internalizing capacity by cancer cells, make these agents ideal candidates for delivery of therapeutics and radioactive probes for diagnosis of various types of cancer. Secondly, L-peptides are cheaper to produce and can be easily manipulated to optimize affinity for a particular receptor. Their short biologic half-life because of proteolysis in plasma, may be circumvented by selectively incorporating D and nonproteinogenic amino acid residues.[15]

The only known peptide ligand targeted against EGFRvIII is PEPHC1,[16] HFLIIGFMRRALCGA, PEPHC1 was developed using the computer program AMINOMAT. This program identifies peptides that are hydropathically complementary to the target sequence. PEPHC1 had binding ability and selectivity to EGFRvIII. Very recently, we identified the amino acid residues in PEPHC1 important for binding to the tumor-specific receptor EGFRvIII.[17] Our results indicated that the amino acid residues at the N-terminus of PEPHC1 are essential for the binding to the mutated receptor. To identify novel peptide ligands targeted against EGFRvIII, which eventually could be radioactively labeled for diagnosis of various types of cancer, we designed a combinatorial peptide library.

Combinatorial peptide chemistry is a powerful tool for identifying new receptor binding peptides, where no prior knowledge about naturally-occurring ligands is required. For a recent review of cancer-targeting peptides identified from combinatorial chemistry, see Aina et al.[12]

By using this strategy, it is possible to rapidly synthesize and screen thousands to millions of different peptides, compared to the time and resource consuming "one-at-the-time" synthesis.[18] Currently, there are five main methods for creating peptide libraries[19]; (i) biological libraries such as phase display, (ii) spatially addressable libraries or solution phase libraries, (iii) synthetic library methods using affinity chromatogram selection, (iv) one-bead-one-compound, and (v) synthetic library methods requiring deconvolution such as positional scanning or iterative approach.

A positional scanning synthetic combinatorial library (PS-SCL) consists of sublibraries in which one position is occupied by a defined amino acid residue, the others by mixtures. Each sublibrary is tested in a solution-phase *binding* assay to identify key residues at each defined position. The combination of all amino acids defined in the most active mixtures leads to active individual compounds.[20–23]

The aim of this study was to use a PS-SCL to identify new peptide ligands targeted against the cancer-specific receptor mutation EGFRvIII. Promising peptide ligands can be radiolabeled for evaluation as tracers for positron emission tomography (PET). PET is a powerful imaging modality, using positron emitting radiopharmaceuticals to study metabolic processes in vivo. Because of its high sensitivity, PET can detect very small malignant foci in the human body. Development of novel cancer-specific peptides is of clinical interest to enhance and optimize the current application for early diagnosis and staging of cancer using PET.[24]

MATERIALS AND METHODS

Materials

TentaGel S RAM resin (loading 0.24 meq/g) was purchased from RAPP Polymers (Tübingen, Germany); 1-hydroxybenzotriazole (HOBt), Fmoc-protected amino acids, bovine serum albumin (BSA), biotinamidohexanoic acid N-hydroxysuccinimide ester, acetonitrile, N,N′-diisopropylcarbodimide (DIPCDI), thioanisole, Tween-20, fibronectin, and α-cyano-*p*-hydroxycinnamic acid were purchased from Sigma Aldrich (Hamburg, Germany), piperidine and triisopropylsilane (TIS) were obtained from Fluka (Buchs, Switzerland); Adrenocorticotropic hormone (ACTH) and Substance P were obtained from Sigma (St Louis, USA); phenylisothiocyanate from Pierce (Rockford, USA); trifluoracetic acid (TFA) from Merck (Schuchardt, Germany). Cell culture reagents, and streptavidin-HRP and ortho-phenylenediamine (OPD) were purchased from Invitrogen (Denmark). All starting chemicals were used without further purification.

Peptide Synthesis and Biotinylation of the Peptides

Peptide libraries were synthesized manually in individual syringes using TentaGel RAM resin (114 × 35 mg, loading 0.24 mmol/g)

and Fmoc (9-fluorenylmethoxycarbonyl) solid phase chemistry. Coupling of amino acid mixtures was performed using DIPCDI and HOBt (1.1 equiv. of amino acids in total and 1.1 equiv. coupling reagent) for 2 h.[25] For position O, a four-fold excess of amino acid, DIPCDI and HOBt was employed. Fmoc deprotection was accomplished by treatment with 20% piperidine in NMP for 10 min. For the biotinylation of the peptides, a two-fold excess of *N*-hydroxysuccinimido-biotinamidocaproate ester in DMSO was added to the resin. The mixture was shaken overnight at ambient temperature. The biotinylated peptides were cleaved from the solid support along with the permanent side chain protection groups using TFA/H_2O/TIS/Thioanisol (90:5:2.5:2.5: v/v).

Individual peptides were synthesized on a TentaGel RAM resin (25 mg, loading 0.24 mmol/g) using a four-fold excess of amino acids and coupling reagents. Following biotinylation and cleavage, which was done as described above, the peptides were purified by preparative HPLC and masses verified by MALDI-TOF-MS.

HPLC. Analytical high-performance liquid chromatography was performed using a Waters C_{18}-reverse-phase column (Symmetry® C_{18} 5 μm, 4.6 × 250 mm, Part No. WAT054275, Waters Corp., Milford, MA) on a Waters 600E system equipped with Millennium software. Preparative HPLC was performed on a Vydac C_{18}-reverse-phase column (10–15 μm, 22 × 250 mm, Part No. 218TP101522, VYDAC, Hesperia, CA).

MALDI-TOF MS. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a VG Tof Spec E Fisons instrument (Fisons Instruments, Beverly), using α-cyano-*p*-hydroxycinnamic acid as matrix. Substance P and adrenocorticotropic hormone were used as calibrants.

Cell Culture

Three cell lines were used for the binding assay: NR6W-A, expressing the wildtype EGFR; NR6M, expressing EGFRvIII; and NR6, expressing neither of the receptors. NR6 is a variant of the Swiss 3T3 murine fibroblast cell line[26] and, NR6M and NR6W-A are cell lines generated by transfection of NR6.[27,28]

Binding Assay

The binding assay was a modification of a previously described assay.[16]

The concentrations of NR6W-A, NR6M, and NR6 cells were 1×10^4 cells/ml on fibronectin-coated well in 96-well plate. The cells were incubated overnight at 37°C. On the next day, the wells were washed three times with phosphate buffered saline (PBS) and blocked with 20 μl PBS-BSA (PBS, 10 mg/ml BSA). To each well was added the appropriate peptide mixture or peptide (concentration range 500–1000 μM) in phosphate buffer containing 0.5% DMSO) and incubated at 4°C for 1 h. The wells were then washed three times with PBST (PBS with 0.05% tween-20), and 100 μl streptavidin-conjugated horseradish peroxidase (HRP 250 μg/L) was added to each well, followed by incubation for a further 1–2 h at ambient temperature and four rinses with PSBT. *o*-Phenylenediamine (ODP, 100 μl) in citrate-phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide was then added to each well, followed by incubation for 10–20 min at ambient temperature in the dark. The color reaction was stopped by adding 100-μl 1*M* H_2SO_4 to each well. The absorbance was measured using colorimetric detection at 492 nm (ELISA reader, Dynatech MR5000).

RESULTS AND DISCUSSION

The cancer-specific EGFRvIII is expressed in several kinds of cancer, in particular, ovarian, glioblastomas, and breast cancer, but not in normal tissue. The mutated receptor results from the fusion of exon 1 to exon 8 of the EGFR gene, which results in a novel glycine at the junction.[10] Therapeutic approaches have included MAbs directed toward this junction. However, antibodies have some drawbacks mainly because of the excessive molecular mass (150 kDa).[13]

Cancer-specific cell surface ligands, such as peptides and peptidomimetics, are excellent alternatives to antibodies as cancer targeting agents. They have been identified from combinatorial libraries and can be used for diagnostics, imaging, and drug delivery.[12]

We used a PS-SCL to identify new receptor-binding peptides targeted against EGFRvIII, which eventually could be radiolabeled, for example, 4-[^{18}F]fluorobenzoic acid, for use in cancer research. The peptides were designed as hexamers because this is the upper size limit for practical purposes.[29] The library consisted of six individual sublibraries, which may be represented: H-O_{1-6}XXXXX-NH_2, O being one of the 19 proteinogenic amino acids (cysteine omitted) and X an equimolar mixture of these. The resulting library consisted of 114 mixtures in total. The peptide mixtures were synthesized by Fmoc chemistry and biotinylated at the N-terminus using *N*-hydroxysuccinimido-biotinamidocaproate. The 114 sublibraries were tested for binding to the EGFRvIII receptor in a biotin-streptavidin binding assay. Three different cell lines, NR6M, NR6W-A, and NR6 were employed. These cells express EGFRvIII, EGFR, and neither of the receptors, respectively.

The result from each sublibrary was examined to identify the most active amino acid at each defined position.

The results are shown in Figure 1.

The determining factors in selecting active compounds for further testing were relative binding to the NR6M cell line and selectivity for EGFRvIII. Thus, sublibraries that showed binding to the NR6M cell line with an Abs_{492nm} greater than 0.3 and had a 1.5-fold selectivity for EGFRvIII over the wildtype EGF receptor and the EGF receptor negative cell lines were considered for selection.

At the first position of the sublibrary, H-OXXXXX-NH_2, the mixtures defined with Arg and Phe were selected. The Arg sublibrary, H-RXXXX-NH_2, was found to have the highest relative binding to EGFRvIII, followed by H-FXXXXX-

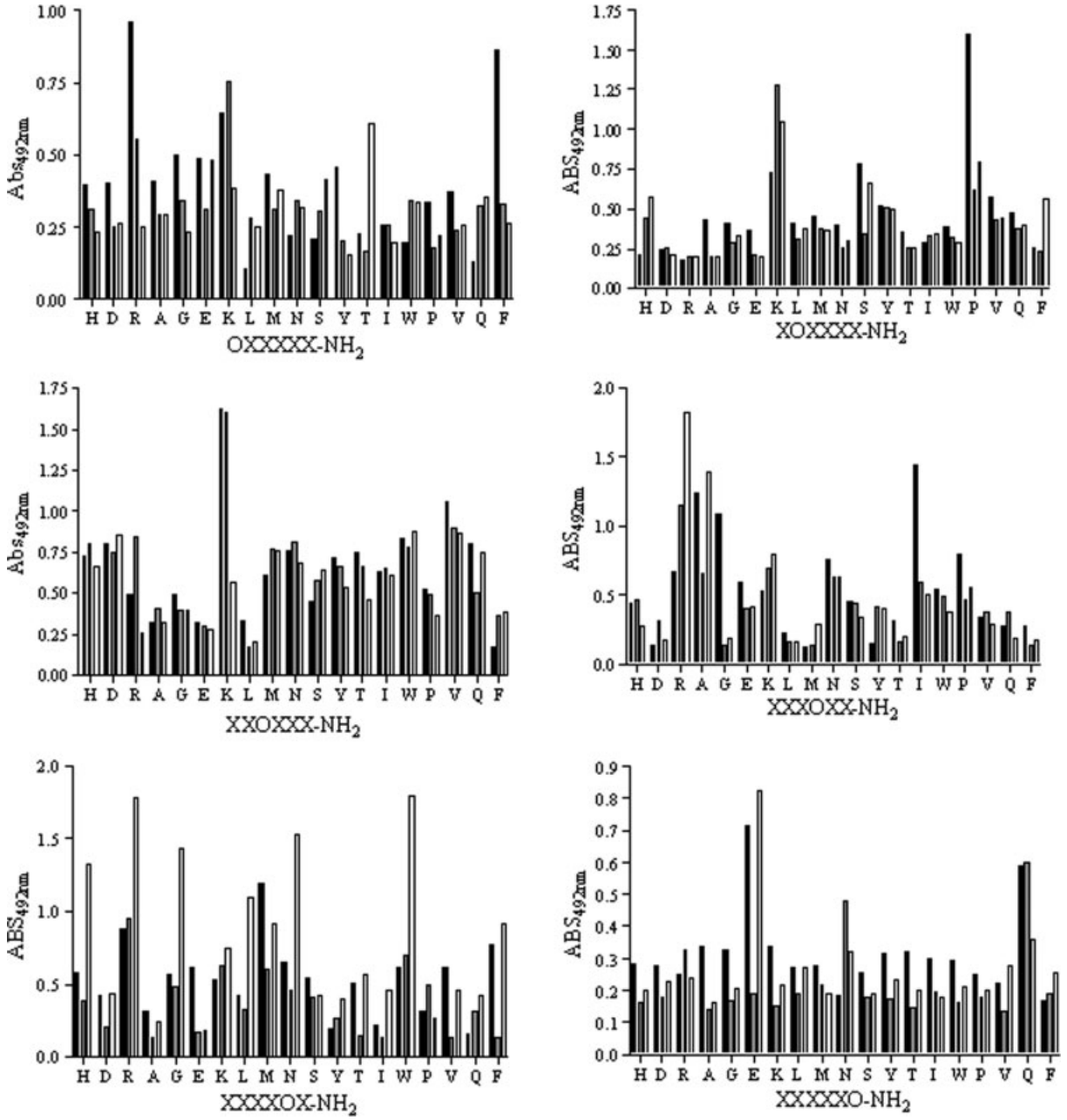

FIGURE 1 Binding results of the peptide mixtures of the six sublibraries, using NR6M (EGFRvIII, black), NR6W-A (wild-type EGFR, gray), and NR6 (EGF receptor negative, white) cell lines. O represents the defined position, and the *x*-axis represents to the amino acids occupying this position for the specific peptide mixture.

NH_2, which was 10% lower. However, Phe showed a 2.5-fold selectivity for EGFRvIII over the wild-type EGF receptor and the EGF receptor negative cell lines. The selectivity of Arg for the mutated receptor was less than 2-fold. Furthermore, Tyr showed some activity at this position, which is not surprising considering its similarity to Phe. We also noted that the mixtures containing the hydrophobic amino acids Val, Gly, Pro, Met, and Ala had some selectivity for EGFRvIII. Surprisingly, neither Leu nor Trp displayed activity.

At position 2, H-XOXXXX-NH_2, the most active mixture was defined with Pro, which showed both an excellent relative binding and a 2-fold selectivity for the mutated receptor over both the wild-type EGF receptor and the EGF receptor negative cell lines. We also chose the mixture defined with

Ala, H-XAXXXX-NH_2 which only displayed a moderate relative binding to the EGFRvIII receptor. However, this mixture showed ~2-fold selectivity for the NR6M cell line over NR6W-A and NR6 cell lines.

At position 3, H-XXKXXX-NH_2 had the highest relative binding to EGFRvIII. We observed a 2.5-fold selectivity for the mutated receptor over the EGF receptor negative cell lines. However, the mixture showed equal selectivity for EGFRv III and the wild-type EGF receptor. The mixture defined with Leu at the third position, H-XXLXXX-NH_2 displayed low relative binding to the EGFRvIII receptor. However, this mixture was selected over Lys because it showed ~2-fold selectivity for the NR6M cell line over NR6W-A and NR6 cell lines.

Of the amino acids defined at the fourth position of the sublibrary, H-XXXOXX-NH_2, Ile, was found to have the highest relative binding to EGFRvIII, followed by H-XXXGXX-NH_2 with an ~10% lower binding. Gly and Ile showed a 6-fold and 2.5-fold selectivity for the mutated receptor over the wild-type EGF receptor and the EGF receptor negative cell lines.

The mixture defined with Met at the fifth position, H-XXXXMX-NH_2, showed the highest relative binding to EGFRvIII. However, the sublibrary displayed an approximately equal selectivity for EGFRvIII and the EGF receptor negative cell line. Instead, the mixture having Glu at this position, H-XXXXEX-NH_2, was selected. This mixture showed ~2.5-fold selectivity for the NR6M cell line over the NR6W-A and NR6 cell lines. Furthermore, we noted that 13 of the defined positions of the sublibrary H-XXXXOX-NH_2 were more selective to the EGF receptor negative cell line than EGFRvIII. This was not the case for the other five sublibraries.

At position 6, H-XXXXXE-NH_2 had the highest relative binding to EGFRvIII. However, the sublibrary showed a 1.1-fold and 4-fold selectivity for the EGF receptor negative cell lines over the mutated receptor and the wild-type EGF receptor. Furthermore, Gln showed a high relative binding but equal selectivity for EGFRv III and the wild-type EGF receptor. Instead, the mixture defined with Ala at the sixth position, H-XXXXXA-NH_2, was selected because this sublibrary had 2-fold selectivity for the mutated receptor over the wild-type EGF receptor and the EGF receptor negative cell lines.

We also observed that the mixtures containing the hydrophobic amino acids Val, Gly, Pro, Met, Trp, and Leu had some selectivity for EGFRvIII.

Eight promising peptides were identified from the six sublibraries; H-FALGEA-NH_2, H-FALIEA-NH_2, H-RALIEA-NH_2, H-RALGEA-NH_2, H-FPLGEA-NH_2, H-FPLIEA-NH_2, H-RPLGEA-NH_2, and H-RPLIEA-NH_2. The peptides were synthesized and tested for binding to N6RM, N6RW-A, and N6R cell lines using the biotin-streptavidin assay.

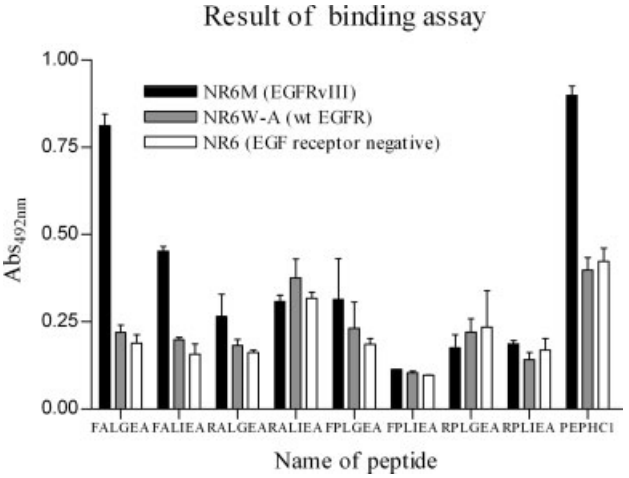

FIGURE 2 Binding results of the eight individual peptides identified from PS-SCL using NR6M (black), NR6W-A (gray), and NR6 (white) cell lines. The results are presented as mean ± S.D. of five experiments.

The results are shown in Figure 2. Six of the eight tested peptides showed selectivity for the mutated receptor. Our best candidate, H-FALGEA-NH_2, displayed a relative binding to EGFRvIII, which was comparable to PEPHC1. However, H-FALGEA-NH_2 showed a 3-fold selectivity for the mutated receptor over the wild-type EGF receptor and the EGF receptor negative cell lines. This was better than PEPHC1, which showed a 2-fold selectivity. For H-FALIEA-NH_2, a 2-fold selectivity for the mutated receptor was also observed. However, the relative binding was approximately half of that of PEPHC1.

Four of the peptides, H-RALGEA-NH_2, H-FPLGEA-NH_2, H-FPLIEA-NH_2, and H-RPLIEA-NH_2, only showed a modest relative binding to the mutated receptor and an ~1.1-fold selectivity for EGFRvIII over the wild-type EGF receptor and the EGF receptor negative cell lines.

The two best EGFRvIII binding peptides identified from the combinatorial library, H-FALGEA-NH_2 and H-FALIEA-NH_2, differ only in position 4, indicating that these residues are of importance for the binding to the mutated receptor.

As previously mentioned, we have reported an alanine scan of PEPHC1 to identify the amino acid residues important for binding to EGFRvIII.[17] Our results indicated that the amino acid residues at the N-terminus of PEPHC1 are essential for the binding to the mutated receptor. Furthermore, we synthesized six truncated peptide analogs derived from the N-terminus of PEPHC1, H-HFIIL-NH_2, H-HFIILG-NH_2, H-HFIILGF-NH_2, H-HFIILGFM-NH_2, H-HFLIIGFMR-NH_2, and H-HFLIIGFMRR-NH_2. We found that H-HFIIL-NH_2

and H-HFIILG-NH_2 showed almost three-fold lower binding to the mutated receptor than PEPHC1. The first truncated peptide to show acceptable binding to EGFRvIII was H-HFIILGF-NH_2, which is in good agreement with the results found in this study. The N-terminus of PEPHC1, H-HFIILGF-NH_2, has a high degree of similarity with H-FALGEA-NH_2 and H-FALIEA-NH_2. Our data obtained in this study indicate that a hydrophobic motif, in which at least five out of six amino acids are hydrophobic, is necessary for binding to EGFRvIII.

CONCLUSION

Using a positional scanning synthetic combinatorial library, we have identified two hexapeptides, H-FALGEA-NH_2 and H-FALIEA-NH_2, which selectively bind to the cancer-specific mutation EGFRvIII. The best EGFRvIII binding peptide H-FALGEA-NH_2 showed a relative binding to EGFRvIII, which was comparable to PEPHC1. However, H-FALGEA-NH_2 showed a 3-fold selectivity for the mutated receptor over the wild-type EGF receptor and the EGF receptor negative cell lines. This was better than the EGFRvIII specific peptide PEPHC1, which showed a 2-fold selectivity.

Our best candidate H-FALGEA-NH_2 will be radioactively labeled and evaluated as an imaging agent for PET investigation for diagnosis, staging, and monitoring of therapy of various types of cancer.

The authors thank the referees for valuable comments and suggestions. Furthermore, the authors thank Ms. Jette Petersen and Ms. Mette Villingsø for providing excellent technical assistance.

REFERENCES

1. Pedersen, M. W.; Meltorn, M.; Damstrup, L.; Poulsen, H. S. Ann Oncol 2001, 12, 745–760.
2. Ge, H.; Gong, X.; Tang, C. K. Int J Cancer 2002, 98, 357–361.
3. Lorimer, I. A. J Curr Cancer Drug Targets 2002, 2, 91–102.
4. Modjtahedi, H.; Moscatello, D. K.; Box, G.; Green, M.; Shotton, C.; Lamb, D. J.; Reynolds, L. J.; Wong, A. J.; Dean, C.; Thomas, H.; Eccles, S. Int J Cancer 2003, 105, 273–280.
5. Omidfar, K.; Rasaee, M. J.; Modjahedi, H.; Forouzandeh, M.; Taghikhani, M.; Golmakani, N. Tumor Biol 2004, 25, 296–305.
6. Kuan, C.-T.; Wikstrand, C. J.; Bigner, D. Endocr Relat Cancer 2001, 8, 83–96.
7. Archer, G. E.; Sampson, J. H.; Lorimer, I. A. J.; McLendon, R. E.; Kuan, C.-T.; Friedman, A. H.; Friedman, H. S.; Pastan, I. H.; Bigner, D. D. Clin Cancer Res 1999, 5, 2646–2652.
8. Yang, W.; Wu, G.; Barth, R. F.; Swindall, M. R.; Bandyopadhyaya, A. K.; Tjarks, W.; Tordoff, K.; Moeschberger, M.; Sferra, T. J.; Binns, P. J.; Riley, K. J.; Ciesielski, M. J.; Fenstermaker, R. A.; Wikstrand, C. J Clin Cancer Res 2008, 14, 883–891.
9. Trembath, D. G.; Lal, A.; Kroll, D. J.; Oberlies, N. H.; Riggins, G. J Mol Cancer 2007, 6, 30–43.
10. Li, G.; Wong, A. J. Expert Rev Vaccines 2008, 7, 977–985.
11. McGregor, D. P. Curr Opin Pharmacol 2008, 8, 1–4.
12. Aina, O. H.; Liu, R. W.; Sutcliffe, J. L.; Marik, J.; Pan, C. X.; Lam, K. S. Mol Pharm 2007, 4, 631–651.
13. Reubi, J. C. Endocr Rev 2003, 24, 389–427.
14. Shadidi, M.; Sioud, M. Drug Resist Update 2003, 6, 363–371.
15. Toth, F.; Farkas, J.; Toth, G.; Wollemann, M.; Borsodi, A.; Benyhe, S. Peptides 2003, 24, 1433–1400.
16. Campa, M. J.; Kuan, C.-T.; O'Connor-McCourt, M. D.; Bigner, D. D.; Patz, E. F. Biochem Biophys Res Commun 2000, 275, 631–636.
17. Hansen, C. L.; Hansen, P. R.; Pedersen, N.; Poulsen, H. S.; Gillings, N.; Kjær, A. Chem Biol Drug Des 2008, 72, 273–278.
18. Fenniri, H. Combinatorial Chemistry: Practical Approach, The Practical Approach Series; Oxford University Press: New York, 2003, p 476.
19. Lam, K. S.; Lebl, M.; Krchnák, V. Chem Rev 1997, 97, 411–448.
20. Houghten, R. A.; Pinilla, C.; Giulianotti, M. A.; Appel, J. R.; Dooley, C. T.; Nefzi, A.; Ostresh, J. M.; Yu, Y.; Maggiora, G. M.; Medina-Franco, J. L.; Brunner, D.; Schneider, J. J Comb Chem 2008, 10, 3–19.
21. Houghten, R. A.; Pinilla, C.; Appel, J. A.; Blondelle, S. E.; Dooley, C. T.; Eichler, J.; Nefzi, A.; Ostresh, J. M. J Med Chem 1999, 42, 3743–3778.
22. Pinilla, C.; Appel, J. R.; Borràs, E.; Houghten, R. A. Nat Med 2003, 9, 118–122.
23. Rubio-Godoy, V.; Pinilla, C.; Dutoit, V.; Borras, E.; Simon, R.; Zhao, Y.; Cerottini, J.-C.; Romero, P.; Houghten, R.; Valmori, D. Cancer Res 2002, 62, 2058–2063.
24. Okarvi, S. M. Cancer Treat Rev 2008, 34, 13–2625.
25. Rønn, L. C. B.; Olsen, M.; Østergaard, S.; Kiselyov, V.; Berezin, V.; Mortensen, M. T.; Lerche, M. H.; Jensen, P. H.; Soroka, V.; Saffells, J. L.; Doherty, P.; Poulsen, F. M.; Bock, E.; Holm, A. Nat Biotechnol 1999, 10, 1000–1005.
26. Pruss, R. M.; Herschman, H. R. Proc Natl Acad Sci USA 1977, 74, 3918–3921.
27. Wells, A.; Welsh, J. B.; Lazar, C. S.; Wiley, H. S.; Gill, G. N.; Rosenfeld, M. G. Science 1990, 247, 962–964.
28. Batra, S. K.; Castelino-Prabhu, S.; Wikstrand, C. J.; Zhu, X.; Humphrey, P. A.; Friedman, H. S.; Bigner, D. D. Cell Growth Differ 1995, 6, 1251–1259.
29. Lebl, M.; Krchnák, V.; Sepetov, N. F.; Seligmann, B.; Strop, P.; Felder, S.; Lam, K. S. Biopolymers 1995, 3, 177–198.

Reviewing Editor: Alfred Wittinghofer