# Peptide Arrays for Screening Cancer Specific Peptides

**ARTICLE** *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2010

Impact Factor: 5.64 · DOI: 10.1021/ac1003085 · Source: PubMed

*Anal. Chem.* **2010,** *82,* 7533–7541

# Peptide Arrays for Screening Cancer Specific Peptides

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**In this paper, we describe a novel method to screen peptides for specific recognition by cancer cells. Seventy peptides were synthesized on a cellulose membrane in an array format, and a direct method to study the peptide−whole cell interaction was developed. The relative binding affinity of the cells for different peptides with respect to a lead 12-mer p160 peptide, identified by phage display, was evaluated using the CyQUANT fluorescence of the bound cells. Screening allowed identification of at least five new peptides that displayed higher affinity (up to 3-fold) for MDA-MB-435 and MCF-7 human cancer cells compared to the p160 peptide. These peptides showed very little binding to the control (noncancerous) human umbilical vein endothelial cells (HUVECs). Three of these peptides were synthesized separately and labeled with fluorescein isothiocyanate (FITC) to study their uptake and interaction with the cancer and control cells using confocal laser scanning microscopy and flow cytometry. The results confirmed the high and specific affinity of an 11-mer peptide 11 (RGDPAYQGRFL) and a 10-mer peptide 18 (WXEAAYQRFL) for the cancer cells versus HUVECs. Peptide 11 binds different receptors on target cancer cells as its sequence contains multiple recognition motifs, whereas peptide 18 binds mainly to the putative p160 receptor. The peptide array−whole cell binding assay reported here is a complementary method to phage display for further screening and optimization of cancer targeting peptides for cancer therapy and diagnosis.**

Many conventional chemotherapeutic agents, such as, anthracyclines, alkylating agents, and antimicrotubules, can cause toxic side effects and have low specificity for tumor cells.[1] Targeting chemotherapy drugs to the cancer cells can help improve the outcome of existing cancer therapies.[2−4] Several strategies have been used for the diagnosis of cancer as well as for targeting tumor cells for delivery of chemotherapeutic drugs.[5−9] These strategies include the use of engineered antibodies,[3,6] tumor-homing peptides,[5,7] and aptamers.[8,9] Among these approaches, the use of antibodies has received a favorable attention for a number of years. More recently, peptides have also gained recognition as targeting agents for the delivery of cytotoxic drugs to the tumor site.[10]

A number of peptides have been identified by peptide phage display for targeting different tumors and cell types.[2,11−13] Among these, tumor homing peptides RGD[2,14,15] and NGR[16−19] sequences have received particular attention. These peptides target the $\alpha_v\beta 3$ integrin and aminopeptidase N receptors, respectively, in the tumor cells and vasculature.[2,15] A dodecapeptide GE11 has also been reported that binds to the epidermal growth factor receptor (EGFR) overexpressed by tumors.[11,20] Another 12-mer peptide p160, with a yet unidentified receptor, has been shown to bind MDA-MB-435, MCF-7, and WAC-2 human cancer cells strongly and specifically.[21,22] The phage displaying p160 peptide bound to several tumor cell lines such as Wac-2, SH-EP, Tet21N, and MDA-

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10.1021/ac1003085 
Published on Web 08/27/2010

MB-435 and showed no binding to control cell lines like 293, HaCaT, and 184A epithelial cells.[13] Further, in vivo biodistribution experiments in tumor-bearing mice showed better uptake and retention of p160 peptide after organ perfusion by tumor as compared to normal organs.[22]

Peptide p160, therefore, shows considerable promise in the development of targeted drug delivery systems. The prerequisites for the use of an agent as targeting vehicle are its selective binding to the tissue of interest and its limited uptake by the healthy tissues. In this context, peptides identified from the phage display for targeting cancer cells can be further improved for better and selective binding by chemical manipulation of their structures.[23] For instance, peptide arrays can be used for screening a library of designed peptides to complement random phage display screening. Short peptides covalently bound to a solid surface display specific binding affinity to the cells[24,25] and such peptide/protein arrays are being used for several applications in the biomedical and biotechnology fields.[26,27]

The objective of this study is, therefore, to develop a peptide array−whole cell binding assay for screening p160-derived peptides that bind specifically to the cancer cells. Two human cancer cell lines, MDA-MB-435 and MCF-7, as well as control human umbilical vein endothelial cells (HUVECs) were chosen for screening the peptide array library. The cell selection was based on a previous study that reported preferred affinity, binding, and internalization of p160 to MDA-MB-435 and MCF-7 cells over HUVECs.[22] A library of 70 peptides (derivatives of p160) was synthesized in an array format on cellulose membrane. Peptide−cell interactions were observed using fluorescence of the bound cells. At least five peptides were found that displayed higher binding (1.7−2.7 times) to the cancer cells compared to p160. Three of these peptides that showed highest affinity were synthesized and labeled with fluorescein isothiocyanate (FITC) to confirm the results of the peptide array−whole cell binding assay. Interaction of the FITC-labeled peptides with the cells was studied using confocal laser scanning microcopy as well as flow cytometry techniques. Two peptides, **11** (RGDPAYQGRFL) and **18** (WXEAAYQRFL), showed enhanced uptake by the cancer cells compared to p160 peptide and showed very little affinity for the control HUVEC cells.

## MATERIALS AND METHODS

**Chemical and Reagents.** Wang resin, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt), and the Fmoc amino acids were purchased from NovaBiochem (San Diego, CA) while piperidine was from Caledon. CyQUANT and FITC dyes were obtained from Invitrogen (Eugene, Oregon, USA). *N*,*N*′-Diisopropylcarbodiimide (DIC), *N*,*N*-dimethylformamide (DMF), *N*-methyl morpholine (NMM), trifluoroacetic acid (TFA), and all other reagents were purchased from Sigma-Aldrich. All commercial reagents and solvents were used as received with no further purification.

**Equipment.** Peptide array was made using a semiautomatic robot AutoSpot ASP222 (Intavis AG, Germany). Solid phase synthesis of peptides on Wang resin was done using manual synthesis. HPLC purification and analysis were carried out on a Varian Prostar HPLC system (Walkersville, MD) using Vydac C18 semipreparative (1 × 25 cm, 5 $\mu$m) and analytical (0.46 × 25 cm, 5 $\mu$m) columns. Peptides were detected by UV absorption at 220 nm. Mass spectra were recorded on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager spectrometer (Applied Biosystems) or Waters micromass ZQ. Imaging experiments were done using a Kodak Image Station 4000 M (USA) and Carl Zeiss microscope (Göttingen, Germany), and confocal laser scanning microscopy was with a Zeiss 510 LSMNLO confocal microscope (Carl Zeiss Microscope systems, Jena, Germany). Fluorescence-activated cell-sorting (FACS) experiments were performed on a Becton-Dickinson Facsort and analyzed by DakoCytomation Summit software.

**Peptide Array Synthesis.** Seventy peptide sequences in duplicates ranging in length from 9 to 12 amino acids were synthesized in an array format on a cellulose membrane using an AutoSpot robot. Peptide arrays were synthesized on an amino-PEG500 cellulose membrane-UC540 (Intavis, Germany). DIGEN software (Jerini Biotools GmbH, Berlin, Germany) provided with the instrument was used for designing the arrays. The surface of the membrane is derivatized with a polyethylene (PEG) linker and a free amino terminal group. The Spot synthesis method was used to build the peptide arrays on the free amino terminal group.[28] Briefly, Fmoc amino acids activated with HOBt and DIC for 15 min were spotted on the membrane in 60 nL aliquots per spot by a robotic syringe. The concentration of each peptide (spot) was controlled by the amount of the liquid delivered during the reaction steps. By delivering 60 nL of the activated amino acids (0.25 mM/mL) at intervals of 8.0 mm, a loading of 0.4 $\mu$mol/cm$^2$ was achieved. After coupling of the Fmoc amino acid, the membrane was removed from the synthesizer and was treated with acetic anhydride (2%) to cap any free remaining amino groups. The membrane was then washed and treated with 20% piperidine in DMF for the Fmoc group deprotection. After washing with DMF and IPA, the membrane was air-dried and carefully repositioned on the robotic synthesizer to repeat the next coupling cycle. These steps were repeated for each amino acid until the end of the sequence. At the end, all peptides were N-terminally acetylated. The C-terminal end of the peptide was anchored to the surface of the amino-PEG500 cellulose membrane through a $\beta$-alanine linker. The final removal of side-chain protecting groups was performed by treating the membrane with a cocktail of reagents comprised of 15 mL of TFA, 15 mL of dichloromethane (DCM), 0.9 mL of triisopropylsilane, and 0.6 mL of water. The membrane was allowed to react with the cocktail solution in a polypropylene box with a lid for about 3 h. After extensive washing with DCM, DMF, and ethanol, the membrane was dried with cold air and stored in a sealed bag at −20 °C until use.


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The peptides synthesized on the cellulose membrane were characterized by preparing a small control library of 9-mer peptides on a $\beta$-alanine membrane with a cleavable linker (Intavis, Germany). Peptides were cleaved from the membrane using an ammonia gas procedure.[29] After the cleavage of the side-chain protecting groups with TFA/triisopropylsilane/$H_2O$ and air oxidation of the cysteine SH groups to form the disulfide bond, the cellulose membrane was placed in a desiccator over a saturated solution of ammonia in THF. The membrane was exposed to ammonia gas overnight at 25 °C. Subsequently, the peptide spots were punched out using a 96 well puncher, and each spot was placed in an eppendorf tube containing 30% acetonitrile (ACN)/water (150 $\mu$L). The solution was sonicated for 30 min to elute the peptide from the membrane. The resulting peptide solutions were characterized by MALDI-TOF mass spectrometry and analytical RP-HPLC. The sequence and characterization of the peptides from the control library are shown in Figure S1 (Supporting Information). Peptides were found to be >95% pure.

**Cell Lines.** All cell lines were cultivated at 37 °C in a 5% $CO_2$ incubator. The human cancer cell line MDA-MB-435 was cultured in RPMI 1640 with Glutamax containing 10% fetal calf serum (FCS; Invitrogen, Karlsruhe, Germany), 100 IU/mL penicillin, and 100 IU/mL streptomycin. The human breast cancer cell line MCF-7 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax containing 10% FCS (Invitrogen). HUVECs, kind gift from the laboratory of Sandra Davidge, University of Alberta, were cultivated using Endothelial Cell Growth Medium (EGM, LONZA) containing 20% FCS, 2 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 2 ng/mL basic fibroblast growth factor (Roche Diagnostics, Mannheim, Germany).

**Peptide Array-Cell Binding Assay.** The peptide array membrane was soaked in ethanol for 30 s to prevent any precipitation of hydrophobic peptides, followed by its incubation in sterile phosphate-buffered saline (PBS; pH 7.4) for 30 min. The cells were seeded directly on a culture dish ($75 \times 10^3$ cells/mL) containing the peptide array membrane for 4 h in serum free media, in order to prevent the proteolytic effect of serum on the peptides. After washing the nonbound cells, the membrane was frozen at −80 °C for 2 h. The membrane was thawed at room temperature followed by incubation with the CyQUANT dye for 30 min following the manufacturer's protocol. It was washed three times with PBS, each for 5 min by shaking on an automatic shaker. The membrane was scanned using a Kodak imager at 465 nm excitation and 535 nm emission, and the net fluorescence intensity of each peptide spot was quantified using Kodak Molecular Imaging Software Version 4.0. The binding affinity of the cells for each peptide (spot) was determined by subtracting the net fluorescent intensity of the peptide itself (autofluorescence). The background cell binding outside the peptide spot was minimal. An external standard (set of peptides) was used to calibrate the fluorescence intensity between scans performed on the same day and on different days. After each cell-binding experiment, the bound cells were removed by first washing with ethanol for 5 min, followed by treatment with 0.1 N HCl for 20 min. The peptide array membrane was regenerated by washing with DMF ($4 \times 20$ min), ethanol ($3 \times 3$ min), and finally drying in air. Each cell-binding experiment was repeated twice. The results are presented as average fluorescence intensity (±standard deviation) of two duplicate peptide spots, two scans, and two different experiments. The relative cell adhesion ratio for each peptide analogue was calculated as the ratio of the average fluorescence of the peptide analogue divided by that of the p160 peptide.

**Synthesis of FITC-Labeled Peptides.** The FITC-labeled peptides, FITC-p160, FITC-**11**, FITC-**18**, and FITC-**40** and the unlabeled peptides, p160, **11**, cyclic (c)-RGD, and NGR were synthesized by manual solid phase peptide synthesis (SPPS) using Fmoc coupling protocols on Wang or Trityl resin (0.1 mmol).[30] After the peptide synthesis, $\beta$-alanine (spacer) was conjugated to the N-terminal amino group followed by FITC coupling. FITC was coupled in the dark for 20 h followed by extensive washing of the resin. FITC-labeled peptide was cleaved from the resin, along with the deprotection of the amino acid side chains by TFA treatment. The crude cleaved peptides were precipitated by cold diethyl ether followed by their purification using reversed-phase HPLC. The mass of the products was determined by MALDI-TOF mass spectrometry.

**Fluorescence Microscopy.** MDA-MB-435 cells (50 000) were cultured on the top of coverslip at 37 °C for 24 h. The medium was removed and replaced with 1 mL of fresh serum free medium, containing FITC-labeled peptides at a concentration of $10^{-5}$ mol/L. The cells were incubated with the peptides for 30 min at 37 °C. After incubation, the medium was removed and the cells were washed thrice with 2 mL of serum free medium. The cells were fixed on ice with 2% formaldehyde for 20 min. The formaldehyde was removed by washing with medium (three times). The coverslips were put on slides containing one drop of 4',6-diamidino-2-phenylindole (DAPI)-Antifade (Molecular Probes) to stain the nucleus. The cells were imaged under the fluorescence microscope (Zeiss) using green and blue filters with 20× magnification.

The samples prepared for fluorescence microscopy were also used for visualization by confocal microscopy. Confocal laser scanning microscopy was performed with a Carl Zeiss inverted confocal microscope with a 100× oil immersion lens. Confocal stacks were processed using the Carl Zeiss LSM 5 Image software, which also operates the confocal microscope. For the competitive binding, the same experiment was carried out in the presence of unlabeled p160 peptide ($10^{-4}$ mol/L) as a competitor.

**Flow Cytometry Analysis.** Fluorescence-activated cell-sorting (FACS) analysis was used to evaluate the binding of the FITC-labeled peptides to the human cancer cell line MDA-MB-435 and the control HUVEC cells. The MDA-MB-435 and HUVEC cells were placed into 6-well plates at a density of $10^6$ and $3 \times 10^5$, respectively, in 3 mL of culture medium at 37 °C for 24 h. The culture medium was replaced by 1 mL of fresh serum-free medium, containing FITC-labeled p160, **11**, **18**, and **40** peptides at a concentration of $10^{-5}$ mol/L. Cells were incubated with the peptides for 30 min at 37 °C. The media was then

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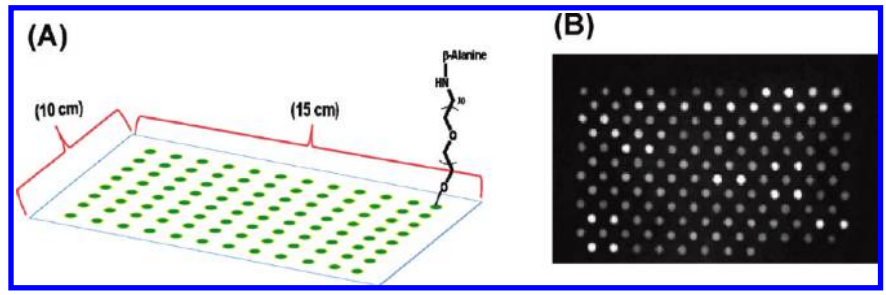


**Figure 1.** (A) Schematic of a cellulose membrane (amino-$PEG_{500}$-UC540) showing the functionalization on the surface for spot synthesis. The surface has a loading capacity of 400 nmol/$cm^2$ delivering a peptide concentration of 50 nmol/spot (12.56 $mm^2$). (B) An image of the peptide array at $\lambda_{ex}$ = 465 nm and $\lambda_{em}$ = 535 nm showing 70 peptide spots in duplicates.

removed and the cells were washed three times with ice-cold PBS to remove the unbound peptide. The cells were then scrapped from the wells using a manual scrapper. The cells were transferred to centrifuge tubes and centrifuged at 1000 rpm for 7 min. The pellet was resuspended in FACS buffer (PBS with 5% FCS and 0.09% sodium azide), washed once more, and then resuspended again in FACS buffer. Untreated cells were subjected to similar steps without any peptide treatment to detect autofluorescence of the cells. The samples were then subjected to the FACS instrument, Becton-Dickinson Facsort to acquire data. The data was analyzed by DakoCytomation Summit software. For the determination of dissociation constant ($K_d$), MDA-MB-435 cells ($10^6$) were incubated with varying concentration of peptide **FITC-18** (0−100 $\mu$M) and the samples were treated as above; the experiment was repeated. The fluorescence intensity of the peptide−cell complex was plotted against peptide concentration. The data was fitted to one binding site equation using the numerical simulation/least-squares fitting program DYNAFIT[31] as described before.[30]

Competitive peptide binding assays were performed by incubating MDA-MB-435 cells with FITC-labeled peptide **11** in the presence of a 100-fold excess unlabeled **11** or c-RGDfK. After incubation for 30 min at 37 °C, the cells were washed with ice-cold PBS. Thereafter, FACS analysis was performed as described above. A similar experiment was repeated for FITC-**18** in the presence of excess unlabeled **18**. All the experiments for the binding assay were repeated 2−4 times.

## RESULTS AND DISCUSSION

**Peptide Array Synthesis.** A library of 70 peptides was synthesized in an array format on a cellulose membrane using SPOT synthesis.[28] The surface of the cellulose membrane derivatized with a polyethylene (PEG-10) linker and a free amino terminal group (Figure 1A) allowed an easy synthesis of peptide arrays. Peptides were synthesized in duplicates by covalent conjugation to the free amino functional group using a stepwise Fmoc−SPPS procedure. Each peptide was synthesized at a concentration of ~50 nmoles which was spread on the membrane in a spot with a diameter of 4 mm. Each spot was separated from the next peptide (spot) by 8 mm evenly distributing 140 peptide spots on the membrane surface as shown in Figure 1B. The sequence for the peptide library is shown in Table 1.

**Peptide Library Design.** Peptide p160 (**1**) is a linear 12-mer peptide carrying a net charge of zero. Peptide sequences based on the p160 sequence were designed for selective binding to cancer cells (Table 1). The designed peptide sequences ranged from 9-mer to 11-mer where the first two N-terminal amino acids (Val-Pro) of p160 sequence were deleted. Askoxylakis and co-workers have shown that the N-terminal Val and Pro residues do not contribute toward cancer cell binding, in particular, neuroblastoma WAC2 cell binding.[21] The first set of peptides, **1**−**5**, in the library was the control peptides where peptide **1** is p160, **2** is p160 with Nle substituted for Met4, and **5** is the peptide with tumor homing RGD motif. Peptides **3** and **4** are the negative controls where four important C-terminal residues from p160 have been deleted. The second set of peptides, **6**−**13**, is the fusion sequences containing RGD and/or NGR incorporated into the p160 sequence. The rationale behind this design strategy was to make the peptide more specific for the cancer cells by targeting multiple receptors overexpressed on the surface of the cancer cells. Peptides **6** and **7** have an RGD(E) in the N-terminal region, whereas **8** and **9** have Q(N)GR motif introduced toward the C-terminal. Peptides **10**−**13** have both the RGD(E) and the Q(N)GR motifs present in the sequence.

The third set of peptides were 10-mers and represent Ala scan (**14**−**23**), where **14** is essentially p160 with the first two amino acids deleted and Met replaced with Nle. Ala scan was performed to determine the importance of each residue in binding. The next set of peptides, **24**−**50**, involves replacement of the N- (Trp1 or Nle2) or C-terminal (Phe9 or Leu10) residues with different amino acids. For instance, Trp1 was substituted with conservative mutations such as Leu, Gln, Tyr, Phe, 4-chloro-Phe, and D-Phe or orthogonal amino acids like acidic Glu or basic Lys residues. Likewise, Nle2 was substituted with Leu, D-Leu, Gln, Thr, Glu, or Lys. The C-terminal Phe9 and Leu10 were similarly replaced to give peptides **38**−**50**. Finally, peptides **51**−**70** were the 9-mers where three N-terminal residues from p160 sequence were deleted. It has been shown previously that an 8-mer peptide (p160-8-1) with four N-terminal residues deleted from the p160 sequence shows better affinity than p160 for the cancer cells.[21] Therefore, up to four N-terminal residues in p160 are not critical for binding and can be safely removed. Peptides **52**−**70** are the same as **32**−**50** with the N-terminal Trp deleted.

**Peptide Array-Whole Cell Binding Assay.** Peptides attached to the solid support (cellulose membrane) were directly screened for cell binding. The cellulose membranes used in this study (provided by Intavis) are acid hardened for improved stability (pH 1−14) and have greater distance to cellulose carrier, and there is a hydrophilic spacer (PEG) which reduces the background. These properties of the membrane make the peptide arrays synthesized on them suitable for direct cell binding assays. Further, the peptide

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**Table 1. Amino Acid Sequence and the Cell Adhesion Capacity of the p160 Based Peptide Array Library[a]**

| Peptide # | Amino Acid Sequence | Relative Cell Adhesion | | Peptide # | Amino Acid Sequence | Relative Cell Adhesion | |
|---|---|---|---|---|---|---|---|
| | | *MB-435* | *MCF-7* | | | *MB-435* | *MCF-7* |
| **1** (p160) | VPWMEPAYQRFL | 1.0 | 1.0 | **38** | WXEPAYQR**E**L | 1.2 | 2.1 |
| **2** | VPWXEPAYQRFL | 1.3 | 1.4 | **39** | WXEPAYQR**L**L | 1.3 | 1.4 |
| **3** | VPWMEPAY | 0.5 | 0.4 | **40** | WXEPAYQR**K**L | 2.7 | 2.7 |
| **4** | VPWXEPAY | 0.7 | 0.8 | **41** | WXEPAYQR**Q** | 1.4 | 1.5 |
| **5** | GRGDS | 1.1 | 1.1 | **42** | WXEPAYQR**Y**L | 1.6 | 1.8 |
| **6** | **RG**EPAYQRFL | 1.7 | 1.5 | **43** | WXEPAYQR**F***L | 0.7 | 1.7 |
| **7** | **RGD**PAYQRFL | 1.5 | 1.9 | **44** | WXEPAYQR**f**L | 1.1 | 1.9 |
| **8** | WXEPAYQ**G**RFL | 1.0 | 1.6 | **45** | WXEPAYQRF**E** | 1.6 | 1.5 |
| **9** | WXEPAY**NG**RFL | 1.0 | 1.8 | **46** | WXEPAYQRF**l** | 1.7 | 2.0 |
| **10** | **RG**EPAYQ**G**RFL | 1.7 | 1.9 | **47** | WXEPAYQRF**K** | 2.2 | 2.8 |
| **11** | **RGD**PAYQ**G**RFL | 1.7 | 1.8 | **48** | WXEPAYQRF**Q** | 1.2 | 1.5 |
| **12** | **RG**EPAY**NG**RFL | 1.4 | 1.7 | **49** | WXEPAYQRF**T** | 0.8 | 1.5 |
| **13** | **RGD**PAY**NG**RFL | 1.0 | 2.1 | **50** | WXEPAYQRF**F** | 1.1 | 1.8 |
| **14** | WXEPAYQRFL | 1.0 | 1.7 | **51** | XEPAYQRFL | 1.1 | 1.2 |
| **15** | **A**XEPAYQRFL | 1.1 | 1.2 | **52** | **E**EPAYQRFL | 1.1 | 1.2 |
| **16** | W**A**EPAYQRFL | 0.9 | 1.3 | **53** | **L**EPAYQRFL | 1.2 | 1.2 |
| **17** | WX**A**PAYQRFL | 1.6 | 1.9 | **54** | **l**EPAYQRFL | 1.0 | 0.9 |
| **18** | WXE**A**AYQRFL | 2.2 | 2.7 | **55** | **K**EPAYQRFL | 0.9 | 1.6 |
| **19** | WXEPA**A**QRFL | 0.9 | 1.7 | **56** | **Q**EPAYQRYL | 0.8 | 1.0 |
| **20** | WXEPAY**A**RFL | 1.1 | 1.8 | **57** | **T**EPAYQRYL | 0.8 | 1.0 |
| **21** | WXEPAYQ**A**FL | 1.1 | 1.5 | **58** | XEPAYQR**E**L | 1.0 | 1.1 |
| **22** | WXEPAYQR**A**L | 1.0 | 1.2 | **59** | XEPAYQR**L**L | 0.9 | 1.0 |
| **23** | WXEPAYQRF**A** | 1.3 | 1.6 | **60** | XEPAYQR**K**L | 1.6 | 1.7 |
| **24** | **E**XEPAYQRFL | 1.6 | 1.1 | **61** | XEPAYQR**Q**L | 0.2 | 0.95 |
| **25** | **L**XEPAYQRFL | 0.6 | 1.2 | **62** | XEPAYQR**Y**L | 0.6 | 1.0 |
| **26** | **K**XEPAYQRFL | 1.2 | 1.9 | **63** | XEPAYQR**F***L | 0.9 | 1.4 |
| **27** | **Q**XEPAYQRFL | 0.9 | 1.2 | **64** | XEPAYQR**f**L | 0.8 | 1.1 |
| **28** | **Y**XEPAYQRFL | 1.5 | 1.8 | **65** | XEPAYQRF**E** | 0.9 | 1.4 |
| **29** | **F**XEPAYQRFL | 1.2 | 1.3 | **66** | XEPAYQRF**l** | 0.9 | 0.9 |
| **30** | **F***XEPAYQRFL | 1.0 | 1.3 | **67** | XEPAYQRF**K** | 1.2 | 2.0 |
| **31** | **f**XEPAYQRFL | 0.7 | 1.8 | **68** | XEPAYQRF**Q** | 0.5 | 1.0 |
| **32** | W**E**EPAYQRFL | 1.5 | 2.1 | **69** | XEPAYQRF**T** | 0.6 | 0.7 |
| **33** | W**L**EPAYQRFL | 1.6 | 1.8 | **70** | XEPAYQRF**F** | 0.8 | 1.2 |
| **34** | W**l**EPAYQRFL | 1.4 | 1.5 | | | | |
| **35** | W**K**EPAYQRFL | 1.6 | 1.8 | | | | |
| **36** | W**Q**EPAYQRFL | 1.5 | 1.4 | | | | |
| **37** | W**T**EPAYQRFL | 0.7 | 1.6 | | | | |

[a] The C-terminus of the peptides is covalently attached to the cellulose membrane. An amino acid replacement or insertion of an amino acid in the p160 sequence is highlighted in bold as well as underlined. X stands for norleucine; F* refers to 4-chlorophenylalanine, and the lower case letter denotes D-amino acid. The relative cell adhesion is the average ratio of fluorescent intensity of a peptide divided by that of **1** (p160).

array membrane can be regenerated after the cell binding assay, allowing repetitive use of the same peptide array. The peptide arrays on cellulose membranes show remarkable affinity toward different binding moieties and have been used mainly for studying peptide−protein and peptide−antibody interactions.[32−36] Okochi et al. studied the direct interaction of peptides on cellulose membrane with cells.[37] However, the authors punched individual peptide spots into the 96-well plates followed by cell adhesion and cell labeling. The peptide array−whole cell binding assay described here provides a new method for screening a library of peptides for binding to different cell types.

The peptides were screened for specific binding to two human cancer cell lines MDA-MB-435 and MCF-7. HUVEC was used as a control cell line. MDA-MB-435 is a melanoma cell line that was considered as a model breast cancer cell line for several years,[38] whereas MCF-7 is an adenocarcinoma human breast cell line.[39] The cells were allowed to bind directly to the peptides, and the bound cells were labeled with the CyQUANT dye. The use of CyQUANT dye makes this assay extremely sensitive, as this dye has very low intrinsic fluorescence and shows large fluorescence enhancement and high quantum yield upon nucleic acid binding.[40] In the assay, CyQUANT allowed detection of less than 1000 cells. Using a representative 9-mer peptide on the cellulose membrane, we found that CyQUANT allowed detection of as low as 1000 cells


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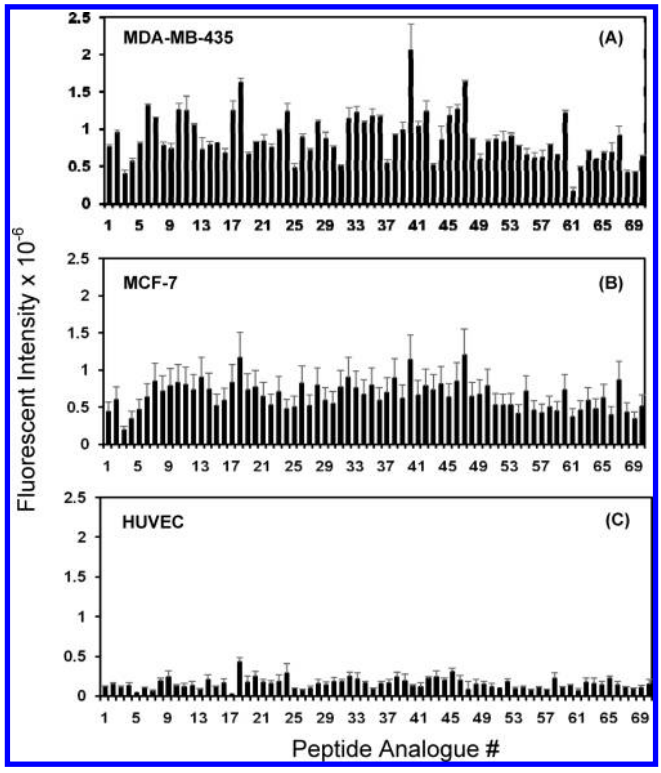


**Figure 2.** Average CyQUANT fluorescence intensity of the peptide bound cells. MDA-MB-435 (A), MCF-7 (B), and HUVEC (C) cells (75 $\times$ $10^3$ cells/mL) were incubated with the peptide array for 4 h at 37 °C, followed by labeling the cells with the CyQUANT dye. The fluorescence intensity was measured using a Kodak imager at 465 nm excitation and 535 nm emission. The results are presented as mean fluorescence intensity $\pm$ SD.

when imaged using the Kodak imager, while the other dyes, such as CFSE and DAPI, were not able to detect cells bound in this range or enhanced the intrinsic fluorescence of the peptide upon cell binding (Figure S2, Supporting Information). In comparison, the CyQUANT dye showed minimal enhancement of the intrinsic fluorescence of the peptides upon cell binding.

The relative cell adhesion of the peptides was estimated on the basis of the fluorescence of the bound cancer or control cells as shown in Figure 2. All the peptides were compared to the wild type p160, and the relative cell adhesion ratio for each peptide binding to cancer or control cells was determined. The relative adhesion ratio for the cancer cells is listed in Table 1. The affinity of the peptides for the two cancer cell lines followed similar trend (Figure 2A,B), whereas the HUVEC cells showed very little binding to all the peptides (Figure 2C). On the basis of the cell adhesion ratio, several peptides were identified that displayed better cell binding compared to the p160. Five peptides that displayed highest affinity (1.7−2.7-fold compared to p160) for the MDA-MB-435 and MCF-7 cells were **10**, **11**, **18**, **40**, and **47**. Among the fusion peptides (**6**−**13**), **6**, **7**, **10**, and **11** showed superior binding to MDA-MB-435 cells. For the MCF-7 cells, most of the fusion peptides (**7**−**13**) showed high cell adhesion. Peptide **11** was chosen for further investigation, as it showed low affinity for the HUVEC cells (relative cell adhesion ratio 0.7).

From the alanine scan peptides (**14**−**23**), peptide **18** displayed highest affinity for the cancer cells relative to p160. The relative cell adhesion ratio was 2.2 and 2.7 for MBA-MB-435 and MCF-7 cells, respectively. In this peptide, Pro has been substituted

| | |
|---|---|
| RGDPAYQGRFL | **11** |
| WXE**A**AYQRFL | **18** |
| WXEPAYQR**K**L | **40** |
| VPWMEPAYQRFL | **1** (p160) |

**Figure 3.** Amino acid sequences of the peptide identified from the peptide array showing a better binding profile for the cancer cells. The changes in the peptide sequence compared to the wild type p160 are highlighted in bold. The sequence of p160 is shown for comparison.

with Ala. Perhaps, the Pro residue can also be replaced with other amino acids delivering a peptide with a better binding spectrum compared to p160. Zhang et al. found, using phage display, that peptides p161 and p25 with sequence very similar to p160 but with Pro substituted with Gln or Met bound to a variety of tumor cells and showed no binding to the normal cells.[13]

From the peptides that have mutations in the N- or C-terminal residues (**24**−**70**) with respect to p160, several peptides displayed enhanced binding to the cancer cells. Two peptides that showed the highest affinity (up to 2.2−2.8-fold) for the cancer cells compared to p160 in this category were **40** and **47**. In these peptides, a C-terminal hydrophobic Phe or Leu was replaced with a basic Lys residue making these analogues positively charged (net charge +1). Peptides **32**−**36,** where the N-terminal Nle was substituted, also showed relatively better binding (1.4−2.1-fold) compared to p160. The 9-mer peptides **52**−**70**, in general, showed either the same or less binding to the cancer cells compared to p160, suggesting the importance of the N-terminal Trp residue. Only two 9-mer peptides, **60** and **67**, showed higher binding (range 1.2−2.0-fold) than p160. The results show that the increase in the net positive charge of the peptides in the C-terminus increases cell adhesion. This may be due to the electrostatic interactions between the peptides and the cancer cells. However, introducing a positive charge (basic residue) in the N-terminal region, such as peptides **26** and **35**, does not increase binding affinity to the same extent as observed for **40** and **47**. Peptide **40** was chosen for further investigation.

**Affinity and Specificity of Selected Peptides.** Three selected peptides, **11**, **18**, and **40**, identified from the peptide array−cell binding screening process were further investigated (Figure 3). Fluorescence based experiments, such as flow cytometry and confocal microscopy, were used to monitor the affinity and specificity of the selected peptides during in vitro cell binding experiments. Peptides (p160, **11**, **18**, and **40**) were labeled with FITC in the N-terminus via a $\beta$-alanine linker (Figure S3, Supporting Information). FITC-labeled peptides were incubated with the MDA-MB-435 cancer cells or the HUVEC cells (as negative controls) for 30 min at 37 °C to characterize the peptide−cell binding.

The fluorescence microscopy images of the MDA-MB-435 cells showed that the peptide analogues uniformly surrounded the nuclei of the cells (Figure 4A−C). The FITC-labeled peptides, FITC-**11**, FITC-**18**, and FITC-**40**, bound to the surface of the cells and were also present in the cytoplasm. The binding of the peptides to the cells was further confirmed using flow cytometry. Figure 4D,E shows the fluorescence for the different peptides bound to MDA-MB-435 and HUVEC cells, respectively. FITC-labeled p160 was used as a positive control. In general, MDA-MB-435 cells showed significantly higher FITC fluorescence than the HUVEC cells when bound to FITC-labeled peptides. This

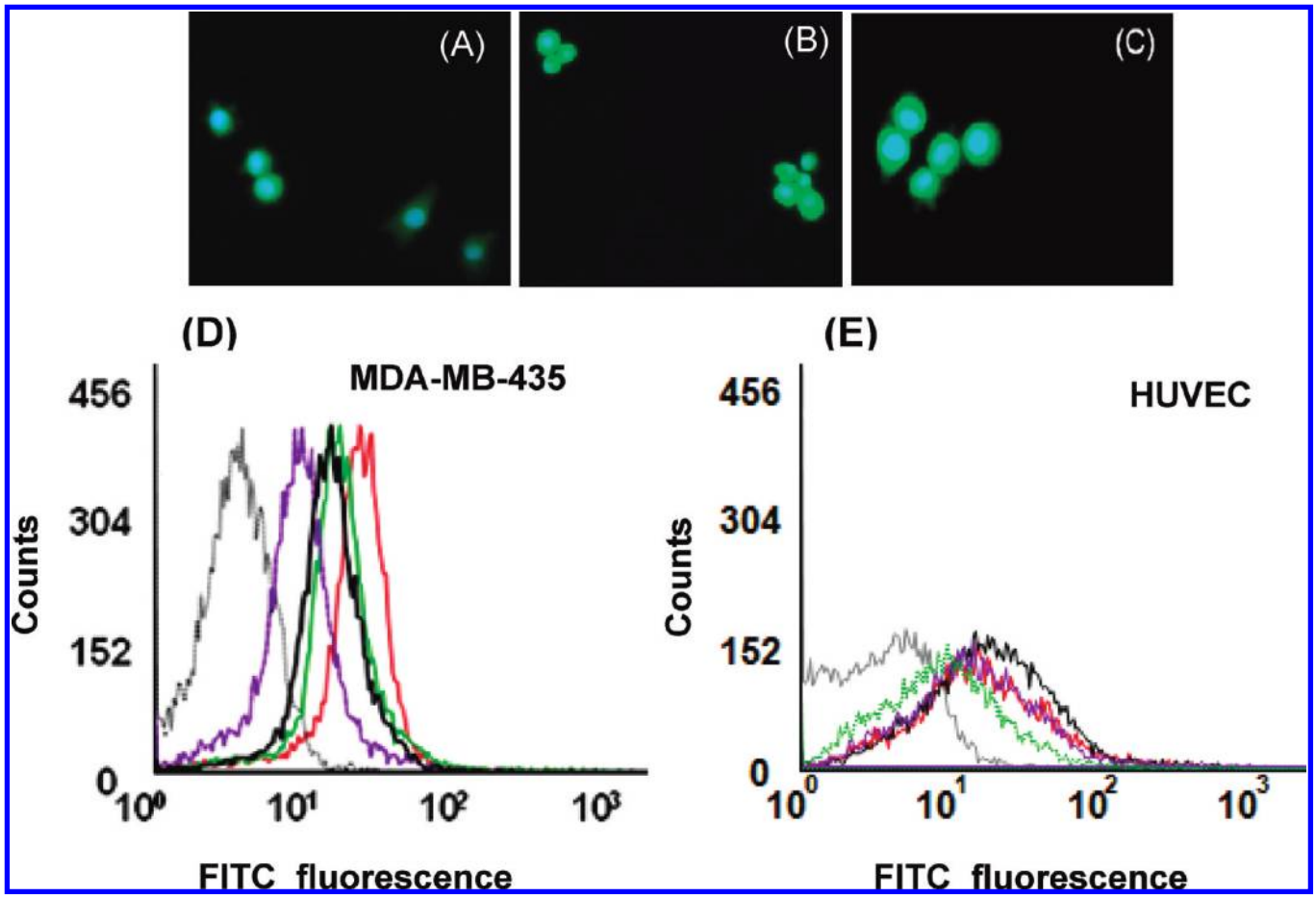


**Figure 4.** Fluorescence microscopy images of MDA-MB-435 cells after incubation with (A) FITC-**11**, (B) FITC-**18**, and (C) FITC-**40** for 30 min at a peptide concentration of $10^{-5}$ mol/L. Cell nuclei were stained blue with DAPI. Peptide uptake by the MDA-MB-435 cells (D) or HUVEC cells (E) measured by flow cytometry. The peptides ($10^{-5}$ mol/L), FITC-**1** (p160, black), FITC-**11** (green), FITC-**18** (red), and FITC-**40** (purple) were incubated with the cells for 30 min at 37 °C. Autofluorescence of the cells is shown in gray.

shows specific binding of the peptides to the cancer cells over HUVEC cells. MDA-MB-435 cells showed highest FITC fluorescence when bound to peptide **18**, followed by **11**, p160, and the lowest for **40**. These results indicate that peptides **18** and **11** have higher affinity for the MDA-MB-435 cells compared to p160. Peptide **40** showed weak binding compared to p160, in contrast to what was observed in the peptide array−cell binding assay (relative cell adhesion ratio 2.7). This is most likely due to the high autofluorescence of peptide **40** (Figure 1B) which was enhanced after cell binding.

In a competition experiment, 100-fold excess of unlabeled **18** caused an up to 85% decrease in the FITC fluorescence of the FITC-**18** bound cells (Figure S4, Supporting Information). Similar competitive experiments for peptide **11** showed very different results. As shown in Figure 5A, only a slight decrease (3%) in the fluorescence was observed when the cells were incubated with FITC-**11** in the presence of 100-fold excess unlabeled **11**. However, a substantial (48%) decrease in FITC fluorescence was observed when FITC-**11** was incubated in the presence of unlabeled c-RGDfK (100-fold). The fluorescence was not completely wiped out in the presence of either excess **11** or c-RGDfK peptides, suggesting multiple binding sites for **11** on the cancer cells. Peptide **11** is a fusion peptide with RGD and QGR sequences inserted in the p160 sequence. It may, therefore, bind to different receptors, for instance, the $\alpha_v\beta3$ integrin, aminopeptidase N (CD13), and the putative p160 receptor on the cancer cells. However, this needs to be validated by further experiments, such as overexpression of putative peptide **11** targets in HUVECs or knockdown of putative peptide **11** targets in MCF-7 and MDA-MB-435 cells which will lead to peptide binding in the former and loss of binding in the later cells. MDA-MB-435 and MCF-7 cancer cells are known to express $\alpha_v\beta3$ integrin; however, expression of APN (CD13) by these cells is minimal.[19,41,42]

Cancer cell binding and uptake was also confirmed by visualization using the confocal microscopy. Figure 5B shows the z-stack scan of peptide FITC-**11** bound to the MDA-MB-435 cells. The scan shows that peptide is present at the surface as well as inside the cells. Figure 5C shows similar images of FITC-**11** bound to cells in the presence of 10-fold excess unlabeled p160. A decrease in the fluorescence is observed when p160 is present as a competitor. This decrease in fluorescence is of the same order as observed with unlabeled **11** as a competitor using flow cytometry (Figure 5A). These results, along with the FACS experiments, confirm that peptide **11** binds to multiple receptor

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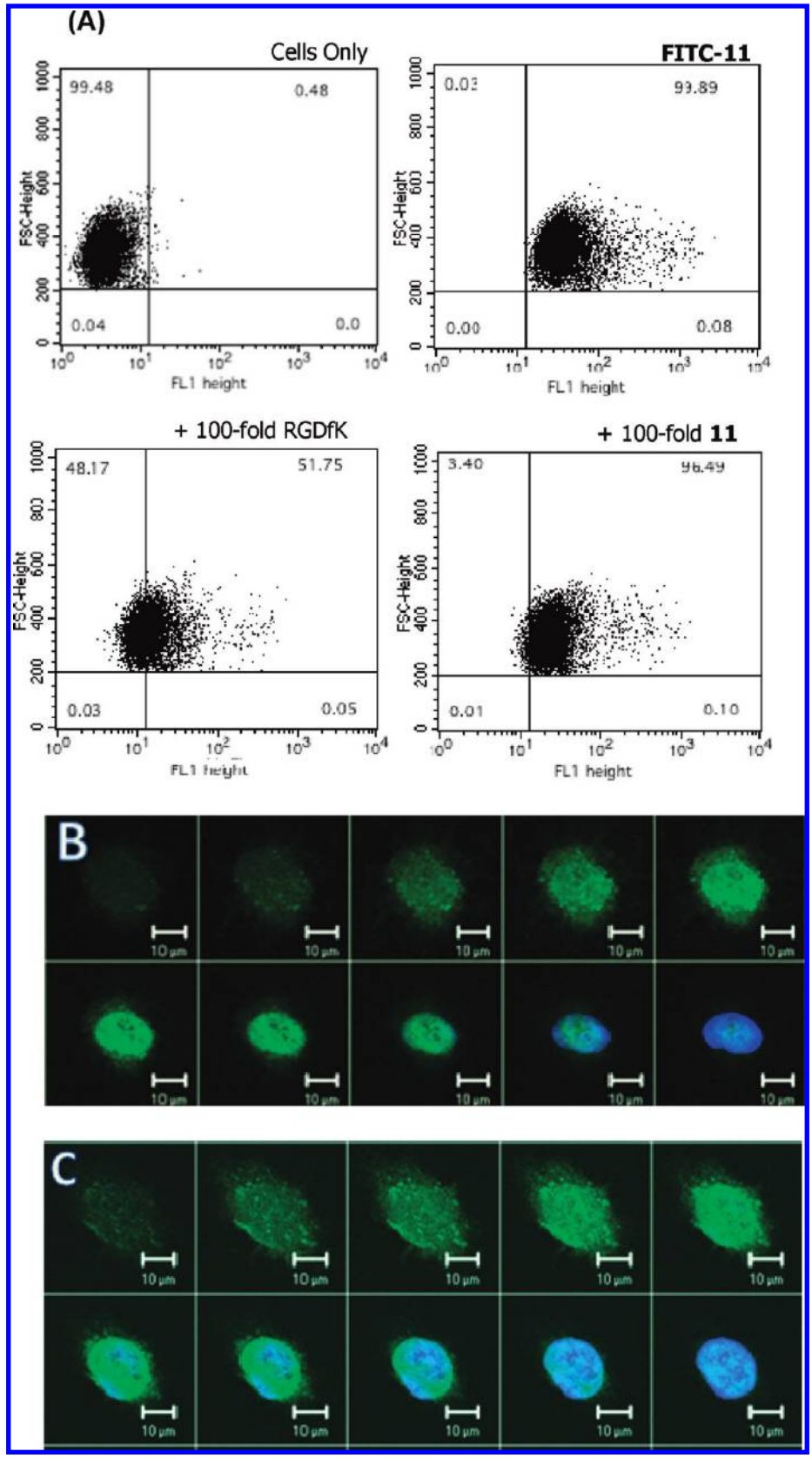


**Figure 5.** (A) FACS analysis for the competitive binding of the peptides, showing autofluorescence of MDA-MB-435 cells (top left), fluorescence of cells after incubation with $10^{-5}$ mol/L FITC-**11** (top right), and fluorescence of cells after incubation with FITC-**11** ($10^{-5}$ mol/L) in the presence of 100-fold excess **11** (bottom right) or c-RGDfK (bottom left). (B and C) Fluorescence images showing binding and internalization of FITC-**11** by MDA-MB-435 cells. Cells were incubated for 30 min with (B) FITC-**11** ($10^{-5}$ mol/L) alone or (C) in the presence of unlabeled p160 ($10^{-4}$ mol/L). Cell nuclei were stained blue with DAPI ($\lambda_{ex}$ = 360 nm, $\lambda_{em}$ = 460 nm) and overlaid with FITC−peptide fluorescent images ($\lambda_{ex}$ = 494 nm, $\lambda_{em}$ = 520 nm). Ten slices from the top to the middle of the cells were extracted using the z-stack scan mode of the confocal fluorescence microscope. Scale bar is 10 $\mu$m.

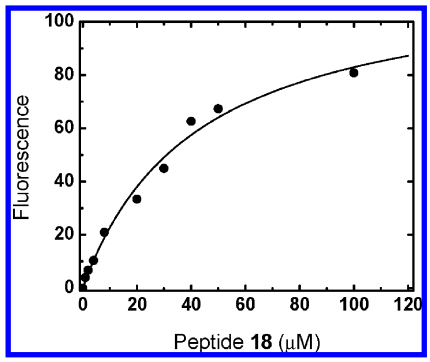


**Figure 6.** Binding of FITC-**18** to MDA-MB-435 cells ($10^6$) as observed by flow cytometry. Shown is fluorescence of the cell-bound peptide as a function of different peptide concentrations. The symbols are experimental data points whereas the line was calculated by fitting data to one binding site equation as described before.[30]

sites. Thus, targeting multiple receptors with a fusion peptide such as **11** can lead to peptides with higher affinity.

The binding of **18** to the cancer cells is presumed to be very similar to the p160 peptide, as there is single mutation in **18** compared to p160. Peptide **18** showed almost complete removal of fluorescence due to cell bound FITC-**18** in the presence of unlabeled **18** (Figure S4, Supporting Information). Similar receptor mediated specific binding of p160 by the MDA-MB-435 and MCF-7 cancer cells was demonstrated by Askoxylakis et al.[22] A large decrease in cell binding was observed when $^{125}$I-labeled p160 ($10^{-6}$ mol/L) was allowed to bind cancer cells in the presence of unlabeled p160 as a competitor. The authors also show internalization of the radio-labeled and FITC-labeled p160, which was inhibited in the presence of unlabeled p160.

Finally, peptide **18** shows about 3-fold better affinity compared to p160 (Figure 4D) and, in general, peptides like **18** bind to cancer cells in the low micromolar range. As shown in Figure 6, peptide **18** recognizes the MDA-MB-435 cells with an apparent $K_d$ of 41.89 ± 7.1 $\mu$M. This may seem to be a high concentration range in comparison to the other targeting moieties recently reported.[8,9,43] The HER-2 and EGFR specific affibodies and the cancer-specific aptamers bind to the cells in the nanomolar range. However, it is important to note that our peptides are very small (10-mers) compared to affibodies (58 amino acids)[43] and aptamers (39−85 nucleotides).[8,9] These peptides can behave like small molecule drugs and present less in vivo problems related to large molecules such as immunogenicity and toxicity. Currently, peptides **11** and **18** are being conjugated to micelles for targeted delivery of anticancer drugs to cancer cells in vitro and in a mouse model. We have recently demonstrated that micelles surface coated with RGD peptide show increase in the therapeutic efficacy of doxorubicin for sensitive and resistant cancers.[44] Our preliminary experiments (unpublished results) show that p160 conjugated micelles display 10 times more uptake by the MDA-MB-435 cancer cells compared to RGDfK coated micelles. The two peptides (**11** and **18**) identified here using the peptide array−cell screening method hold a huge potential in targeted cancer drug delivery. These peptides are identified without the knowledge of a biomarker or receptor on the cell surface. The results suggest that such peptides can not only be used for targeting a diseased cell but also be used for the diagnosis without the complete understanding of the molecular processes involved in the disease.

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## CONCLUSION

In conclusion, we have developed a new method to screen peptides for specific recognition by MDA-MB-435 and MCF-7 cancer cells using peptide array−whole cell interactions. A peptide array of 70 peptide sequences was designed on the basis of p160 peptide, a cancer cell targeting peptide identified by phage display. Peptides ranging in length from 9-mer to 12-mer were synthesized on a cellulose membrane. A direct peptide array−cell binding experiment allowed identification of several new peptides that showed higher affinity for the MDA-MB-435 and MCF-7 cancer cells compared to the wild type p160. These peptides showed very little affinity for the control HUVECs. Three peptides, namely, **11**, **18**, and **40**, that displayed the highest affinity for the cancer cells were synthesized and labeled with FITC. The cancer cell binding ability of these selected peptides was confirmed using fluorescence imaging and flow cytometry. FITC-**11** and FITC-**18** displayed high affinity for the cancer cells compared to the FITC-p160 peptide. In a competition experiment, the fluorescence for FITC-**18** was eliminated in the presence of an excess of unlabeled **18**. On the other hand, the fluorescence for FITC-**11** was only 3% reduced in the presence of unlabeled **11** and was reduced to half (48%) in the presence of an excess of unlabeled RGDfK, suggesting multiple binding sites for **11**. These results suggest the presence of a specific receptor on cancer cells and can help in the identification of a specific biomarker for cancer. The peptide array−cell binding assay established in this study is not only useful for the identification of cancer targeting peptides but also useful for the generation of diagnostic tools for cancer.

## ACKNOWLEDGMENT

The authors thank Samar Hamdy and Xiao-Bing Xiong for their help in the cell culture experiments. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC). The infrastructure support from the Canada Foundation for Innovation (CFI) is also acknowledged.

## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 3, 2010. Accepted August 6, 2010.

AC1003085