

AD _____

Award Number: DAMD17-99-1-9271

TITLE: Novel Peptide/Protein Delivery System Targeting erbB2-Overexpressing Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Dihua Yu, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson
Cancer Center
Houston, Texas 77030

REPORT DATE: August 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20011212 146

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 00 - 31 Jul 01)	
4. TITLE AND SUBTITLE Novel Peptide/Protein Delivery System Targeting erbB2-Overexpressing Breast Cancer Cells			5. FUNDING NUMBERS DAMD17-99-1-9271	
6. AUTHOR(S) Dihua Yu, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas M.D. Anderson Cancer Center Houston, Texas 77030 E-Mail: dyu@notes.mdacc.tmc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words)				
14. SUBJECT TERMS Breast Cancer, Penetratin, ErbB2-targeting, Peptide delivery, Novel therapies				15. NUMBER OF PAGES 20
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

TABLE OF CONTENTS

Contents	Page
Cover	1
Standard form (SF) 298	2
Table of contents	3
Introduction	4
Body	4
Key Research Accomplishments	6
Reportable Outcomes	6
Conclusions	6
References	7
Appendices	8-19
Figures 1-11	

Introduction

It has been well recognized that the next frontier in molecular medicine is the delivery of therapeutics. Among the biological therapeutics, peptides/proteins are especially difficult and challenging to deliver. A peptide sequence localized within a 13-amino acid domain of Tat (named "penetratin"), when linked to other peptides or proteins, was able to carry attached peptide or protein into the cells when they were added to cell culture medium (1). We **hypothesized** that the unique property of penetratin can be utilized for delivery of therapeutic peptides/proteins. It should be noted that after the funding of this Idea Award, a report published in *Science* by Steven Dowdy's laboratory demonstrated that penetratin can deliver functional β -galactosidase protein (120 kD) to all tissues in mice (2). This report indicated that our basic hypothesis is right. However, although this report showed the high efficiency of the penetratin system they used, the reported penetratin system clearly has no selectivity or targeting ability that will limit its application for breast cancer treatment. The major goal of our Idea proposal is **to develop a new penetratin-based peptide delivery system that specifically targets ErbB2-overexpressing breast cancer cells**. We have proposed three Specific Aims to fulfill our major goal. During the first funding year, we focused on Aim1, i.e. **to establish and optimize the penetratin delivery system for targeting**. We have identified a shortened penetratin sequence (P3) that delivers to the cytoplasmic subcellular compartment of cells but not the nucleus. During the second funding year, we first continued to work on Aim 1 and finished task 3. We then did not work on Aim 2 as originally planned, but focused on Aim3, i.e. to develop penetratin-based delivery system **targeting specifically ErbB2-overexpressing breast cancer cells**. We made this change because: a) after the report published in *Science* by Steven Dowdy's laboratory, we realized that targeting is the most critical issue we have to resolve to fulfill our final research goal; and b) with the fast progress in understanding ErbB2 biology, better ErbB2 interfering peptides are emerging, we will test other ErbB2 interfering peptides along with the proposed ESP. We had some progress delay in our first funding year due to unexpected difficulties in labeling the peptides with FITC (please see our progress report for the first year). In our second funding year, we suffered additional delay from Storm Allison (activity loss of peptides stored in the freezer that was warmed-up due to loss of power). Nevertheless, we have made substantial progress (see below). We have achieved the goal of developing penetratin-based delivery system **targeting specifically ErbB2-overexpressing breast cancer cells**. Our effort has pointed a very positive direction for the next grant-support year, when we will test effects of various ErbB2 signal-blocking peptides using the ErbB2-targeting penetratin delivery system.

Body

During the second funding year, we have performed the following studies:

Objective 1. To establish and optimize the penetratin delivery system.

Task 3: Systemic study of penetratin delivery system.

As shown in Figure 1 (and the previous report), Peptide3 (FITC- β -Ala-YGRKKRRQR) were observed predominantly in cytosol with decreased translocation ability compared to P1 and P2. Thus, P3 should give reduced non-specific binding than P1 and P2. To confirm that the fluorescence signals of P3 are from inside the cells but not from cell membranes, we removed the image haze using the 2-D deconvolution function (Figure 2). This confirmed that

P3 is indeed translocated inside the cell but not stuck on the cell membrane. Compared to P1, (the original penetratin), P3 has attenuated translocation, which gives reduced non-specific binding. Thus, P3 is an ideal penetratin sequence for conjugating to an ErbB2- targeting peptide. We further tested the translocation kinetics of P3 and found that the translocation of P3 is concentration-dependent in ErbB2-overexpressing SKBr3 breast cancer cells (Figure 3). The pattern of P3 translocation is similar among ErbB2 low expressing MDA-MB-435 cells versus ErbB2-overexpressing BT-474 and SKBr3 cells at either higher concentrations of P3 (Figure 4) or lower concentrations of P3 (Figure 5). This indicated that P3 itself has no targeting ability.

To investigate if the penetratin translocation is reversible, SKBr3 cells were treated with 20nM FITC-P3 for 15 min. The cells were then incubated in fresh medium without P3 for various time intervals (0, 30, 60, or 180 min, respectively) before observation (Figure 6). The fluorescence intensities were decreased with increased incubation time in fresh medium. The same tendency was observed in MDA-MB-435 cells (Figure 7). Thus, P3 translocation seems reversible.

Aim 3: To develop the penetratin-based delivery system targeting specifically ErbB2-overexpressing breast cancer cells

The major obstacle in using the powerful penetratin delivery system *in vivo* is its lack of specificity. We hypothesized that we can increase the specificity to ErbB2-overexpressing breast cancer cells by optimizing penetratin sequence (Aim 1) and conjugating with an ErbB2/HER2/neu binding peptide (Aim 3). We originally proposed to use the ErbB2 extra-cellular domain binding protein (ECDBP) that we identified through peptide library screening. A recent publication identified an anti-HER2/neu peptide mimetic (AHNP) that specifically and efficiently binds to ErbB2/HER2 (3). We, therefore, compared the binding affinity between ECDBP and AHNP. We found that AHNP binds to ErbB2/HER2 at higher affinity than ECDBP. Therefore, we decided to conjugate AHNP to the optimized P3 penetratin sequence.

Tasks 7, 8: Synthesis and evaluation of AHNP-conjugated penetratin peptides.

Since we experienced difficulties labeling the P1, P2, and P3 peptides with FITC last year, we synthesized biotin-labeled P3-AHNP. The company, who synthesized the P1, P2, P3 peptides for us last year, had technical difficulties in synthesizing the P3-AHNP and eventually decided to give-up. We then worked with Dr. Martin Campbell in our institution who modified an amino acid in the AHNP and modified synthesis conditions. We were able to synthesize the peptides. Biotin-labeled P3 without AHNP was also synthesized to serve as a control peptide.

When added to cells and detected using 2-D deconvolution microscopy, AHNP-conjugated P3 demonstrated better translocation ability than that of P3 specifically in ErbB2-overexpressing 435.eB cells but not in the parental ErbB2 low-expressing MDA-MB-435 cells (Figure 8A and B). This is further shown by quantification of the average fluorescence intensities in cytoplasmic regions (Figure 8C). Notably, the average fluorescence intensities in nuclear regions were similarly low among all four samples. The targeting capacity of the AHNP-conjugated P3 for ErbB2-overexpressing breast cancer cells was also confirmed in SKBr3 cells along with 435.eB cells (Figure 9). To demonstrate P3-AHNP penetration depends on ErbB2 expression level, we stained ErbB2 expression side-by-side with P3-AHNP penetratin staining (Figure 10). Cells with stronger ErbB2 staining (435.eB) indeed showed stronger P3-AHNP translocation.

AHNP was developed based on a recombinant humanized anti-HER2 monoclonal antibody rhuMAb4D5 (Herceptin). If the enhanced translocation in ErbB2-overexpressing cells was due to AHNP, pre-treatment of ErbB2-overexpressing breast cancer cells with Herceptin should compete P3-AHNP binding and reduce the binding of P3-AHNP to these cells. Indeed, when we pretreated ErbB2-overexpressing cells with Herceptin (Figure 11), P3-AHNP translocation were hindered in both ErbB2-overexpressing 435.eB and SKBr3 cell lines. Taken together, these data demonstrated that we have achieved our goal of targeting P3 delivery to ErbB2-overexpressing breast cancer cells.

Key Research Accomplishments

1. We have chemically synthesized the biotin-labeled and AHNP-conjugated Penetratin 3 peptides.
2. We have demonstrated that the P3-AHNP system has increased delivery specificity to ErbB2-overexpressing breast cancer cells *in vitro*.
3. We demonstrated that as low as 2nM of P3-AHNP is enough to show efficient delivery and specific targeting ErbB2-overexpressing breast cancer cells *in vitro*.

Reportable Outcomes:

-manuscripts, abstracts, presentations

We are not at the stage of writing manuscript yet. We expect that we will submit an abstract to AACR annual meeting in 2002.

-patents and licenses applied for and/or issued

We intend to patent our findings in the future.

-degrees obtained that are supported by this award None

-development of cell lines, tissue or serum repositories None

-informatics such as databases and animal models None

-funding applied for based on work supported by this None

-employment or research opportunities applied for and/or
received on experiences/training supported by this award

30% salary of a post-doctoral fellow and 100% salary of a Graduate Research Assistant

Conclusions:

Although we suffered additional delay from Storm Allison (loss of peptide activities), we have made substantial progress. Mainly, we have achieved the goal of developing penetratin-based delivery system **targeting specifically ErbB2-overexpressing breast cancer cells**. Our effort has pointed a very positive direction for the next grant-support year, when we will test effects of various ErbB2 signal-blocking peptides using the ErbB2-targeting penetratin delivery system.

We have a request to bring to the attention of the USAMRMC. As indicated above, we experienced difficulties labeling the peptides with FITC (please see our progress report of last year). In our second funding year, we suffered additional delay from Storm Allison (difficulties synthesizing peptides and loss of peptide activities). It will be very difficult for us to finish all the proposed research by July 31, 2002. Therefore, we respectfully ask that the USAMRMC grant us a one-year non-funded extension (i.e., without additional funds) of this Idea award to July 31, 2003. This will allow us time to fulfill all of our major goals plus testing additional ErbB2 interfering peptides.

References:

1. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. Tat-mediated delivery of heterologous proteins into cells, *Proc. Natl. Acad. Sci. USA*. *91*: 664-668, 1994.
2. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. In vivo protein transduction: delivery of a biologically active protein into the mouse, *Science*, 1999.
3. Park, B. W., Zhang, H. T., Wu, C., Berezov, A., Zhang X., Wang, Q., Kao, G., O'Rourke, D. M., Greene, M., I., and Murali, R. Rationally designed anti-HER2/neu peptide mimetic disables P185^{HER2/neu} tyrosine kinases in vitro and in vivo. *Nature Biotechnology* 18, 194-198 (2000)

Appendices

Please see 11 figures on the following pages.

Figure 1 SKBr3 cells

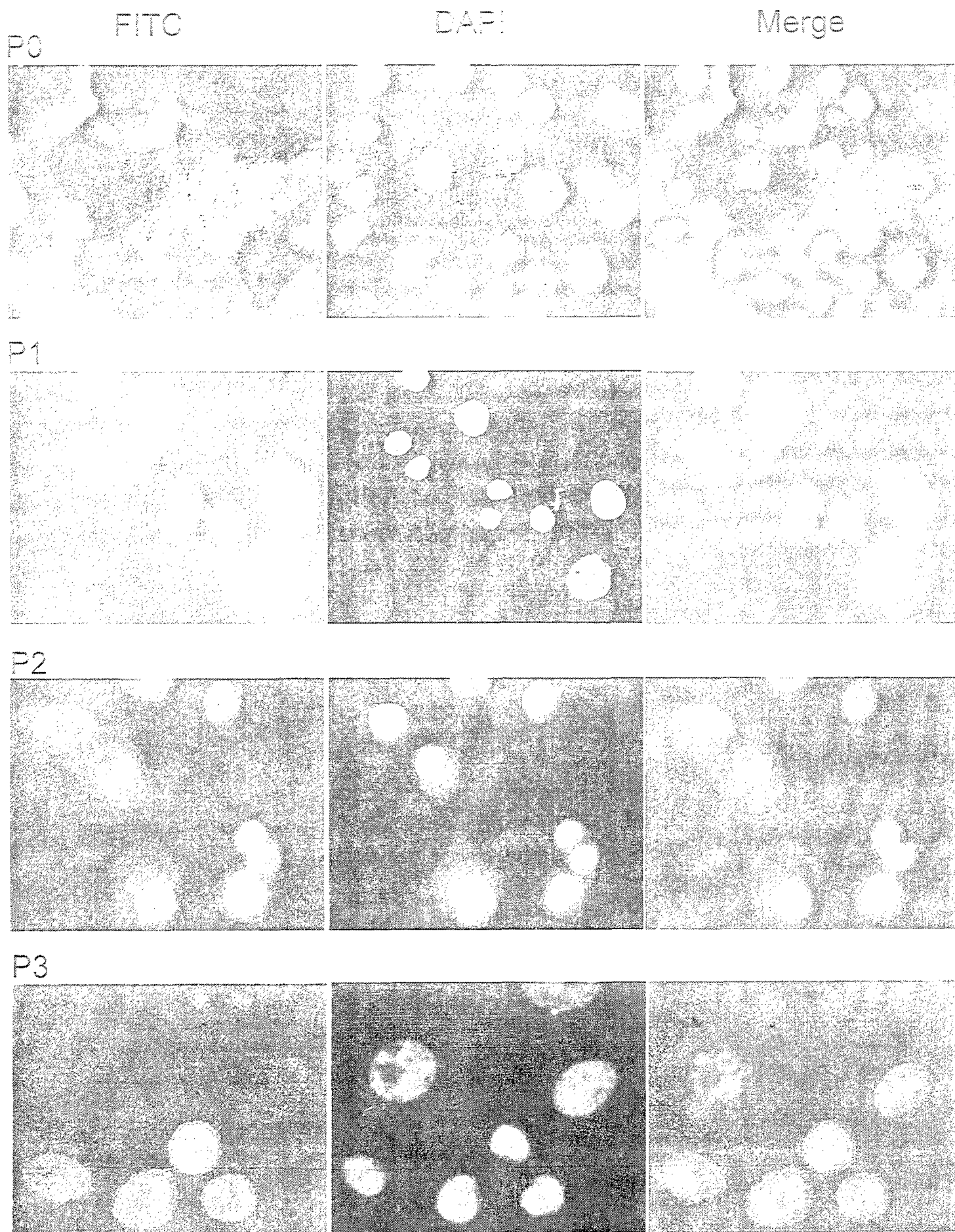


Figure 2 SKBr3 cells

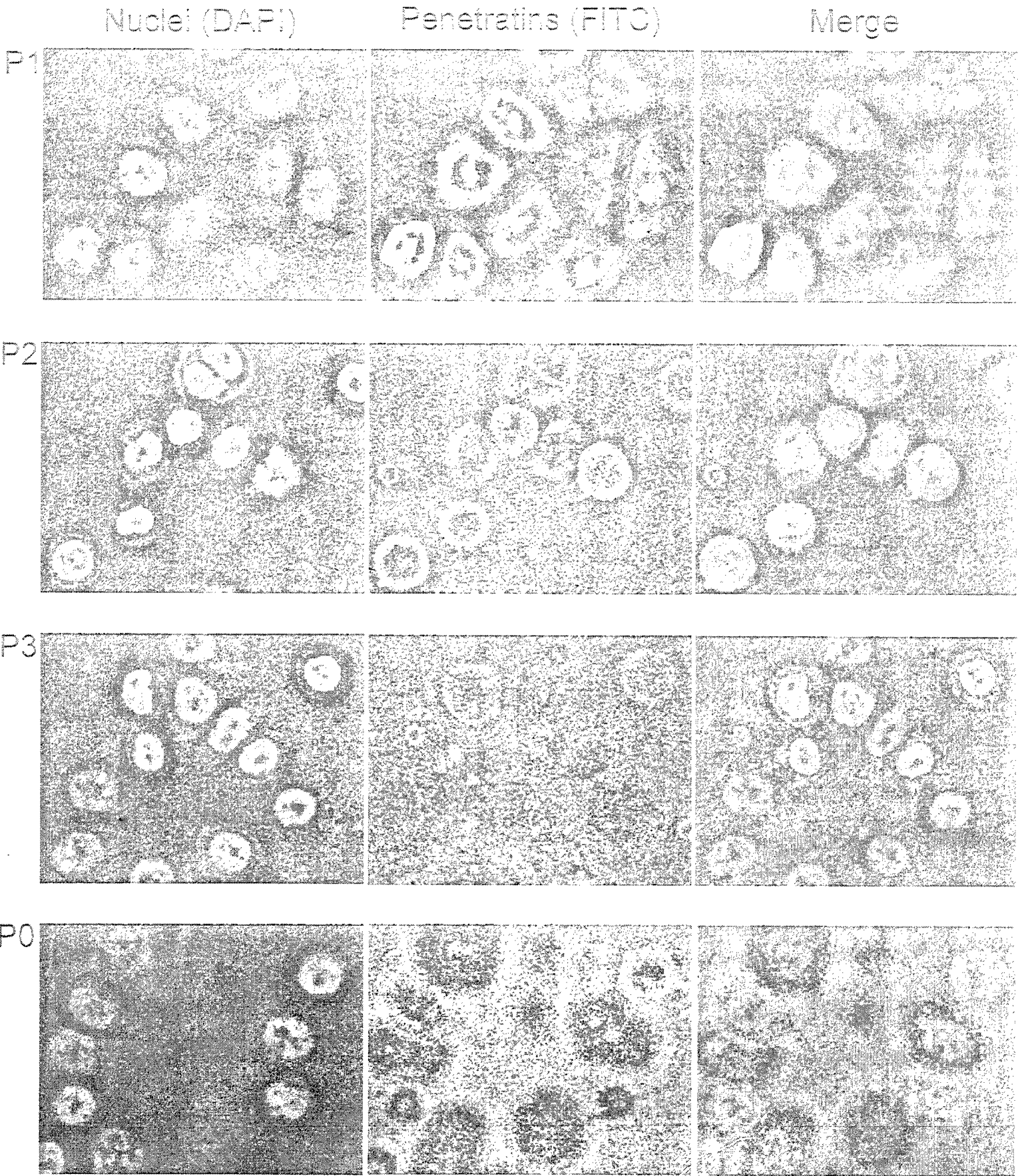


Figure 3 SKBr3 cells

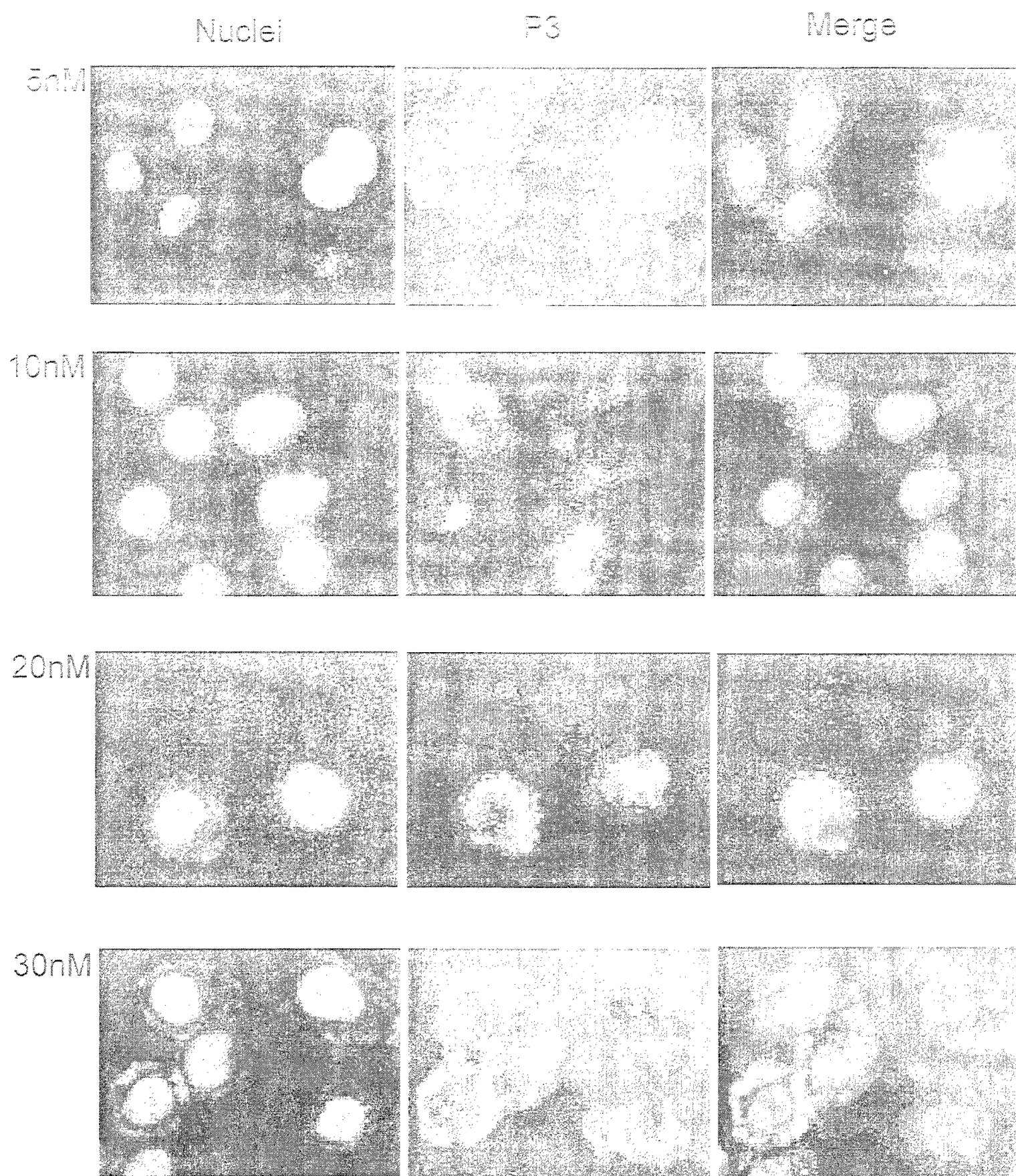


Figure 4

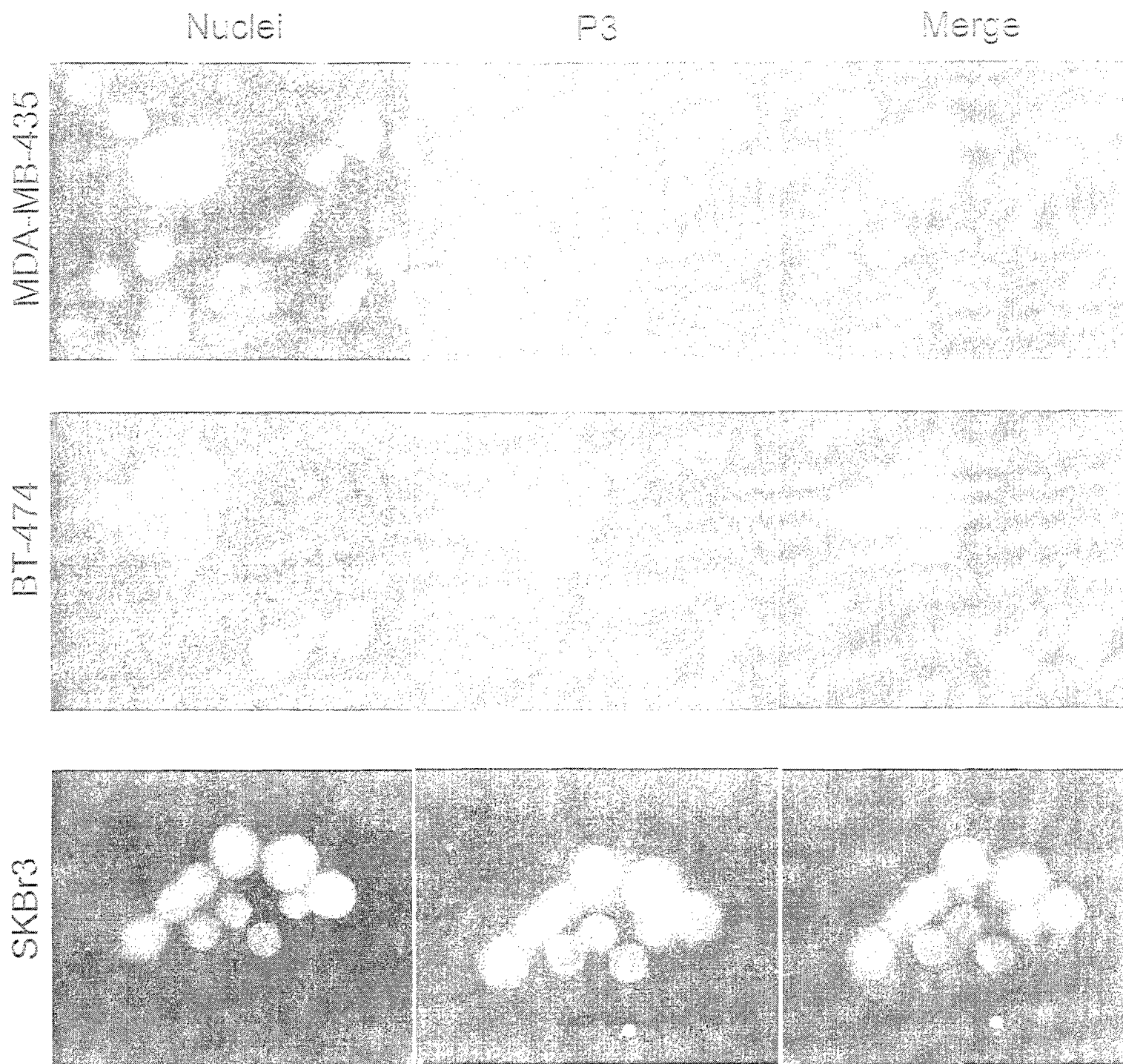


Figure 1 SKBr-3 cells

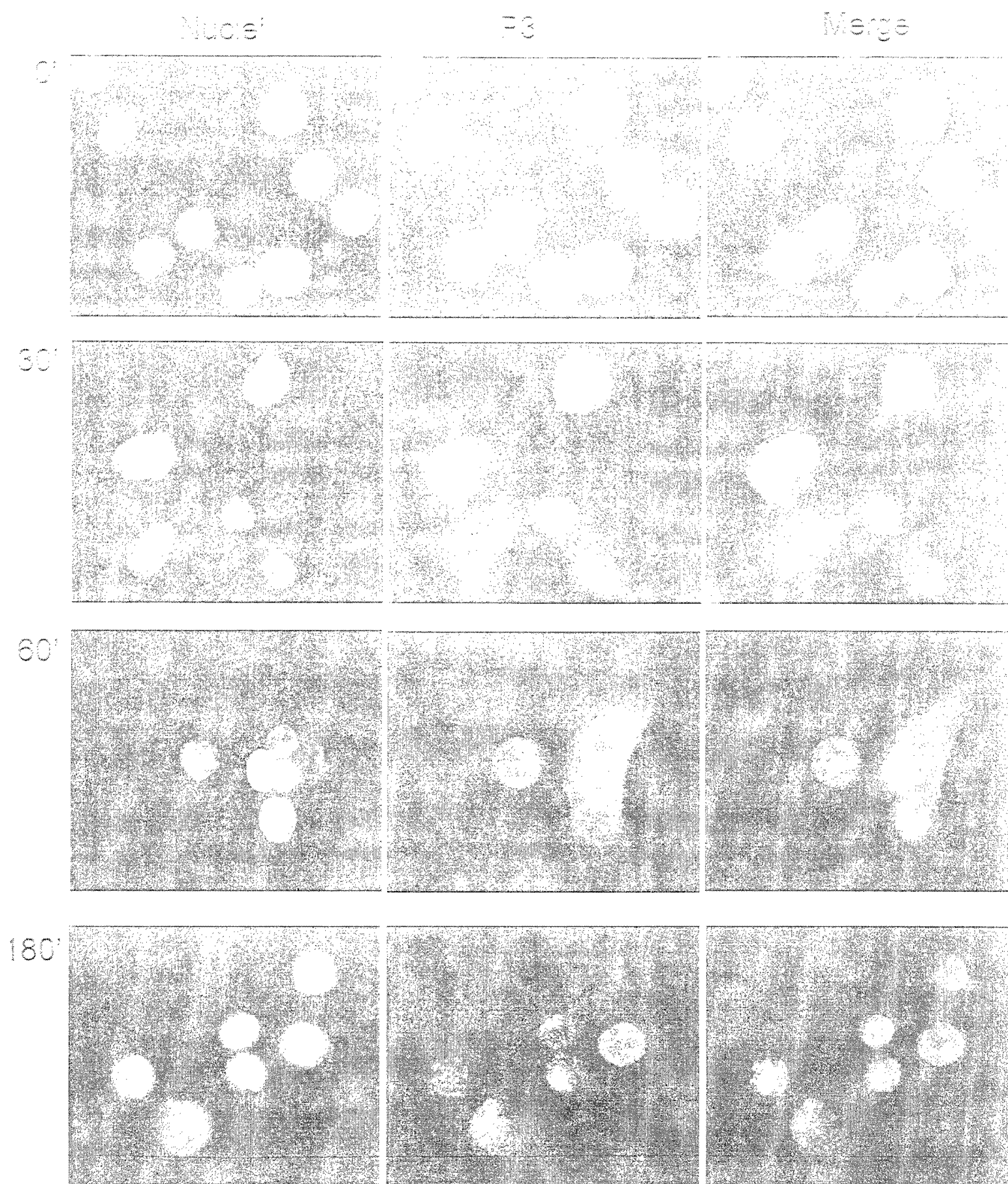


Figure 7. MD-AMB-455 cells

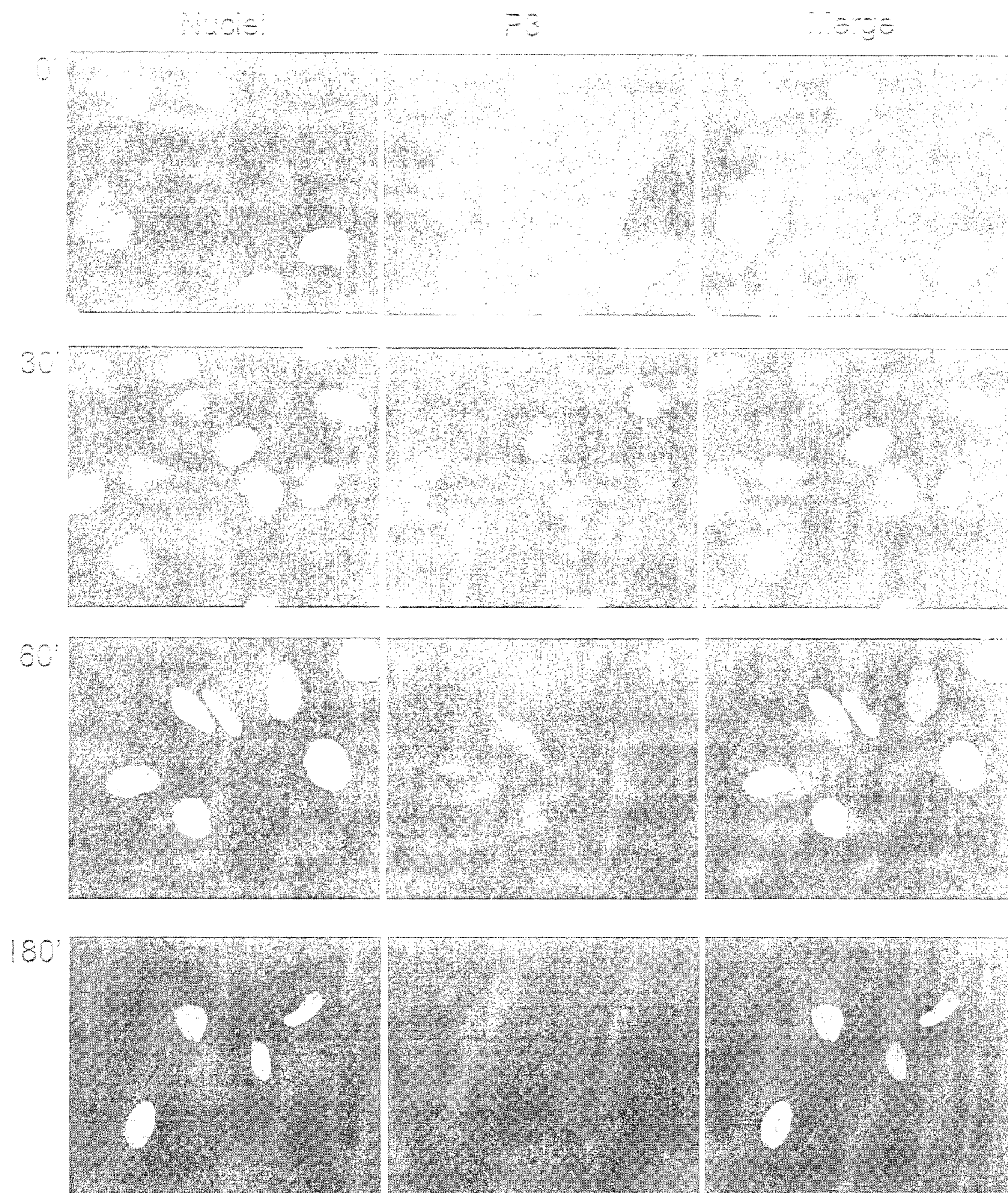
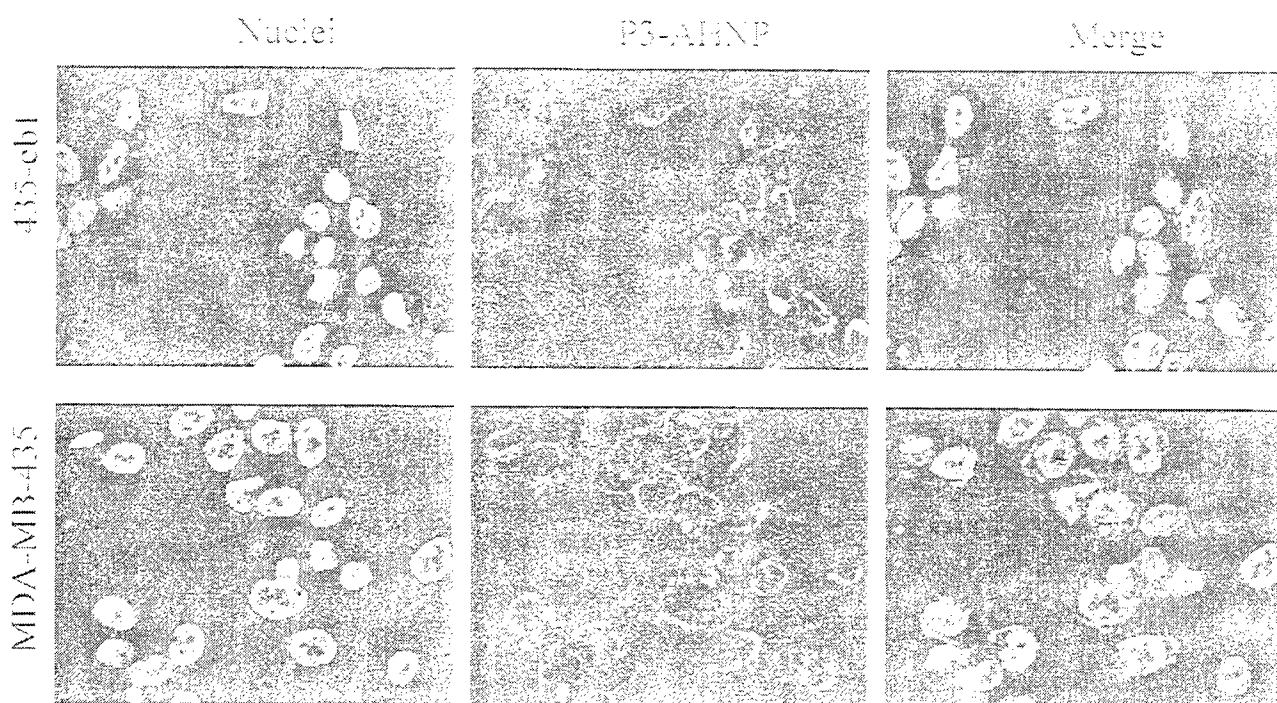


Figure 8

A. P3-AHNP



B. P3

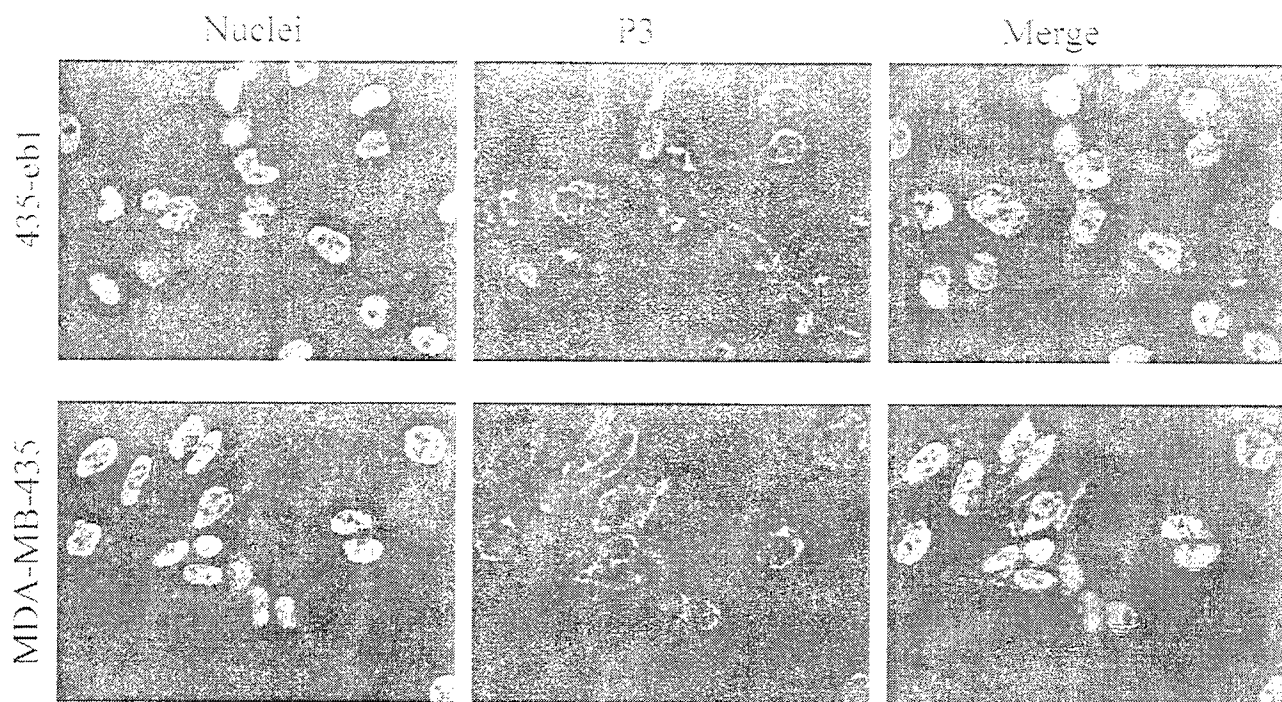


Figure 8C

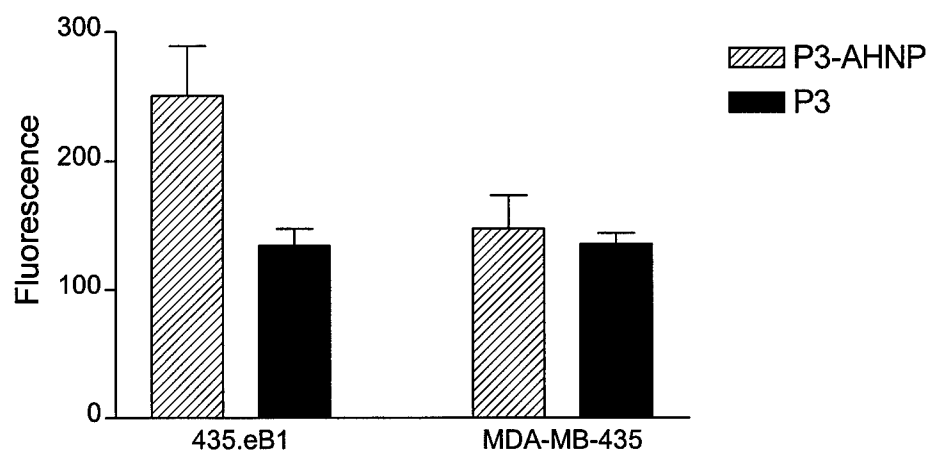


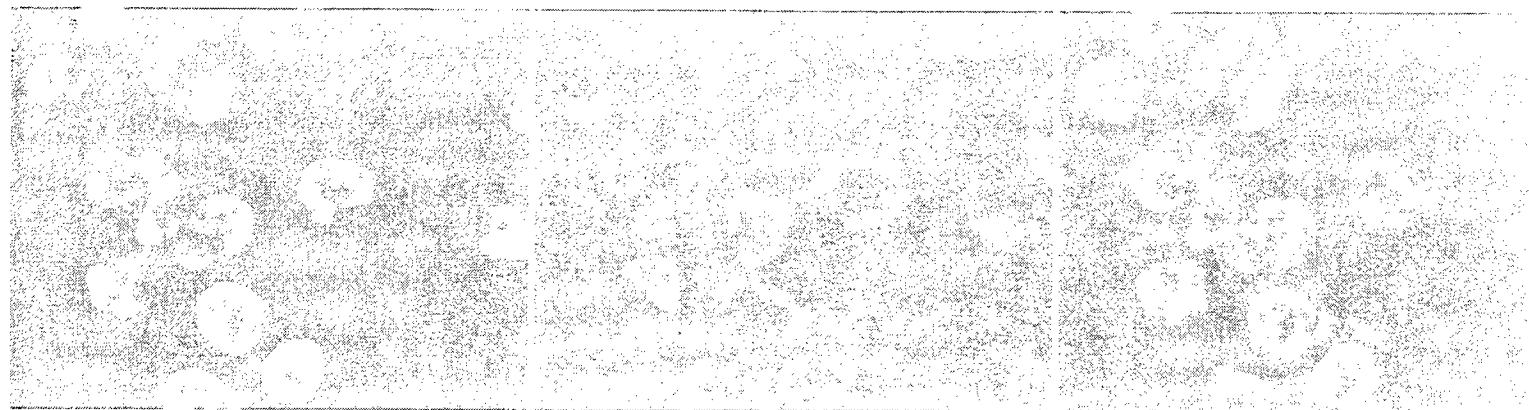
Figure 3

Nuclei

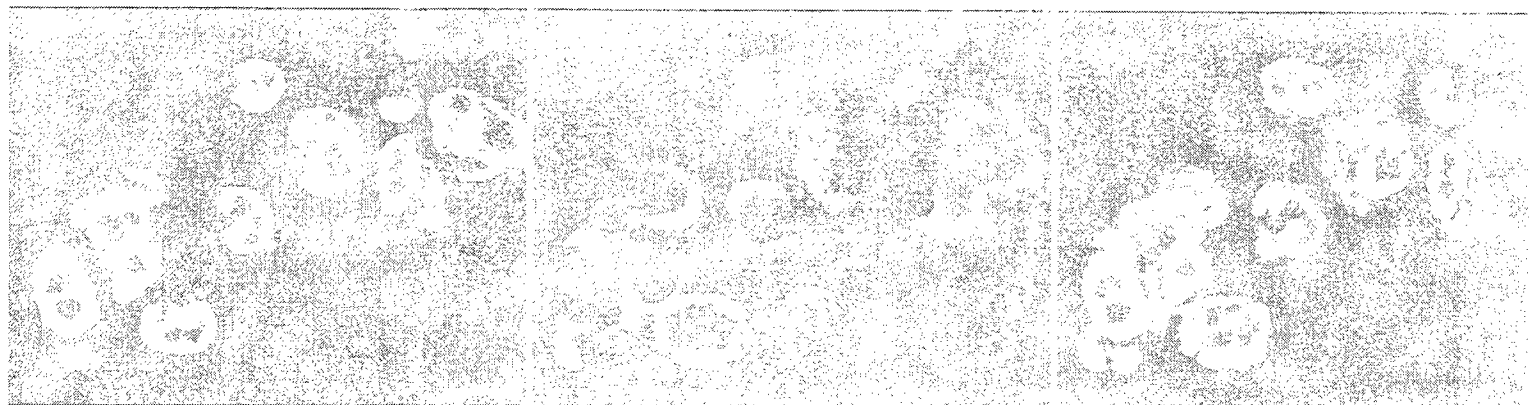
P3-AHKF

Merge

A. MDA-MB-435



B. 435.eB



C. SKBr3



D. SKBr3 (without deconvolution)

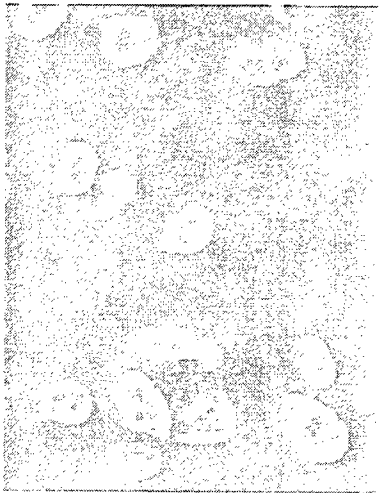
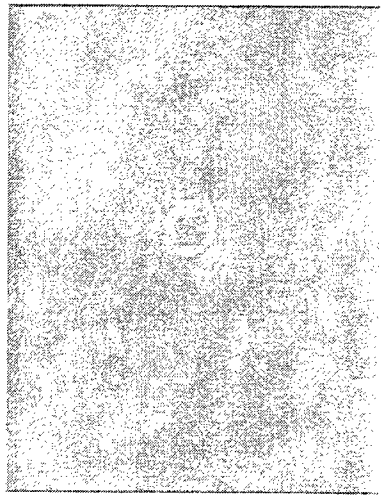
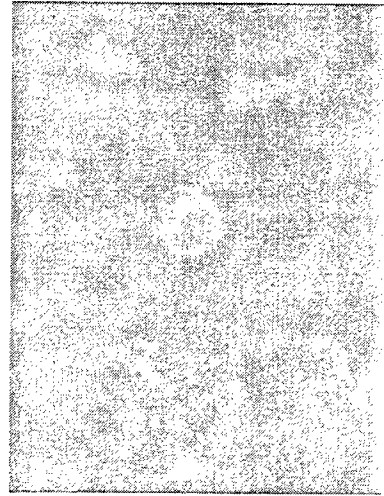
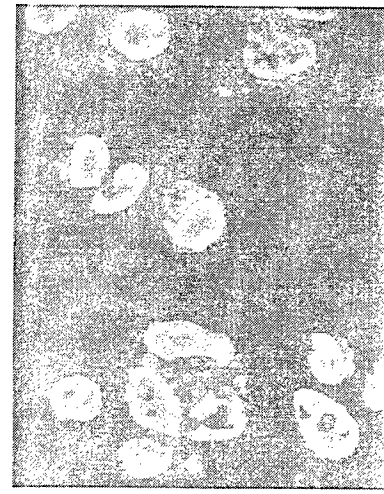


Merge

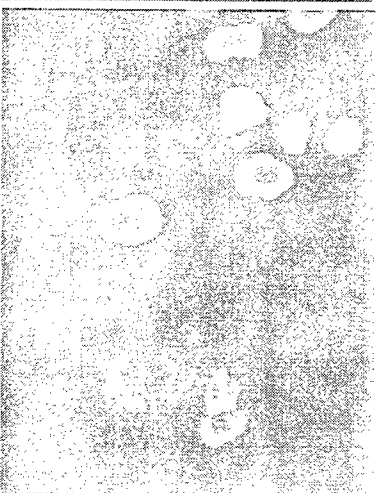
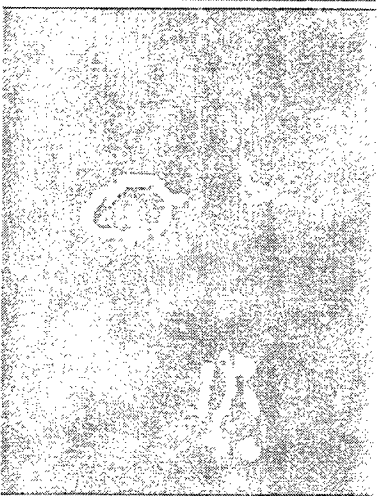
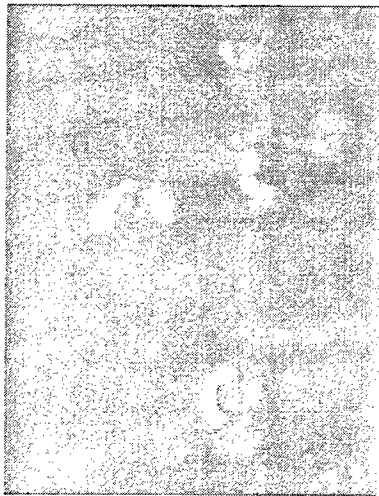
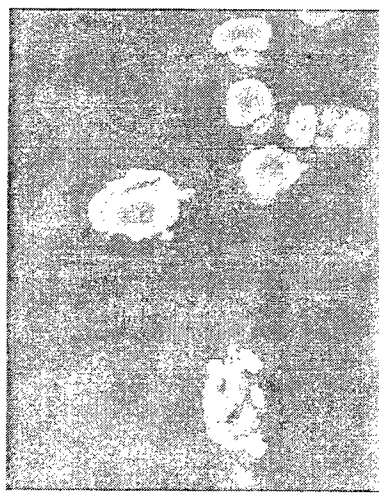
P3-AINP

ErbB2

Nuclei



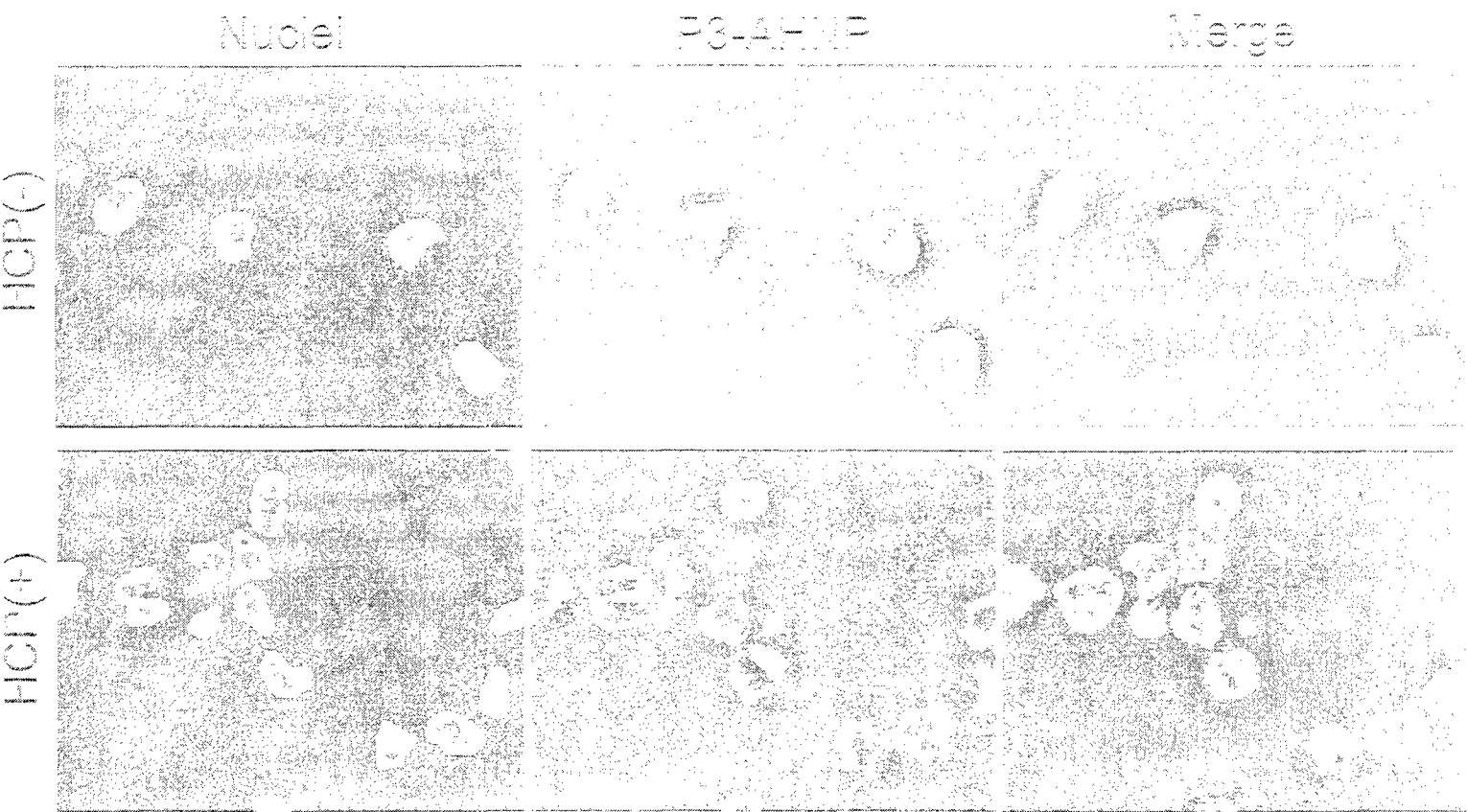
MDA-MB-435



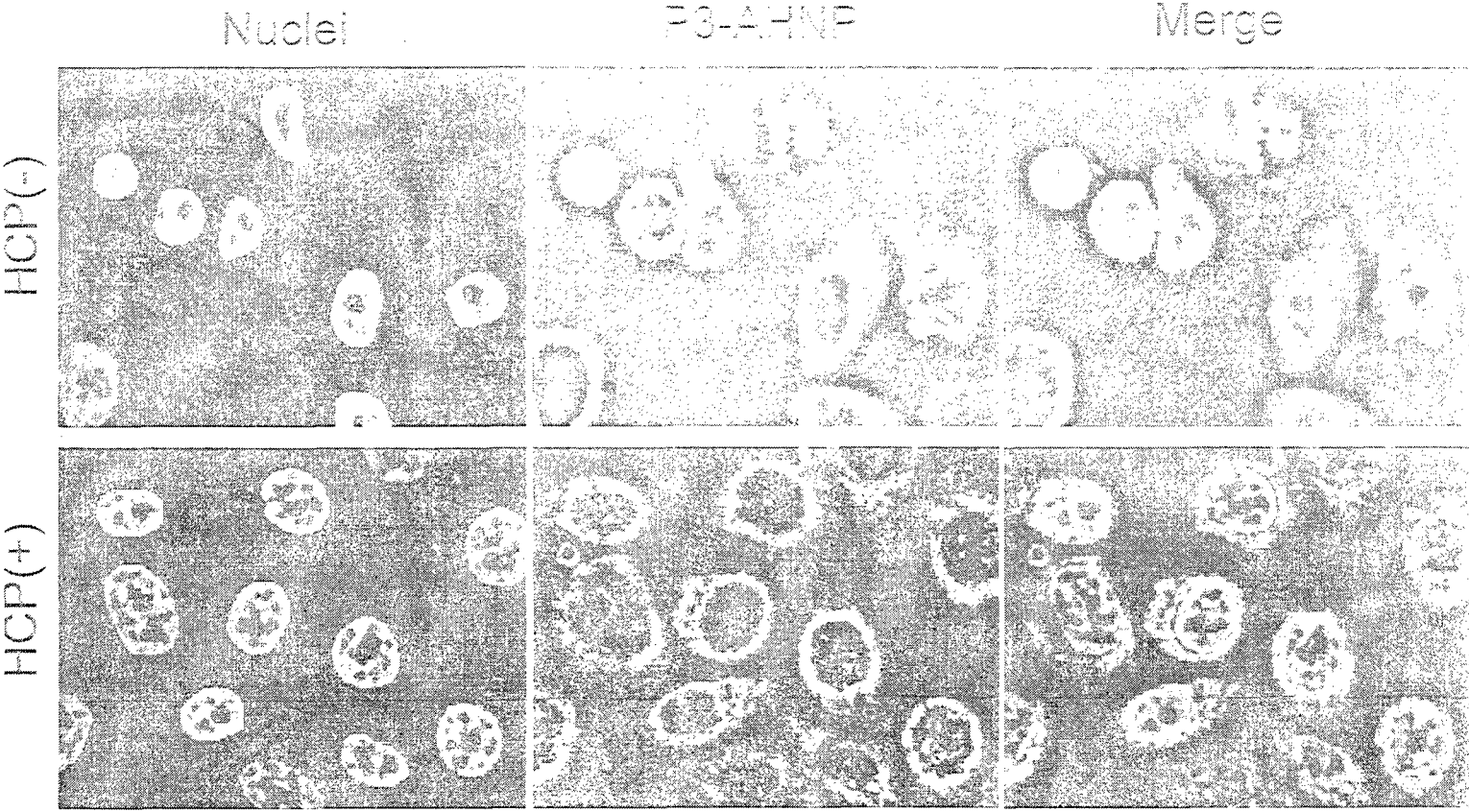
435.93

Figure 11

A. 435.e3



B. SKBr3



Legend

Figure 1

SKBr3 cells grown on sterile CultureSlides (Becton Dickinson, NJ) were incubated with Peptide0 (FITC- β -Ala-YGKKKKKKKKK, negative control), Peptide1 (FITC- β -Ala-YGRKKRRQRRR, positive control), Peptide2 (FITC- β -Ala-YGRKKRRQRR), and Peptide3 (FITC- β -Ala-YGRKKRRQR) respectively at 20nM for 15 min. Cells were then fixed with 4% paraformaldehyde/PBS and washed with PBS 20 times. In this and the following figures, samples were stained with DAPI for 5 min. These cells were photographed by Nikon ECLIPSE E400 at $\times 400$ magnification in MetaMorph Imaging System (Universal Imaging Corporation, PA). P1 distributed in nuclear as well as cytoplasmic region. P2 showed similar translocation pattern with decreased extent compared to P1. P3 were observed predominantly in cytosol with further decreased translocation ability.

Figure 2

SKBr3 cells were prepared as described in Figure 1 and incubated with P0, 1, 2, and 3 respectively at 1 μ M for 15 min. The acquired images were processed in MetaMorph using the 2D deconvolution function to remove image haze.

Figure 3

SKBr3 cells were treated with P3 at 5, 10, 20, and 30nM respectively for 15 min. The fluorescence increased with increasing concentrations of P3.

Figure 4

MDA-MB-435, BT-474, and SKBr3 cells were prepared as described in Figure 1 and incubated with P3 at 20nM for 15 min. The fluorescence images showed similar translocation patterns and extents among different cell lines.

Figure 5

Same experiment as Figure 4 with 5nM P3.

Figure 6

SKBr3 cells were incubated with 20nM P3 for 15 min. Cells were then washed with PBS twice and incubated in fresh medium. The time points shown on the left of figure (0', 30', 60', and 180') indicate the duration the cell had stayed in the fresh medium before fixation and observation. The FITC signals decreased with increased duration of fresh medium incubation.

Figure 7

Same experiment as figure 6 using MDA-MB-435 cells.

Figure 8

Two sets of MDA-MB-435 and 435-eB1 cells were incubated with 2nM of biotin-YGRKKRRQR-AHNP (P3-AHNP) (A) or biotin-YGRKKRRQR (P3) (B) for 15 min respectively. After PBS washing for 3 times, cells were fixed in 4% paraformaldehyde followed by 10 times of PBS washing. Cells were then permeabilized with 0.5% Triton X-100/PBS and washed. Samples were incubated DTAF-conjugated Streptavidin (Jackson ImmunoResearch, PA) for 1 hr in room temperature. Two-D

deconvolution and quantification of fluorescence signals were performed on MetaMorph Imaging System. The average intensity (integrated intensity per unit area) in the cytoplasmic region was calculated and plotted in panel C.

Figure 9

MDA-MB-435(A), 435.eB(B), and SKBr3(C &D) cells were treated with 20nM biotin-P3-AHNP for 15min followed by DTAF-streptavidin (green) and DAPI (blue) treatment. A-C were processed with 2D deconvolution; panel D shows the same field as panel C without deconvolution. P3-AHNP showed decreased translocation in erbB2 low-expressing MDA-MB-435 cells compared to erbB2 over-expressing 435.eB and SKBr3 cells.

Figure 10

MDA-MB-435 and 435.eB cells were treated with 20nM biotin-P3-AHNP for 60 min. After PBS washing for 3 times, cells were fixed in 4% paraformaldehyde followed by 10 times PBS washing. Cells were then permeabilized with 0.5% Triton X-100/PBS and washed. After 1hr of Ab3 (c-neu) (Oncogene Research Products, MA) incubation, samples were incubated in Texas Red-conjugated anti-mouse IgG and DTAF-conjugated Streptavidin (Jackson ImmunoResearch, PA) for 1 hr in room temperature. The acquired images were processed in MetaMorph using the 2D deconvolution function

Figure 11

435.eB (A) and SKBr3 (B) cells were incubated in 20nM of biotin-P3-AHNP for 30 min with (HCP(+)) or without (HCP(-)) 4 μ g/ml (27.2 μ M) Herceptin pre-treatment. In both cell lines, P3-AHNP translocation extents were attenuated with Herceptin pre-treatment.