Design and Synthesis of Tubulin-derived Peptides as Molecular Imaging Probes that Target Tumor Cells.

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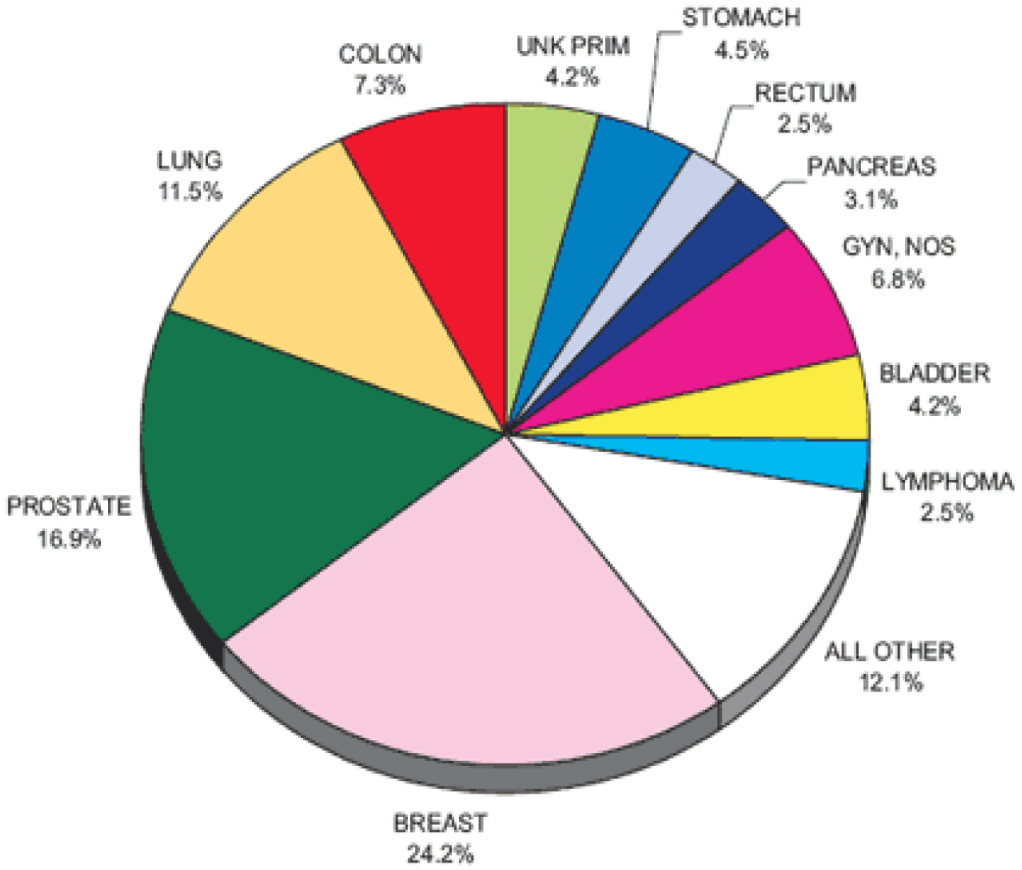
Western

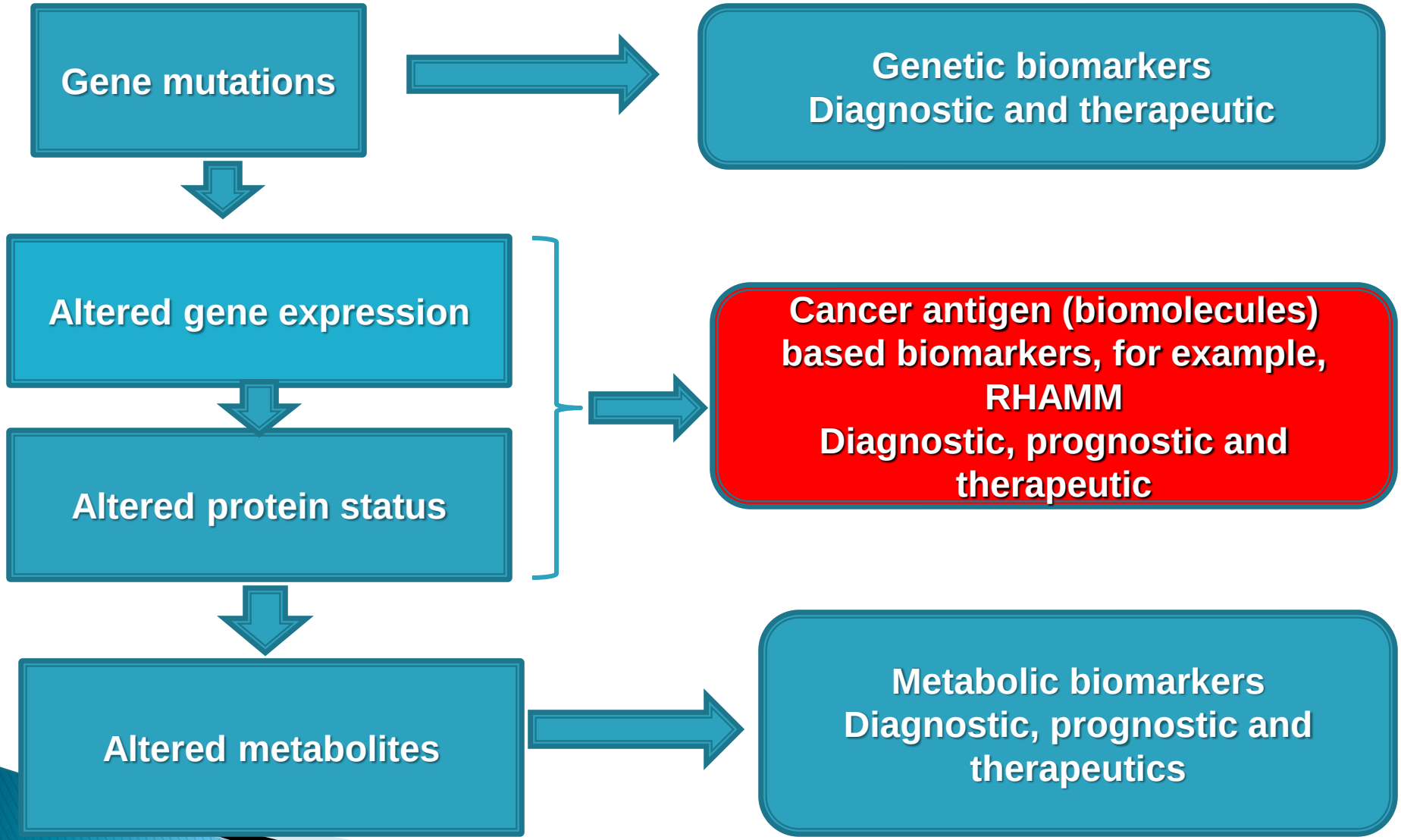
Frequency of Cancer

2012 Distribution of Primary Diagnosis



Cancer is a leading cause of morbidity and death worldwide. Survival rates for this disease could be significantly increased with improved and earlier diagnosis.

Cancer cells display a broad spectrum of genetic alterations, that include:



Applications:

►Technologies to recognize and understand the signatures of normal cells and how these become cancerous, can be useful for early cancer detection, diagnosis, and treatment.

►Biomarkers are invaluable tools for cancer detection, diagnosis, patient prognosis and treatment selection.

Available Techniques:

►Genetics, genomics, proteomics, many non invasive imaging techniques allow measurement of several biomarkers.

►However, limitations of screening techniques exist: they are not sensitive or specific enough for early detection of the cancer, they have a short half life, rapid renal clearance, give false positive results.

The goal of our research:

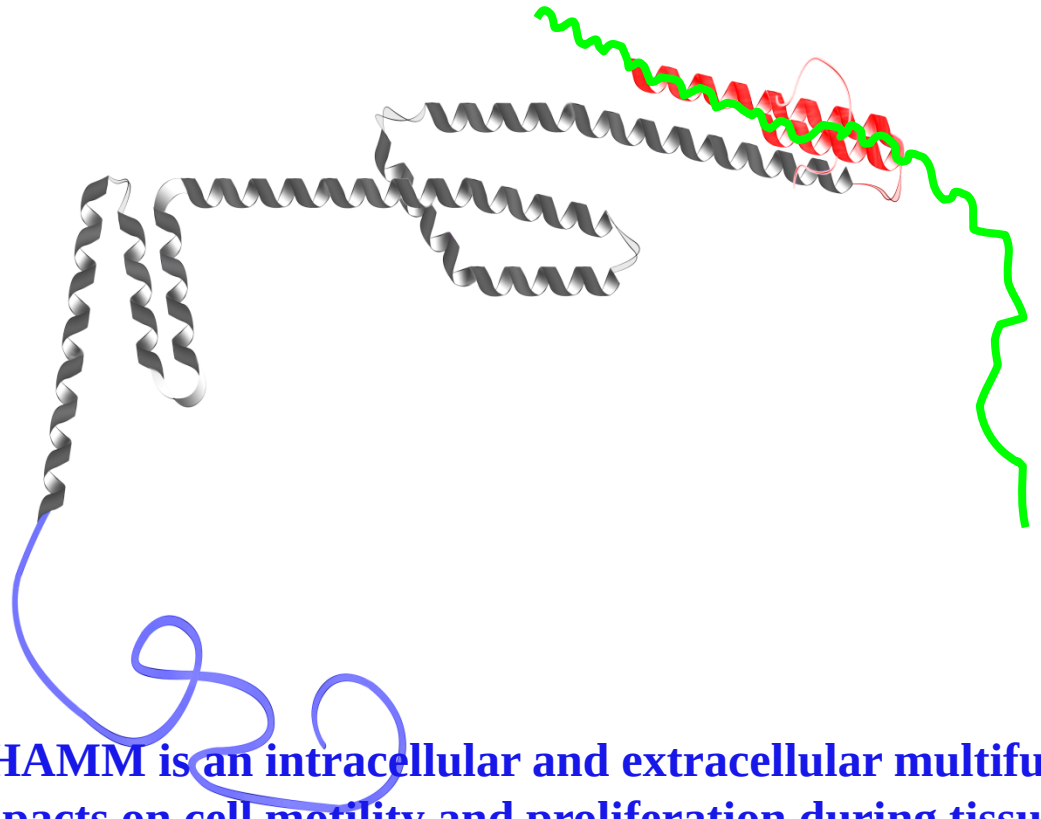
- To design specific, selective peptides that target Rhamm and use them for diagnostic or therapeutic purposes of cancer decease.

OUTLINE:

1. Design and Synthesis Tubulin-Derived Peptides as Novel High Affinity Ligands for the RHAMM/HMMR*
2. Evaluation of specificity and selectivity of Tubulin-Derived Peptides
3. Uptake of Tubulin-Derived Peptides by breast and prostate cancer cells

* In collaboration with Kenneth Virgel N. Esguerra (Luyt lab)

RHAMM -Receptor hyaluronan mediated motility, a coiled-coil protein



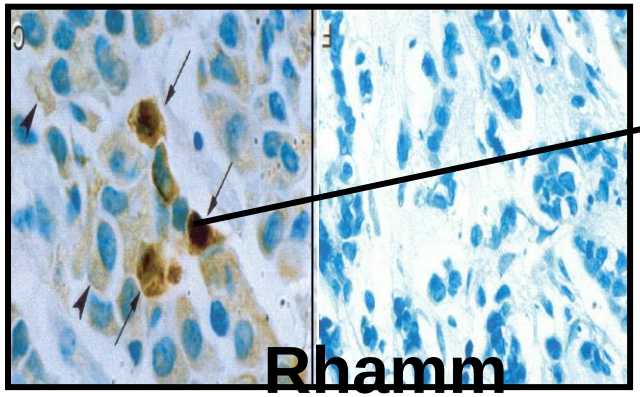
RHAMM is an intracellular and extracellular multifunctional protein, which impacts on cell motility and proliferation during tissue repair and disease processes such as cancer, diabetes and arthritis.

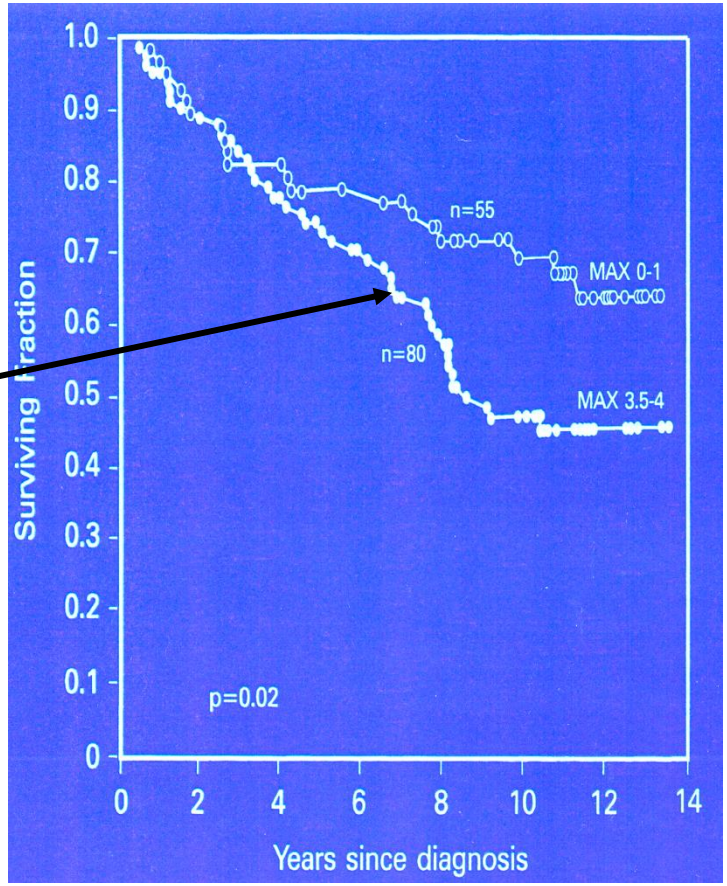
Extracellular RHAMM binds to HA fragments and partners with CD44 to activate signalling cascades such as MAPK.

Intracellular RHAMM binds to tubulin/ERK1 and controls mitotic spindle/interphase microtubule dynamics.

RHAMM is involved in proliferation, motility, migration, invasion, mitotic spindle formation in tumor cells.

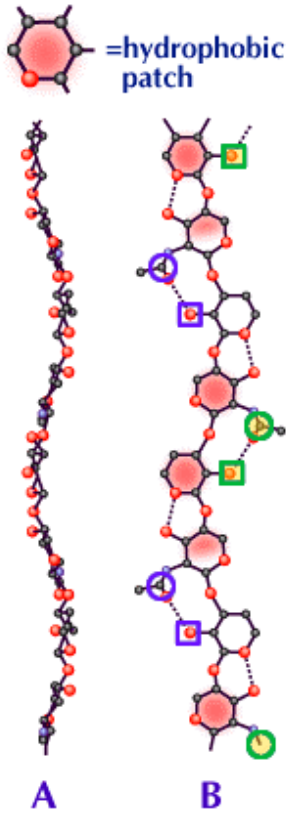
RHAMM is overexpressed in aggressive cancer cells, such as breast, prostate cancer cells, solid and blood tumors, myeloid leukemia, multiple myeloma and usually overexpression of RHAMM correlates with poor outcome.





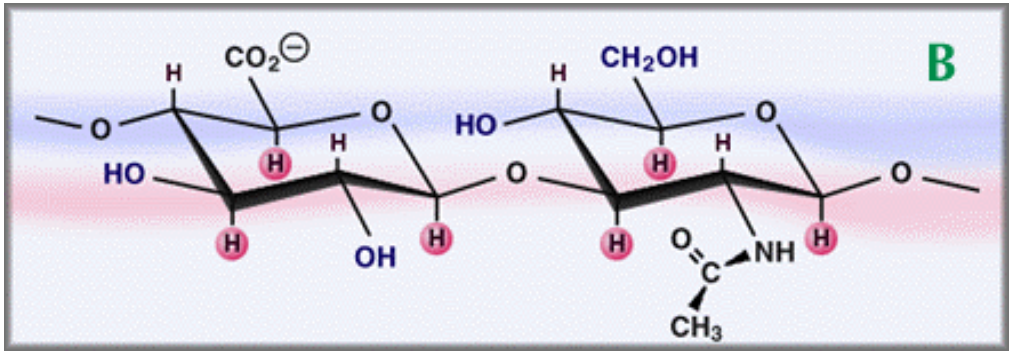
RHAMM hyper-expression in breast cancer cell subsets predicts poor clinical outcome and increased risk of metastasis

Hyaluronan is a polysaccharide and a natural ligand of Rhamm .



Computer model of hyaluronan

Chemical structure of hyaluronan



HA is a polydisperse glycosaminoglycan consisting of dimeric repeats of D-glucuronic acid and *N*-acetylglucosamine.

Functions of Hyaluronan:

It performs complex structural and signalling functions.

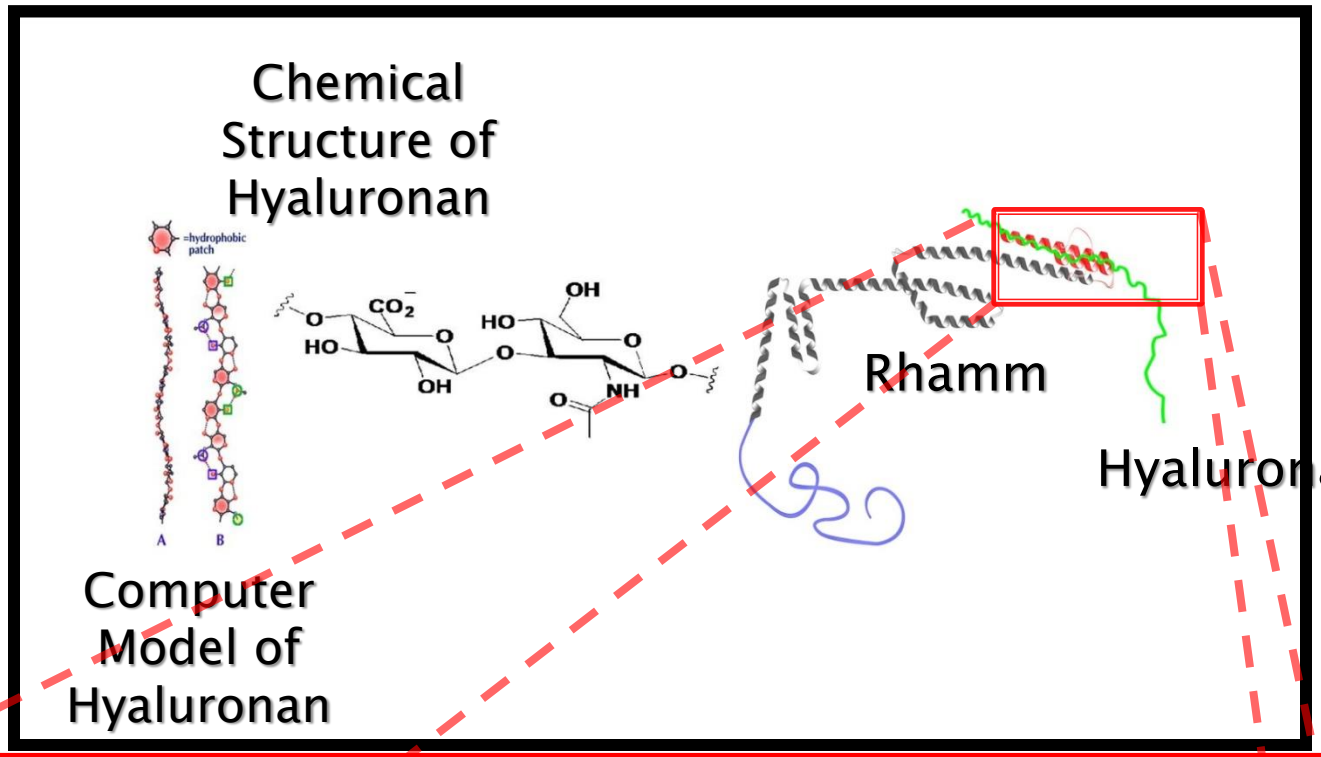
Hyaluronan (HA) fragments promote innate immune responses during tissue repair and are integral to progression of diseases such as cancer. Usually increased accumulation of this polysaccharide within cancer cells is a prognostic factor for poor outcome.

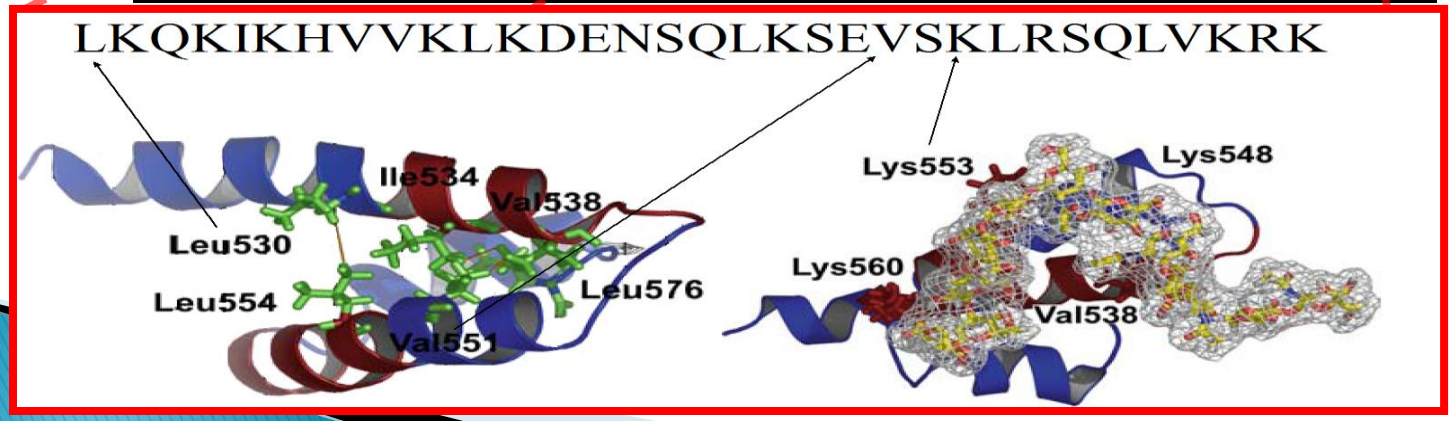
HA acts through different receptors, including Rhamm and CD44, LYVE1, TLR2,4.

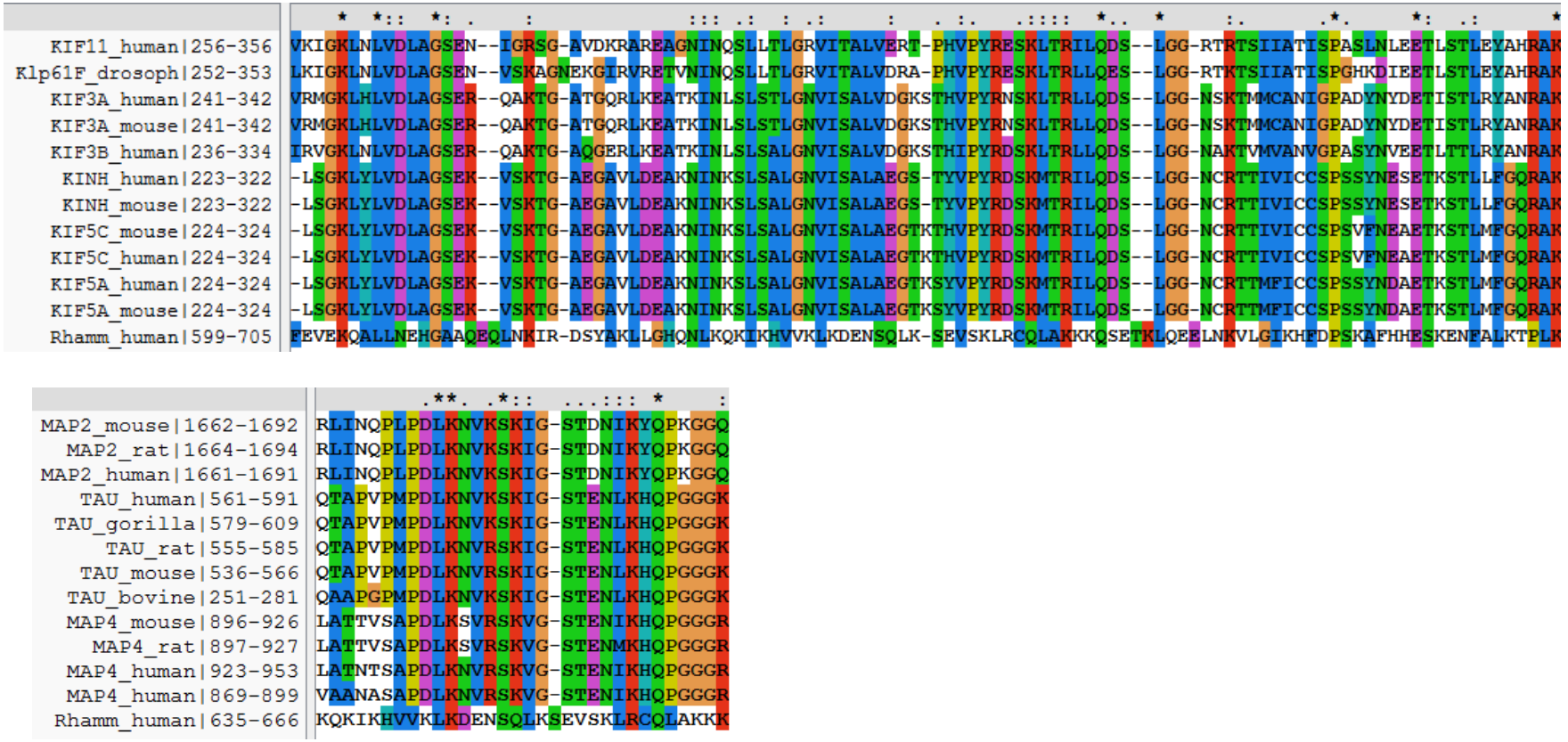
These functions are determined by the size of the HA polymer.
Interactions between these receptors and the smaller HA polymers activate signalling cascades, which contribute to cell migration, cell survival and cell proliferation particularly during repair processes and inflammation.

HA:RHAMM interactions require clusters of positively charged amino acids arranged in a helix.

Rhamm-HA Binding Model

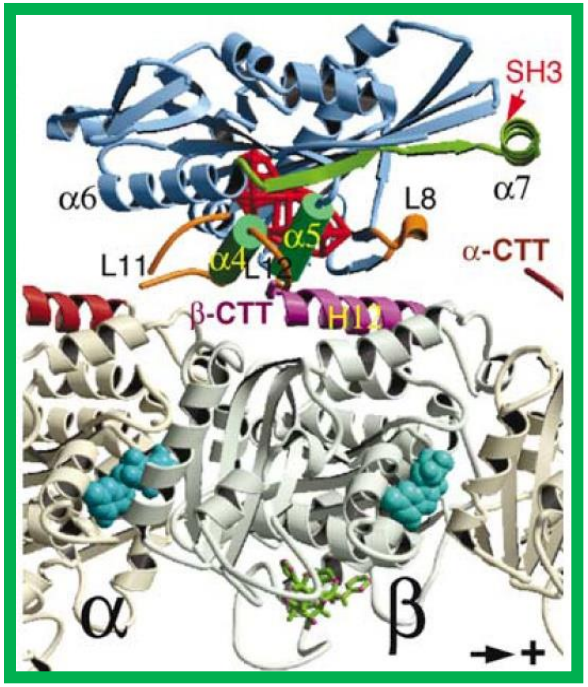




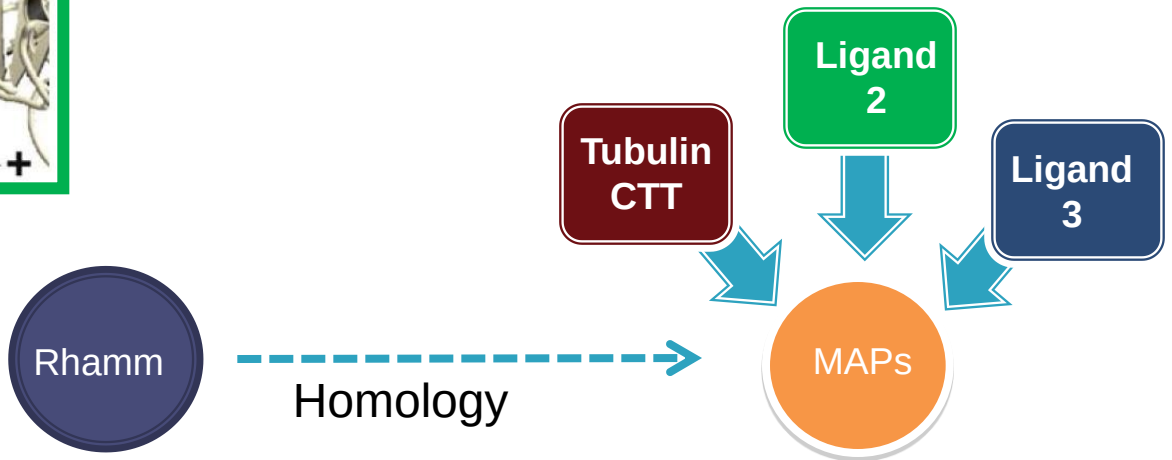


Supplemental Figure 1: Sequence alignment of the HA-binding domain of Rhamm against: (A) motor domain of microtubule motor proteins.(B) tubulin binding domain of microtubule associated proteins (MAPs). Using ClustalX2, each sequence is aligned only to Rhamm. The HA binding domain of Rhamm is boxed in red. Identical, conserved and semi-conserved residues are denoted by (*), (:), and (.), respectively.

Tubulin Binding Domain



- Kinesins and MAPs bind α- and β-tubulin
- Specifically bind to the carboxy terminal tail (CTT) of tubulins
- RHAMM also binds directly to alpha and beta tubulin monomers and polymers



Maxwell *et al*. Mol. Bio. Cell (2003) 14, 2262-2272

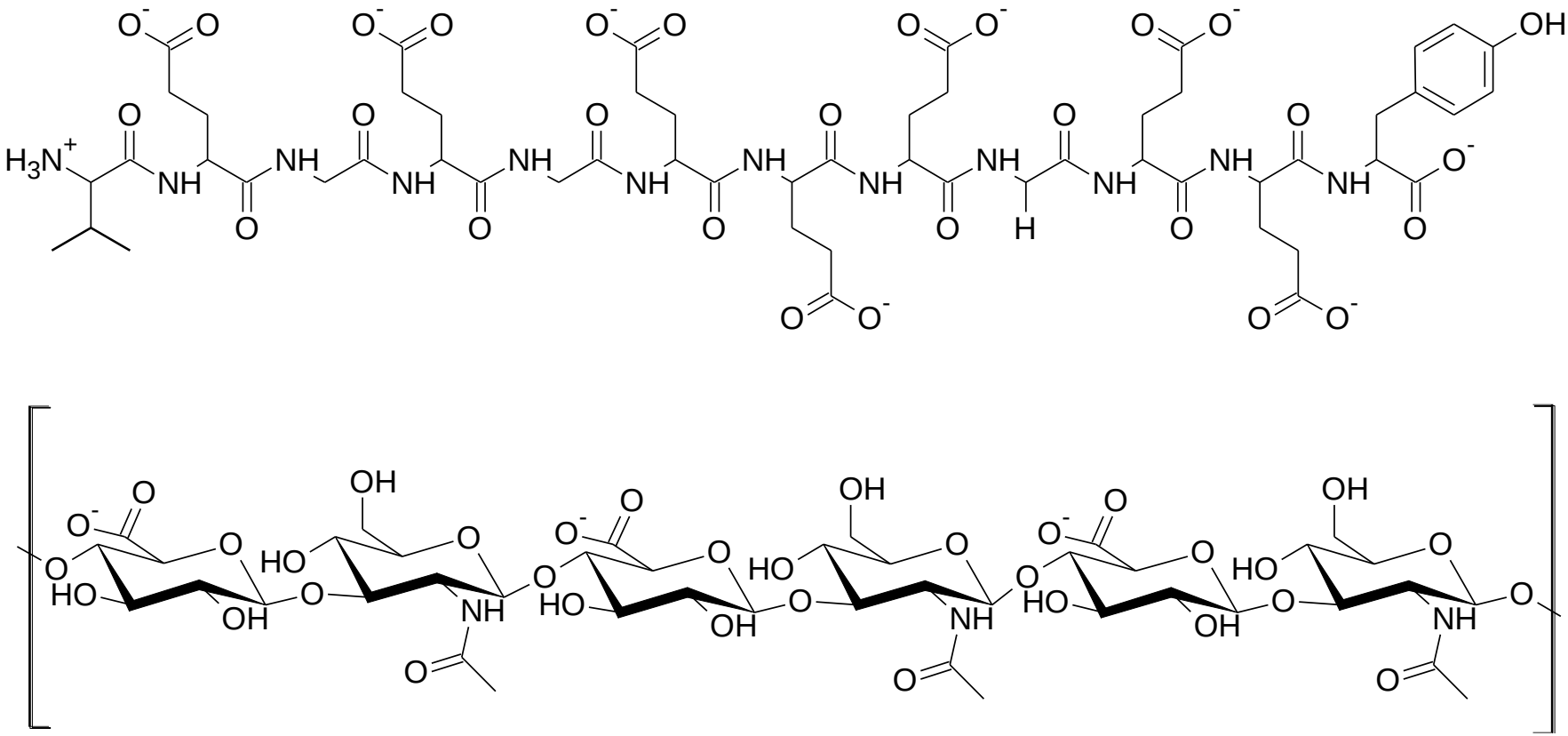
Inclan et al. J. of Cell Sci. (2000) 114, 413-422

Skiniotis et al. EMBO J. (2004) 23, 989-999

Tubulin-binding site of Rhamm:

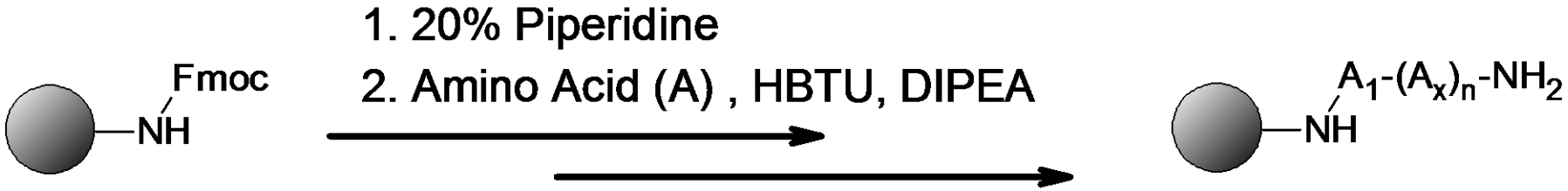
1. Pull-down assay showed that Rhamm binds to α- and β-tubulin subunits*.
2. Rhamm has tubulin binding site, which overlapps with hyaluronan binding site
3. Therefore, it was postulated that synthetic peptides derived from carboxy terminal tails of alpha or beta-tubulin can be used to target Rhamm.

*Tolg C., Hamilton S…. Turley E.A. J. Biol. Chem., 2010, 285 (34):26461-74.



Chemical structure of hyaluronan (bottom) and a putative hyaluronan peptide mimic (top), indicating the carboxylates required for binding to Rhamm.

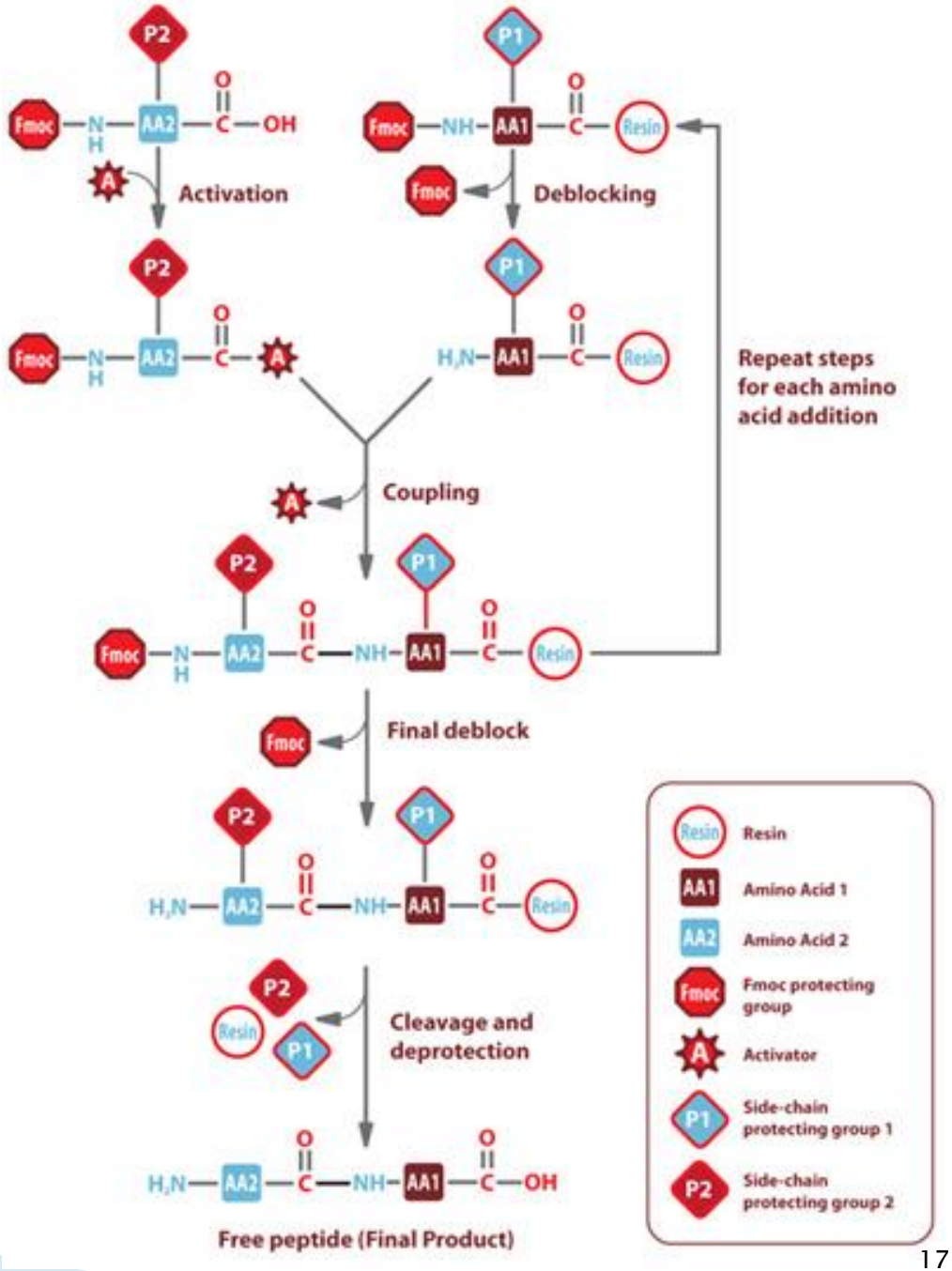
Synthesis of peptides according to Fmoc protocol*



* Ken Esguerra, Lyut's Lab

Peptide Synthesis

- Peptides corresponding to α- and β-tubulin sequences were synthesized, using standard Fmoc protocols.

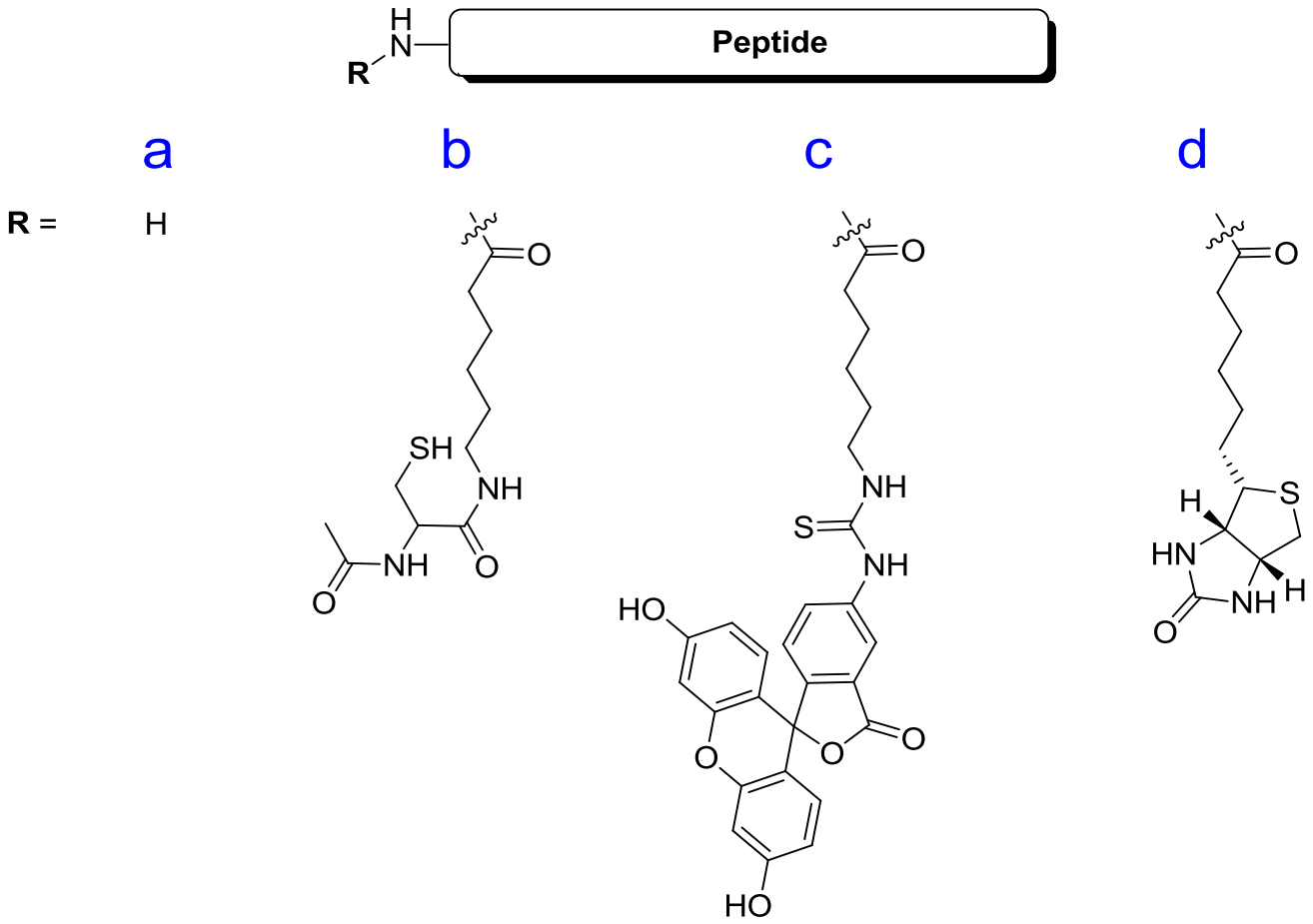


Analysis of synthesized tubulin-derived peptides using ESI-MS and RP HPLC

B

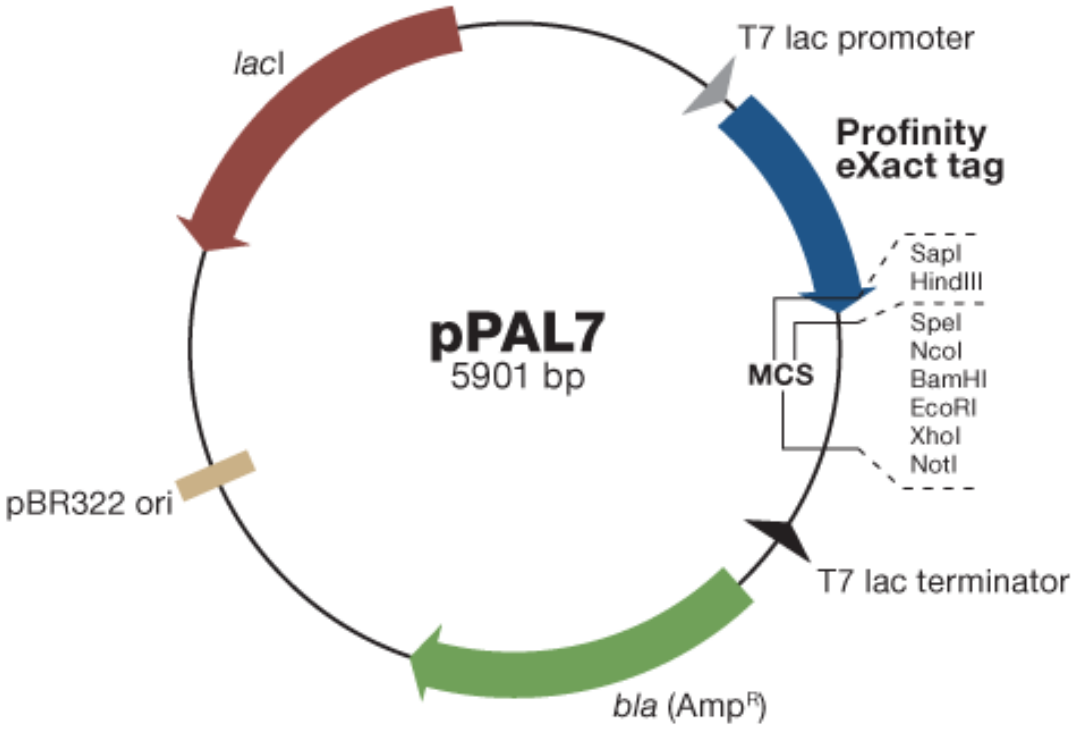
Compound	Sequences	Tubulin Fragment	Type	Calculated M/Z*	Observed M/Z	Purity (%)
1	DSADGEDEGEEY	αIa (438-449)	CTT	658.2$[M+2H]^{2+}$	657.7 $[M+2H]^{2+}$	98
2	VEGEGEEEGEEY	αIa (439-451)	CTT	677.7 $[M+2H]^{2+}$	677.6 $[M+2H]^{2+}$	98
3	SVEAEAEEGEEY	αIIIc (439-450)	CTT	671.3 $[M+2H]^{2+}$	670.7 $[M+2H]^{2+}$	97
4	IDSYEDEDEGEE	αIVa (437-448)	CTT	715.2$[M+2H]^{2+}$	714.7 $[M+2H]^{2+}$	99
5	DSFEEENEGEEF	αVIII (438-449)	CTT	730.8$[M+2H]^{2+}$	730.3 $[M+2H]^{2+}$	97
6	LEKDYEEVGVDS	αIa (427-439)	H12	691.8$[M+2H]^{2+}$	691.3 $[M+2H]^{2+}$	99
7	GEFSEAREDMAA	αIa (416-426)	H12	653.3$[M+2H]^{2+}$	656.3 $[M+2H]^{2+}$	98
8	FVHWYVGEGMEE	αIa (404-415)	H12	741.9$[M+2H]^{2+}$	741.3 $[M+2H]^{2+}$	99
9	GEFEEEEGEDEA	βIIa (433-445)	CTT	685.2$[M+2H]^{2+}$	684.7 $[M+2H]^{2+}$	98
10	EEDFGEEAEEEA	βIa (433-444)	CTT	691.8 $[M+2H]^{2+}$	691.9 $[M+2H]^{2+}$	99
11	GEFEEEAEEEVA	βIV (433-444)	CTT	684.3 $[M+2H]^{2+}$	683.8 $[M+2H]^{2+}$	97
12	EAFEDEEEEIDG	βVI (407-418)	CTT	706.3 $[M+2H]^{2+}$	705.8 $[M+2H]^{2+}$	99
13	SNMNDLVSEYQQ	βIIIa (413-424)	H12	714.4 $[M+2H]^{2+}$	713.8 $[M+2H]^{2+}$	99
15	FTEAESNMNDLV	βIIIa (408-419)	H12	685.2 $[M+2H]^{2+}$	684.8$[M+2H]^{2+}$	99
16	RPDYISWGTQEQ	γI (440-439)	CTT	740.4 $[M+2H]^{2+}$	740.4 $[M+2H]^{2+}$	98
17	VQQLIDEYHAAT	γI (428-439)	H12	693.2 $[M+2H]^{2+}$	693.8 $[M+2H]^{2+}$	95

General structure of modified tubulin-derived peptides used for evaluation.



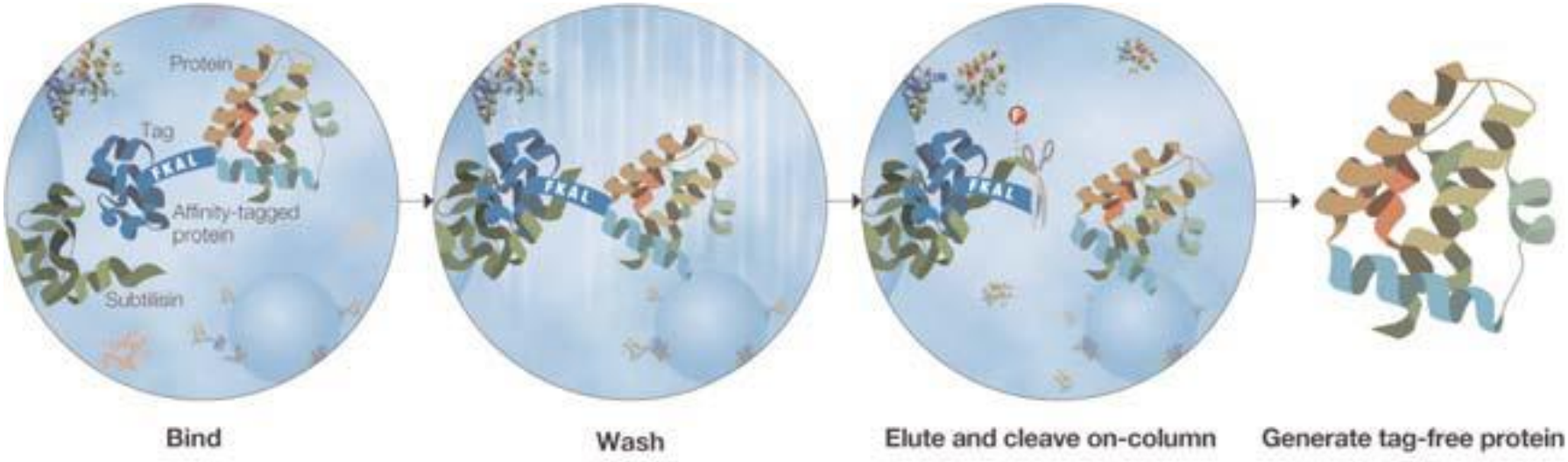
The figure shows the general structure of tubulin-derived peptides conjugated to N-acyl cysteine (b), FITC (c), and biotin (d).

Expression of Rhamm-CT (aa. 706-767, M.w. 7.2 kDa, pI = 10.1)



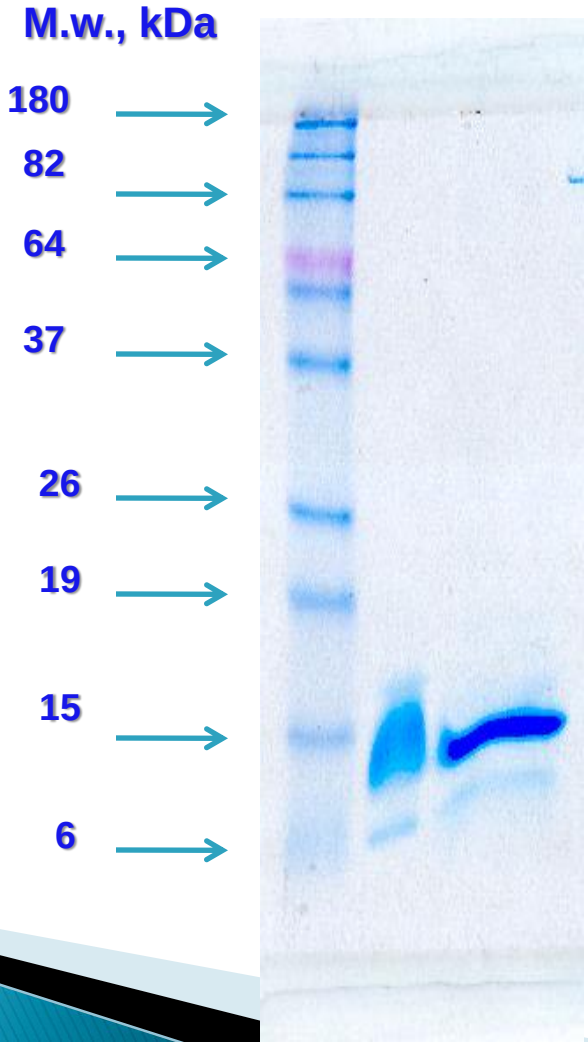
Rhamm-CT was cloned into the pPAL7 vector and expressed in *E.coli* as a fusion protein.

Purification of recombinant protein Rhamm-CT, using the Profinity eXact Fusion-Tag System (BioRad)



Recombinant protein Rhamm-CT (aa. 706-767, M.W. 7.2 kDa, pI = 10.1) purified from *E. coli*.

Coomassie stained
10 % SDS-PAGE

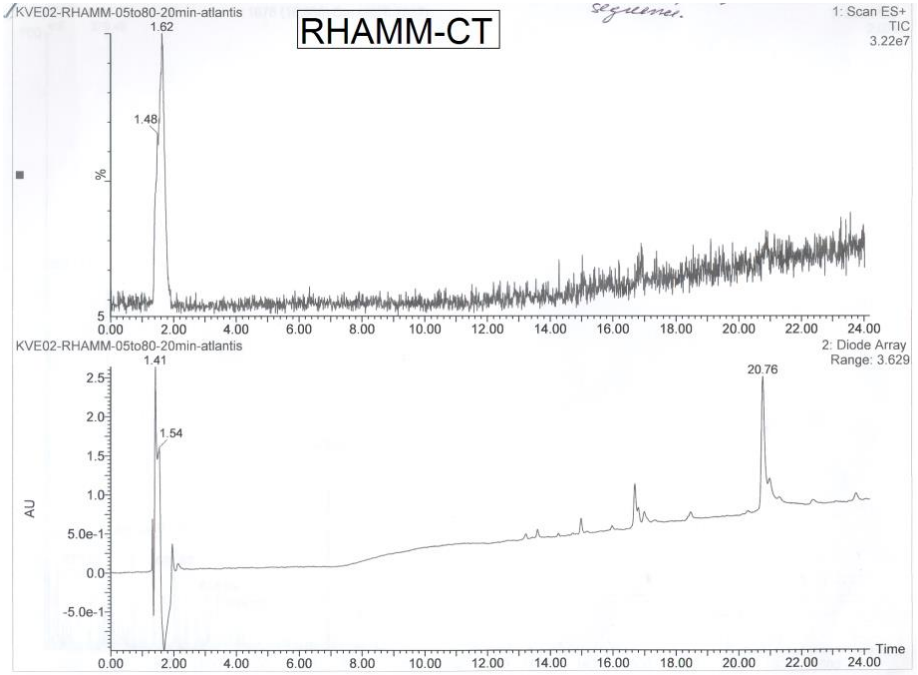


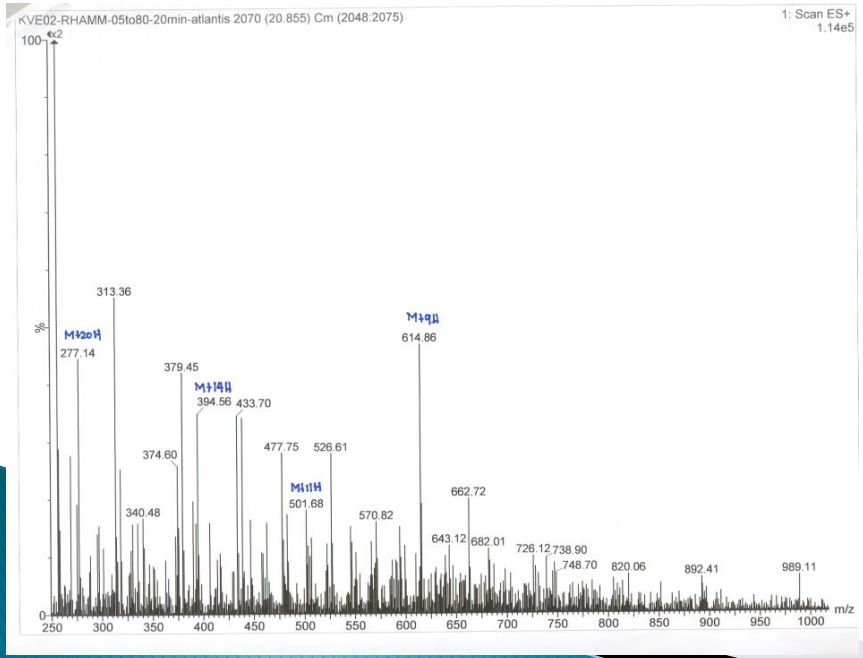
Rhamm

Western blot

Mass spec analysis confirmed the sequence of RHAMM-CT

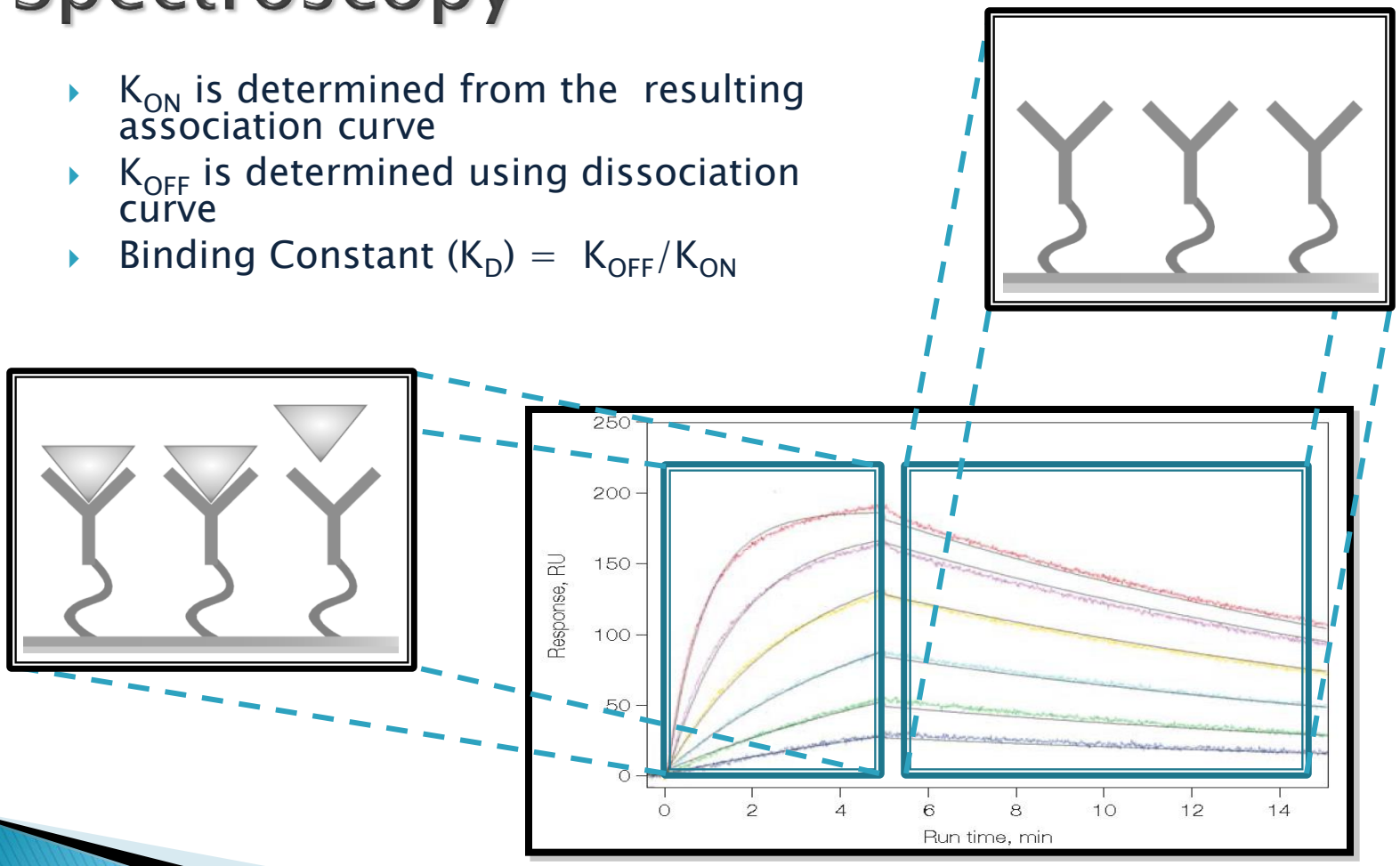
Mass spec analysis RHAMM-CT confirmed the sequence of RHAMM-CT



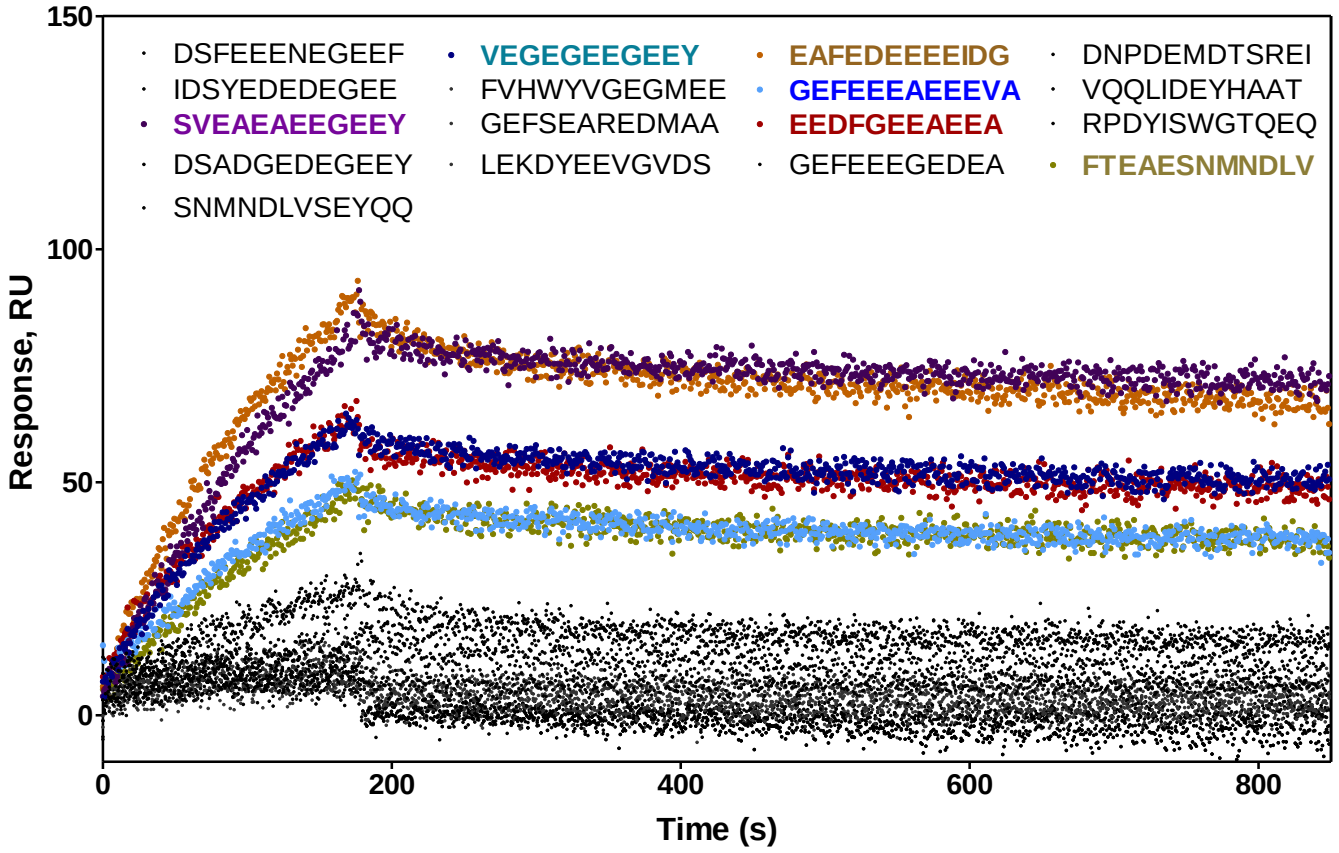


Surface Plasmon Resonance (SPR) Spectroscopy

- K_{ON} is determined from the resulting association curve
- K_{OFF} is determined using dissociation curve
- Binding Constant (K_D) = K_{OFF}/K_{ON}



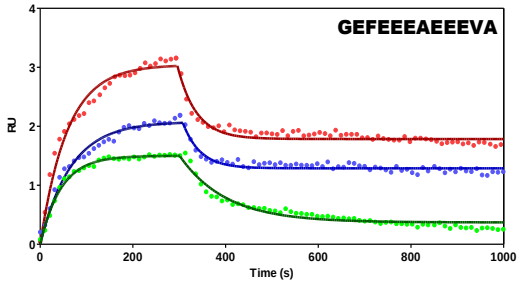
Screening of Tubulin-Derived Peptides against Rhamm via Surface Plasmon Resonance Spectroscopy

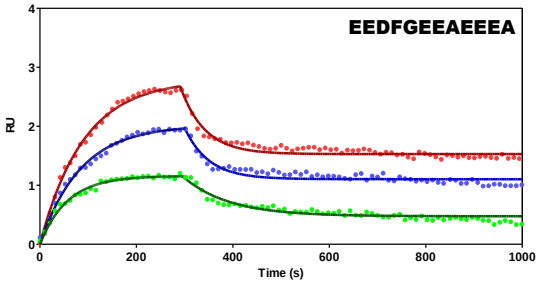


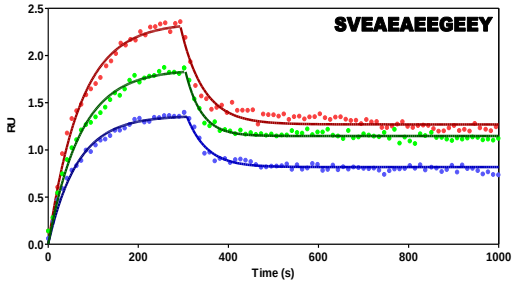
Screening generated 6 peptides (coloured traces) which show high affinity to Rhamm. Black traces represent low affinity peptides.

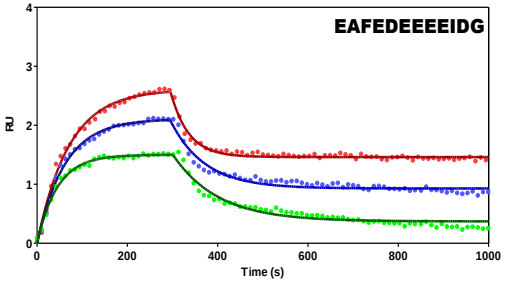
ProteON sensor Chip Surface Chemistry (Bio-RAD).

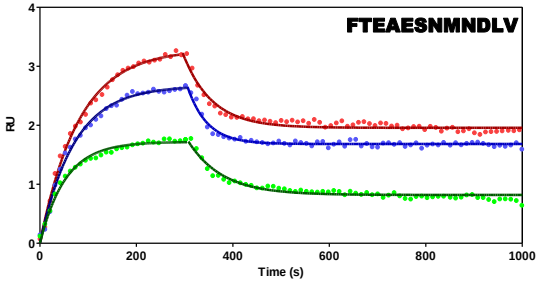
Seven sets of sensograms showing global fits to each specific peptide-Rhamm interaction.

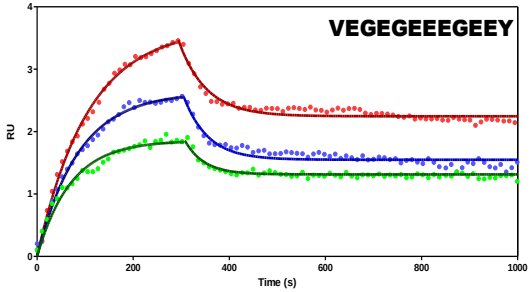


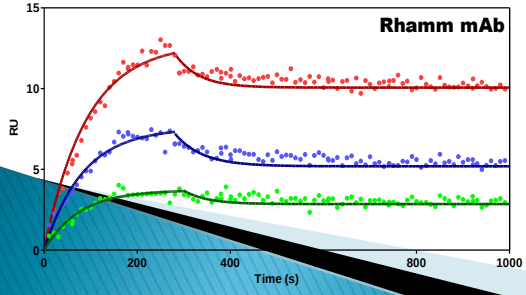


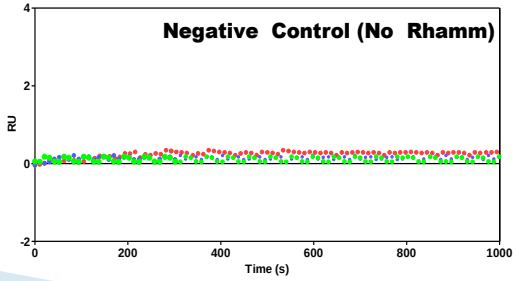


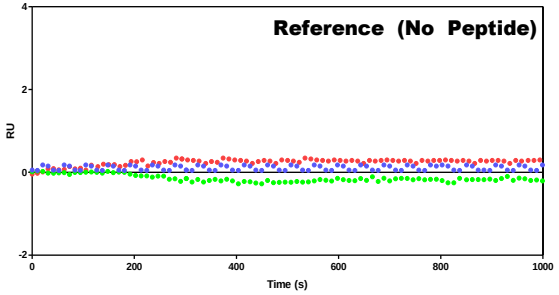










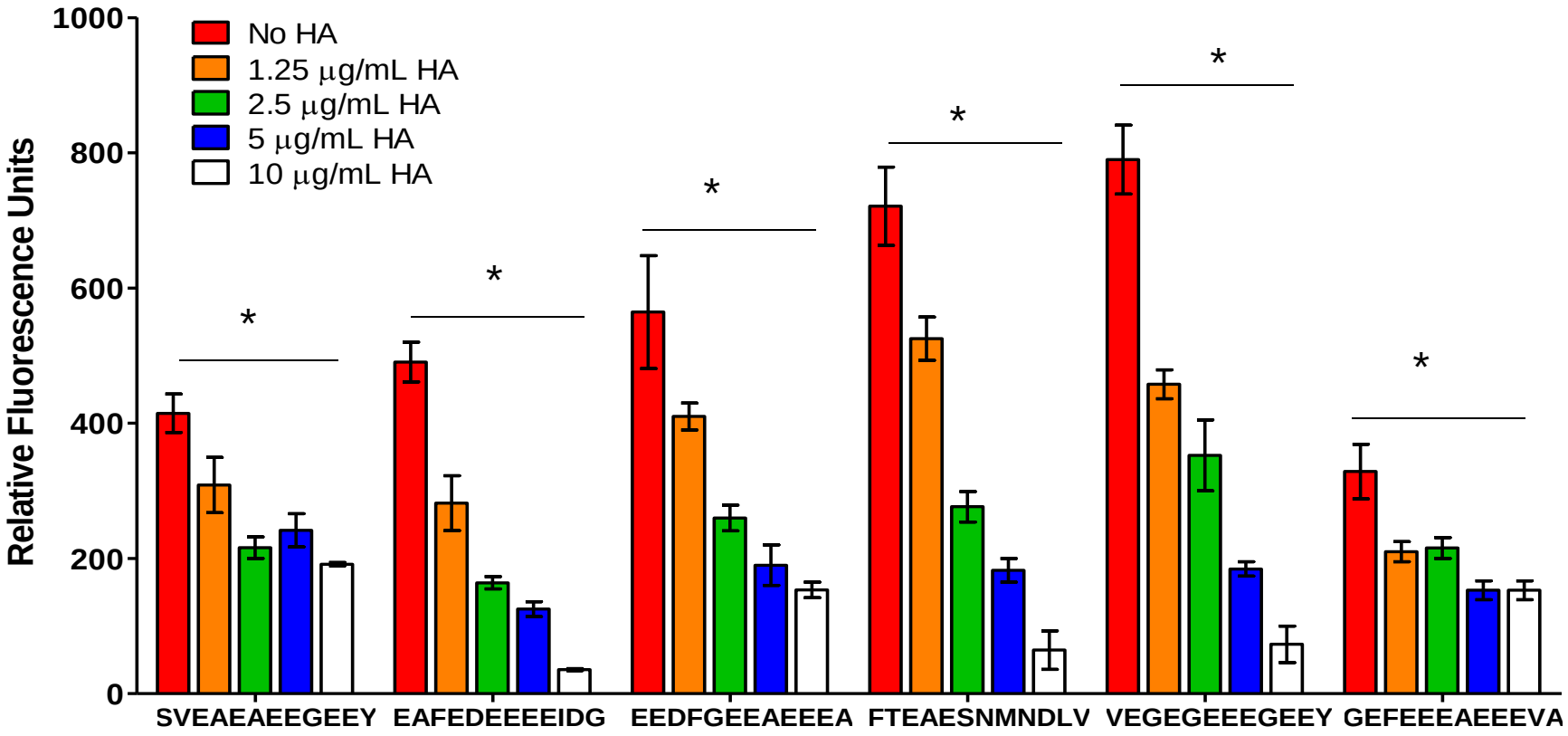


1000 nM 750 nM 500 nM

Calculated K_D of tubulin-derived peptides against RHAMM

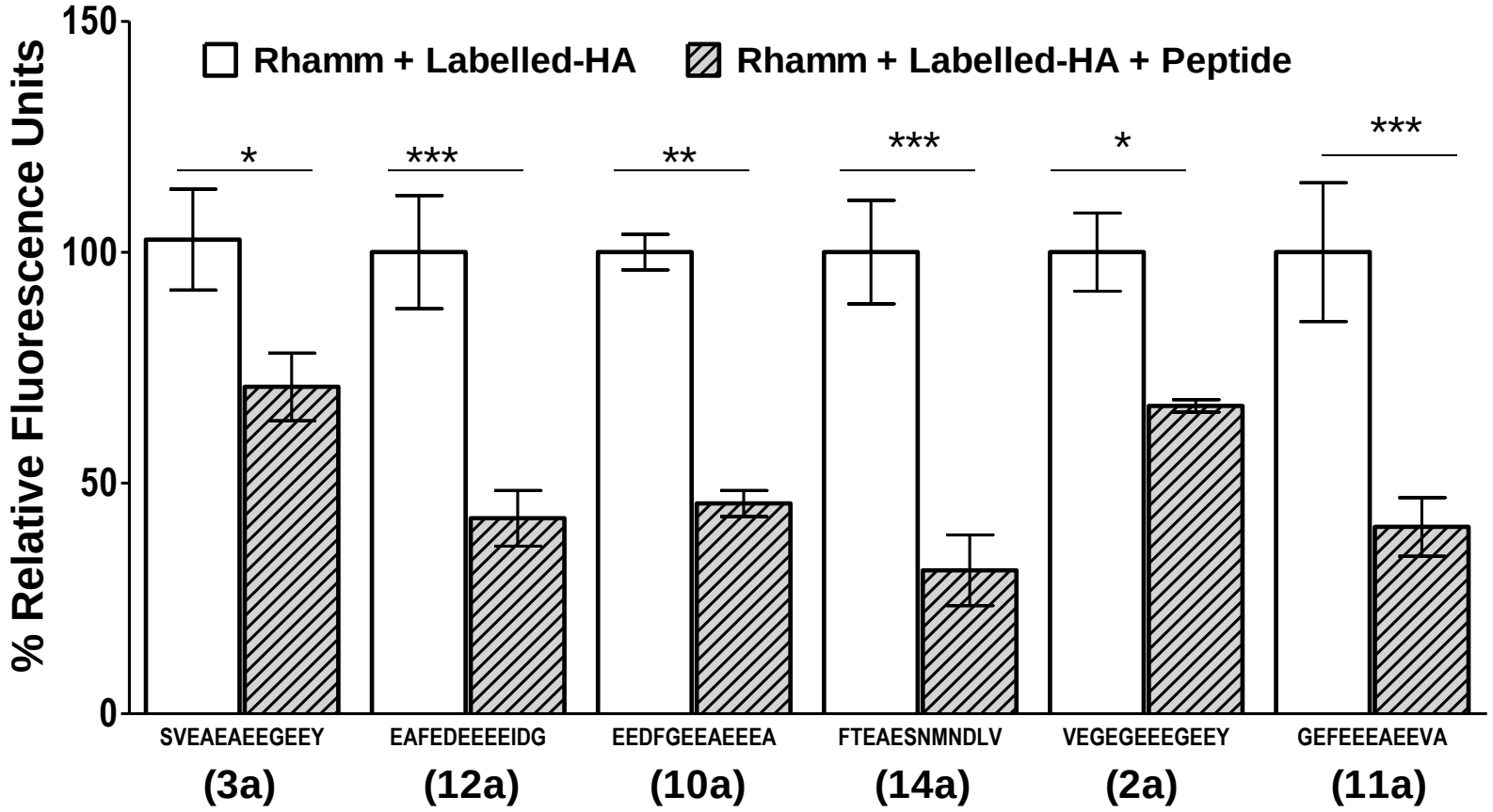
Sequence	SVEAEAE EGEEY	GEFEEEAE EEVA	VEGEGEEE GEEY	EEDFGEEA EEEA	EAFEDEEEEI DG	FTEAESNMN DLV
Ave. K_D (nM)	331.1 ± 24.5	130 ± 12.9	24.2 ± 0.4	32.6 ± 1.1	211.3 ± 8.6	30.2 ± 1.5

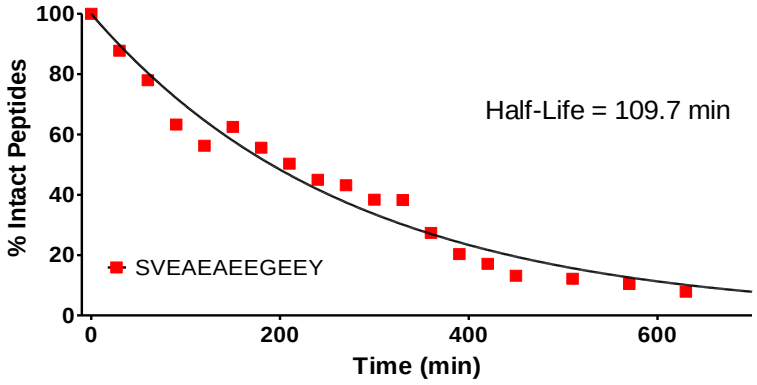
Specific binding of Tubulin-derived peptides to RHAMM

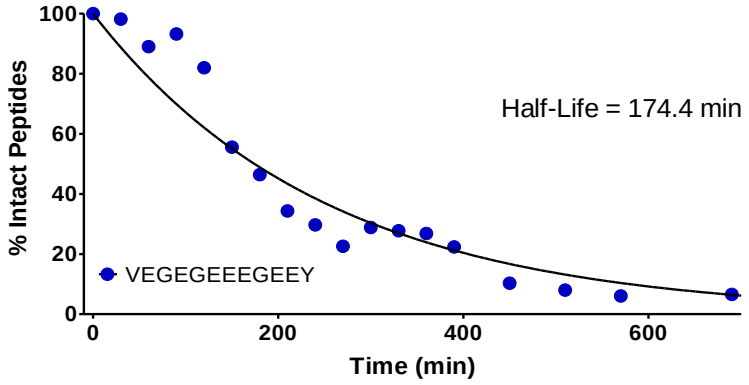


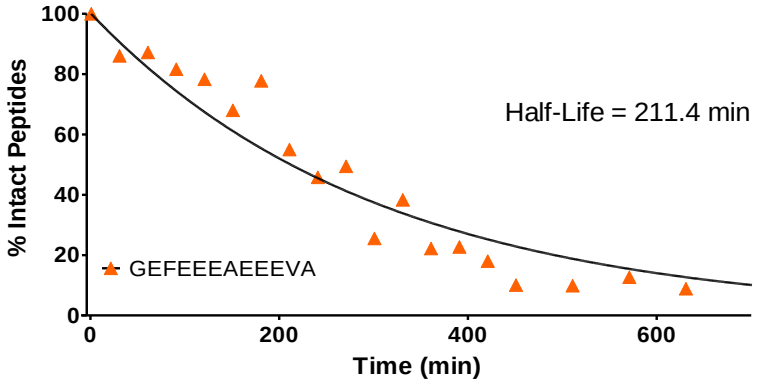
Competitive ELISA displacement assay of fluorescein-conjugated “Hit” peptide to immobilized Rhamm.

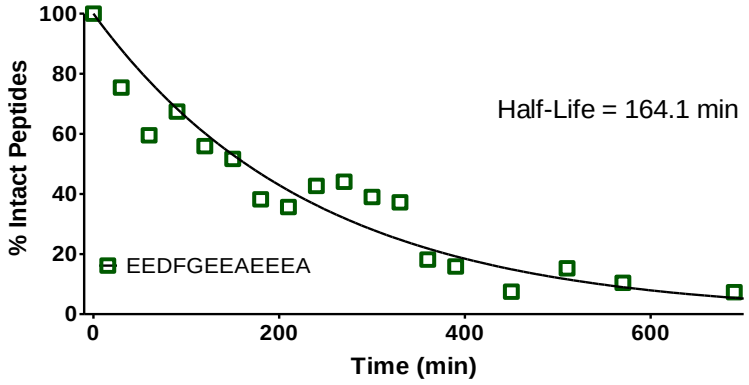
Competitive displacement of dye-labelled HA by non-labelled tubulin-derived peptides, using ELISA assay.

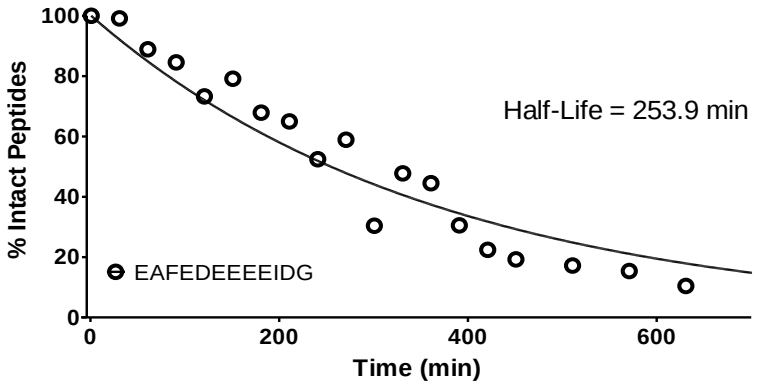


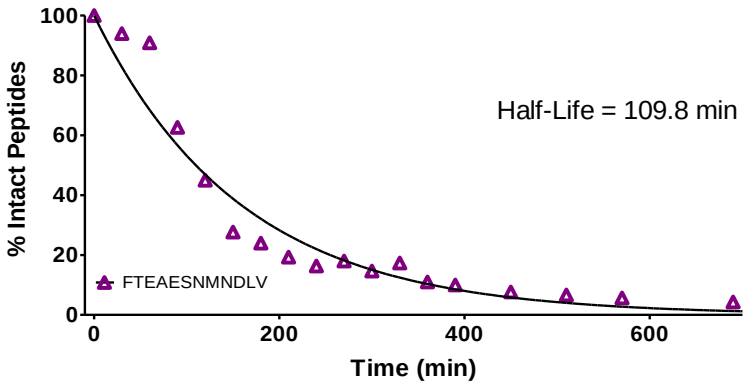








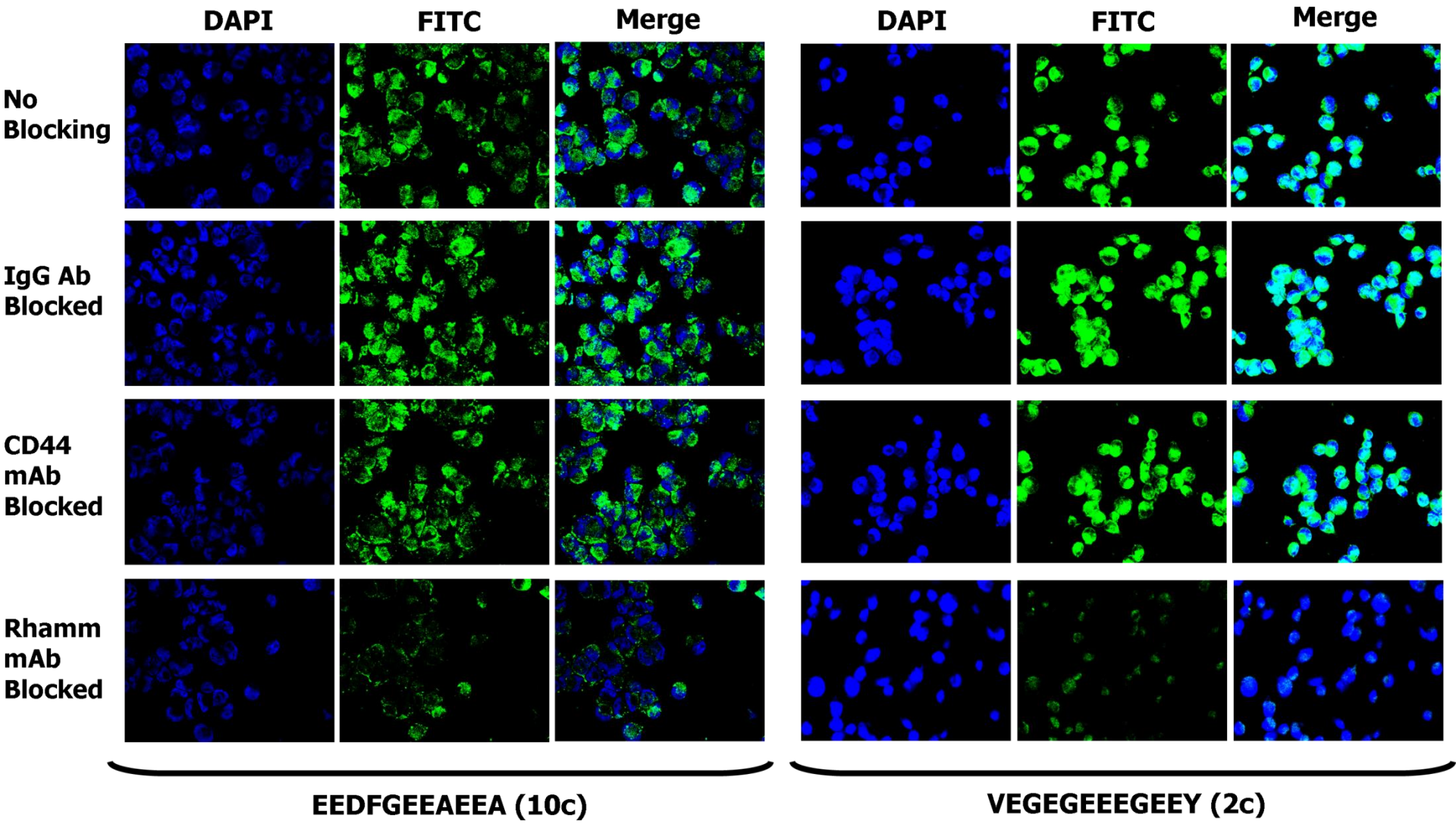




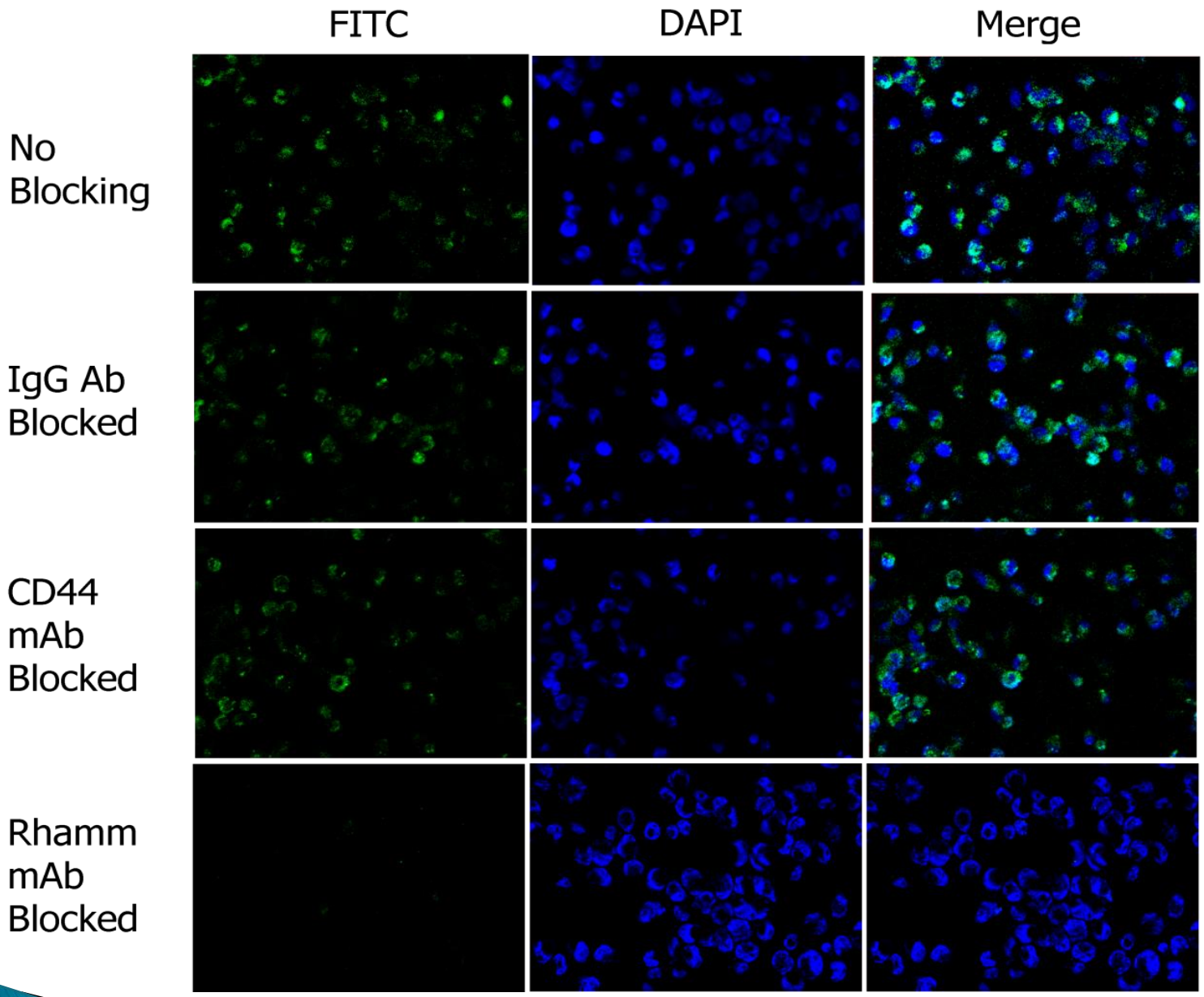
Serum stability study of six tubulin-derived peptides under physiological conditions in fetal bovine serum.

Cellular uptake has been studied, using 3 peptides:

1. **EEDFGEEAEEEA, peptide #35**
2. **VEGEGEEEGEEY, peptide#37**
3. **FTEAESNMNDLV, peptide #40**

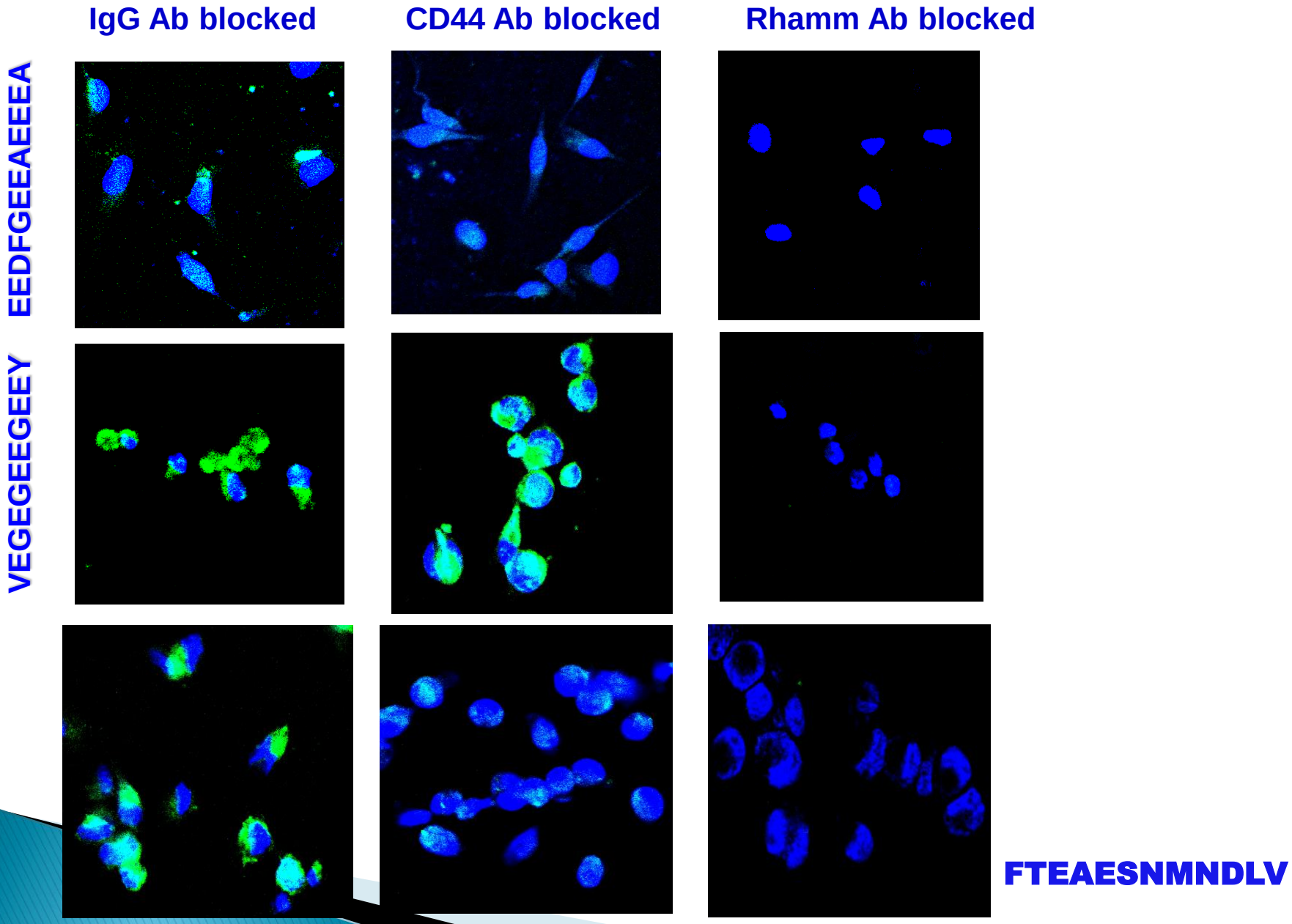


Visualization of uptake of fluorescein-conjugated peptides in breast tumor cells using fluorescence microscopy.

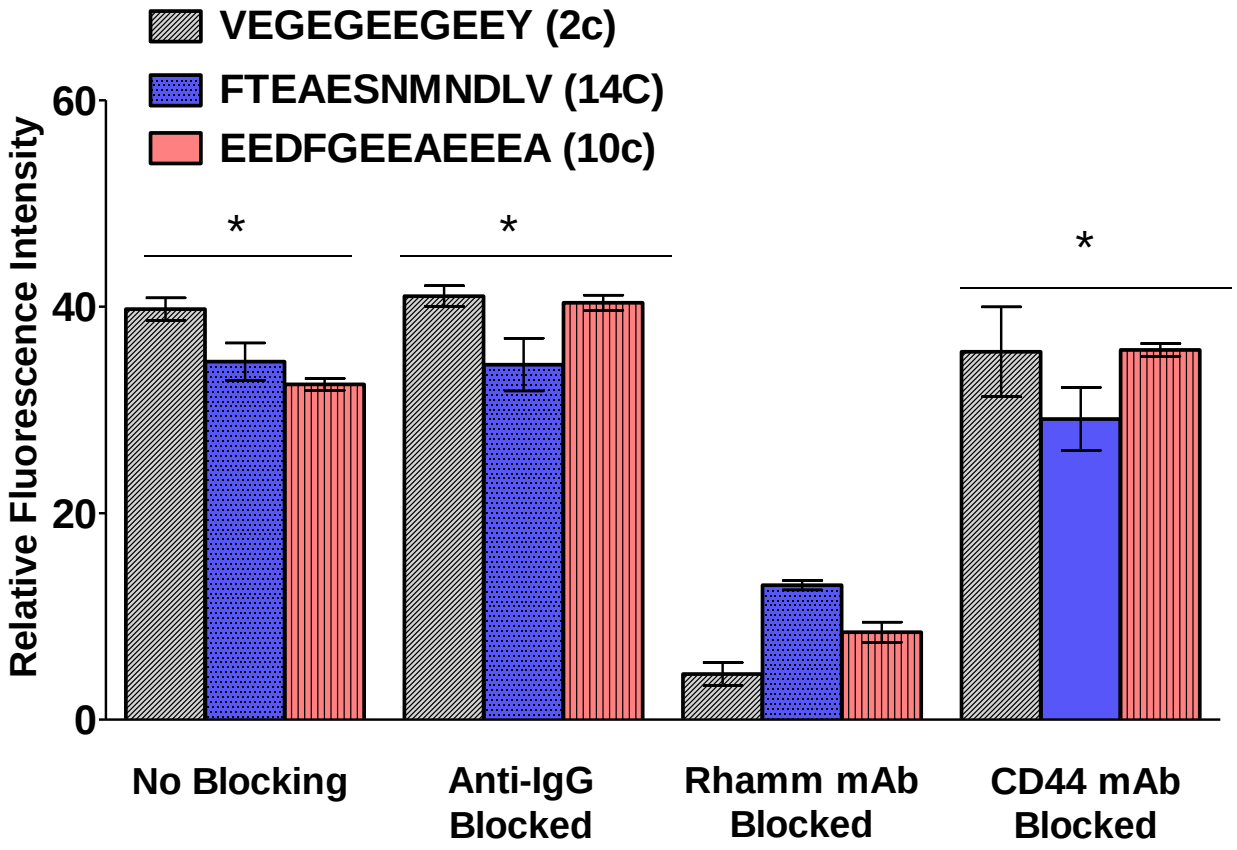


Visualization of uptake of fluorescein-conjugated FTEAESNMNDLV in breast tumor cells using two-channel fluorescence microscopy.

Cellular uptake of HA-mimetic Peptides to Rhamm-Expressing Breast cancer cells (MDA-MB-231).



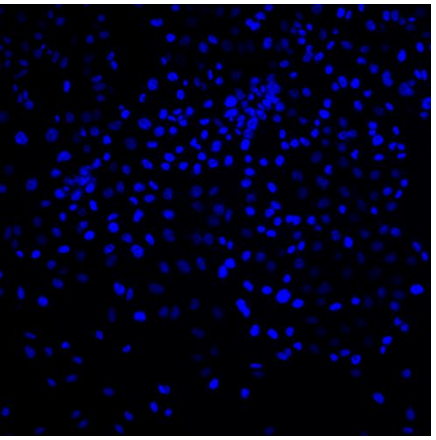
Quantification of uptake FITC-peptides in MDA-231-MB cells.



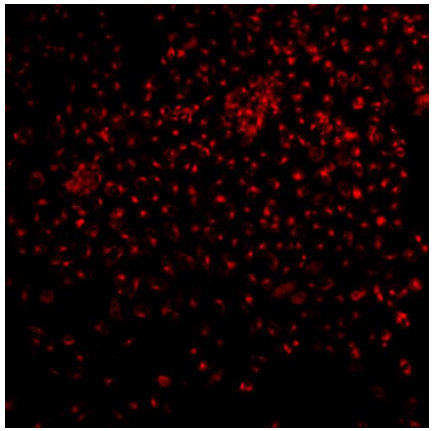
Using ImageJ software, ROI corresponding to the cancer cell bodies (1048, n = 3) were selected. Mean fluorescence of each ROI was obtained using 8-bit images and represented as a bar graph. Data were analyzed using one-way ANOVA.

HACy5.5 uptake in Prostate cancer cells (PC3M-LN4).

DAPI

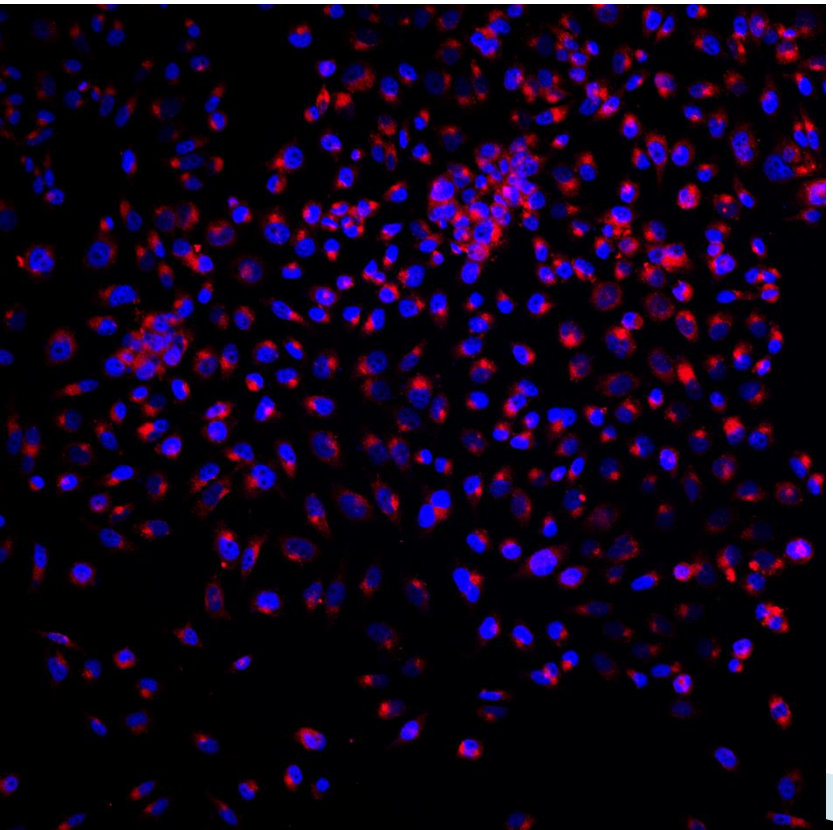


Cy5.5.

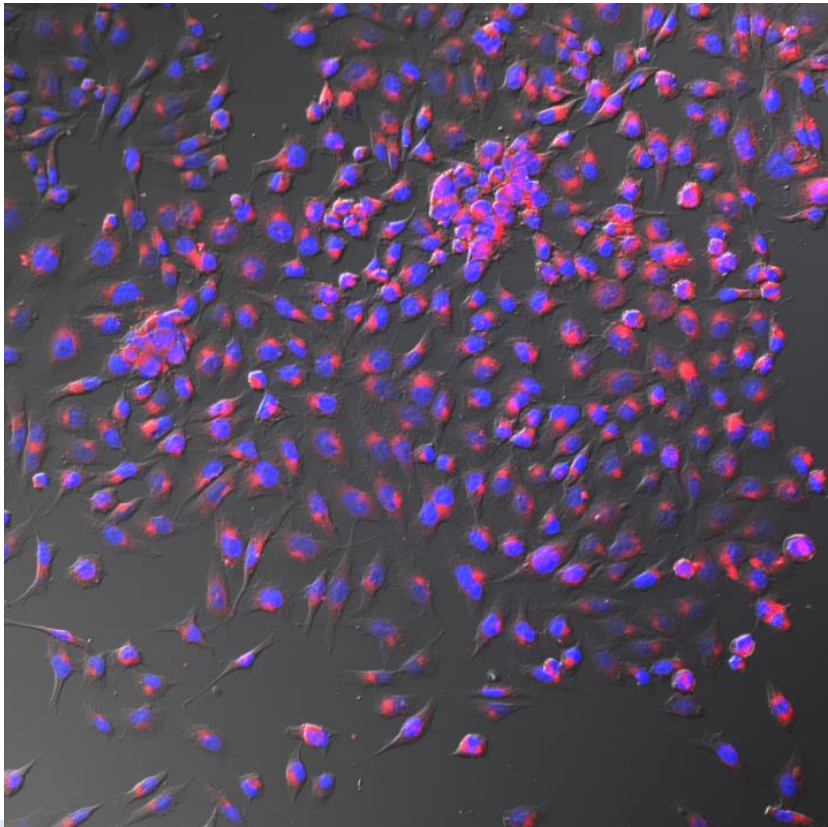


Magnification x20

MERGE



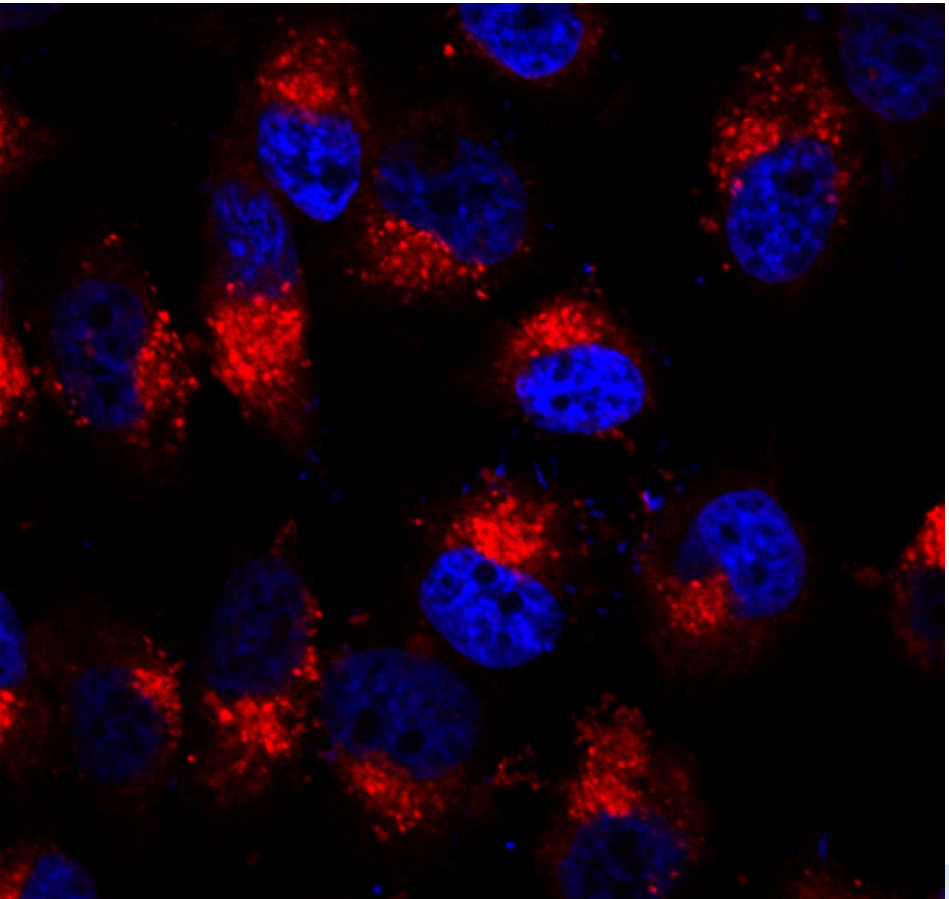
Merge with TD1 channel



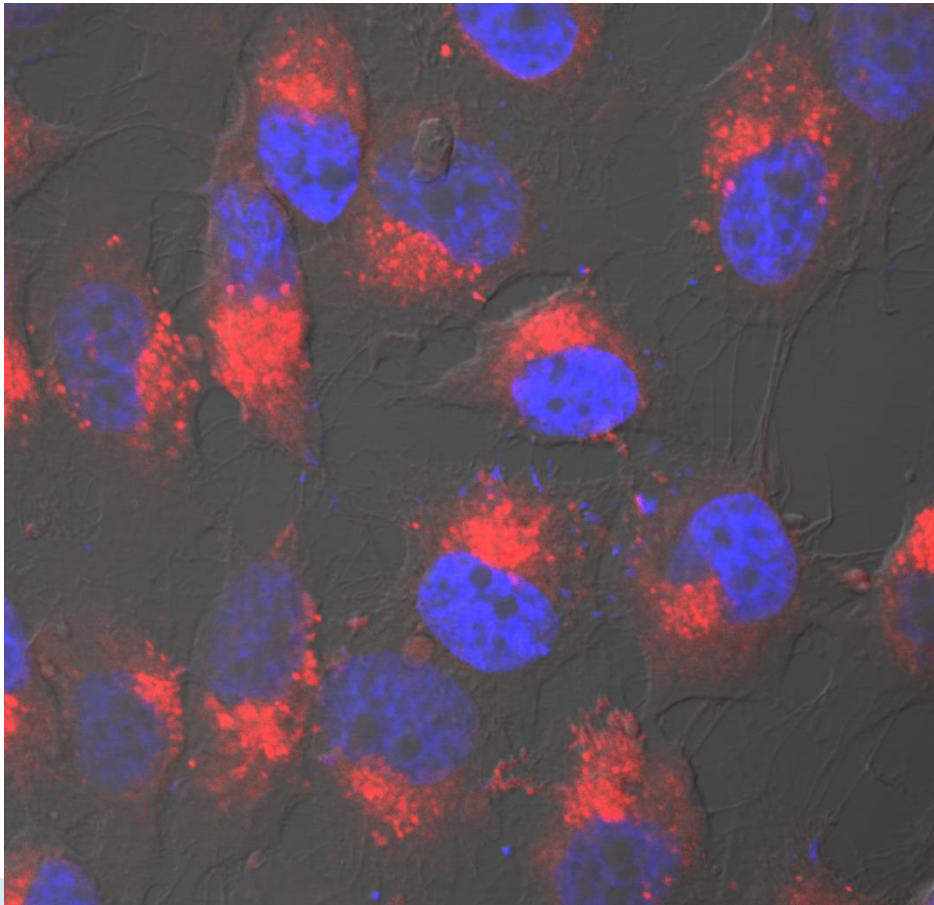
HACy5.5 uptake in Prostate cancer cells (PC3M-LN4).

Magnification x60

MERGE

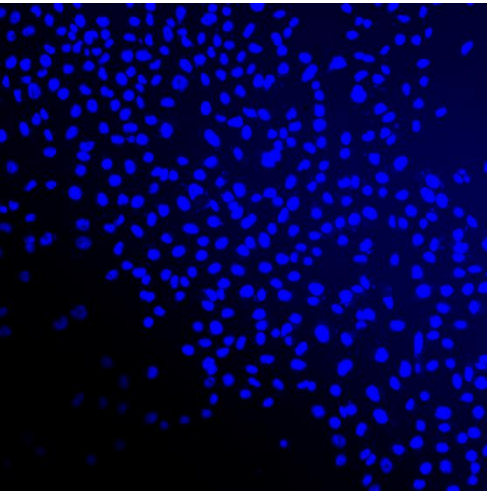


MERGE with TD1 channel

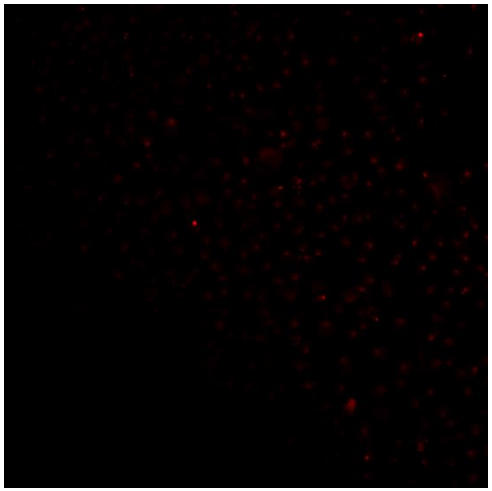


Blocking HACy5.5 uptake with Rhamm Ab in Prostate cancer cells.

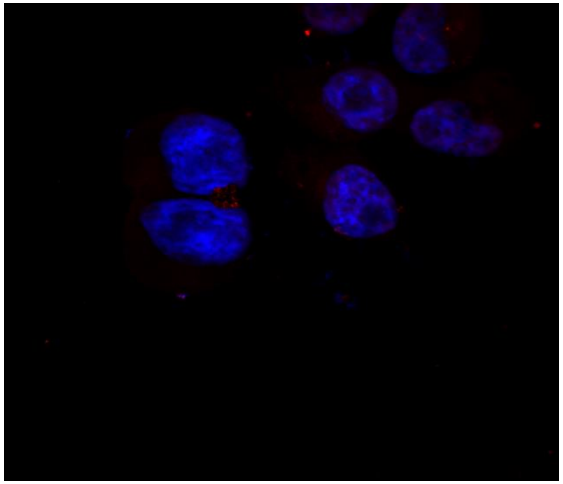
DAPI



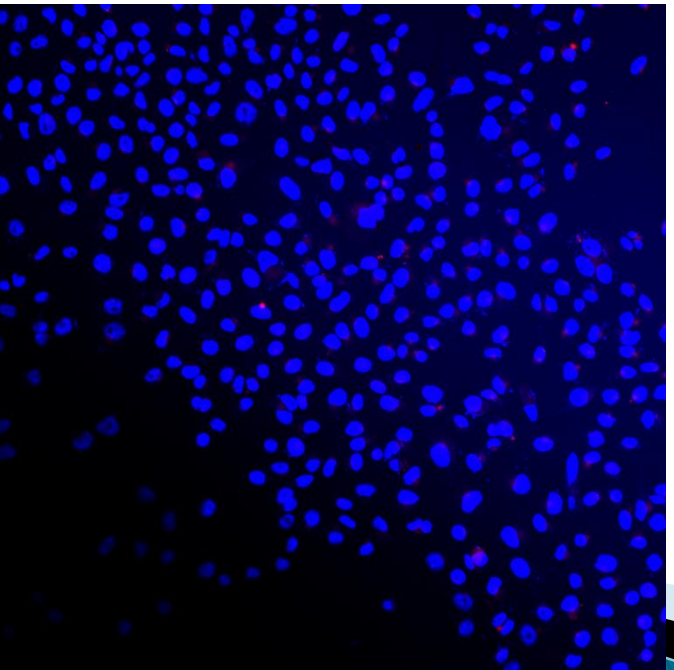
Cy5.5



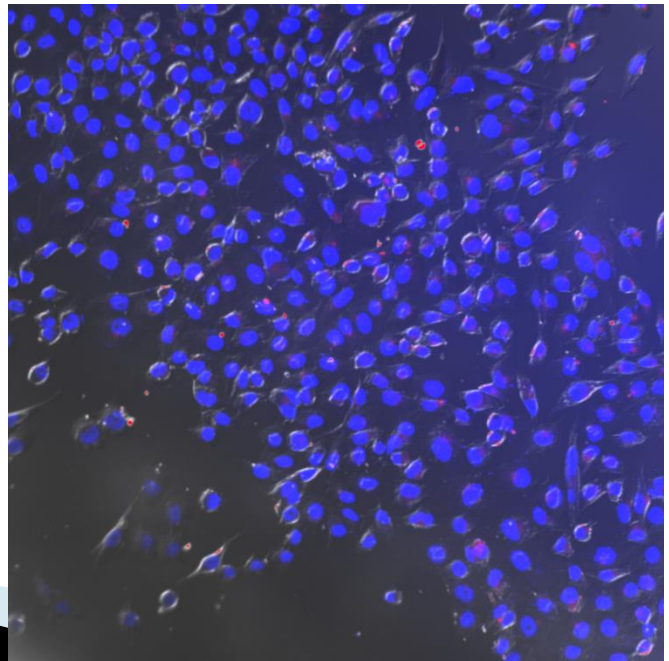
Magnification x60



MERGE

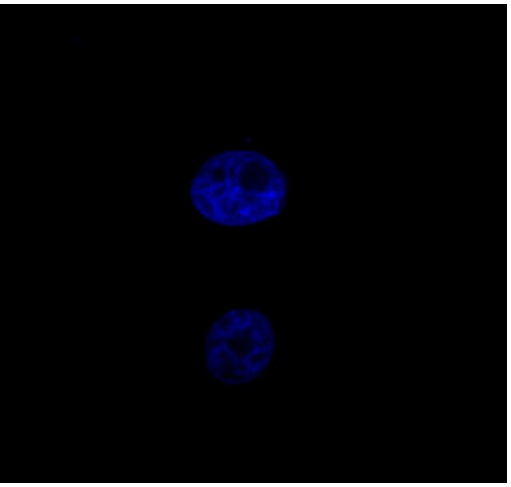


MERGE with TD1 channel

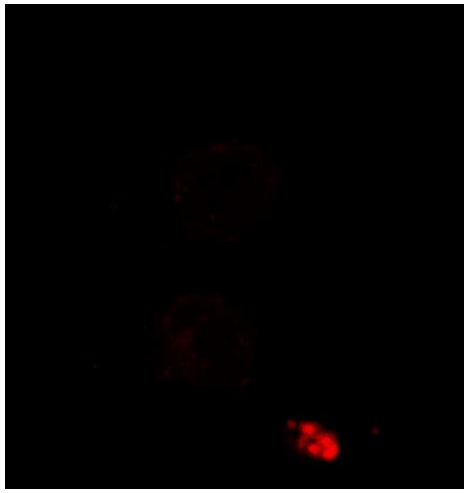


Blocking HACy5.5 uptake with non-labeled HA in Prostate cancer cells.

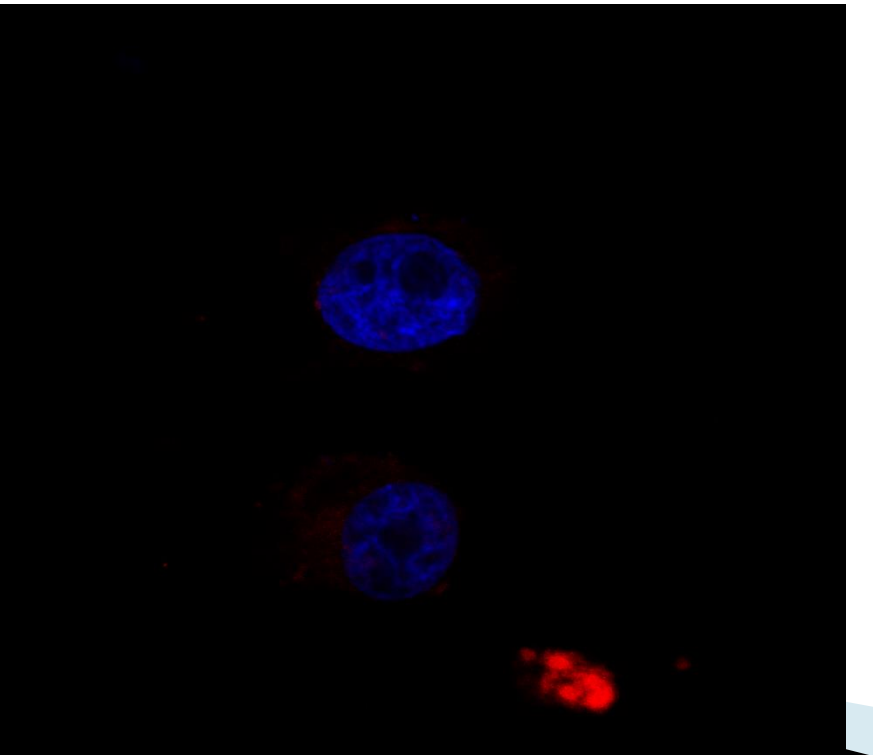
DAPI



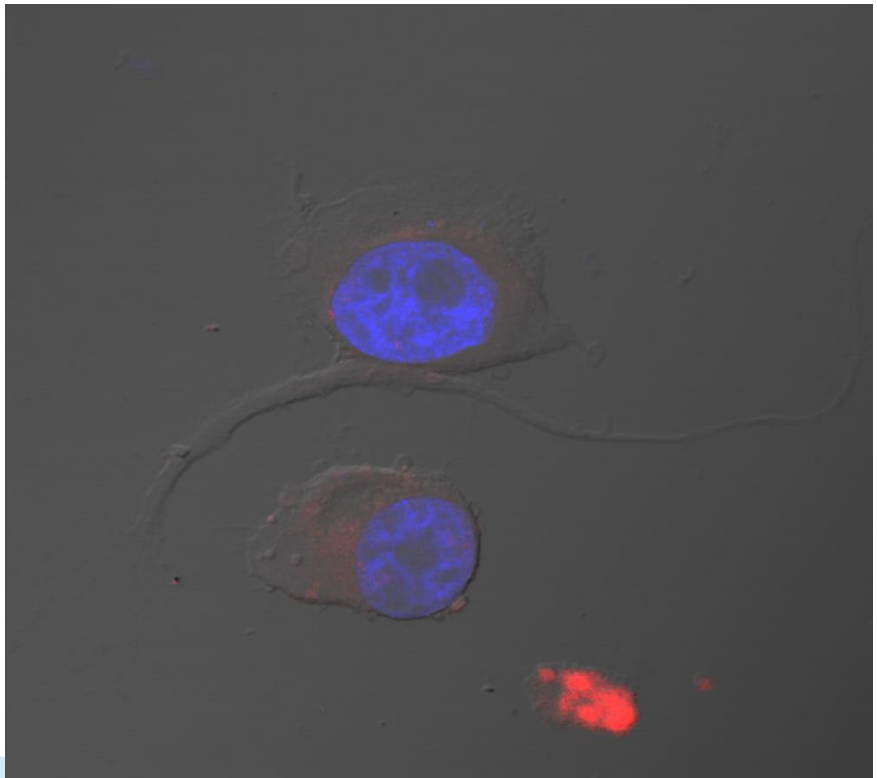
Cy5.5



MERGE

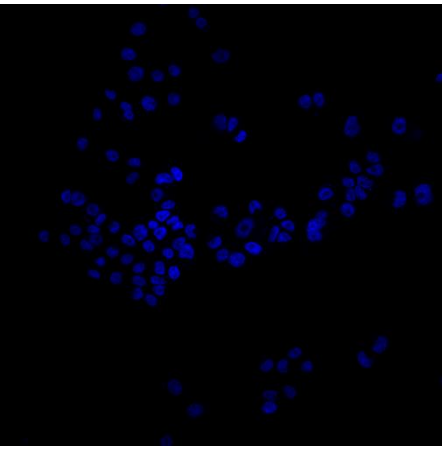


MERGE with TD1 channel

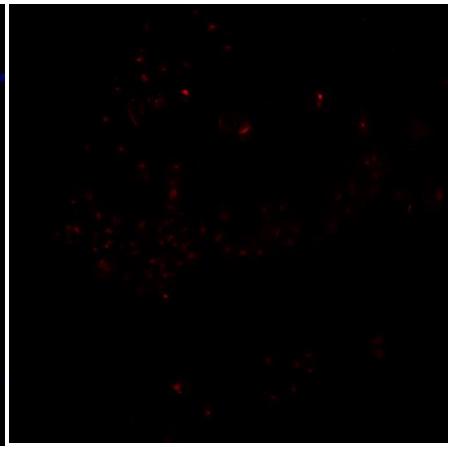


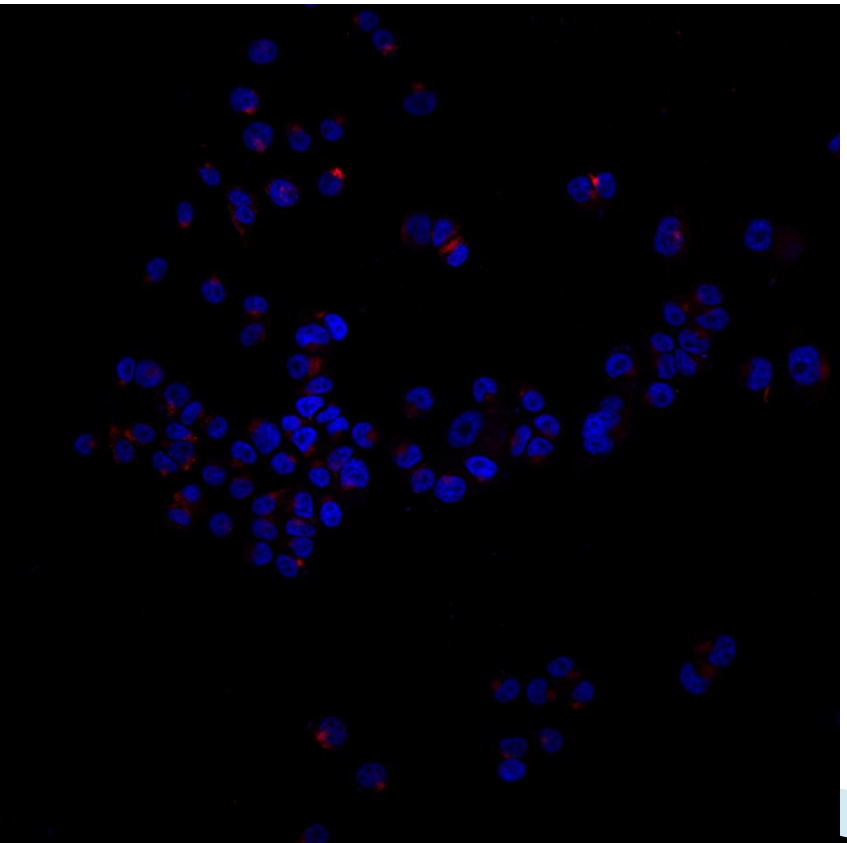
Blocking HACy5.5 uptake with Peptide VEGEGEEEGEEY in Prostate cancer cells.

DAPI



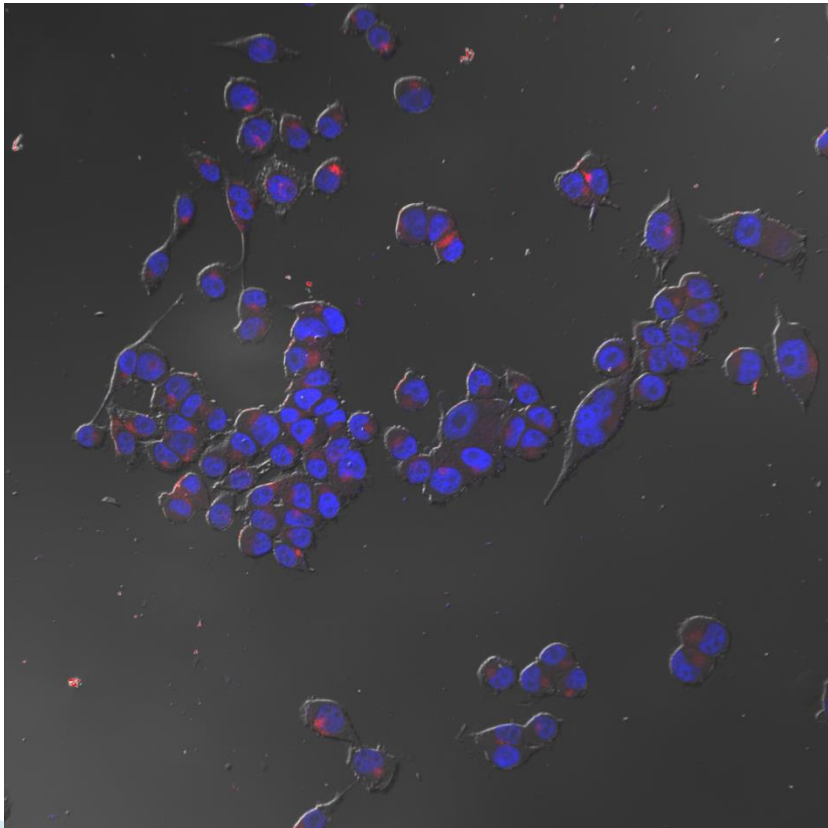
Cy5.5.





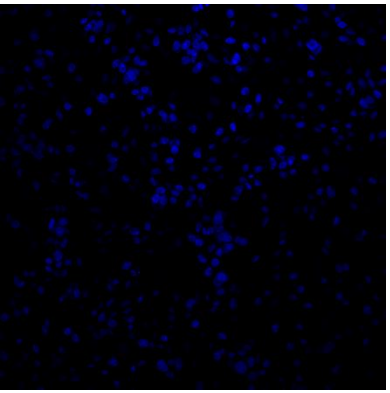
MERGE

MERGE with TD1 channel

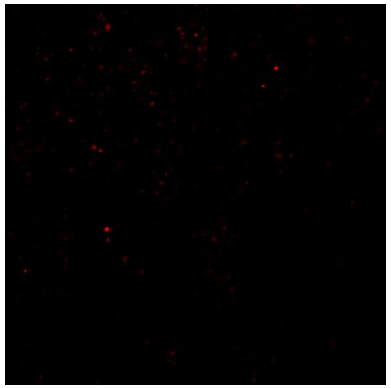


Blocking HACy5.5 uptake with Peptide EEDFGEEAEEEA in Prostate cancer cells.

DAPI

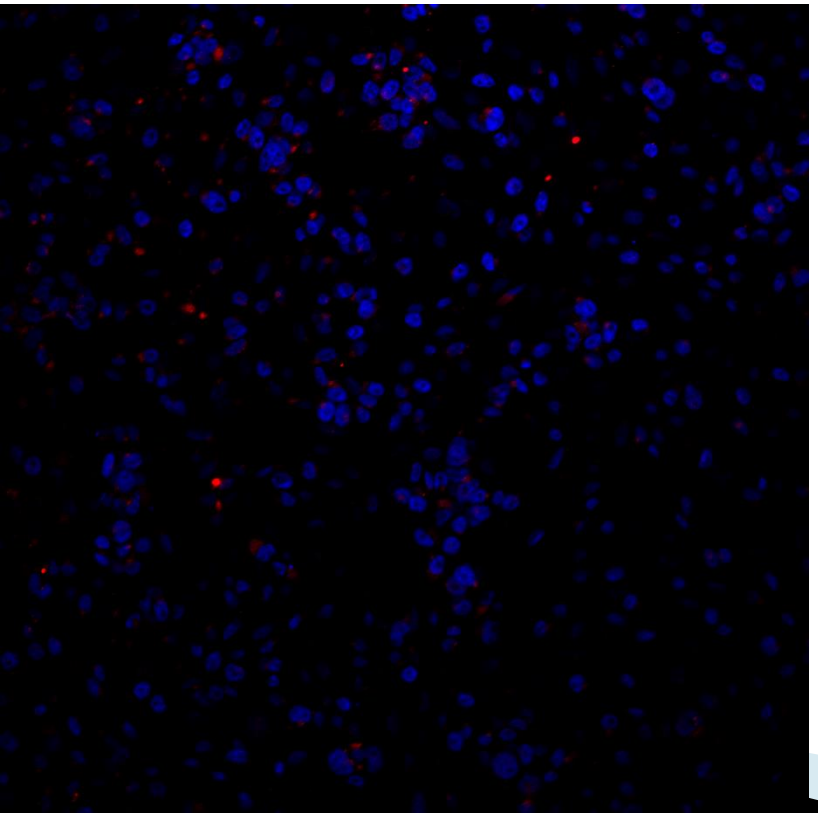


Cy5.5



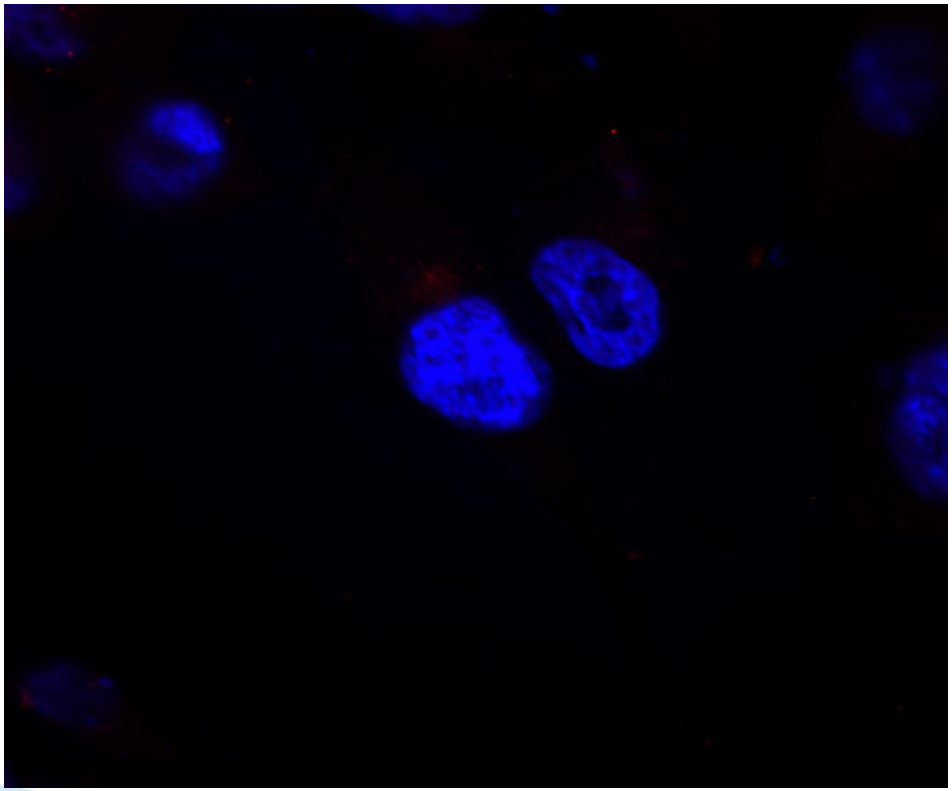
Magnification x20

MERGE



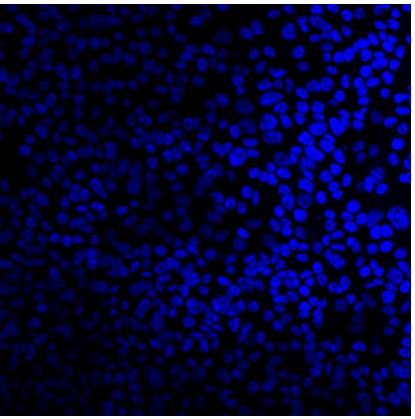
MERGE

Magnification x60

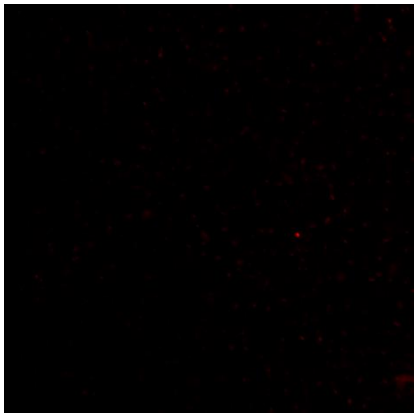


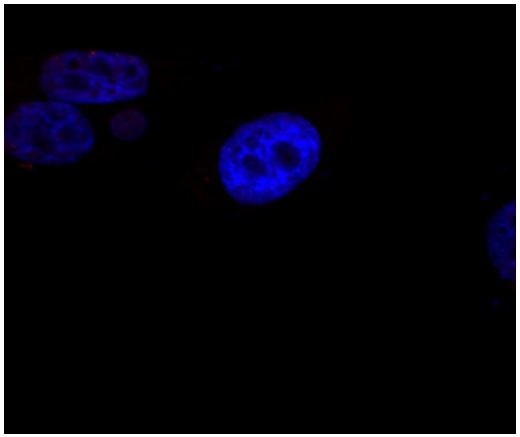
Blocking HACy5.5 uptake with Peptide FTEAESNMNDLV in Prostate cancer cells

DAPI



Cy5.5

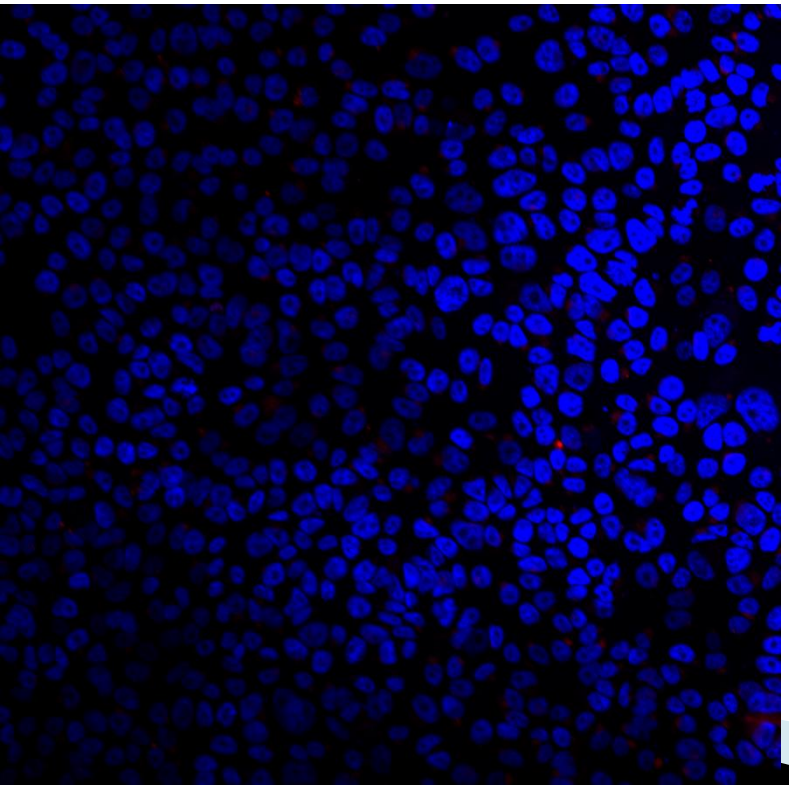




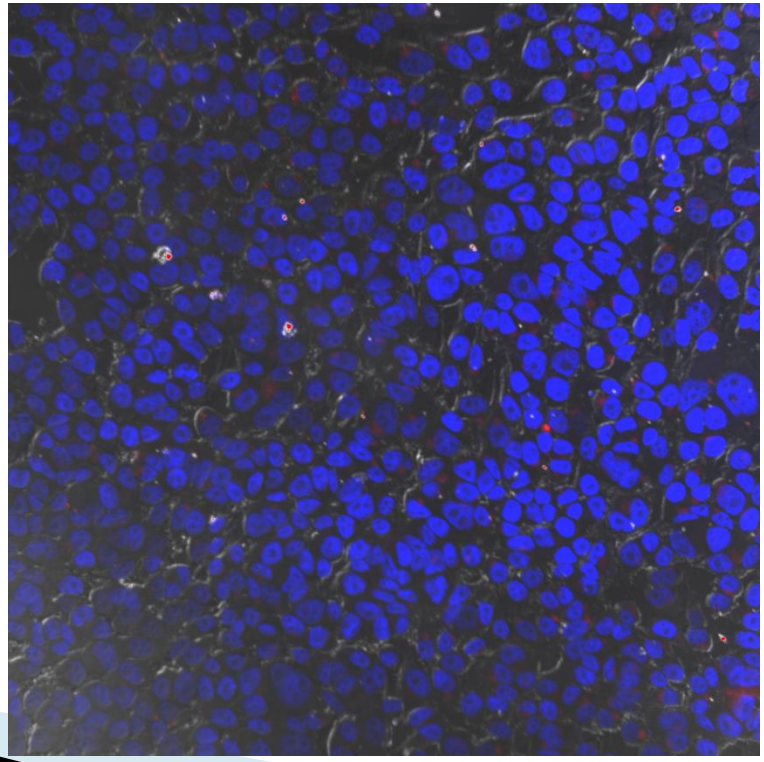
Magnification x60

MERGE

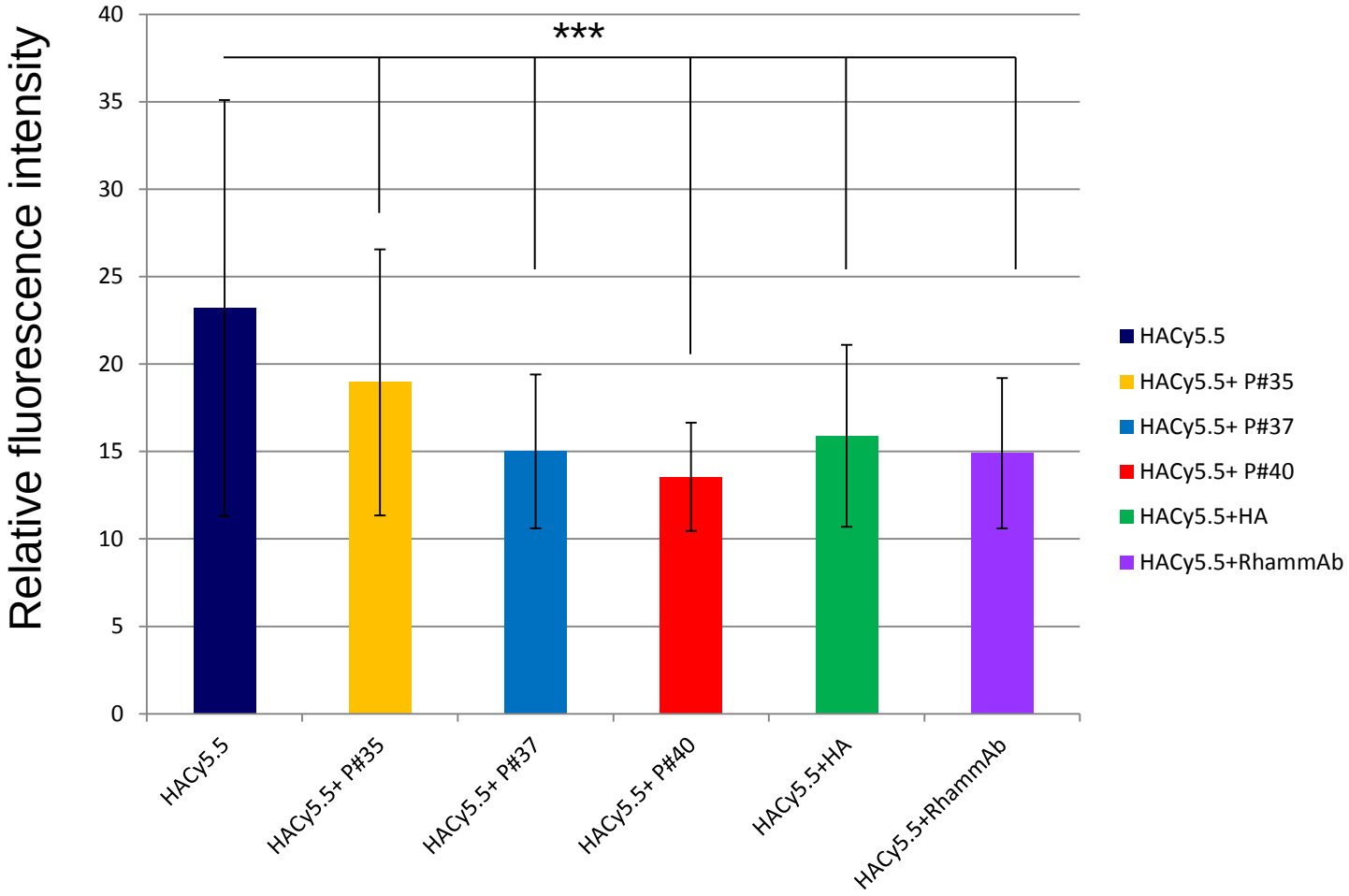
Magnification x20



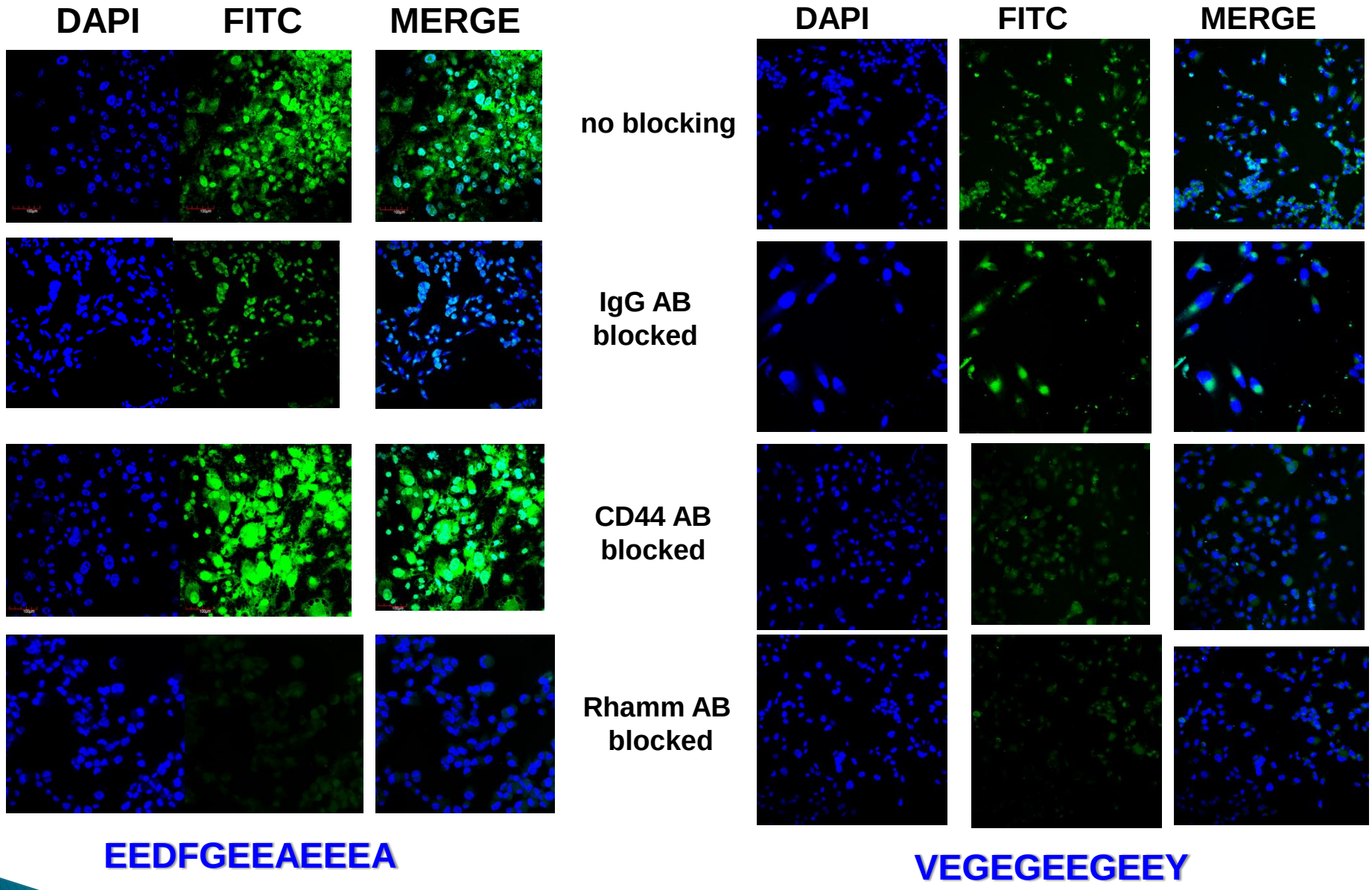
MERGE with TD1 channel



Quantification of cellular HA uptake in PC3M-LN4 cells.

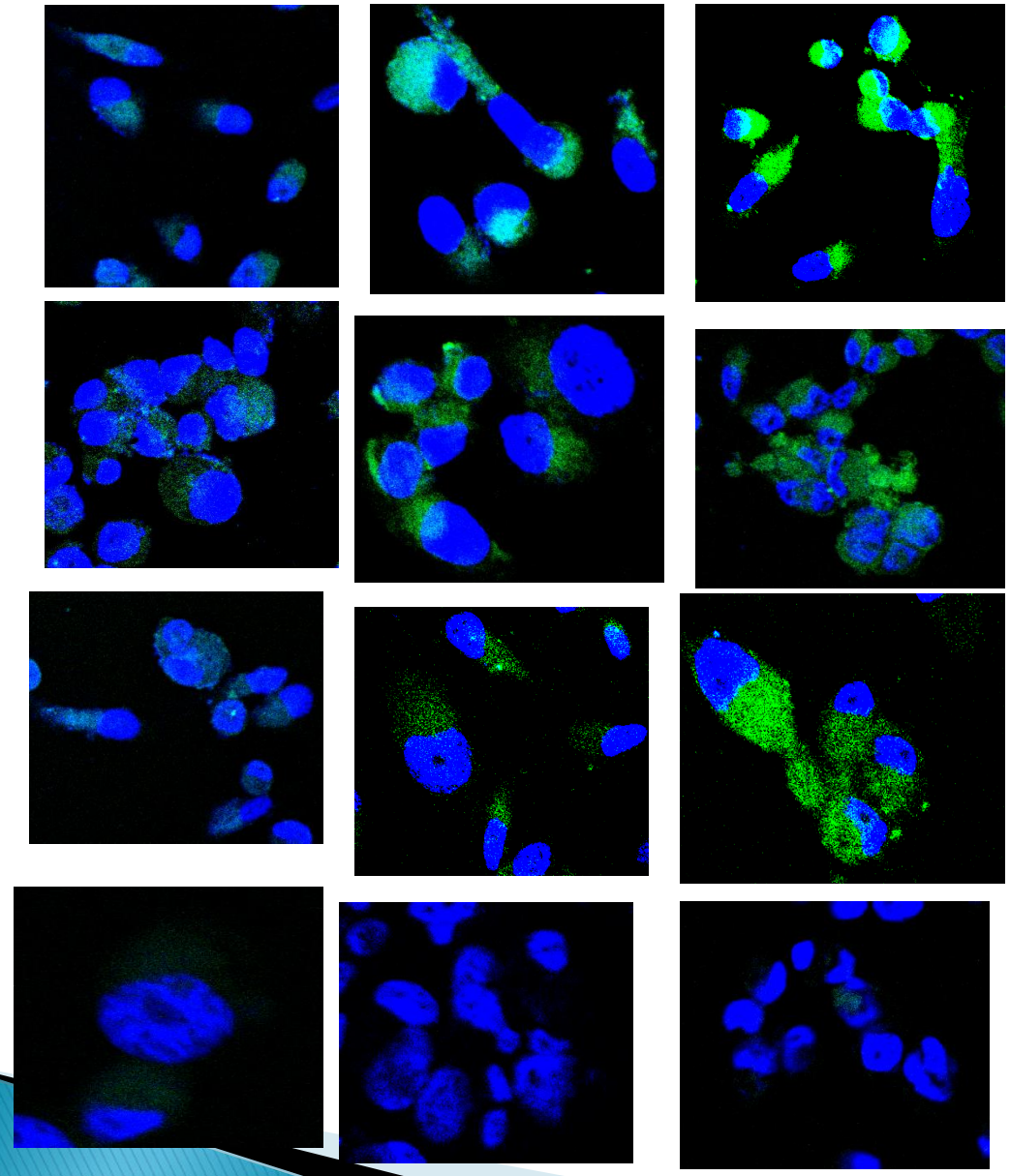


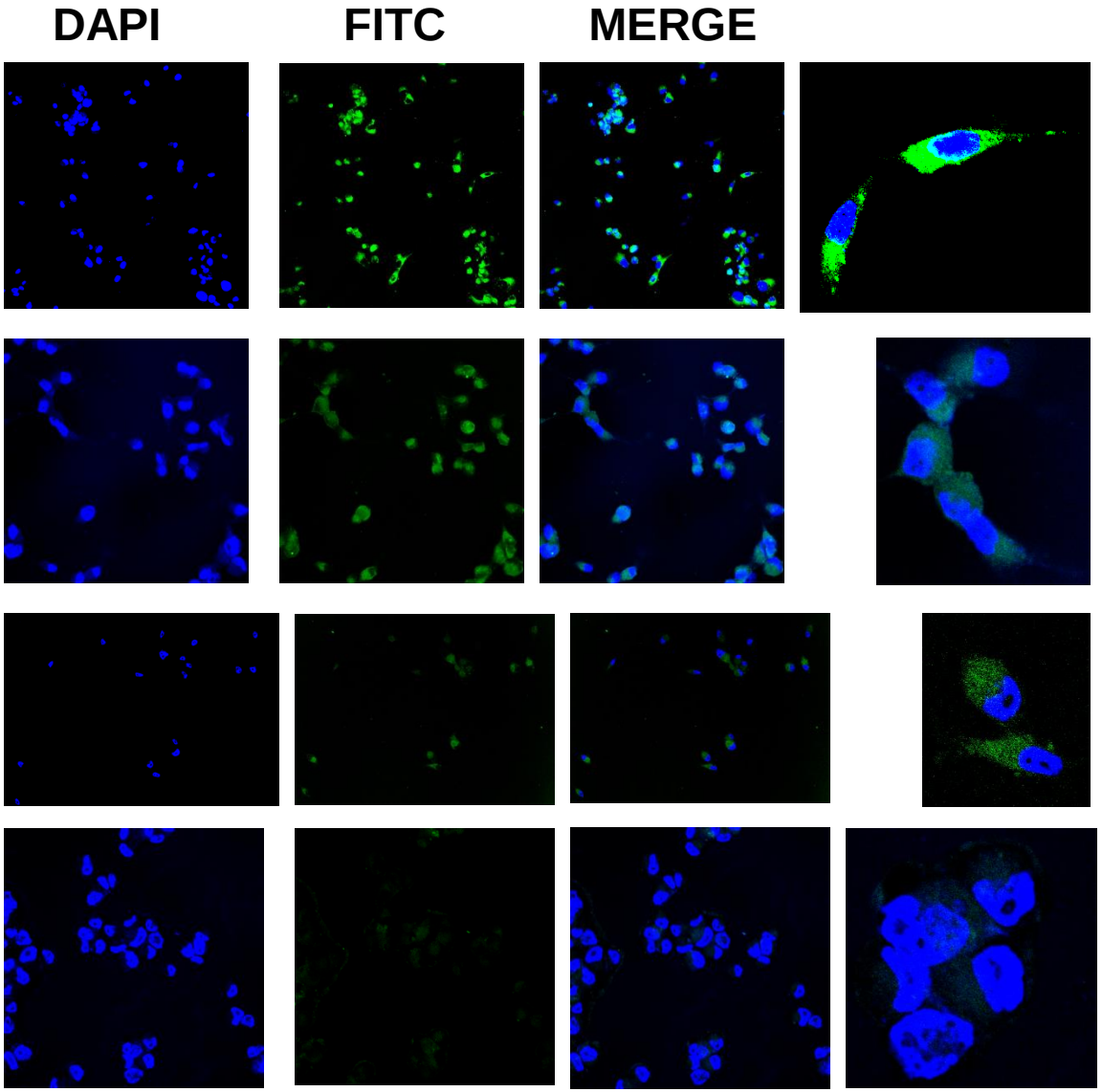
Using ImageJ software, ROI corresponding to the cancer cell bodies (4 610 , n = 3) were selected. Mean fluorescence of each ROI was obtained using 8-bit images and represented as a bar graph. Data were analyzed using one-way ANOVA.



Visualization of uptake of FITC-conjugated peptides in prostate cancer cells (PC3mLN4) using fluorescence microscopy.

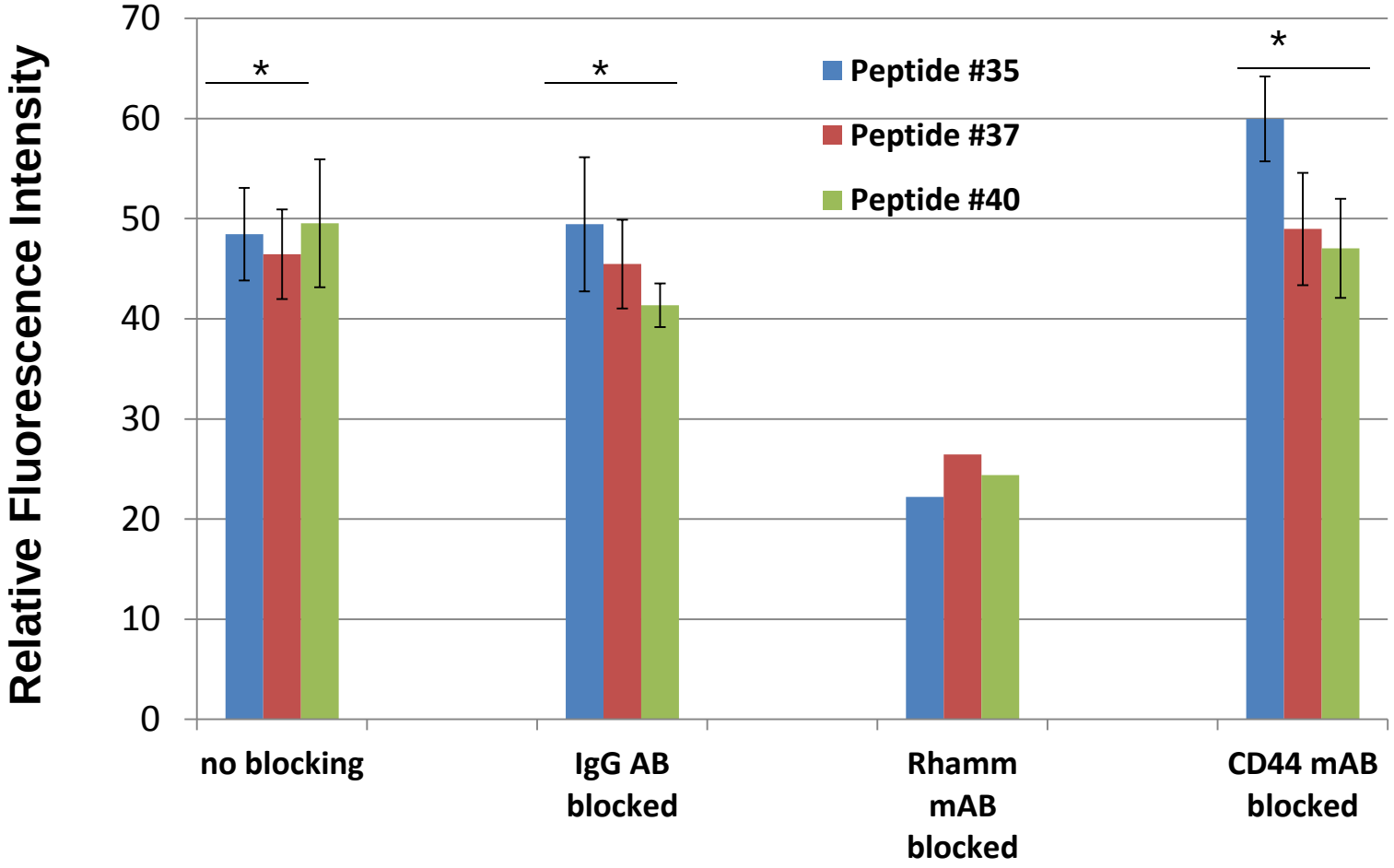
Cellular uptake of Tubulin-derived Peptides to Rhamm-Expressing Prostate cancer cells (PC3mLN4).





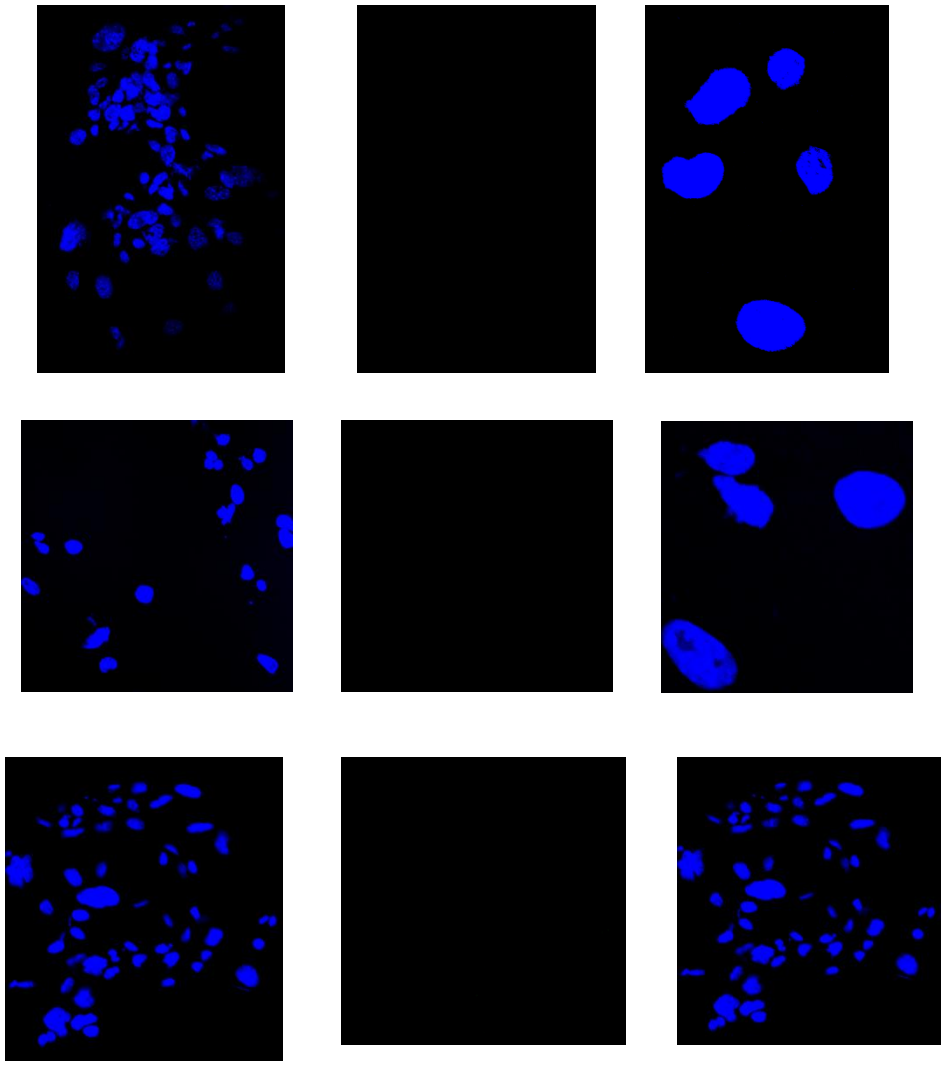
Visualization of uptake of FITC-conjugated FTEAESNMNDLV in prostate cancer cells (PC3mLN4) using fluorescence microscopy.

Quantification of uptake FITC-peptides in PC3mLN4 cells



Using ImageJ software, ROI corresponding to the cancer cell bodies (6 158, n = 4) were selected. Mean fluorescence of each ROI was obtained using 8-bit images and represented as a bar graph. Data were analyzed using one-way ANOVA (* - $p<0.05$).

Cellular uptake of Tubulin-derived Peptides in Knockout fibroblasts, Rhamm (-/-).



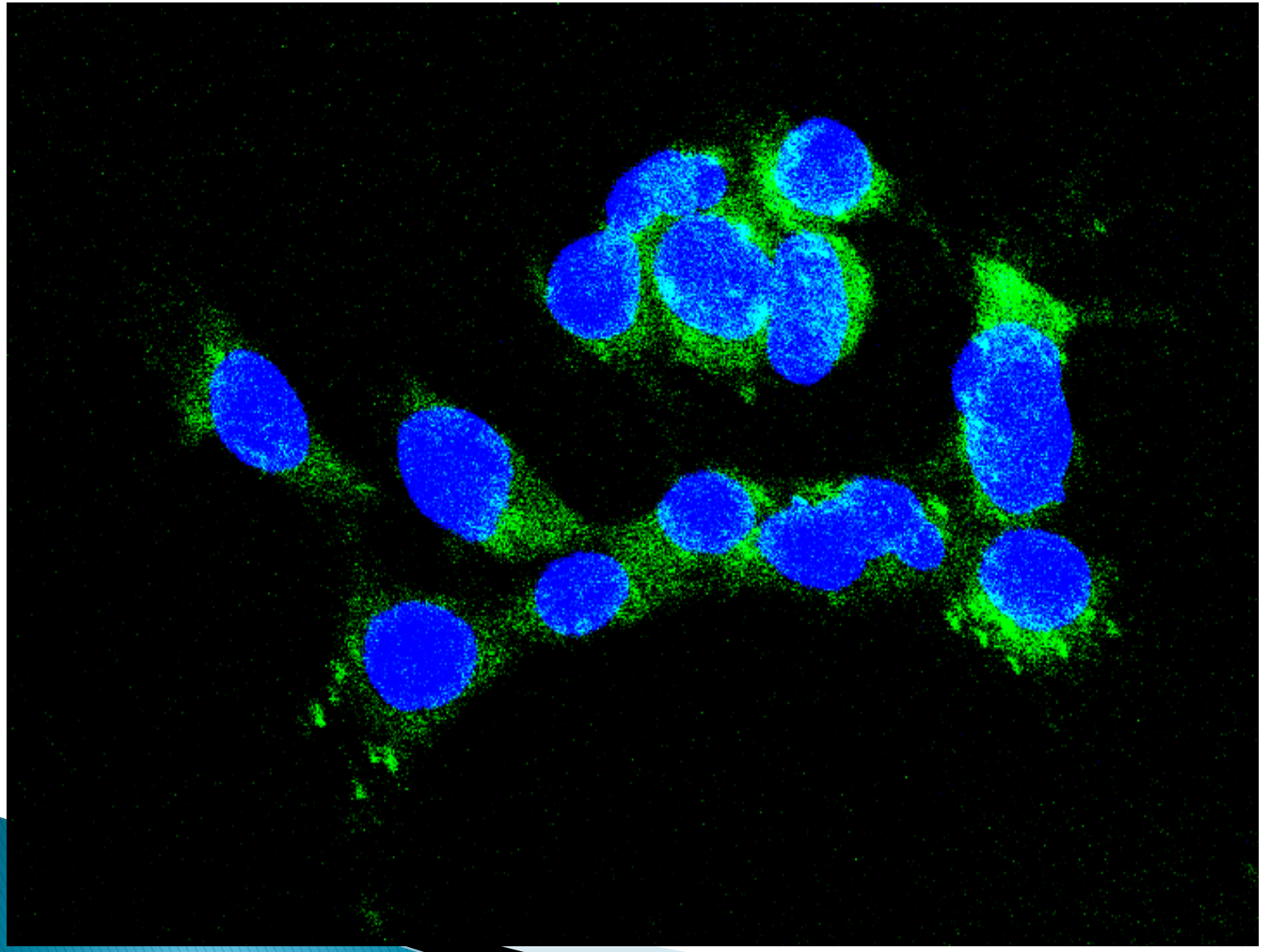
Peptide EEDFGEEAEEEA

Peptide VEGEGEEEGEEY

Peptide FTEAESNMNDLV

DAPI FITC MERGE

Cellular uptake Peptide VEGEGEEEGEEY by Ko (Rhamm -/-) transfected with full length of Rhamm).



Summary:

1. Tubulin-derived peptides were synthesized.

2. Tubulin-derived peptides demonstrated specific, selective interaction with Rhamm.

3. Uptake tubulin-derived peptides was specific in breast and prostate cancer cells.

4. Peptides showed moderate stability in bovine serum, which is long enough to facilitate *in vivo* imaging.

CONCLUSIONS

- **1. Here we report an approach for identifying peptide ligands that bind to RHAMM.**
- **2. We designed peptides that mimic HA oligosaccharides in their negative charge, nanomolar affinity and specificity for RHAMM.**
- **3. Our results demonstrated the selective cellular uptake tubulin-derived peptides and ability to block RHAMM:HA interactions in cells.**
- **4. We propose that these probes will permit the selective detection of highly aggressive progenitor cells in primary tumors.**

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Prof. Eva Turley

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Research Unit

cancer care ontario | action cancer ontario

Ontario Institute for Cancer Research
science → discoveries → solutions

Department of Defense
Congressionally Directed Medical Research Programs

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Thanks for your ATTENTION!

Q&A