London Health Sciences Centre
London Regional Cancer Program

Организация и методики проведения доклинических испытаний противоопухолевых соединений в London Health Science Centre, Laboratory regional Cancer Research Program, London, ON, Canada.

Akentieva Natalia, PhD

Research projects:

1. Design and synthesis tubulin-derived peptides as novel molecular imaging probes that target cancer cells.(NIH grant, USA; Ontario Institute of Cancer Research (OICR), Canada).

Achievements:

Tubulin-derived peptides were synthesized.

Peptides demonstrated specific, selective interaction with Rhamm.

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

Our results demonstrated the selective cellular uptake peptides and ability to block RHAMM:HA interactions in cells.

We conclude, that these probes will permit the selective detection of highly aggressive progenitor cells in primary tumors.

2nd Project. CHARACTERIZATION of HYALURONAN-MIMETIC PEPTIDES as ANTI-CANCER AGENTS.

Achievements:

HA-mimetic peptides inhibited the cell viability and proliferation of breast and prostate cancer cells.

HA-mimetic peptides induced apoptotic and modulated necrotic death in breast and prostate cancer cells.

HA-mimetic peptides inhibited the invasion of breast and prostate cancer cells.

Peptide D completely blocked the development cancer in mice.

3d Project. Study the therapeutic effect of Rhamm-derived peptides for treatment diabetes 1 and 2.

Achievements:

Naor D., **Akentieva N.P.,** Turley E**. Rhamm-derived peptide for treatment of diabetes. International Patent 12459. June, 2013.** Yissum Research Development company of the Hebrew University of Jerusalim, Israel.

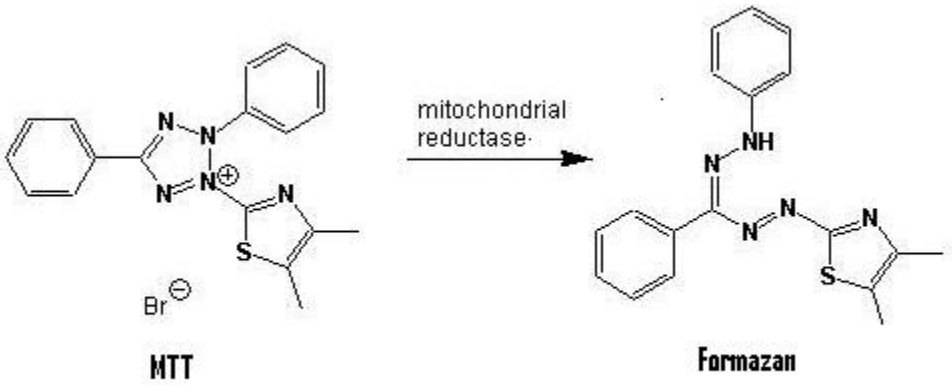
Collaboration with Johnson&Johnson company for production Rhamm as therapeutic agent for treatment diabetes.

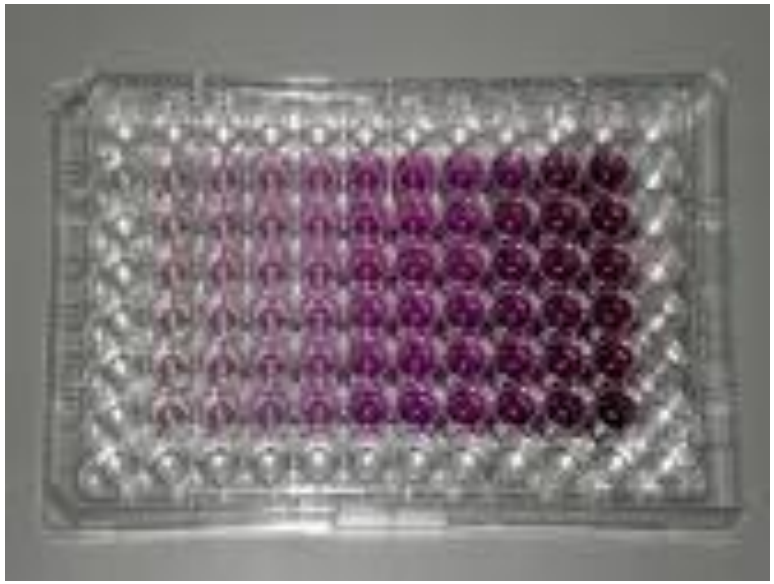
Methods for screening anti-cancer drugs: *in vitro* and *in vivo*

- Cell-based screening assays, *in vitro* models:

- 1. MTT assay
- 2. PI assay
- 3. Cell-viability assay

Principle MTT Assay:

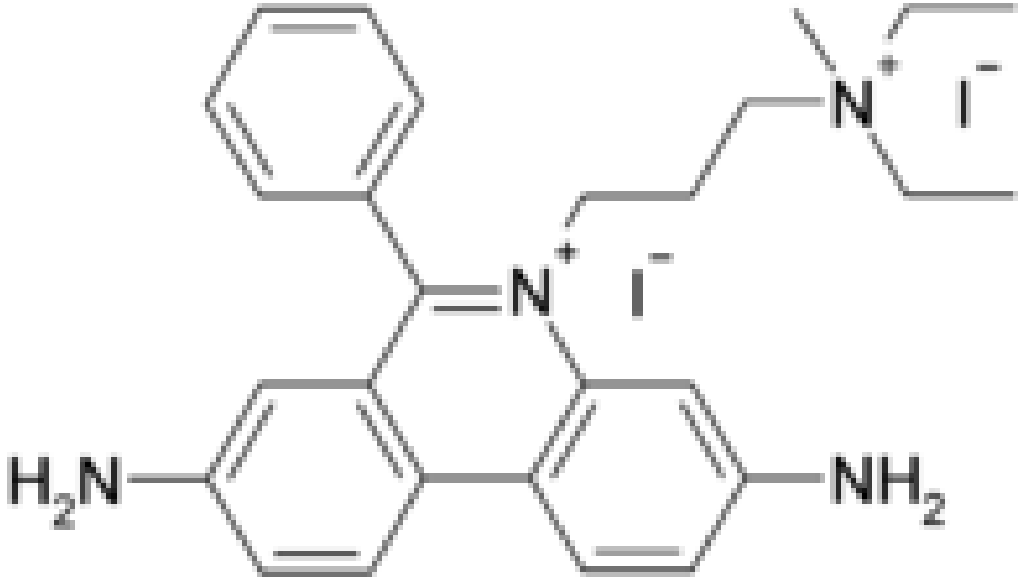




A microtiter plate after an MTT assay. Increasing amounts of cells resulted in increased purple colouring.

PI Assay

Fluorescence detection system: It includes the propidium iodide (PI) assay staining for DNA content. P**ropidium iodide** (or PI) is a fluorescent molecule, can be used to stain cells. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535ʌnm and the emission maximum is 617ʌnm. Propidium iodide is used as a DNA stain for both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis,and microscopy to visualise the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. This assay offers the most advantages, such as high sensitivity and easy handling.



Cell viability Assay-AlamarBlue

The Fast, Simple and Reliable Reagent to Assess Cell Health

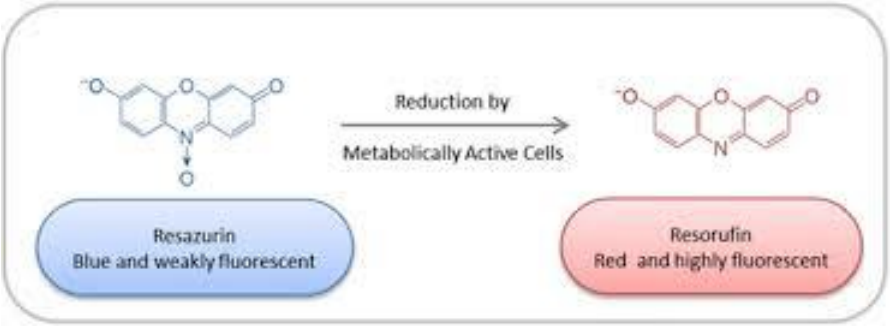
- alamarBlue® is designed to provide a rapid and sensitive measure of cell proliferation and cytotoxicity in various human and animal cell lines, bacteria and fungi.
- It works on suspended as well as attached cell lines. alamarBlue® is a non-toxic, ready-to-use reagent offering you a simple, safe and economical way to get the reliable results you need.

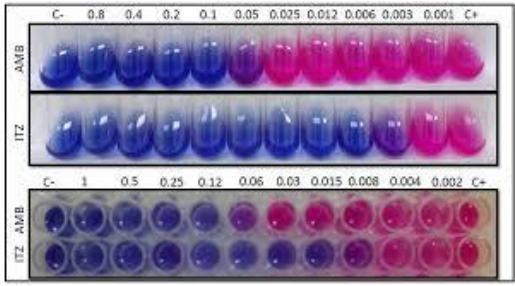
How does alamarBlue® work?

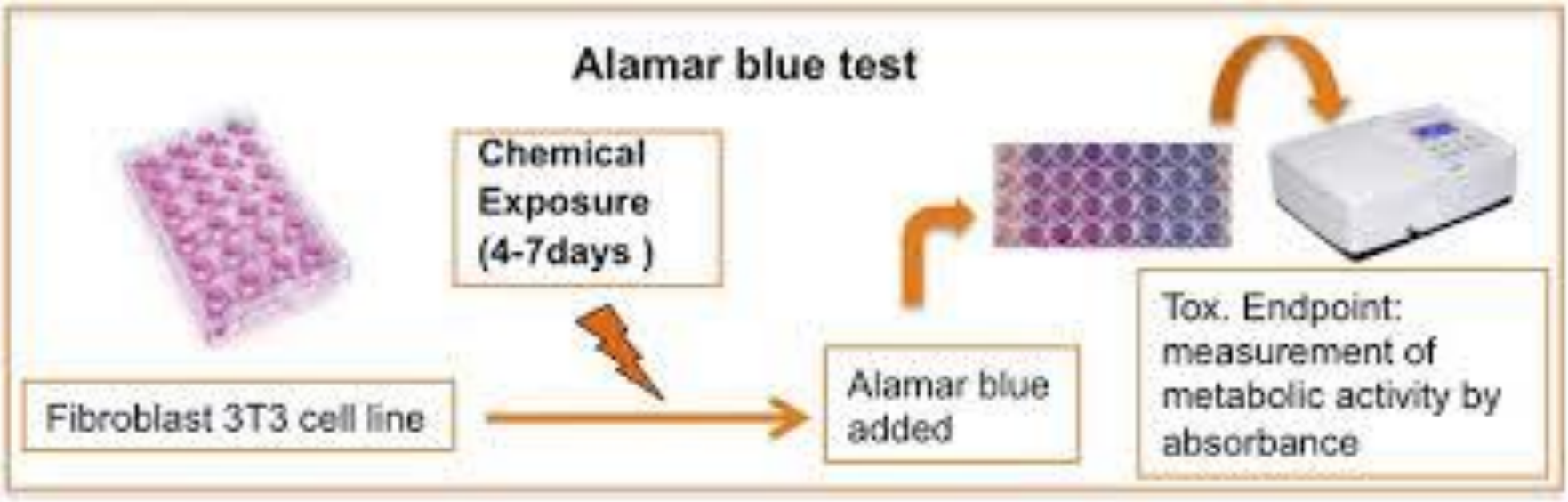
The active ingredient of alamarBlue®, resazurin is a non-toxic, cell permeable dye that is blue and weakly fluorescent.

It is used as an oxidation-reduction (REDOX) indicator that undergoes colorimetric change in response to cellular metabolic reduction. The reduced form resorufin is pink and highly fluorescent.

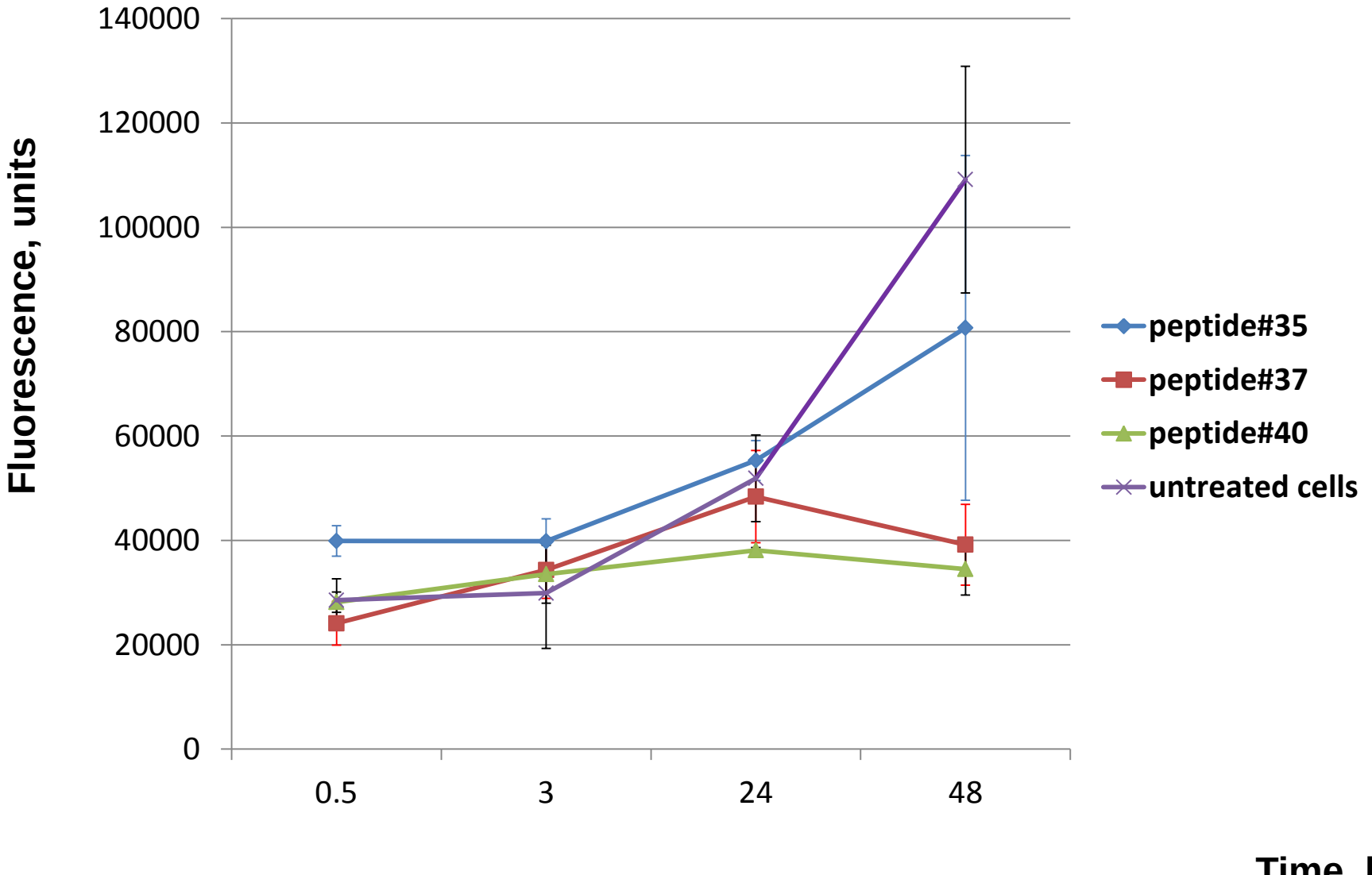
AlamarBlue assay:







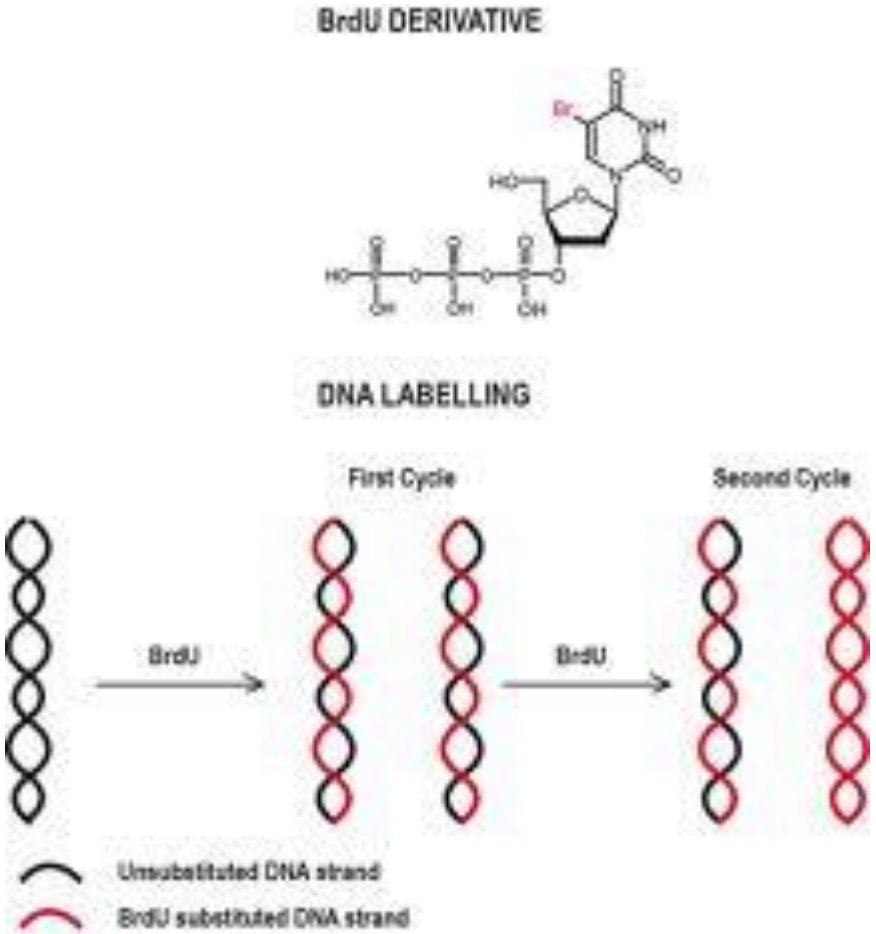
Cell viability of HA-mimetic peptides-treated prostate cancer cells (PC3mLN4).

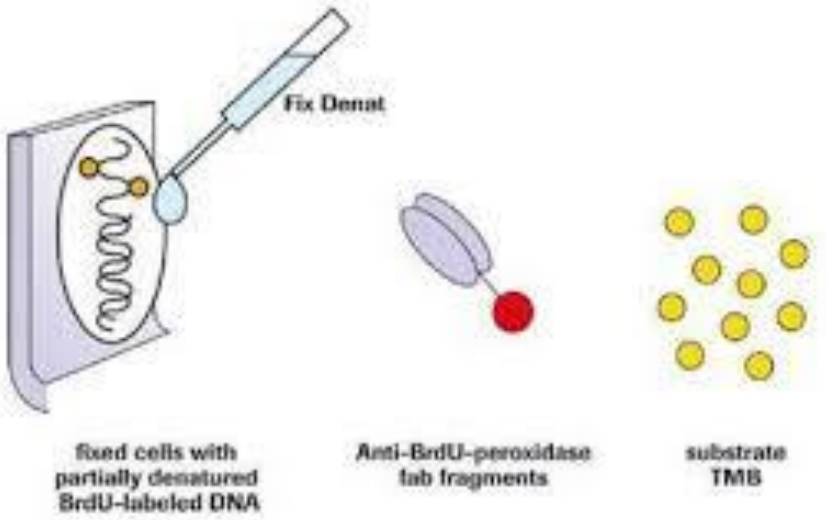


BrdU assay

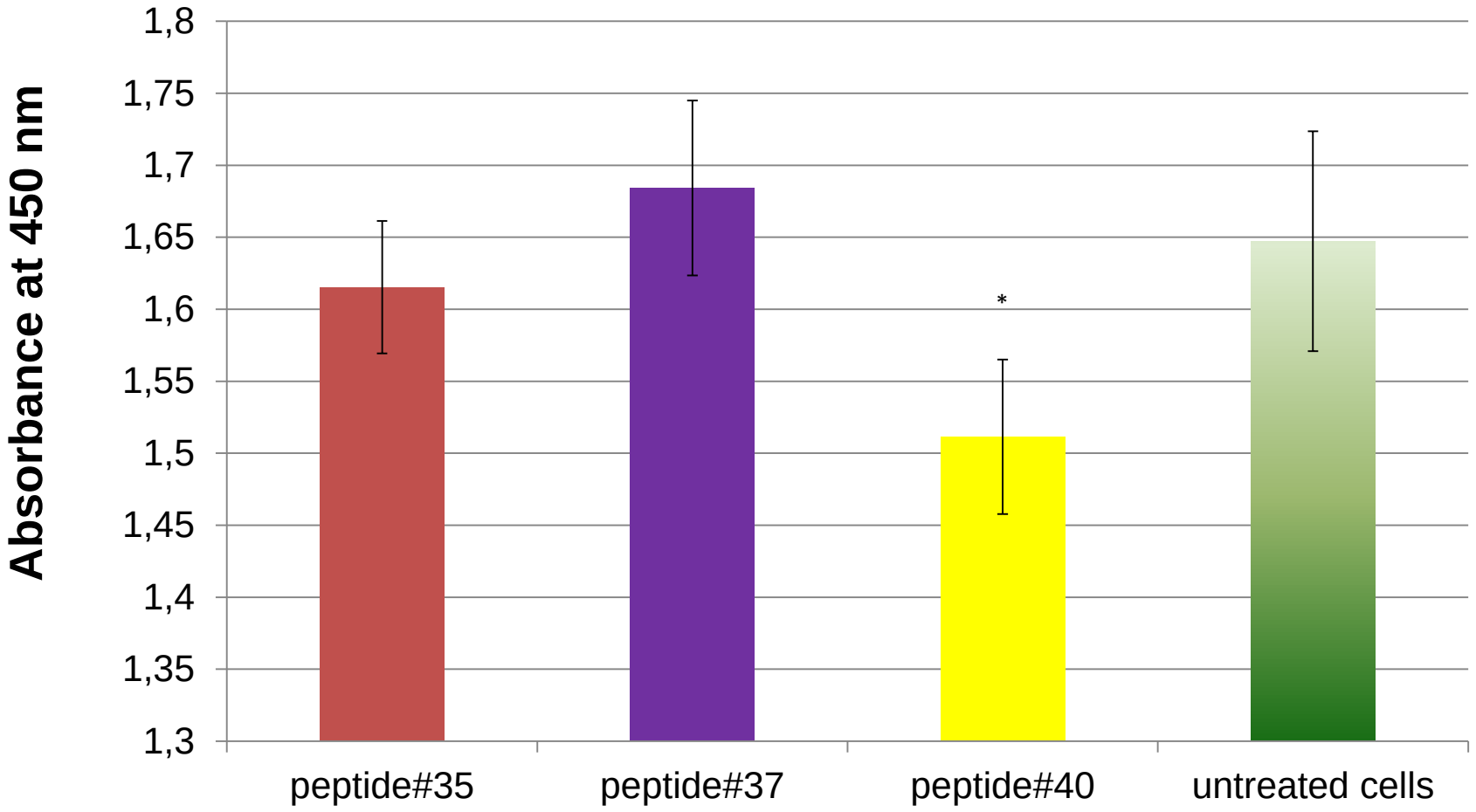
- **Principle:**
- Cellular proliferation requires the replication of genomic DNA. Thus, monitoring DNA synthesis is an indirect parameter of cell proliferation, as well as being suitable for the study of the regulation of DNA synthesis.
- The assay is based on the detection of BrdU incorporated into the genomic DNA of proliferating cells.

How BrdU assay works?





Effect HA-mimetic peptides on proliferation prostate cancer cells (PC3M-LN4), using BrdU assay.



Data were analyzed using one-way ANOVA, means are significant different at * $p<0.05$.

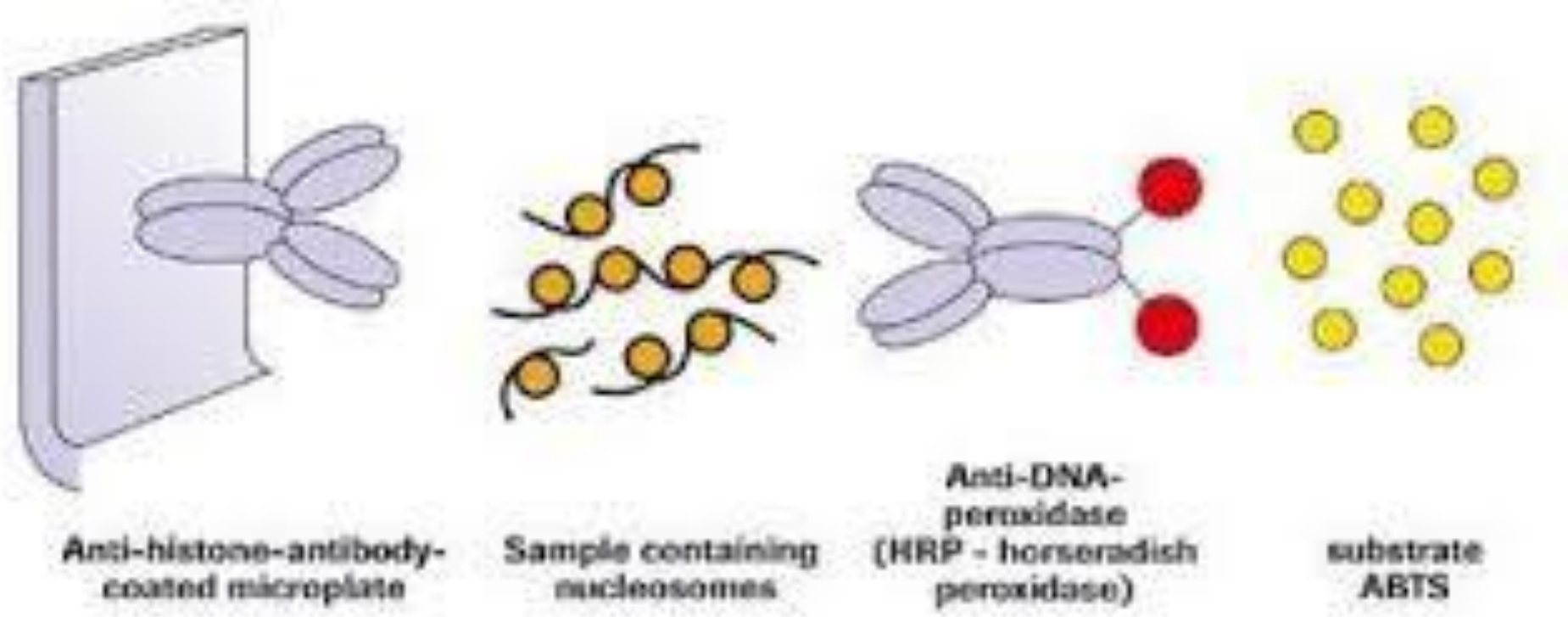
Biochemical Enzyme screening assays, *in vitro* models

- Biochemical assays are compared to cellular assays “target-driven” and provide the means for evaluating high numbers of compounds.
- Suitable approach is enzyme linked immunoadsorbent assays (ELISA) and other enzyme-based colorimetric methods.
- Prominent targets for this assay are the protein kinases, caspases, the histone-component of the mono-, oligonucleosomes (histones H1, H2A, H3 an H4) and DNA component of the nucleosomes, MMTs and other enzymes.

Apoptosis&necrosis assay

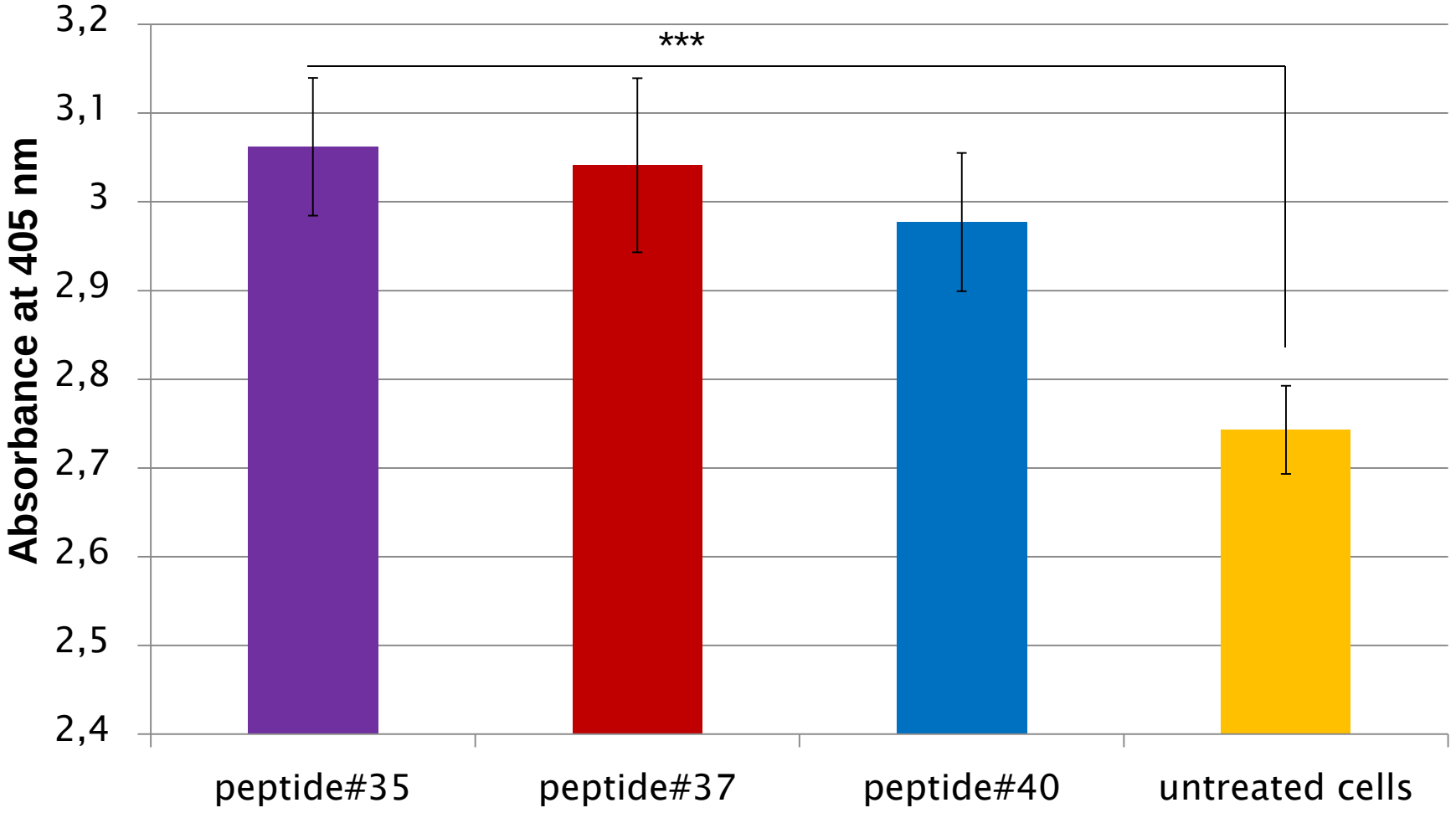
- **A cell death detection ELISA^{PLUS} assay** was performed to detect the type of cell death induced by anti-cancer agents.
- The assay is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal Ab directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.

Principle:



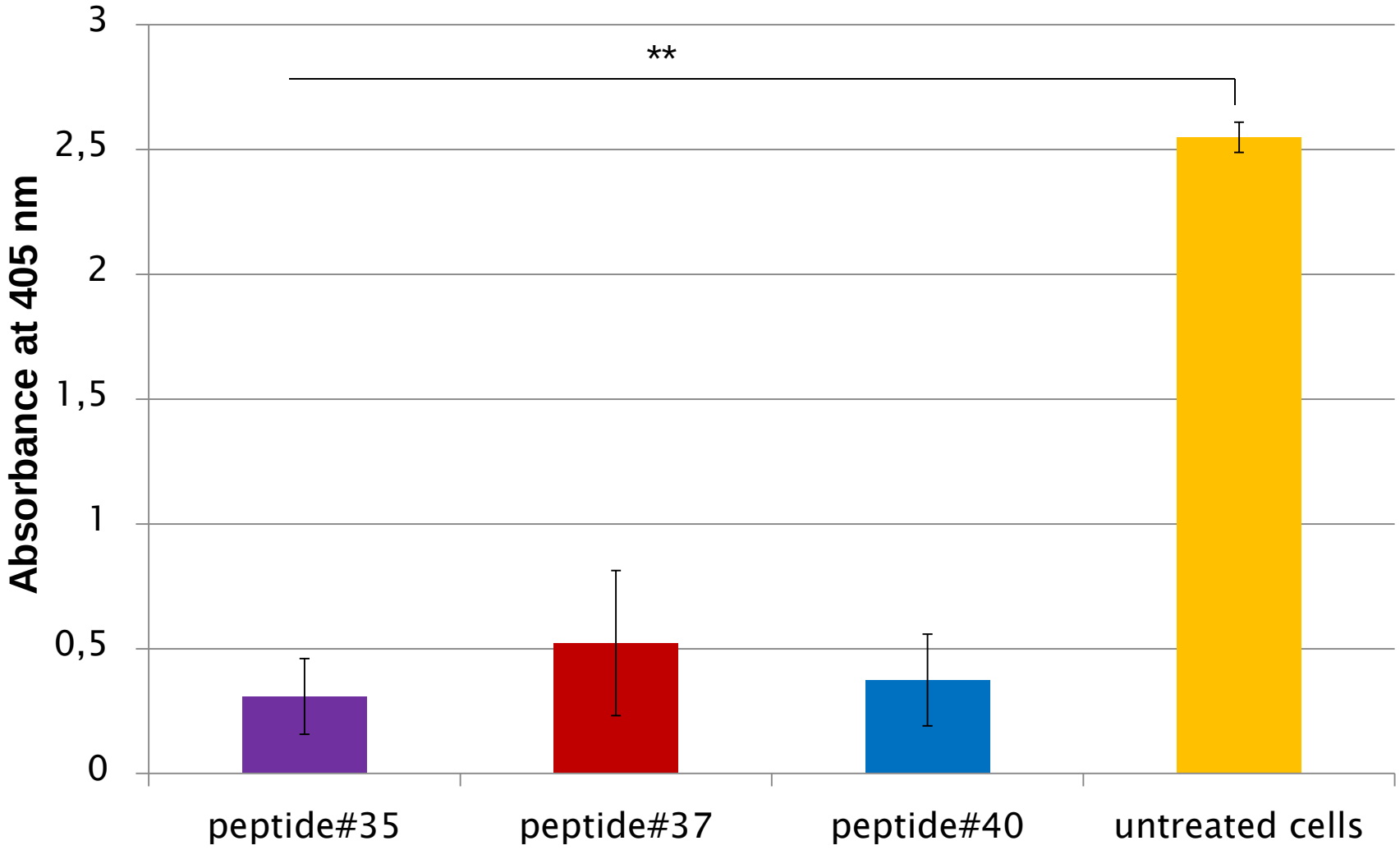
•During the incubation period, the anti-histone Ab binds to the histone-component of the mono-, oligonucleosomes (histones H1, H2A, H3 an H4) and DNA component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated plates via its biotinylation

Induction of apoptosis of PC3mLN4 cells by HA-mimetic peptides.



Data were analyzed using one-way ANOVA, means are significant different at (***- $p<0.05$).

Inhibition of necrosis of PC3mLN4 cells by HA-mimetic peptides.



Data were analyzed using one-way ANOVA, means are significant different at (**- $p<0.05$).

MCF 7 cells

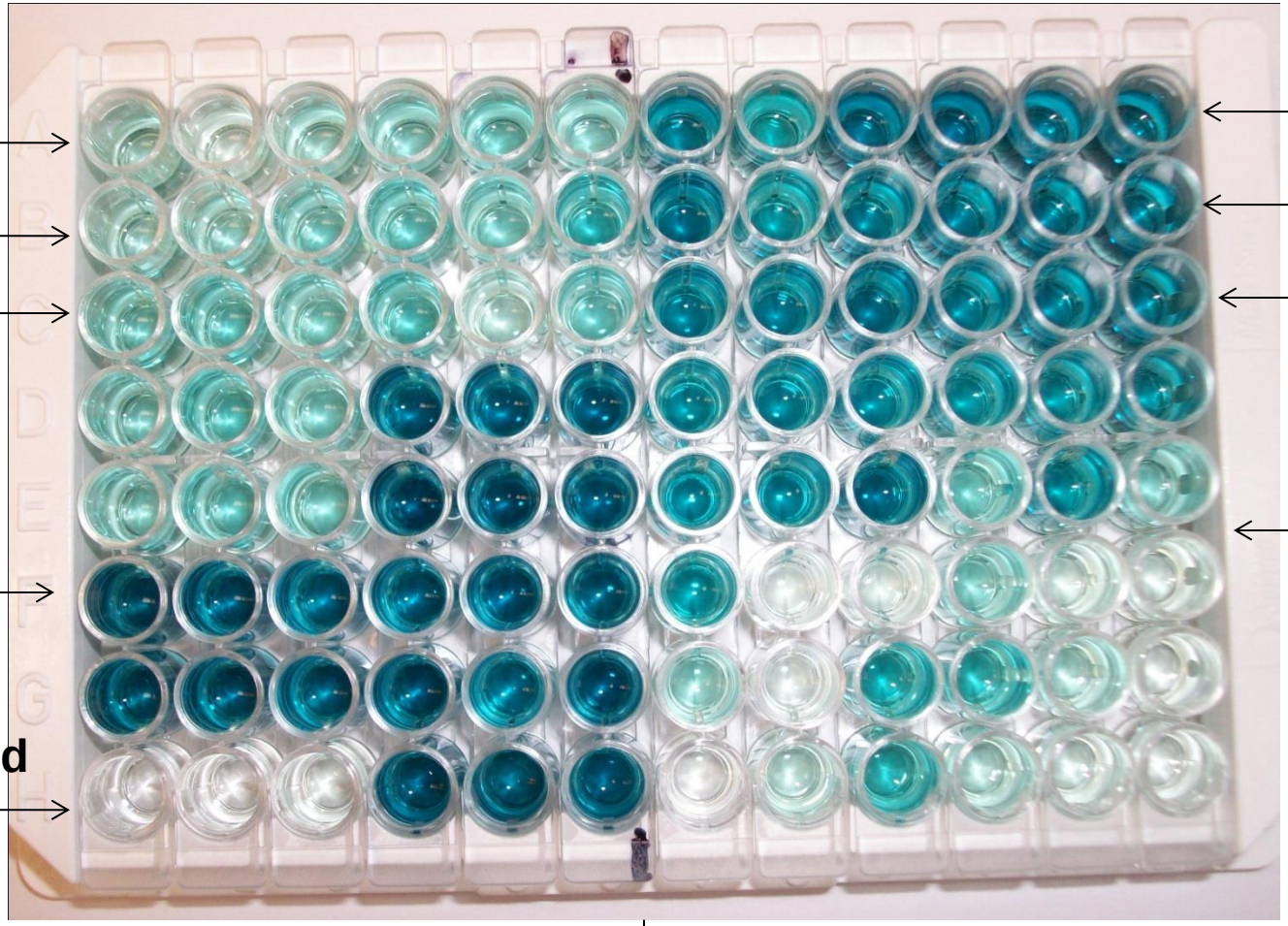
PC3mLN4 cells

apoptosis

P#35

P#37

P#40



necrosis

background

apoptosis

P#35

P#37

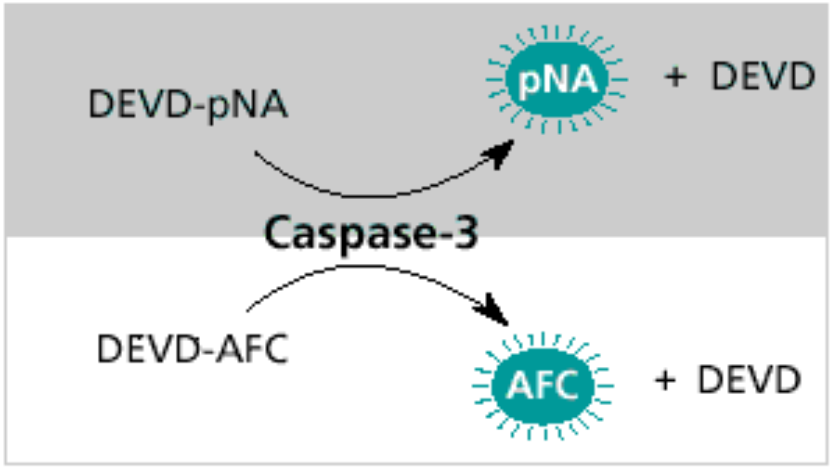
P#40

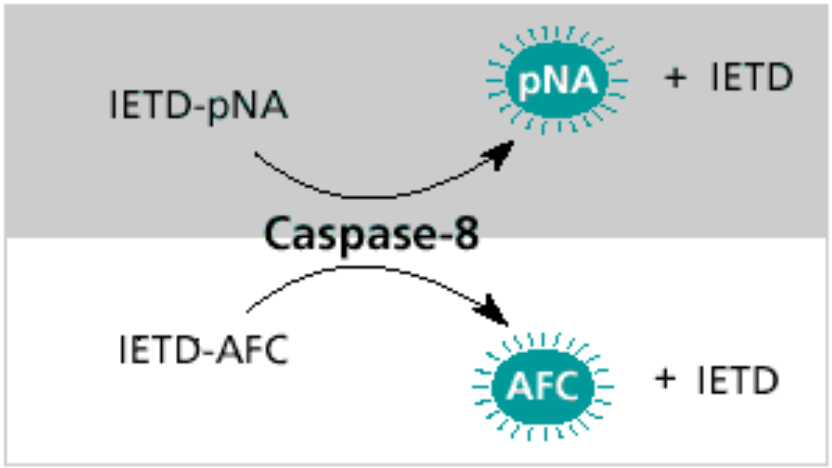
necrosis

Caspase-3 assay

- Caspases play important roles in apoptosis and cell signaling. The activation of caspase is important for the initiation of apoptosis. Caspase is identified as a drug-screening target.
- The caspase-3 colorimetric protease assay provides a simple and convenient means for quantitating caspases (responsible for apoptosis) that recognize the amino acid sequence, DEVD.

PRINCIPLE:

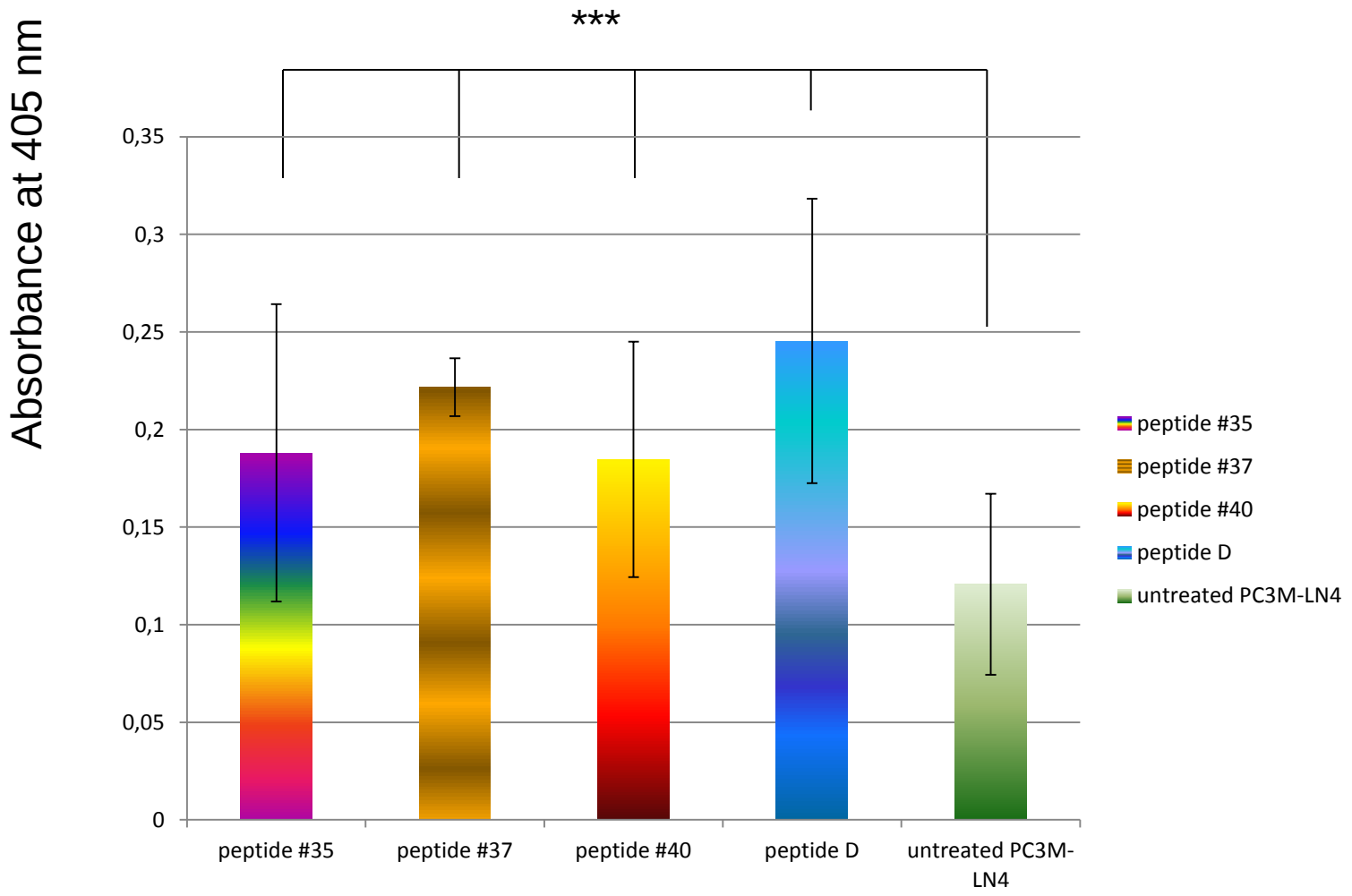




The substrate, DEVD-*p*NA, is composed of the chromophore *p*-nitroanilide (*p*NA), and a synthetic tetrapeptide, DEVD (Asp-Glue-Val-Asp), which is the upstream amino acid sequence of the caspase-3 cleavage site in PARP. Upon cleavage of the substrate by caspase-3 or related caspases, free *p*NA light absorbance can be quantified using a spectrophotometer or a microplate reader at 400 or 405 nm.

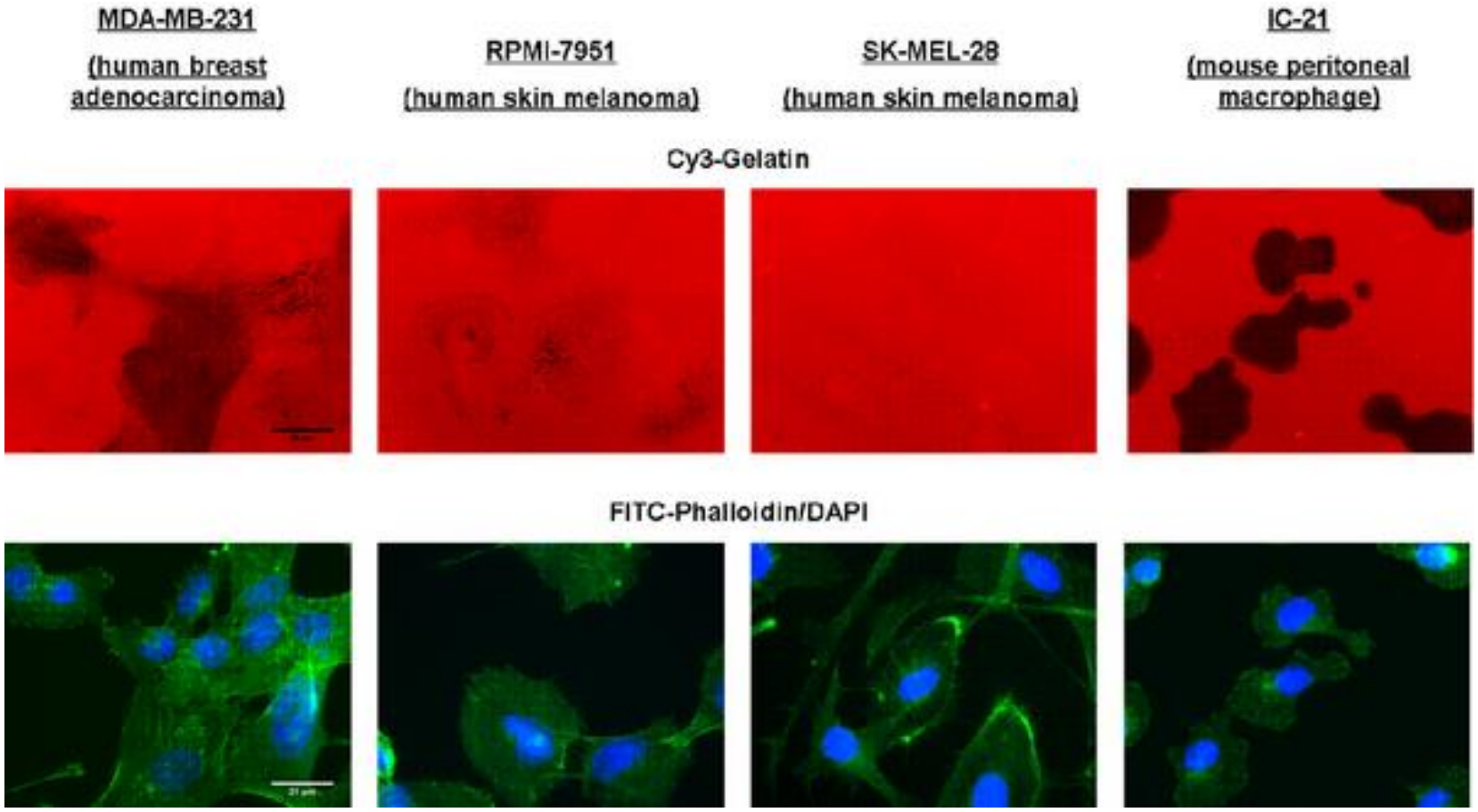
Comparison of the absorbance of *p*NA from apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity.

Effect of HA-mimetic peptides on caspase-3 activity in PC3M-LN4 cells.



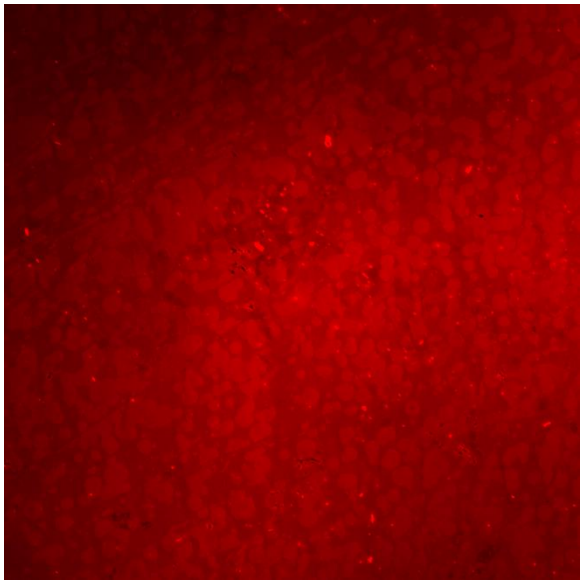
Invasion assay. Fluorescent gelatin degradation assay for investigating invadopodia formation. This method based on confocal microscopy.

- This assay allows to study effect of anti-cancer drugs on invasion of cancer cells, on invadopodia formation. The QCM™ Gelatin Invadopodia assay (red) kit was used.

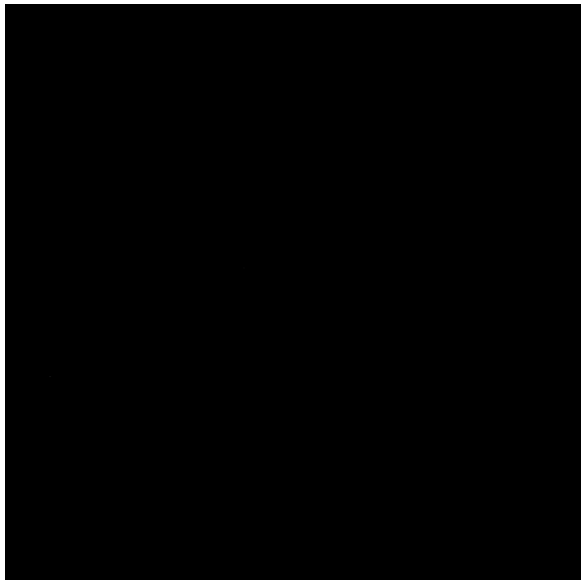


ImageJ software analysis of images

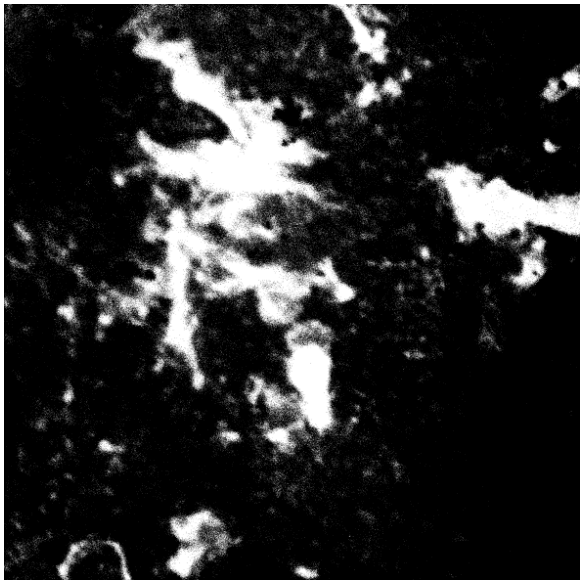
Cy3-labeled gelatin, no cells



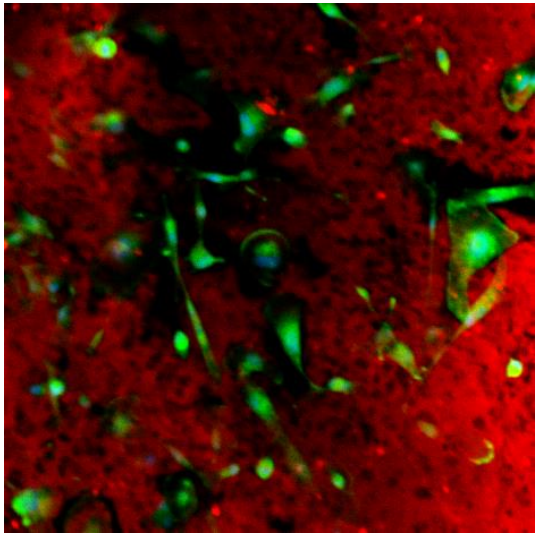
No degradation area,
no invadopodia formation



Degraded area-white



Analysis invadopodia in invasive tumor cells. Fluorescent gelatin (Cy3) degradation and phalloidin/DAPI staining of MDA–MB-231 cells. Magnification x20.



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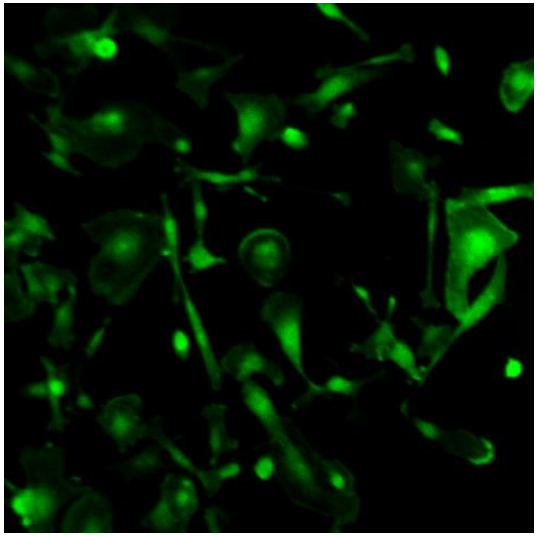
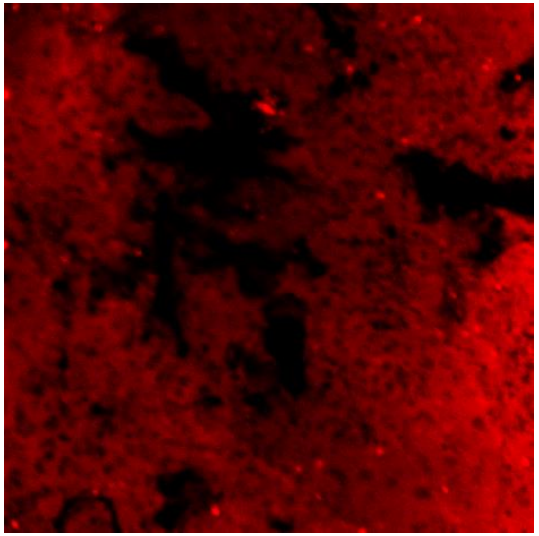
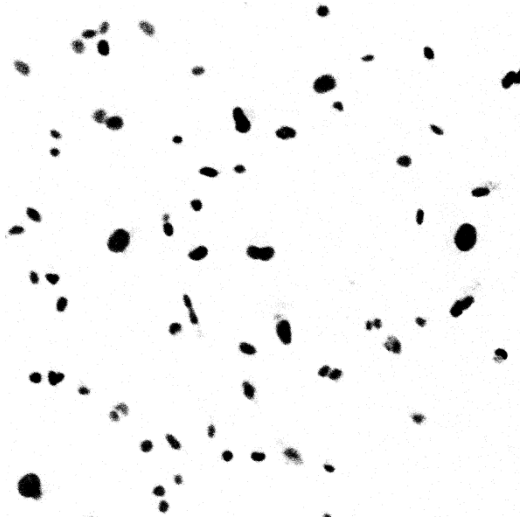


FITC



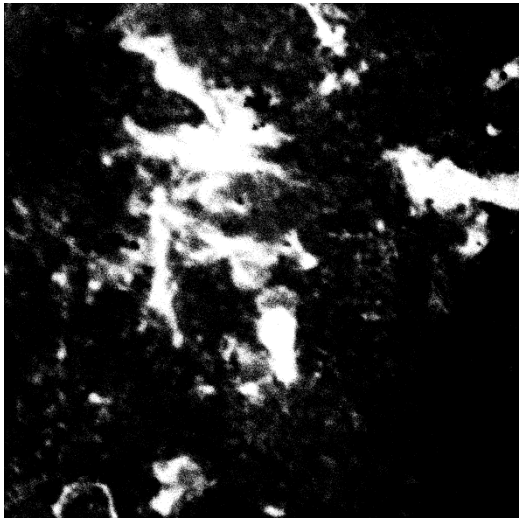
Cy3

Number of cells

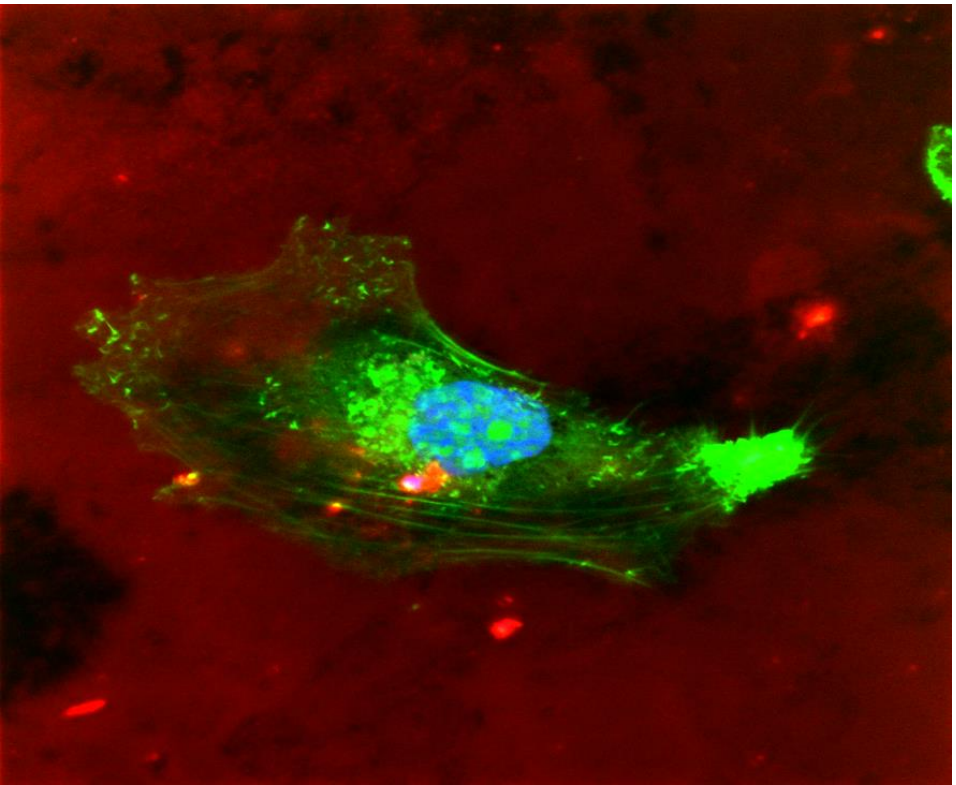


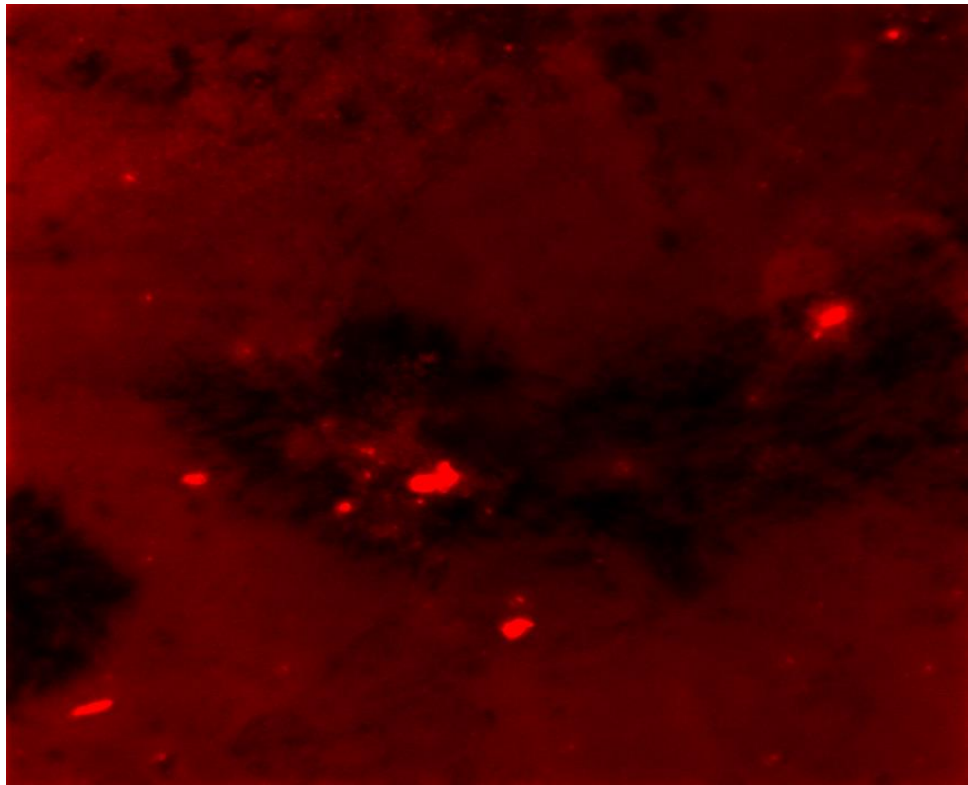
Cell area

Degraded area-white

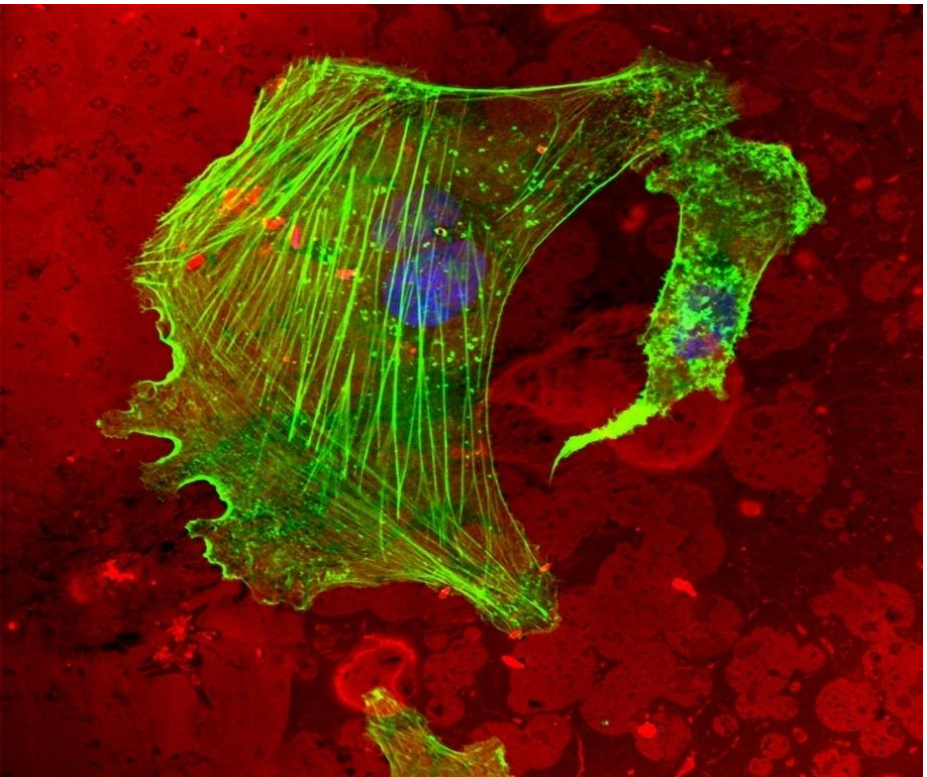


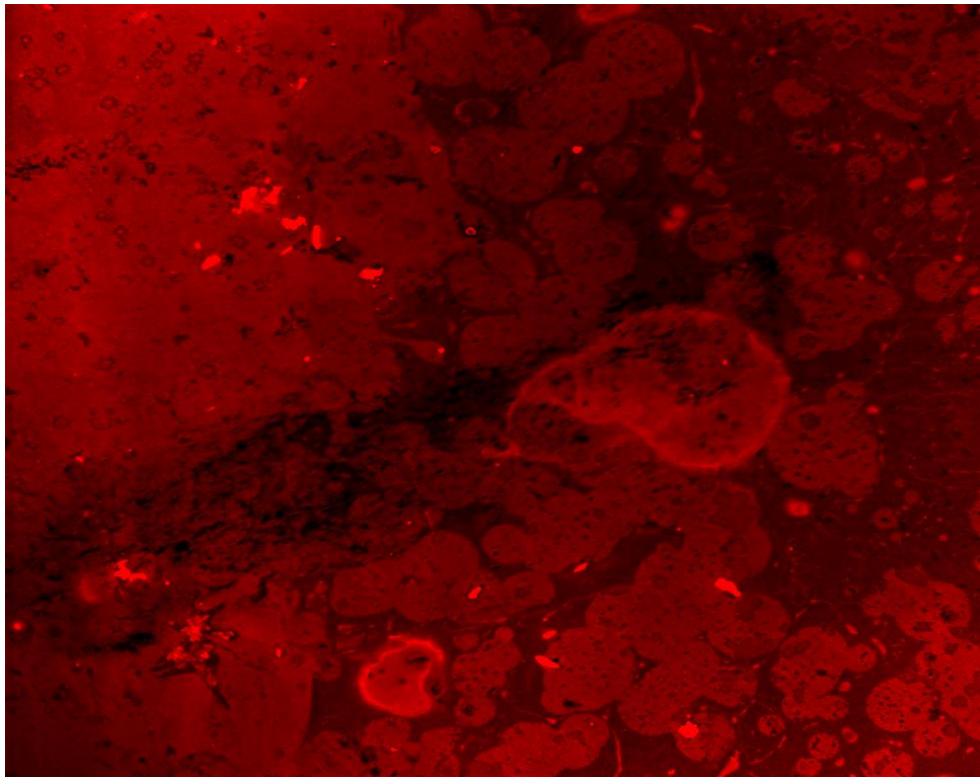
Fluorescent gelatin (Cy3 labeled) degradation and FITC - phalloidin/DAPI staining of MDA–MB-231. Magnification x60 cells.

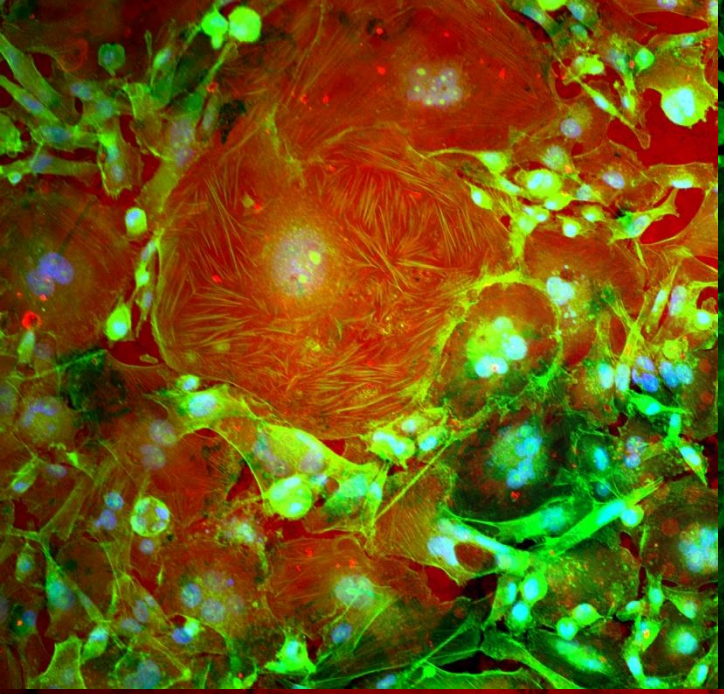


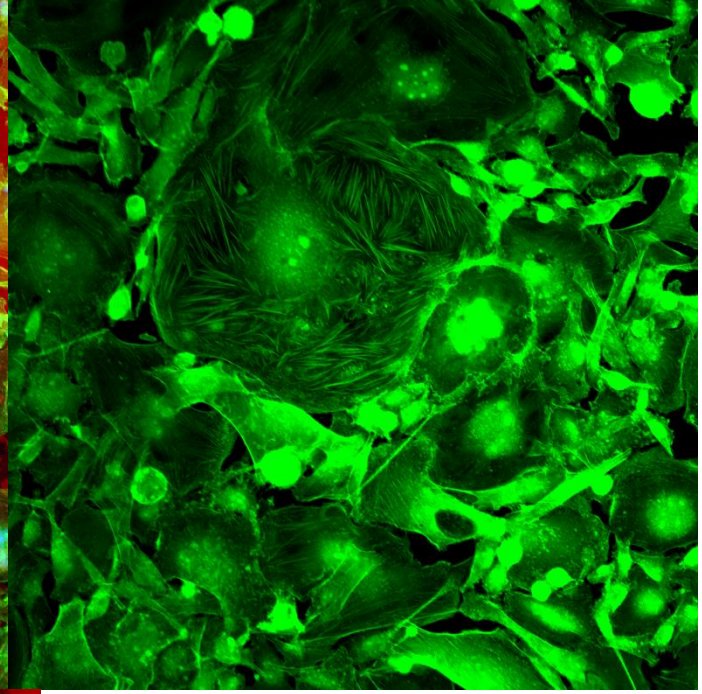


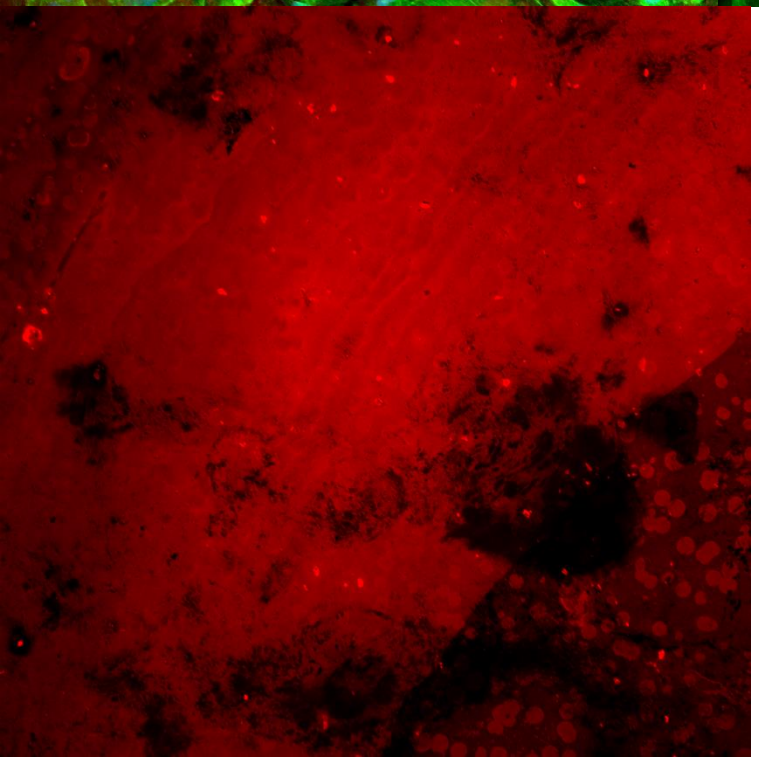
Fluorescent gelatin (Cy3 labeled) degradation and phalloidin/DAPI staining of MDA–MB-231. Magnification x60 cells.



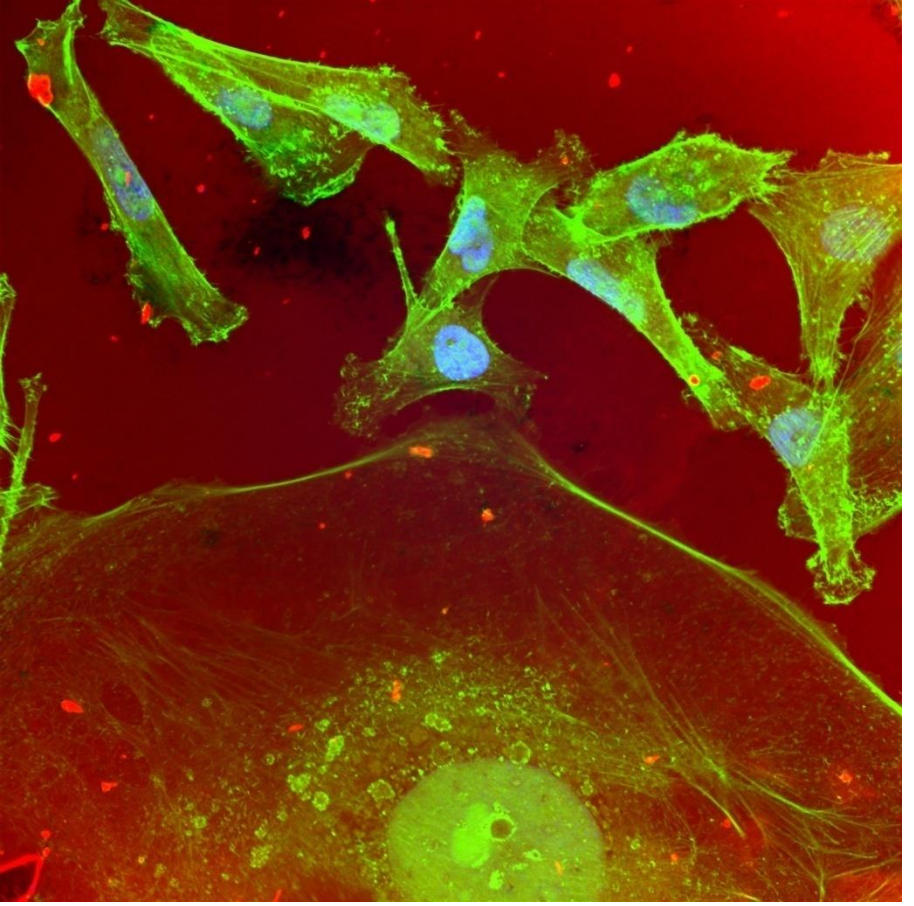


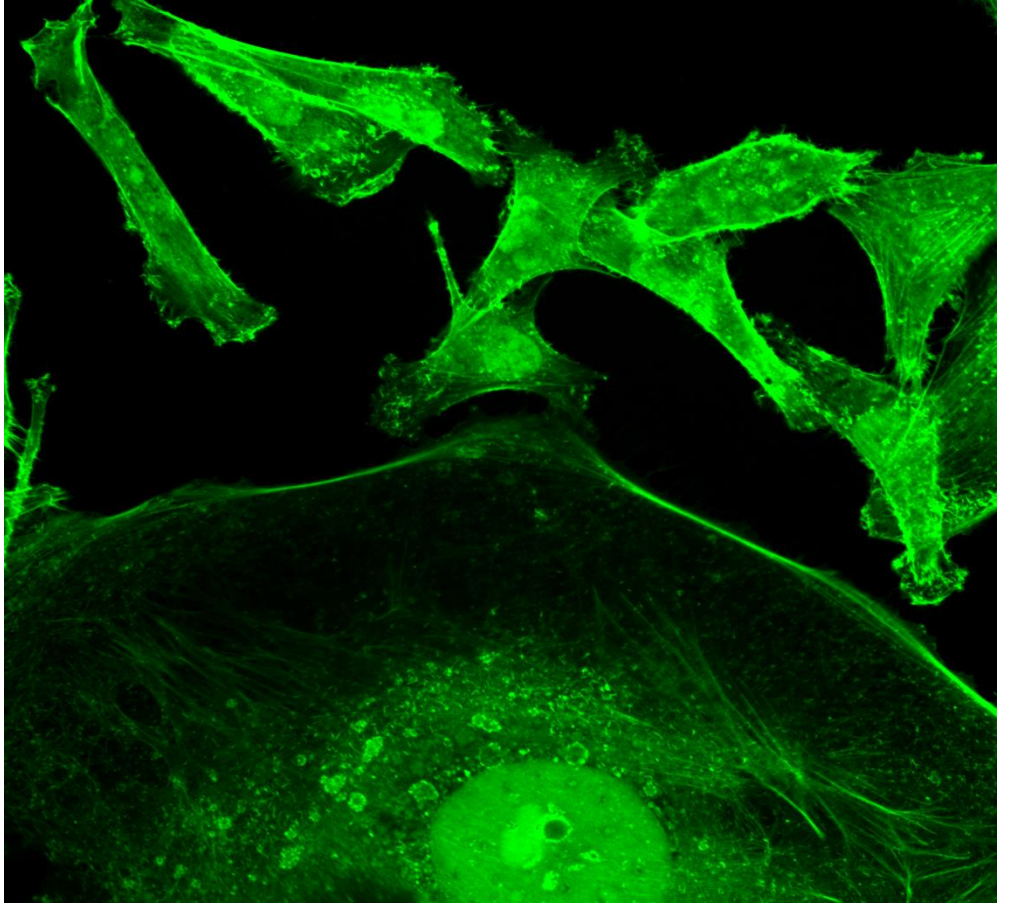






Modulation of gelatin degradation by HA-mimetic peptides. Assess activity of HA-peptide #35 on invadopodia formation. Magnification x20.





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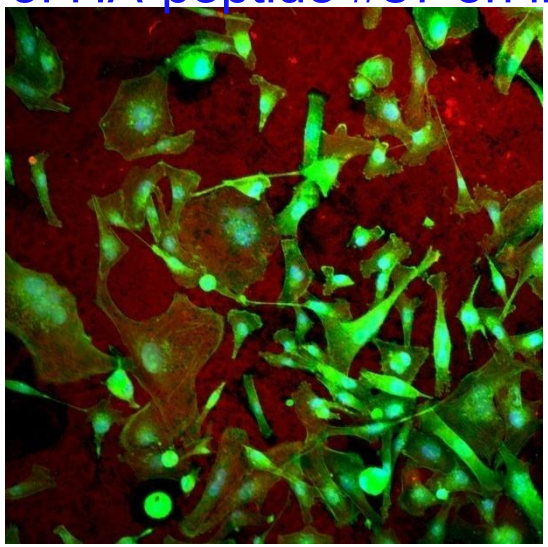
Modulation of gelatin degradation by HA-mimetic peptides. Assess activity of HA-peptide #35 on invadopodia formation MDA-MB 231 cells. Magnification x60.

Modulation of gelatin degradation by HA-mimetic peptides. Assess activity of HA-peptide #37 on invadopodia formation. Magnification x20.



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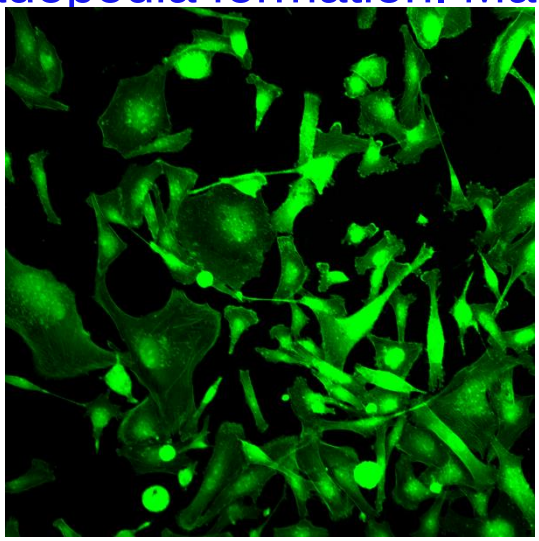

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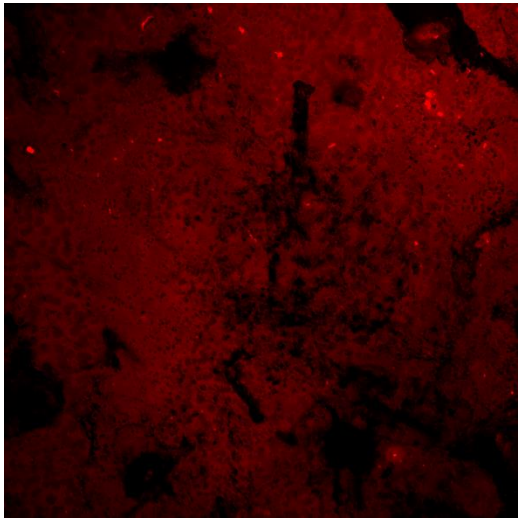
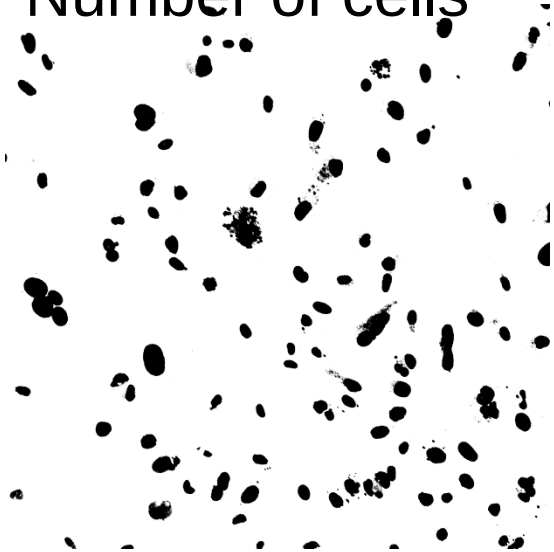


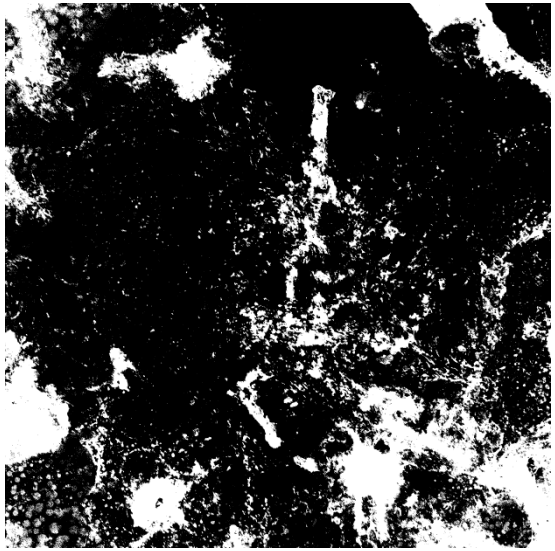
Cy3

Number of cells



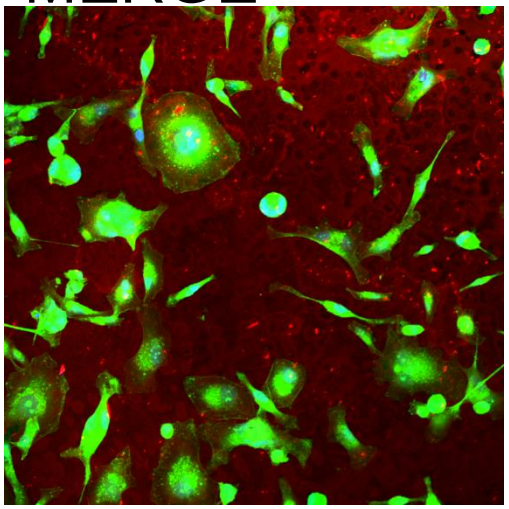
Cell area

Degraded area-white

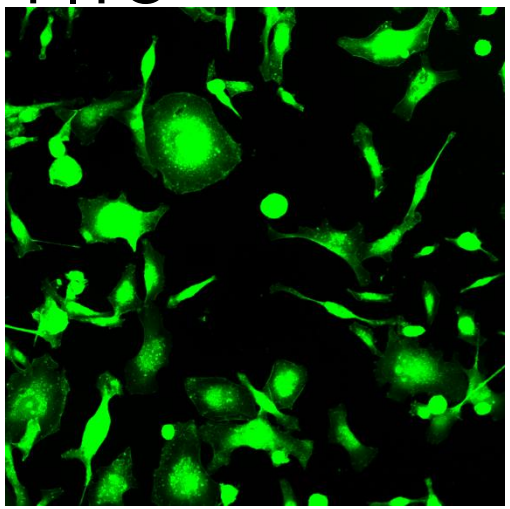


Modulation of gelatin degradation by HA-mimetic peptides. Assess activity of HA-peptide #40 on invadopodia formation. Magnification x20.

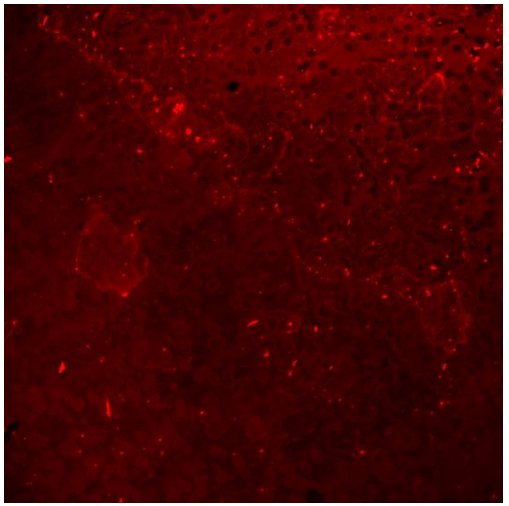
MERGE



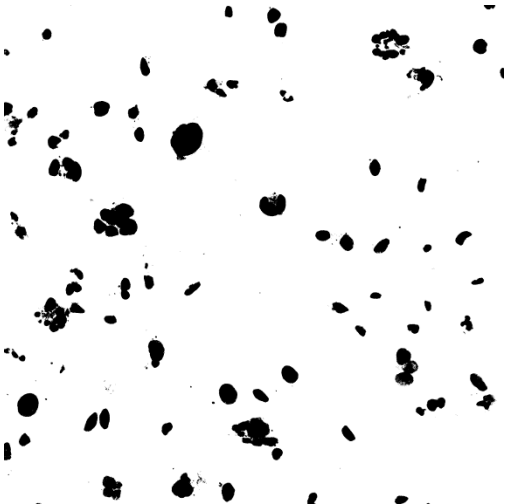
FITC



Cy3

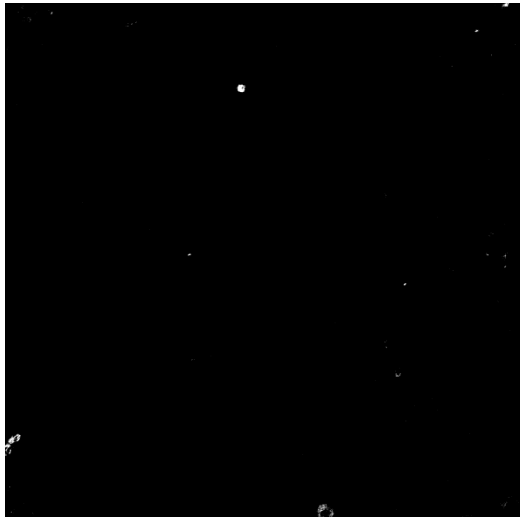


Number cells



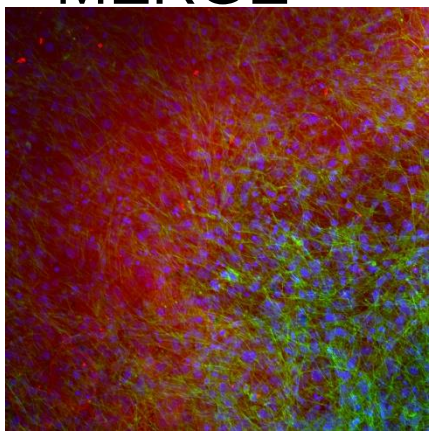
Cell area

Degraded area-white

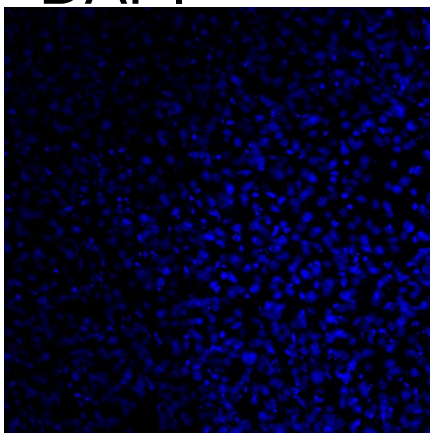


Analysis invasion in normal 10 T 1/2 cells. Fluorescent gelatin (Cy3) degradation and phalloidin/DAPI staining of 10 T 1/2 cells. Magnification x20.

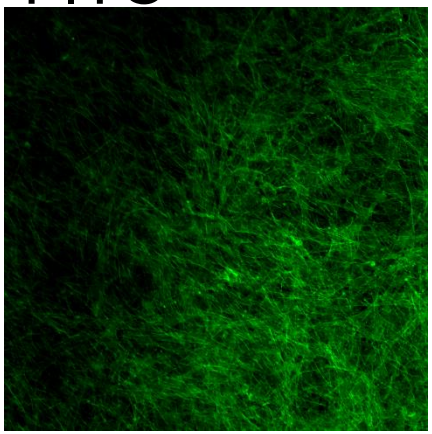
MERGE



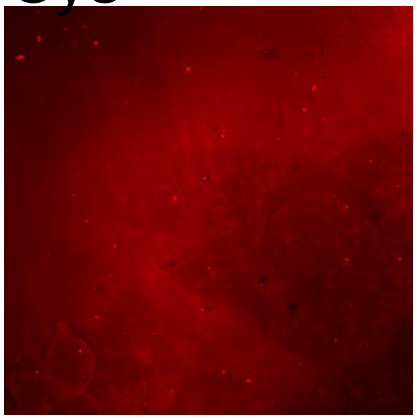
DAPI



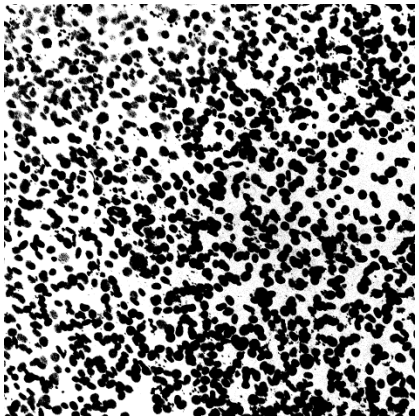
FITC



Cy3

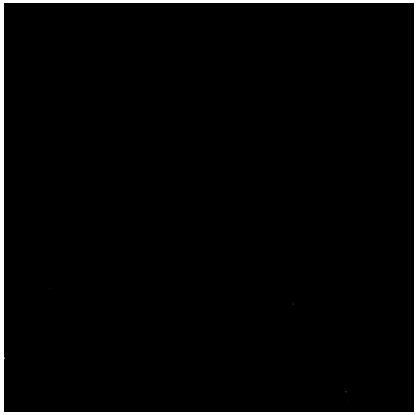


Number of cells

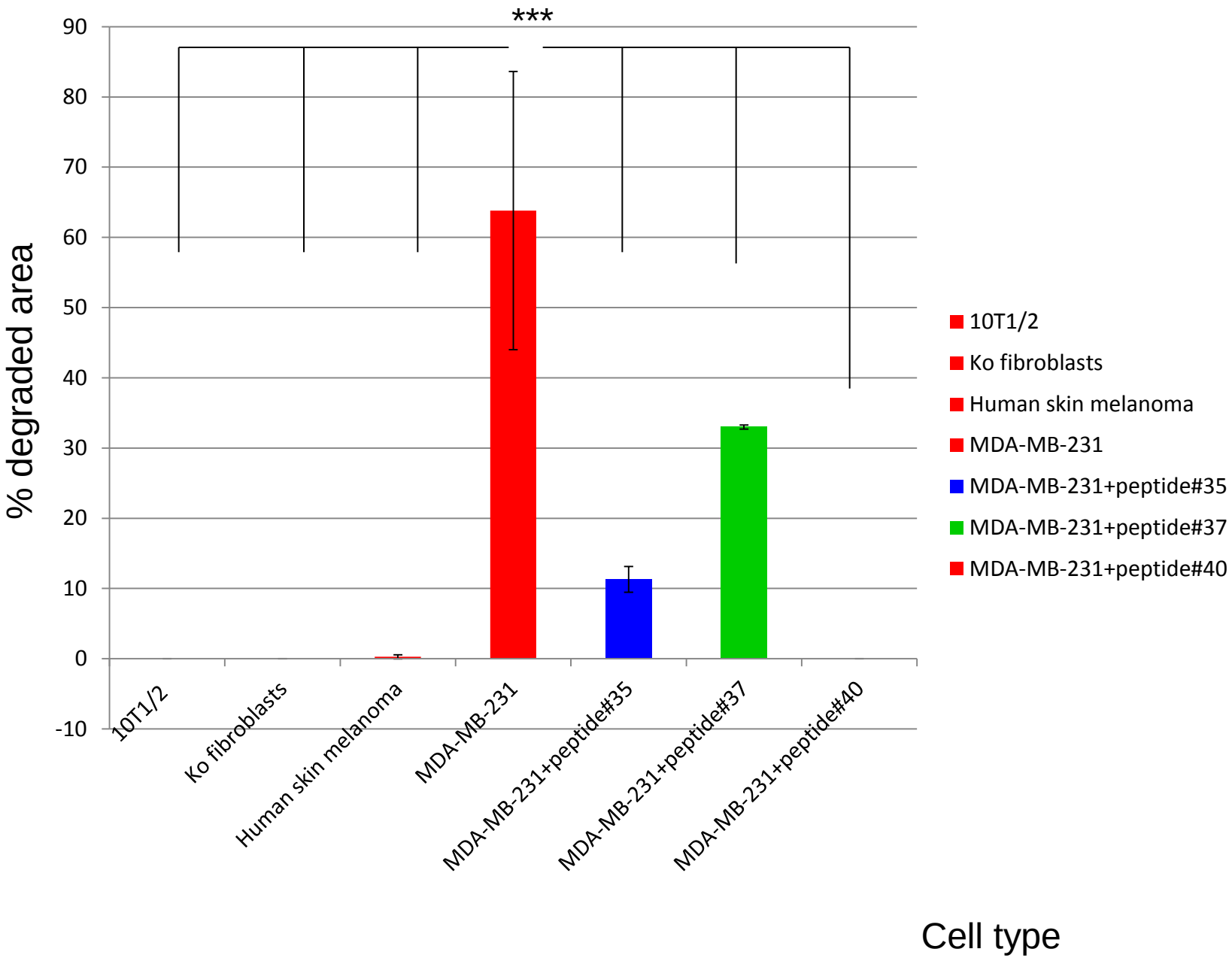


Cell area

Degraded area-white



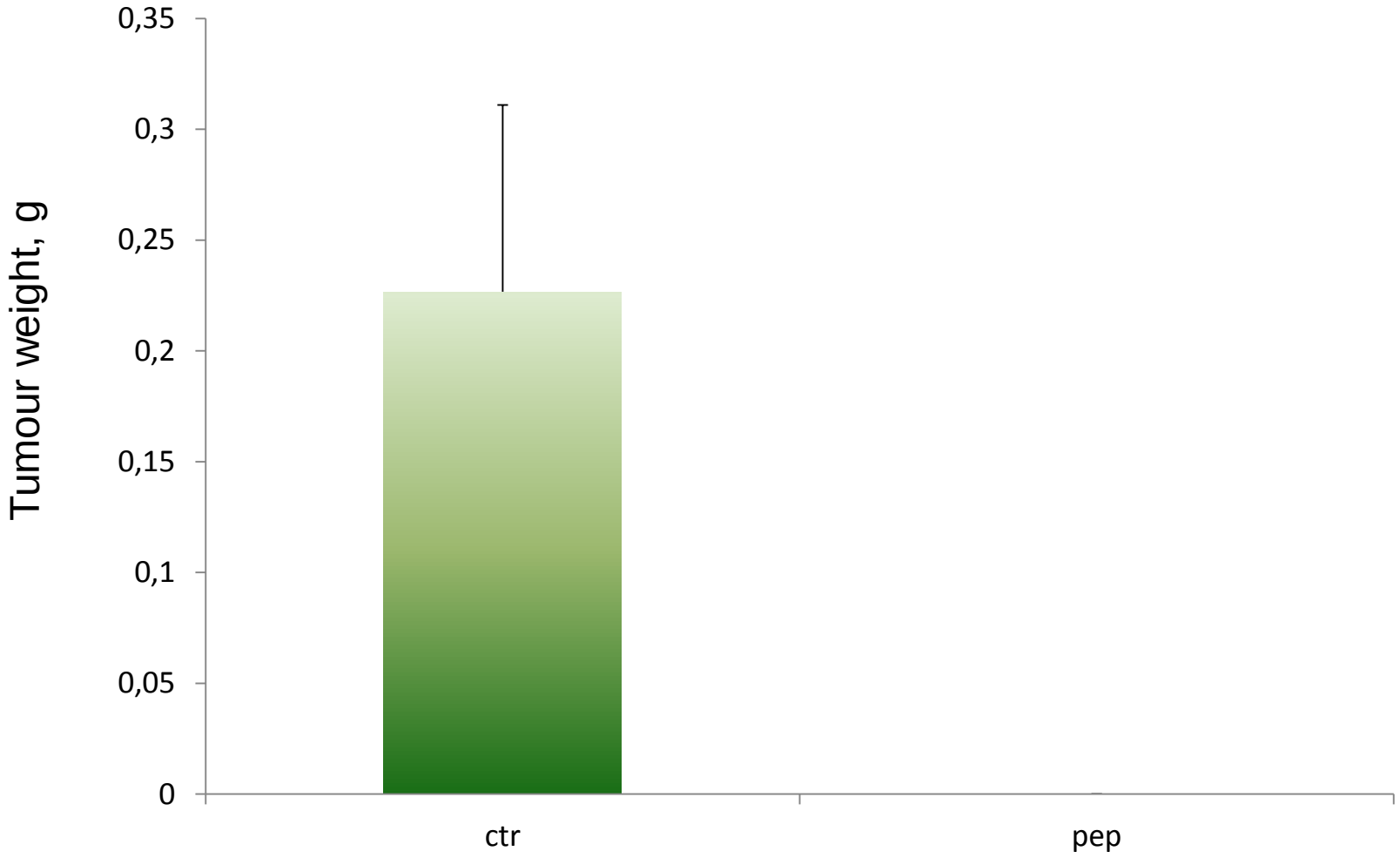
Cy3-Gelatin: % Degradation area of total cell area.



Methods screening anti-cancer drugs *in vivo*

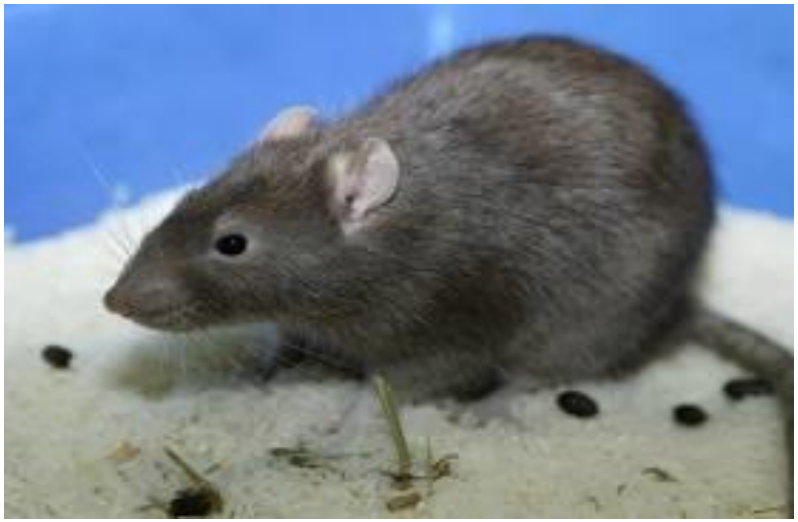
1. Growth prostate cancer cells, 2 millions.
2. Treatment cells with peptides, blank-untreated cells
3. Injection cells in mice, check the development tumors every week by palpation
4. After 2 months mice were killed, weight the tumors
5. Analysis development tumors compare with blank mice.

Effect peptide D (#135) on development prostate tumor in mice.



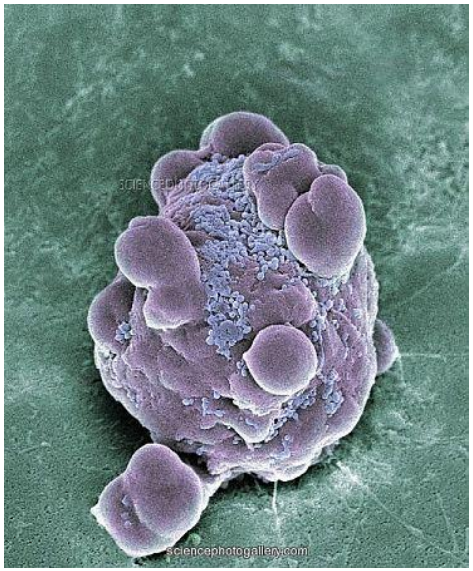
Study effect of apoptotic peptides on “shrinkage” tumor.

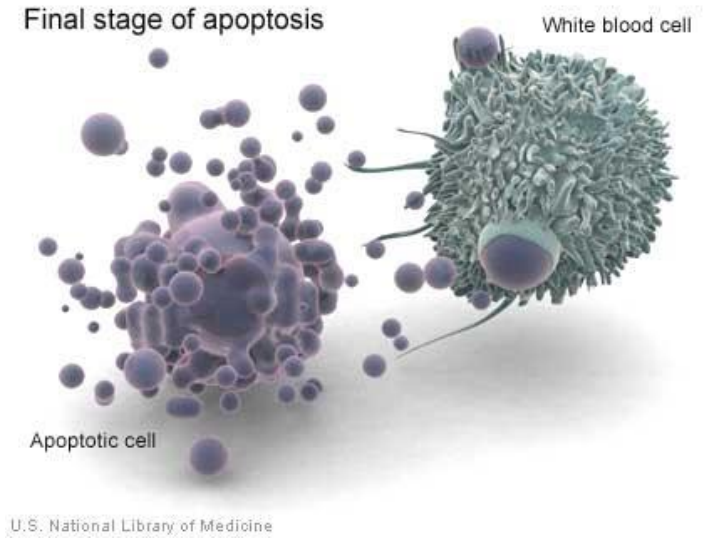
1. Take mice xenografts with developed tumors.
2. Use perfusion system, pump peptides inside&around the tumor.
3. Measure the size tumor during the time, then kill mice and weight of tumor.
4. Compare size and weight with untreated tumors.



+ apoptotic peptides= estimate “shrinkage” tumor

Study effect of pro-apoptotic peptides on shrinkage cancer.





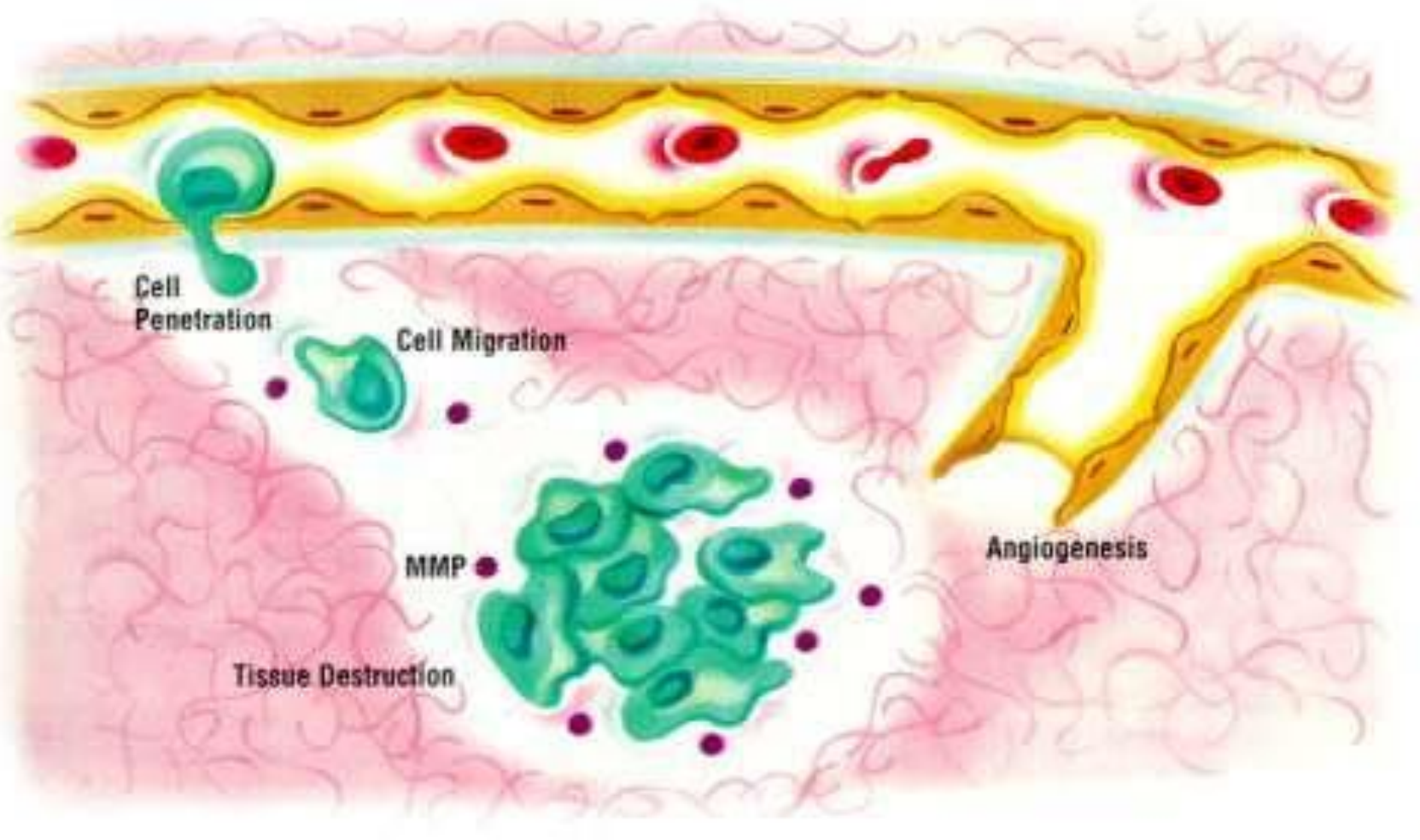
Screening of NO derivatives.

NO can have varied roles in the **tumor environment influencing DNA repair, cell cycle, and apoptosis.** It can result in antagonistic actions including DNA damage and protection from cytotoxicity, inhibiting and stimulation cell proliferation, and being both anti-apoptotic and pro-apoptotic.

Genotoxicity due to high levels of NO could be through direct modification of DNA (nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation and DNA strand breakage) and inhibition of DNA repair enzymes (such as alkyltransferase and DNA ligase) through direct or indirect mechanisms.

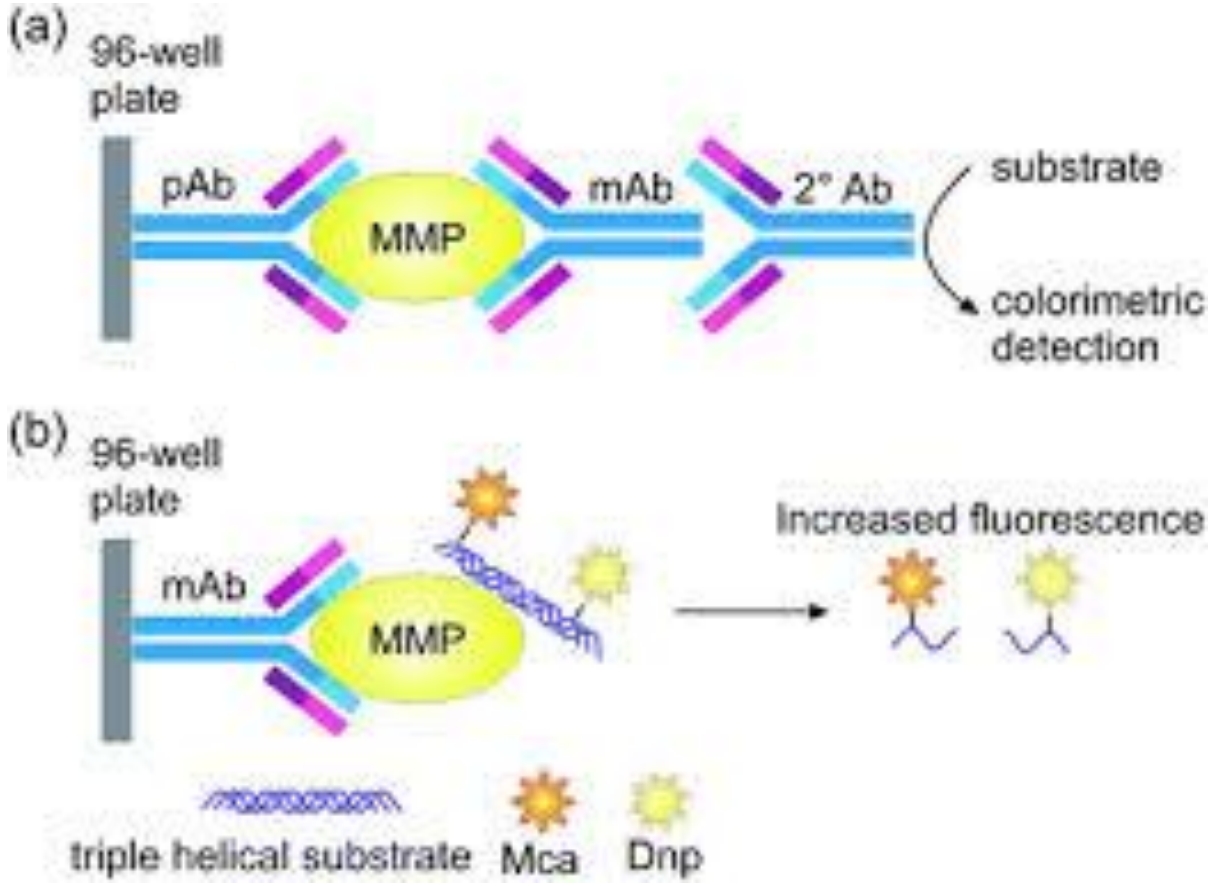
NO can stimulate angiogenesis and can also promote metastasis by increasing vascular permeability and upregulating matrix metalloproteinases (MMPs).

MMPs have been associated with several functions including cell proliferation, migration, adhesion, differentiation, angiogenesis and tumor cell metastasis. Therefore, the MMTs assays are important for anti-cancer drug screening.



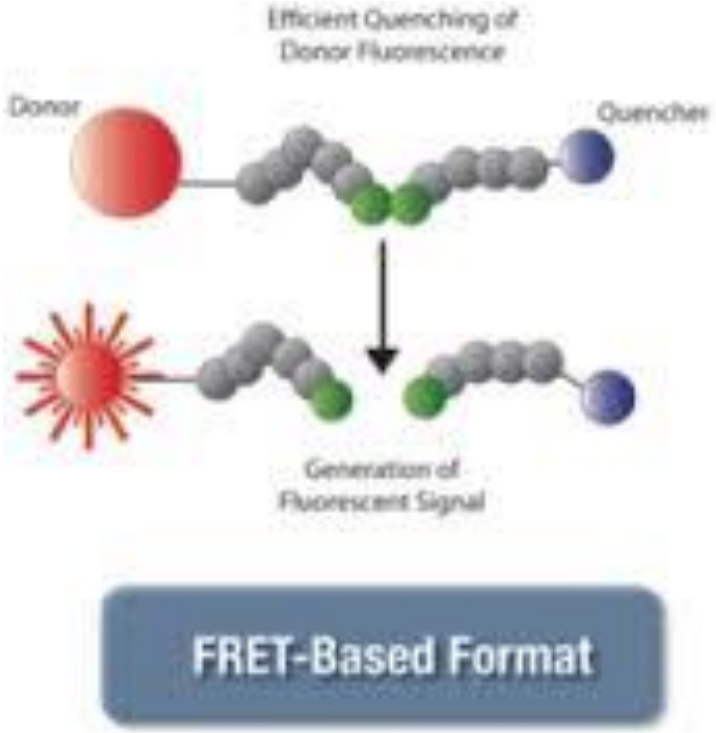
Metalloproteinase (MMPs) assays.

It is concluded that the assay for MMPs will permit the evaluation of these proteases as prognostic markers in cancer. Results from model tumour systems suggest that either increased levels of certain metalloproteases (MMPs) or decreased levels of their inhibitors correlate with invasion and metastatic potential. The levels of MMPs can be measured by enzyme-linked immunosorbent assay.



The matrix metalloproteinases (MMPs) assay

Amplite™ Universal Fluorimetric MMP Activity Assay Kit uses a Tide Fluor™ 2 (TF2)/Tide Quencher™ 2 (TQ2) fluorescence resonance energy transfer (FRET) peptide as a generic MMP activity indicator. It is designed to check the general activity of an MMP enzyme and to screen MMP inhibitors. In the intact FRET peptide, the fluorescence of TF2 is quenched by TQ2. After cleaved into two separate fragments by MMPs, the fluorescence of TF2 is recovered.



Myeloperoxidase (MPO) assay.

Myeloperoxidase (MPO), most abundantly present in neutrophils and monocytes, is a green hemoprotein having peroxidase activity.

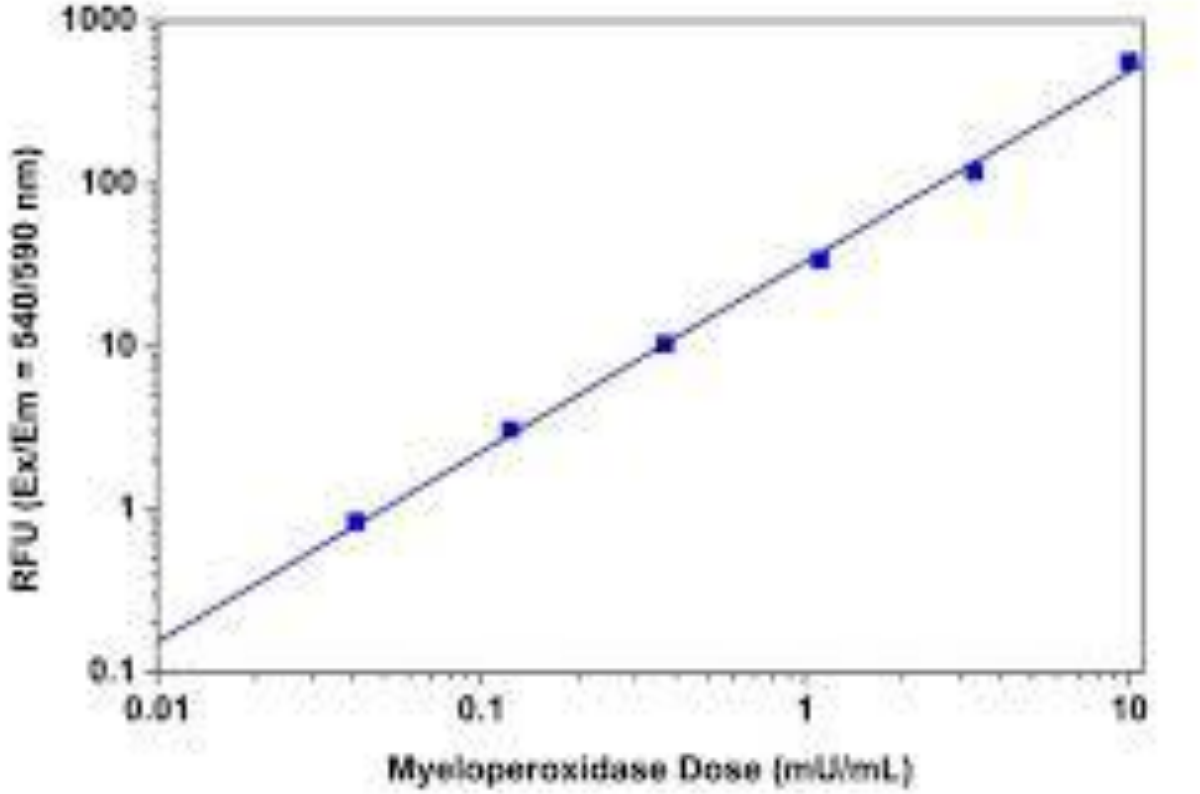
It catalyzes the reaction of hydrogen peroxide and halide ions to form cytotoxic acids and other intermediates; and plays an important role in the oxygen-dependent killing of tumor cells and microorganisms. There are considerable interests in the development of therapeutic MPO inhibitors.

Taurine chloramine thus formed is then allowed to react with 5-thio-2-nitrobenzoic acid (TNB). TNB has a chromophore that has maximal absorbance at 412 nm while its reaction product with taurin chloramine, 5-5-dithiobis(2-nitrobenzoic acid) or DTNB is colorless. By following the decrease of absorbance at 412 nm, MPO activity is (linearly) enhanced.

Amplite™ Myeloperoxidase Assay Kit provides a quick and sensitive method for the measurement of myeloperoxidase in solution and in cell lysates.

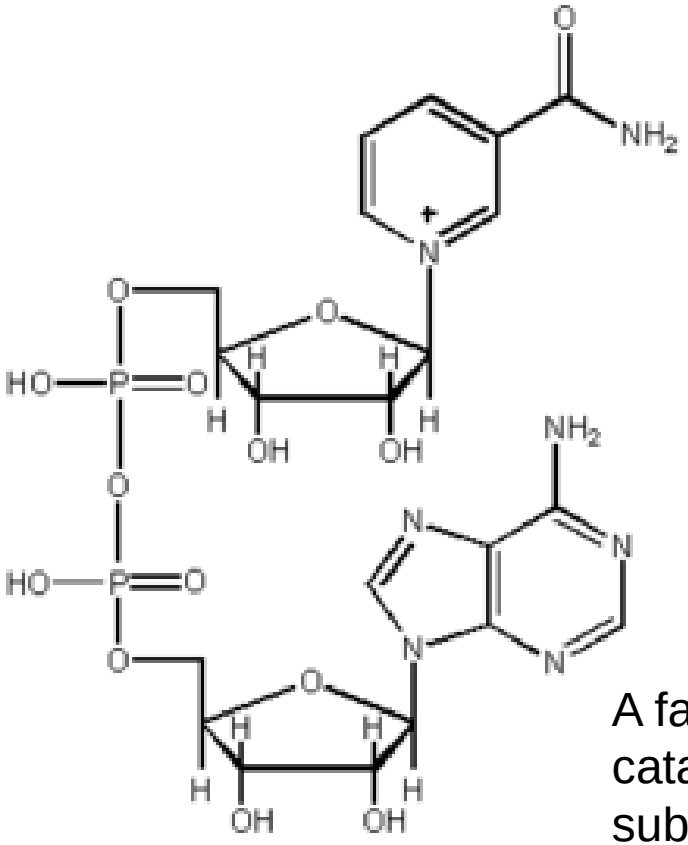
The kit uses our Amplite™ Red substrate which enables a dual recordable mode. The signal can be easily read by either a fluorescence microplate reader at Ex/Em = 540/590 nm or an absorbance microplate reader at ~576 nm.

The kit can be automated for high throughput screenings of MPO inhibitors.

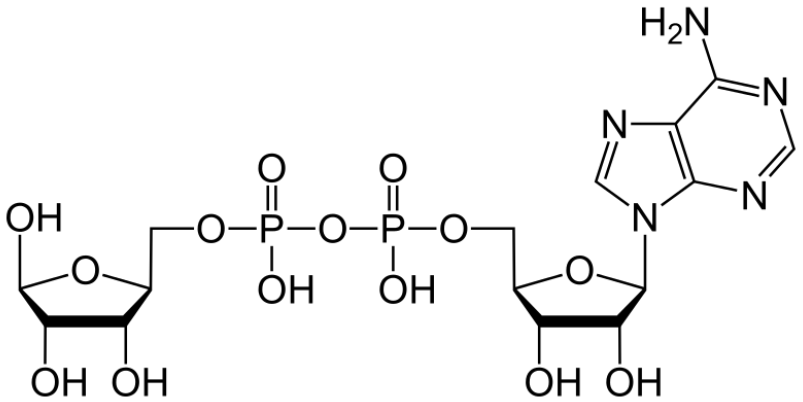


High-throughput screening of small molecules as potential PARP inhibitors.

The enzyme poly(adenosine 5'-diphosphate (ADP)-ribose) polymerase (PARP-1) catalyzes the formation of (ADP)-ribose polymers on a variety of protein acceptors in a NAD+ -dependent manner.



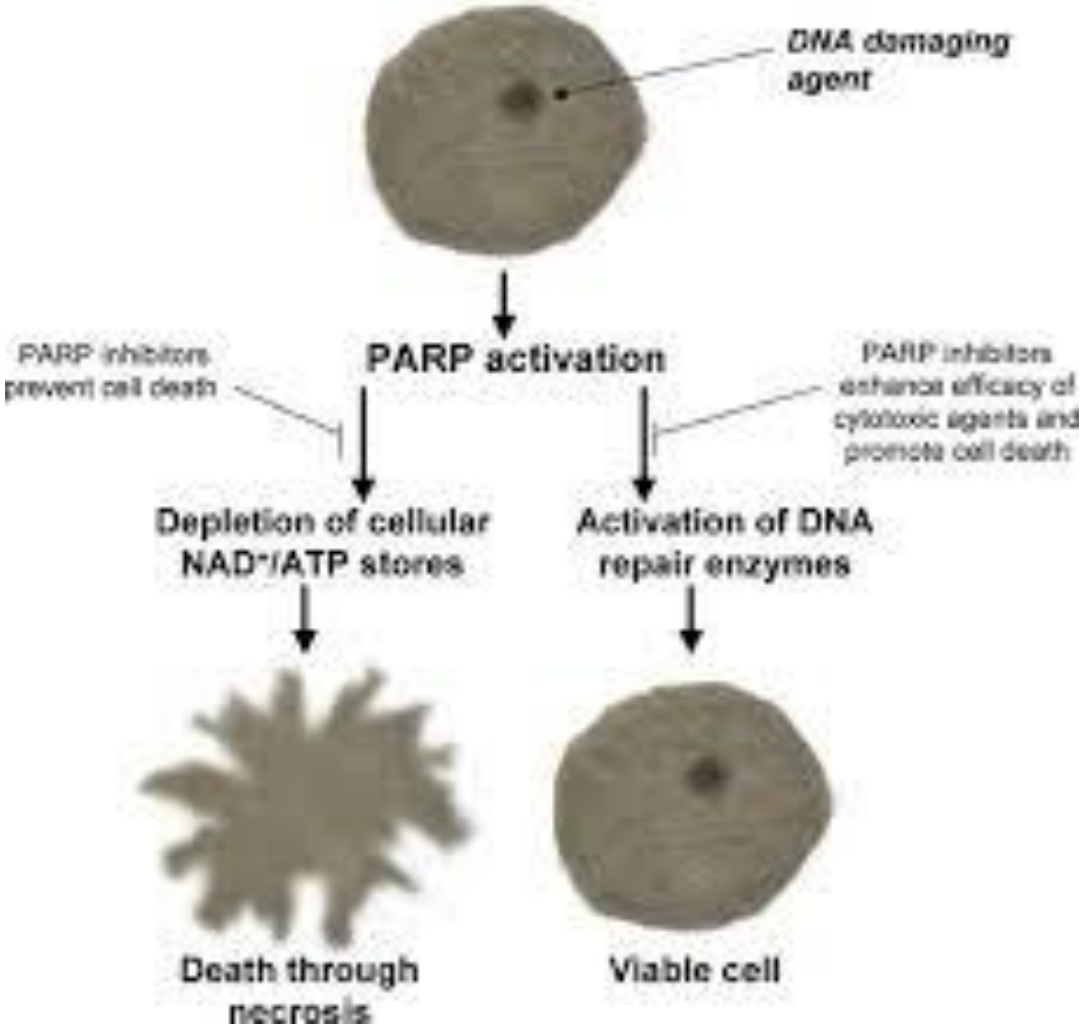
NAD+

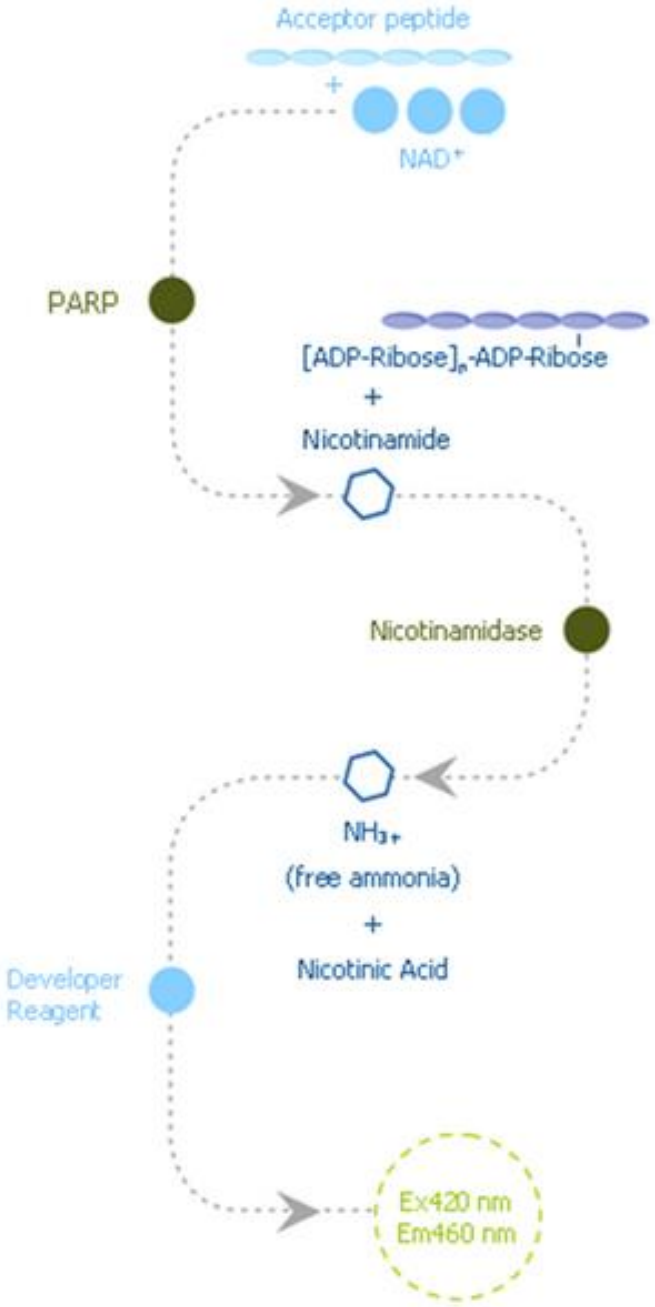


ADP

A family of poly(ADP-ribose) polymerases (PARPs) catalyzes the addition of ADP-ribose moieties onto substrate proteins via cleavage of NAD+ and liberation of nicotinamide.

Due to this dual role of PARP in the cell, small-molecule inhibitors of the PARP family of enzymes have been widely investigated for use as potentiators of anticancer therapies and as inhibitors of neurodegeneration and ischemic injuries.

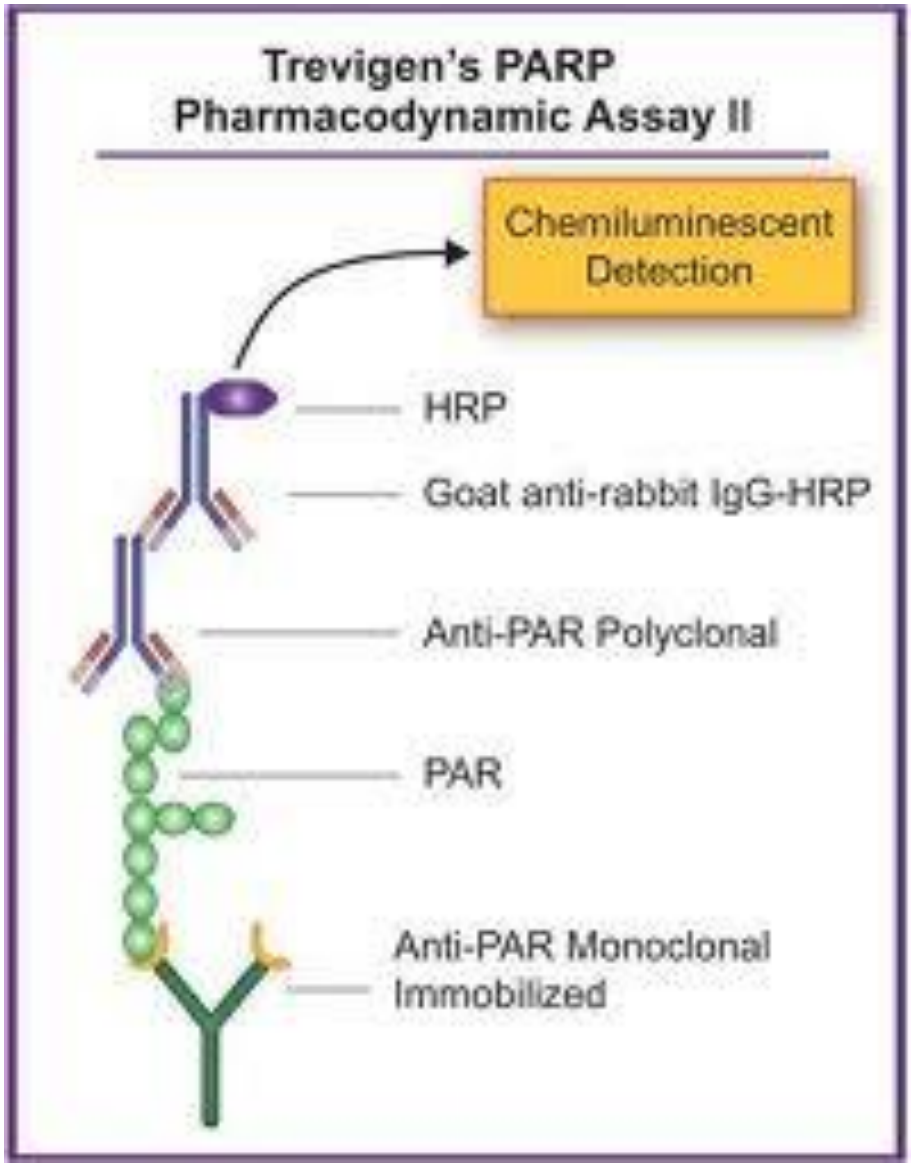




An enzymatic assay for poly(ADP-ribose) polymerase-1 (PARP-1) via the chemical quantification of NAD(+).

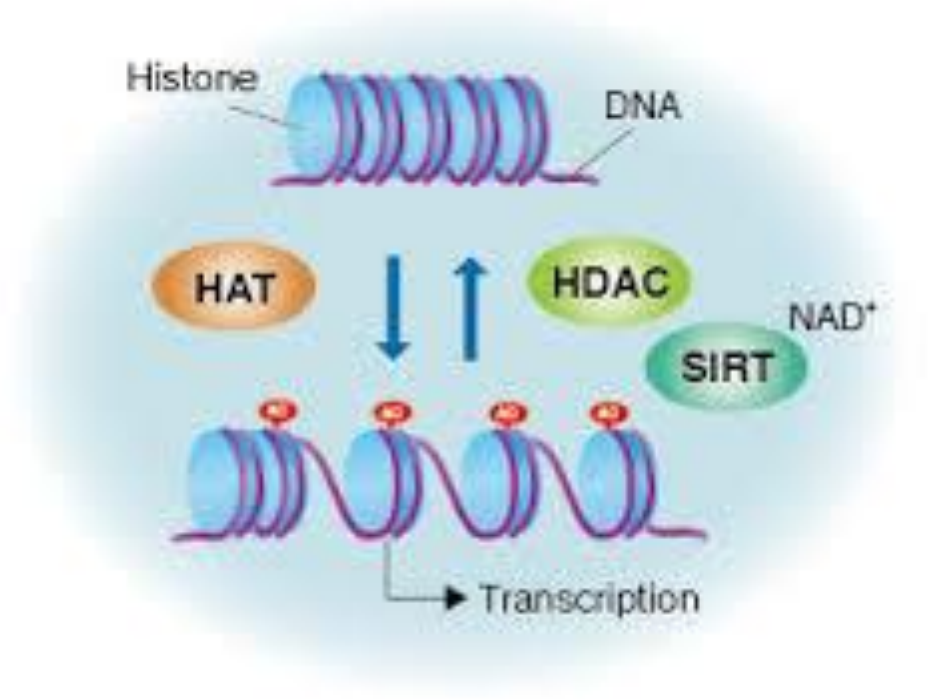
Assays for PARP activity and inhibition is a highly sensitive, inexpensive, and operationally simple assay for the rapid assessment of PARP activity that relies on the conversion of NAD+ into a highly fluorescent compound.

Immunoassay for PARP, using antibodies to poly(ADP-ribose).



Histone deacetylases (HDAC) are a class of enzymes

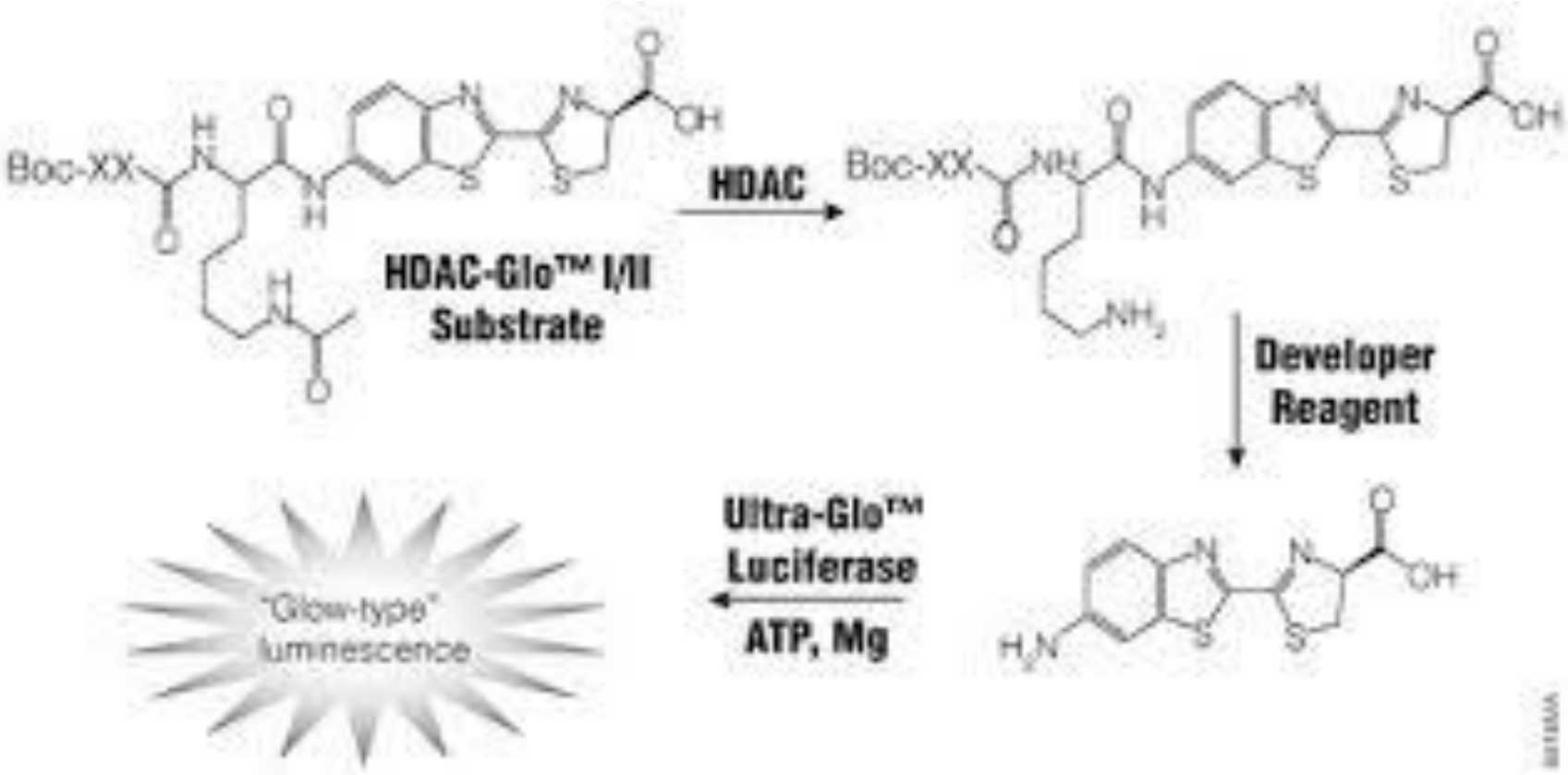
that remove acetyl groups from a ε-N-acetyl lysine amino acid on a histone. Deacetylation restores the positive electric charge of the lysine amino acids, which increases the histone's affinity to the negatively charged phosphate backbone of DNA. This process generally down regulates DNA transcription by blocking the access of transcription factors. HDAC inhibitors are being studied as a treatment for cancer.



Amplite™ Fluorimetric HDAC Activity Assay Kit

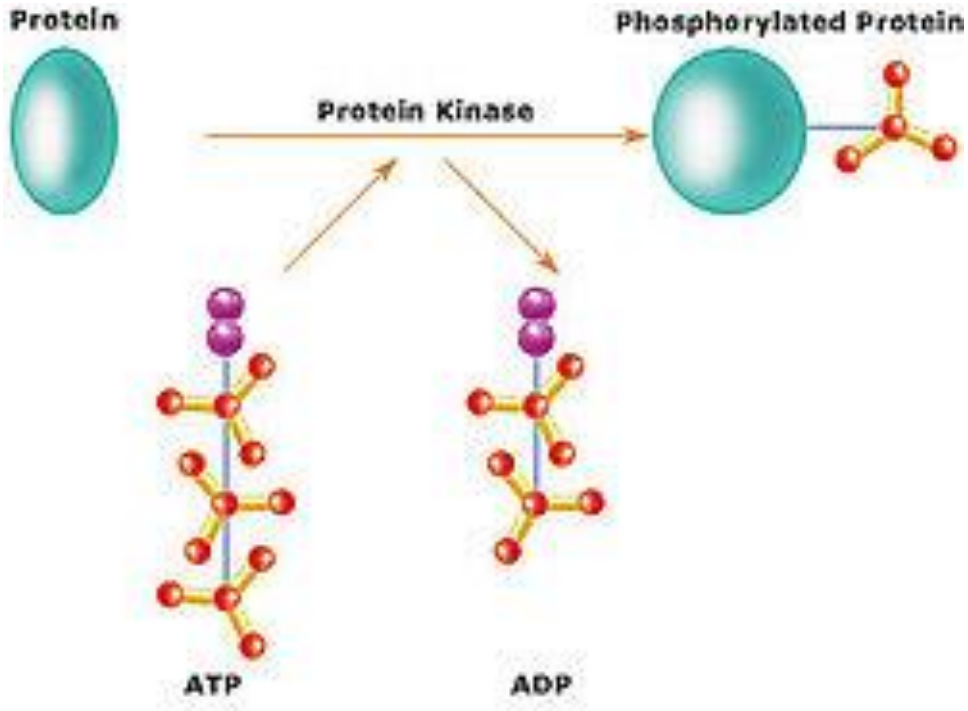
provides a quick, convenient, and sensitive method for the detection of HDAC activity. This kit uses our non-peptide HDAC Green™ substrate .

This kit can be used for measuring HDAC activity in cell lysates or HDAC inhibitor screening with cell extracts or purified enzymes. HDAC activity is monitored with excitation at 490 nm and emission at 525 nm.



Screening protein kinase inhibitors. Protein kinases assay.

Family of protein tyrosine kinases has been implicated in the promotion of cancer cell invasion and metastasis. Protein kinases are the enzymes that transfer a phosphate group from a phosphate donor to an acceptor amino acid in a substrate protein. Kinases are of great interest to researchers involved in drug discovery. Most of the commercial protein kinase assay kits are based on monitoring either the phosphopeptide formation or the ATP depletion.

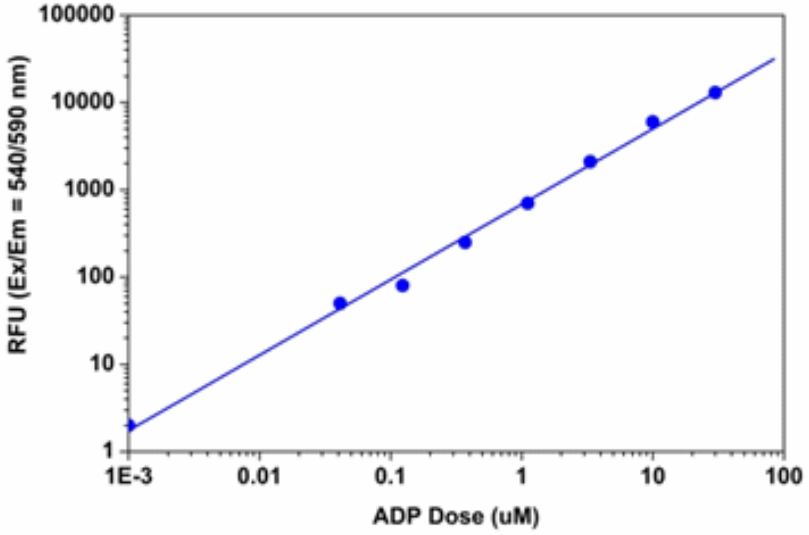


The Amplite™ Universal Fluorimetric Kinase Assay Kit

is based on monitoring ADP (ADP sensor, light sensitive) formation, which is directly proportional to enzyme phosphotransferase activity and is measured fluorimetrically.

This enzyme-coupled kit provides a fast, simple, and homogeneous assay to measure kinase activities.

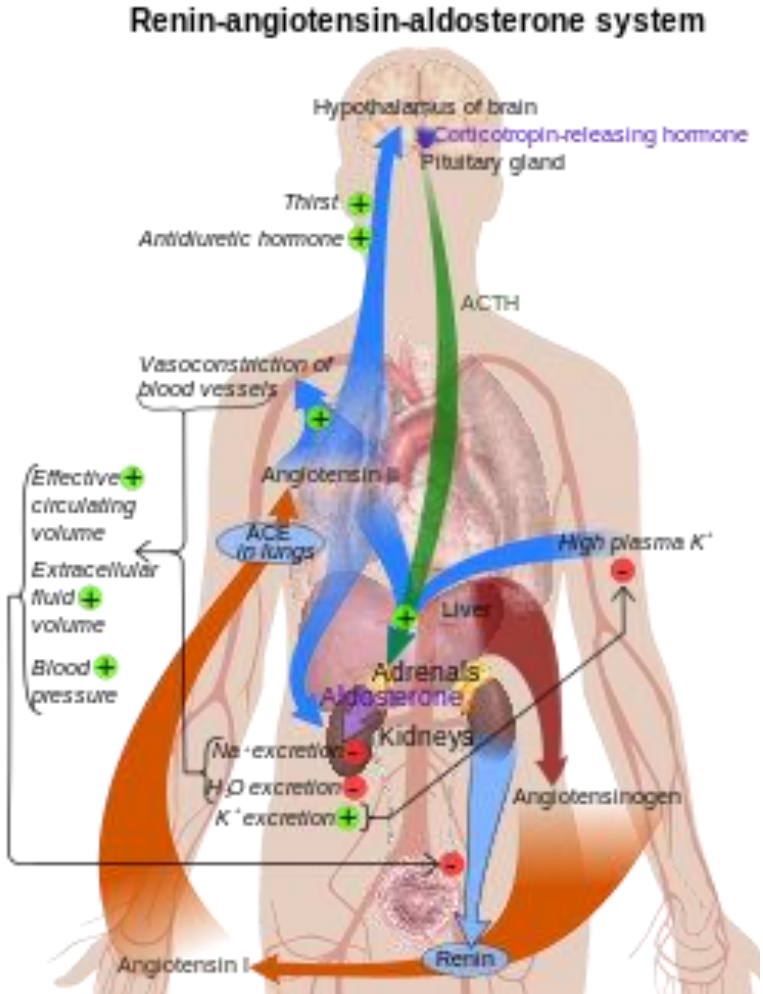
Its characteristics of high sensitivity (<0.3 μM ADP) and broad ATP tolerance (1-300 μM) make it an ideal kit for determining kinase kinetics and for screening and identifying kinase inhibitors.



ADP dose response was measured with the Amplite™ Universal Fluorimetric Kinase Assay Kit in a solid black 384-well plate. As low as 0.3 μM ADP can be detected with 30 minutes incubation.

Renin assay.

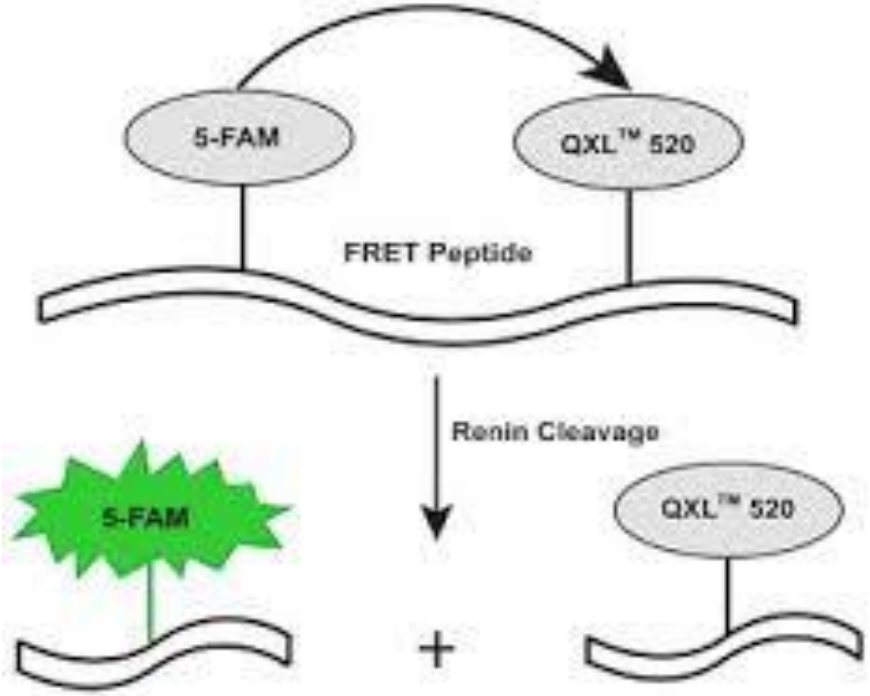
Renin is an enzyme that participates in the body's renin-angiotensin system (RAS). It regulates blood pressure and electrolyte homoeostasis. Renin has been identified to be an attractive target for the treatment of hypertension.

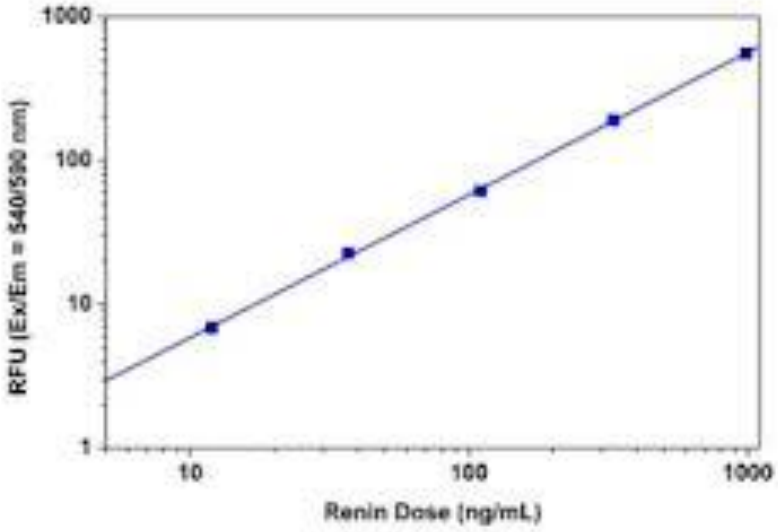


The Amplite™ Renin Assay Kit

provides a convenient assay for high throughput screening of renin inhibitors and renin activity using proprietary iFluor™ 488/TQ™ 520 fluorescence resonance energy transfer (FRET) peptide.

In the FRET peptide, the fluorescence of iFluor ™488 is quenched by TQ™ 520. Upon cleavage into two separate fragments by renin, the fluorescence of iFluor™ 488 is recovered, and the fluorescent signal can be easily monitored by a fluorescence microplate reader at Ex/Em = 490/520 nm.





Proteosome 20S Activity assay.

The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis.

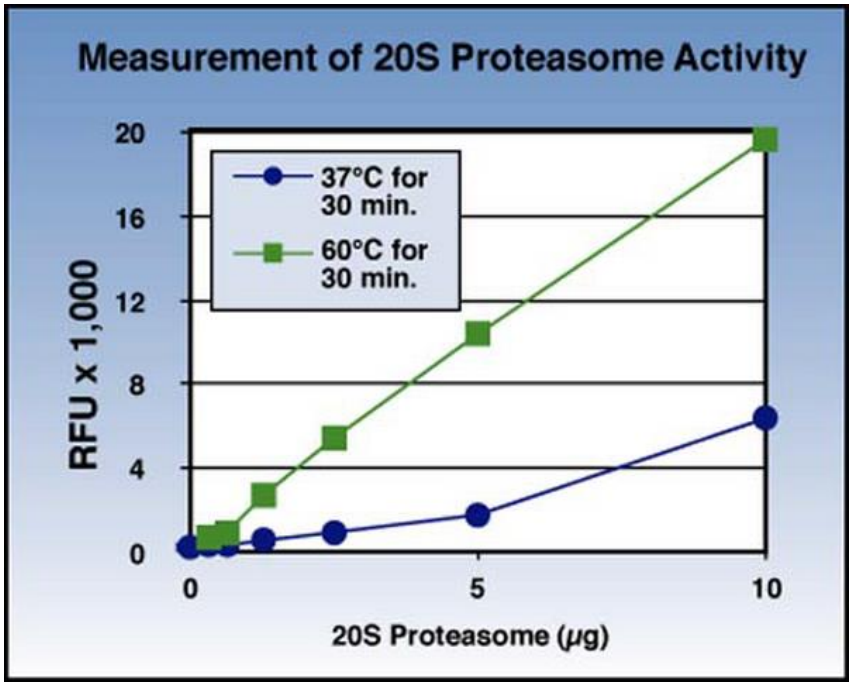
The most common form of the proteasome in this pathway is the 26S proteasome, an ATP-dependent proteolytic complex, which contains one 20S (700-kDa) core particle structure and two 19S (700-kDa) regulatory caps.

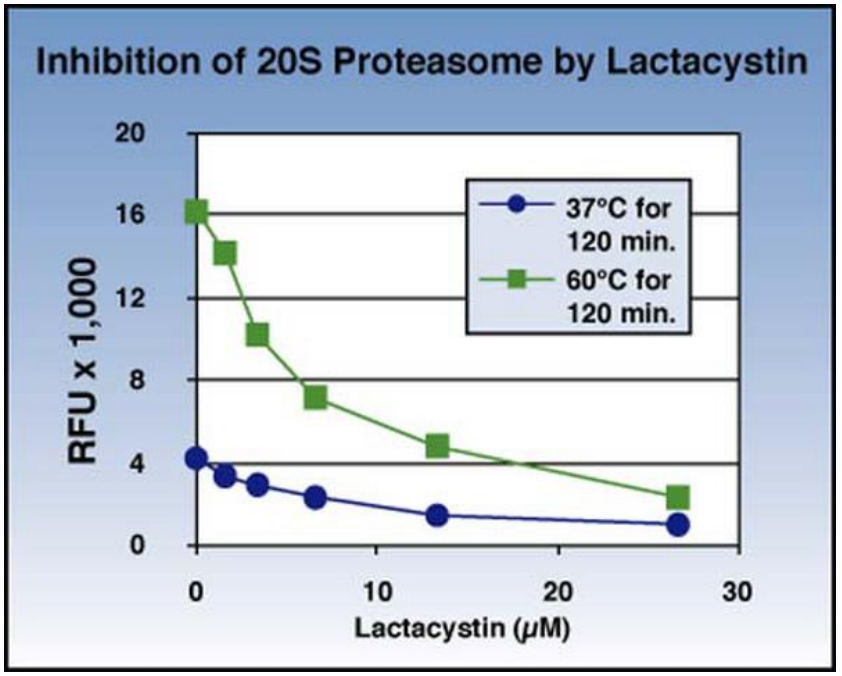
The 20S core contains three major proteolytic activities including chymotrypsin- like, trypsin-like and caspase-like activities. It is responsible for the breakdown of the **key proteins involved with apoptosis, DNA repair, endocytosis, and cell cycle control.**

Amplite™ Fluorimetric Proteasome 20S Activity Assay Kit

is a homogeneous fluorescent assay that measures the chymotrypsinlike protease activity associated with the proteasome complex in cultured cells.

This kit uses **LLVY-R110** as a fluorogenic indicator for proteasome activities. Cleavage of LLVY-R110 by proteasome generates strongly **green fluorescent R110** that is monitored fluorimetrically at 520-530 nm with excitation at 480-500 nm. The assay can be readily adapted for high-throughput assays to evaluate the proteasome activities or screen the inhibitors in cultured cells or in solution.





Thank you for your attention!

Q&A