DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of cell membrane biophysical properties and dynamics in the uptake of cell-penetrating peptides and the formation of local ligand concentration gradients

by Gabriella Tóth

Supervisors: Dr. Gyula Batta, Prof. Dr. Péter Viktor Nagy

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

Debrecen, 2026

The role of cell membrane biophysical properties and dynamics in the uptake of cell-penetrating peptides and the formation of local ligand concentration gradients

By Gabriella Tóth, Biology MSc

Supervisor: Prof. Dr. Péter Viktor Nagy, DSc
Dr. Gyula Batta, PhD

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Defense Committee**:	Prof. Dr. László Csernoch, DSc
Reviewers:	Dr. Barbara Zsebik, PhD
	Dr. Szilvia Veszelka, PhD
Members of the Defense Committee:	Dr. Beatrix Dienes, PhD
	Dr. Tamás Visnovitz, PhD

The PhD Defense takes place at the University of Debrecen, Faculty of Medicine, Department of Obstetrics and Gynaecology lecture hall
at 11:00 on 23rd March 2026

1 Introduction

1.1 Cell-penetrating peptides (CPP)

In the treatment of cancer and other difficult-to-treat diseases, both biotechnology and medicine strive to increase the effectiveness of drug molecule targeting. Most traditional chemotherapeutic agents, such as cyclophosphamide or paclitaxel, easily pass through cell membranes, but for many new molecules targeting intracellular or intraorganellar structures, the double phospholipid layer forms an impenetrable barrier. This is due to the inner hydrophobic layer of the membrane, which selectively inhibits the entry of larger, polar molecules. In addition, the effectiveness of cancer therapies is also limited by a lack of cell specificity. Small, electrically neutral or non-polar molecules, such as oxygen or carbon dioxide, easily pass through the membrane, while ionic or hydrophilic particles enter through channels and transport proteins. However, macromolecules larger than 500 Da, such as peptides, proteins, or nucleic acids, cannot spontaneously cross the membrane and require active or facilitated transport mechanisms. Several methods have been developed to overcome these challenges, such as liposomes, nanoparticles, viral vectors, and electroporation. One of the most significant breakthroughs was the discovery of cell-penetrating peptides. These oligopeptides consist of 8-30 amino acids and are able to pass directly through the cell membrane or enter cells by endocytosis. The first such peptides were identified based on their ability to enter cells, such as the TAT48-60 sequence derived from the HIV-1 TAT protein or penetratin derived from the Drosophila Antennapedia transcription factor. Derossi and colleagues described the translocation properties of the short 16-amino acid peptide pAntp(43–58), also known as penetratin, which corresponds to the third helix of the Antennapedia homeodomain. A few years after the discovery of the ability of TAT and the Antennapedia homeodomain to enter cells, several peptides with similar translocation abilities were identified, collectively referred to as SPPs (cell-penetrating peptides). SPPs typically have amphipathic, hydrophobic, and cationic properties, which determine their ability to enter cells. For therapeutic applications, these peptides can be made even more effective through chemical modifications that increase their stability and selectivity. Since their discovery, cell-penetrating peptides have been increasingly used in basic research on various cell types as effective transfection tools, and they are also present in translational research. In the latter area, SPPs have proven to be crucial in certain therapeutic methods: they have resulted in increased concentrations of drug molecules in tissue and cell regions that are difficult to access, thereby

enhancing therapeutic efficacy. SPPs have thus become a promising drug delivery system capable of delivering various molecules (proteins, DNA, antibodies, contrast agents) into cells with low cytotoxicity.

Despite the enormous potential of SPPs, their current clinical application is limited by their low bioavailability, short half-life, and lack of specificity.

1.2 Plasmamembrane

The study of biological membranes is an extremely complex field, partly due to their molecular structure and composition, and partly due to the wide range of their functions. The diversity of membrane functions is also reflected in their varied appearance: they range from nanometer-sized lipid-protein domains to micrometer-sized organelles with curved or tubular structures. The plasma membrane basically consists of a double layer of phospholipids, which are amphipathic lipid molecules. These molecules have a hydrophilic head and a hydrophobic tail. The hydrophilic heads are oriented toward the outer, aqueous surface of the membrane, while the hydrophobic tails are oriented toward the interior of the membrane. Key transmembrane proteins such as enzymes, receptors, and transporters are partially or completely embedded in this double layer. The diversity of lipids and the composition of proteins and other non-lipid elements (e.g., sterols, carbohydrates) determine the specific biological functions of membranes. Carbohydrates are present in the form of glycolipids and glycoproteins. The classic structure of plasma membranes was described by Singer and Nicolson's "fluid mosaic model" developed more than 50 years ago. This model assumed that the lipid bilayer of eukaryotic membranes forms a disordered, fluid "sea" in which lipid and protein molecules are arranged chaotically. This idea dominated membrane research for decades, but it also limited it, as the model was often applied to all other membranes. In 2014, Nicolson himself modified the original idea and gave the revised theory the name "dynamic complex model" or "modified fluid mosaic model." According to this, cell membranes are actually mosaic complexes consisting of numerous microdomains in which protein and lipid clusters form more ordered, heterogeneous areas. This modified approach also led to the discovery of "lipid rafts." In 1997, Simons and Ikonen proposed the concept of lipid rafts: small (10-200 nm), dynamic areas rich in sterols, glyco- and sphingolipids, where the density of lipid molecules is increased around certain proteins. It is important to note that the structure of the plasma membrane does not serve as the basis for all other membranes. For example, the lipid composition of chloroplast and mitochondrial membranes differs significantly from that of the plasma membrane. The plasma membrane is a complex system consisting of two layers of lipids and proteins organized into

nanodomains. Although both layers are asymmetrical, the connection between them is extremely strong, as evidenced by the fact that the nanodomains formed in the outer and inner layers are identical. The lipid composition of the plasma membrane is extremely diverse, depending on the organism, cell type, cell cycle stage, and environmental factors. The domains that define the plasma membrane can be classified into three main groups:

1. Membrane lipids and associated and integral membrane proteins. Lipid composition: the main lipids of the plasma membrane are glycerolipids, sphingolipids, and sterols. In addition, the lipid membrane also carries proteins. Based on their location, they can be divided into two large groups: integral membrane proteins and peripheral membrane proteins. Integral membrane proteins are embedded in the phospholipid bilayer, while peripheral membrane proteins (PMPs) include proteins that are directly attached to the lipid membrane and located on one side of the membrane (true peripheral membrane proteins) as well as those that bind indirectly through interactions with integral membrane proteins (membrane-associated proteins). The components of the membrane do not form a homogeneously distributed structure but are characterized by asymmetry. Flippases, floppases, and scramblases play an important role in this process.
2. Lipid rafts or nanodomains: Areas of concentrated lipids, known as lipid rafts or nanodomains, are created through the asymmetric distribution of lipids. This asymmetry is particularly noticeable between the two layers: there are more anionic lipids on the cytosolic side, while the extracellular side mostly contains neutral lipids. This asymmetry creates different electrostatic surfaces that influence the binding of proteins to the membrane and the activity of integral proteins. Lipid rafts are not equilibrium structures, but have different compositions: they are rich in cholesterol and sphingolipids and can be associated with palmitoylated proteins (e.g., ankyrin-G) and GPI-anchored proteins. They can exist simultaneously in different sizes and in different spatial and temporal forms. Their lifetime can range from microseconds and milliseconds to seconds. This versatility confirms that lipid rafts can undergo significant rearrangement in response to various biological stimuli.
3. The actin cytoskeleton and membrane-bound proteins: the network of actin filaments is located beneath the cell membrane and functions as a "grid," dividing the membrane area into compartments. The actin network provides a physical framework that promotes the structural cohesion of nanodomains. Transmembrane proteins are located in the membrane, and their cytoplasmic parts often collide with the actin cytoskeleton. This "collision" mechanism not only restricts the movement of proteins but also slows

down the diffusion of surrounding lipid molecules, contributing to the orderly, nanodomain-based organization of the membrane.

1.3 Entry into the cell: direct translocation and endocytosis

Initially, it was thought that cell-penetrating peptides (SPPs) mainly enter cells by direct translocation through the membrane. Direct penetration, also known as membrane transduction, is a unique, energy-independent process that can occur at low temperatures and in the presence of endocytosis inhibitors. This mode of entry is based on the interaction between positively charged cell-penetrating peptides (SPPs) and negatively charged membrane components. This interaction is followed by peptide entry, which occurs through transient pore formation or membrane destabilization. Two pore formation models have been described: toroidal pores and barrel-shaped pores. Other mechanisms are based on membrane destabilization, which is described by the "carpet-like" model and the inverse micelle mechanism. However, it later became apparent that certain experimental methods, such as cell fixation, cast doubt on the results. According to the latest accepted data, endocytosis is the main route of entry for most SPPs and SPP complexes. Endocytosis is a naturally occurring, energy-consuming process in which the cell engulfs external molecules. There are different types, such as macropinocytosis and clathrin- or caveolin-mediated endocytosis. The dominant pathway depends on the size and physicochemical properties of the molecule. While SPPs that enter by non-endocytic means go directly into the cytosol, peptides that enter by endocytosis enter the endolysosomal system. In order to reach their target and exert their biological effect, they must be released from the endolysosomes to avoid lysosomal degradation. Endolysosomal release is one of the main limiting factors for effective intracellular drug delivery. The entry mechanism of SPPs is as follows: they bind to the membrane through electrostatic interaction, as they are positively charged and the membrane phospholipids are negatively charged. They then undergo a conformational change (helix structure) and, as a result, integrate into the membrane. Their internalization can occur through endocytosis or direct translocation. After entry, they ensure delivery to the intracellular target: the peptides reach the cytoplasm, nucleus, or other intracellular target organelles and deliver the transported molecule to its destination. As research progresses, more and more information is being gathered about these processes. It has been shown that electrostatic interactions between SPPs and the lipid bilayer cause conformational changes in the peptides, which lead to their incorporation into the membrane and changes in their physical properties.

1.4 Factors influencing cellular uptake: dipole potential

The transport of charged substances across the plasma membrane is influenced by three types of membrane potential: the transmembrane potential, the surface potential, and the dipole potential. Dipole potential is an electrical potential inside the lipid membrane created by the favorable arrangement of water molecules associated with the membrane and the dipole side chains of lipids. This potential is in the range of 200–300 mV, which is several times greater than the transmembrane potential, and its strength can reach 108−109 V/m. As a result, the dipole potential has a significant effect on the conformation of transmembrane proteins, membrane binding, and transport of molecules. One of the most important determinants of dipole potential is the cholesterol content of the membrane. Cholesterol increases the potential both directly (through its own dipole moment) and indirectly, as it enhances the ordering of lipids and water molecules and modifies the dielectric constant of the membrane. Therefore, the potential is higher in the lipid raft domains of the membrane, which are rich in cholesterol. Statins reduce cellular cholesterol levels by inhibiting HMG-CoA reductase, thereby also reducing the dipole potential of the plasma membrane. These drugs are widely used to treat high cholesterol (hypercholesterolemia) because they effectively reduce the risk of cardiovascular disease. Atorvastatin, for example, is more effective than other statins because it can achieve the same LDL cholesterol-lowering effect at lower doses. Atorvastatin is present in its active form, unlike simvastatin or lovastatin, which first require enzymatic activation. Based on the principles described above, it can be assumed that membrane potentials influence the entry of positively charged penetratin into cells. Although the number of studies is limited, it has already been shown that the non-physiological elimination of transmembrane potential inhibits the uptake of positively charged SPPs. This confirms the critical role of membrane potentials in the intracellular transport of drugs.

1.5 Ligands

The plasma membrane not only forms a controlled, semipermeable boundary between the cell and its environment, but also contains numerous membrane proteins. Although the first step in transmembrane signal transduction is the binding of ligands to receptors, traditional research has typically focused on processes occurring on the cytoplasmic side of the membrane, such as phosphorylation. However, modern quantitative biophysical methods are increasingly revealing the complexity of receptor oligomerization, which is regulated by protein-protein interactions, membrane domains, and cytoskeletal dynamics. At the same time, ligand binding

itself can be highly complex: receptor affinity depends on the type of ligand and on the binding state of the receptor dimer, which leads to cooperative binding. A further difficulty in the binding of growth factors is the complex and dynamic structure of the plasma membrane. This aspect has long been overlooked, as even quantitative analyses were based on the simplifying assumption that the ligand is homogeneously distributed and uniform in the extracellular space. However, the study of ligand binding influenced by the membrane has led to the description of a number of unexpected phenomena. One such phenomenon is the apparent increase in receptor affinity, which may be caused by receptor clustering or hindered diffusion, facilitating the re-binding of dissociated ligands. In some cases, even the initial assumption that the ligand concentration in the extracellular space is uniform has been refuted. A ligand is a molecule that binds specifically to a receptor or other biomolecule, thereby eliciting a biological response. Ligands include proteins, hormones, drugs, neurotransmitters, and smaller molecules that can modify the conformation of a receptor by binding to it. As the concept evolved, the ligand-receptor relationship was initially understood as a simple association of rigid bodies, but later the decisive role of conformational changes became apparent. This conformational change can activate or inhibit the function of the receptor and indirectly influence the regulation of cell function. Ligand-receptor interactions are usually reversible and are mediated by various non-covalent interactions. Depending on where they work, ligands can be intracellular, binding to receptors inside cells (like estrogen), or extracellular, acting through receptors on the cell surface (like insulin or neurotransmitters). The interaction between ligands and their targets forms the basis of many chemical and biological processes.

1.6 Cell surface receptors: receptor tyrosine kinases

Cell surface receptors are embedded in the membrane: they are located on the outer surface of the cell, but are still found in the plasma membrane, and specifically bind extracellular hydrophilic ligands or other signaling molecules. Cell surface receptors can be classified into four main groups based on their function and mechanism of action: ion channel-linked receptors, G protein-linked receptors, enzyme-linked receptors, and adhesion receptors (or regulated intracellular signal transduction receptors). Enzyme-linked receptors are transmembrane proteins whose ligand-binding domain is located on the extracellular side of the plasma membrane. Their cytosolic portion either has independent enzymatic activity or is directly associated with an enzyme. They are characterized by the fact that each subunit usually contains a single transmembrane segment. These receptors were primarily identified based on

their role in mediating cellular responses to extracellular ligands, as they are key in regulating cell growth, division, differentiation, and survival. Currently, six main classes are known: receptor tyrosine kinases, tyrosine kinase-associated receptors, receptor-like tyrosine phosphatases, receptor serine/threonine kinases, receptor guanylate cyclases, and histidine kinase-associated receptors. Each group performs a wide range of biological functions. The largest family is that of receptor tyrosine kinases, which use ATP to catalyse the phosphorylation of specific tyrosine side chains on target proteins. The activation of receptor tyrosine kinases begins with the binding of the ligand to the extracellular domain. The inhibition of abnormal activation of tyrosine kinases, which can be caused by overexpression, mutation, or autocrine stimulation, is currently a central element of cancer therapy research. Numerous therapeutic agents are being developed specifically to block these pathological signaling mechanisms. Receptor tyrosine kinases are unique in that they function both as cell surface transmembrane receptors and as enzymes with kinase activity. Their structure consists of three main parts: a multi-domain extracellular ligand-binding region, a single hydrophobic transmembrane helix, and a cytoplasmic domain. Regulatory sequences are attached to both the N-terminal and C-terminal ends of the kinase domain. During the activation of receptor tyrosine kinases, ligand binding induces dimerization, which allows mutual transphosphorylation of the cytoplasmic regions.

The first receptor tyrosine kinase to be identified was the epidermal growth factor receptor (EGFR).

1.7 Epidermal growth factor receptor (EGFR) and ErbB2

The epidermal growth factor receptor is a member of the ErbB receptor family, which belongs to the superfamily of receptor tyrosine kinases. The ErbB family comprises four receptors: EGFR (also known as ErbB1 or HER1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4). EGFR is a single transmembrane domain protein consisting of several structural elements: an extracellular ligand-binding domain, a transmembrane region, a juxtamembrane segment, a kinase domain, and a C-terminal regulatory "tail." EGFR can activate several intracellular signaling pathways, including the Ras/Raf/MAP kinase pathway. Its activation is triggered by various extracellular ligands, such as epidermal growth factor (EGF). EGFR was one of the first receptors for which ligand-induced dimerization was described as a fundamental molecular mechanism of transmembrane signaling. Epidermal growth factor itself is a polypeptide consisting of 53 amino acids and is one of the most important representatives of

growth factors. It exerts its effects through a plasma membrane receptor, EGFR, with a molecular weight of approximately 170 kDa. The tissue concentrations of EGF and EGFR are modulated by endocrine factors such as thyroid hormones, estrogen, testosterone, and growth hormone, suggesting that certain growth and developmental effects of these hormones may be mediated by EGF. ErbB2 is a transmembrane tyrosine kinase whose overexpression on the cell surface is associated with tumorigenesis and poor prognosis in breast cancer. ErbB2 is not capable of ligand binding on its own, but primarily acts as a preferred heterodimerization partner for ligand-activated sister receptors, thereby amplifying mitogenic signaling. This peculiarity can be explained structurally by the "open" conformation of the extracellular domain, which mimics the activated, ligand-bound state of EGFR. Two conflicting models have been proposed to explain the endocytic trafficking of ErbB2. One model suggests that ErbB2 is efficiently recycled after basal endocytosis and that the same fate applies to activated EGFR–ErbB2 heterodimers. The other model believes that dimers containing ErbB2 remain on the cell surface and avoid endocytosis. Both approaches emphasize the importance of ErbB2 endocytic trafficking for normal function and oncogenesis associated with overexpression. Although ErbB2 endocytosis is of paramount importance in cancer therapy, the details of the process are poorly understood. Antibody assays suggested that ErbB2 is capable of inducing endocytic degradation like EGF-stimulated EGFR. However, subsequent experiments with chimeric receptors have shown that EGFR is unique in that other ErbB receptors, including ErbB2, do not possess this property. It has been demonstrated that the ErbB2 cell surface pool is dynamic: it undergoes basal endocytosis followed by rapid and efficient recycling.

Fluorescently labeled ligands are often used to study these receptors and mechanisms. TAMRA-EGF is one such molecule: epidermal growth factor is labeled with the fluorescent dye tetramethylrhodamine (TAMRA). Labeling is performed in a site-specific manner using TAMRA-aminophenylalanine incorporated into the protein, which allows for precise fluorescent tracking and thus examination of EGFR using fluorescence microscopy and other fluorescence-based methods.

1.8 Formation of ligand concentration gradient and its examination by FCS measurement

Certain parameters, such as the classical equations used to determine the binding affinity of ligands to target receptors, are based on the assumption that ligands diffuse freely, are evenly distributed, and their concentration in the immediate vicinity of the receptor is the same as in the general (bulk) aqueous phase. However, it is known that drug molecules can interact

directly with the plasma membrane, which can lead to an increase in local ligand concentration in the vicinity of the receptors. For ligands that incorporate into the lipid bilayer, it has been suggested that local concentrations may significantly exceed those measured in the bulk phase due to the formation of "reservoirs" in the membrane. Ligand diffusion from the membrane and interactions at the interface between the membrane and the surrounding aqueous medium may also contribute to higher ligand concentrations in the immediate vicinity of the cell membrane. Fluorescence correlation spectroscopy (FCS) detects intensity fluctuations occurring during the diffusion of fluorescent molecules in a small (~0.25 fL) confocal volume, and then, based on their autocorrelation analysis, the concentration and diffusion coefficient of the molecules under investigation can be determined. FCS is based on the fluctuation-dissipation theorem and provides an opportunity to study the kinetic and thermodynamic properties of fluorescently labeled molecules in solution. The method is based on the analysis of spontaneous fluctuations in the detected fluorescence intensity: the temporal relaxation of the fluctuations provides information about the kinetic characteristics of the system, while their amplitude provides information about the thermodynamic parameters. FCS can also be used in systems that are in a permanent steady state, whether in equilibrium or non-equilibrium conditions. Although the average concentration in these systems remains constant over a long period of time, spontaneous fluctuations occur continuously at the local level as a result of Brownian motion (spatial movement of molecules) and chemical reactions (Poisson processes). Molecules continuously enter and exit the small volume under investigation, causing temporary changes in local concentration. If the molecules are fluorescently labeled, this also results in fluctuations in fluorescence intensity. The operating principle of FCS is based on the fact that fluorescently labeled molecules emit fluorescence photons when excited by a laser. These photons are collected by the microscope objective and transmitted to the optical system. The returning light still contains the excitation radiation, which is separated by a dichroic mirror: only emission photons with longer wavelengths are allowed to pass through. The light selected in this way passes through an emission filter, which ensures that only fluorescence reaches the detector. The signal then enters the pinhole of the confocal system, which filters out photons arriving outside the focal plane, creating an extremely small detection volume in the femtolitre range. Finally, the light reaches a highly sensitive avalanche photodiode (APD), which converts individual photons into electrical signals, allowing the intensity fluctuations resulting from the number and movement of molecules to be recorded. The key to this method is precisely this small, well-defined detection region created by confocal optics. An autocorrelation function is calculated from the fluorescence intensity for different time delays, and the resulting curve is

fitted to appropriate theoretical models. This modeling allows the number of molecules, their diffusion time, and parameters that refer to different interactions or states of the molecules (e.g., triplet state) to be determined.

2 Aims

I examined membrane phenomena, the biophysical properties of membranes, and their circulation from several perspectives. Concerning the effects exerted on the uptake of cell-penetrating peptides, we set the following goals:

- Increasing the efficiency of cell penetration of cell-penetrating peptides by altering the dipole potential, an important factor in membrane potential
- Increasing cell penetration and endosomal release using atorvastatin
- To investigate the role of endocytosis as a mechanism of cell entry in the penetration of SPPs
- To determine the extent of lysosomal degradation of fluorescently labeled cell-penetrating peptides after cell entry
- Determination of the cell entry efficiency of modified β-penetratin
- Determination of the extent of β-penetratin "self-inactivation"
- Determination of the threshold concentration measured during β-penetratin cell uptake
- Investigation of the aggregation ability of penetratin using FRET measurement
- Investigation of the aggregation ability in light of its effect on the entry efficiency of cell-penetrating peptides

In the second phase of my work, I examined how EGF, which is a membrane-impermeable ligand, affects the ligand concentration in the extracellular space after binding through its active, membrane-related functions:

- Determination of ligand concentration by fluorescence correlation spectroscopy (FCS), taking into account the distance measured from the plasma membrane
- Consequences of extracellular matrix digestion in terms of membrane traffic and ligand concentration
- The effect of ion channels on membrane traffic and the observed peak ligand concentration near the membrane
- Investigation of the TAMRA-EGF concentration gradient in cells and giant plasma membrane vesicles
- Application of a BODIPY-based molecular rotor for viscosity measurement

3 Materials and methods

3.1 Experiments aimed at increasing the cellular uptake of cell-penetrating peptides

3.1.1 Cell lines

We conducted our experiments using two different cell lines: MDA-MB-231 and SKBR-3.
MDA-MB-231 is a human breast cancer cell line that is often used to model triple-negative breast cancer (TNBC). TNBC is characterized by the absence of estrogen receptor, progesterone receptor, and HER2 expression, and therefore does not respond to hormonal therapies or HER2-inhibiting treatments.
The SKBR-3 cell line is also derived from human breast cancer and is an important model for HER2-positive breast cancer research. This cell line is characterized by overexpression of the HER2 gene, which plays a key role in the biology of HER2-positive tumors.
Both cell lines were cultured in DMEM medium according to their specifications, supplemented with 10% fetal bovine serum (FBS), 50 µg/ml gentamicin, and 2 mM L-glutamine. The cells were grown in cell culture flasks at 37 °C in a thermostatically controlled incubator containing 5% CO_2 and were passaged every 2–3 days, depending on the degree of confluence.
For microscopic measurements, cells were cultured in 8-well chambers until they reached ~80% confluence. For flow cytometric analysis, cells were grown in T-25 or T-75 culture flasks, depending on the number of cells required. In these cases, measurements were performed in cell suspension. This required the removal of cells from the bottom of the flasks, which was achieved using a trypsin-EDTA-PBS solution (0.05% trypsin, 0.02% EDTA).

3.1.2 Cell-penetrating peptides

Penetratin was synthesized by our collaboration partner, István Mándity (Semmelweis University, Budapest), on TentaGel R RAM resin. The peptide was prepared using standard Fmoc chemistry in Merrifield's solid-phase synthesis. Half of the penetratin was labeled overnight with AlexaFluor532 N-hydroxysuccinimidyl ester. The other half was reacted with 5(6)-carboxynaffluorescein N-succinimidyl ester. The labeled peptides were deprotected, removed from the resin, and the crude products were prepared by filtration and precipitation. They were purified by preparative reverse-phase HPLC on a C18 column and then freeze-dried. The purity of the products was checked by analytical reverse-phase HPLC on a C18 column,

while the authenticity of the peptide was verified by Bruker electrospray ionization mass spectrometry. These methods also made it possible to examine the modified version of penetratin. Hereinafter, I will refer to this peptide as β-penetratin; its structure cannot be published due to the planned patent application.

3.1.3 Use of substances affecting dipole potential

We examined the effect of various substances on the dipole potential of the membrane: phloretin reduces the dipole potential in the membrane, while 6-ketocholestanol increases the sterol content of the membrane and thus also the dipole potential. In addition, Pluronic acid aids in the solubilization of hydrophobic molecules. We used DAPI to map the ratio of live to dead cells. The MDA-MB231 and SKBR-3 cells were first incubated with dipole potential-modifying agents for 10 minutes, then a solution containing 1μg/ml DAPI was added for 5 minutes at room temperature. In addition, we used two different fluorescently labeled penetratins, AlexaFluor 532 and Naftrofluorescein, at a concentration of 5 μM. Incubation took place at 37°C for 20 minutes, so we also increased the temperature of the treatment solutions before their application. We performed the measurement using a BD FACS Aria III flow cytometer, omitting the washing step.

3.1.4 Investigation of the effect of atorvastatin on dipole potential

Atorvastatin reduces membrane dipole potential by reducing sterol content through inhibition of cholesterol biosynthesis. Twenty-four hours after placing MDA-MB231 and SKBR-3 cells in flasks, the cells were treated with atorvastatin. Atorvastatin was used in four concentrations: 1 nM, 10 nM, 100 nM, and 10 μM, with an incubation time of 48-72 hours. DAPI was used at a concentration of 1 μg/ml, and AlexaFluor 532 and Naftrofluorescein-labeled penetratin were used at a concentration of 5 μM. Incubation was performed at 37°C for 20 minutes. Without washing, we measured as described in previous experiments, under the same excitation and detection conditions, using a BD FACS Aria III flow cytometer.

3.1.5 Blocking the endocytosis with Dyngo4a dynamin inhibitor

To inhibit endocytosis, we used the dyngo4a dynamin inhibitor, which inhibits dynamin-dependent endocytosis, as a result of which fluorescently labeled cell-penetrating peptides can only enter cells via dynamin-independent endocytosis or direct membrane penetration. Dyngo4a was used at four different concentrations: 5 μM, 10 μM, 20 μM, and 40 μM, with an incubation time of 15 minutes. The action of the dynamin inhibitor was monitored using

Transferrin AFDye 647, which was used in a concentration of 50 µg/ml in all cases, with an incubation time of 15 minutes. The concentration of penetratin labeled with AlexaFluor532 and naphthofluorescein was not changed, but they were added to the cells immediately before measurement, together with the DAPI solution. Subsequently, we initially detected 5 measurement points using the FACS Aria flow cytometer, and later replaced this measurement method with continuous measurement.

3.1.6 Examination of lysosomal degradation of cell-penetrating peptides

During the investigation of the lysosomal degradation of cell-penetrating peptides, we examined the possibility that the fluorescently labeled cell-penetrating peptide is released from the endolysosomal system after entry. If it remains trapped and degraded in the lysosomes, only the released fluorophores can pass through the endolysosomal system membrane, so only the fluorescence intensity of the dye can be detected in the cytoplasm. The experiment was performed with the cysteine protease inhibitor E-64d. The cells were treated with 10 nM atovastatin for 48 hours, and phloretine was also applied for 10 minutes. After the appropriate treatments, the protease inhibitor was added to the samples at a concentration of 10 mM. After a 5-minute incubation period, the fluorescently labeled cell-penetrating peptides and DAPI-containing solution were added to the cells, and the measurement was immediately started on the flow cytometer.

3.1.7 Examination of β-penetratin's cellular uptake using confocal laser scanning microscopy and flow cytometry

We wanted to examine the entry of modified β-penetratin into cells based on tests with α-penetratin, and then compare the results. During the experiment, the cells were attached to an 8-well chamber. Incubation with AlexaFluor532 and naphthofluorescein-labeled penetratin solution took 20 minutes, while treatment with DAPI took 5 minutes. Afterwards, we washed out the excess fluorescent solution with PBS and took images using a Zeiss LSM880 confocal laser scanning microscope.

For flow cytometric measurements, the cells were in a cell suspension, as described above. We did not change the concentration or treatment time of penetratin labeled with AlexaFluor532 and naphthofluorescein. The measurement was performed using a FACS Aria flow cytometer.

3.1.8 Investigation of the self-quenching properties of β-epentartin

The results obtained with flow cytometry and microscopy using β-penetratin labeled with naphthofluorescein differ significantly from those obtained with penetratin labeled with AlexaFluor532. A possible explanation for these differences is the phenomenon of self-quenching. We performed our measurements in two different media: PBS as a hydrophilic medium and trifluoroethanol (TFE) as a hydrophobic medium. Our samples contained AlexaFluor532 free dye, AlexaFluor532-labeled α and β-penetratin, and naphthofluorescein free dye, naphthofluorescein-labeled α and β-penetratin. We prepared five different concentrations of solutions from the samples: 20 μM, 40 μM, 60 μM, 80 μM, and 100 μM. Measurements were performed using a Yobin Yvon Fluorolog spectrofluorometer.

3.1.9 Determination of the threshold concentration for penetratin uptake

We used fluorescently labeled versions of α and modified β-penetratin on two different cell types. We examined the uptake of Alexa Fluor532-penetratin and NF-penetratin at seven concentrations: 0 μM, 100 nM, 500 nM, 1 μM, 3 μM, 5 μM, and 10 μM. The measurements were performed using flow cytometry without an incubation time.

3.1.10 Examination of the aggregation ability of penetratin using FRET measurements

FRET measurements were performed using a confocal laser scanning microscope to examine the aggregation ability of fluorescently labeled penetratins. We used penetratins labeled with AlexaFluor532 and Cy5: these fluorophores form a good pair for FRET measurements. We incubated the cells for 20 minutes at 37°C with 5 μM donor (AlexaFluor532)-labeled and 5 μM acceptor (Cy5)-labeled unmodified (α) and modified β-penetratin.

3.2 Investigation of local ligand concentration gradients induced by plasma membranes

3.2.1 Cell lines

We used two cell lines for the experiments: CHO cells that did not endogenously express ErbB proteins. The F1-4 cell line stably expresses GFP-tagged EGFR, which we used to characterize EGFR-specific effects without the influence of other ErbB proteins. The F1-4_ErbB2 cell line was created by transient transfection of F1-4 cells with CFP-tagged ErbB2, and we used it to examine the possible effects of co-expression of ErbB2.

3.2.2 Determination of the concentration and diffusion constant of fluorescent EGF near the cell membrane using fluorescence correlation spectroscopy

F1-4 cells were incubated in the presence of 10 nM fluorescently labeled TAMRA-EGF, and the concentration of the peptide growth factor was measured at distances of 100 μm from the plasma membrane: at every 1 μm distance from the membrane up to 20 μm, then at every 10 μm up to 100 μm. The measurements were performed using a Nikon confocal and STORM super-resolution microscope.

3.2.3 Inhibition of cell membrane turnover

This inhibition was achieved using a three-component cocktail containing latrunculin B, para-amino blebbistatin, and myristoylated dynamin inhibitor peptide (DIP), which inhibited actin polymerization and myosin and dynamin-related activity. The drugs were used at concentrations of 5 μM, 50 μM, and 50 μM to inhibit membrane trafficking. The cells were incubated with these drugs for 60 minutes at 37°C, and the drugs were present at the concentrations mentioned earlier throughout the experiment. The measurements were also performed using the FCS technique.

3.2.4 Digestion of extracellular matrix

For digestion of the extracellular matrix, cells were treated with 20 μg/mL collagenase (C8176, Sigma) and 20 U/mL hyaluronidase for 90 minutes at 37°C. Then, F1-4_ErbB2 cells expressing EGFR and ErbB2 were incubated with 10 nM TAMRA-EGF. The measurement ranged from the membrane to 100 μm. Images were taken every micrometer through 20 μm, then every 10 μm up to 100 μm above the plasma membrane. The method and settings used previously were employed for the measurement.

3.2.5 Blocking ion channels

Cells were treated with naringenin to inhibit the activity of the two-pore ion channel 2. Naringin was applied at a concentration of 1 mM for 30 minutes at 37°C, and the inhibitor was present throughout the entire duration of the FCS measurement. The FCS measurement was performed as described above.

3.2.6 TAMRA-EGF concentration gradient studied in the presence of GPMVs

GPMVs were prepared by osmotic vesiculation. The cells were washed twice with hypotonic washing buffer and then incubated for 12 hours at 37°C in hypertonic vesiculation buffer. The

supernatant containing GPMVs was distributed into an 8-well poly-L-lysine-coated chamber. The vesicles were incubated with 10 nM TAMRA-EGF, and the number of particles (N) in the confocal volume and their diffusion coefficient were determined using FCS, as described above.

3.2.7 Viscosity measurement with a BODIPY-based molecular rotor

The fluorescence lifetime of molar rotor molecules increases in viscous environments. Based on this, we added 8-Phenyl-BODIPY 505/515 to F1 4_ErbB2 cells and measured the fluorescence lifetime of the dye using the TCSPC method.

4 Results

4.1 Effect of phloretin, 6-ketocholestanol, and atorvastatin on the dipole potential

A reduced dipole potential significantly enhanced both the overall cellular uptake of penetratin and its concentration in non-acidic compartments in SKBR-3 and MDA-MB-231 cells. One of the most prominent results regarding penetratin uptake was the cytosolic concentration (based on NF-penetratin intensity), which increased by approximately 50% with phloretin. In contrast, 6-ketocholestanol, which significantly elevated the dipole potential, exerted no statistically significant effect on either the total cellular uptake of penetratin or its endo-lysosomal release. Treatment of MDA-MB-231 cells with atorvastatin at concentrations ranging from 1 nM to 10 μM significantly enhanced the endo-lysosomal release of penetratin, while having only a minor effect on total uptake. In contrast, atorvastatin had a weaker impact in SKBR-3 cells, and at lower concentrations (1 nM, 10 nM) did not reach statistical significance. This can be explained by the minor reduction in cholesterol content in this cell line. At higher atorvastatin concentrations (100 nM and 10 μM), penetratin uptake was significantly reduced in SKBR-3 cells. This result can be attributed to atorvastatin-induced inhibition of endocytosis. Significantly, the extent of penetratin escape from endo-lysosomal compartments was significantly increased in these cells even when total uptake was lower, as indicated by the nearly twofold increase in the NF/AlexaFluor532 intensity ratio.

4.2 Penetratin enters cells via endocytosis

Dyngo4a, an inhibitor of dynamin, was used to assess the contribution of endocytosis. Although dynamin inhibition does not necessarily block all forms of endocytosis, these experiments point to a dominant role of endocytosis in penetratin uptake. Compared with

untreated cells, Dyngo4a significantly reduced the total cellular content and cytoplasmic concentration of fluorescent penetratin by 50–80%.

4.3 Lysosomal degradation of penetratin is negligible even under an altered dipole potential

To determine whether lysosomal degradation contributes to the increase in NF fluorescence, penetratin uptake experiments were performed in the presence of aloxistatin (E-64d), an inhibitor of lysosomal cysteine proteases. The results showed that penetratin degradation does not significantly contribute to the observed changes in fluorescence intensity within the 20-minute measurement window. We propose that substantial lysosomal degradation of internalized peptides requires longer incubation times. Therefore, the contribution of lysosomal degradation to cytosolic accumulation of penetratin can be considered negligible, even under conditions of altered dipole potential induced by atorvastatin or phloretin.

4.4 Concentration-dependent uptake of α-penetratin and β-penetratin assessed by confocal laser scanning microscopy and flow cytometry

Confocal microscopy of fluorescent penetratin derivatives previously revealed that AlexaFluor532-penetratin exhibits strong fluorescence even in endosomes where NF fluorescence is quenched, indicating that NF fluorescence originates from penetratin outside the endo-lysosomal compartments. Based on this, we assume that AlexaFluor532-penetratin and NF-penetratin fluorescence reflect the total cellular uptake and the endo-lysosomal escape, respectively. We aimed to compare these findings with β-penetratin experiments and validate them with flow cytometry.

AFDye532-penetratin fluorescence was proportional to the total cellular uptake of penetratin. NF-penetratin fluorescence in non-acidic compartments (mainly cytosol) reflected cytosolic concentration, while the NF/AFDye532 ratio revealed the efficiency of endo-lysosomal escape. Confocal microscopy demonstrated that β-penetratin displayed a more homogeneous intracellular distribution than α-penetratin in both cell lines, suggesting more efficient uptake and endo-lysosomal escape, particularly for AlexaFluor532-labeled penetratin.

Flow cytometry confirmed these results, showing significantly higher fluorescence intensities for β-penetratin compared with α-penetratin, corresponding to greater uptake and endo-lysosomal release. β-penetratin exhibited approximately fourfold higher fluorescence intensity than α-penetratin for AlexaFluor532-labeled peptides in both cell lines. Uptake was further

identified as a concentration-dependent process with a threshold around 5 μM: below this concentration, uptake remained low, while above it, uptake sharply increased, suggesting cooperative effects of peptide accumulation in the membrane and/or initiation of endocytosis.

4.5 Self-quenching of NF-labeled penetratin

The results showed that NF exhibited self-quenching, as its fluorescence intensity in both aqueous (PBS) and hydrophobic (TFE) environments deviated from linearity with respect to molar concentration. Conjugation to penetratin reduced this quenching, likely by reducing self-aggregation. However, significant self-quenching reappeared when conjugated to β-penetratin, indicating a higher aggregation potential of β-penetratin compared to α-penetratin. AlexaFluor532 did not exhibit substantial self-quenching in free or conjugated form, likely due to the absence of aggregation. However, mild quenching was observed in β-penetratin conjugates in both aqueous and hydrophobic media.

4.6 Higher aggregation potential of β-penetratin

Aggregation was investigated using FRET between AlexaFluor532- and Cy5-labeled penetratins. Since FRET efficiency increases when donor and acceptor are in proximity due to molecular interactions, higher FRET efficiency reflects higher aggregation. The FRET efficiency of β-penetratin was nearly fourfold higher than that of α-penetratin, indicating a substantially greater aggregation potential, which may underlie its enhanced uptake efficiency.

4.7 Determination of fluorescent EGF concentration and diffusion coefficient near the plasma membrane using FCS

Near the membrane, the EGF concentration exhibited a peak extending ~5 μm from the surface, reaching three times higher than in the bulk solution. A similar peak was observed in F1-4_ErbB2 cells, whereas F1-4 cells displayed an additional distal peak (~10–20 μm from the membrane) of comparable magnitude. Based on particle numbers and diffusion coefficients, the membrane-proximal peak did not correlate with changes in diffusivity. In contrast, the distal peak coincided with a local minimum in the diffusion coefficient. Computational analysis indicated that the observed reduction in diffusivity was sufficient to generate the distal EGF concentration peak. Thus, the distal peak arises from locally hindered diffusion, whereas the membrane-proximal peak is independent of this mechanism.

4.8 Inhibition of membrane trafficking eliminated the membrane-proximal peak

A three-component cocktail containing latrunculin B, para-amino blebbistatin, and a myristoylated dynamin inhibitory peptide abolished the membrane-proximal peak. Still, it induced a new, broader peak that negatively correlated with diffusivity. This indicates that the newly observed peak arises through a distinct mechanism from the previously detected one.

4.9 Ion channel inhibition also eliminated the membrane-proximal peak

The results with the trafficking inhibitor cocktail suggest that membrane trafficking contributes to the formation of the proximal peak. However, monensin, a widely used inhibitor of exocytosis, did not abolish this peak. This may explain how rapid cycles of endocytosis and exocytosis give rise to the proximal peak: endocytosed content may be immediately re-exocytosed after concentration within shrinking endosomes. Two-pore ion channels may also contribute, as treatment with naringenin significantly reduced the proximal peak in F1-4_ErbB2 cells without affecting the distal peak. Notably, the shape of the distal peak differed slightly from earlier observations.

4.10 Ligand concentration is homogeneous around GPMVs from F1-4 cells

GPMVs, which resemble the plasma membrane compositionally but lack trafficking, exhibited homogeneous ligand distribution in F1-4 cells. In contrast, GPMVs from F1-4_ErbB2 cells displayed a membrane-proximal peak, inversely correlating with diffusivity. This suggests that locally hindered diffusion caused by extracellular matrix components deposited during vesicle formation generates this peak. These findings highlight the requirement of active trafficking in live cell membranes for the formation of membrane-proximal EGF concentration peaks.

4.11 Extracellular matrix digestion abolished the distal peak

Correlation analysis of EGF concentration and diffusivity in F1-4_ErbB2 cells treated with collagenase and hyaluronidase revealed that enzymatic digestion abolished the distal peak but induced a strong proximal peak coinciding with a pronounced reduction in the diffusion coefficient. We attribute this to ECM degradation and deposition of its fragments on the plasma membrane.

4.12 A local viscosity maximum causes a distal ligand concentration peak

BODIPY-based molecular rotor fluorescence lifetime measurements indicated a local maximum in microviscosity ~10–20 μm above the membrane of F1-4_ErbB2 cells, whereas in cell-free medium the lifetime remained constant. Upon enzymatic ECM digestion, this high-viscosity region shifted closer to the membrane, consistent with the observed relocation of the diffusivity minimum. These findings demonstrate that the distal ligand concentration peak in F1-4_ErbB2 cells is driven by a local maximum in viscosity, likely due to the presence of extracellular matrix components.

5 Discussion

Transport across the cell membrane is the starting point for many cellular biological processes. In my work, I focused on two phenomena in which membrane-related processes play a key role in their regulation. First, I investigated the entry of cell-penetrating peptides (CPPs) into cells. Although the ability of CPPs to cross membranes has long been known, their effectiveness is greatly limited by the difficulty of their release from endolysosomes. Therefore, in the first part of my research, I studied the biophysical properties of the plasma membrane and the factors that influence the efficiency of CPP uptake. Our observations indicate that the high dipole potential of the membrane hinders the complete cellular uptake of penetratin and its release from the endolysosomal system. This was confirmed by the temporal evolution of the intracellular intensity of AlexaFluor532- and NF-labeled penetratin. The effect of the dipole potential is most likely exerted through the incorporation of the peptide into the cell membrane, as it modifies its properties. It is known that the incorporation and penetration of peptides and small hydrophobic molecules into membranes is strongly dependent on the dipole potential. Although its effect on endocytosis is presumably minor, the binding of the peptide to the membrane may also vary depending on the dipole potential. Analysis of the biophysical properties of the membrane revealed that, of the characteristics examined, only the dipole potential provides sufficient information about the extent of penetratin uptake. The positive charge of penetratin offers a reasonable explanation for why positive dipole potential limits its passage through the membrane, and this principle may also apply to other cationic CPPs. Treatment with phloretin and atorvastatin reduced the membrane dipole potential and significantly increased the cytoplasmic concentration of penetratin. It should be noted that the use of atorvastatin at nanomolar concentrations is consistent with clinically used doses, so the statin-enhanced penetration potential has potential medical significance. Interestingly,

although atorvastatin reduced the dipole potential to a greater extent, it only promoted release from endolysosomes, while phloretin enhanced total cell uptake. In the latter case, it can be assumed that during the short 10-minute incubation period, phloretin does not reach the intracellular membranes in sufficient quantities; therefore, the dipole potential of the endolysosomes remains unchanged. However, three days of atorvastatin treatment is long enough to reduce cholesterol levels significantly and, as a result, decrease the dipole potential of endolysosomal membranes. The duality between the slight decrease in dipole potential induced by phloretin and the considerably increased uptake of penetratin suggests that physiological dipole potential strongly limits uptake. This is supported by the fact that the dipole potential increased by 6-ketocholestanol had little effect on penetratin uptake. At the same time, the minor decrease caused by phloretin was sufficient to enhance uptake. Atorvastatin caused a significant reduction in dipole potential but did not increase the total cellular concentration of penetratin. The rapid increase in intensity of AlexaFluor532-penetratin and the prolonged signal change of NF-penetratin suggest that initial uptake occurs by endocytosis, which was not modified by either treatment. Thus, total penetratin uptake did not increase during atorvastatin treatment, indicating inhibition of endocytosis. This is particularly important because during endocytosis, molecules enter the endolysosomal system, where they are susceptible to degradation, which may prevent them from reaching their intended target site. However, our results show that the extent of lysosomal degradation is negligible for fluorescently labeled penetratin, regardless of changes in dipole potential.

In addition to treatments that affect the biophysical properties of the membrane, we also explored other strategies to enhance CPP uptake efficiency. Within this framework, we examined a structurally modified cell-penetrating peptide synthesized by a research group at the Institute of Organic Chemistry at Semmelweis University (details cannot be published). Our studies showed that the modified β-penetratin alone, without treatments affecting its dipole potential, enters cells much more efficiently, but is more prone to aggregation (based on FRET and self-quenching experiments). The homogeneous, vigorous signal intensity indicates that it is easily released from the endo-lysosomal system and reaches the cytoplasm. This supports the medical applicability of CPPs, as peptides used as carriers for therapeutic purposes must have similar efficacy and properties.

Our results shed new light on the concept of the plasma membrane. What was once regarded as a static boundary surface can now be described, in light of current findings, as a dynamic system that continuously changes in space and time. In this study, we not only provided

evidence for how this dynamism modifies ligand concentrations in the immediate vicinity of the membrane, but also analyzed the potential biological consequences of this phenomenon.
To interpret these findings in the context of cell biology, one must ask: *which mechanisms are capable of generating stable concentration gradients?* Based on physical principles and prior observations, gradients may arise in two ways:
(1) from local variations in diffusivity, i.e., spatial heterogeneity in the diffusion coefficient; or
(2) from continuous local production, as is well established in the case of morphogens.
Since diffusion alone acts to dissipate concentration differences, it cannot establish a stable gradient. However, a persistent, spatiotemporally stable pattern of diffusion coefficients can sustain a gradient. Such patterns may arise from (1) switch-like, locally regulated conformational changes; (2) viscosity gradients; or (3) size-dependent variations in the diffusion constant near membrane surfaces. The latter possibility can be excluded here, as it is relevant only for molecules much larger than EGF and is limited to the nanometer scale. In contrast, our results revealed gradients detectable over micrometer distances. Membrane fluctuations are also unlikely, as they would have resulted in altered autocorrelation functions, which were not observed. Moreover, EGF is not known to undergo conformational changes that affect diffusivity, excluding that mechanism as well. Similarly, local viscosity gradients cannot account for the membrane-proximal EGF peak, since it did not correlate with the diffusion coefficient. Taken together, these considerations suggest that the most plausible explanation for the membrane-proximal EGF concentration peak is the presence of a continuous, local source.
It is essential to note that neither reversible equilibrium binding to receptors nor interactions with the lipid bilayer can maintain a long-lasting concentration gradient. Without changes in diffusivity, any peak would equilibrate. Thus, maintaining the gradient requires a continuous source. From a biophysical perspective, the following statements hold for a membrane-proximal ligand gradient:

- It cannot arise spontaneously, as this would violate the second law of thermodynamics;
- Its formation necessarily involves an active biological process (consistent with the absence of such gradients in GPMVs);
- Its temporal stability implies that this active process leads to a biological steady state.

Our experiments, therefore, aimed to identify which processes are capable of generating a temporally stable, membrane-proximal ligand gradient. Upon inhibition of actin polymerization, myosin and dynamin activity, and membrane trafficking using a specific cocktail, the membrane-proximal peak disappeared, while a new, distinct peak emerged. In

untreated F1-4 cells, the concentration peak was located within ~5 μm of the membrane and showed no correlation with the diffusion coefficient. After inhibitor treatment, however, the peak extended to ~10 μm and exhibited an inverse correlation with the diffusion coefficient. This phenomenon can be explained by the exocytic effect of latrunculin B, which induces the rapid release of vesicles. Although the identity of the released molecules remains unknown, the negative correlation between diffusion coefficient and particle number clearly indicates the presence of a diffusion barrier.

To explain the persistence of the membrane-proximal peak, we must assume that membrane trafficking—specifically, the re-exocytosis of endocytosed material—provides the necessary replenishment. Our simulations supported the notion that both receptor-bound and free ligands can reappear and sustain the gradient. This is further corroborated by the finding that monensin, an inhibitor of classical exocytic pathways, had no effect, suggesting that the membrane-proximal EGF peak is most likely maintained by dynamic membrane trafficking. Such trafficking may occur via various mechanisms—"kiss-and-run," "open-and-closed," or "flicker" exocytosis—where vesicles do not fully form or do not release their contents completely. A similar mechanism has been described for PECAM, which is functionally removed from the membrane without endocytosis and then reappears on the cell surface within a short time. These processes could provide sufficient turnover to sustain the membrane-proximal EGF peak.

Based on our results, we propose that the shrinkage of endosomes or macropinosomes followed by exocytosis underlies this phenomenon. Two-pore ion channels are already known to play a role in endosomal maturation and shrinkage, and the observation that naringenin—a channel inhibitor—significantly reduced the membrane-proximal EGF peak further supports this hypothesis.

In addition to the proximal peak, we identified a second, more distal concentration maximum. This distal EGF peak can be explained by increased local viscosity, as it inversely correlated with the EGF diffusion coefficient. Our calculations confirmed that the observed reduction in the diffusion coefficient was sufficient to account for the concentration peak. The increased fluorescence lifetime of the BODIPY molecular rotor in this region further supported the idea that higher microviscosity slows down diffusion. Although the precise source of this elevated viscosity could not be determined, the shift in peak position upon collagenase and hyaluronidase treatment in F1-4_ErbB2 cells indicates a connection with the extracellular matrix. Interestingly, in our CHO-derived cell lines, this distal EGF peak was observed only in

ErbB2-expressing cells, although in other cell types it may also occur independently of ErbB2 expression.

In summary, we have demonstrated that the distribution of a growth factor near the plasma membrane is not homogeneous. We identified two distinct concentration peaks: one associated with active membrane trafficking and another with locally hindered diffusion. These phenomena significantly alter ligand concentrations near the plasma membrane compared with bulk concentrations and may thus fundamentally modulate dose-dependent cellular responses. We conclude that our findings are essential to consider in the interpretation of quantitative cell biological experiments, particularly those investigating the stimulation of transmembrane receptors.

6 Summary

The study of cell-penetrating peptides (CPPs) is motivated by the fundamental barrier posed by the phospholipid bilayer of the plasma membrane, which is impermeable to most molecules. CPPs are short oligopeptides capable of translocating across the membrane independently and of delivering otherwise non-permeable molecules into cells. This dissertation focuses on *penetratin*, which originates from the Drosophila *Antennapedia* transcription factor. Our experimental findings can be summarized as follows:

- Using the inhibitor Dyngo4a, we demonstrated that at a concentration of 5 µM, penetratin enters cells primarily through dynamin-dependent endocytosis.
- The uptake of penetratin is dependent on the membrane dipole potential: atorvastatin enhances endo-lysosomal release by reducing it, whereas 6-ketocholestanol inhibits entry by increasing membrane rigidity.
- The peptide accumulates in endosomes and lysosomes; however, inhibition of cysteine proteases indicated that lysosomal degradation is not substantial.
- β-penetratin exhibits significantly more efficient cellular uptake, attributable to its increased aggregation potential, as confirmed by FRET and fluorescence quenching assays.

Classical models of ligand binding assume a homogeneous distribution of ligands in the extracellular space. Our results, however, demonstrate that plasma membrane dynamics and the extracellular matrix (ECM) generate inhomogeneous distributions and membrane-proximal concentration peaks. This directly influences the activity of receptors such as the epidermal growth factor receptor (EGFR). By fluorescence correlation spectroscopy, we detected an EGF concentration peak 10–20 µm away from the membrane, coinciding with a local minimum of the diffusion constant, attributable to the ECM (as shown by collagenase and hyaluronidase treatment). In contrast, a concentration peak detected within 1–5 µm of the membrane was found to be a consequence of active membrane trafficking. It was present exclusively in living cells (absent in GPMVs).

Taken together, the results of these two subprojects clearly indicate that plasma membrane dynamics and the local organization of the ECM fundamentally determine the biophysical environment of transmembrane transport and ligand binding.

7 New findings/results

In terms of regulating the transport of cell-penetrating peptides, one of the most important findings of the research is that the membrane dipole potential is the key biophysical parameter that limits the cellular uptake and endolysosomal release of cationic, positively charged SPPs such as penetratin. A significant pharmacological modulation possibility has been confirmed: phloretin and atorvastatin reduce the dipole potential, thereby increasing the cytoplasmic concentration of penetratin. The clinical relevance of this lies in the fact that atorvastatin, even at nanomolar doses currently used in clinical practice, promotes the release of peptides from endolysosomes through cholesterol reduction.
Furthermore, a successful structural modification shows promising results: we have created a new, modified β-penetratin variant that enters the cytoplasm more efficiently without external treatment, although it shows an increased tendency to aggregate.

The second part of the study presents evidence that the cell membrane is not a static interface, but a dynamic, rapidly changing structure, which causes the distribution of ligands, in this case EGF, in the membrane environment to be non-homogeneous. Two separate concentration peaks were identified:

- A peak caused by active membrane turnover, located at a distance of 0–5 μm from the membrane: This gradient is maintained by continuous exocytosis and endocytosis, the so-called "kiss-and-run" mechanism. Research has revealed that this process is linked to the maturation of endosomes and the functioning of two-pore ion channels.
- Diffusion barrier peak: The concentration maximum further away from the membrane (10-20 μm) is caused by an increase in local microviscosity and the structure of the extracellular matrix (ECM), which slows down diffusion.

The results highlight that during cell biological processes, receptors encounter these local "peaks" rather than the average ("bulk") solution concentration. Although the phenomenon of concentration peaks has been described before, the identification of the underlying cellular biological processes is linked to our project. This realization fundamentally modifies our previous models of dose-dependent cellular responses and transmembrane signal transduction.

8 Appendix

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/482/2025.PL
Subject: PhD Publication List

Candidate: Gabriella Tóth
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. *Nagyné Szabó, Á. T., **Tóth, G.**, Szatmári, T., Mocsár, G., Rebenku, I., Szöllősi, J., Nagy, P.: Local ligand concentration gradients induced by the plasma membrane.
   iScience. 28 (7), 1-12, 2025.
   DOI: http://dx.doi.org/10.1016/j.isci.2025.112954
   IF: 4.1 (2024)
2. Batta, G., Kárpáti, L., Henrique, G. F., **Tóth, G.**, Tarapcsák, S., Kovács, T., Zákány, F., Mándity, I. M., Nagy, P.: Statin-boosted cellular uptake and endosomal escape of penetratin due to reduced membrane dipole potential.
   Br. J. Pharmacol. 178 (18), 3667-3681, 2021.
   DOI: http://dx.doi.org/10.1111/bph.15509
   IF: 9.473

List of other publications

3. Yousef, M., Szabó, I., Murányi, J., Illien, F., Soltész, D., Bató, C., **Tóth, G.**, Batta, G., Nagy, P., Sagan, S., Bánóczi, Z.: Cell-Penetrating Dabcyl-Containing Tetraarginines with Backbone Aromatics as Uptake Enhancers.
   Pharmaceutics. 15 (1), 1-22, 2023.
   DOI: http://dx.doi.org/10.3390/pharmaceutics15010141
   IF: 4.9

Total IF of journals (all publications): 18,473
Total IF of journals (publications related to the dissertation): 13,573

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

19 August, 2025

*Ágnes Nagyné Szabó and Gabriella Tóth contributed equally.