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Efficient peptide-mediated delivery of oligonucleotides into mammalian cells

Master's Thesis (30 ECTS)

Curriculum Bioengineering

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Abstract

Efficient peptide-mediated delivery of oligonucleotides into mammalian cells

The clinical application of therapeutic nucleic acids has been limited due to the challenges encountered during their delivery into cells. A challenge that is very difficult to overcome is the entrapment of internalized nucleic acid in endo-lysosomal compartments. Other cellular processes that can negatively influence the functional delivery of nucleic acid therapeutics are exocytosis and autophagy. In this work, series of compounds known to enhance endosomal escape, modulate exocytosis and autophagy were added to nanoparticles prepared of splice-correcting oligonucleotide (SCO) and cell-penetrating peptide (CPP), and SCO activity was evaluated in a luciferase assay. Some of the selected compounds such as imipramine and L-leucyl-L-leucine methyl ester increased transfection efficiency with minimal cytotoxicity, which advocates their potential use for increasing efficacy of nucleic acid therapeutics in the future.

Keywords: Cell-penetrating peptide, transfection, oligonucleotide, endosomal escape.

CERCS: B190, B210, P310, P320.

Oligonukleotiidide efektiivne transport imetajarakkudesse peptiidide abil

Põhiliseks takistuseks terapeutiliste nukleiinhapete laialdaseks kliiniliseks kasutuseks on nukleiinhapete ebapiisav viimine sihtmärgini ja rakkudesse. Suurimaks takistuseks on siinkohal nukleiinhappe kinnijäämine endosoomidesse ning sellele järgnev degradeerimine lüsosoomides. Teisteks rakulisteks protsessideks mis võivad negatiivselt mõjutada terapeutiliste nukleiinhapete produktiivset transfektsiooni on eksotsütoos ja autofaagia. Käesolevas töös viidi rakkudesse splaissingut korrigeerivat oligonukleotiidi (SKO) rakkudesse penetreeruva peptiidi (RPP) abil, lisades SKO-RPP osakestele aineid, mis võiksid suurendada nende vabanemist endosoomidest, moduleerida eksotsütoosi ja autofaagiat, ning SKO aktiivsust hinnati reporter-rakuliinis luminestsentsi järgi. Mõned testitud ainetest, näiteks imipramiin ja L-leutsüül-L-leutsiin metüülester, suurendasid produktiivse transfektsiooni efektiivsust, olemata toksilised rakkudele, mis vihjab nende potentsiaalsele terapeutilisele kasutusele tulevikus nukleiinhappe transpordil.

Võtmesõnad: Rakkudesse penetreeruvad peptiidid, transfektsioon, oligonukleotiidid, vabanemine endosoomidest.

CERCS: B190, B210, P310, P320.

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TERMS, ABBREVIATIONS AND NOTATIONS

ASO – antisense oligonucleotide

CPP – cell-penetrating peptide

DPBS – Dulbecco's Phosphate-Buffered Saline

EACC – ethyl(2-(5-nitrothiophene-2-carboxamido)thiophene-3-carbonyl)carbamate, inhibitor of autophagy

EE – early endosomes

FDA – Food and Drug Administration

LE – late endosomes

LLOMe – L-leucyl-L-leucine methyl ester

NP – nanoparticle

ON – oligonucleotide

ROC-325 – 1-((2-((2-((7-chloroquinolin-4-yl)amino)ethyl)(methyl)amino)ethyl)amino)-4-methyl-9H-thioxanthen-9-one, inhibitor of autophagy

RT – room temperature

SCO – splice-correcting oligonucleotide

siRNA – small interfering RNA

INTRODUCTION

Oligonucleotide (ON) therapeutics have attracted a lot of attention as a promising tool for altering gene expression in a wide spectrum of pathologies. However, limited cellular delivery has prevented many candidate oligonucleotides and other nucleic acid-based macromolecular therapeutics from reaching clinical application. Transfection, which is the process of delivering nucleic acids into the cell, is still in need of serious improvements to ensure efficient uptake and functional delivery of nucleic acid drugs to target sites in cytosol and nucleus. Over the years, different transfection methods have been developed, including the viral (biological), non-viral (chemical) and physical methods, each of which has certain advantages and disadvantages.

One group of such non-viral vectors that has emerged in the last decades consists of cell-penetrating peptides (CPPs). The first discovery of such peptides was based on a viral protein. In 1988, Tat (trans-activator of transcription) of the human immunodeficiency virus 1 (HIV-1) was discovered and shown to have membrane-penetrating properties. A few years later, the *Drosophila* antennapedia transcription factor proteins were shown to be able to penetrate cell membrane and enter cells. These discoveries imply that short sequences of these proteins have the ability to penetrate cell membrane. CPPs are cationic and/or amphipathic peptides usually 5 to 40 amino acids in length and have been shown to enhance the delivery of a wide variety of bioactive cargoes, including plasmid DNA (pDNA), small interfering RNA (siRNA), messenger RNA (mRNA) and splice-correcting oligonucleotide (SCO), both *in vitro* and *in vivo* in a non-toxic manner.

The cellular uptake mechanism of CPPs is still not understood in all details, but it is now widely accepted that CPPs-cargo complexes usually utilize different endocytic pathways for internalization. Because of this, CPPs and their cargos often stay entrapped in the endosomal compartments, which limits the bioavailability of CPP-cargo complexes, similarly to other non-viral vectors. Other cellular events that can limit the bioavailability of CPP-cargo nanoparticle are exocytosis and autophagy.

The aim of this work was to increase the efficiency of CPP-based delivery of oligonucleotides by altering the normal intracellular endo-lysosomal trafficking of nanoparticles and modulating other cellular processes such as exocytosis and autophagy.

1 LITERATURE REVIEW

1.1 Nucleic acid-based therapeutics

The notion that inherited genetic disorders caused by abnormal gene products can be treated by transferring a normal copy of a gene into the cell of a living organism was conceived about five decades ago (Friedmann and Roblin 1972). In recent times, methods of transferring nucleic acids into cells have attracted the attention of scientists because of their great potential to treat genetic diseases. From the public health perspective, the application of these methods in disease prevention is seen in the efforts of Moderna Therapeutics and Pfizer/BioNTech to develop mRNA vaccines to combat the ongoing COVID-19 pandemic (Anderson *et al*. 2020, Rossi 2020).

Clinical application of macromolecular drugs developed to treat genetic diseases is now a reality as seen in the recent approval of several nucleic acid-based therapeutics by United States Food and Drug Administration (FDA) (Bost *et al*. 2021).

Unlike conventional drug molecules that usually target proteins, nucleic acid drugs regulate gene expression by inhibiting, adding, replacing, or editing at the DNA or RNA level to produce therapeutic effect. The transfer of foreign nucleic acids into cells to counteract dysfunctional genes is a promising way to attain greatly targeted, long-lasting and possibly curative therapeutic effects in genetic diseases. However, employing nucleic acid therapeutics is challenging because they are easily degraded by nucleases, can contribute to activation of immune system and have unsuitable physicochemical characteristics such as large size and negative charge, that prevent their efficient transfer into cells (Jayesh *et al*. 2021).

There are various types of nucleic acids that can have therapeutic effect when delivered into cells. These include antisense oligonucleotides (ASOs), small interfering RNA (siRNA), micro RNA (miRNA), plasmid DNA (pDNA), messenger RNA (mRNA), and others (Jackson *et al*. 2006, Davis *et al*. 2006).

1.1.1 Oligonucleotides as therapeutics

Oligonucleotides (ON) are short nucleic acid sequences, and usually 15 to 30 base pairs long ONs are used in therapeutic approaches. Synthetic ONs can contain various chemical alterations that are introduced to modify their characteristics in a suitable way, e.g. to increase

their stability against cellular nucleases and affinity to target sequence. ONs have low toxicity and can associate with their targets with remarkable specificity that is based on sequence complementarity, i.e. they can be designed to bind any RNA sequence, whether in pre-mRNA, mRNA, ribonucleoproteins, or miRNA. The pharmacokinetics of oligonucleotides can be altered by modifying functional groups in its backbone, while the target specificity is dependent on the nucleotide sequence (Khvorova and Watts 2017).

Short regulatory oligonucleotides such as siRNAs, antisense, splice redirection, decoy ONs etc., are fast evolving method for altering gene expression *in vitro* and *in vivo* (Margus *et al*. 2012). There are some other applications of oligonucleotides. For example, ONs can be designed to assemble in a specific 3D conformation that associates with proteins. These nucleic acid structures are called aptamers and can be used for protein detection or for preventing protein-protein interactions, and also as targeting ligands that specifically associate with a specific protein on targeted cells.

1.1.2 Mechanism of action of splice-correcting oligonucleotides

Oligonucleotides used for the redirection/correction of splicing are short, single-stranded ONs. They modulate alternative splicing by binding to the complementary pre-mRNA sequence in cell nucleus, sterically blocking the binding of splicing complex (spliceosome) and thus preventing aberrant splicing (Dominski and Kole 1993).

About 20–30% of all disease-causing mutations affect pre-mRNA splicing (Faustino *et al*. 2003). Mutations that change alternative splicing have often been associated with different diseases such as cystic fibrosis, β-thalassemia, muscular dystrophies, and different types of cancer (Sazani and Kole 2003). Several methods have been developed for the treatment of splicing defects, among which one of the most promising is applying ONs for silencing these mutations.

Most of the splicing redirection experiments *in vitro* have been conducted in HeLa pLuc 705 cell line, human cervical cancer-derived cell line that is stably transfected with luciferase-encoding gene interrupted by a mutated β-globin intron 2 (Margus *et al*. 2011). This mutation introduces an aberrant pre-mRNA splicing site, thus leading to the synthesis of non-functional luciferase (Figure 1). However, binding of the complementary oligonucleotide to the aberrant splicing-inducing site restores the normal pre-mRNA splicing and functional luciferase is expressed (Kang *et al*. 1998).

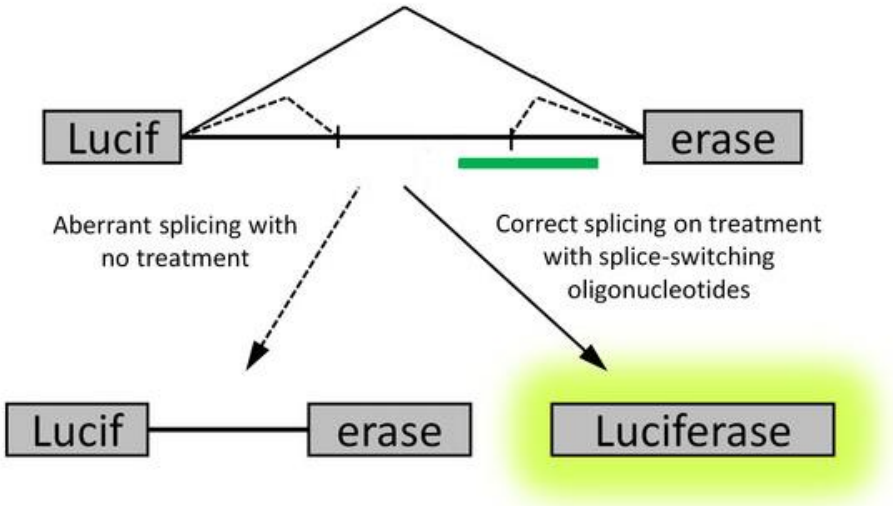

Figure 1. Illustration of the mechanism of action of splice-correcting oligonucleotide (SCO). In the abscence of SCO, abnormal splicing occurs, leading to the synthesis of non-functional luciferase, as shown by the left arrow in the diagram. But upon treatment, SCO (green) modulates alternative splicing by binding to the complementary pre-mRNA sequence and sterically blocking the binding of splicing complex (spliceosome) and thus preventing aberrant splicing, hence, functional luciferase (yellow) is produced (Hande *et al*. 2019).

1.1.3 Problems with the delivery of oligonucleotides

There are significant bottlenecks that hinder the extensive use of ONs and other nucleic acid-based therapeutics, which must be overcome during drug design to ensure functional delivery into cells. There are several requirements that have to be fulfilled by therapeutic oligonucleotides to become drug candidates.

First of all, the oligonucleotide must have suitable pharmacokinetic properties for *in vivo* application, i.e. sufficiently long half-life and stability to nucleases. In addition, the oligonucleotide molecules should not induce an undesired immune response.

Secondly, the oligonucleotide must be able to enter cells in sufficient concentrations. For that, it has to cross biological lipid membranes to reach its site of action and should be able to efficiently target specific cells and avoid others. The specificity of the drug to the desired tissues must be high and toxicity must be low, and off-target effects must be minimal (Bost *et al*. 2021).

Finally, the ON drug must be easily scalable and affordably produced to be a usable therapeutic with a predictable behavior across the population.

Some of these challenges have been surmounted to a great extent. To enhance cellular uptake and enable efficient transfection of oligonucleotides and other nucleic acid molecules into cells, multiple delivery agents, such as cationic lipids, cell-penetrating peptides and polymers have been developed.

Also, the stability of oligonucleotides to enzymatic degradation can be improved by chemical modification of sugar-phosphate backbone as well as by altering nitrogenous bases of the nucleic acid. As steric blocking of the mutated site does not imply recruitment of any cellular enzymes, splice-switching oligonucleotides can be largely modified. One of the commonly used modifications is phosphorothioate (PS). In the PS backbone, the nonbridging oxygen of the phosphodiester linkage is replaced with a sulphur atom. In addition to increased stability, the PS backbone also induces an increase in tissue uptake (Eckstein 2014).

The deoxyribose in DNA and the ribose in RNA can be altered to enhance the stability of ON against nucleases to ensure a longer half-life in organism ranging from days to weeks (Geary *et al*. 2015). The most common modification of the sugar residue is the 2′-O-methyl (2′-O-Me). In this modification, a methyl group is attached to the hydroxyl group of ribose (Bost *et al*. 2021). Other common modifications include 2′-O-methoxyethyl (2′-O-MOE) and 2'-fluoro RNA (2′-F-RNA). The carbohydrate can also contain modifications which "lock" the nucleotide into its north (C3′-endo/C2′-exo) conformation. Bridging the 2′-O to the C4′ with a methylene link leads to an increase in enzymatic stability. The locked nucleic acid (LNA) approach has been applied to modify the structure of siRNAs, gapmers, splice-switching ONs, antagomirs etc (Jepsen *et al*. 2004).

1.1.4 Oligonucleotides that are already in clinics

Extensive research in the field of oligonucleotide drugs has resulted in successful clinical trials of some ON therapeutics. The advancement in oligonucleotide/nucleic acid-based drug delivery methods and identification of genetic targets is promoting rapid growth of the field and use of these therapies in clinics. Current FDA-approved oligonucleotide therapeutics are shown in the table below (Table 1).

Table 1. Current FDA-approved oligonucleotide-based therapeutics. Oligonucleotide therapeutics that have reached clinics by the year 2021 are listed, with the year of their approval by FDA, indication, class and chemical modifications that they contain (adapted from Bost *et al.* 2021).

Drug name (alternative name)	FDA approval	Indication	Class	Chemical modifications
Vomivirsen (Vitravene)	1998	CMV retinitis	DNA	PS backbone, 5′ CpG motif
Macugen (Pegaptanib)	2004	retinal AMD	aptamer	PS, 3′-3′ dTcap; 2′-OMe purine ribose sugars; 2′-F pyrimidine ribose sugars; PEG conjugation
Kynamro (Mipomersen)	2013	HoFH	gapmer	PS backbone, 2′-O-MOE 5-mer regions
Exondys 51 (Eteplirsen)	2016	DMD	SSO	PMO
Defitelio (Defibrotide)	2016	sVOD	mixed	PO backbone; ss and ds
Spinraza (Nusinersen)	2016	SMA	SSO	PS backbone; 2′-O-MOE; 5-mC
Onpattro (Patisiran)	2018	hATTR	siRNA	ds; 2′-OMe uridines; LNP encapsulation
Tegsedi (Inotersen)	2018	hATTR	gapmer	2′-O-MOE
Givlaari (Givosiran)	2019	AHP	siRNA	ds; partial 2′-F, partial 2′-OMe, partial PS; GalNAc conjugation
Golodirsen (Vyvondys 53)	2019	DMD	SSO	PMO
Viltepso (Viltolarsen)	2020	DMD	SSO	PMO
Oxlumo (Lumasiran)	2020	PH1	siRNA	ds; partial 2′-F, partial 2′-OMe, partial PS backbone; GalNAc conjugation
Amondys 45 (Casimersen)	2021	DMD	SSO	PMO

Abbreviations: CMV, cytomegalovirus; PS, phosphorothioate; AMD, age-related macular degeneration; 2′-OMe, 2′-O-methyl; 2′-F, 2′-fluoro; PEG, polyethylene glycol; HoFH, homozygous familial hypercholesterolemia; 2′-O-Moe, 2′-O-methoxyehtyl; DMD, Duchenne muscular dystrophy; PMO, phosphorodiamidate morpholino oligomer; sVOD, severe hepatic veno-occlussive disease; PO, phosphodiester; SMA, spinal muscular atrophy; hATTR, hereditary transythyretin amyloidosis; LNP, lipid nanoparticle; AHP, acute hepatic porphyria; GalNAc, N-acetylgalactosamine; PH1, primary oxaluria type 1; FGF2, fibroblast growth factor.

1.2 Transfection types

Transfection is a method of transferring exogenous nucleic acids into eukaryotic cell to alter its gene expression pattern. It can be achieved by inhibiting translation of certain mRNA sequences, altering splicing of endogenous pre-mRNA or by introducing additional copies of a gene in order to enhance its expression (Chow *et al*. 2016). Over the last three decades, transfection has been widely applied for studying cellular processes and molecular mechanisms of diseases (Arnold *et al*. 2016). Understanding the molecular pathway of disease is

crucial for discovering specific biomarkers that may be used to diagnose and prognose it (Roser *et al*. 2018). Transfection, as already mentioned, is also an important part of development of nucleic acid-based therapeutics that can be used to treat inherited genetic diseases (Yao *et al.* 2008). Current techniques in the field of biology allow nucleic acids of different types and sizes to be transferred into cells.

Most broadly, transfection can be grouped into two types: stable and transient transfection (Kim and Eberwine 2010). Stable transfection implies maintaining prolonged expression of transgene by incorporating exogenous DNA into the host genome (Lufino *et al.* 2008). In contrary, in the case of transient transfection, exogenous nucleic acid is not integrated into the host cell's genome. Instead, it is maintained freely in cytosol or nucleus, where it is either translated in order to provide cells with additional copies of the protein of interest, or acts by other mechanisms such as inhibiting expression of certain genes or altering splicing (Riedl *et al*. 2018).

Another way to classify transfection techniques is by dividing them into viral, non-viral, or hybrid methods (Figure 2).

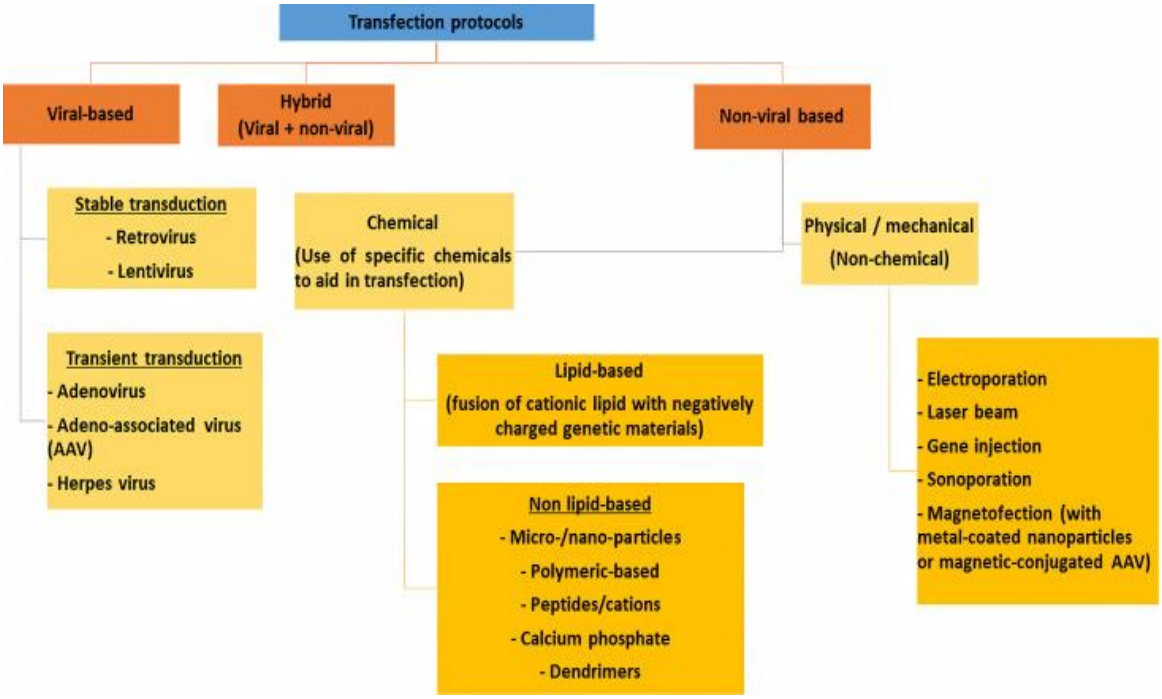

Figure 2. Different transfection protocols. Transfection methods can be divided into viral-based, non-viral based or combination of both (hybrid). For viral and non-viral methods, more detailed division and most widely used methods are listed (Chong *et al*. 2021).

1.2.1 Cell-penetrating peptides as transfection agents

Cell-penetrating peptides (CPPs) are short peptides that usually contain fewer than 40 amino acids. CPPs can be cationic, amphipathic and hydrophobic, and are able to enter cells by various mechanisms, usually endocytosis. Due to their positive charge, CPPs can condense negatively charged nucleic acids into nanoparticles in a non-covalent manner and are also able to facilitate the intracellular delivery of covalently conjugated bioactive cargos, including nucleic acids and low molecular weight drugs, at the same time displaying low cytotoxicity.

In general, when coupled to a large cargo molecule, such as proteins or pDNA, or short nucleic acids, such as SCOs, siRNA or miRNA, CPPs transport their cargo into cells mainly by endocytosis. The process consists of (a) complexation between the cargo and peptide, (b) association with the cellular plasma membrane followed by internalization, (c) trafficking and endosomal escape, and (d) cytoplasmic or nuclear localization (Langel 2007).

There are several ways to increase the uptake and stability of CPP-cargo complexes *in vitro* and *in vivo*, including chemical modifications of the structure of known CPPs or rational design of novel CPPs.

1.2.2 Cellular uptake and endosomal escape of oligonucleotides

The uptake of oligonucleotides, as well as of CPP-ON complexes, usually occur in three steps: association, internalization, and intracellular trafficking.

Association occurs when the ON comes in contact with proteins on the cell membrane. As nucleic acids are negatively charged due to the phosphodiester linkages, ONs are unlikely to associate with negatively charged cell membrane. However, nucleic acids with PS backbone have higher binding affinity to the cell surface proteins.

In 1997, it was discovered that scavenger receptors on endothelial cells were able to bind certain ON species (Wolfe and Trejo 2007). The class A scavenger receptors (SCARAs) have been identified as the main binding target of nanoparticles formed of CPP and ONs that contain 2′-OMe modifications (Ezzat *et al*. 2015).

After association, internalization of the ON occurs. This can happen via multiple pathways, but it usually involves the following stages: initially, there is the accumulation of material into a distinct patch on the cell membrane, and finally, the protruding and pinching off the

membrane takes place which induces the membrane to move inward, becoming an endosomal vesicle (Mayor and Pagano 2007).

The internalization of naked ON as well as of CPP-ON complexes usually involves entrapment in endosomes. Particles must escape from the endosomal compartment to reach their target site in the cytosol or nucleus. Endosomal entrapment is the major obstacle in the delivery of oligonucleotides. A remarkable amount of current research has focused on modulating the association, internalization, and intracellular trafficking to induce escape of nanoparticles from endosomal compartments. This has become the major focus in the delivery of oligonucleotides and other nucleic acid-based therapeutics.

Clathrin-mediated endocytosis is a well-studied internalization route that can lead to funtional delivery of nucleic acids. Clathrin was discovered in 1976 as a coat protein composed of heavy and light chains (Pearse 1976). The protein plays an essential role in endocytosis as it triggers the formation of membrane patches which bud inward (Bonifacino 2003). The clathrin-coated membrane buds eventually become endosomal vesicles via action of dynamin GTPases (Cocucci *et al.* 2014).

Other non-clathrin mediated internalization processes can also encourage oligonucleotide activity. One of such is caveolae-mediated endocytosis, which has been well studied. Caveolin pits are invaginations in the cell membrane which are encountered in several, but not all, types of cells and contain at least one protein from caveolin family. They are involved in endocytosis, and also the maintenance of membrane tension and cell surface area (Keren 2011). Caveolin pits can endocytose a wide variety of cargo molecules (Mayor and Pagano 2007) and, similarly to clathrin-dependent endocytosis, depend on dynamin for vesicle formation.

Macropinocytosis is also an internalization pathway, but does not usually require the association of nanoparticles with cell membrane. Macropinocytosis is a process that involves the ruffling of the plasma membrane and formation of lamellopodia, leading to “engulfing” of a portion of the extracellular environment. The internalized volume is sufficient for nonspecific solute molecules to enter from the extracellular environment. Intracellular vesicles that form during macropinocytosis, i.e. macropinosomes, are much larger than in the case of other endocytic pathways, having diameter of over 1 μm (Kirkham and Parton 2005).

Multiple entry routes play a role in funtional CPP-cargo delivery, but eventually, all internalization pathways lead to the formation of early endosomes. As regards ON delivery, this

often leads to the entrapment of ONs in endosomal vesicles irrespective of the particular entry way used.

Intracellular trafficking usually varies from one cell type to another and is one of the most crucial steps that influences the pharmacological activity of ONs (Juliano and Carver 2015). After internalization, CPP-cargo is trafficked via early endosomes and late endosomes into lysosomes (Gruenberg and Maxfield 1995). Different steps of the trafficking can be studied by the time-dependent colocalization with specific markers such as EEA1 and Rab5 for early endosomes, and Rab7/9 or LAMP-1 for late endosomes and lysosomes.

Endocytic vesicles usually fuse with early endosomes (EEs) after pinching off from the plasma membrane (Wang *et al*. 2016). Early endosomes may then move their cargo towards late endosomes (LEs) into lysosomes or multivesicular bodies (MVBs). Cargo can also be directed towards the cell membrane via recycling endosomes for exocytosis (Figure 3) (Juliano 2018).

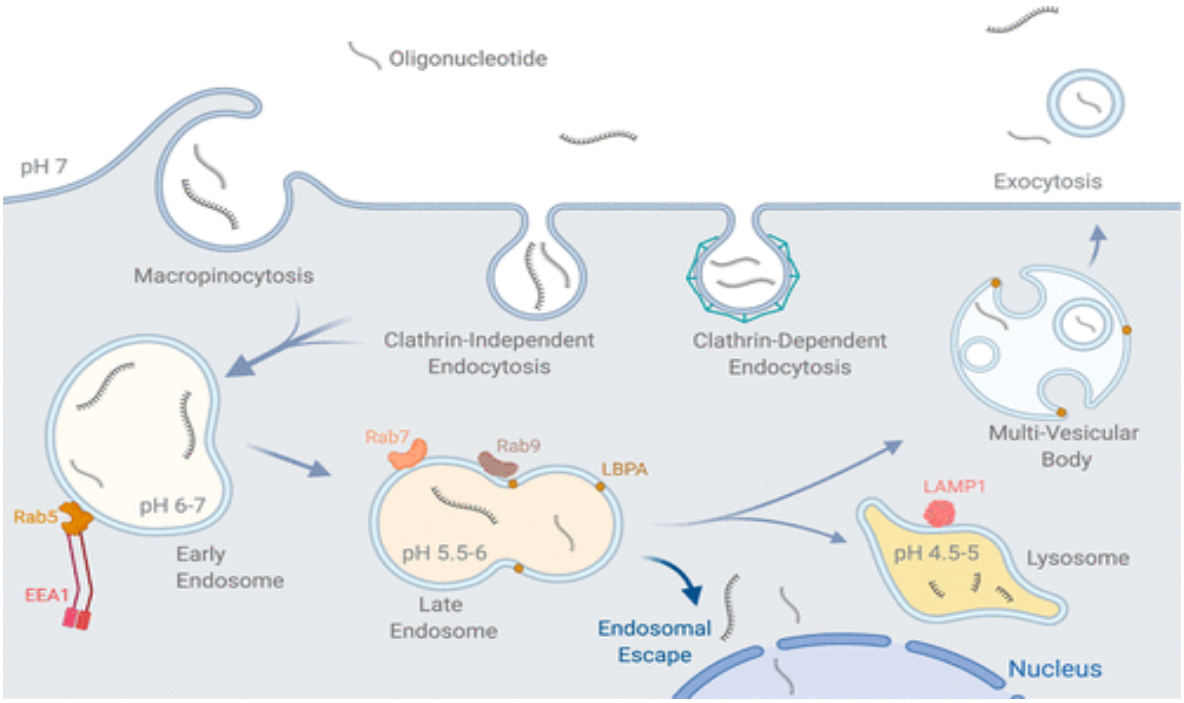

Figure 3. Endocytic uptake and endosomal escape of ONs. The well known internalization routes of ONs are clathrin-dependent endocytosis, caveolin-dependent endocytosis, and macropinocytosis. ON is subsequently trafficked via the early endosome, late endosome and to the lysosome or to the multivesicular bodies (MVB), or is exocytosed. Late endosome membrane remodeling and transition to MVB or lysosome have been indicated as likely points of endosomal escape. Commonly used endosomal markers are shown. Abbreviations: Rab, Ras-superfamily protein; EEA1, early endosome antigen 1; LBPA, lysobisphosphatidic acid; LAMP1, lysosomal-associated membrane protein 1 (Bost *et al*. 2021).

The endosomal trafficking of nanoparticles involves a decrease in luminal pH as endosome compartments mature and eventually converge with lysosomes with an acidic pH between 4 and 5 (Pillay *et al*. 2002). Acidic environment and lysosomal enzymes, hydrolases, mediate degradation of most molecules that get entrapped in lysosomes. Because of that, for efficient transfection, nucleic acids have to escape from endosomes at early stages of trafficking via endo-lysosomal pathway in order to avoid eventual degradation upon entrapment in these acidic vesicles.

1.2.3 Enhancement of endosomal escape

The greatest obstacle to the clinical application of oligonucleotides is their endosomal entrapment. As a result of this, ON is unable to get to the cytosol or nucleus of its target cells in sufficient concentrations (Juliano *et al.* 2008). One strategy that can be used to induce escape of ONs from endosomes is the addition of endosomolytic compounds to nanoparticles.

Endosomolytic compounds enhance endosome membrane rupture and escape of CPP-ON nanoparticles by buffering the pH in the endosomal lumen as the pH drops during maturation of the vesicle. This buffering enhances osmotic pressure in the lumen, that leads to the swelling of the endosome, inducing membrane rupture and escape of nanoparticles into the cytosol. Endosomolytic compounds are often used as additives to enhance the activity of CPP-ON nanoparticles to promote robust endosomal escape (El Andaloussi *et al*. 2011, Lehto *et al*. 2011). The widely used endosomolytic compound is chloroquine (Pelt *et al*. 2018). Chloroquine has high activity *in vitro* and *in vivo* (Yang et al. 2015), enhancing endosomal escape in the range of 40 to 100 µM (Lönn *et al*. 2016).

In general, more work is needed to identify endosomolytic compounds with considerable risk-benefit ratio that can be used as additives to CPP-ON nanoparticles. In this study, we tested several compounds that could enhance endosomal escape of CPP-ON nanoparticles, leading to an increase in biological effect of the ON (see Table 2 in the Materials and Methods section).

1.2.4 Inhibition of autophagy and exocytosis

Autophagy is a cellular process in which a part of the cytoplasm is isolated inside a double membrane vesicle, called autophagosome, that eventually fuses with the lysosome for degradation (Yang and Klionsky 2010). It is an important pathway for maintaining cellular homeostasis and integrity by neutralizing unwanted cytosolic compounds and dysfuntional organelles under physiological conditions.

Autophagy can be categorized into three major classes: macroautophagy, microautophagy, and chaperone-mediated autophagy. Microautophagy involves the separation of bulk portion of cytoplasm or organelles inside the lysosomal lumen by septation, invagination, or protrusion of the lysosomal membrane. The exact mechanism of macroautophagy is not well understood.

Research has shown that the autophagy pathway is triggered during the cellular uptake of CPP-ON nanoparticle delivery. This implies that autophagy process may influence the efficacy of nucleic acid transfection, as nanoparticles can be trapped in autophagosomes and eventually degraded (Dowaidar *et al.* 2017). Thus, the rationale for use of autophagy modulating agents is enhancing the functional delivery of the ON.

Exocytosis is an active transport of internalized molecules out of the cell. Endocytosed ON can be redirected to the extracellular environment via exocytosis (Figure 3), that leads to loss/reduction of the amount of active ON at site of action, and biological activity is greatly affected. Thus, compounds that inhibit or reduce exocytosis can be added to CPP-ON nanoparticles to enhance efficiency of productive delivery of the ON.

In this study, we tested several compounds that could reduce autophagy or exocytosis, leading to an increase in productive delivery of CPP-ON nanoparticles and enhancement of biological effect of the ON (see Table 2 in the Materials and Methods section).

1.2.5 Putative mechanism of calcium and magnesium ions

Recent research has demonstrated that calcium and magnesium ions have the potential to increase the transfection efficiency and biological activity of CPP-SCO nanoparticles (Maloverjan *et al*. 2022). Calcium has been previously used in nanoparticle formulations to condense nucleic acids and enhance the transfection of viral DNA and siRNA (Graham *et al*. 1973, Baoum *et al*. 2012). Calcium and magnesium are involved in regulating metabolic pathways and internalization of various molecules into cells. Calcium ions play a critical

role in endocytosis (Santos *et al*. 2017). Additionally, peptide-mediated transfection involves a transient increase in intracellular calcium concentration, and sequestration of extracellular calcium ions is known to reduce internalization and biological activity of CPP-SCO nanoparticles (Melikov *et al*. 2015). Calcium supposedly acts via enhancing the endosomal escape of nanoparticles, but its exact mechanism of action is not well understood. In this study, adding of calcium chloride to the CPP-ON nanoparticles was used as a positive control.

2 THE AIMS OF THE THESIS

The aim of the thesis was to enhance the functional delivery of SCO and, alternatively, siRNA by altering the normal intracellular trafficking of CPP-ON nanoparticles through endo-lysosomal pathway and modulating other cellular events that can lead to degradation and loss of activity of the ON. This work was divided into the following steps:

1. Selecting compounds that could increase functional delivery and biological effect of ON. The compounds selected for screening are known to induce endosomal escape of oligonucleotides or to modulate other cellular events that can lead to loss or reduction of activity of ON, such as exocytosis and autophagy.
2. Screening of the series of selected compounds (endosomal escape enhancers, autophagy inhibitors and exocytosis inhibitors) in splice-correcting assay in HeLa pLuc 705 reporter cell line and selecting the best ones.
3. Testing the biological activity and toxicity of the best-performing compounds in another cell line with impaired splicing.
4. Assessing the ability of the best-performing compounds to enhance the biological effect of another type of nucleic acid, siRNA.

3 EXPERIMENTAL PART

3.1 Materials and methods

3.1.1 Materials

Oligonucleotides and cell-penetrating peptide:

Splice-correcting oligonucleotides, SCO-705 (5'-CCUCUUACCUCAGUUACA-3') and SCO-654 (5'-GCUAUUACCUAAACCCAG-3'), siRNA against luciferase and negative siRNA were purchased from Microsynth, Switzerland. Lipofectamine RNAiMAX Reagent was purchased from Invitrogen, USA. The CPP used was PepFect14 (PF14), a stearylated amphipathic peptide from the PepFect family. CPP was obtained from PepScan, the Netherlands. 1 mM peptide stock solutions were prepared and stored at -20 °C.

Cell culture solutions and plastic:

Dulbecco's Modified Eagle Medium (DMEM) used was purchased from Sigma-Aldrich; Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and magnesium and trypsin-EDTA solution were purchased from Corning, USA. 6 cm cell culture plates were purchased from Corning, 24-well plates were obtained from VWR, USA, and 96-well plates from Corning.

Solutions and reagents used for luminescence and fluorescence measurements:

The firefly luciferase substrate solution contained luciferin (LH2), DTT, CoA, ATP, $MgCO_3$ and $MgSO_4$ and was prepared and stored at -25 °C. Cell Culture Lysis Reagent was obtained from Promega, USA and stored at 4 °C. Ready-to-use Cell Proliferation Colorimetric Reagent, WST-1 was obtained from BioVision, USA and stored at -25 °C. Hoechst 33258 dye stock solution (1 mg/mL) was purchased from Sigma-Aldrich and stored at -25 °C. Paraformaldehyde (PFA) was purchased from Sigma-Aldrich and stored at 4 °C.

Compounds added to the CPP-ON nanoparticles:

$CaCl_2$, $MgCl_2$ and chloroquine were purchased from Sigma-Aldrich and Applichem, Germany. The series of compounds studied in this project were selected from Medchemexpress bioactive compound screening library and purchased from Sigma-Aldrich and Cayman chemical company, USA and stored at 4 °C. The series included: polysorbate 60, polysorbate 80, retro-2, L-leucyl-L-leucine methyl ester monohydrochloride (LLOMe), spermidine, 1-

pyrenebutyric acid, dantrolene sodium salt, wortmannin, rottlerin, bafilomycin A1, brefeldin A, EACC, ROC-325, autophinib, N-benzylguanidine hydrochloride, vacuolin-1, mevinolin (lovastatin) and imipramine hydrochloride (Table 2). Stock solutions of these compounds in DMSO with different concentrations were prepared and stored at 4 °C.

Table 2. Compounds added to the CPP-ON nanoparticles in this study. The compounds used were added for three different purposes: promoting endosomal escape of the nanoparticles, inhibiting autophagy or inhibiting exocytosis. All of these mechanisms can result in an increase of functional delivery of the nucleic acid cargo.

Name of a compound	Mechanism of action	Reference
Autophinib	Autophagy inhibitor	Robke *et al*. 2017
Bafilomycin A1	Autophagy inhibitor	Yuan *et al*. 2015
Brefeldin A	Autophagy inhibitor	Wang *et al*. 2016
Chloroquine	Endosomal escape	Lönn *et al*. 2016
Dantrolene sodium	Endosomal escape	Chitkara *et al*. 2018
EACC	Autophagy inhibitor	Vats and Manjithaya 2019
Imipramine hydrochloride	Exocytosis inhibitor	Catalano *et al*. 2020
L-leucyl-L-leucine methyl ester monohydrochloride (LLOMe)	Endosomal escape	Repnik *et al*. 2017
Mevinolin	Exocytosis inhibitor	Mailman *et al*. 2011
N-benzylguanidine hydrochloride	Endosomal escape	Kong *et al*. 2020
Polysorbate 60	Endosomal escape	Kaur *et al*. 2014
Polysorbate 80	Endosomal escape	Göppert and Müller 2005
Pyrenebutyric acid	Endosomal escape	Lai *et al*.2017
Retro-2	Endosomal escape	Valérie *et al*. 2020
ROC-325	Autophagy inhibitor	Carew *et al*. 2017
Rottlerin	Endosomal escape	Duchardt *et al*. 2007
Spermidine	Endosomal escape	Tang *et al*. 2017
Vacuolin-1	Exocytosis inhibitor	Cerny *et al*. 2004
Wortmannin	Autophagy inhibitor	Moon *et al*. 2015

3.1.2 Cell culturing and seeding

Two splice-switch reporter cell lines, HeLa pLuc 705 and HeLa EGFP 654, were used. For siRNA assay, luciferase-expressing cell line U87 MG-Luc2 was used. The cells were grown at 37 °C and 5 % of CO_2 in DMEM that contained 4.5 g/L of glucose, 10 % (v:v) of fetal bovine serum (FBS) and 1 % (v:v) of penicillin-streptomycin solution (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were grown on 6 cm cell culture plates. Splitting of cells was performed every 48 hours. For detaching cells from plate, trypsin-EDTA solution was used. Cells were washed with DPBS without calcium and magnesium ions. For experiments, cells were seeded on 24-well plates and 96-well plates. All experiments with cells were performed in a sterile cell culture hood.

3.1.3 Nanoparticle preparation and transfection

For formation of nanoparticles, CPP and SCO were mixed at molar ratio (MR) 5 in Milli-Q (MQ) water in 1/10th of final volume. After 15 minutes incubation at room temperature (RT), $CaCl_2$ or $MgCl_2$ solutions and the series of compounds under study were added at various concentrations. After 15 min, solutions were diluted with pre-warmed growth medium 10-fold to reach the final volume, and applied to the cells. SCO was applied at a final concentration of 100 nM and CPP of 500 nM.

For CPP-siRNA nanoparticle formation, MR 17 (CPP:siRNA) in MQ water was used. The concentration of siRNA on cells was 15 nM and CPP was 255 nM. As a negative control, negative (non-targeting) siRNA was used. As a positive control, nanoparticles were prepared using Lipofectamine RNAiMAX Reagent, according to the manufacturer's protocol. PF14-siRNA nanoparticles were prepared in the same way as PF14-SCO nanoparticles, diluted 10 times with growth medium and incubated with cells for 48 h before luminescence measurement.

3.1.4 Luciferace assay

HeLa pLuc 705 cells at 20,000 cell per well, were plated on a 96-well plate 24 h prior to transfection. CPP-SCO-705 nanoparticles were prepared and cells were transfected as described above. After 24 h of incubation, cells were washed with DPBS and lysed by adding 20 µL of cell culture lysis buffer and incubating samples at -25 °C for 20 minutes until freezing of the solutions. Then, solutions were thawed at RT, 70 µL of luciferase substrate solution per well was added, solutions were transferred to white 96-well plate and luminescence intensity was measured, using the 96 Microplate Luminometer (Promega) and GloMax software.

For siRNA assay, luciferase-expressing cell line, U87 MG-Luc2 was used. 10,000 cells per well were plated on a 96-well plate, and next day, CPP-siRNA nanoparticles were prepared as described above. Cells were incubated with siRNA-containing solutions for 48 hours. After it, lysis and measurement of luminescence was performed as described above.

3.1.5 Flow cytometry

HeLa EGFP 654 cells at 10,000 cell per well were plated on a 96-well plate 24 h prior to transfection. Next day, the growth medium was replaced with medium containing CPP-SCO-654 nanoparticles prepared with the addition of the most efficient compounds selected from the series under study. Nanoparticles of PF14 and SCO-654 were prepared as described above. Cells were incubated with the nanoparticles for 24 h at 37 °C. Cells were washed with PBS, trypsin was added and cells were incubated at 37 °C for 3 to 5 minutes until detachment. Then, trypsin was diluted with PBS and the plate was placed on ice. Next, DAPI solution in PBS at a final concentration of DAPI 0.5 µg/mL was added to distinguish between live and dead cells. Nuclei were stained for at least 5 minutes and cells were resuspended with a pipette. Next, flow cytometry of cell suspensions was performed with Attune NxT Flow Cytometer, detecting DAPI (λ_{ex} 405 nm) and EGFP (λ_{ex} 488 nm). For each sample, 5000 events were analyzed.

3.1.6 Cell viability assay

To evaluate the cytotoxicity of nanoparticles, cell viability was measured spectrophotometrically, using WST-1 assay. HeLa pLuc 705 cells (20,000 cells per well), HeLa EGFP 654 (10,000 cells per well) or U87 MG-Luc2 (10,000 cells per well) were plated on a 96-well plate and transfected the next day with the PF14-SCO or PF14-siRNA nanoparticles prepared with the most efficient compounds as described above, at a volume of 70 µL per well. After 24 h (or 48 h, in the case of U87 MG-Luc2 cells) of incubation, 7 µL of WST-1 solution was added to each well and cells were placed at 37 °C. After 3 hours of incubation, absorbance of the resulting solutions was measured at 440 nm with reference wavelength 650 nm, using an Infinite M200 PRO (Tecan) microplate reader. Absorbance of cell-free WST-1 containing growth medium was taken for blank and subtracted from all other values. Absorbance of untreated cells was taken for 100% viability.

3.1.7 Confocal microscopy

HeLa EGFP 654 cells at 50,000 cells per well were plated in a 24-well plate on cover glasses with a diameter of 12 mm (Menzel-Gläser, Germany) 24 hours before transfection. Then, cells were transfected as described above, with the nanoparticles prepared with the addition of the best performing compounds from the series screened, using SCO-654. The next day,

cells were washed with DPBS and incubated for 25 minutes with 4% PFA solution in PBS at RT for fixing cells. Next, cells were washed with DPBS and incubated at RT for 10 minutes with Hoechst 33258 dye (at 1 µg/mL solution in DPBS) for staining nuclei. Finally, cells were mounted to glass slides in 30% glycerol. Samples were analyzed using Olympus FluoView FV1000 (Olympus, Japan) confocal microscope. For each solution, two samples were prepared and at least 2 images per sample were obtained. EGFP (λ_{ex} 488 nm) and Hoechst 33258 (λ_{ex} 405 nm) signals were analyzed, using 60× objective with water immersion.

3.1.8 Statistical analysis

All statistical analyses were performed using Prism 8.0.1 (GraphPad Software, USA). Each dataset is shown as mean + SEM. Statistical significance of differences between the values of each study group was analyzed using one-way ANOVA with post-hoc Dunnett's test at a significance level of 0.05.

3.2 RESULTS

3.2.1 Chloroquine, PS80, imipramine, vacuolin and LLOMe markedly enhance biological effect of SCO

Compounds of interest (n = 19) were divided into five series for screening. In each series, untreated cells were used as a negative control and PF14-SCO nanoparticles prepared with addition of 3 mM calcium and magnesium chloride, that were previously shown to significantly enhance efficiency of SCO (Maloverjan *et al.* 2022) were used as a positive control.

The first series of compounds included chloroquine and polysorbate 80 (PS80). Chloroquine is an endosome-destabilizing compound (Lönn *et al.* 2016) that is known to increase transfection efficiency of multiple compounds, which can get entrapped and degraded in the endo-lysosomal compartments. PS80 is a detergent that has been shown to enhance efficiency of lipid nanoparticles, increasing penetration through physiological barriers, including blood-brain barrier (Göppert and Müller 2005).

PF14-SCO nanoparticles were prepared as described above, with addition of PS80 and chloroquine at different concentration, and applied to cells (Figure 4). Chloroquine was also added to SCO at various concentrations without PF14 in order to see, if it could significantly improve efficiency of naked SCO. When prepared, particle-containing solutions were diluted with cell culture medium and applied on cells. The splice-correcting acivity was measured by luminescence after 24 h of incubation.

It can be clearly seen that chloroquine, as well as calcium, significantly increased the efficiency of transfection (Figure 4). Chloroquine at 30 µM was the most efficient, while at 50 µM, the splicing efficiency was reduced, probably due to toxicity. PS80 had a positive, though not significant effect at 0.1 mg/mL, which was low compared to chloroquine and calcium. SCO-chloroquine only solutions had little or no effect when applied on cells, implying that PF14 is necessary for transport of nanoparticles across cell membrane. Chloroquine at 30 µM and 50 µM and PS80 at 0.1 mg/mL were selected and used for further analysis in this work.

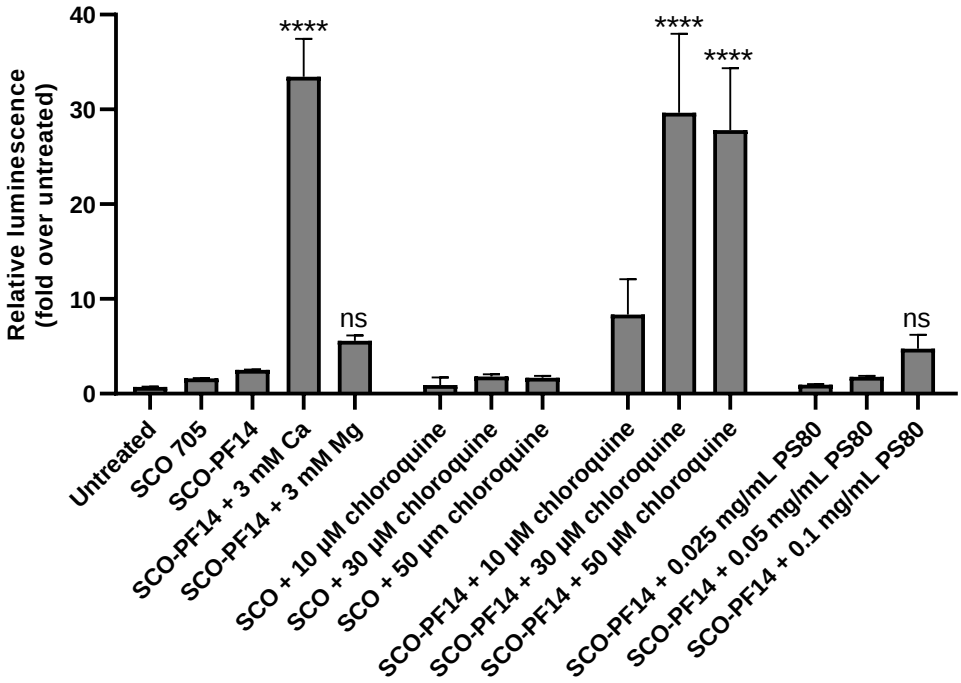

Figure 4. Transfection of HeLa pLuc 705 cells with PF14-SCO-705 nanoparticles associated non-covalently, with addition of chloroquine, polysorbate 80 (PS80), calcium and magnesium ions at various concentrations. Untreated cells were used as a negative control. PF14+SCO+calcium nanoparticles were used as a positive control. Transfection efficiency was measured with luciferase assay and luminescence values were normalized to untreated cells. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, **** p-value < 0.0001, ns – not significant.

The second analyzed series of agents included Brefeldin A, EACC, ROC-325, autophinib and N-Benzylguanidine hydrochloride. These compounds were added to PF14-SCO nanoparticles at various concentrations and resulting solutions were applied on cells, as well as ones prepared with calcium and magnesium ions (Figure 5). Splice-correcting activity of SCO was measured with luciferace assay.

It can be seen that only the diavalent metal ions (calcium and magnesium) had considerable effect on transfection efficiency and biological activity of SCO. EACC (Vats and Manjithaya 2019), ROC-325 (Carew *et al*. 2017), autophinib (Robke *et al*. 2017) and brefeldin A (Wang *et al*. 2016) are known to inhibit autophagy. As stated in the literature review, autophagy is a cellular process that can lead to degradation and loss activity of PF14 nanoparticles. As

indicated by the results of this study, inhibiting this process has a minimal effect on transfection efficiency. N-benzylguanidine hydrochloride, a compound that could promote cellular uptake and endosomal escape of nanoparticles, also had almost no effect on transfection efficiency. None of the compounds from the second series was selected for further experiments.

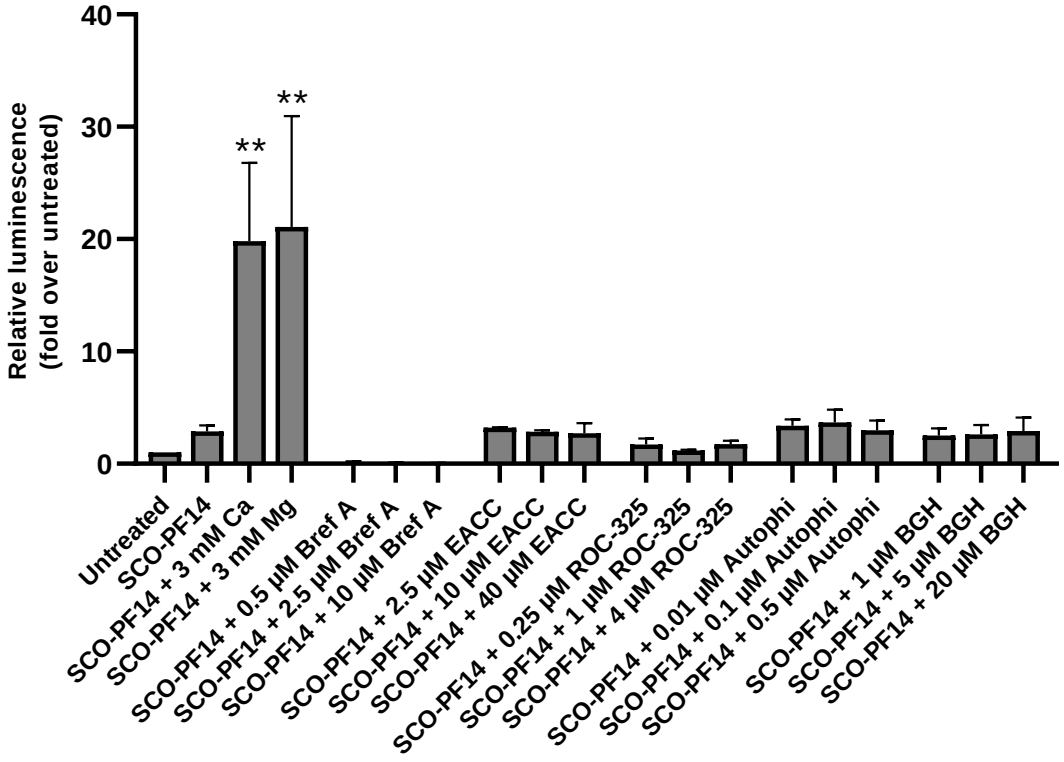

Figure 5. Transfection of HeLa pLuc 705 cells with PF14-SCO-705 nanoparticles associated non-covalently, with addition of Brefeldin A (Bref A), EACC, ROC-325, autophinib (Autophi), N-benzylguanidine hydrochloride (BGH), calcium and magnesium ions at various concentrations. Untreated cells were used as a negative control, PF14+SCO+calcium ions were used as a positive control. Transfection efficiency was measured with luciferase assay and luminescence values were normalized to untreated cells. Each dataset represents mean + SEM of three independent experiments. The data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, ** p-value < 0.005.

The third series of compounds included pyrenebutyric acid, dantrolene, rottlerin, wortmannin and bafilomycin A1 (Figure 6). Bafilomycin A1 (Yuan *et al*. 2015) and wortmannin (Moon *et al*. 2015) are know to inhibit cellular autophagosome-lysosome fusion and interfere with degradation in lysosomes. In addition to it, bafilomycin is a reversible inhibitor of

vacuolar H^+-ATPase (V-ATPase), which inhibits endosome acidification, that could also reduce endo-lysosomal degradation of the oligonucleotide. These two autophagy inhibitors had negligible effect on efficiency. Rottlerin, a protein kinase C inhibiitor, was shown to modulate cell-penetration of CPPs (Duchardt *et al*. 2007). Pyrenebutyric acid, which is known to facilitate translocation of CPPs across membranes, and dantrolene, which has been previously shown to encourage endosomal escape of nanoparticles had little or no effect on transfection efficiency.

As only calcium and magnesium ions showed increased efficiency when added to nanoparticles, none of the compounds from the third series was selected for further experiments.

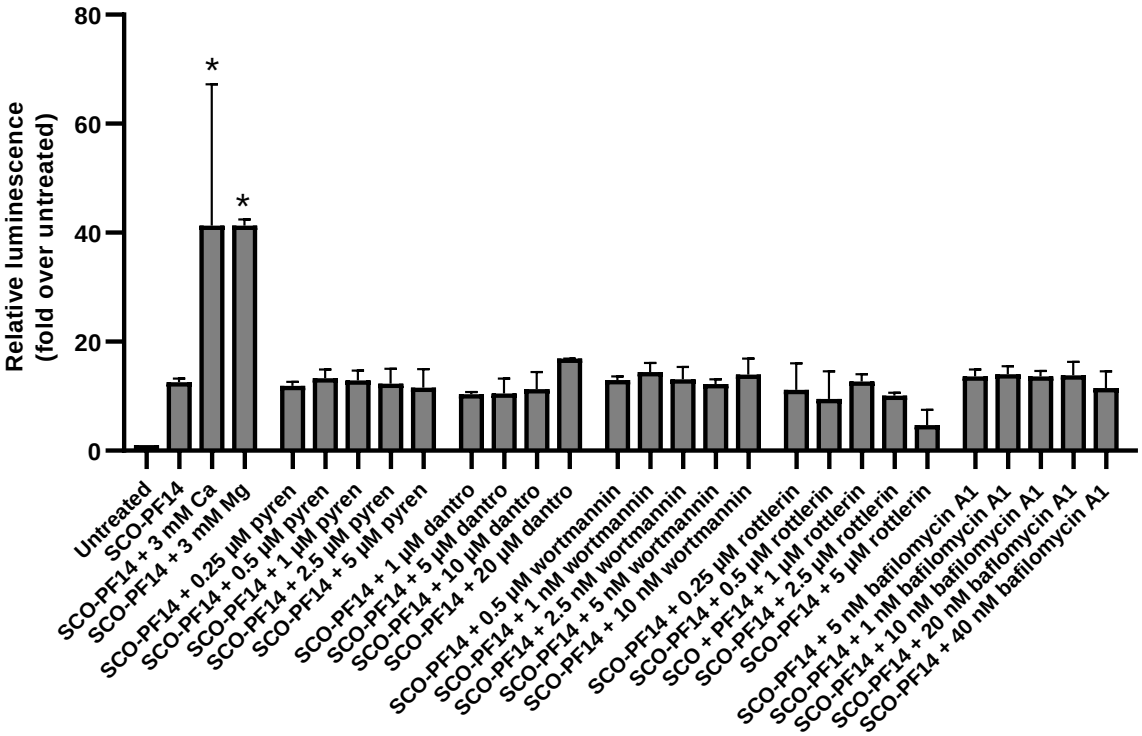

Figure 6. Transfection of HeLa pLuc 705 cells with PF14-SCO-705 nanoparticles associated non-covalently, with the addition of pyrenebutyric acid (pyren), dantrolene (dantro), wortmannin, rottlerin, bafilomycin A1, calcium and magnesium ions at various concentrations. Untreated cells were used as a negative control, PF14+SCO+calcium nanoparticles were used as a positive control. Transfection efficiency was measured with luciferase assay and luminescence values were normalized to untreated cells. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, * p-value < 0.05.

In the fourth series of tested inhibitors, vacuolin, imipramine and mevinolin were added to PF14-SCO nanoparticles at different concentrations and applied on cells, as well as calcium and magnesium ions (Figure 7). Imipramine is known to inhibit exocytosis and extracellular vesicle formation (Catalano *et al.* 2020). Vacuolin is a potent and cell-permeable lysosomal exocytosis inhibitor (Cerny *et al.* 2004). It is also known to inhibit autophagy.

Imipramine and vacuolin had a positive, though not significant effect on the splice-correcting activity of SCO. The optimal effect was reached when applying 40 µM imipramine and 2.5 µM vacuolin. Mevinolin (also known as Lovastatin) that reduces cholestrol levels in cells and modulates permeability of membranes had no positive effect on transfection efficiency. Imipramine and vacuolin were selected for further analysis.

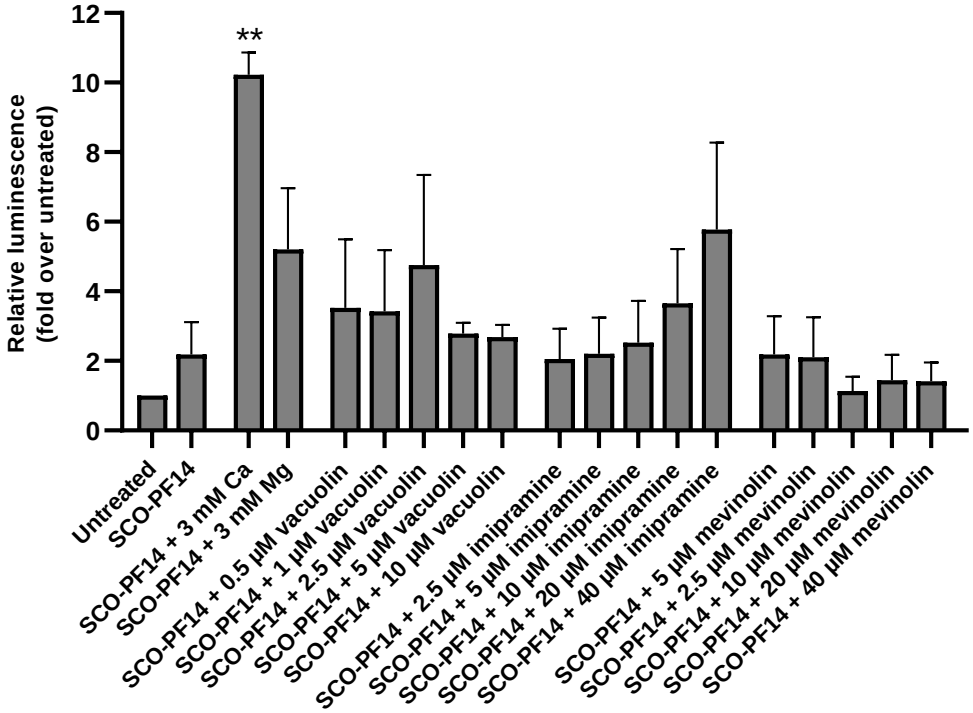

Figure 7. Transfection of HeLa pLuc 705 cells with PF14-SCO-705 nanoparticles associated non-covalently, with addition of vacuolin, imipramine and mevinolin at various concentrations, as well as calcium and magnesium ions. Untreated cells were used as a negative control. PF14+SCO+calcium nanoparticles were used as a positive control. Transfection efficiency was measured with luciferase assay and luminescence measurements were normalized to untreated cells. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, ** p-value < 0.005.

Finally, the fifth series included L-leucyl-L-leucine methyl ester (LLOMe), spermidine, polysorbate 60 (PS60) and retro-2 (Figure 8). LLOMe is know to induce lysosomal membrane permeabilization which encourages the escape of nanoparticles (Repnik *et al*. 2017).

LLOMe had a strong positive effect on the splice-correcting activity of SCO, as seen in Figure 8, and the highest effect was observed at 0.5 mM concentration. Retro-2, an endosomal escape inducer of oligonucleotides in retrograde transport, and PS60, an analogue of PS80, had negligible effect on transfection efficiency of PF14-SCO nanoparticles. Spermidine increased expression of luciferase to some extent, but the effect was much weaker than that of LLOMe and calcium ions. LLOMe was selected from this series and used for further experiments.

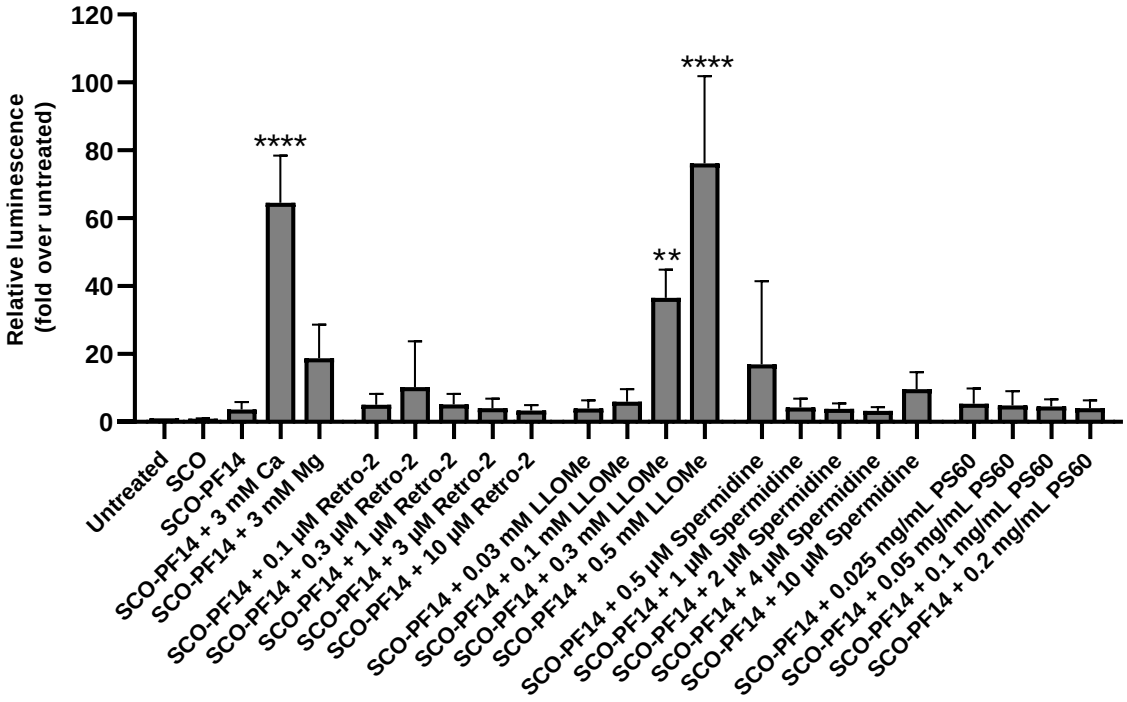

Figure 8. Transfection of HeLa pLuc 705 cells with PF14-SCO-705 nanoparticles associated non-covalently, with addition of Retro-2, L-leucyl-L-leucine methyl ester (LLOMe), spermidine and Polysorbate 60 (PS60) at various concentrations, as well as calcium and magnesium ions. Untreated cells were used as a negative control. PF14+SCO+calcium ions particles were used as a positive control. Transfection efficiency was measured with luciferase assay and luminescence values were normalized to untreated cells. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, ** p-value < 0.01, **** p-value < 0.0001.

3.2.2 Chloroquine, LLOMe and imipramine have the most prominent positive effect on the PF14-mediated transfection of SCO

The most efficient compounds selected during the screening, i.e. chloroquine, LLOMe, vacuolin, imipramine and PS80, were analysed side-by-side in order to compare their efficiency with each other and with calcium and magnesium ions (Figure 9). 40 µM imipramine, 30 µM chloroquine and 0.5 mM LLOMe significantly and most strongly increased splice-correcting activity of SCO. These concentrations were selected as optimal ones. When the concentration was increased further, as seen with imipramine at 60 µM and chloroqiune at 50 µM, SCO activity was reduced, probably due to toxicity. To assess for possible synergy or additive effects of calcium with these most efficient compounds, calcium ions were used together with imipramine, chloroquine and LLOMe, respectively, and added to PF14-SCO nanoparticles, followed by transfection into cells. The results showed no additive or synergistic effect on SCO activity.

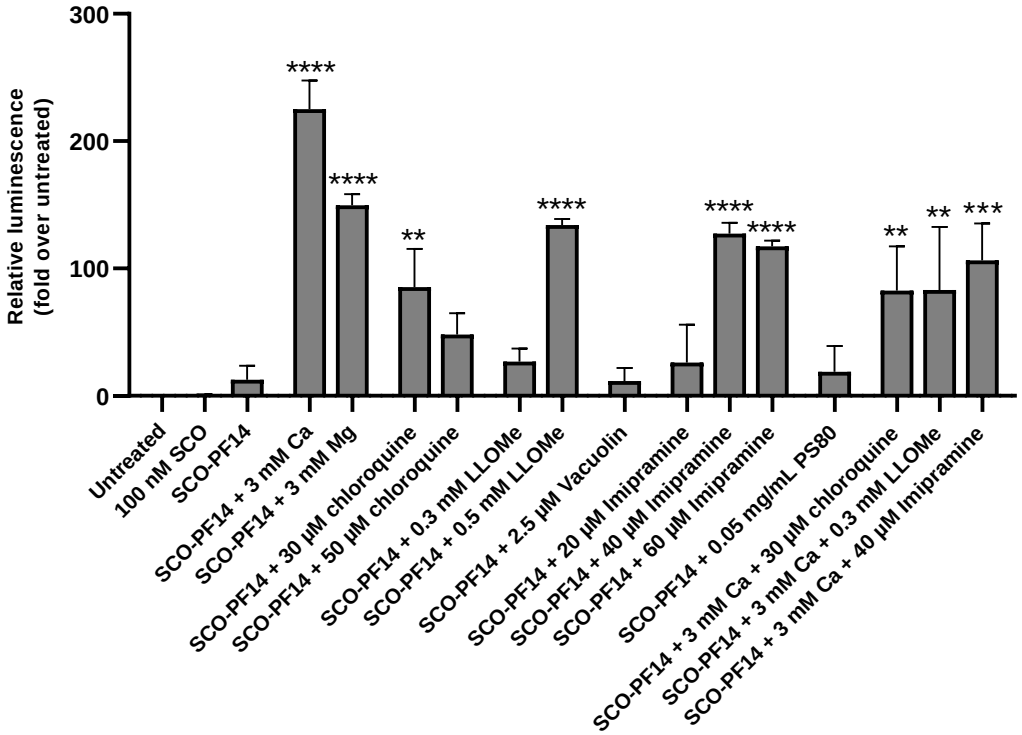

Figure 9. Transfection of HeLa pLuc 705 cells with PF14-SCO-705 nanoparticles formed non-covalently, with addition of the most efficient compounds selected from the screening. PF14+SCO+calcium particles were used as a positive control. Untreated cells were used as a negative control. Transfection efficiency was measured with luciferase assay and luminescence values were normalized to untreated cells. Each dataset represents mean + SEM of 3 independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

3.2.3 The most efficient compounds enhance splicing correction in HeLa EGFP 654 reporter cell line

After selecting the compounds that could significantly enhance the efficiency of SCO-705 transfected with PF14 into HeLa pLuc 705 cells, we decided to test these compounds in another reporter cell line with aberrant splicing, i.e. HeLa EGFP 654, in order to assess the universality of their application. In this cells line, the introduced EGFP gene is interrupted by the β-globin intron 2, that contains a mutation in position 654, which leads to aberrant splicing. When splice-correcting oligonucleotide SCO-654 is efficiently delivered into the cells, it masks the aberrant splicing-inducing site, enabling production of enhanced green fluorescent protein, EGFP.

The addition of the most efficient compounds to PF14-SCO-654 nanoparticles resulted in an increased splice-correcting activity of SCO in HeLa EGFP 654 cell line (Figure 10). In Figure 10, A, B and C have low intensity of green flourescence which indicates very little or no splice-correcting activity in cells. These are the untreated control cells, cells treated with SCO only, and cells incubated with PF14-SCO nanoparticles, respectively. However, in D, E and F, a high intensty of green flourescence can be seen, which indicates substantially higher splice-correcting activity of the respective nanoparticle. Nanoparticles in D, E and F were supplemented with calcium ions, magnesium ions and chloroquine, respectively. These compounds enhanced the activity of SCO-654, leading to the expression of green flourescent protein at easily detectable concentration. In addition, cells with nanoparticles in G, H and I, which contained LLOMe, bafilomycin A1 and imipramine, respectively, expressed considerable amount of GFP, too, but the signal intensity was lower, compared to calcium and magnesium.

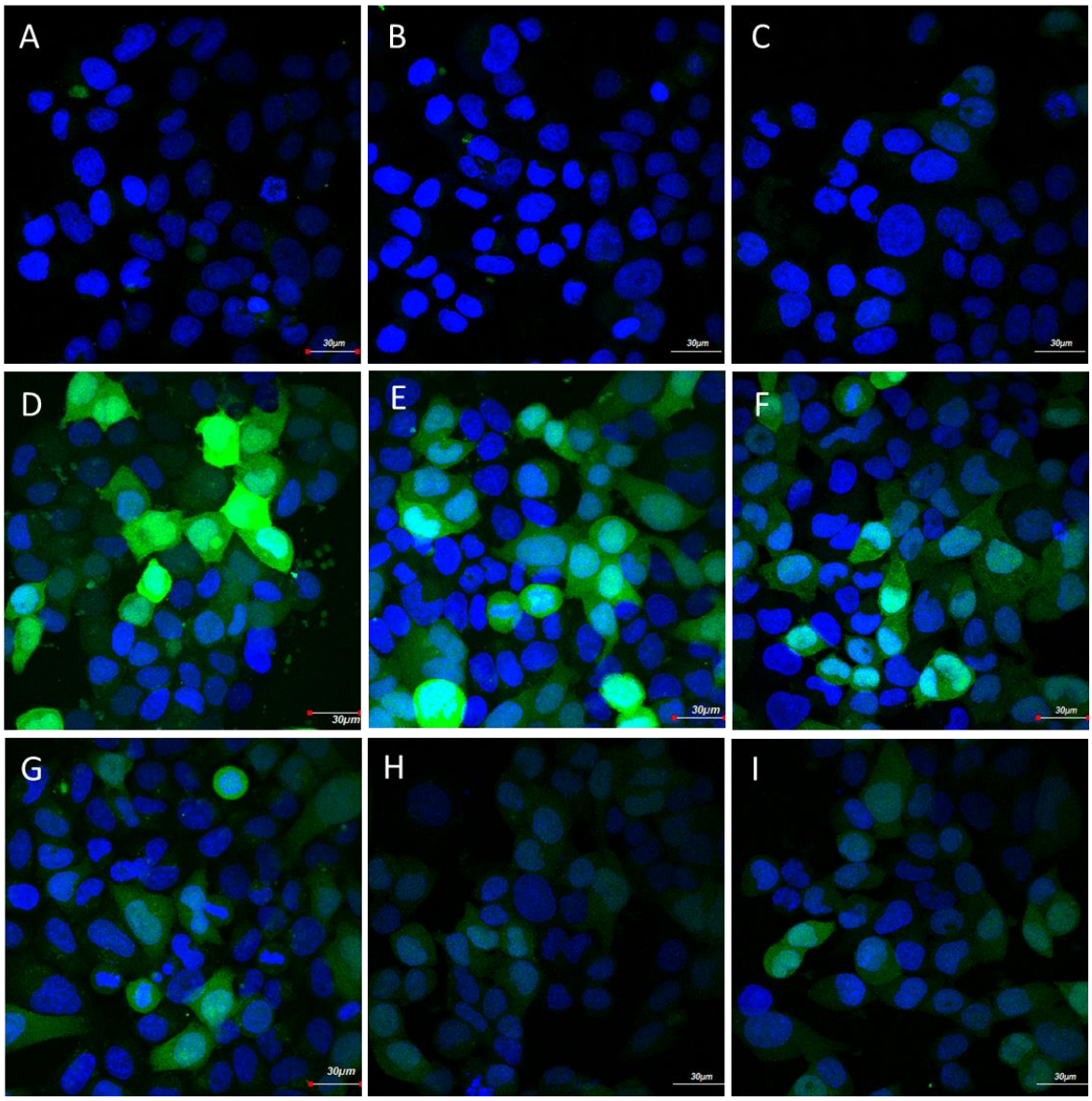

Figure 10. Confocal microscopy images showing the delivery of splice-correcting oligonucleotide into HeLa EGFP 654 cells with PF14 nanoparticles. (A) represents untreated cells, (B) is cells treated with only SCO-654, (C) cells treated with PF14-SCO, (D) cells treated with SCO-PF14 + 3 mM calcium chloride, (E) cells treated with SCO-PF14 + 3 mM magnesium chloride, (F) cells treated with SCO-PF14 + 30 µM chloroquine, (G) cells treated with SCO-PF14 + 0.5 mM LLOMe, (H) cells treated with SCO-PF14 + 10 nM Bafilomycin A1 and (I) cells treated with SCO-PF14 + 40 µM imipramine. One representative of three independent experiments is shown. Scale bar is 30 µM. The cell nuclei were stained with Hoechst 33258 dye (blue). In images D, E, F, G, H and I, the enhancement of EGFP expression (green) by the addition of cacium ions, magnesium ions, chloroquine, LLOMe, bafilomycin A1 and imipramine respectively, to PF14-SCO nanoparticles can be seen.

The effect of the most efficient compounds on SCO delivery into HeLa EGFP 654 cells with PF14 was further examined with flow cytometry in order to quantitatively analyze the efficiency of the compounds under study (Figure 11). Result in Figure 11 demonstrates that these compounds have a similar level of activity in both splice-switching reporter cell lines. The most efficient compounds such as chloroquine, LLOMe, imipramine as well as calcium and magnesium ions have similar positive effect when added to PF14-SCO nanoparticles formed with both SCO-705 and SCO-654. Also, the addition of calcium to PF14-SCO nanoparticle containing imipramine, chloroquine and LLOMe showed no synergistic or additive effect on SCO activity.

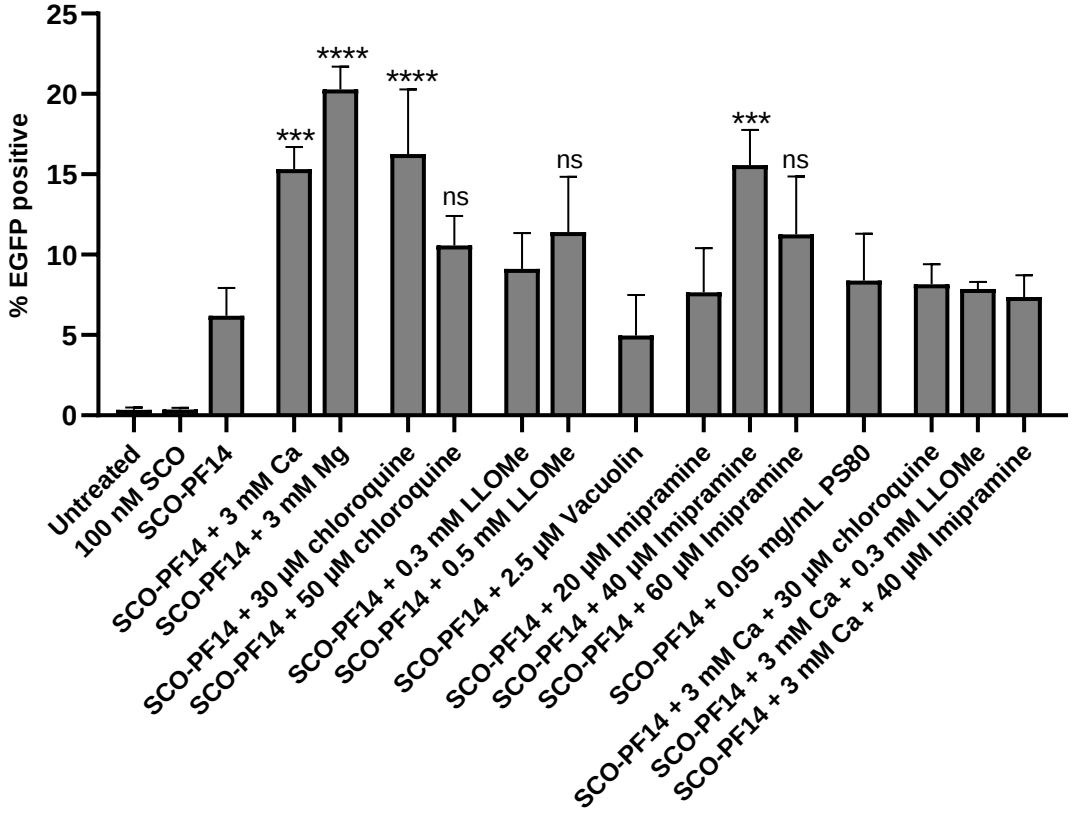

Figure 11. Flow cytometry analysis showing the percentage of EGFP-positive cells among population of untreated cells, cells treated with naked SCO, PF14-SCO nanoparticles, and PF14-SCO nanoparticles prepared with the addition of chloroquine, LLOMe, vacuolin, imipramine, PS80, calcium and magnesium ions. Calcium ions were also added to PF14 nanoparticles containing chloroquine, LLOMe and imipramine to check for probable additive or synergistic effects. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to PF14-SCO, *** p-value < 0.001, **** p-value < 0.0001, ns – not significant.

3.2.4 Among selected most efficient compounds, only LLOMe is toxic to HeLa pLuc 705 cells

Most efficient compounds were added to nanoparticles of 100 nM SCO-705 and 500 nM PF14 and applied on HeLa pLuc 705 cells to evaluate their cytotoxicity using WST-1 assay (Figure 12). Brefeldin A was highly toxic to HeLa pLuc 705 cells and was later used as a positive control of cytotoxicity. The results of measuring cell viability in Figure 12 show that the used compounds did not interfere with the viablity of HeLa pLuc 705 cells, except for LLOMe, that reduced cell viability by about 50%. In addition, chloroquine showed significant, though not so high toxicity, reducing cell viability by about 20%. However, the highest efficiency of PF14-SCO nanoparticles was reached when applying chloroquine at 30 µM. Thus, the toxicity of chloroquine can be avoided by keeping its concentration low, at the same time maintaining high efficiency.

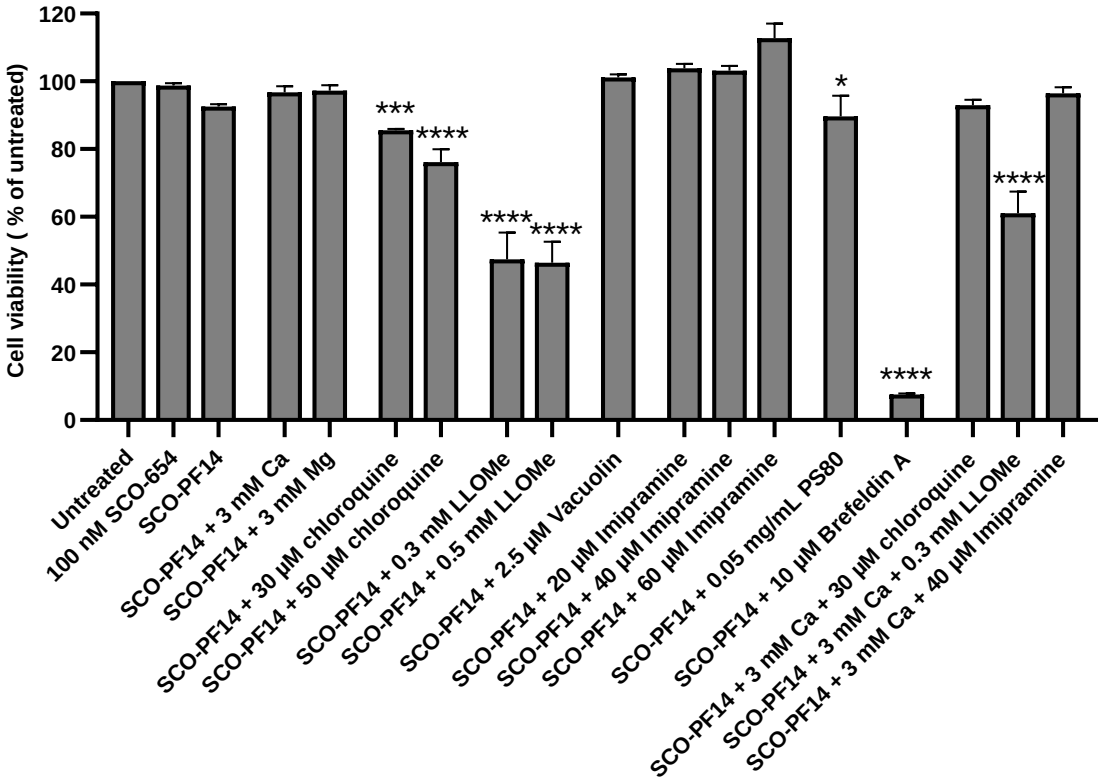

Figure 12. Cytotoxicity of the most efficient compounds to the HeLa pLuc 705 cells. Viability of the HeLa pluc 705 cells was evaluated using WST-1 assay, and absorption of untreated cells was taken for 100% viability. Brefeldin A was used as a positive control. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to untreated cells, * p-value < 0.05, *** p-value < 0.001, **** p-value < 0.0001.

The cytotoxicity of the most efficient compounds added to nanoparticles was next tested in another cell line, HeLa EGFP 654, using the WST-1 assay (Figure 13). Most of the compounds had little or no cytotoxic effect in this cell line and even brefeldin A, that showed high cytotoxicity in HeLa pLuc 705 cells and was used as a positive control of toxicity, was only slightly toxic, reducing cell viability by about 20% at 10 µM concentration.

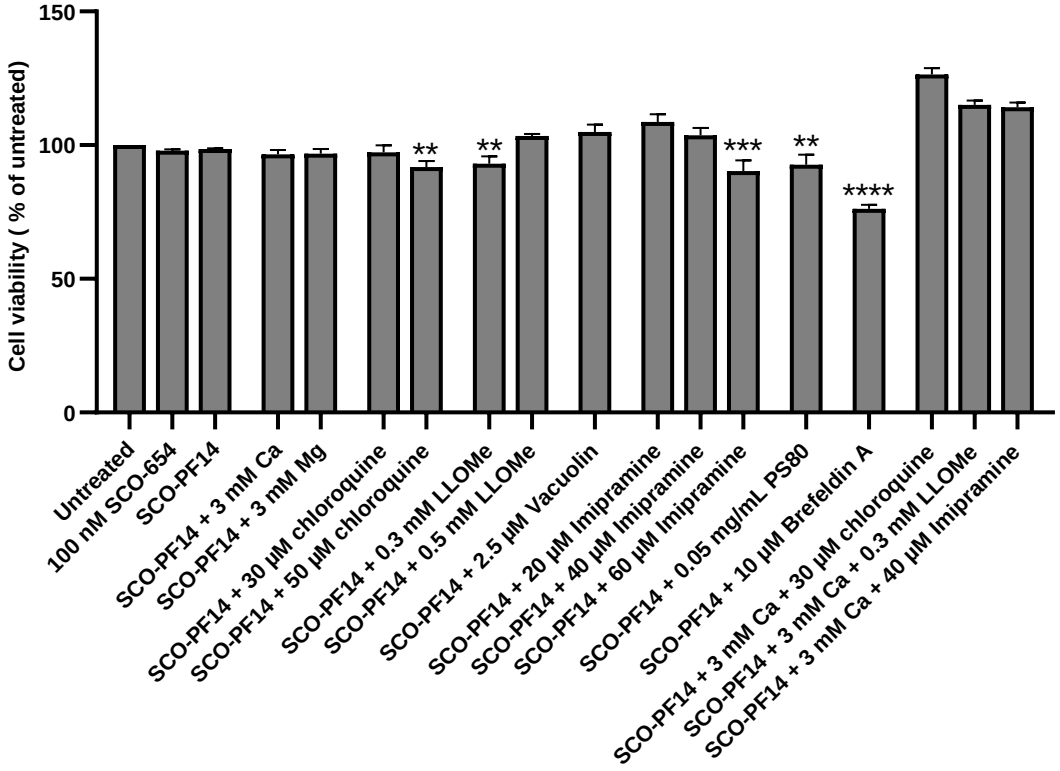

Figure 13. Cytotoxicity of the most efficient compounds to HeLa EGFP 654 cells. Viability of HeLa EGFP 654 cells was evaluated using WST-1 assay, and absorption of untreated cells was taken for 100% viability. Each dataset represents mean + SEM of three independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to untreated cells, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

3.3 DISCUSSION

Cell-penetrating peptides (CPPs) have shown great potential for transporting nucleic acid molecules of various sizes across cellular membranes.

The cellular membrane transport and uptake mechanism of CPPs is not completely understood, but it is generally accepted that CPPs, when associated with their cargo, use various endocytic pathways for entering the cell (Lundin *et al.* 2008). As a consequence, CPPs and their cargo molecules usually get entrapped in the endosomal vesicles and are translocated through early endosomes, late endosomes to lysosomes for degradation. Endosomal entrapment and eventual degradation in lysosomes limits the bioavailability of CPP-nucleic acid nanoparticles. A similar phenomenon can also be observed in the case of other non-viral vectors (Pack *et al*. 2005), being a major obstacle that has hindered wide clinical application of therapeutic nucleic acids.

In recent years, a lot of attention and research has been focused on intracellular trafficking of CPP-ONs in the endolysosomal pathway. This is a dynamic pathway, characterized by series of fusion and fission steps of membranes in cells. Hence, there might be a chance to trigger membrane destabilization, which leads to the escape of ON, increasing its functional delivery and biological activity.

Also, the activity of internalized CPP-ON complexes can be decreased by other cellular processes such as exocytosis and autophagy as described in the literature review above, that may even lead to complete loss of biological activity of ON.

With the ON delivery challenges (endosomal entrapment, exocytosis and autophagy) in mind, we attempted to proffer a solution in this research work by using compounds that have the potential to induce escape of ON from endosomes and suppress cellular exocytosis or autophagy, with the goal of increasing the efficiency of CPP-mediated transfection and bioavailability of the oligonucleotide.

During the course of this project work, we tested and characterized several previously identified small molecules (listed in the materials and methods section) which have the potential to enhance transfection efficiency of ONs with PF14. To test the efficiency of compounds, we used HeLa pLuc 705 reporter cell line, in which the higher level of productive transfection of splice-correcting oligonucleotide results in the higher expression level of functional luciferase protein. The characteristics of compounds we are looking for include high activity in a low micromolar range and minimal toxicity to the cells. This is always a delicate

balance to pursue, as there is a correlation between toxicity and potency for most compounds that enhance delivery.

The results of this functional screening (Figures 4 to 8) showed that majority of the compounds, when included in PF14-SCO nanoparticles, exhibited little or no effect on splice- switching activity of SCO, when compared to nanoparticles containing only SCO and PF14. Only chloroquine, L-leucyl-L-leucine methyl ester (LLOMe), imipramine, polysorbate 80 and the previously identified divalent ions, calcium and magnesium (Maloverjan *et al.* 2022) increased the functional delivery of ON when added to PF14-SCO nanoparticles, as measured by quantitation of the functional luciferase (Figure 9). An increase in activity induced by the compounds was concentration-dependent, and for each compound, an optimal concentration could be detected, after which a reduction in functional luciferase production was observed, probably due to cytotoxicity.

Chloroquine is a well-known endosomolytic compound and its mechanism of action is based on buffering of endosomal pH during maturation of endosomes. Chloroquine is able to buffer the lumenal pH of the endosomal vesicles by accepting protons at acidic pH (Wojnilowicz *et al*. 2019). Under normal circumstances, the pH in endosomal lumen would decrease, turning interior more acidic, while in the presence of chloroquine that associates protons, more protons must be pumped into the endosomal lumen in order to achieve the same drop in pH. The resulting accumulation of ions in the lumen may induce osmotic swelling of the endosomes and ultimate membrane rupture or leakage of SCO.

LLOMe is known to induce rapid and complete lysosomal membrane permeabilization at sub-apoptotic concentrations (above 0.25 mM and below 4 mM in HeLa cells) (Repnik *et al*. 2017). Membrane permeabilization potentially leads to the escape of SCO, before degradation in lysosomes, with a greater possibility of reaching the target site.

Imipramine is considered to inhibit exocytosis of internalized SCO by interfering with translocation of acidic sphingomyelinase in membranes.

Polysorbate 80 is a widely used surfactant and emulsifier in various products that, in addition, has the potential to promote the cellular uptake by association with receptors on cell surface and to induce cell membrane permeation because of its destabilizing actions on membrane proteins.

Another reporter cell line, HeLa EGFP 654, was used to verify, if the increased splice-correction activity of SCO observed upon the addition of compounds shown in Figure 9 to

PF14-SCO nanoparticles, is reproducible in a cell line having another splicing defect and expressing another reporter protein, EGFP. Confocal microscopy images in Figure 10 show a high signal (green flourescence) when chloroquine, imipramine, LLOMe, calcium and magnesium were added to PF14-SCO-654 nanoparticles. This indicates higher splice-correcting activity, that resulted in higher expression of EGFP, in contrast to the untreated cell, SCO only, and PF14-SCO nanoparticles, which showed almost no green flourescence. Also, flow cytometry performed using HeLa EGFP 654 cell line (Figure 11) revealed an analogous degree of efficiency of the compounds under study to that in HeLa pLuc 705 (Figure 9). It implies that the effects of these compounds are reproducible in another cell line, and are of universal nature. Furthermore, calcium ions were added to PF14-SCO nanoparticles containing imipramine, chloroquine and LLOMe, respectively, to evaluate possible synergistic or additive effect on SCO activity. However, results both in HeLa pLuc 705 and HeLa EGFP 654 show no increased activity of SCO, suggesting that the mechanism triggered by these compounds are similar or cellular pathways employed get saturated already by using one of them, or the mechanisms provoked by these inducers counteract each other.

The cytotoxicity studies (Figures 12 and 13) revealed that imipramine, chloroquine, PS80 and LLOMe did not impair the viability of HeLa EGFP 654 cells. However, in HeLa pLuc 705 cells, LLOMe and chloroquine were toxic to some extent, when compared to untreated cells. Brefeldin A was the most toxic to HeLa pLuc 705 cells among used compounds, and was used as a positive control of toxicity in this cell line. However, HeLa EGFP 654 cells tolerated Brefeldin A rather well and their viablity was only slightly impaired.

The most efficient compounds selected in this study were also added to PF14-siRNA nanoparticles to test their effect on another type of nucleic acid cargo in luciferase expressing cell line, U87 MG-Luc2. The positive effects obeserved in HeLa cell lines did not entirely reproduce in this cell line and assay. Also the impact of these compounds on the viability of U87 MG-Luc2 cells was generally higher than to HeLa cells. This also complicated drawing solid conclusions regarding the efficiency of the multicomponent nanoparticles, because in the case of this assay, both the increase in efficiency and toxicity lead to the reduction in the luminescence signal. Therefore, the results about the potentiation of the PF14-siRNA nanoparticles effect with selected compounds in U87 MG-Luc2 cells were added as supplementary material.

SUMMARY

Low molecular weight compounds that interfere with the normal intracellular endo-lysosomal trafficking have a potential to enhance the efficiency of CPP-mediated delivery of nucleic acids. Substances known to induce endosomal escape such as chloroquine and LLOMe, as well as inhibitors of exocytosis, such as imipramine, were added to self-assembling nanoparticles of cell-penetrating peptide PepFect14 (PF14) and splice-correcting oligonucleotide (SCO), and the biological effect of SCO in cells was measured in a splice-correcting assay. From 19 analyzed compounds, five increased the splice-correcting activity of SCO, when added to PF14-SCO nanoparticles, and were selected for further studies. These included chloroquine, LLOMe, imipramine, vacuolin and PS80. Autophagy inhibitors analysed in this study had a minimal, if any, effect on transfection efficiency. Depending on the cell line used, the most efficient compounds used had either little or no cytotoxicity. To sum up, a good understanding of the fate of internalized nanoparticles and the molecular mechanisms that are implicated in the cell during CPP-mediated delivery of nucleic acids is essential for increasing efficiency of transfection. Further studies are definitely required in this regard, including screening and testing novel compounds known to induce endosomal escape and inhibit exocytosis and autophagy.

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Appendix

Transfection of PF14-siRNA nanoparticles supplemented with the most efficient compounds under study into U87 MG-Luc2 cell line

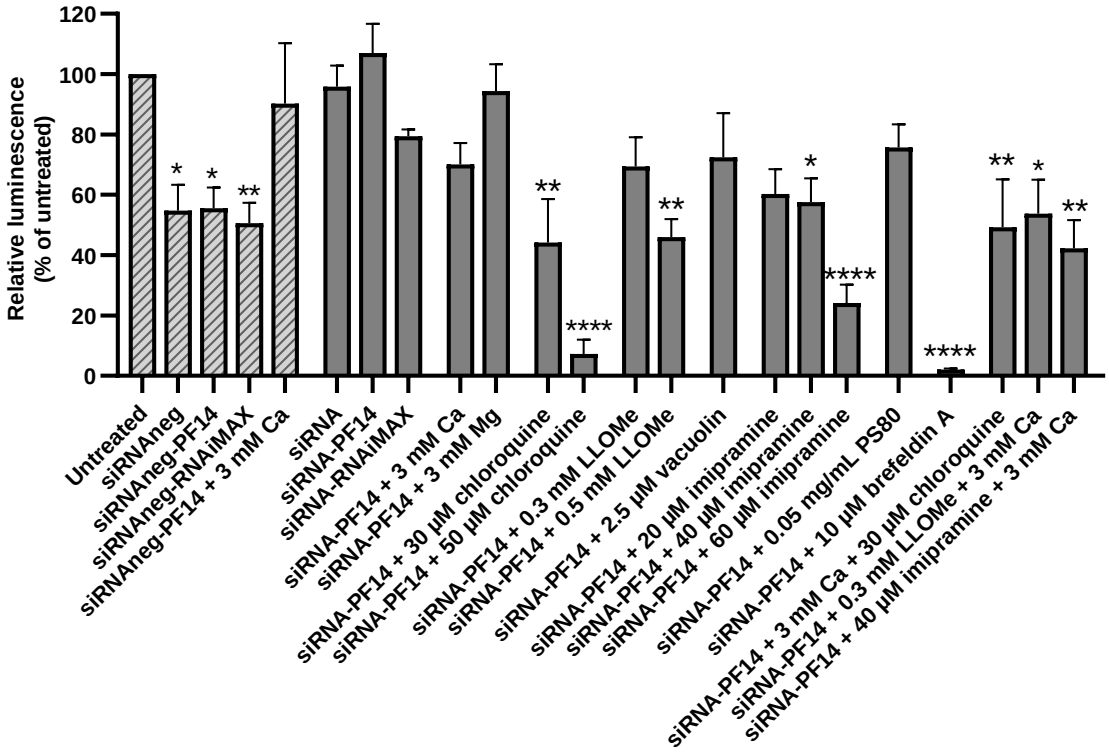

Figure 14. Transfection of U87 MG-Luc2 cell line with non-covalently associated PF14-siRNA nanoparticles, supplemented with the most efficient compounds selected from the screening. Transfection efficiency was measured with luciferase assay and luminescence values were normalized to untreated cells. Each dataset represents mean + SEM of 3 independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant reduction compared to untreated cells, * p-value < 0.05, ** p-value < 0.01, **** p-value < 0.0001.

Cytotoxicity of the selected most efficient compounds in PF14-siRNA nanoparticles in U87 MG-Luc2 cell line

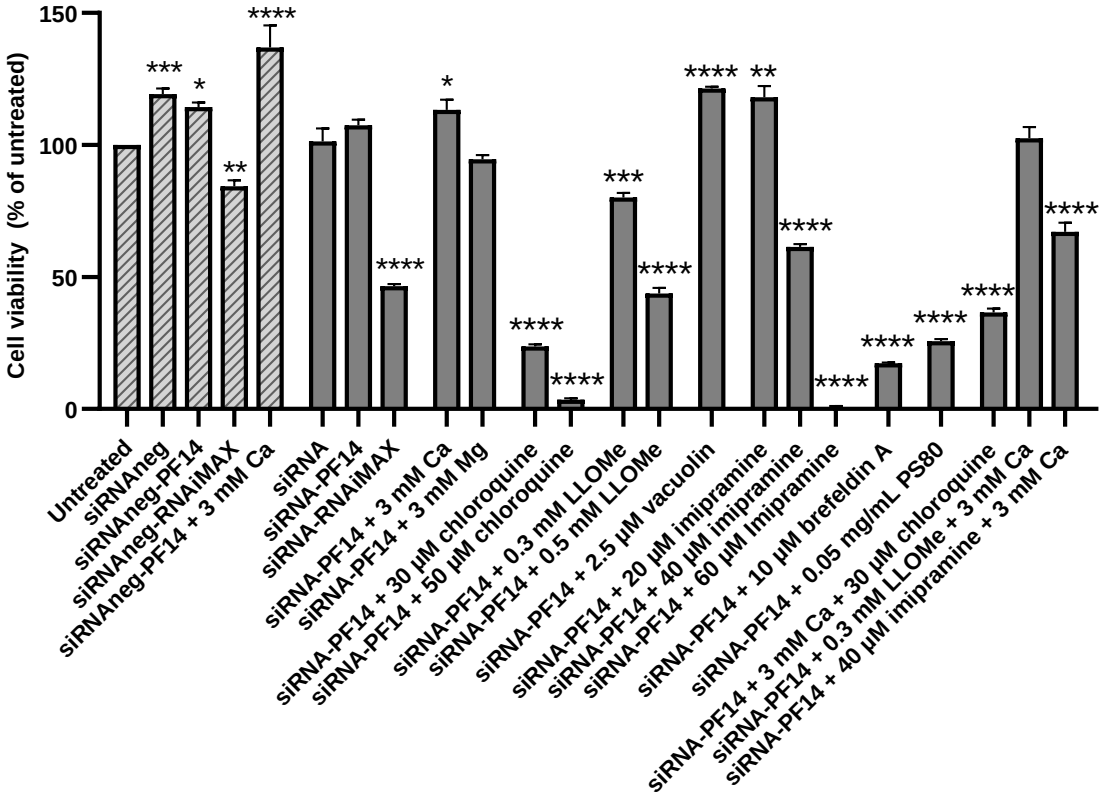

Figure 15. Viability of U87 MG-Luc2 cells upon addition of differently prepared nanoparticles. Viability of U87 MG-Luc2 cells was evaluated using WST-8 assay, and absorption of untreated cells was taken for 100% viability. Each dataset represents mean + SEM of at least 3 independent experiments. Data was analyzed by one-way ANOVA with Dunnett's test. The asterisks show statistically significant difference compared to untreated cells, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

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