Πανεπιστήμιο Θεσσαλίας

Τμήμα Βιοχημείας και Βιοτεχνολογίας

Διπλωματική Εργασία

"Ενδοκυτταρικό βακτήριο *Simkania negevensis* και σηματοδότηση του κυττάρου ξενιστή"

"Intracellular bacterium Simkania negevensis and host cell signalling"

Δημήτριος Παπακηρύκος του Δημητρίου

Έτος 2021

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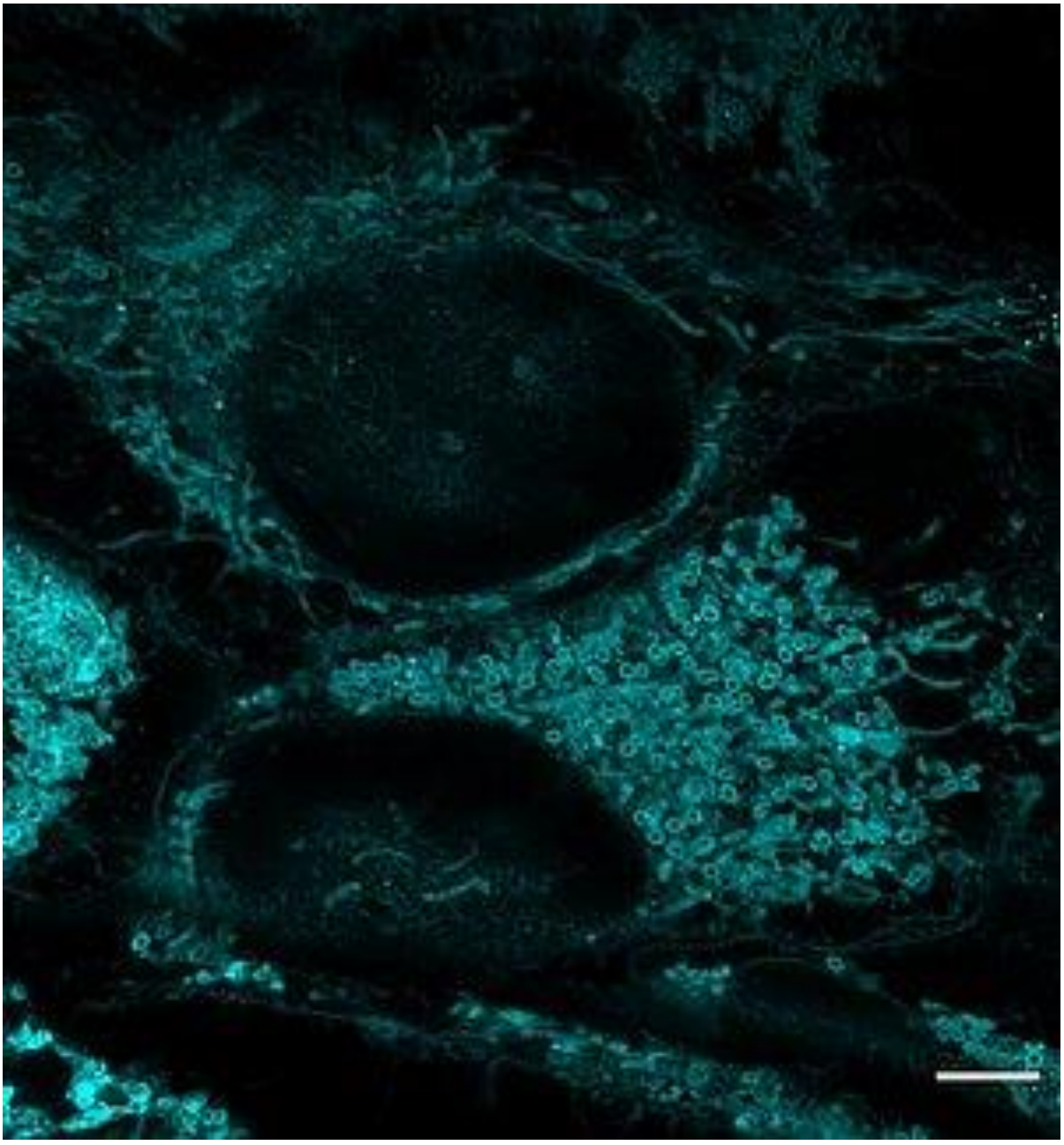

Confocal fluorescence images of HeLa cells infected with *Simkania negevensis* and labeled with clickable C6 ceramide after 4x expansion of the samples. This image is 48 h after infection. The developing infection vacuole filled with dividing reticulate bodies is visible, as well as intertwining mitochondria and ER membranes. The image was constructed by Tobias Kunz.

ACKNOWLEDGMENTS

I would like to cordially thank Prof. Vera Kozjak-Pavlovic for giving me the opportunity to work in her group at the Department of Microbiology, University of Würzburg. There, I learned and mastered new scientific techniques, I got enriched with state of the art knowledge and I practiced my diligence and fortitude. Moreover, I would like to thank the PhD student Jonas Weinrich for bidding his time on guiding me through the experiments and responding whenever I had any inquiry. Of course it would be a felony not to mention all the other staff of the lab - Eva-Maria Hörner, Kristýna Pekárková, Eva Rühl, Ravisha Rawal and Dr. Tobias Kunz - that were on all occasions affable to me and had erudition in their field. I shall make also a nod to the whole chair of microbiology for the pleasant and constructive atmosphere.

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1. List of abbreviations

ATF6: Activating Transcription Factor 6

ATP: Adenosine Triphosphate

BiP: Binding Immunoglobulin Protein

Bp: Base pairs

BSA: Bovine Serum Albumin

cDNA: complementary DNA

CTP: Cytidine Triphosphate

DAPI: 4′,6-diamidino-2-phenylindole

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic Acid

E. coli: *Escherichia coli*

FCS: Fetal Calf Serum

FUNDC1: FUN14 domain-containing 1

GAPDH: Glyceraldehyde 3-phosphate Dehydrogenase

GFP: Green fluorescent protein

GTP: Guanosine Triphosphate

IRE-1: Inositol-requiring Enzyme 1

LC3: Microtubule-associated proteins 1A/1B light chain 3B

MOI: Multiplicity Of Infection

OMM: Outer Mitochondrial Membrane

ON: Overnight

PBS: Phosphate-buffered Saline

PCR: Polymerase Chain Reaction

PERK: Protein Kinase R-like Endoplasmic Reticulum Kinase

PFA: Paraformaldehyde

RFP: Red Fluorescent Protein

PI: Post infection

RNA: Ribonucleic acid

RPM: Rotations Per Minute

RT: Room Temperature

SnCV: Simkania-Containing Vacuole

Sne: *Simkania negevensis*

SnGroEL: *Simkania negevensis* GroEL

SREBPs: Sterol Regulatory Element Binding Proteins

TAE: Tris-Acetate-EDTA

Tm: Melting temperature

Tom40: Translocase of the Outer Mitochondrial Membrane 40

Tris: Tris-hydroxymethyl-aminomethane

TTP: Thymidine Triphosphate

UPR: Unfolded Protein Response

2. Abstract

The bacteria of the Chlamydiae phylum have concerned humans for a long time because many species are pathogenic with the famous representative *Chlamydia trachomatis*, which causes sexually transmitted disease. In the current research two independent hypotheses were studied regarding the bacterium *Simkania negevensis*. This organism develops intracellularly in the host cell and it requires many components from it. Therefore, the first hypothesis is if the infection in HeLa cells is affected when various inhibitors (desipramine, myriocin and ARC39) that block the synthesis of ceramide are applied, and the second is to clone a mitophagy reporter with the ultimate purpose to detect when mitophagy occurs in HeLa cells during *S. negevensis* infection. Mitophagy is a process that involves the selective degradation of defective mitochondria under potentially harmful conditions, like an infection by a microorganism.

For the detection of mitophagy, the cloning of the mitophagy reporter, a mitochondria-targeted RFP-GFP fusion construct, was performed, using the cloning technique DISEC-TRISEC. Regarding ceramide inhibitors, a series of experiments was carried out to quantify efficacy of infection in the presence of the inhibitors, such as immuno-labeling for immunofluorescent microscopy and western blot analysis. In addition, in order to have a sufficient amount of bacteria for the experiments the preliminary preparation of big amounts of *Simkania* was performed.

All the inhibitors used had to some extent inhibitory action against *S. negevensis*, without causing any toxicity to the cells. The inhibitor ARC39 seems to be the stronger inhibitor, where with only 10 μM concentration, in 3 days post infection (PI) it almost completely inhibits the infection. Regarding desipramine and myriocin, comparison was not possible because of the variability in the infection rate of the control cells. Also the results on the western blots for ARC39 and desipramine show that they are effective inhibitors. However, it is necessary to emphasize that most of the data extracted had a high standard deviation and low statistical significance and therefore they cannot be considered reliable and precise. Desipramine and myriocin results look in principle more conclusive than the experiments with ARC39.

Περίληψη

Τα βακτήρια της επικράτειας *Chlamydiae* απασχολούν τον άνθρωπο εδώ και πολύ καιρό γιατί πολλά είδη από αυτήν είναι παθογόνα, όπως το διάσημο εκπρόσωπο *Chlamydia trachomatis*, το οποίο προκαλεί σεξουαλικώς μεταδιδόμενα νοσήματα. Στην παρούσα έρευνα μελετήθηκαν δύο ανεξάρτητες υποθέσεις σχετικά με το βακτήριο *Simkania negevensis*. Αυτός ο οργανισμός αναπτύσσεται ενδοκυτταρικά στο κύτταρο ξενιστή και απαιτεί πολλά συστατικά από αυτό. Επομένως, η πρώτη υπόθεση είναι εάν η μόλυνση στα κύτταρα HeLa επηρεάζεται όταν εφαρμόζονται διάφοροι αναστολείς (δεσιπραμίνη, μυριοκίνη και ARC39) που εμποδίζουν τη σύνθεση του κεραμιδίου και η δεύτερη είναι η κλωνοποίηση ενός ανιχνευτή μιτοφαγίας με απώτερο σκοπό τον προσδιορισμό του πότε συμβαίνει μιτοφαγία σε κύτταρα HeLa κατά τη διάρκεια μόλυνσης από *S. negevensis*. Η μιτοφαγία είναι μια διαδικασία που περιλαμβάνει την επιλεκτική αποικοδόμηση ελαττωματικών μιτοχονδρίων κάτω από δυνητικά επιβλαβείς συνθήκες, όπως μια μόλυνση από έναν μικροοργανισμό.

Για την ανίχνευση της μιτοφαγίας, πραγματοποιήθηκε η κλωνοποίηση του ανιχνευτή μιτοφαγίας RFP-GFP, ενός κατασκευάσματος σύντηξης το οποίο στοχεύει τα μιτοχόνδρια, χρησιμοποιώντας την τεχνική κλωνοποίησης DISEC-TRISEC. Όσον αφορά τους αναστολείς κεραμιδίου, διεξήχθη μια σειρά πειραμάτων για να ποσοτικοποιηθεί η αποτελεσματικότητα της μόλυνσης παρουσία των αναστολέων, όπως ανοσο-επισήμανση για μικροσκοπία ανοσοφθορισμού και στύπωμα western. Επιπλέον, για να υπάρχει επαρκής ποσότητα βακτηρίων για τα πειράματα έγινε η προκαταρκτική προετοιμασία μεγάλων ποσοτήτων *Simkania*.

Όλοι οι αναστολείς που χρησιμοποιήθηκαν είχαν σε κάποιο βαθμό ανασταλτική δράση έναντι του *S. negevensis*, χωρίς να προκαλούν καμία τοξικότητα στα κύτταρα. Ο αναστολέας ARC39 φαίνεται να είναι ο ισχυρότερος αναστολέας, όπου μόνο με συγκέντρωση 10 μM, σε 3 ημέρες μετά τη μόλυνση (PI) αναστέλλει σχεδόν πλήρως τη μόλυνση. Όσον αφορά τη δεσιπραμίνη και τη μυριοκίνη, η σύγκριση δεν ήταν δυνατή λόγω της μεταβλητότητας στο ποσοστό μόλυνσης των κυττάρων ελέγχου. Επίσης τα αποτελέσματα στα western blots για το ARC39 και τη δεσιπραμίνη δείχνουν ότι είναι αποτελεσματικοί αναστολείς. Ωστόσο, είναι απαραίτητο να τονιστεί ότι τα περισσότερα από τα δεδομένα που εξήχθησαν είχαν υψηλή τυπική απόκλιση και χαμηλή στατιστική σημασία και ως εκ τούτου δεν μπορούν να θεωρηθούν αξιόπιστα και ακριβή. Τα αποτελέσματα της δεσιπραμίνης και της μυριοκίνης είναι κατά βάση πιο πειστικά από τα αποτελέσματα με το ARC39.

3. Introduction

3.1 General information on *Simkania negevensis*

The bacterium *Simkania negevensis* is a gram-negative bacterium that was discovered in 1993, in the laboratory of Kahane in Israel as a contaminant in human cultures, and has not been studied thoroughly, hence the literature on it is very limited. It belongs to the order Chlamydiales and is an obligate intracellular organism. Structurally and functionally, it exhibits similar characteristics to *Chlamydia trachomatis*, the bacterium that is largely responsible for the homonymous sexually transmitted disease as well as the infection of eye called trachoma. Thus, at a laboratory level, similar techniques and methodologies can be applied to some extent. So far it has not been shown to have pronounced pathological effects because several people infected with it have been recorded having no symptoms [2]. On the other hand it has been linked with respiratory diseases to a portion of people, like pneumonia to adults and bronchiolitis to infants [2]. *S. negevensis* develops for up to 15 days, a big time span in comparison to other bacteria of the Chlamydiae class. Nevertheless, the infected cells lyse in average three days post infection with *S. negevensis* by a so far unexplored mechanism. Moreover, as almost all intracellular bacteria its development takes place inside a specialised compartment within the host cell. This compartment, which helps the bacterium to develop and replicate, is called *Simkania*-containing vacuole (SnCV) has two basic characteristics in the case of *S. negevensis*. Firstly, it is only one single web-like vacuolar system instead of many small spindle-like vacuoles and secondly it recruits and is in contact with many organelles, such as the endoplasmic reticulum (ER), peroxisomes, lysosomes and mitochondria. The surface of the SnCV is lined with the endoplasmic reticulum. Some of the host metabolites that contribute to its development are cofactors, specific amino acids, nucleotides, and lipids. One category of lipids that are important for *S. negevensis* growth are sphingolipids. From all potential pathogens, fungi can synthesize sphingolipids by themselves, whereas most of viruses and bacteria “steal” them from the host. [20]

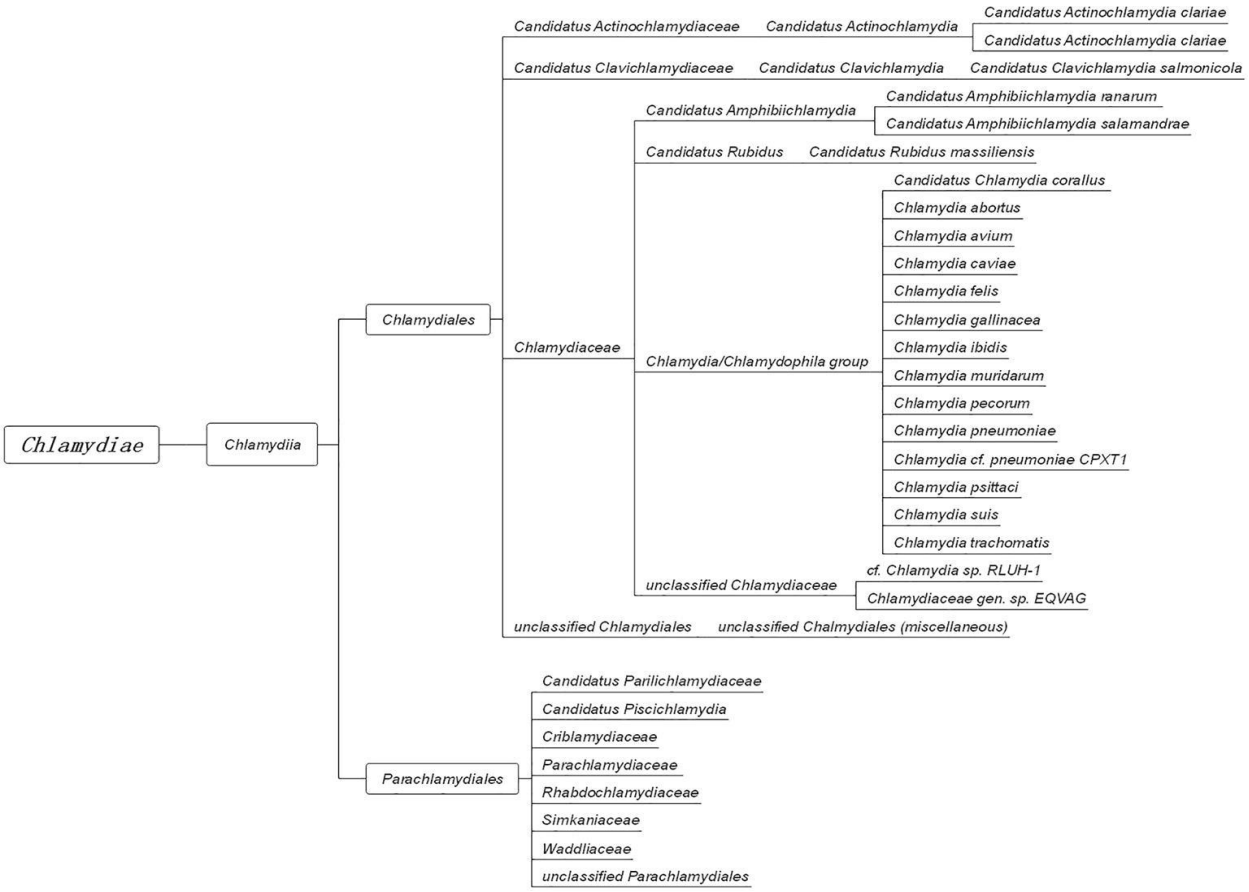


Figure 1. Phylogenetic tree of Chlamydiae. *Simkania negevensis* belongs to the family Simkaniaceae. From "Clear Victory for Chlamydia: The Subversion of Host Innate Immunity", Chen et al. [33]

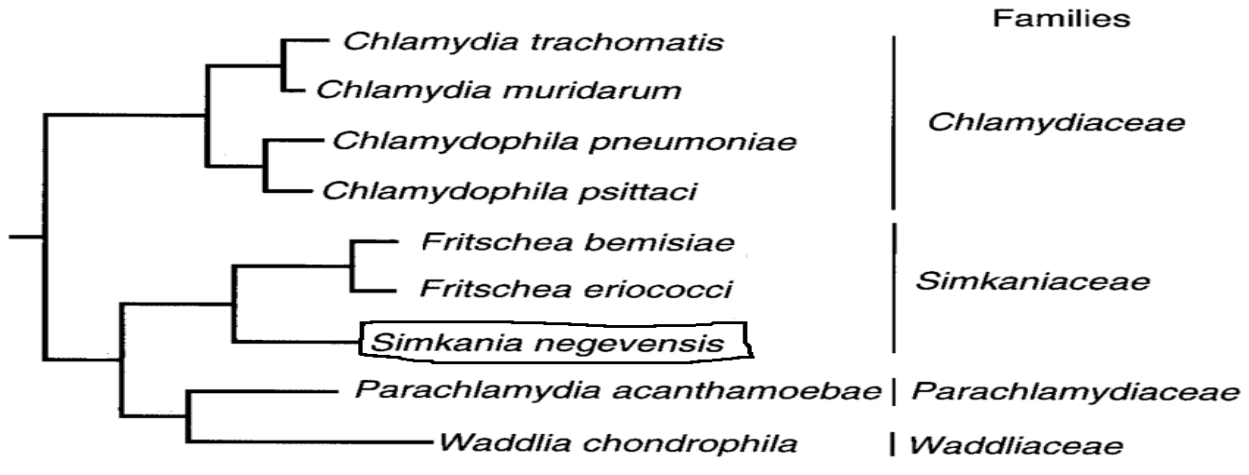


Figure 2. *Simkania negevensis* is the only species from the *Simkania* genus. Adapted from "Phylogenetic evidence for two new insect-associated Chlamydia of the Family Simkaniaceae", M.L. Thao et al. [32]

3.2 Mechanism of infection

S. negevensis exists in two forms, the elementary bodies (EB) and the reticulate bodies (RB), similar to *Chlamydia*. The EB are smaller and infectious, that means they can enter the host cell and can convert to RB in the SnCV inside the host cell. The RB are bigger, non-infectious but metabolically active and they replicate.

For bacterial development, ceramide is of special importance, considering the high concentration of this sphingolipid found in *Simkania* [22]. Ceramide is the central molecule in the sphingolipid metabolism and it is constructed via three methods, the *de novo*, the salvage and the sphingomyelin hydrolysis pathway. Ceramide can be transferred through the retrograde transport mechanism from the plasma membrane to the Golgi apparatus (where complex lipids are synthesized) and from there to the ER [22]. The retrograde transport is one of the major lipids provider methods for the SnCV. The SnCV is created by these lipids, chiefly cholesterol and sphingomyeline [22]. This might explain the close contact between the SnCV and the ER.

After infection, bacteria induce ER stress, but are also capable of suppressing ER stress response. ER is necessary, among other things, for protein synthesis and modification, such as folding. ER has at its disposal three main sensors, the activating transcription factor 6 (ATF6), the protein kinase R-like endoplasmic reticulum kinase (PERK) and the inositol-requiring enzyme 1 (IRE-1), which when they perceive an incongruity in the normal protein production of the cell, they induce the unfolded protein response (UPR) [16]. This mechanism, except that it is correlated with innate immune response, restores protein homeostasis by increasing the folding capacity of proteins and decreasing the translation of proteins or leads to apoptosis if the damages are irreversible. *S. negevensis* triggers the increase of the chaperone binding immunoglobulin protein (BiP) in the host cell that confines UPR, thus inhibiting ER stress [16]. This characteristic of *S. negevensis* plays pivotal role for its survival, because via ER stress, autophagy or apoptosis can get prompt, which have devastating results both for the cell and the pathogen [16].

Another important organelle for the development of *Simkania* are mitochondria, even though their precise interaction is not well characterized. Mitochondria are recruited to the surface of the SnCV through their contacts with the ER. It appears that they need them in the early stages for their development but roughly 3 days post infection, fragmentation of mitochondria can be observed [16]. The translocase of the outer mitochondrial membrane 40 (Tom40), an import channel of the mitochondrial outer membrane which serves as a non-ATP requiring pore in the outer membrane of mitochondria for sorting and translocation of proteins, appears to be imperative for the development of SnCV. Lastly, peroxisomes contribute as well to the development of SnCV, due to the important role in lipid metabolism [16].

Eventually, after roughly 3 days of infection, when the bacteria have been developed sufficiently, they exit the cell. The exact mechanism is not known but it is probably similar to *C. trachomatis* which has two potential pathways, spontaneous lysis of the inclusion and the plasma membrane of the cell (fast method) or a method called extrusion (slow method) [34]. Extrusion is the gradual protuberance of the inclusion to the plasma membrane and has the unique trait of leaving the cell intact. It has been proven nevertheless that *Simkania* requires a set of proteases to egress the cell, hinting that they also utilize the lysis mechanism as well as myosin II, a key mediator of contractile ring formation in cytokinesis, a mechanism akin to extrusion. Upon exit of the cell, the bacteria which are again transformed into the EB infectious form, attach to adjacent cells in order to commence a new infection cycle [34].

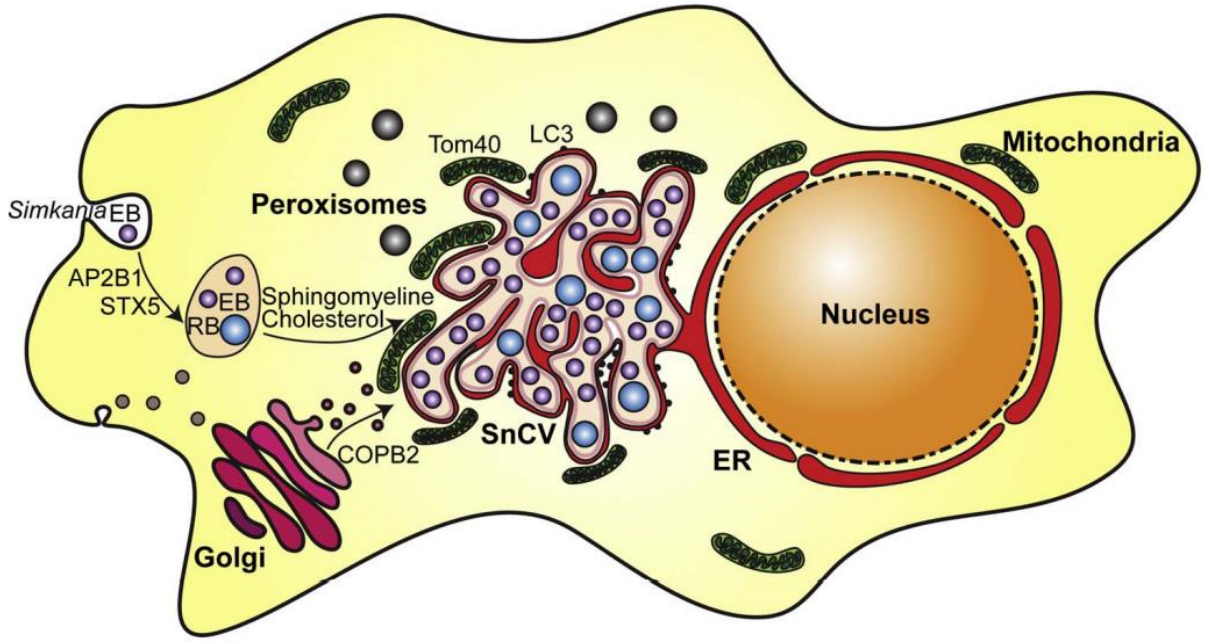


Figure 3. *Simkania* exists in two forms, the infectious elementary bodies (EB) and the replicative reticulate bodies (RB). Inside the host cell it forms, via host molecules like cholesterol and sphingomyelin a *Simkania* containing vacuole (SnCV), that is adjacent to endoplasmic reticulum (ER) and mitochondria. LC3 is an autophagy marker on the surface of the SnCV. COPB2, AP2B1, STX5, Tom40 and some peroxisomal proteins are host cell proteins necessary for the development of this vacuole. From "The role of host cell organelles in the development of *Simkania negevensis"*, V. Kozjak-Pavlovic et al. [16]

3.3 Sphingolipids

Lipids constitute an essential component for the survival of living organisms. They are in general to a small degree soluble in water, due to lack of polarizing atoms like N, P, S and O, but are very soluble in organic solvents like methanol. There is no universal classification for lipids, nevertheless they could be categorised based on structural properties. Some lipids for example contain the molecule sphingosine as backbone while others contain glycerol or they can contain different amount of fatty acids. Membranes need lipids like phosphatidylcholine, main phospholipid of cell membranes, because they form a protective permeability barrier. However, they also have other traits and one good example of that is cholesterol, which except being a major lipid component of eukaryotic membranes that influences membrane rigidity, is also involved in diverse cellular processes including signal transduction, gene transcription, protein function and degradation, endocytic and Golgi trafficking, and intra-organelle membrane contact site formation. Without cholesterol important substances like vitamin D cannot be produced [35].

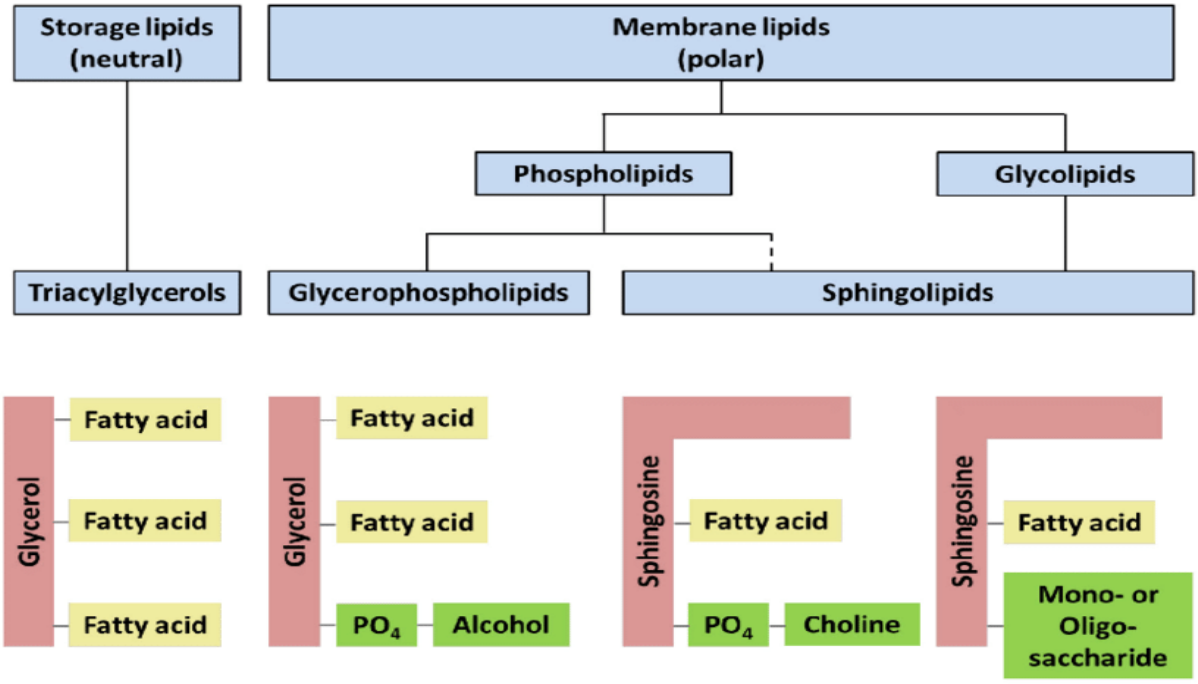


Figure 4. Categories of lipids. The majority of lipids can be separated depending on if the backbone they contain is sphingosine or glycerol. Sphingolipids have sphingosine connected with one fatty acid and either a phosphocholine group (e.g sphingomyelins) or saccharides (e.g gangliosides). The only variant group in sphingolipids is the fatty acid. From "Multiple routes of phosphatidylethanolamine biogenesis ensure membrane integrity of *Toxoplasma gondii"*, Anne Hartmann [31]

During the last years, research is carried out by scientists about sphingolipids to a great extent. Sphingolipids are a class of lipids that contain the sphingoid base sphingosine. Sphingosine was named after J. L. W Thudichum in 1884 for its "enigmatic" properties. The analytical functions of sphingolipids remain a conundrum.

Function of sphingolipids are manyfold and are not only limited to their role as structural components of the cell, but also extend to them being major players in various cell signalling pathways [19].

Some sphingolipids, like sphingomyelins comprise roughly 25% of lipids of the myelin sheath that surrounds neurons. As such, they play salient role in brain function and signal transduction. There are also other functions of sphingolipids as: a) antimicrobial agents, b) the regulation of cholesterol efflux receptors, ABCA1 and ABCG1 and c) the formation of lipid rafts [36,19].

Specifically:

a) Sphingolipids have partially contradictory effects upon bacterial infection, because on one hand they participate in the immune vigilance and the autophagy machinery and on the other hand they help in the formation of the inclusion of various intracellular bacteria [36]. For example sphingolipids take part in the ubiquitination of the pathogen *Salmonella enterica* containing vacuole and therefore can act as antimicrobial agents. Also *Legionella pneumophila* disrupts sphingolipids to elude apoptosis. Other bacteria, such as *Staphylococcus epidermitis*, *Micrococcus luteus* and *Staphylococcus aureus* are eliminated by the mechanisms induced by sphingosine [36].

b) Sphingolipids, more specifically their *de novo* composition, regulate the outflow of cholesterol from the membrane receptors ABCA1 and ABCG1. When their *de novo* composition is increased, the efflux of cholesterol is decreased, but the synthesis is increased, mediated by the sterol regulatory element binding proteins (SREBPs) [19].

c) Cholesterol and sphingolipids do not diffuse freely into the cytoplasmic membrane but accumulate in small perforated areas, lipid rafts. These rafts are regions of high cell membrane organization and important for signal transduction and protein transport. These rafts on the other hand can be utilized by intracellular bacteria, like *S. negevensis*, to construct vacuoles inside the host necessary for their survival [39].

The composition of sphingolipids:

Ceramide (N-acylated form of sphingosine) is the key to the biosynthesis of sphingomyelin and other types of sphingolipids. The de novo synthesis of sphingolipids begins with a condensation reaction catalyzed by serine palmitoyltransferase, the most crucial enzyme in the procedure, which uses serine and palmitoyl-CoA as substrates to synthesize 3-keto sphinganine. 3-keto sphinganine is reduced to sphinganine. This reaction is catalyzed by the 3-keto sphinganine reductase. Sphinganine is then acylated by the enzyme dihydroceramide synthase to form dihydroceramide. This is followed by the formation of a double bond in the 4-5C carbons in the backbone of sphingosine by dihydroceramide desaturase resulting in the formation of the ceramide. Based on this, the 4 basic sphingolipids are formed, which are the structural basis of the most complex ones. These are: sphingomyelin, ceramide 1-phosphate (C1P), galactosylceramide and glucosylceramide. In order to construct longer sphingolipids it is necessary for the cell to transport ceramide from the organelle that it is generated, the endoplasmic reticulum, to Golgi apparatus via the ceramide transport protein (CERT) [37].

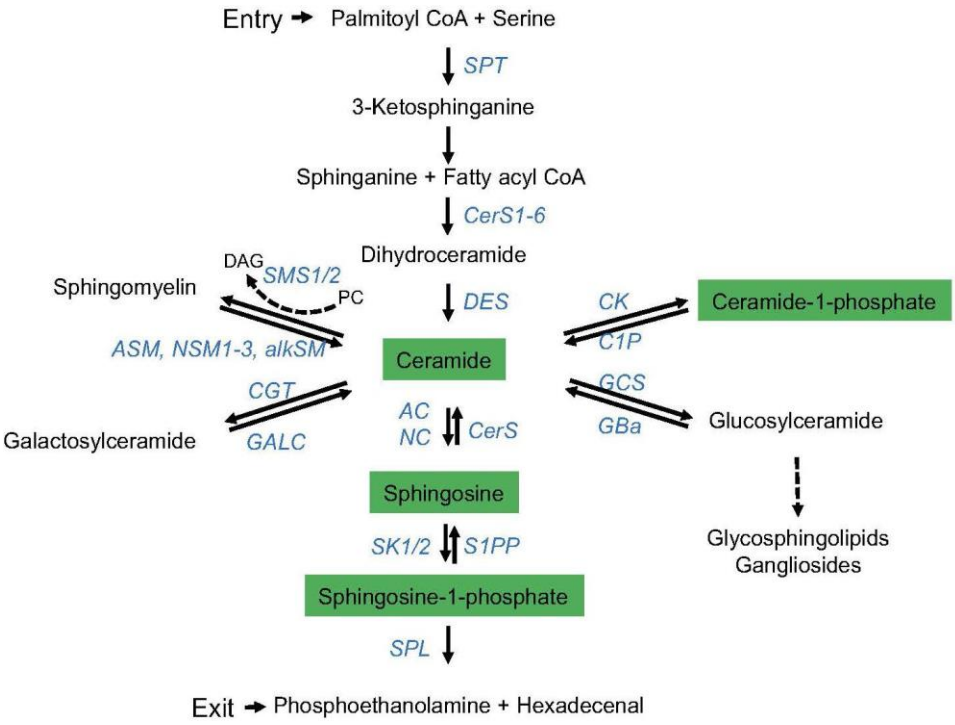


Figure 5. The sphingolipid metabolic pathway. SPT is the initial enzyme of de novo ceramide synthesis. From ceramide 4 basic structures are produced: glucosylceramide, ceramide-1-phosphate, galactosylceramide and sphingomyelin, from which the most elaborate ones are generated. Glucosylceramide yields glycosphingolipids and gangliosides while galactosylceramide can yield sulfatides. From "Sphingomyelin Breakdown in T Cells: Role of Membrane Compartmentalization in T Cell Signaling and Interference by a Pathogen", Avota, E. [37]

Figure 6. Molecular structure of ceramide. Adapted from https://en.wikipedia.org/wiki/Sphingolipid

Furthermore, two more pathways other than the *de novo* synthesis also exist. The salvage pathway, which occurs in the acidic lysosomes is in a sense the opposite of the de novo pathway. Long chain sphingolipids are degraded to shorter ones that lead ultimately to the formation of ceramide. For example from glycosphingolipids are removed first the saccharides with exohydrolases and then depending on the sphingolipid other cleaving enzymes will regenerate or ”salvage” ceramide. Acid sphingomyelinase cleaves the phosphocholine group from sphingomyelin yielding ceramide. Ceramide can further be converted to sphingosine with acid ceramidase by cutting off the fatty acid residue. Vice versa sphingosine can yield ceramide with the contribution of ceramide synthase. The latter pathway is the immediate conversion of sphingomyelin that is found in close proximity of mitochondria to ceramide via sphingomyelinase (see figure 6). [30]

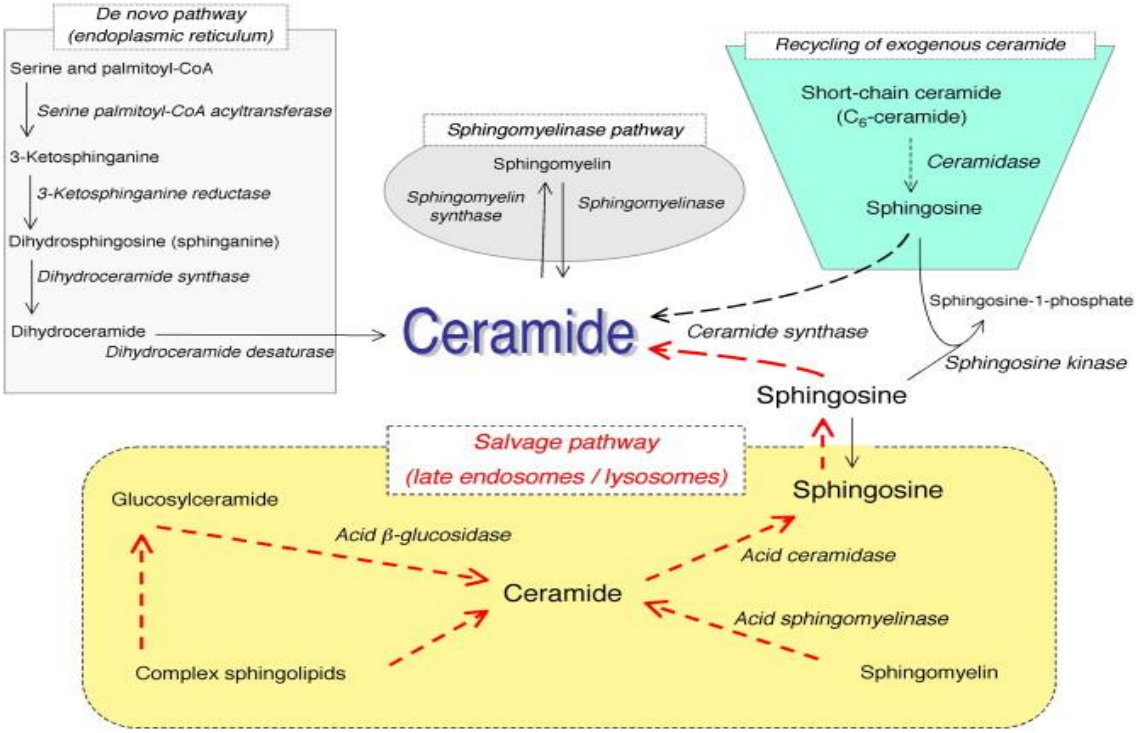


Figure 7. Ceramide synthesis. The scheme shows concisely metabolic pathways for ceramide synthesis composed of the sphingomyelinase pathway, the de novo pathway and the salvage pathway. Dotted lines indicate the pathway of ceramide synthesis resulting from recycling/salvaging sphingosine. From "The sphingolipid salvage pathway in ceramide metabolism and signaling", Kitatani K [30]

3.4 Sphingolipid metabolism enzyme inhibitors

Desipramine:

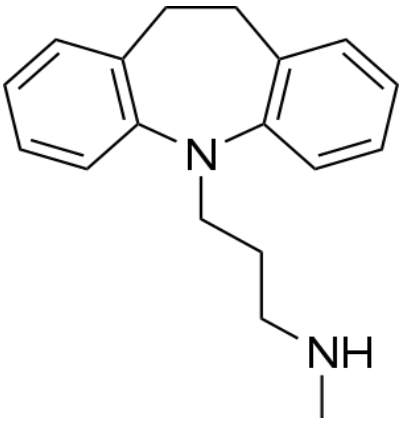


Figure 8. Molecular structure of desipramine. From https://en.wikipedia.org/wiki/Desipramine

This synthetic tricyclic compound is a drug against depression. Desipramine exerts its antidepressant actions by inhibiting the sodium-dependent serotonin transporter and the sodium-dependent noradrenaline transporter thus blocking the reuptake from the neuronal synapse of serotonin and noradrenaline respectively. As a consequence, the levels of serotonin and norepinephrine remain increased, relieving the symptoms of depression. [8]

Another use of desipramine is that it indirectly and non-specifically inhibits acid sphingomyelinase (ASM), an enzyme that catalyzes the hydrolysis of sphingomyelin to phosphorylcholine and ceramide (salvage pathway of ceramide synthesis). Desipramine, like other functional inhibitors of acid sphingomyelinase (FIASMA) is a weak base and accumulates in acidic compartments like the lysosome, because it becomes protonated at the acidic pH. Due to the positive charge, it can then no longer cross the membrane. While the lipophilic moiety of desipramine is anchored in the lysosomal membrane, the protonated, positively charged portion is exposed to the lumen, thus altering the electrostatic properties of the inner lysosomal membrane. As a consequence, ASM gets detached and repelled from the lysosomal membrane and thus trapped inside the lysosome and then is degraded by intralysosomal proteases. [9]

Ceramide induces apoptosis, when is transported to mitochondria. Other than that, ceramide and other sphingolipids are shown to be necessary for the survival and growth of many intracellular bacteria that create vacuoles inside the host cell, like *Simkania negevensis* [9]. Consequently, it would be interesting to research how the infection would proceed if ASM inhibitors, like desipramine, were applied but also what would be the effect on apoptosis due to the reduction of ceramide.

ARC39:

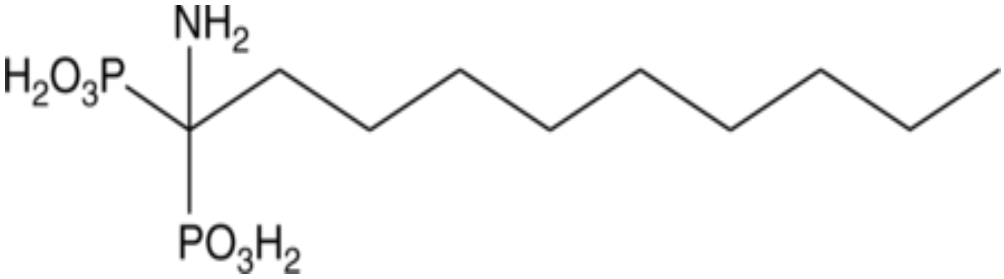


Figure 9. Molecular structure of ARC39.

Adapted from https://www.biomol.com/products/chemicals/biochemicals/1-aminodecylidene-bis-phosphonic-acid-sodium-salt-cay13583-1

(1-aminodecane-1,1-diyl)diphosphonic acid (ARC39) is, in contrary to desipramine, a direct inhibitor of ASM (enzyme that takes part in the lysosome pathway of ceramide synthesis). It is selective for acid sphingomyelinase over neutral sphingomyelinase. It is a synthetic biphosphonate compound with high affinity and specificity (IC50 = 20 nM). Hence, it is expected to have very strong inhibitory effect in the infection of *Simkania negevensis* at HeLa cells. [13]

Myriocin:

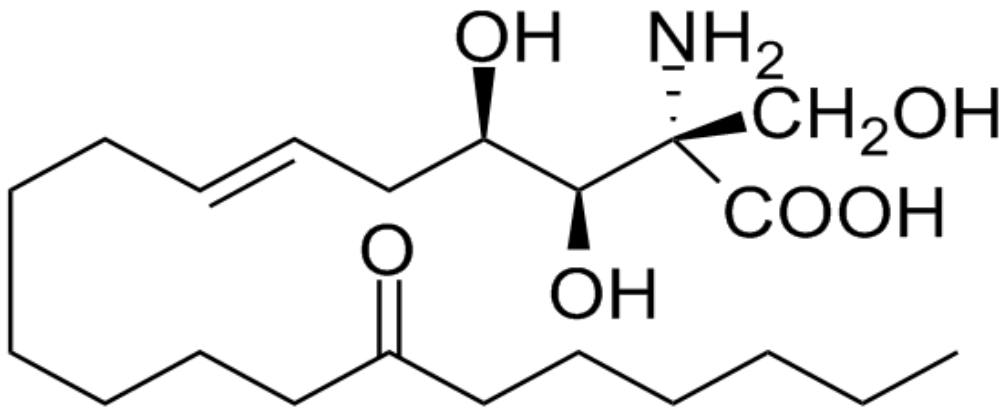


Figure 10. Molecular structure of myriocin. From https://en.wikipedia.org/wiki/Myriocin

Myriocin is a non-proteinogenic amino acid derived from certain thermophilic fungi, like *Isaria sinclairii*. As a molecule itself, it has no therapeutical uses but it has been shown that it possess potent immunosuppressant effect. Besides, Fingolimod, a synthetic drug used to overcome the symptoms of multiple sclerosis, is developed by modifying myriocin. It is a direct and potent inhibitor of serine palmitoyltransferase (SPT). The human enzyme is a heterodimer consisting of two monomeric subunits known as long chain base 1 and 2 (LCB1/2) encoded by separate genes. It participates in the de novo synthesis of ceramide in endoplasmic reticulum. It catalyzes the chemical reaction:

palmitoyl-CoA + L-serine $\rightleftharpoons$ CoA + 3-dehydro-D-sphinganine + CO_2

This is the first and pivotal step for the generation of sphingosine, the precursor of many sphingolipids. Consequently, by inhibiting the regulator of sphingolipids, like ceramide, myriocin can have many impacts, one of them may be the confinement of insulin resistance in human skeletal muscles. Therefore, it might be a promising novel drug, in cases that depletion of sphingolipids from cells is required. [14]

3.5 Mitophagy

Autophagy is a catabolic indispensable process that helps the cells to dispose of any substances that may pose threat to the cellular health and integrity. It was thought that it is a non-selective procedure, meaning that it does not designate precisely the contents to be eliminated but has a wider spectrum. However, recently it has been shown that in a lot of cases it aims only at a specific target. Hence, there are many sub-types such as ribophagy (removal of ribosomes), pexophagy (peroxisomes), xenophagy (intracellular pathogens) and mitophagy (removal of mitochondria). Mitophagy is the selective degradation procedure of impaired or superfluous mitochondria, which are generated by many factors such as cancer, a bacterial infection or oxidative stress. It is a highly conserved mechanism among eukaryotes. [27]

The process commences with the engulfment of defective mitochondria by a membrane called isolation membrane, the exact origin of which is not yet known but it is expected to originate from the ER, forming the mitophagosomes. These vesicles are subsequently conjugated with lysosomes forming mitolysosomes, hence the mitochondria are degraded by enzymes inside the lysosome, like acidic hydrolases. There are two pathways that stimulate mitophagy; the PINK1/Parkin–dependent and PINK1/Parkin–independent pathway. In the first method under specific stimulus, like oxidative stress, PINK1 accumulates in the outer mitochondrial membrane instead of the inner mitochondrial membrane. As a result Parkin is activated and through signal transduction microtubule-associated proteins 1A/1B light chain 3B (LC3) indirectly targets the mitochondria with the help of ubiquitin and promotes the formation of mitophagosome. In the PINK1/Parkin-independent pathway LC3 exerts its action swifter and directly without ubiquitin (see figure 10). [29]

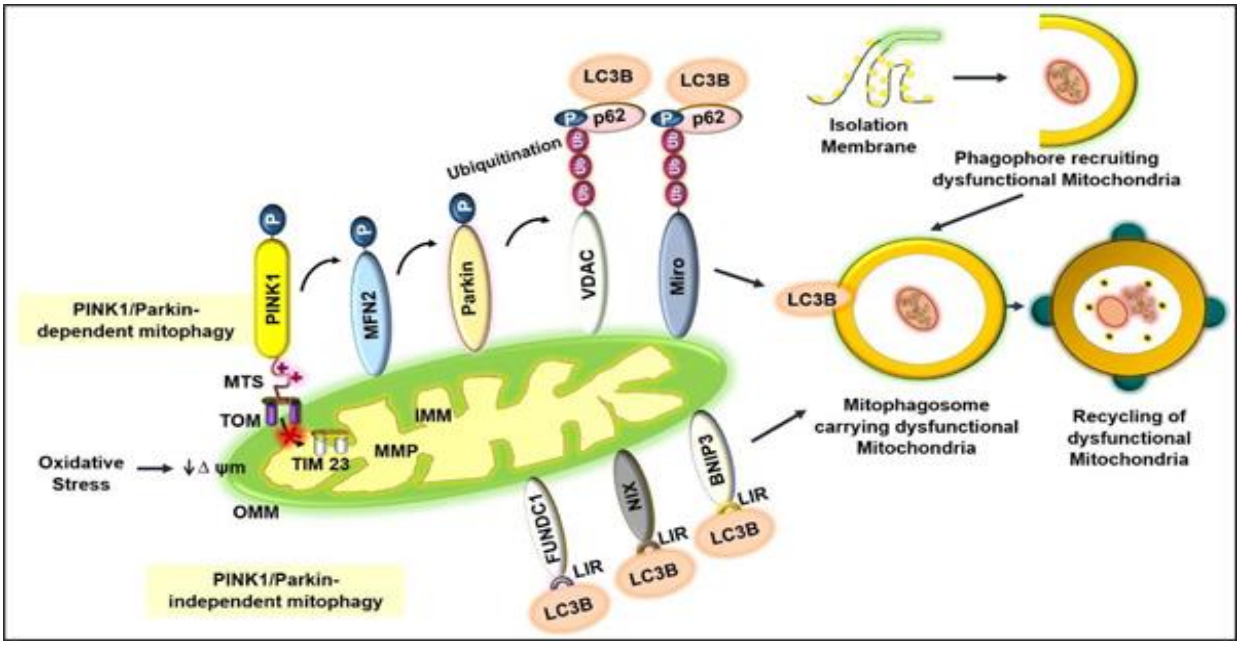


Figure 11. Mechanisms of mitophagy: PINK1/Parkin-dependent and PINK1/Parkin-independent pathways. In the PINK1/Parkin dependent mitophagy, PINK1, due to various conditions such as bacterial infection or oxidative stress, accumulates in the outer mitochondrial membrane (OMM) and connects with the translocase of outer membrane (TOM). PINK1 indirectly activates Parkin, which takes part in the ubiquitination of various OMM proteins. As a consequence p62 adaptor protein attaches these ubiquitinated proteins leading to the recruitment of LC3B, which initiates mitophagy. In the independent pathway LC3B binds to OMM proteins directly. The first step in mitophagy is the encapsulation of dysfunctional mitochondria by the isolation membrane (mitophagosome) and then fusion with lysosomes. From "The Emerging Role of Mitophagy in Kidney Diseases", Bhatia D and Choi ME [29]

4. Aims

The purpose of this project is to study the development of *Simkania* during primary infection in HeLa229 cells and the ability to infect other cells in presence of various inhibitors of sphingolipid metabolism and transport, like ARC39, desipramine and myriocin. Furthermore another goal is to clone a reporter gene for mitophagy using GFP-RFP in *E.coli* with the DISEC/TRISEC technique.

5. Materials and Methods

5.1 Materials

- Glass Pipettes
- T-75 flasks
- Mechanical pipettes of 0.2-2 , 10, 100, 200, 1000 µl
- Falcon tubes of 15 + 50 ml
- 12-well plates
- 6-well plates
- Eppendorf tubes of 1.5 + 2 ml
- Coverslips
- Glass beaker
- Coverslips 15mm
- 26G x 1" syringes- B|BRAUN
- 20G X 1 ½ " syringes- B|BRAUN
- Microscope slides- VWR
- PVDF hydrophobic membrane for western blotting
- stainer (coomassie): 40% ethanol, 7% acetic acid, 0.2% coomassie R-250
- destainer (coomassie): 30% ethanol, 10% acetic acid
- 10x SDS running buffer: TRIS 30.3 gr., glycine 144 gr, SDS 10 gr, 1litre dH_20
- 1x RPMI 1640 medium (gibco) +10% FBS inactivated (no Hepes, no P/S)
- 1x RPMI 1640 medium (gibco) +5% FBS inactivated (no Hepes, no P/S)111
- 1x TrypLE^{TM}Express (gibco) dissociation reagent
- PFA solution: 4% PFA in PBS
- 1 kb DNA ladder GeneRuler (Thermo Fisher Scientific) for electrophoresis
- 6x DNA loading Dye (Thermo Fisher Scientific)
- 1x DPBS=dulbecco's phosphate buffered saline (Gibco)
- PageRuler prestained protein ladder 10 to 180 kDa (Thermo Fisher Scientific)
- PureYield™ Plasmid Midiprep System - Promega Corporation
- Nucleospin plasmid Miniprep – macherey nagel
- GeneJET Gel extraction kit from thermofisher (for DNA purification):
  - Binding buffer (guanidine thiocyanate)
  - Wash buffer (+add 225 ml ethanol 96%)
  - dH_20 instead of elution buffer

- Running gel 12.5% (SDS-PAGE):

  - 6.9 ml acrylamide/bis{rotiphorese gel 30(37,5:1) from ROTH}
  - 3.5 ml 1.875 Tris pH=8.8
  - 0.17 ml 10% (w/v) SDS
  - 0.3 ml dH_20
  - 100 µl 10% (w/v) APS
  - 10 µl TEMED

- Stacking gel:

  - 0.83 ml acrylamide/bis{rotiphorese gel 30(37,5:1) from ROTH}
  - 0.5 ml 0.8M Tris pH=6.8
  - 50µl 10% (w/v) SDS
  - 3.55 ml dH_20
  - 50 µl 10% (w/v) APS
  - 10 µl TEMED

- Pure isopropanol 100%

- 10x transfer buffer:

  - 24 gr. Tris
  - 113 gr. Glycine
  - 2 gr. SDS
  - 1 liter dH_20

- 1x transfer buffer (20% ethanol):

  - 10 ml 10x transfer buffer
  - 20 ml ethanol
  - 70 ml dH_20

- 50x TAE buffer (agarose and polyacrylamide electrophoresis):

  - 242.28 gr. Tris (2 M)
  - 18.61 gr. EDTA (0.05 M) pH= 8.5
  - 57.1 ml acetic acid (1 M)

- 1x TAE buffer (agarose and polyacrylamide electrophoresis):
  - 20 ml 50 x TAE
  - 1000 ml dH_20

- Agarose gel 1.5%: 100ml 1x TAE buffer, 1.5 gr. agarose LE (Genaxxon bioscience)

- HD green plus DNA stain (INTAS)

- Blocking solution (for staining after western blot): 5% skimmed milk powder(saliter) in TBS

- TBS: 0.1M Tris(12.11 gr.), 9% Nacl (90 gr.) pH=7.5, 1000 ml dH_20

- PBS: 5.8 gr. Nacl, KCL 0.2 gr., Na_2HPO_4 1.15 gr, KH_2PO_4

• Primary antibody from rabbit SnGroel in TBS
• Primary antibody from rabbit GAPDH in TBS
• Restriction enzymes EcoRI (10U/µl) and NOTI (10U/µl) - Thermofisher scientific
• Buffer O (10X) - Thermofisher Scientific
• 10x buffer klenow - Thermofisher Scientific
• 10x BSA - Thermofisher Scientific
• 10x T4 DNA ligase buffer - Thermofisher Scientific
• 5x buffer T4 polymerase - Thermofisher Scientific
• T4 DNA ligase (5U/µl) - Thermofisher scientific
• DNA Polymerase I, Large (Klenow) Fragment (10U/µl) - Thermofisher scientific
• T4 polymerase (5U/µl) - Thermofisher scientific
• Taq-polymerase(5U/µl) - Thermofisher scientific
• Secondary antibody from rabbit coupled with horseradish peroxidase
• Secondary antibody from rabbit coupled with fluorescent Alexa488
• Secondary antibody from rabbit coupled with fluorescent rbCy3
• Secondary antibody from rabbit coupled with fluorescent GFP
• Primer mycoplasma GPO-1(forward) - sigma aldrich
• Primer mycoplasma MGSO(reverse) - sigma aldrich
• sucrose phosphate glutamate (SPG medium)
• LB (Luria Bertani) media solution: 10 g tryptone, 10 g sodium chloride (NaCl),5g yeast in 900ml distilled water
• Triton solution: 0.2% triton in PBS
• Mowiol solution: ➢ 6,0 g Glycerin ➢ 2,4 g Mowiol 4-88 ➢ 6,0 ml destilliertes Wasser, steril ➢ 12,0 ml 0,2 M Tris-HCl (pH 8,5)

5.2 Methods

5.2.1 Cell Culture

A preliminary step of seeding cells is required before commencing almost every experiment. For seeding, initially HeLa cells in a T75 flask with RPMI medium 10% inactive (in.) FBS are split using 1.5 ml of trypsin solution, after sucking out the previous medium. Trypsin is a serine protease that dissociates the cells by breaking down proteins that help them to adhere to the bottom of the flask. Incubation follows for 5 minutes (5% CO2, 37ºC). Afterwards, RPMI medium 10% in. FBS is added to the flask. Finally after some dilutions we can transfer the cells to well plates (seeding). After one day in 37ºC and 5% CO_2 to let them multiply, the cells are ready.

Cell line	Characteristics
HeLa229	The cell line that was used hereby was HeLa229. All the cells from HeLa cell line derive from a 31 year old black woman Henrietta Lacks, which was admitted in the john Hopkins hospital in Baltimore, USA, because she was afflicted by cervical cancer, which ultimately killed her on 1951. These cells became the first human cell line that could achieve immortality in vitro. One drawback that HeLa cells have is that they must get split from the flask every two to three days otherwise they get overpopulated and die. Also the overall use of cells descending from a specific original cultivation does not have a big time span partially due to accumulation of toxic waste. On the current research this cell line was used.
HBE	HBE (human Bronchial epithelial) cells are used thoroughly in research for the study of respiratory diseases and the pathology of lungs .The primary function of the bronchial epithelium is to act as a defensive barrier aiding the maintenance of normal airway function. They also help in the lubrication of lungs and the preservation of humidity. They form the interface between the external environment and the internal milieu, making it a major target of inhaled insults. On the current research these cells were not used.
HUVEC	HUVEC (Human umbilical vein endothelial cells) derive as the name indicates from the endothelium of veins from the umbilical cord of humans. They are used for many research purposes including coagulation, angiogenesis etc. They are cheap and they can be isolated relatively easy with techniques like the explant method. On the current research these cells were not used.

Table 1. Cell lines

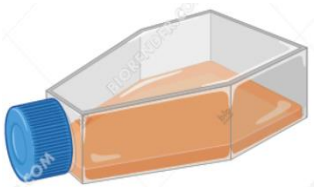
5.2.2 *Simkania negevensis* Preparation (*Simkania* Prep)

Simkania is a bacterium that requires a lot of work to cultivate. One of the factors contributing to that is that it only replicates intracellularly. For example if *Simkania* is extracted from the freezer (-80 °C) it is not advisable to freeze them again in contrast to *Escherichia coli*, which they grow easily on an LB medium plate and you can use them multiple times theoretically. The procedure of making *simkania* stocks requires diligence and requires a lot of time, roughly two weeks, more time than most of the other relative bacteria. In order to start the procedure we need to have a stock of *simkania*.

First step is the primary infection where we pipette usually 10 μl of *simkania* into the flask with the Hela cells (it is required a confluence of about 75%). The flask contains RPMI medium with 5% in. FBS. After that follows incubation for 6 hours at the incubator (35 ºC, 5% CO_2). After 6 hours the medium is changed with new with the same constitution. After the three days of infection, the cells are detached from the bottom of the flask with cell scrapers. Then they are transferred to a falcon with 7.5 ml sterile glass beads, where this falcon is vortexed for 3 minutes. This contributes to the lysis of the cells and the extraction of their inner content. Then the lysed cells are extracted and they are put into a new falcon. Then follows centrifugation at 600g/4^{0}C/10 min. This contributes to the creation of two layers; the heavier debris of the cells will attach to the bottom of the falcon (pellet), whereas the bacteria will be at the liquid (supernatant). Subsequently is the secondary infection. More specifically, seeded 6x 6-well plates with new HeLa cells will be inoculated with 2 ml per well of liquid (1 ml cell suspension from the centrifugation supernatant + 1 ml new RPMI medium 5 % in. FBS). After 6 hours or ON changing of medium (again RPMI 5% in. FBS). Again after this step there is incubation at 35ºC, 5% CO_2 for 3 days. Secondary infection is very crucial to give more time to bacteria to grow in greater proportions.

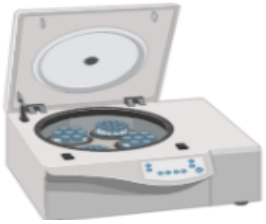
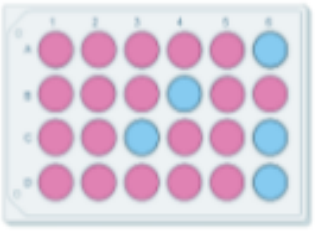
Then similar procedure follows; detaching of cells from 6 well plates, vortexing them for 3 minutes with 7.5 ml glass beads in a falcon tube, the lysed cells from the vortexing are centrifuged at 600 g /4 ^{0}C/10 min. After that the supernatant which should include the bacteria is again centrifuged at 20000 g /4 ^{0}C/10 min. Now the pellet must include the bacteria, because it is heavier compared to other inner components of the cell, so the supernatant is discarded. The pellet is dissolved in 5 ml SPG medium, which is a transport medium (sucrose phosphate glutamate). Then again centrifuge at 20000 g /4 ^{0}C/10 min to get more pure bacteria. Then again we dissolve the new pellet with 2.5 ml SPG medium. Last steps include mixing the solution with a syringe of G20 and a syringe of G26. After that the mixture is distributed equally to 25 sterile eppendorf tubes (100 μl each) and stored at -80^{0}C.

Primary infection

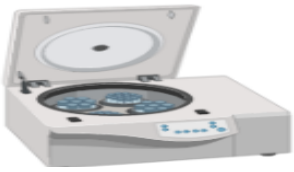


+ -> ->

Secondary infection


-> -> ->



-> dissolution of pellets in SPG medium-> Store in eppendorfs at -80^{0}C

Figure 12. Concise representation of *Simkania* Prep

5.2.3 Verification techniques for *Simkania* Prep

Two verification techniques must be implemented in order to find out if the prep is successful. One is checking for contamination of mycoplasma, a vast category of bacteria that lacks cellular wall and they exist in abundance everywhere and the other is the determination of MOI **(**multiplicity of infection). For the mycoplasma checking, a common part of DNA of the genome of all mycoplasmas will be multiplied with PCR, using the primers GPO-1 (forward) and MGSO (reverse). The product after PCR is expected to be around 700 bp. For the PCR is required as samples a positive control (Sne Prep with mycoplasma), a negative control (distilled water sample) and then the Prep under examination. Firstly, a *Simkania* aliquot is boiled for 5 minutes at 98°C. Each PCR tube must contain 50 μl: 5 μl sample, 1 μl GPO-1 (forward primer), 1 μl MGSO (reverse primer), 0,5 μl Deoxynucleoside triphosphates (dNTPs), 5 μl 10x Taq buffer, 0.5 μl Taq polymerase, 37 μl distilled H_2O.

For the procedure's facilitation a master mix was made. This mix contains in an eppendorf tube all the quantities of the components of PCR except the templates multiplied by 4 (the number of samples is 3, but we increment by 1 for safety reasons). Namely: 4 μl GPO-1, 4 μl MGSO, 2 μl dNTPs, 20 μl 10x Taq buffer, 2 μl Taq polymerase, 128 μl distilled H_2O.

Afterwards, 45 μl of master mix are added to each of the 3 PCR tubes that contain 5 μl of the different samples (total 50 μl).

PCR settings:

3 min. 94^0C

---

1 min. 94^0C

1 min. 55^0C 39 cycles

1 min. 72^0C

---

10 min. 72^0C

∞ min. 4^0C

After the PCR is concluded, electrophoresis takes place. In a 2% agarose gel are loaded 7 μl of 1 Kb DNA marker and the 10 μl of the mixture of 6x loading dye (containing bromophenol blue and xylene cyanol) and of each sample (we blend 10 μl of each eppendorf with 2 μl of 6x loading dye, so that it gets diluted to 1x). For the electrophoresis the device is set at 140 Volt and 400 mA (constant) for 30 minutes. Lastly, the gel is imaged under UV light.

The MOI experiment is crucial to determine how much quantity from a Prep should be added to a group of cells so that they get sufficiently infected. MOI indicates the ratio of the numbers of bacterial particles to the numbers of the host cells in a given infection medium. Simplifying it, MOI of 1 means that every cell from the total amount of HeLa cells in a flask is infected in average with 1 bacterium. Sometimes smaller amounts are required, for instance 5 μl of the prep and other times higher amounts, more than 20 μl to achieve the desired MOI of 1, depending on how successful the prep was. First step is to seed cells in a 12-well plate with no coverslips overnight. Next day the medium is exchanged to the infectious one (RPMI in 5% in. FBS). Then in this order *Simkania* is added to the 12 wells: 1, 2.5, 5, 7.5, 10, 12.5, 15, 20, 25 and 30 μl. Two different alternatives were tested, one was to centrifuge the 12-well plate immediately after infection (910g/60 minutes/35^{o}C) so that the bacteria can precipitate to the bottom and thus contaminate efficiently the cells and the other is without centrifugation. After 3 days, the cells are fixed with 4% paraformaldehyde (PFA) for about 1 hour. PFA fixes proteins chiefly through the formation of di-sulphide bridges between adjacent cysteine residues. This causes the disruptment of the normal structure of essential proteins which kills the cells. Fixation is essential prior to immunostaining because it permeabilizes the cells and hence antibodies can enter them. Last step is the staining and imaging procedure, where antibodies and fluorescent dyes are utilized. The purpose is to find the well that corresponds to the quantity of *Simkania* that renders roughly MOI of 1.

5.2.4 *Simkania* primary infection of HeLa cells with the presence of sphingolipid inhibitors

The experiment is triplicate and commences with the seeding of HeLa cells of two 12-well plates with coverslips and two 12-well plates without coverslips. Only 18 wells are going to be occupied from each of the two groups. Next day, the desired inhibitor concentrations are being prepared depending on the inhibitor according to the fundamental equation $C_1V_1=C_2V_2$:

Desipramine:

Stock desipramine: C= 100 mM

Desired concentrations:

For 30 µM: total volume will be 18 ml -> $C_1V_1=C_2V_2$ -> V_{stock}= 5.4 µl (need to be added to 18 ml RPMI 5% in. FBS)

For 15 µM: 5 ml of RPMI 5% in. FBS + 5 ml of 30 µM solution are mixed

For 5 µM: 8 ml of RPMI 5% in. FBS + 4 ml of 15 µM solution are mixed

For 1 µM: 8 ml of RPMI 5% in. FBS + 2 ml of 5 µM solution are mixed

ARC39:

For ARC39 less concentration is examined.

Stock ARC39: C= 1 mM

Desired concentrations:

For 10 µM: total volume will be 22 ml -> $C_1V_1=C_2V_2$ -> V_{stock}= 220 µl (need to be added to 21.78 ml RPMI 5% in. FBS)

For 7.5 µM: 3 ml of RPMI 5% in. FBS + 9 ml of 10 µM solution are mixed

For 5 µM: 3 ml of RPMI 5% in. FBS + 6 ml of 7.5 µM solution are mixed

For 1 µM: 8 ml of RPMI 5% in. FBS + 2 ml of 5 µM solution are mixed

Consequently, 3 wells per concentration are filled with 1 ml in each well, 3 wells that will get infected later are filled just with medium with 1 ml in each well as a positive control and 3 wells that will not get infected later are filled with the highest concentration (30 μM in the case of desipramine and 10 μM in the case of ARC39) with 1 μl per well as a negative control. This process is repeated both for the plates with and without coverslips. After 3 hours follows infection with 5 μl *Simkania* stock per well and immediately centrifugation of the well plates (910 g/1 hour/35^{0}C). After 6 hours the medium mixed with the proper amount of inhibitors for each concentration as already explained, is exchanged. The purpose of the experiment is to study the action of inhibitor 3 days post infection, so for the next 2 days the medium will be exchanged roughly every 24 hours.

On the third day the cells with the well plates that have coverslips are fixed, then immuno-labeled for GroEL and finally are imaged under the fluorescent microscope under a 40x objective. On the other hand, the cells from the well plates with no coverslips are incurred the western blot procedure for *Simkania negevensis* GroEL (SnGroEL) and GAPDH detection. GroEL is a heat shock protein, also known as molecular chaperonin, present in many bacteria. It contributes to the proper folding of proteins, especially under stressful for the cell situations. It is kindred to the HSP60 family proteins of eukaryotes, due to their endosymbiotc provenance. The molecular weight of SnGroEL is 55 kDa. It is chosen for the antibody processes as detector for the quantification of *Simkania* because it is expressed abundantly. GAPDH is an enzyme that catalyzes the sixth step of glycolysis and has a molecular weight of 37 kDa. In addition other studies suggest that it has more cellular functions in initiation of apoptosis, in signal transduction and other non-metabolic tasks [38]. It is a housekeeping gene, that is it is constitutively expressed at high levels stably in all cells of the organism, under both physiological and pathological conditions. Notwithstanding, its expression rate may vary under certain situations. Consequently, due to this sufficiently stable expression it is implemented as a loading control for western blot.

5.2.5 Fluorescence microscopy

This method requires a well-plate with cells inside. Independently from the experiment we want to carry out, for example determination of MOI in *Simkania* preps or tracking the efficiency of inhibitors in infected cells with *Simkania*, the main steps are the same. Initially, 500 μl of Triton solution are added in every well of the well plates, that were fixed with PFA and contained infected HeLa cells, for 45 minutes under shaking conditions. This solution is actually a detergent that is used to permeabilize the membranes of living cells, so that the antibody can enter through the cell membrane. Afterwards, 300 μl of blocking solution (2% goat serum in PBS) is applied for 1 hour under shaking conditions in room temperature to each well to block unspecific binding of the antibody. Then 300 μL per well of primary antibody anti-SnGroEl solution (anti-SnGroEL in blocking solution 1:800) is added. Incubation in cold room (4ºC) overnight. Next day, after washing down with PBS, 300 μl of secondary antibody solution is added to each well, which consists of secondary antibody coupled with fluorescent rbCy3 (red) or rbAlexa488 (green) with a ratio of 1:1600 in blocking solution, DAPI fluorescence which attaches to the DNA and hence can mark the nucleus with a ratio of 1:3000 in blocking solution. The secondary antibody binds to the primary antibody. Incubation is 1 hour in room temperature. Lastly, the well-plate can be directly imaged in the fluorescence microscope in the case of determination of MOI experiment. In the case of the inhibitor experiment, as mentioned before there are coverslips in each well of the well plates, in which the cells are fixed. So slides are being constructed before imaging. In order to achieve that, we drop one droplet of mowiol solution in an ordinary microscope slide and glue the coverslip with the sample in that solution. A mounting medium like mowiol is used because it fixes the specimen on a microscope slide and also prevents its dehydration. Upon capturing of the images, they were processed with the image processing tool Fiji (Image J).

5.2.6 Western blot

Western blot is probably the most thoroughly used protein-analytical technique for the detection of a specific protein or proteins in a sample. Other similar techniques include dot blot, which basically bypasses the step of separation with SDS and enzyme-linked immunosorbent assay (ELISA), in which quantification can also be implemented. There are a lot of variations in western blot concerning some properties like the membrane for the transfer being used (nitrocellulose, PVDF), the percentage of the density of SDS-gel, the type of western blot (semi-dry,wet), e.t.c. However the main steps are the same in all forms of western blot:

-separation by molecular weight in a SDS-gel electrophoresis of the proteins

-transfer of the proteins to a hydrophobic membrane that has microporous substrates to which proteins bind

- detection of the protein of interest using a label with antibodies

Before commencing the western blot procedure, it is necessary to prepare the samples. Initially in the 12-well plates (18 wells) with no coverslips that were constructed with the Sne infection with the presence of inhibitors procedure, the medium is removed and 100 μl of 2x lemli buffer is added to each well. Afterwards, the cells are scraped with a scraper thoroughly so that all cells get lysed. Then the content of every well is added to an eppendorf tube and consequently the tubes are heated up for 5 minutes at 98^0C, so that the bacteria get neutralised. The samples are being loaded to an SDS-gel with 14% running gel. For the SDS gel it is needed both a running gel 14% and above that a stacking gel. The running gel is used to separate the proteins and the stacking gel is used to condense the proteins so that all proteins are lined up to begin at the same moment at the running gel. The SDS denatures the electrophoretic separation of proteins, so that all proteins bind to SDS and thus have the same negative charge. Hence, the separation is based only on the molecular weight. Each gel has 10 pockets using combs. The gel is placed inside an electrophoresis chamber, where electrical current is applied (24mA per gel, 48 mA for 2 gels and so on, 220 V, Amber=constant) for about one hour. Of note the experiment is triplicate as explained before, so there are 3 gels (72mA). In the empty pockets, lemli buffer was added to reduce lateral diffusion which may create a smile shape, a phenomenon that distorts the movement of the adjacent filled pockets. The marker is used always to mark the approximate position of our protein of interest.

The layout in the pockets is as follows:

For the desipramine:

Lemli	Marker (10 to 180 kDa)	+Sne(control) des.	1 μM des.	5 μM des.	15 μM des.	30 μM des.	30 μM des. -Sne	lemli	lemli
15 μl	5 μl	15 μl	15 μl	15 μl	15 μl	15 μl	15 μl	15 μl	15 μl

For the ARC39:

lemli	Marker (10 to 180 kDa)	+Sne(control)	1 μM ARC39	5 μM ARC39	7.5μM ARC39	10 μM ARC39	10 μM ARC39 -Sne	lemli	lemli
15 μl	5 μl	15 μl	15 μl	15 μl	15 μl	15 μl	15 μl	15 μl	15 μl

After SDS-electrophoresis, the gel with the separated now proteins is extracted and is inserted into a structure. This structure contains filter papers, which help bringing the surface of the membrane (in our case it was used PVDF membrane because it is more sensitive in attaching even small proteins and it is also more resilient and can be reused) and the gel closer so that the transfer of proteins is more efficient. The order of the "sandwich" is as follows from the positive electrode to the negative: thin, thick, thin filter paper, membrane, gel, thin, thick, thin filter paper. It is imperative that the membrane is placed between the gel and the positive electrode so that the negatively charged proteins migrate from the gel onto the membrane. Thereafter, the device for western blot is set at 20 V, 125 mA per gel for 90 minutes. After transfer, a dye called coomassie is added to stain the proteins (stainer) so that the transfer can be confirmed. Afterwards, destainer is added to remove all coomassie that did not bind to the proteins. A subsequent complete destaining with EtOH also takes place. Before the final step -adding the antibodies(primary and secondary)- it is necessary to use a blocking solution that will block all the unspecific binding of the antibodies, so that the antibody binds only to the antigen of interest. For our cause, milk in TBS was used as a blocking solution for 1 hour. Milk has a lot of proteins like casein that bind everywhere in the membrane, where proteins have not been attached. Thus, the antibodies will bind only to the specific target proteins because there is no room in the membrane to bind somewhere else.

There are two proteins we want to detect, one is GroEL (55 kDa) and the other is GAPDH (37 kDa), so the membrane is being cut horizontally at the 40 kDa mark roughly. The upper part of the membrane (which contains the GroEL) is incubated with the anti-GroEL antibody inside a falcon and the lower part (which contains the GAPDH) is incubated with the anti-GAPDH antibody inside another falcon. Both incubations take place in the cold room (4^0C) overnight (ON) for an efficient binding.

The next day the secondary antibody, which is coupled with the enzyme horseradish peroxidase, is being added simultaneously to all the membranes for 1 hour in room temperature (RT). Because the primary and the secondary antibody come from the species of rabbit we do not need separate secondary antibody for GroEL and GAPDH. It is self-evident that washing steps with TBS are carried out between incubations to minimize non-specific signal on the immunoblot. Final step is the visualisation of the proteins. In order to achieve that we pour 1 ml of ECL solution per blot and the membrane is immediately imaged. The ECL western blotting substrate is a very sensitive non-radioactive, enhanced luminol-based chemiluminescent substrate for the visualisation of horseradish peroxidase. ECL is based on the emission of light during the horse radish peroxides oxidation and hydrogen peroxide catalyzed oxidation of luminol. The emitted light is captured by a camera inside the imaging machine, where absolute darkness prevails, and indicates the presence of the antigen. Also it is essential beforehand to take a photo of the marker with the door of the imaging machine opened so that light can enter.

5.2.7 Mitophagy examination: Cloning procedure DISEC-TRISEC (di- and trinucleotide-sticky-end cloning of PCR amplified DNA) of RFPGFP (insert) to preseq-pCDNA3 (vector)

The aim in this experiment is to insert the tandem sensor RFP-GFP which has the red fluorescent protein (RFP) and green fluorescent protein (GFP) into the preseq-pCDNA3 vector. As a template RFP-GFP-LC3 construct was implemented. Microtubule-associated proteins 1A/1B light chain 3B (LC3) protein plays a critical role in mitophagy, and the localization of this protein to mitophagosomes can be used as a general marker for the formation of mitophagosomes during stimulation of mitophagy. The environmental pH inside mitophagosome is neutral and both GFP and RFP emit signal. Marker molecules like LC3 promote the conjugation of the lysosome with the mitophagosome thus shaping the mitolysosome. The acidic hydrolases of the lysosome degrade the ingredients inside mitolysosome. GFP is sensitive and loses the signal in this acidic pH of the mitolysosome, leaving only red fluorescence from RFP which is insensitive.

Briefly, the cloning method counts on the generation of complementary di- or trinucleotide sticky ends at the linearized vector and the PCR-amplified DNA. These are generated by the 3'→5' exonuclease activity of T4 DNA polymerase for the insert (trim reaction) and by Klenow polymerase for the vector (fill-in reaction). The Klenow polymerase is a large protein fragment produced when DNA polymerase I from E. coli is enzymatically cleaved by the protease subtilisin. It maintains the 5' → 3' polymerase activity and the 3' → 5' exonuclease activity for removal of precoding nucleotides and proofreading, but loses its 5' →3' exonuclease activity. Bacteriophage T4 encodes a DNA polymerase that also catalyzes DNA synthesis in a 5' → 3' direction and has 3' → 5' exonuclease activity.

Concisely, the steps are as follow:

- Scission of the plasmid pcDNA3 by NotI + EcoRI (1)
- Fill in reaction (vector) with klenow polymerase (2)
- PCR with iProof polymerase for the amplification of insert (3)
- Trim reaction (insert) with T4 DNA polymerase (4)
- Ligation with T4 DNA Ligase (5)
- Transformation of DH5a *E.coli* with ligation product (also have vectors without insert as control) (6)
- Poolscreening of colonies that received the plasmid containing the insert. Taq polymerase was utilised for PCR (7)
- Plasmid extraction from positive colonies
- Transfection of HeLa229 cells with the recombinant plasmid
- Imaging through fluorescence microscopy of transformed HeLa229 cells

All purifications that will be mentioned carried out according to GeneJET Gel extraction kit from thermofisher.

1) Total volume = 70 µl: 15 µl DNA (vector), 1 µl EcoRI (10 U/µl), 1 µl NotI (10 U/µl), 7 µl buffer O (10x), 46 µl dH_2O. Afterwards purification takes place to a total volume of 30 µl.

pCDNA3-PresequenceM vector sequence: It will be cut by NOTI (**5' GC↓GGCCGC 3'**) and EcoRI (**5' G↓AATTC 3'**) (yellow marking), green marking = starting codon, magenta marking = presequence

5'GGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAAT
TAATACGACTCACTATAGGGAGACCCAAGCTATGCTGGGCGCCGCTCTCCGCCGCTGCGCTGTGACCG
CAACCACCCGGGCCGACCCTCGAGGCCTCCTGCACTCCGCCCGGATCCCCGGCCCCGCCGTGGCTATC
CAGTCAGTTCGCTGCTATTCCGAATTCTGGAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCT
AGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCT 3'

The insert will be inserted between the EcoRI and NotI cutting sites of the pCDNA3-PresequenceM vector.

2) Fill-in reaction is performed for 30 minutes at RT, total volume= 50 µl: 2.5 µl dATP (20mM), 2.5 µl dGTP (20mM), 1.25 µl klenow (10U/µl), 5 µl 10x buffer klenow, 30 µl DNA (cut vector), 8.75 µl dh_2O. Afterwards purification takes place to a total volume of 20 µl.

3) We need around 2.5 µg DNA (insert). Total volume= 50 µl: 0.5 µl plVTHM-mRFP-GFP-ratLC3, 0.5 µl primer (f) RFPGFP, 0.5 µl primer (r) RFPGFP, 0.5 µl dNTPs, 2 µl MgCl2, 10 µl 5x PCR buffer, 0.5 µl iProof polymerase, 35.5 µl dH2O. Afterwards purification takes place to a total volume of 30 µl.

PCR settings:

30 sec. 98^0C

---

10 sec. 98^0C

30 sec. 61^0C 30 cycles

1 min. 72^0C

---

10 min. 72^0C

∞ min. 4^0C

4) Trim reaction is performed for 30 minutes at 12ºC, then 10 minutes at 75 ºC and then immediately on ice. Total volume=40 μl: 4 μl dCTP (20mM), 4 μl 10x BSA, 8 μl 5x buffer T4 polymerase, 0.5 μl T4 polymerase, 3.5 μl dH_2O, 20 μl DNA (insert). Afterwards purification takes place to a total volume of 30 μl.

5) For ligation we need at least 500 ng of vector, so there is ample (20 μl x 96.7 ng/μl=1937 ng). The ratio insert:vector is 3:1. Total volume of DNA (both insert and vector) is 5 μl. The amount of insert and vector needed are calculated according to a complex formula (factors are quantity in ng + length in bp):

Vector volume = total volume DNA/(((vector concentration x insert length x ratio insert:vector)/ insert concentration x vector length))+1)

Insert volume= total volume – vector volume

Vector	Insert
5400 bp	1400 bp
96.7 ng/μl	48.41 ng/μl

Total volume= 20 μl: 2 μl vector, 3 μl insert, 1 μl T4 DNA ligase, 2 μl 10x buffer T4 DNA ligase, 12 μl dH_2O. Afterwards NO purification but storage in the fridge ON (+4ºC).

It is important to make the negative control with the cut + fill-in vector without the insert to observe if it will be put back together without receiving the insert. Normally it should not if the fill-in reaction is successful. Total volume= 2 μl DNA (cut + fill-in vector), 2 μl 10x buffer T4 DNA ligase, 1 μl T4 DNA ligase, 15 μl dH_2O. Afterwards NO purification but storage in the fridge ON (+4ºC).

6) The steps of transformation will be done twice with the control and the recombinant plasmid. Addition of 100 μl DH5a E. coli + 15 μl ligation product to a new eppendorf tube. Thereafter, incubation for 30 min. on ice, then 90 sec. at 42ºC (this is the crucial step where the cells take up the plasmids) and then incubation again on ice for 2 min. Afterwards, addition of 1 ml LB medium sterile and incubation on shaking conditions 850 rpm, at 37ºC for 30-45 min. Afterwards centrifugation at 4000 rpm/3 min and shedding of the most supernatant and then mixing of the pellet (which has the bacteria) with the supernatant that remained (around 100 μl). Then follows addition of 10 μl from the mixed eppendorf tube to the LB agar plate containing the antibiotic Ampicillin with a pipette. A roller soaked in EtOH (for sterile reasons) helps to spread the bacteria on the plate. Lastly, the plates are placed in the 37^0C room ON so that they can grow and next day they are put in the cold room (4^0C) so their development gets suspended.

7) From the overnight culture of transformed *E. coli* colonies, 25 random colonies from the plate were chosen, transferred onto a new LB agar plate and subjected into PCR with Taq polymerase together with the negative control. Total volume= 50 µl: 0.5 µl T7 primer (f), 0.5 µl rev_dim_cl primer (r), 5 µl 10x buffer Taq polymerase, 0.5 µl dNTPs, 0.25 µl Taq polymerase, 38.25 µl dH_2O, 5 µl DNA

PCR settings:

2 min. 94^0C

---

20 sec. 94^0C

30 sec. 49^0C 30 cycles

2 min. 72^0C

---

10 min. 72^0C

∞ min. 4^0C

The product expected at the transformed bacteria is roughly 500 bp. There should be no product from the negative control.

Unfortunately, no positive colonies were observed so the experiment terminated here. However the following step includes the extraction of the plasmids from the positive colonies. Afterwards, HeLa229 cells would be transfected with the recombinant plasmids that contained the insert with RFP (red) and GFP (green) and then imaged via the fluorescent microscope.

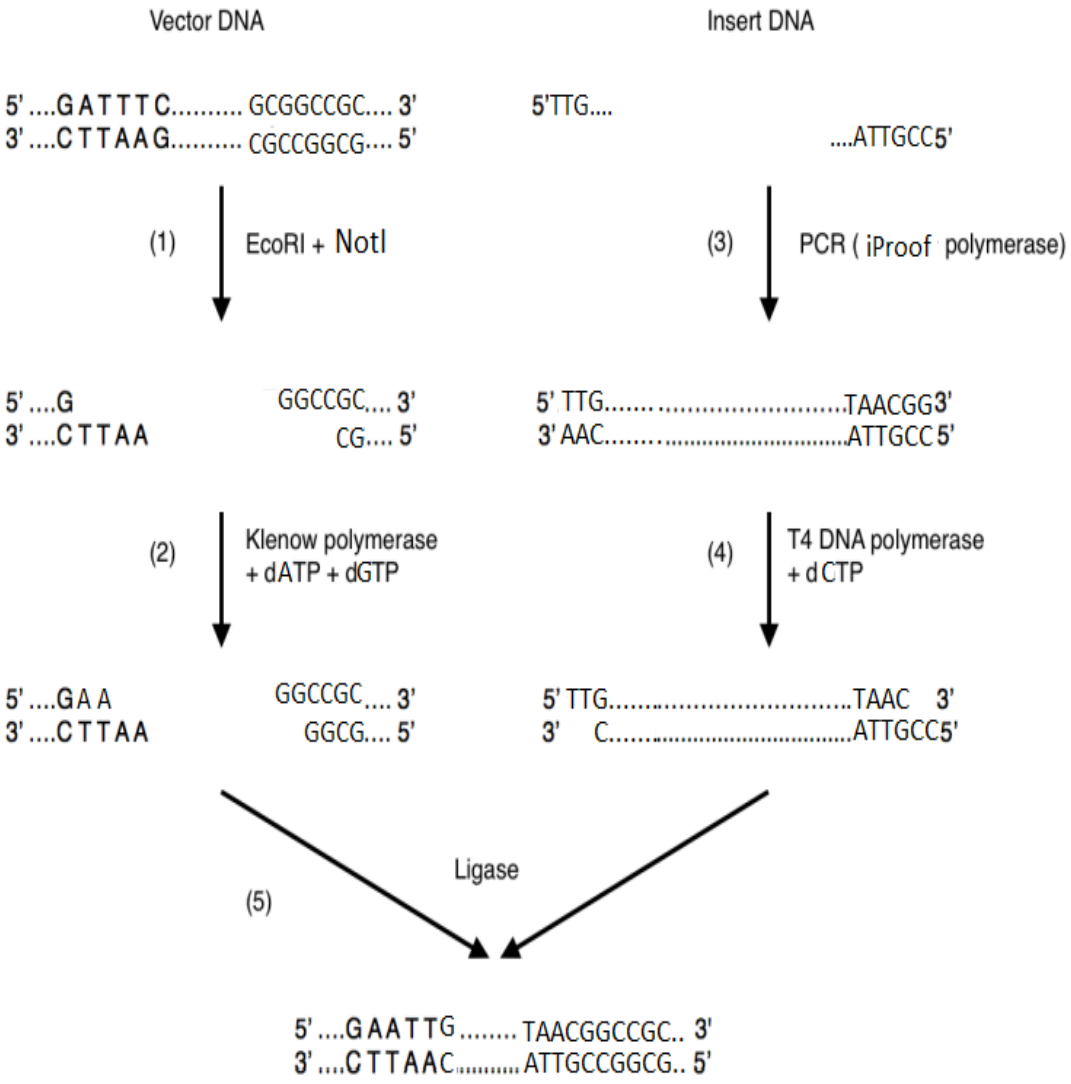


Figure 13. Schematic representation of DISEC/TRISEC method to clone pLVTHM-mRFP-GFP-ratLC3. The numbers correspond to the aforementioned steps that described comprehensively.

5.2.8 Plasmid DNA extraction (Midiprep of Promega Corporation) from *E.coli*

In experiments where plasmids are being used for various biotechnological applications is necessary to have purified plasmids. The yielding with midipreps is usually around 100-200ug of plasmid DNA from 50ml bacteria culture. One of the best strains to use for optimal transformation is the DH5a *E. coli*, which were engineered by Douglas Hanahan. They have three basic mutations that make them so competent, recA1, endA1 and Δ*(lacZ)*M15. RecA1 is a mutation that entails the inactivation of recombinases and the subsequent inhibition of homologous recombination. That means reduced occurrence of unwanted recombination in cloned DNA which is desired. The endA1 mutation inactivates an intracellular endonuclease to prevent it from degrading the inserted plasmid. Δ*(lacZ)*M15, which is a deletion mutation, is the alpha acceptor allele needed for blue-white screening with many lacZ based vectors. Initially, *E. coli* cells are extracted from the cell culture and placed into two 50ml falcon tubes with LB medium where they undergo centrifugation at 5100g/12min/RT. The supernatant is removed and 5 ml of cell resuspension solution are used to suspend the pellet with the cells. Afterwards for 3 minutes the cells are incubated in 5 ml of cell lysis solution in room temperature. After lysis is occurred, 8.3 ml of neutralisation solution are added again for 3 minutes in room temperature, a solution that neutralizes the resulting lysate and creates appropriate conditions for binding of plasmid DNA to the silica membrane column. Precipitated protein, genomic DNA, and cell debris are then pelleted by a centrifugation step in 14.000g/15min/RT and the supernatant is loaded onto the silica binding column. This is a pivotal step because due to centrifugation the majority of chromosomal DNA will form a pellet while the plasmid DNA will remain soluble. It is necessary however before pouring the supernatant to place a clearing column above the binding column and this construct must be placed in the vacuum device that will pump the liquid, as shown in the image below. When all liquid is absorbed, the clearing column is removed and 5 ml of endotoxin removal solution are added to the binding column that has now the plasmid DNA to remove endotoxins, proteins, RNA and endonucleases. Finally, plasmid DNA is eluted by 600 μl of dh_2O in an eppendorf tube via the vacuum again after it is washed with 20 ml of column wash solution. Of note, the purified plasmid DNA must be stored in the freezer (around -20^oC).

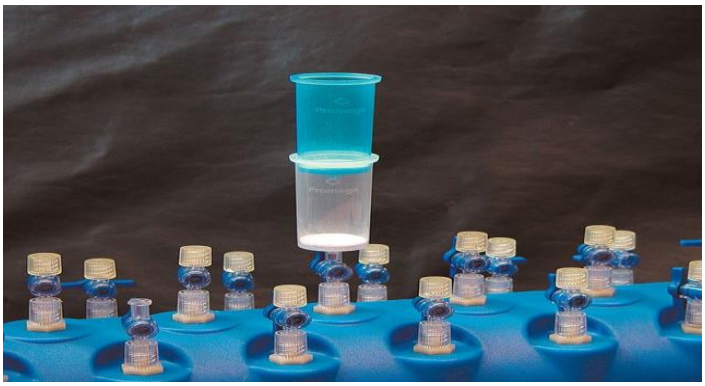


Figure 14. Assembly of Lysate Clearing Column (blue) and DNA Binding Column (white) for use with the PureYield™ Plasmid Midiprep System DNA Purification by Vacuum protocol of Promega Corporation. From Promega corporation [28]

5.2.9 Preparation of cell culture stocks

This method is significant for the storage of important samples, like specially modified cells that have improved properties. First step is the removal of the previous RPMI medium in 10% in. FBS from the T75 flask and the addition of thrypsin solution 1.5 ml. After 5 minutes the solution is mixed with 6.5 ml of new medium of the same constitution with the old one and is transferred to a falcon tube. The tube is centrifuged at 800g/5min/RT and after the supernatant is removed, the pellet containing the cells is resuspended with 3 ml freezing medium. The freezing medium contains dimethyl sulfoxide (DMSO), a polar, aprotic organic solvent that is commonly used as a cryoprotectant because of its membrane penetrating and water displacement properties. It is added to reduce ice formation and thereby prevent cell death during the freezing process. Finally, 1 ml is added in each cryotube, tubes that are resilient and robust, and they are transferred for storage in the freezer (-80^0C).

5.2.10 plasmid DNA extraction (Miniprep of Macherey-Nagel) from *E.coli*

This method is very similar with the midiprep technique, with the difference that the productivity of pure plasmid DNA is smaller, roughly 5-50 μg from 3 ml cell culture of the DH5a strain of *E. coli*. Of course the yielding depends also from the available cell strain. Firstly, 1-5 ml of cultured *E. coli* in LB medium are centrifuged at 11.000g/30sec/RT. The supernatant is discarded and 250 μl of cell resuspension buffer (A1) is added to suspend the pellet. This buffer containing Tris and EDTA helps by denaturing the DNA, disrupting the cellular membrane so the lysis occurs smoother and inhibiting endonucleases, enzymes that degrade DNA. Thereupon, 250 μl of buffer A2 are added and the mixture is incubated for up to 5 minutes in room temperature. Next 300 μl of buffer A3 are added and centrifuge follows at 11.000g/10 minutes/RT. It is pivotal to mix gently A2 and A3 buffers and not vortex or pipette up and down due to possible shearing of genomic DNA. This can lead to contamination of plasmid DNA with bits of genomic DNA. The supernatant now contains the plasmid DNA and it is transferred to a silica binding column which is connected with a collection tube. This construct is centrifuged at 11,000g/1min./RT to get rid of lysate debris. After flowthrough from collection tube is discarded, 500 μl AW buffer and after 600 μl of buffer A4 are used with centrifugation at 11,000g/1min./RT to wash the membrane. Lastly, the binding column is placed in a new, sterile 1.5 ml eppendorf tube and the plasmid DNA is eluted with 50 μl dH_2O by centrifugation at 11,000g/1min./RT. Once more, the eppendorfs are stored in the freezer (-20^0C).

5.2.11 DNA purification

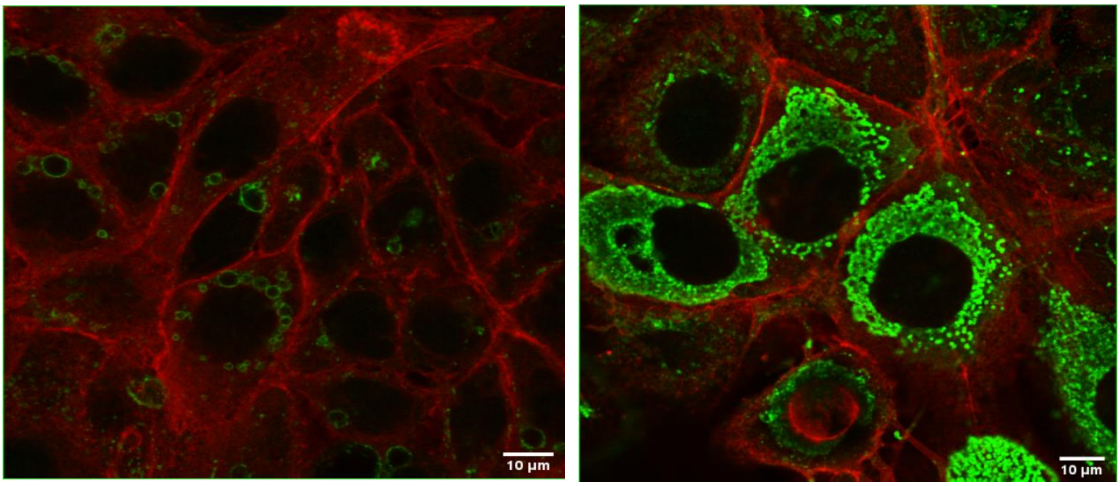
Usually, after a PCR, in experiments including cloning procedures DNA, purification is required, so that the experiment can proceed. First step is adding by 1:1 volume the binding buffer to the DNA under purification in the PCR tube. After thorough mixing, the mixture is poured in a specially constructed column with a collection tube. The binding buffer helps the mixture to stick to the silica membrane column. Afterwards follows quick centrifugation for 1 minute and the flowthrough is discarded. Thereafter, 700 μl of washing buffer are added and quick centrifuged for 1 minute to clean the membrane. The empty column is centrifuged again for 1 minute after the flowthrough is discarded. Ultimately, designated amount of dh_2O is added and centrifuged for 1 minute depending on how much volume and how much diluted our DNA we want to be. Now the throughflow is harvested and with a help of a spectrometer the exact concentration of pure DNA is measured at 260 nm, because UV light is absorbed the most by nucleic acids in this wavelength. Caution is to be taken also for the ratio A260/280, which shows possible contamination with various substances absorbing at 280nm, i.g phenols, and 260/230, which indicates possible contamination with substances absorbing at 230nm, i.g carbohydrate carryover. An A260/280 ratio of ~1.8 is considered as pure for DNA and a ratio of ~2.0 is accepted as pure for RNA in general. The A260/230 on the other hand should be in the range of 2.0–2.2.

6. Results

6.1 Cell lines

Here are cited the cell lines with images.

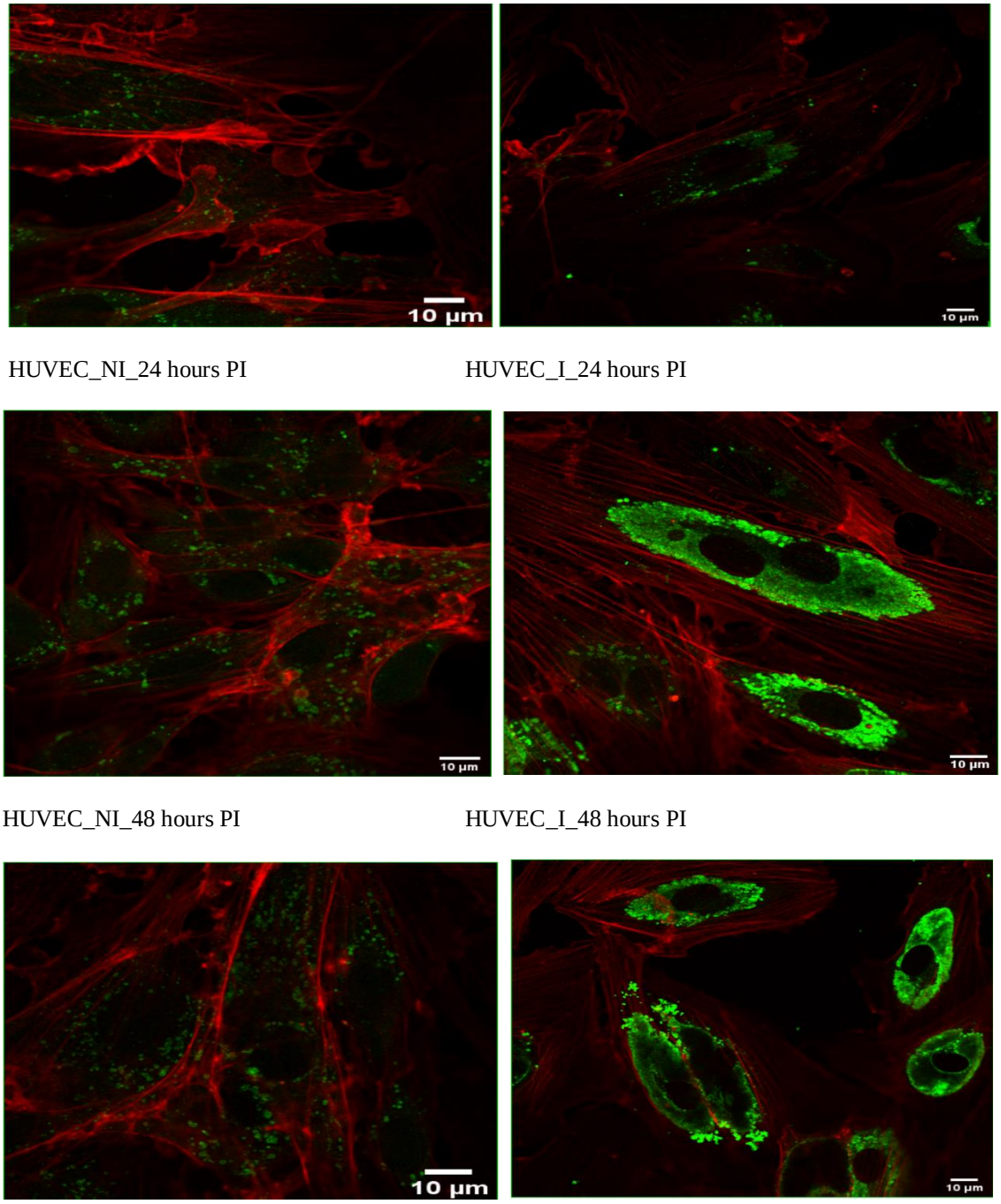
HBE cell line:



HBE_NI_ 0 hours PI HBE_I_ 0 hours PI

Figure 15. Pictures were recorded with confocal microscope. HBE cells infected by Sne (MOI 1) zero hours PI (post infection). The red stain indicates actins, proteins that form microfilaments in the cytoskeleton. The green stain on the left side where the NI (not infected) are, is unspecific binding of the antibody, specifically an anti-*S. negevensis* (Sn) GroEL primary antibody, followed by staining with fluorophore-coupled secondary antibody. Courtesy of Dr. Vera Kozjak-Pavlovic.

HUVEC cell line:



HUVEC_NI_24 hours PI HUVEC_I_24 hours PI

HUVEC_NI_48 hours PI HUVEC_I_48 hours PI

HUVEC_NI_72 hours PI HUVEC_I_72 hours PI

Figure 16. Pictures were recorded with confocal microscope. HUVEC infected by Sne (MOI 1) 24, 48 and 72 hours PI (post infection). The red stain indicates actins, proteins that form microfilaments in the cytoskeleton. The green marks on the left side where the NI (not infected) are, is unspecific binding of the antibody, specifically an anti-*S. negevensis* (Sn) GroEL primary antibody, followed by staining with fluorophore-coupled secondary antibody. Courtesy of Dr. Vera Kozjak-Pavlovic.

HeLa cell line:

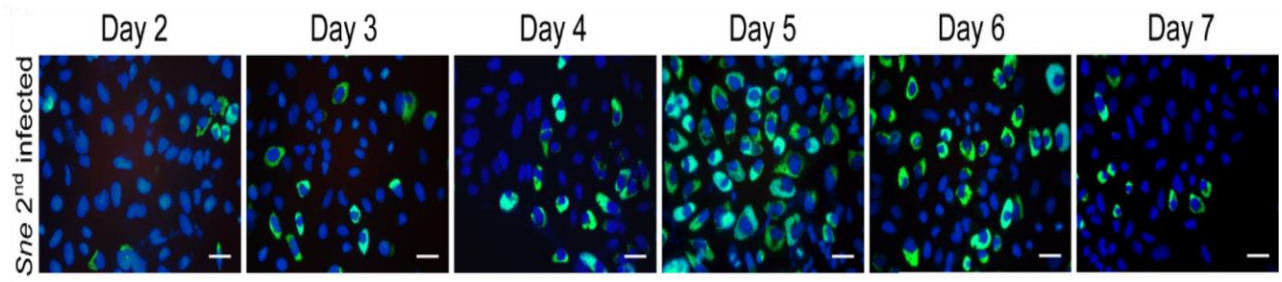


Figure 17. Secondary infection of HeLa cells with *S. negevensis* for 2-7 days. Staining used was DAPI (blue) and anti-SnGroEL antibody (green). Scale bar corresponds to 100µm. Adapted from "Modulation of Host Cell Death and Lysis Are Required for the Release of *Simkania negevensis"*, Koch et al. [11]

6.2 PCR for mycoplasma on *Simkania* prep + Determination of MOI

As it is observed in the figure 18 the product of our positive control is at 700 bp (a bit below 750bp marker band) as it should be, according to the primers used. Primers for mycoplasma used were MGSO and GPO-1. There is no other band, thus the *Simkania* stock produced was free of mycoplasma.

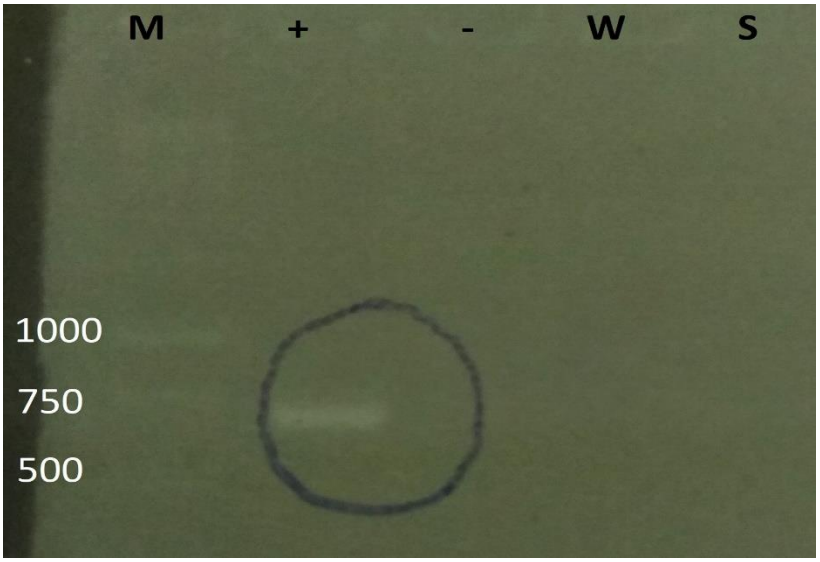


Figure 18. Result of electrophoresis. M (marker), + =positive control rendering signal (previous stock known it has mycoplasma), - =negative control (stock known it is free of mycoplasma), H_2O =another negative control (not necessary), S =our prep of interest

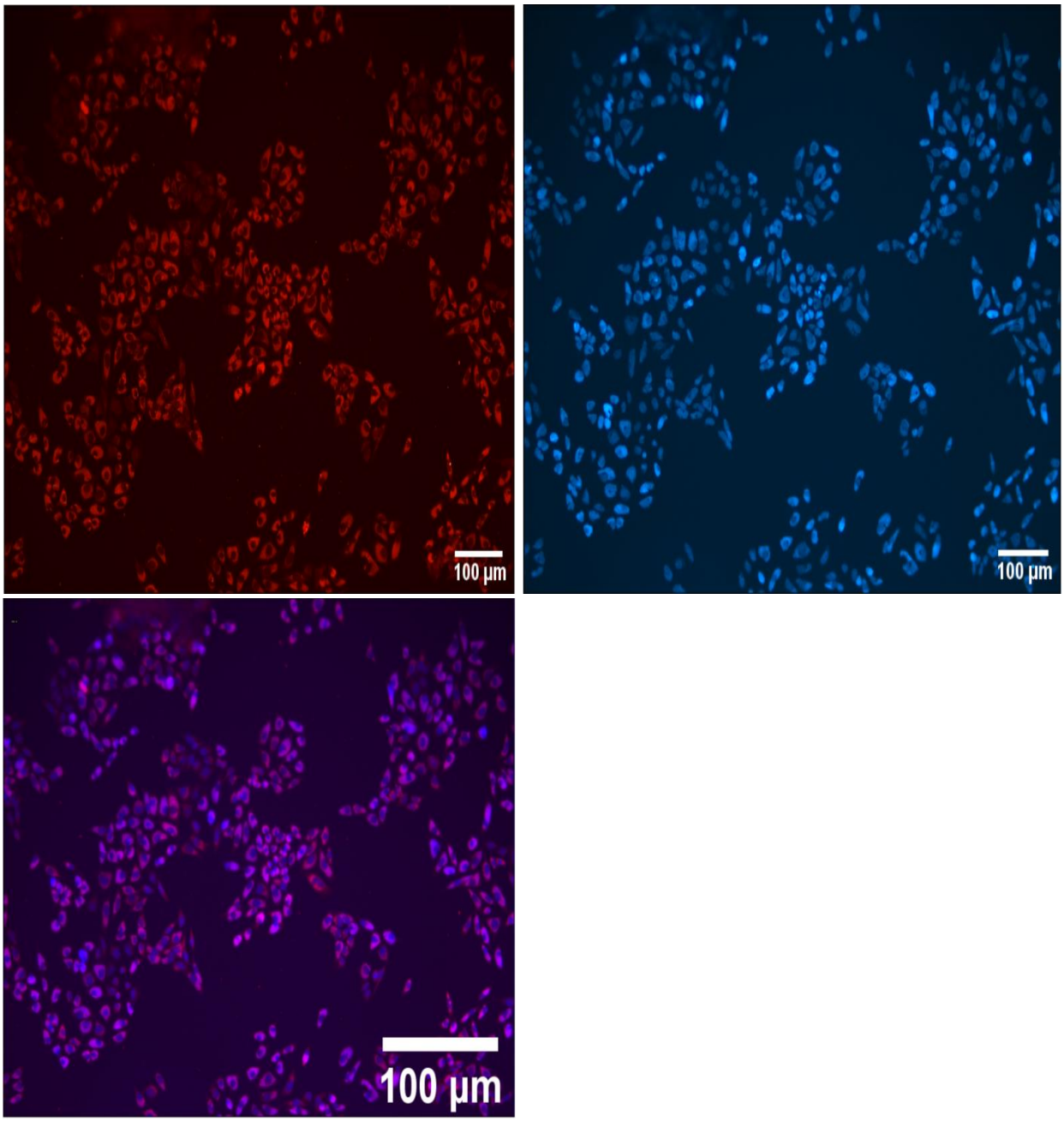


Figure 19. Infection 20 µl *Simkania* with centrifugation for determination of MOI. In the upper left image GroEL proteins have been stained with a secondary antibody coupled to Cy3 showcasing the bacteria. In the upper right image is the same spot but now depicts DNA, meaning the nuclei of HeLa cells and the DNA of *Simkania*, through DAPI staining. The lower photo is the merged representation of the two channels (blue and red). This is a procedure that helps detecting infected cells. In this particular Prep, at 20 µl there is MOI=1.

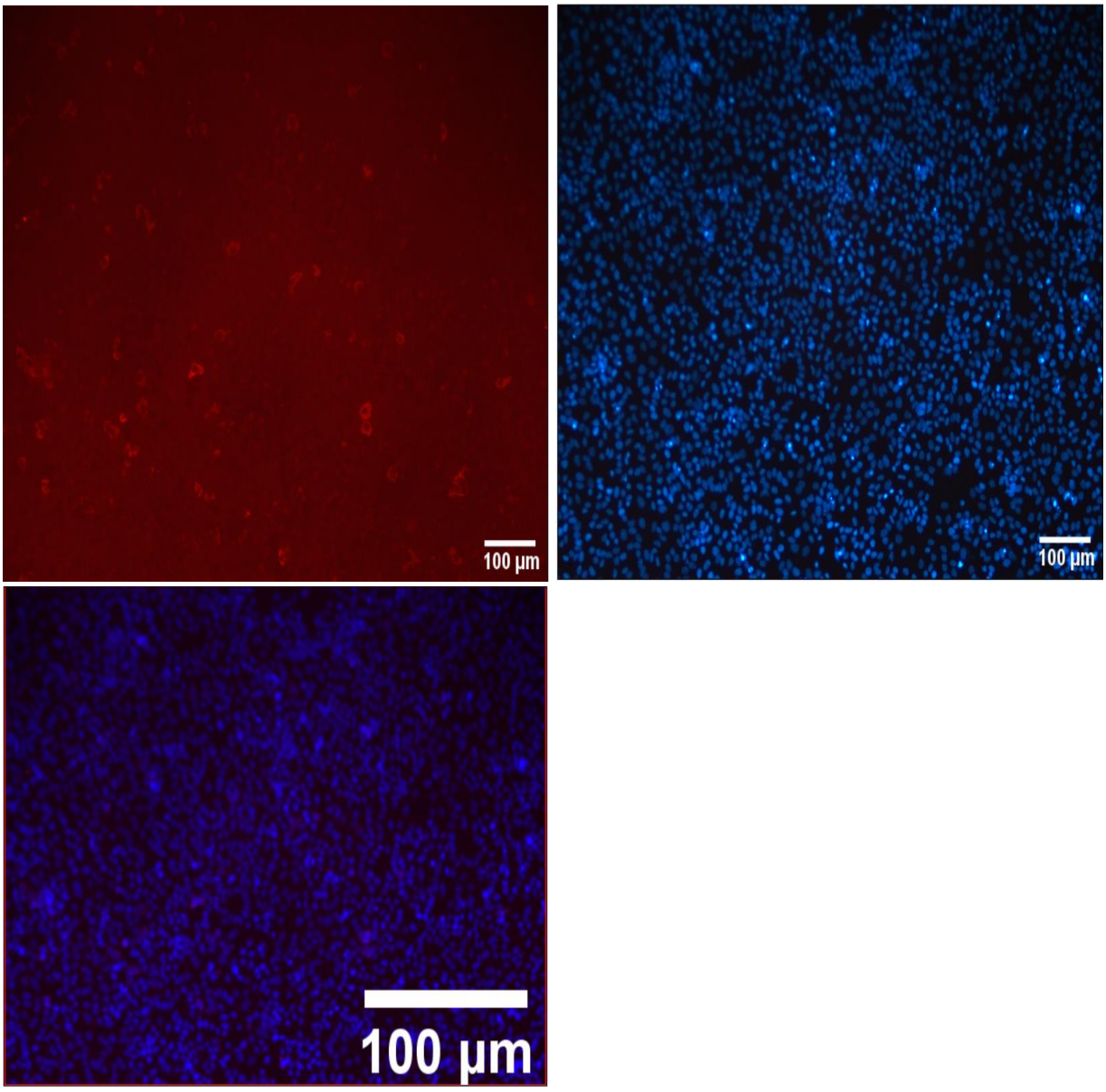


Figure 20. Infection 20 µl *Simkania* without centrifugation for determination of MOI. In the upper left image GroEL proteins have been stained with the dye Cy3 showcasing the bacteria. In the upper right image is the same spot but now depicts the nuclei of HeLa cells through DAPI staining. The lower photo is the merged representation of the two channels (blue and red). This is a procedure that helps detecting the infected cells.

As it is observed in the figure 19, almost every cell is infected at 20µl of the *Simkania* Prep with *Simkania*, which means it is not a good Prep because we need as less amount as possible to achieve MOI of one (5µl is suitable). As it can easily be observed without centrifugation (figure 20) only a very limited amount of cells is infected. Hence, centrifugation immediately after infection is always essential.

6.3 Fluorescence Microscope

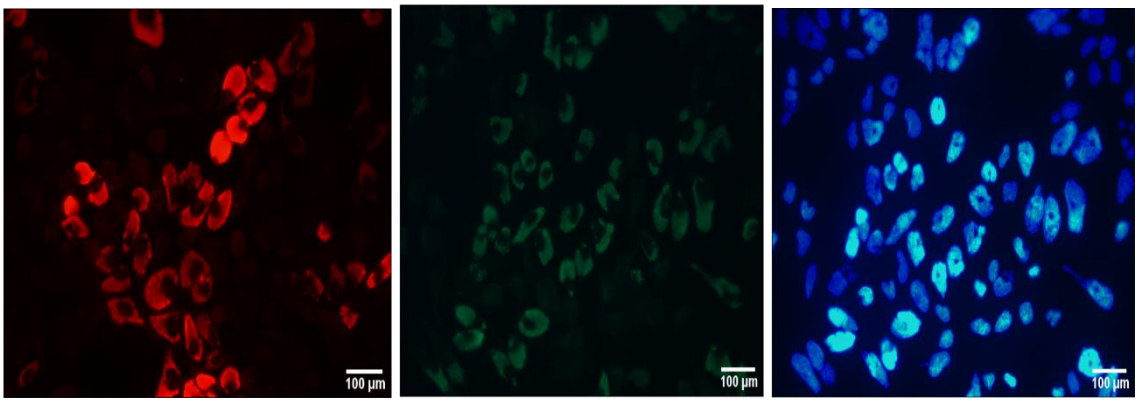


Figure 21. A sampling of three images that represent infected HeLa cells. In the first image (far left) with red colour is the fluorescent Cy3 that stains the SnGroEL protein and in the second image the green fluorescent Alexa488 also stains the SnGroEL protein. In the third image is the blue fluorescent DAPI that stains the nuclei of HeLa229 cells. *Simkania* can also be observed surrounding the nuclei.

6.4 Myriocin inhibitor

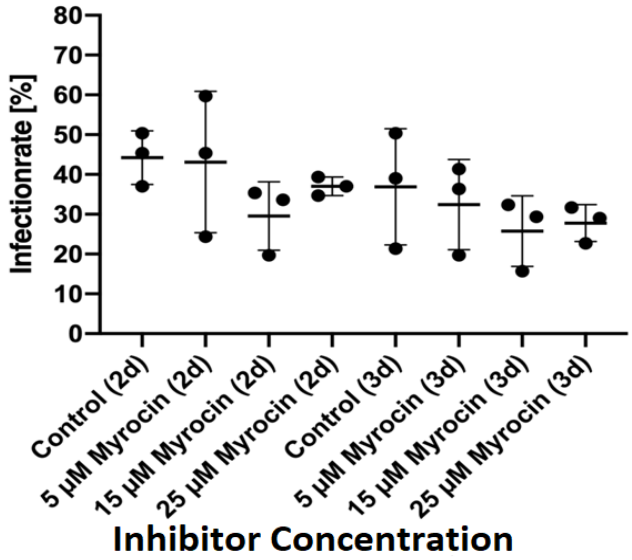


Figure 22. Infection rate of HeLa229 cells from *Simkania negevensis* in comparison with the increment of the inhibitor concentration two and three days post infection. The black lines between the three dots is the mean of the three values for each concentration. The smaller black lines at the edges is the standard deviation.

As it can be observed from this triplicate experiment in figure 22, at 15 μM there is the most reduction on infection rate at around 30% 2 days post infection (PI) and 27% 3 days PI. The controls derive from wells that were only HeLa cells infected with *Simkania* and no myriocin. Infection rate is calculated by dividing the infected cells by the number of total cells. At 25 μM we should expect a lower infection rate but the chart shows otherwise. This is maybe because of the fact that above a certain concentration the infection rate does not change significantly. Also we can notice a lower infection rate at 3 days PI instead of 2 days PI, because as the days pass the infection rate normally reduces. All in all, there is unfortunately not much evidence that the gradual increment of myriocin decreases the infection rate. There is a high standard deviation, a statistic that measures the dispersion of a dataset relative to its mean. As a consequence the results are not significant. There is a trend visible and the inhibitor is somewhat efficient but it is difficult to extract any further conclusions from these results.

6.5 ARC39 inhibitor

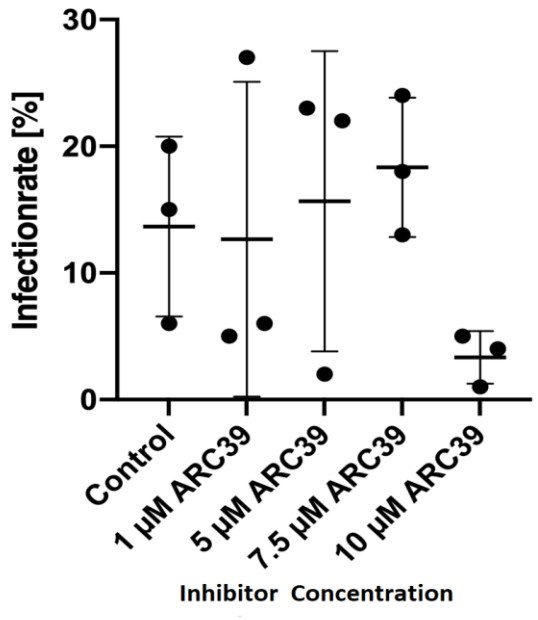


Figure 23. Infection rate of HeLa229 cells from *Simkania negevensis* in comparison with the increment of the inhibitor concentration ARC39 three days post infection. The black lines between the three dots represent the mean of the three values for each concentration. The smaller black lines at the edges is the standard deviation.

The results with the ARC39 inhibitor (figure 23) produce high standard deviation. The control, which is the infected cells without inhibitor, has a very low infection rate and has also a lot of deviation between the three technical replicates of the same experiment thus it is difficult to extract any precise conclusions for the other measurements. The only result that has statistical significance is the 10 µM concentration and it seems to have very strong inhibitory effect. The first three concentrations, 1, 5 and 7.5 µM, appear to gradually increment the infection rate, something that could indicate that in very small concentration ARC39 is not deterrent for *Simkania.*

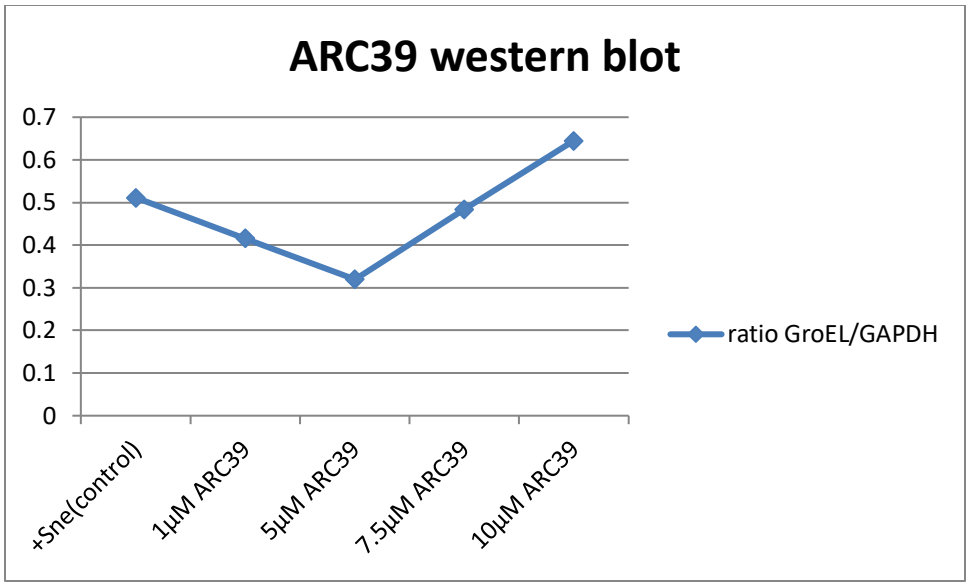


Figure 24. ARC39 western blot. The numbers on the vertical axis represent the ratio of the density of the bands on the blot of GroEL divided by the density of the bands on the blot of GAPDH. By rule, the bigger ratio indicates more infection. On the horizontal axis is the control on the bottom left, which encompasses only infected cells without the inhibitor, and after that, each of the different concentrations tested.

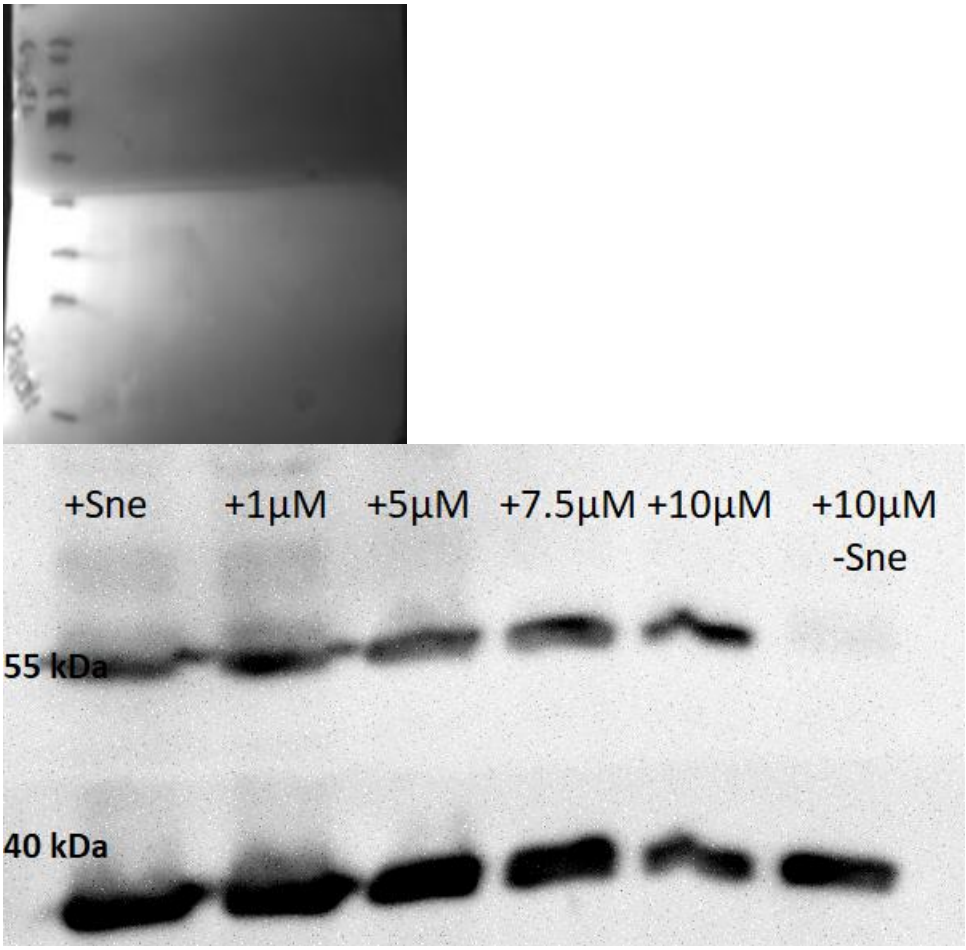


Figure 25. Result of western blot in the imaging machine. The first image (from top) is the marker that will help identify our proteins of interest. In the second image where there is the membrane from the blot of infected HeLa cells with the inhibitor ARC39, two main lines can be observed. The upper line is the GroEL and the lower line the GAPDH, exposure time 3 minutes. As anticipated GAPDH signal emits in every case and that is the desired result because it is used as a loading control of the cell population. First band is the control and after follow increasingly concentrations of inhibitor. The vertical axon depicts the size of the proteins (molecular weight) in the specific line. The last band is used to check if ARC39 has any cytotoxic effects.

For the western blot of ARC39 there was only one blot that produced results in the imaging machine from the triplicate experiment (figure 24). There is pronounced consecutive reduction of *Simkania* at the concentrations 1 and 5 μM ARC39 indicating the effectiveness of the inhibitor. Nonetheless, at 7.5 and 10 μM there is contradictory results because the ratio is increased, even above the control. This is due to the fact that there were not many cells in these wells, since the GAPDH bands were sparse, something that cannot be observed in the chart but affects the results. When there are very few cells, no reliable results can get extracted.

6.6 Desipramine inhibitor

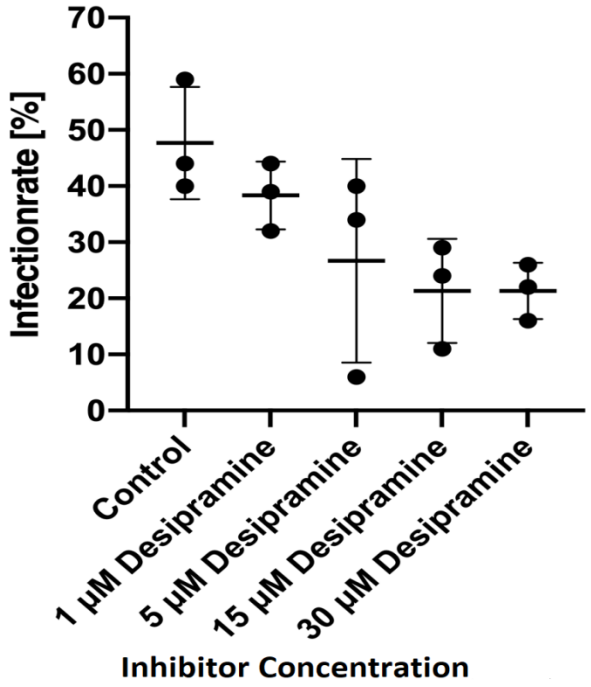


Figure 26. Infection rate of HeLa229 cells with *Simkania negevensis* in comparison with the increment of the inhibitor concentration desipramine three days post infection. The black lines between the three dots respresent the mean of the three values for each concentration. The smaller black lines at the edges is the standard deviation.

The results on desipramine (figure 26) seem more reliable than ARC39 but there is still a high standard deviation in most measurements and also on the 5 μM there is no statistical significance meaning that the numbers provided could be random results. It is observed that the increase of concentration of desipramine decreases gradually the infection rate, reaching the lowest infection rate of about 22% mean value with concentrations 15 and 30 μM. There is also high infection rate in the control (mean value about 50%) giving the opportunity of better results.

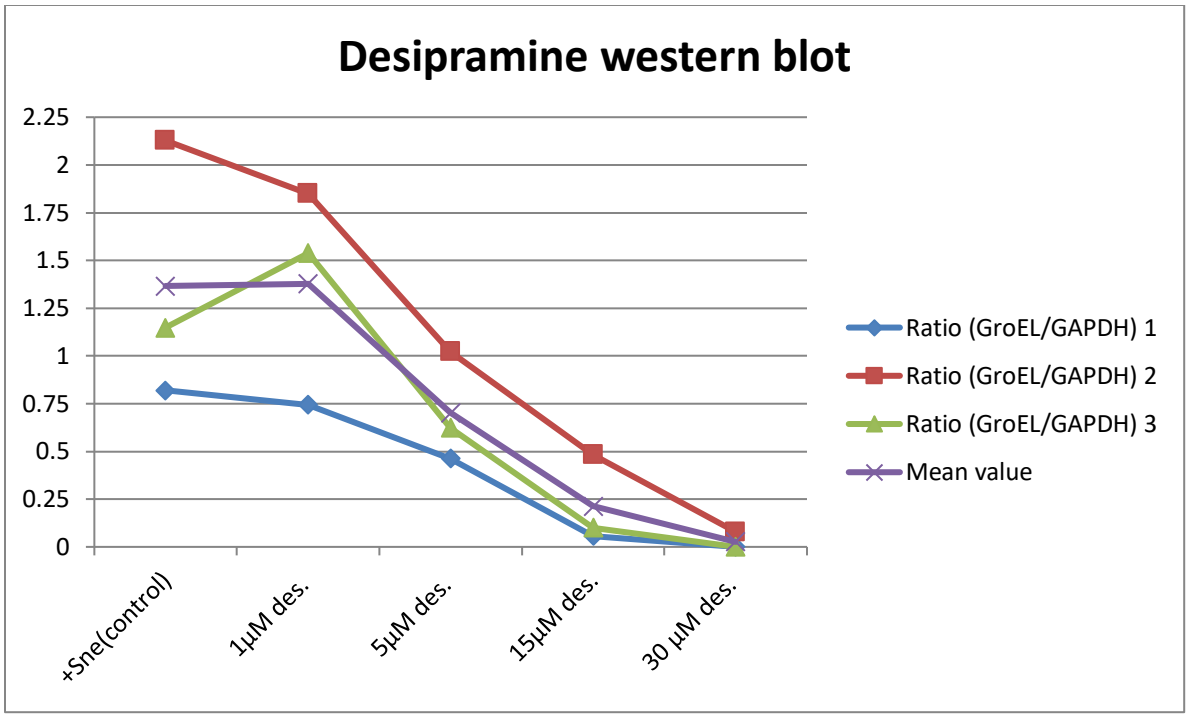


Figure 27. Western blot. The numbers on the vertical axis represent the ratio of the density of the bands on the blot of GroEL divided by the density of the bands on the blot of GAPDH. The experiment was triplicate, hence the 3 lines and the fourth (purple) is the mean value of all three. On the horizontal axis is the control, which encompasses only *Simkania* without the inhibitor, and after that each of the different concentrations tested.

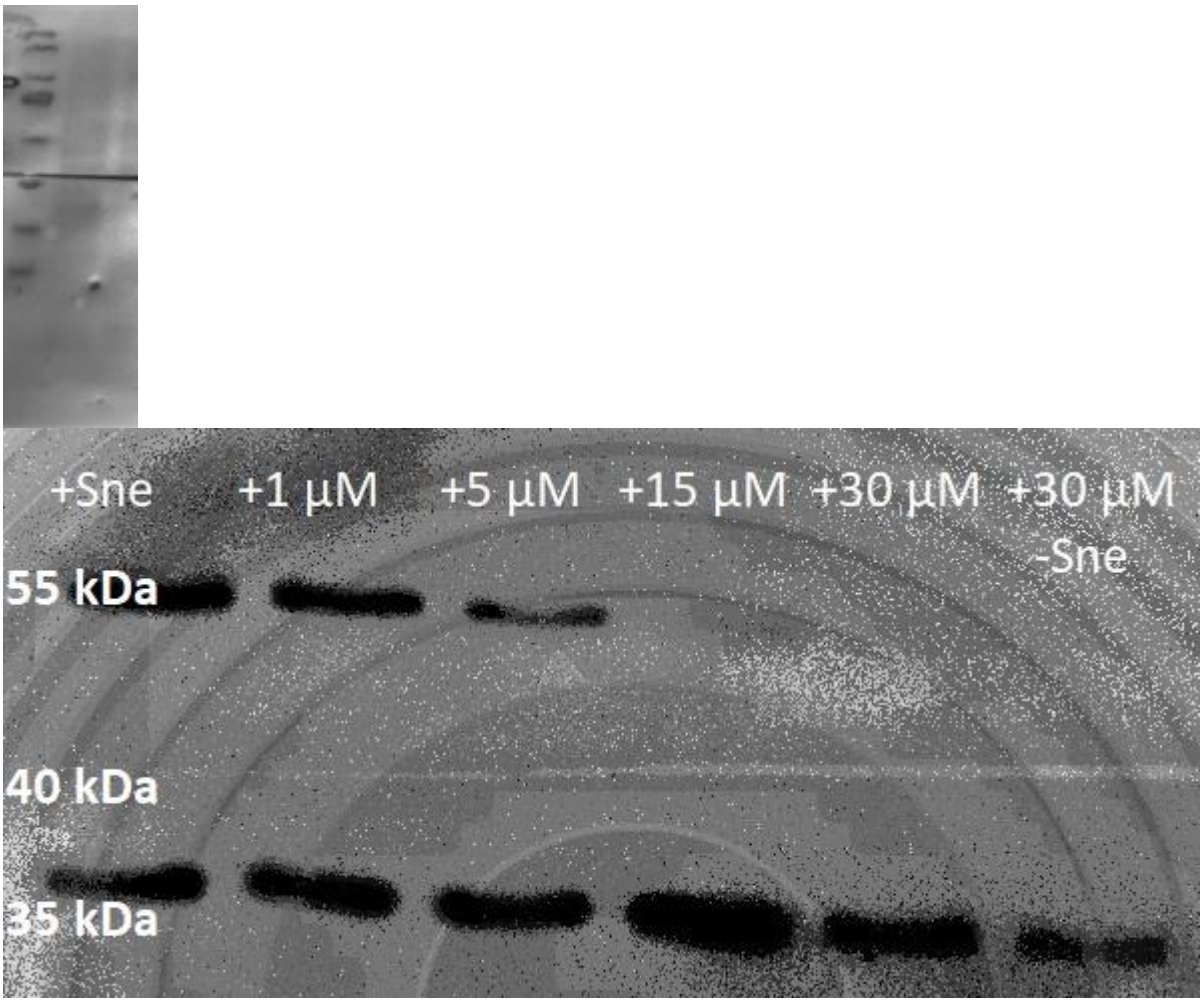


Figure 28. Result of a western blot in the imaging machine. The first image (from top) is the marker that will help identify our proteins of interest. In the second image where there is the membrane from the blot of infected HeLa cells with the inhibitor desipramine, two main lines can be observed. The upper line is the GroEL and the lower line the GAPDH, exposure time 3 minutes. As anticipated GAPDH signal emits in every case and that is the desired result because it is used as a loading control of the cell population. First band is the control and after follow increasingly concentrations of inhibitor. The vertical axon depicts the size of the proteins (molecular weight) in the specific line. The last band is used to check if desipramine has any cytotoxic effects.

For the desipramine blot there is consecutive trend (figure 27). The higher the concentration of the inhibitor the higher the reduction. Specifically from 15 µM the mean ratio has dropped to less than 0.25 and at 30 µM the ratio is almost 0. Nevertheless there is no statistical significance, especially on the control, because there is an apparent declination on the three values. Other than that it is noteworthy that in all three cases there is pronounced reduction on the infection.

7. Discussion

Concerning the flaws of the experiments, a high standard deviation is observable in a lot of cases. Even after many attempts in triplicate experiments to acquire statistically significant results, the situation in each of the wells with the same conditions and treatment seems to vary. Also it is pivotal that in experiments the control must be statistically significant and has high infection rate because all data will get compared according to that. There are also problems sometimes with the amount of HeLa cells seeded being too low initially or with their replication mechanisms. This has as result less cells to be provided for *S. negevensis* to infect and thus alter the outcome. Consequently, the comparison between wells of different concentration of inhibitor cannot occur, since there is different amount of original cells. Moreover, in order to check if merely the inhibitors are sufficient to decrease the infection, like myriocin that inhibits the de novo synthesis, the substances that are downstream of SPT for example (sphingosine +dihydroceramide) could be supplied to the cells after the inhibitor to check if the infection will proceed normally. Concerning the cloning procedure DISEC/TRISEC, there was a probable issue with the poolscreening PCR because no product could be detected. One reason for that is maybe that the primers generated a big product after poolscreening that has more than 1000 bp. Many times the production of such a molecule is not feasible in practice so it is advisable to obtain products that are up to 500 bp. However even with other primers that produce smaller products, like rev_dim_cl (r) and T7 (f), nothing could be detected. Collectively, there are many variables that could go wrong in this experiment, especially when so many enzymes are used. It is noteworthy to mention that this is a safer and more efficient method in general for cloning than the conventional way because the insert is not cut by restriction enzymes, thus when it contains a restriction site (in our case for example) it will not get cut and ruin the experiment. Also the 5‘-overhanging ends at the plasmid created by the Klenow polymerase are non-complementary and consequently there is no fear of relegation of the plasmid, without receiving the insert.

There is not much data published about these three inhibitors on *Simkania negevensis* infection. Regarding desipramine it was shown for four other intracellular bacteria, *Anaplasma phagocytophilum, Coxiella burnetii, Chlamydia trachomatis* and *Chlamydia pneumonia* that it exhibit antibacterial properties and this ability is correlated on the pathogen dependency on LDL cholesterol (Jason A Carlyon et al., 2019). Pro rata, due to inactivation of acid sphingomyelinase by desipramine, deficiency of ceramide and therefore LDL cholesterol may also affect negative *Simkania.* Another study indicated that the use of myriocin in *C. trachomatis* results in loss of inclusion membrane integrity due to the absence of sphingomyelin, early redifferentiation to infectious elementary bodies, and premature release of infectious bacteria (Beatty et al., 2009). Hence, more research is needed about the precise effect of myriocin in intracellular bacteria, like *Simkania.* All in all, it seems that because intracellular bacteria have similar mechanisms of infection, they are affected some in a smaller and some to a greater extent by the absence of sphingolipids.

About the DISEC/TRISEC it has been implemented efficiently for the cloning of a 0.8 kb cDNA fragment encoding the protein YptC1 from the algae *Chlamydomonas reinhardtii* and a 1.4 kb cDNA fragment encoding the protein a-tubulin from the green algae *Volvox carteri* (Schmitt et al., 1993). The insert in this experiment was also 1400 bp, so that indicates that the experiment was feasible and realistic.

8. Conclusion

As described, *S. negevensis* is a complex bacterium that has a lot of morphological and functional properties that we are not yet aware of. Studies on that bacterium will help us to unveil information about intracellular bacteria that will help us produce vaccines and drugs. The present cohort had as a purpose to observe, whether or not various sphingolipid inhibitors alter the survival chances of *Simkania* inside HeLa cells. As it was explained in the introduction it has been proved that sphingolipids and in general lipids contribute to some extent on the proliferation of these bacteria [16]. However, it is necessary to check if sphingolipids are obligatory or not, meaning if *Simkania* finds ways to overcome this issue, for example by producing sphingolipids by themselves. Unfortunately there is not much literature about this subject to compare but on the other hand it adds in to the uniqueness of this project.

All in all, with ARC39 there is a pronounced reduction of the infection rate of *Simkania* at higher concentrations, but in smaller ones there is not much of a difference. Concerning myriocin there is a decrease of infection until a certain point (until 15 μM) but then more concentration does not seem to have further effect. Probably the whole project requires more time and I would like to repeat some of the experiments I did in the future to verify with higher reliability if the results will be reproducible so that safer results can get extracted. Also in the outlook is to try these inhibitors on a secondary infection so we can compare it with the primary infection, so that we can examine the effect on establishment and replication. Furthermore, it would be interesting to study the effect of another inhibitor, HPA12, a ceramide analogue that inactivates ceramide transport protein (CERT). This protein is necessary for the transfer of ceramide from the production place, endoplasmic reticulum, to Golgi organelle for the generation of more complex sphingolipids. It is shown by a study that the size and the number of *Chlamydia psittaci* inclusions is significantly reduced by HPA-12 treatment (Heuer et al., 2017). Again, there is not much data published about that but it is expected a lower infection rate on the secondary infection.

Furthermore, i would like to repeat the DISEC/TRISEC cloning procedure after making some tweaks until a successful transformation is achieved because it will be useful to understand the mitophagy mechanism in a more insightful way using the tandem RFP-GFP construct. If this is achieved, then we can observe the consequences of the infection on mitochondria.

9. Bibliography:

1. Ambros, P. F., & Karlic, H. I. (1987). Chromosomal insertion of human papillomavirus 18 sequences in HeLa cells detected by nonisotopic in situ hybridization and reflection contrast microscopy. Human Genetics, 77(3), 251–254
2. Vouga, M., Kebbi-Beghdadi, C., Liénard, J., Baskin, L., Baud, D., & Greub, G. (2018). What is the true clinical relevance of *Simkania negevensis* and other emerging Chlamydiales members? New Microbes and New Infections, 23, 1–5
3. HARRY TOWBIN et. al. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications , National Academy of Sciences, 76(9), 4350–4354
4. Burnette, W. N. (1981). "Western Blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A., Analytical Biochemistry, 112(2), 195–203
5. Bryant, F. R. (1988). Construction of a recombinase-deficient mutant recA protein that retains single-stranded DNA-dependent ATPase activity. Journal of Biological Chemistry, 263(18), 8716–8723
6. Chan, W., Verma, C. S., Lane, D. P., & Gan, S. K. (2013). A comparison and optimization of methods and factors affecting the transformation of *Escherichia coli*. Bioscience Reports, 33(6)
7. Tatsumi, M., Groshan, K., Blakely, R. D., & Richelson, E. (1997). Pharmacological profile of antidepressants and related compounds at human monoamine transporters. European Journal of Pharmacology, 340(2-3), 249–258
8. LEONARD, B. E. (1987). A Comparison of the Pharmacological Properties of the Novel Tricyclic Antidepressant Lofepramine with its Major Metabolite, Desipramine: A Review. International Clinical Psychopharmacology, 2(4), 281–297
9. Kornhuber, J., Tripal, P., Reichel, M., Mühle, C., Rhein, C., Muehlbacher, M., Gulbins, E. (2010). Functional Inhibitors of Acid Sphingomyelinase (FIASMAs): A Novel Pharmacological Group of Drugs with Broad Clinical Applications. Cellular Physiology and Biochemistry, 26(1), 9–20
10. Chelsea L Cockburn, Ryan S Green, Sheela R Damle, Rebecca K Martin, Naomi N Ghahrai, Punsiri M Colonne, Marissa S Fullerton, Daniel H Conrad, Charles E Chalfant, Daniel E Voth, Elizabeth A Rucks, Stacey D Gilk, and Jason A Carlyon (2019). Functional inhibition of acid sphingomyelinase disrupts infection by intracellular bacterial pathogens. Life Science Alliance, 2 (2)
11. Koch, R.-D., Hörner, E.-M., Münch, N., Maier, E., & Kozjak-Pavlovic, V. (2020). Modulation of Host Cell Death and Lysis Are Required for the Release of *Simkania negevensis*. Frontiers in Cellular and Infection Microbiology, 10

12. Beckmann, N., Sharma, D., Gulbins, E., Becker, K. A., & Edelmann, Bã¤. (2014). Inhibition of acid sphingomyelinase by tricyclic antidepressants and analogons. Frontiers in Physiology, 5
13. Naser, E., Kadow, S., Schumacher, F., Mohamed, Z. H., Kappe, C., Hessler, G., Carpinteiro, A. (2020). Characterization of the small molecule ARC39, a direct and specific inhibitor of acid sphingomyelinase in vitro. Journal of Lipid Research, 61(6), 896–910
14. Miyake, Y., Kozutsumi, Y., Nakamura, S., Fujita, T., & Kawasaki, T. (1995). Serine Palmitoyltransferase Is the Primary Target of a Sphingosine-like Immunosuppressant, ISP-1/Myriocin. Biochemical and Biophysical Research Communications, 211(2), 396–403
15. Moro, C., & Capel, F. (2019). Regulation of Skeletal Muscle Metabolism by Saturated and Monounsaturated Fatty Acids. Nutrition and Skeletal Muscle, 347–358
16. Kozjak-Pavlovic, V., Herweg, J.-A., & Rudel, T. (2018). The role of host cell organelles in the development of *Simkania negevensis*. International Journal of Medical Microbiology, 308(1), 155–160
17. Merrill, A. H., Schmelz, E.-M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. J., Wang, E. (1997). Sphingolipids—The Enigmatic Lipid Class: Biochemistry, Physiology, and Pathophysiology. Toxicology and Applied Pharmacology, 142(1), 208–225
18. Worgall, T. S. (2007). Sphingolipids: major regulators of lipid metabolism. Current Opinion in Clinical Nutrition and Metabolic Care, 10(2), 149–155
19. Worgall, T. S. (2011). Sphingolipid Synthetic Pathways are Major Regulators of Lipid Homeostasis. Sphingolipids and Metabolic Disease, 139–148
20. Jian Wang, Yi-Li Chen, Yong-Kang Li, Ding-Kang Chen, Jia-Fan He and Nan Yao. (2021). Functions of Sphingolipids in Pathogenesis During Host–Pathogen Interactions. Frontiers in Microbiology, 12
21. Robertson, D. K., Gu, L., Rowe, R. K., & Beatty, W. L. (2009). Inclusion Biogenesis and Reactivation of Persistent *Chlamydia trachomatis* Requires Host Cell Sphingolipid Biosynthesis. PLoS Pathogens, 5(11)
22. Herweg, J.-A., Pons, V., Becher, D., Hecker, M., Krohne, G., Barbier, J., … Mehlitz, A. (2015). Proteomic analysis of the *Simkania*-containing vacuole: the central role of retrograde transport. Molecular Microbiology, 99(1), 151–171
23. Koch-Edelmann, S., Banhart, S., Saied, E. M., Rose, L., Aeberhard, L., Laue, M., … Heuer, D. (2017). The cellular ceramide transport protein CERT promotes *Chlamydia psittaci* infection and controls bacterial sphingolipid uptake. Cellular Microbiology, 19(10)
24. Dietmaier, W., Fabry, S., & Schmitt, R. (1993). DISEC-TRISEC: di- and trinucleotide-sticky-end cloning of PCR-amplified DNA. Nucleic Acids Research, 21(15)

25. Hybiske, K., & Stephens, R. S. (2007). Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. Proceedings of the National Academy of Sciences, 104(27)
26. Palikaras, K., Lionaki, E., & Tavernarakis, N. (2018). Mechanisms of mitophagy in cellular homeostasis, physiology and pathology. Nature Cell Biology, 20(9), 1013–1022
27. Riley, J. S., & Tait, S. W. G. (2016). Mechanisms of mitophagy: putting the powerhouse into the doghouse. Biological Chemistry, 397(7)
28. Promega Corporation; https://worldwide.promega.com/products/nucleic-acid-extraction/plasmid-purification/pureyield-plasmid-midiprep-system/?catNum=A2492
29. Bhatia D, Choi ME (2019). The Emerging Role of Mitophagy in Kidney Diseases. J Life Sci (Westlake Village). 1(3):13-22
30. Kitatani, K., Idkowiak-Baldys, J., & Hannun, Y. A. (2008). The sphingolipid salvage pathway in ceramide metabolism and signaling. Cellular Signalling, 20(6), 1010–1018
31. Hartmann, A. (2016). Multiple routes of phosphatidylethanolamine biogenesis ensure membrane integrity of *Toxoplasma gondii*
32. Thao, M. L., Baumann, L., Hess, J. M., Falk, B. W., Ng, J. C. K., Gullan, P. J., & Baumann, P. (2003). Phylogenetic Evidence for Two New Insect-Associated *Chlamydia* of the Family Simkaniaceae. Current Microbiology, 47(1), 46–50.
33. Chen, H., Wen, Y., & Li, Z. (2019). Clear Victory for Chlamydia: The Subversion of Host Innate Immunity. Frontiers in Microbiology, 10
34. Hybiske, K., & Stephens, R. S. (2007). Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. Proceedings of the National Academy of Sciences, 104(27)
35. Zampelas, & Magriplis. (2019). New Insights into Cholesterol Functions: A Friend or an Enemy? Nutrients, 11(7)
36. Kunz, T. C., & Kozjak-Pavlovic, V. (2019). Diverse Facets of Sphingolipid Involvement in Bacterial Infections. Frontiers in Cell and Developmental Biology, 7
37. Avota, E., de Lira, M. N., & Schneider-Schaulies, S. (2019). Sphingomyelin Breakdown in T Cells: Role of Membrane Compartmentalization in T Cell Signaling and Interference by a Pathogen. Frontiers in Cell and Developmental Biology, 7
38. Tarze, A., Deniaud, A., Le Bras, M., Maillier, E., Molle, D., Larochette, N., … Brenner, C. (2006). GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. Oncogene, 26(18)
39. Geoffrey, M.C. & Robbert, E.H. (2011). Το κύτταρο: Μια Μοριακή Προσέγγιση. Τόμος δεύτερος [The cell: a molecular approach. Second volume]

10. Appendix

Table 2. Primers for DISEC/TRISEC

Primer	Sequence
RFPGFP (Forward)	5’ TTGGCCTCCTCCGAGGACG 3’
RFPGFP (Reverse)	5’ CCGTTAGAGTCCGGACTTGTACAG 3’
Forward_T7 (forward)	5‘ TAATACGACTCACTATAGGG 3’
Rev_Dim_Cl (reverse)	5’ CAGCTTCACCTTGTAGAT 3‘

Table 3. Primers for mycoplasma

Primer	Sequence
GPO-1 (forward)	5' ACTCCTACGGGAGGCAGCAGTA 3'
MGSO (reverse)	5' TGCACCATCTGTCACTCTGTTAACCTC 3'

Table 4. Sequences DISEC/TRISEC

The plasmid used was the pcDNA3 (5400 bp), which is a mammalian expression vector with a CMV (cytomegalovirus) promoter and has ampicillin resistance. The insert is 1400 bp.

Insert for DISEC/TRISEC: pLVTHM-mRFP-GFP-ratLC3	5'ATGGCCTCCTCCGAGGACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAG TTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCC CCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCCAGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCC CCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACC GTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGG CCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCG AGATCAAGATGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCCGAGGTCAAGACCACCTACATGGCCAAGAAGCCC GTGCAGCTGCCCGGCGCCTACAAGACCGACATCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACA GTACGAGCGCGCCGAGGGCCGCCACTCCACCGGCGCCGGTGGACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGT TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG GGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGT GACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCA TGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTC GAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCT GGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCC GCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCT GGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCCGGACTC 3'
pCDNA3-PresequenceM vector	5'GGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTC ACTATAGGGAGACCCAAGCTATGCTGGGCGCCGCTCTCCGCCGCTGCGCTGTGACCGCAACCACCCGGGCCGACCCTCGA GGCCTCCTGCACTCCGCCCGGATCCCCGGCCCCGCCGTGGCTATCCAGTCAGTTCGCTGCTATTCCGAATTCTGCAGATA TCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCT 3'
Final construct after merging plasmid+insert	5'ATGCTGGGCGCCGCTCTCCGCCGCTGCGCTGTGACCGCAACCACCCGGGCCGACCCTCGAGGCCTCCTGCACTCCGCC CGGATCCCCGGCCCCGCCGTGGCTATCCAGTCAGTTCGCTGCTATTCCGAATTGGCCTCCTCCGAGGACGTCATCAAGGA GTTCATGCGCTTCAAGGTGCGCATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCC CCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCT CAGTTCCAGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGG CTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCG AGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGG GAGGCCTCCACCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGATGAGGCTGAAGCTGAAGGACGG CGGCCACTACGACGCCGAGGTCAAGACCACCTACATGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAAGACCGACA TCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAGCGCGCCGAGGGCCGCCACTCCACC GGCGCCGGTGGACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCT GGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGA AGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTC AGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCAT CTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC TGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTAT ATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCT CGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGT CCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTC GGCATGGACGAGCTGTACAAGTCCGGACTCTAACGG 3'