



COST Action IPLANTA

WG1 Meeting

‘Advance delivery strategies for dsRNA into cells’

5th Sept 2019

**GHENT UNIVERSITY, FACULTY OF
BIOSCIENCE ENGINEERING**

GHENT (BELGIUM)

BOOK OF ABSTRACTS



Wild type

RNAi



COST ACTION CA 15223 - MODIFYING PLANTS TO PRODUCE INTERFERING RNA

WG1 Meeting ‘Advance delivery strategies for dsRNA into cells’

The major target species for RNAi-based pest control are insects, plants, viruses and fungi. Previous research has demonstrated that several barriers related to cellular uptake and endosomal release could impact their sensitivity to RNAi. Hence, understanding the cellular uptake of dsRNA is mandatory and crucial to the development of RNAi-based technologies. Additionally, this WG1 meeting will coordinate the collection and generation of a database, containing information about dsRNA sequences used for RNAi studies in plants, viruses, fungi and insects.



DAY 1 - THURSDAY 6TH Ghent University,

NANOCARRIERS FOR IMPROVED DELIVERY OF DsRNA INTO INSECTS

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RNA interference is a natural mechanism, which is present in all Eukaryotes and which can be exploited for insect pest control. Silencing an essential gene in a target pest organism, by feeding genespecific double stranded RNA, can lead to lethal effects and could lead to an efficient control of the pest. This dsRNA can be applied on the field in different ways, including a classical spray-based approach. A major drawback of this delivery strategy however is the fact that the persistence of dsRNA in the environment is short and the efficacy of RNAi by feeding naked dsRNA in many insect species is variable or low. Several physiological and cellular barriers have been discovered in insects which affect RNAi efficacy. These include nucleolytic degradation of dsRNA in by insect saliva, haemolymph or in the insect gut and also an impaired cellular uptake or endosomal release of the dsRNA. These barriers could be overcome by using specific formulations which could increase the persistence of dsRNA in the environment or the insect body and which could improve cellular uptake.

Here, an overview is presented on the barriers affecting efficient RNAi in insects and on potential formulation strategies to improve RNAi efficacy.



NEW PHYSICAL AND CHEMICAL APPROACHES FOR THE CYTOSOLIC DELIVERY OF BIO-THERAPEUTICS AND NANOPARTICLES INTO CELLS

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Delivery of bio-therapeutics and nanomaterials into living cells is an important step not only for cell studies but also for therapy and bio-imaging. Clear examples are the intracellular delivery of various classes of nucleic acids (siRNA, μ RNA, mRNA, pDNA), peptides and proteins for therapy purposes. As another example, all types of (inorganic/organic) nanoparticles are under investigation as intracellular labels for imaging purposes. Meanwhile it generally accepted that after uptake by cells, nanomaterials typically end up in endo-lysosomal vesicles in which they remain entrapped while they should escape from such compartments and arrive in the cytosolic fluids of the cells. In recent years our team undertook major efforts to understand the biophysics which play a role in (a lack of) escape of nanomaterials from endo-lysosomal vesicles. Very recently we also discovered new chemical strategies (so named 'escape adjuvants') (1) which seems promising to 'liberate' nucleic acids (like siRNA) from endo-lysosomal vesicles into the cytosol. Furthermore we explored physical methods (either light (2,3) or ultrasound (4) driven) which directly deliver bio-therapeutics into the cytosol, thereby bypassing the endo-lysosomal routes. This lecture will explain our recent findings in this area, as reported in a series of recently published papers (1-4). Both pharmaceutical, biological and engineering aspects of our work will be highlighted in the lecture.

References

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3) Cytosolic Delivery of Nanolabels Prevents Their Asymmetric Inheritance and Enables Extended Quantitative in Vivo Cell Imaging



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4) Sonoprinting and the importance of microbubble loading for the ultrasound mediated cellular delivery of nanoparticles

I. De Cock, G.P.R. Lajoinie, M. Versluis, S.C. De Smedt*, I. Lentacker

Biomaterials 2016, 83: 294-307



REPURPOSING CATIONIC AMPHIPHILES FOR IMPROVED CYTOSOLIC DELIVERY OF RNA THERAPEUTICS

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Small interfering RNAs (siRNAs) are attractive therapeutics to reduce the expression of disease-related genes, outperforming traditional small molecule drugs in terms of design, selectivity, and their ability to silence targets previously regarded 'undruggable'. To be functional, siRNAs require delivery into the cell cytosol. However, RNAs do not have optimal drug-like properties as they lack the ability to cross biological membranes. To overcome extra- and intracellular barriers, RNA drugs are typically formulated into polymer- or lipid-based nanoparticles (i.e. nanomedicines). In recent years, many (pre-)clinical trials involving siRNA nanomedicines have demonstrated promising results but also identified many remaining hurdles that limit broad clinical translation. From a cellular delivery perspective, nanomedicines can guide macromolecules like siRNAs into cells through endocytosis, but escape from the endosomal lumen into the cytosol prior to lysosomal degradation remains a major obstacle towards efficient intracellular drug delivery. Despite decades of research, even for the current state-of-the-art nanocarriers such as siRNA-loaded lipid nanoparticles, endosomal escape is a very inefficient process with only ~1% of the internalized dose reaching the cell cytosol. This presentation will describe the repurposing of two distinct cationic amphiphiles, i.e. both low molecular weight cationic amphiphilic drugs (CADs) as well as the lung-related surfactant protein B (SP-B), to improve cellular delivery of small RNA therapeutics. Both approaches significantly promote cytosolic siRNA delivery efficiency, albeit by adopting a different mode-of-action.



SUCKING OF siRNA BY *MACROLOPHUS* BUGS

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RNAi, insect bugs, silencing, plum-tree

Routinely plant health in greenhouse experiments is depending through efficient computing to manage temperature, light, humidity and insect control. Perennial plants grown in greenhouse are commonly infested by pest like Bemisia, mite that are currently challenging any resistance assays and damaging plants. With the fundamental problems raised by these undesired pests, it is possible to keep plants in a good shape through the release of the predator *Macrolophus pygmaeus*. Across different plant clones grown in greenhouse that accumulated siRNA or not, we tested whether these small molecules (RNAi) widely spread in silenced clones were able to move in insect cells. Following feeding time that we managed, leaves and insects were sampled. Using total RNA extracts from both, leaves and insects, followed by appropriate RT/PCR assays using specific primers, we demonstrated that siRNA accumulated in leaves were sucked by *Macrolophus* insects. Whether our goal is currently to track the siRNA accumulated in plants, we successfully learnt more about the theory sizing of RNAi sucked by insect bugs. Although these experiments were focused on the viral siRNA engineered in plum-tree, we demonstrated that the siRNA, small molecules spread in cells, could successfully pass in bugs. These experiments highlighted the involvement of a predator insect obviously fed in young leaves that after biting, they significantly ingest siRNA as natural compounds in plant sieve. These studies showed that a model system involving insects/RNAi is highly relevant to follow up siRNA spread for understanding any application.



RNAI FOR PATHOGEN RESISTANCE IN MAJOR WOODY FRUIT SPECIES

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Most of woody fruit species, like *Vitis* spp. and *Prunus* spp., have high susceptibility to fungal and viral diseases which lead to significant agronomic and economic losses worldwide; therefore, new breeding techniques, such as RNAi, offer the possibility to introduce new clones of the main selected cultivars with increased diseases resistance, in order to reduce environmental impact and improve fruit quality. Thanks to the collaboration with different research groups (UNIVR, UNIBO and University of Riverside) three hpRNAi-based gene constructs have been designed to induce resistance to different fungi and viruses by inducing post-transcriptional gene silencing mechanism in plant. Among them, two target Dicer-like genes (DCL) of *Botrytis cinerea* (Wang et al., 2016) and *Plasmopara viticola* respectively, which are considered major pathogens of grapevine and several agrochemical applications are needed to control these diseases, raising social concern and pressure to develop alternative protection strategies. A third hp-RNAi construct was designed to induce resistance to Plum Pox Virus (PPV), the etiologic agent of Sharka disease, which attacks *Prunus* spp. included peach. Different *in vitro* regeneration and *Agrobacterium*-mediated transformation protocols have been developed for these woody fruit species starting from mature tissues (Sabbadini et al., 2019 a,b), however these procedures are often genotype-dependent and the success of their application to fruit tree plants is often limited by recalcitrance to transformation techniques. Spray-induced gene silencing (SIGS) strategy could represent an alternative tool to the stable expression of hairpin-based gene constructs in plants (Host-Induced Gene Silencing, HIGS), which can be applied to overcome the difficulty in genetic transformation of such agronomical important crops. Different protocols for the exogenous delivery of double strand RNA (dsRNA) molecules against *Botrytis cinerea* and *Plasmopara viticola* on grapevine, and PPV virus on peach are being optimized. Agroinfiltration of *Nicotiana benthamiana* leaves with these constructs is performed to produce dsRNA molecules that will then be assessed as new control means against the target pathogens on grapevine and peach leaves and fruits.



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CONFOCAL LASER SCANNING MICROSCOPY: HISTORICAL PERSPECTIVES AND RECENT DEVELOPMENTS

SKIRTACH A.

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Microscopy is taking a prominent place in further understanding many areas of science. Starting from over four hundred years ago, it has a rich history of fundamental break-through developments in instrumentation, optics, engineering, chemistry, chemical engineering, which ultimately impact biology. Many areas of biology stand to benefit from developments in microscopy, including in plants. In this talk, a brief history of microscopy is overviewed highlighting developments in fluorescence and confocal scanning fluorescence microscopy. Later, a connection is made to the most recent and new technologies driving the progress in biology, including atomic force microscopy. Some application of all types of microscopy are also discussed.



GHENT UNIVERSITY CENTRE OF EXPERTISE ON ADVANCED LIGHT MICROSCOPY DEKEERSMAECKER H.

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As there is an increasing amount of sophisticated and expensive instruments used in research, the need for centralized and shared instruments with support of experts increases. Core facilities fulfil this task thereby increasing accessibility to instruments and ensure training and guidance of researchers. For imaging based research, support by experts is necessary from experimental design to data analysis and interpretation. Therefore, it is essential that researchers are aware of the possibilities core facilities can offer for their research and project proposals.

Here we present the centre of expertise at Ghent University. The Centre for Advanced Light Microscopy at Ghent University is a university-wide core facility clustering expertise with both standard and specialized microscopes to support researchers in conducting microscopy based experiments. The centre aims to provide high end instrumentation and expertise thereby making light microscopy accessible for researchers independently of their experience level. The centre facilitates microscopy experiments to ensure high-quality scientific research where light microscopy plays an indispensable role. The centre features a broad gamma of light microscopy equipment from standard to custom build microscopes. On all instruments, we offer service measurements or access to the equipment to perform your own experiments. To ensure optimal use of our instrumentations, we provide one-to-one expert advice, training and full technical support. No prior experience is required as the facility staff supports researchers throughout the complete process to obtain microscopy based results. Therefore, the instruments are accessible for users of all levels of experience.



LECTINS AND CPP-BASED DSRNA CARRIERS

DE SCHUTTER K.

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Thanks to the specificity and the chemical properties of double stranded RNA (dsRNA), RNA interference (RNAi) has emerged as a very promising candidate in the search for novel, eco-friendly and sustainable pest control strategies. Essential for RNAi is the uptake of the dsRNA molecules into the cells of the pest insect, however, the internalization of naked dsRNAs is not always as evident. To increase the uptake of dsRNA into the cell, one can make use of carrier systems. These systems are designed to allow an efficient delivery of the dsRNAs in the cell by complexing them with a chemical-(e.g. polymers) or peptide carrier (e.g. lectin or cell membrane penetrating peptide). Lectins are carbohydrate binding proteins which can selectively bind to specific glycan structures on the surface of the cell. As many lectins are efficiently internalized after binding, they are interesting candidates for the development of carrier systems. Similarly, cell membrane penetrating peptides are known to facilitate the delivery of various molecular cargo into the cell. Here we present two in-house developed carrier systems to improve delivery of dsRNA into the insect cells.



TRACKING CELLULAR UPTAKE OF dsRNA BY CONFOCAL MICROSCOPY

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Here I present my research on cellular uptake of a guanidine-containing polymer in CF1 cells. Guanidine groups are highly basic, and can interact with the phosphate group of dsRNA or other anionic moieties on the liposomal or cell surface contributing to an efficient uptake in and consequent transport through the cell. Accumulation of fluorescently labeled dsRNA (both naked and complexed with the polymer) inside the cells was observed via confocal microscopy at different time points and confirmed by Image J analysis. Our results suggest that the polymer helps for a faster uptake in CF1 cells, since the complex could already be detected inside the cells after 5 minutes incubation, while the naked dsRNA required at least 30 minutes to be visible inside the cells. To confirm these results, I used Image j to calculate the Corrected Total Cell Fluorescence (CTCF) for each treatment. CTCF results confirmed that the polymer:dsGFP is not only uptaken faster but also in higher quantities.

To further investigate the underlying cellular uptake mechanisms, inhibitors were used to block the clathrin-mediated endocytosis (CME) and caveola mediated endocytosis to elucidate which - and if any - of these pathways are responsible for the uptake of dsRNA and the polyplex. The effects on RNAi efficiency were examined by confocal microscopy of labeled dsRNA and by calculating the CTCF in each treatment. This research sheds light on cellular uptake mechanisms of naked dsRNA and our polymer:dsGFP and this knowledge could lead to further advances in novel dsRNA-delivery methods.